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Improving rumen function

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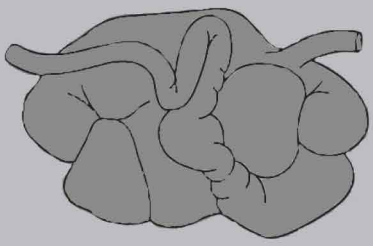

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Preface

Most ruminant animal species were domesticated by humans by 2500 BC. Since then domesticated ruminants have co-migrated with people as they spread across the globe by providing a source of food for survival and reproduction, draft power for migration and later on for cultivation, as well as fibre from which clothing could be fashioned for protection from the environment. Their special relationship with mankind arises from their ability to convert human-indigestible carbohydrate and nitrogen into high quality protein in the form of meat and milk. The ability of both lifeforms to co-exist without competing for the same source of essential macro-nutrients has sustained their relationship over many thousands of years. The capacity of the ruminant to convert vegetation that is indigestible by humans into metabolizable protein and energy for the animal is due primarily to the microorganisms that inhabit the pre-gastric stomach (reticulum, rumen, and omasum) of these herbivores. Although humans realised the benefit of domesticating these animals long ago, it was only during the 20th century that the central role of 'rumen' microorganisms in this process was documented in detail. By mid-century some rumen microorganisms had been isolated and an understanding of the biochemical processes that defined the process of anaerobic fermentation were being published. The first book on rumen microbiology '*The Rumen and Its Microbes*' was written by R. E. Hungate and published in 1966. This classic book provided a description of the rumen microorganisms identified at that time, their physiological activities and requirements as well as the methods that had been developed to cultivate and isolate strict anaerobes. The publication also had a practical dimension in that it discussed this basic knowledge in terms of improving productivity of the ruminant animal and preventing abnormalities in rumen fermentation due to different feeding practises. Even then it was recognised that the discipline of rumen microbiology needed to be relevant to the agricultural sciences and not studied independently. Rumen microbiology continued to flourish following publication of Hungate's book and another book was published In 1988 titled *The Rumen Microbial Ecosystem* (ed. P. N. Hobson) and subsequently a 2nd edition in 1997 (eds P. N. Hobson & C. S. Stewart). These newer books followed the basic format of 'The Rumen and its Microbes' but called on the many experts who had entered the field to contribute specialist chapters on the new microorganisms and biochemical activities that had since been discovered as well as the evolving microbiology techniques.

In the last 25-30 years, microbiology has undergone a revolution in that the field has evolved from culture-based techniques to molecular approaches that

can interrogate complex ecosystems without the requirement for cultivation. Like the previous books focussing on rumen microbiology, this current publication seeks to present the new knowledge on the rumen microorganisms and biochemical pathways that have been discovered with the advent of molecular rumen ecology studies in the 21st century. However more than ever rumen microbiology is being challenged to make itself relevant to the major issues facing the ruminant industries. In particular, the public perception of negative impacts of ruminant production on the environment from methane and nitrous oxide emissions and nutrification of soil and water from urine and manure have been given special attention in the book. Although environmental issues are receiving greater emphasis in rumen research, there is still a demand from industry to increase efficiency of production while providing a satisfying, nutritious and healthy product, all of which can be directly impacted by the rumen ecosystem.

Unique anatomical and physiological adaptations have evolved in some domesticated ruminant species to enable a diverse rumen microbiome to function efficiently on relatively low protein forages high in lignocellulose so that their host can survive and reproduce. Much of the gains in efficiency of ruminant production during the 20th century were achieved with high quality diets and intensive feeding of formulated balanced rations. Ruminant production systems that compete with both humans and feed efficient monogastric livestock for dietary nutrients of high quality will come under greater scrutiny by environmental activists, regulators, as well as the consumer. In the future there needs to be increasing emphasis placed on the unique ability of ruminants to convert lignocellulosic feedstuffs into high quality protein for human consumption so that their rightful place in sustainable livestock production systems can be justified environmentally and economically. This book should therefore provide a reference source for students and researchers as foundational information on rumen microbiology and metabolism for the next 20–30 years of ruminant production given the pressures being faced by climate change.

C. S. McSweeney
R. I. Mackie

Introduction

Major advances in analytical techniques and genomics have transformed our understanding of rumen microbiology. This understanding is of critical importance to livestock production since rumen function affects nutritional efficiency, waste emissions from ruminants (such as methane and nitrous oxide) as well as animal health. This collection reviews what we know about rumen microbiota and the role of nutritional strategies in optimising their function for more sustainable livestock production.

Chapter 1 sets the scene by providing the latest information in the colonisation and establishment of the rumen microbiota. The chapter reviews the establishment of gastrointestinal microbiota in young ruminants and how it can be modulated for promoting health and favouring desirable phenotypes. Case studies of early-life strategies for improving health and production and for reducing enteric methane emissions are also provided. The chapter concludes by providing potential areas for future research and gives examples of resources to use for further information on the subject.

Part 1 Tools to understand the ruminal microbiome

Chapters in the first part of the book summarise advanced methods for analysing the rumen microbiome. Chapter 2 reviews how the gut microbiome can be 'brought to life' in the omics era. Research on the mammalian gut microbiome has in recent years been principally defined by culture-independent analysis of the genetic potential inherent in these microbial communities. However, there has been a renewed interest in culture-based studies of the gut microbiome to increase both the breadth and depth of gut microbial isolates, as these are widely recognised to provide the clearest opportunity to link biological activities with specific microbes. The chapter begins by providing an overview of the methodological approaches widely used to culture fastidious gut microbes, and our evolving understanding of how macro- and micronutrients impact their growth. In addition, alternative culturing approaches are discussed including genomic, genetic and antibody-based isolation strategies that take advantage of the existing wealth of metagenomic data.

Moving on to Chapter 3, this chapter focuses on rumen metabolomics as a tool for discovering and understanding rumen functionality and health. The rumen is a complex ecosystem essential for the health and productivity of the animal. Rumen metabolomics research is generating important data about the metabolites present in the rumen, and the factors affecting the rumen microbiome and metabolome. The chapter begins by providing an overview

of the technologies and extraction techniques for rumen metabolome analysis. It also reviews the factors impacting the composition and functionality of the rumen metabolome. The chapter concludes by providing a section on future trends in this emerging field of research and a summary of the variety of technologies available for metabolomic analysis.

The final chapter of Part 1 reviews mathematical modelling of microbial functionality in the rumen. To benefit from all the data gathered, with new techniques and new data types being introduced continuously, mathematical models need to be constructed that can capture the biological evidence gathered and to predict functionality at the level of the whole rumen. Chapter 4 reviews ways of quantifying rumen microbial functionality at the whole rumen level as well as units and sampling techniques used during mathematical modelling. These can then be used to predict fermentative and digestive processes in the rumen.

Part 2 The rumen microbiota

The second part of the book reviews recent research on the role of different communities of rumen microbiota such as bacteria, archaea, protozoa, anaerobic fungi, viruses and the rumen wall microbial community. As Chapter 5 points out, microbial genome sequencing has had an enormous impact on our understanding of many biological systems, including the identity, relationships and functions of the resident bacteria of the rumen. Since the first rumen bacterial genome was sequenced in 2003, over 500 genomes from cultured isolates and over 5000 genomes from metagenomic data studies have revealed a complex picture of how their genomic repertoire are formed by their ecological interactions during the breakdown of the plant material consumed by the host. Yet this picture remains incomplete since many organisms known to be present are missing from the genomic database. A complete understanding of the rumen microbiome relies on these gaps being addressed. The chapter reviews the power of sequencing a single genome and the curation of a reference genome catalogue for the rumen microbiome. It looks at the application of metagenomic data in identifying novel genomes and key functions in the rumen, as well as the use of genome sequencing to reveal interactions across the rumen microbiome.

Chapter 6 reviews the types of methanogenic archaea found in the rumen. The chapter provides a summary of rumen cultivation studies and molecular surveys of rumen archaeal community composition. It also discusses the current understanding methanogen function via genomic information retrieved from pure cultures of methanogens, re-assemblies of genomes derived from metagenome data sets from mixed enrichment cultures, as well as directly from rumen samples. The chapter concludes by providing an understanding of the

importance of rumen methanogens in methane production and other aspects of rumen function.

Chapter 7 reviews what we know about the ruminal ciliated protozoa, including their taxonomy and population 'types'. It assesses ecological fluctuations in protozoa populations, protozoa interactions in the rumen as well as the effects of protozoal function on ruminant nutrition, health and emissions. The chapter also discusses the challenges of working with rumen protozoa in culture. The chapter includes a case study describing the manipulation of rumen ciliates. It concludes by highlighting current gaps in research and gives examples of key research material and conferences on the subject.

Chapter 8 discusses the life cycle, taxonomy and morphological features of anaerobic fungi. It goes on to provide an overview of all currently known monocentric, polycentric and bulbous genera of anaerobic rumen fungi. Sections on the genomics and meta-omics of anaerobic fungi are also included, followed by a review of the interactions between rumen fungi and other members of the rumen ecosystem. The chapter concludes by emphasising the importance of expanding the research available on anaerobic rumen fungi and directs readers to useful websites and other resources for further information.

As Chapter 9 indicates, despite the sustained research focus on rumen microbial ecology, there is still a relative lack of knowledge surrounding the rumen mobilome including the major factors that contribute to the mobilome (for example viruses and plasmids), and the extent to which the mobilome impacts on rumen function. Viral populations have been shown to co-exist with, and predate on, the rumen microbiota. Non-viral extrachromosomal elements, such as plasmids, are often intrinsically linked with rumen microbial populations. This chapter reviews current understanding of rumen viral populations and extrachromosomal elements, as well as describing carriers of mobile genetic elements, such as extracellular membrane vesicles. The chapter also explores the impact of the mobilome on rumen function.

Chapter 10, the final chapter of Part 2, discusses the rumen wall microbiota community. The rumen microbes are usually divided into three distinct groups based on their habitats: the planktonic microbiota, feed particle associated microbiota, and the epimural microbiota. Among these three groups of microorganisms, the epimural community is the least studied and understood. The members of epimural microbiota are key players in oxygen scavenging, tissue recycling, urea metabolism, and nutrient transportation. The recent development of nucleic acid sequencing techniques has enabled us to better explore the composition and functional of this community. The chapter summarizes the current knowledge on the rumen epimural microbial community including its diversity, ecology, functions, and effects on host physiology.

Part 3 Nutrient processing in the rumen and host interactions

Chapters in Part 3 address the way the rumen processes nutrients such as fibre and protein as well as outputs such as energy, lipids and methane emissions. The first chapter discusses ruminal fibre digestion. Since ruminants obtain most of their energy from their symbiotic microbiota, the efficiency of feed conversion and end-product meat and milk quality is tightly linked to the dynamics and function of the rumen microbiome. Chapter 11 provides an overview of the role of the microbiota in ruminal lignocellulose degradation and the mechanisms they utilize in the decomposition of biomass. It discusses findings from studies on well-known Ruminococcus, Fibrobacter and Prevotella isolates, as well as those from poorly understood and as-yet uncultured Bacteroidetes lineages. Collectively, these approaches have revealed new information related to the hydrolytic capacity of cellulosomes, free enzymes, outer membrane vesicles, polysaccharide utilization loci and large multi-modular enzymes, which are generating deeper insights into the intricate microbial networks that engage in ruminal fibre digestion.

Chapter 12 reviews ruminal protein breakdown and ammonia assimilation. Ruminal nitrogen (N) metabolism has long been associated with effective ruminant degradation of fibre, feed intake and productivity. The chapter focuses on the latest research on N metabolism as well as understanding the process of ammonia assimilation and its regulation in the rumen. An improved understanding of microbial proteolysis and capture of N as microbial protein can then be integrated into nutritional strategies to optimize ruminant animal productivity while minimizing its environmental impact.

Chapter 13 focuses on the factors influencing the efficiency of rumen energy metabolism. It begins by addressing the main pathways of rumen fermentation and how these pathways can be used to produce products such as volatile fatty acids and adenosine triphosphate (ATP) as well as the disposal of metabolic hydrogen. The chapter then reviews the production of methane in the rumen and how modulation of methanogenesis can be used to benefit animal productivity. The factors that can influence the efficiency of microbial growth are also discussed, followed by a review of the interactions between rumen energy and nitrogen metabolism. The chapter concludes by highlighting the importance of research on maximising the energy output of fermentation, controlling the profile of volatile fatty acids, and increasing the efficiency of microbial growth.

The subject of Chapter 14 is understanding rumen lipid metabolism to optimize dairy products and monitor animal health. Rumen lipid metabolism largely determines the fatty acid composition of dairy products. As such, milk fatty acids can be used as indicators of ruminal and hence animal health. Control

of rumen metabolism can also facilitate production of dairy products enriched in unsaturated fatty acids. To better understand the origin of fatty acids in dairy products, the chapter first addresses ruminal metabolism, intestinal digestion, transfer to and fatty acid metabolism in the mammary gland. The chapter then discusses the potential to improve the fatty acid composition of dairy products for enhanced human health, with a particular focus on technologies to protect unsaturated fatty acids from rumen biohydrogenation. Finally, the chapter examines the use of variation in the milk fatty acid profile, induced by changes in the ruminal lipid metabolism, as a monitor of rumen and animal health.

Chapter 15 examines the nutritional factors affecting greenhouse gas production from ruminants and its implications for enteric and manure emissions. Ruminants are significant contributors to global greenhouse gas (GHG) emissions. Mitigating enteric and manure methane (CH_4) production have been explored, but often in isolation of other GHG. Lowering enteric CH_4 emissions can cause unintended increases in GHG from manure. Considering the complexity of rumen and the impact that it can have on manure composition, a whole systems approach is required to assess the value of additives that mitigate enteric CH_4 emissions. The chapter summarises a range of nutritional strategies available for enteric- and manure-CH_4 abatement. Dietary additives including alternative electron acceptors, inhibitors, plant secondary compounds, and carbon (C) derived materials are reviewed for their efficacy as mitigants of overall GHG emissions and are evaluated for how they alter rumen and manure microbiomes.

The next chapter considers host-rumen microbiome interactions and influences on feed conversion efficiency, methane production and other productivity traits. Today, with our ability to assess the composition of the rumen microbial community as a whole, a new holistic view of the microbiome has emerged. This has led to an increased understanding of the role of the microbiome and its components on production efficiency, health, and waste emissions such as methane. Chapter 16 focuses on the recent discovery of the role of the ruminant microbiome on energy harvest, methane emission, and the potential host genetic factors determining its microbial composition and selection.

The final chapter of Part 3, Chapter 17, discusses the rumen as a modulator of immune function in cattle. The rumen and its microbiome play essential roles in supplying key nutrients, such as energy, protein, minerals and vitamins, to the host and also shape the cow's immune system. Rumen health disorders, such as subacute ruminal acidosis, cause ruminal dysbiosis provoking epithelial barrier dysfunction and inflammation. These conditions facilitate rumen- and hindgut-derived lipopolysaccharides (LPS) to translocate into the blood stream, thereby compromising systemic metabolism and immune response. The chapter summarises new research related to rumen health, LPS exposure, and their

role in modulating the systemic metabolome and liver health. It examines the effects of prolonged rumen acidosis episodes on udder health due to the long-term exposure of LPS and highlights the systemic role of LPS in impairing the blood-milk barrier and invading mammary gland tissue.

Part 4 Nutritional strategies to optimise ruminal function

The final group of chapters explore nutritional strategies to optimise rumen function, including the role of pasture, silage, cereal feed, plant secondary compounds and probiotics. Chapter 18 focuses on the role of rumen microbiome in pasture-fed ruminant production systems. Pasture has long been utilised as a feed source for ruminants but the fermentation of forage by ruminants is results in the production of GHGs, which may negatively effect the environment and represents a loss of energy/N for animal performance. The chapter examines the relationship between the rumen microbiome, host feed efficiency and environmental outputs in pasture-based productions systems. The impact of different forages and grassland management practices on rumen microbes are also reviewed.

Chapter 19 assesses the role of silage and concentrate in dairy cow nutrition to improve feed efficiency and reduce methane and nitrogen emissions. Sustainable dairy cow feeding strategies to mitigate GHG and N emissions should make the most of the unique ability of ruminants to convert local human-inedible biomass to high-quality dairy foods. The chapter reviews the potential of silage plant species (grass, forage legumes, maize) and stage of maturity of silage crops as well as forage-to -concentrate ratio to reduce the environmental footprint of dairy cows without compromising animal performance. Dairy cow performance is examined in terms of feed intake, milk yield, feed and N efficiency, and methane emission intensity. The role of concentrate composition (lipids, carbohydrates and protein) is also evaluated. As a case study, the potential of milled rapeseed to reduce environmental footprint of grass silage-based diet is evaluated in practical farm conditions.

The next chapter examines the use of feedlot/cereal grains in improving feed efficiency and reducing by-products such as methane in ruminants. Chapter 20 begins by highlighting the types of cereal grains fed to cattle such as barley, corn, wheat, oats and sorghum. It then addresses the dietary factors affecting methane production by ruminants and the role of starch and forage in methane production. A section on H_2 sinks in the rumen and methane production is also included, followed by a discussion on using cereal grains to improve feed efficiency and reduce methane production. The microbiology of cereal grain fermentation is examined, and the bacteria and archaea involved in this fermentation are also addressed. The chapter also highlights one of the

factors that contributes the most to the degradation of feedstuffs, feed retention time, which is then followed by a review of acidosis and other feed effects.

Chapter 21 considers plant secondary compounds and the beneficial roles they have in sustainable ruminant nutrition and productivity. Plant secondary compounds, also known as phytogenics or phyotochemicals, are secondary metabolites that, when extracted and concentrated, modulate the activity of gut microorganisms. The chapter describes the composition, activity, effects on rumen function and animal performance of three major groups of plant secondary compounds: essential oils, tannins and saponins. The positive effects of these compounds include more efficient use of dietary protein and energy and decreased methane emissions, which can also result in increased milk yield or liveweight gain. The chapter explores current limitations in the use of plant extracts as feed additives in ruminant livestock and how they can be overcome.

The final chapter of the book analyses the use of probiotics as supplements for ruminants. There is an increasing need to promote digestive efficiency and productivity while maintaining animal health and welfare. Probiotics are live microorganisms which confer a benefit for the host when administered in appropriate quantities. Chapter 22 begins by reviewing critical periods in the ruminant lifecycle as targets for probiotics. It then looks at types of probiotics, delivery mechanisms and regulation. The rest of the chapter summarizes and assesses the range of research on the benefits and modes of action of probiotics, starting with their potential in young ruminants. It then considers the role of probiotics in adult ruminants in the areas of feed efficiency, methane production, pathogen control and supporting the immune system.

Chapter 1

Colonization and establishment of the rumen microbiota – opportunities to influence productivity and methane emissions

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1 Introduction

The gastrointestinal (GIT) microbiota of ruminants cannot be dissociated from the host animal. Gut symbiotic microbes have a critical role in the interaction of the host animal with the surrounding environment, providing fundamental nutritional, immunological and protection services. As for other essential body 'organs', the GIT microbiota undergoes a series of development stages from early stages of formation until maturity. Differently from the embryogenesis process; however, the development of the GIT microbiota starts in earnest at birth and it is characterized by a succession of dynamic communities in the early stages of life (Savage, 1977; Jami et al., 2013; Rey et al., 2014). This process of acquisition of various microbial populations and their evolution within the ecosystem is essential for the correct functioning and interaction of the microbiota with the host (Costello et al., 2012).

Determinism is a strong driver dictating the microbial community structure of the GIT of animals as there is a strong selection by the diet, anatomy and

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gut physico-chemical conditions (Ley et al., 2008). Yet, stochastic and historical events also influence the assemblage of the GIT microbiota that may have lasting effects in ruminants (Yáñez-Ruiz et al., 2010; Morgavi et al., 2015; Moraïs and Mizrahi, 2019). In this chapter, we review current information in the establishment of the microbiota in the rumen and posterior intestinal tract in young ruminants and its modulation for promoting health and favouring desirable phenotypes.

2 Establishment of the rumen microbiota

As the composition of the rumen microbiota directly influences the digestive and metabolic performance of the host animal, many studies have explored the microbial colonization of the rumen from birth to adulthood. These include early work using cultural methods (Fonty et al., 1983, 1988) to more recent studies using high-throughput sequencing methods in calves, lambs and goat kids (Jami et al., 2013; Rey et al., 2014; Guzman et al., 2015; Wang et al., 2017b; Abecia et al., 2018; Dias et al., 2018). The developing rumen in the newborn ruminant may provide a unique opportunity to manipulate the symbiotic microbiota for a long-lasting impact in the adult ruminant (Yanez-Ruiz et al., 2015).

2.1 Colonization: from birth (pre-ruminant) to a fully functional rumen

Recent reviews describe the microbial community successions that occur in the rumen from birth to weaning and after, when animals feed exclusively on solid feeds (Malmuthuge et al., 2015; Yanez-Ruiz et al., 2015; Meale et al., 2017a). Functional populations, as well as taxa present in adult rumens, appear very early after birth, in a progressive way and in a defined sequence. Several studies monitored the establishment of the rumen bacterial community in calves from birth to weaning using high-throughput sequencing and qPCR approaches (Jami et al., 2013; Rey et al., 2014; Guzman et al., 2015). They show that rapid changes occur in the composition of the rumen bacterial community during the first days of life. Proteobacteria and *Streptococcus*-related sequences are proportionally abundant in 1–3-day old calves and are rapidly replaced by strictly anaerobic bacterial taxa (Jami et al., 2013). Proteobacteria are then gradually replaced by Bacteroidetes as the animal grows, Firmicutes being present from early age to adulthood (Table 1). These results are in accordance with early studies using culture techniques reporting that aerobic and facultative anaerobic bacteria establish first (Fonty et al., 1987). Notwithstanding, strict anaerobes that are important for function in the mature rumen, such as cellulolytic bacteria and methanogenic archaea are already present in the rumen at 1 or 2 days after birth (Fonty et al., 1987; Gagen et al., 2012; Jami et al., 2013; Guzman et al., 2015). Methanogenic archaea

Table 1 Time line for colonization of the major bacteria phyla from birth to adulthood (values expressed as range of mean percentages)

	Age						
	3 days	7 days	14 days	28 days	42 days	6 months	2 years
Phyla							
Bacteroidetes	13.9–42.6	56.3–56.9	46.0–61.3	49.9–56.3	56.3–74	38.5–55.2	38.5–50.2
Firmicutes	5.1–13.9	13.9–17.5	13.9–34.0	13.9–42.1	10–43.9	36.8–48.9	34.5–56.7
Actinobacteria	0.05–4.9	0.6–4.9	0.9–4.9	0.3–4.9	0.3–4.1	3	3
Fusobacteria	4.7–5.5	4.7–5.3	0.2–0.6	0.2–0.3	0.2–0.4	0.1	0.1
Spirochaetes	0–0.4	0.1–0.4	0.4–2.6	0.4–0.9	0.4	0.7–1.2	0.9–2.5
Fibrobacteres	0–0.3	0–0.3	0.2–0.3	0.3–1.5	0.3–1.6	0.2–1.7	0.5–2.1
Tenericutes	0	0.8	0.2	0.9	1.0	1.0–1.6	1.3–2.3

Data collected from Li et al. (2012), Jami et al. (2013), Rey et al. (2014), Yáñez-Ruzi et al. (2015), Abecia et al. (2018).

can be enumerated in the immature rumen of lambs at 2–4 days, well before the consumption of solid feeds, and after two weeks, their concentration is equivalent to that found in adult animals (Fonty et al., 1987; Morvan et al., 1994). Although not detected by culture, a low-abundant but diverse population of methanogens (predominantly *Methanobrevibacter* spp.) was identified using molecular methods in lambs placed into sterile isolators 17 h after birth (Gagen et al., 2012). A recent study in goat kids also indicated that active methanogens colonized the rumen at one day of life, *Methanobrevibacter*, *Methanosphaera* (both Methanobacteriales order) and Candidatus *Methanomethylophilus* (Methanomassiliicoccales order) being the top three genera (Wang et al., 2017b). There are four major methanogenic orders usually found in the rumen: Methanobacteriales, Methanomicrobiales, Methanosarcinales and Methanomassiliicoccales (Janssen and Kirs, 2008). All these are abundantly present in calves from day 1 to 2 weeks of age, whereas only Methanobacteriales and Methanomassiliicoccales could be qPCR-detected in the mature rumen (Friedman et al., 2017). Based on substrate utilization for methanogenesis, the authors suggest that the early methanogenic community may be characterized by a high activity of methylotrophic methanogenesis, likely performed by members of the order Methanosarcinales. Eukaryotic microorganisms also establish sequentially. Anaerobic fungi can be enumerated in the rumen of lambs by 8–10 days after birth (Fonty et al., 1987). Anaerobic fungi, which are cellulolytic, are thus present in the rumen long before the animal ingests solid feeds regularly. Ciliates are detected from 2 to 3 weeks of age, with *Entodinium* establishing first (15–20 days), then *Polyplastron*, *Eudiplodinium* and *Epidinium* (20–25 days) and finally *Isotricha* (50 days) (Fonty et al., 1988). In contrast to bacteria and archaea, protozoa do not establish when newborns are isolated from their dams shortly after birth (Fonty et al., 1988; Chaucheyras-Durand et al., 2019). In addition, ciliate protozoa require the presence of a complex microbiota to establish (Fonty et al., 1983, 1988). Figure 1 shows the main colonization events by groups of microbes in lamb's rumen throughout the suckling period and up to the end of weaning.

Colonization of the rumen wall by epimural bacteria is also age-related, with sequential diversification of bacterial morphotypes (Rieu et al., 1990). The phylum Proteobacteria is dominant on the rumen epithelium with an important contribution of the genus *Escherichia* (Jiao et al., 2015; Wang et al., 2017a). As for the lumen, the abundance of Proteobacteria associated with rumen epithelium decreases, and that of Firmicutes and Bacteroidetes increases with age (Jiao et al., 2015).

Large differences between digesta and epimural bacterial communities have been observed in the rumen of pre-weaned calves, with higher abundances of Prevotella and lower abundances of Bacteroidetes in digesta compared with epimural bacteria (Malmuthuge et al., 2014).

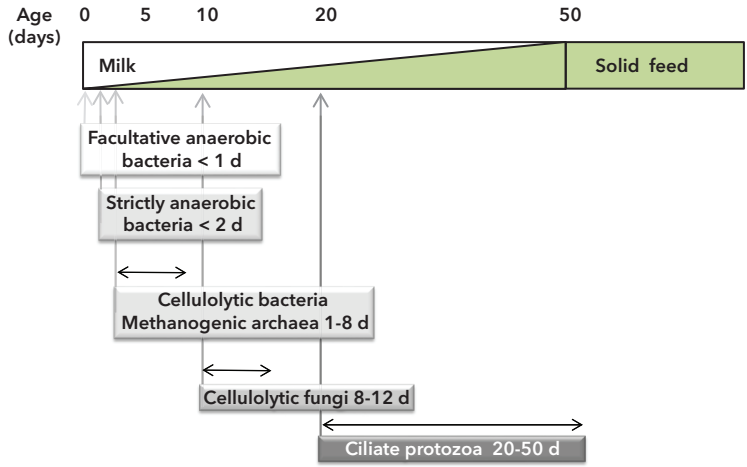

Figure 1 Microbial colonization of the lamb rumen. Colonization of the rumen by microbial groups detected by cultural and molecular methods. Lambs were kept with their dams. Arrows indicate the start of the colonization period.

Although the focus of this chapter is on the rumen compartment, we will mention some aspects of the microbiota of other GIT sections when relevant and/or available. While the rumen microbial community evolution with age has been well studied, information on the other pre-gastric compartments (reticulum and omasum) is scarce. Recent studies analysed fluid samples from these organs in goat kids from 3 to 56 days after birth (Lei et al., 2018) or in calves from birth to 21 days (Yeoman et al., 2018). As for the rumen, Proteobacteria gradually decrease with age while the relative abundance of Bacteroidetes increases.

For the post-gastric compartments, a surprisingly diverse microbiota is also described in the first hours post-delivery (Alipour et al., 2018). Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes dominate the newborn's rectal microbiota but composition rapidly changes in the early postnatal life. Pioneer studies reported *E. coli* and *Streptococcus* to be the firsts to colonize all GIT regions in calves and lambs few hours after birth (Smith, 1965) and culture-independent studies confirmed their high abundance in rectal microbiota one day after birth (Alipour et al., 2018). As for the rumen (Jami et al., 2013), it is assumed that these facultative anaerobes scavenge oxygen and render the environment suitable for strictly anaerobic gut microbes. Lactobacilli take advantage of these conditions and colonize all intestinal sections of one-day-old ruminants (Smith, 1965). Proteobacteria, Firmicutes and Bacteroidetes are the prevalent phyla in all anatomical locations within the first 3 weeks of life (Malmuthuge et al., 2014; Alipour et al., 2018; Yeoman et al., 2018). Firmicutes

dominate distal parts of the GIT in young ruminants (colon and faeces), whereas Bacteroidetes abundance is higher in the reticulum, rumen, omasum and abomasum (Malmuthuge et al., 2014; Yeoman et al., 2018). It should be noted that while Firmicutes and Bacteroidetes are more abundant in luminal contents, Proteobacteria dominate mucosal samples (Yeoman et al., 2018). This niche specialization for mucosa-associated populations is certainly driven by environmental conditions (presence of trace oxygen) and available substrates such as mucins. Interestingly, richness and α-diversity in luminal samples increase with age in most anatomical sections, whereas no such trend is observed for mucosa-associated populations. Nevertheless, mucosa-associated bacterial communities in the small and large intestine are more diverse than digesta-associated communities (Malmuthuge et al., 2014). Though the post-gastric intestinal tract has not received much attention this far, there is evidence that its microbiota plays a crucial role in older animals' health and performance. For instance, lactic acid bacteria increase IgA production by stimulating host's adaptive immune system, boosting calves' active immunity by the time when passive immunity from colostrum decline (Corthésy et al., 2007).

2.2 Interaction host-microbiota

The gastrointestinal tract of mammals has a diverse array of non-specific and specific protective mechanisms to allow it to coexist with resident microbiota (Hooper et al., 2012). The functions of nutrients absorption, symbiotic microbial tolerance and pathogenic microbial barrier, create a conflict in function which requires a complex system of physical, biochemical and cellular mechanisms for protecting the gastrointestinal epithelium and the host against invading agents (Kuhn and Stappenbeck, 2013). Several studies have shown the education process that the immune system needs to go through to deal with microbial loads and this is of particular relevance during early-life stages (Collado et al., 2012; Wu and Wu, 2012). However, the mechanisms involved in the 'tolerance' to the first colonizers of the rumen are largely unknown, partly because the rumen epithelial structure has its own peculiarities as compared to the lower intestinal tract. The rumen has a multi-strated, keratinized epithelium with up to 15 cell layer and no organized lymphoid tissue (Sharpe et al., 1975), which limits the permeability of large molecules such as immunoglobulins. The immunological equilibrium in the rumen is thought to be achieved by a combination of two main mechanisms: (i) the signalling of toll-like receptors (TLRs) (Malmuthuge et al., 2012) and (ii) provision of immunoglobulins (mainly secretory IgA) via saliva (Williams et al., 2009).

(i) TLRs are a family of pattern recognition receptors (PRRs) that play a key role at mucosal surfaces by acting as sensors to detect molecular patterns expressed by both pathogenic and commensal microbiota alike. Cattle express

a similar repertoire of TLRs compared with other mammalian species, as TLR1-10 have been identified and characterized (McGuire et al., 2006) and several studies have shown the relationship between nutritional disturbances (i.e. acidosis) and TLR expression levels (Chen et al., 2012). However, little is known in relation to early-life colonization. The expression of TLR in the GIT of newborn calves is greater than at 6 months of age with the exception of TLR-1 and TLR-3 (Malmuthuge et al., 2012). More recently, Abecia et al. (2017) evaluated the impact of two different management practices in the early life of goat kids (maternal vs. artificial milk feeding) on the immune response of the rumen. They observed that TLRs 1, 2, 5, 8 and 10 displayed an age-dependent expression, consisting of increased gene expression between days 5 and 7 of life and then a subsequent decrease and stabilization. The increase in expression coincided with a rise in VFA concentration and microbial biomass colonization. However, although colonization continued in the following days, this did not trigger higher expression levels. This is in agreement with reports showing downregulation of TLR expression levels in the blood of newborns with increasing age, whereas memory T cells increased in number (Teran et al., 2011; Malmuthuge et al., 2012). The downregulation of TLR activity might also be promoted by the production of butyrate that enhanced barrier function through stable tight junctions signalling (Jiao et al., 2017). Therefore, the few available published studies suggest that downregulation with increasing age might be one mechanism by which the host avoids unnecessary inflammatory responses to commensal microbiota. In suckling calves fed raw milk, the expression of IL-8, IL-10 and claudin-4 in the mucosa of the colon was low compared to calves of the same age fed heat-treated milk (Bach et al., 2017). These calves fed raw milk had a higher Lactobacilli population suggesting that the downregulation observed with increasing age mentioned above is associated with a more developed microbiota.

(ii) Immunoglobulins find their way into the rumen mainly through saliva, particularly IgA that is the major Ig secreted in saliva and also the dominant type in the rumen (Subharat et al., 2015; Abecia et al., 2017). IgA is apparently more resistant to degradation in the rumen compared to IgG, possibly because the secretory component of IgA makes the immunoglobulin more resistant to proteases (Snoeck et al., 2006). Secretory IgA coats commensal bacteria in the GIT of calves (Tsuruta et al., 2012; Fouhse et al., 2017) and the oral microbiota coated with IgA had a greater resemblance to whole rumen microbiota than non-coated oral microbiota, suggesting salivary IgA-coating may be a host-derived mechanism impacting commensal colonization (Fouhse et al., 2017). This contrasts somehow with earlier works conducted on specific rumen microbes for the development of vaccines, which suggest that salivary IgA inhibit certain microbes. However, a recent work supports the hypothesis of Fouhse et al. (2017) by showing that specific immune recognition by IgA

facilitated bacterial adherence to intestinal epithelial cells that mediates stable colonization (Donaldson et al., 2018). This suggests that in addition to its role in pathogen clearance, IgA can drive commensal microbes' acceptance for colonization. This, however, remains to be demonstrated in early-life colonization of the rumen.

Colonization of the rumen induces also a liver response with transcription of genes that can be associated with the development of tolerance to symbiotic microbes (Li et al., 2019). Following the transfer of rumen fluid from an adult cow into lactating calves, the main upregulated genes in the host's liver were linked to immune response, anti-inflammatory response and cell signalling (Li et al., 2019).

2.3 Weaning and stabilization of the rumen microbiota

Important questions addressed in studies on the developing rumen are: what are the main driving factors for a healthy and functional microbiota at weaning (age, pre-weaning diet, late weaning) and at what age the rumen microbiota stabilize and can be considered mature? The weaning transition under standard farm management practices could be a stressful period that reduces intake and growth. The developing rumen of a pre-weaned calf contains the same dominant phyla, Bacteroidetes, Firmicutes and Proteobacteria, as the postweaned rumen, although the relative abundances of these phyla vary with age and stage of development (Li et al., 2012; Jami et al., 2013; Meale et al., 2016; Dias et al., 2018). Generally, the relative abundance of Firmicutes increases after weaning, while that of *Bacteroidetes* declines (Jami et al., 2013; Meale et al., 2016, 2017b). However, the precise evolution with time of these phyla as well as the successions of bacterial families or genera may be different depending on the type of ruminant and there are even differences between studies. The microbial successions also fluctuate depending on management practices, age at weaning or the nature of the feed (Meale et al., 2016, 2017a,b). For example, β-diversity of ruminal microbiota shifted rapidly in early weaned calves (6 weeks), whereas, a more gradual shift was observed in late-weaned calves (8 weeks) (Meale et al., 2017b). The authors concluded that the gradual increase in solid feed consumption of late-weaned calves resulted probably in lesser physiological stress during the weaning transition than that observed in early weaned calves. Also, feeding starter concentrate in addition to milk to pre-weaned calves promoted greater diversity of bacterial taxa known to degrade readily fermentable carbohydrates in the rumen (e.g. *Megasphaera, Sharpea*, and *Succinivribrio*) and favoured *Methanosphaera* instead of *Methanobrevibacter* (Dias et al., 2018). In this last study, the relative abundance of fungi did not change significantly with diet or age, maybe due to high inter-animal variation and low fibre content of the diet. In goats, recent

work showed that the rumen microbial community and the metabolome before and after weaning are clearly different illustrating the impact of the diet (Abecia et al., 2018). Also in goats, variability and instability in the composition of the methanogen community were observed according to the change of diet and age, and stabilization appears to occur at weaning (Wang et al., 2017b). Weaning seems to be a turning point for the rumen with a microbiota structure that resembles that of adult ruminants. Notwithstanding, at lower taxonomical level many microbial taxa differed (Dill-McFarland et al., 2017). The transition from weaning to an adult-like microbiota between weaning can take several months and up to one year of age (Fonty et al., 1988; Dill-McFarland et al., 2017).

2.4 Microbial activities and functions

Most studies to date have used 16S (18S/ITS) rDNA amplicon sequencing for monitoring the rumen microbial colonization. It is important to complete these studies by measuring microbial activities or enumerating functional microbial species to ascertain the establishment of feed-degrading and other functional activities. As already mentioned, cellulolytic bacteria and methanogens are present in the rumen of 1–2-day-old ruminants (Fonty et al., 1987; Guzman et al., 2015). Enzymatic activities critical for utilization of feed nutrients such as plant fibre, proteins and starch are detected in the rumen from the first day of life (Rey et al., 2012; Jiao et al., 2015). These activities increase throughout the first week showing maximal specific activity at around one month of age, which is after the initial stage of microbial colonization is achieved (Jiao et al., 2015). These changes may partially reflect dietary transitions from colostrum to milk or milk replacer, and then to a progressive consumption of solid feeds. As indicated above, methanogenic activity can be measured as early as 2–3 days of life (Morvan et al., 1994; Friedman et al., 2017). The establishment of hydrogenotrophic activity in the rumen of lambs, investigated by culture and isotope-labelling experiments, appeared also to be sequential. Hydrogen-dependent acetogenesis was present in the rumen of 20 h-old lambs while hydrogenotrophic methanogenesis was detected from 30 h of life. Hydrogen-utilizing sulphate-reducing bacteria established by the third day after birth (Morvan et al., 1994). The colonization of reductive acetogens after birth was also explored analysing the presence of genes acetyl-CoA synthase and formyltetrahydrofolate synthetase in the immature lamb rumen. Potential acetogens identified were affiliated with the *Blautia* genus and the Lachnospiraceae family, whereas establishment of methanogenic activity did not substantially affect acetogen diversity in these lambs (Gagen et al., 2012). The functional diversity of the rumen microbiome of the pre-ruminant calf was also explored through metagenomics (Li et al., 2012). More than 8000 putative

Pfam protein families were detected in the rumen of 14-day old calves with up to 60 glycosyl hydrolase families identified, indicating a high metabolic potential for carbohydrate processing long before weaning (Li et al., 2012). An increase in carbohydrate metabolism from 5 to 9 weeks of age in calves inferred from 16S rRNA gene sequences was also suggested (Meale et al., 2017b), although the use of functional metabolic predictions based on taxonomic information should be interpreted with prudence (Vieira-Silva et al., 2016).

2.5 Modes of transmission

It has been conventionally considered that the uterus is a sterile environment and microbial inoculation of the newborn's gut starts immediately after birth, through the vaginal canal, faecal material, colostrum, skin and saliva of the dam as well as from the environment. However, bacterial and archaeal DNA has been detected in the rumen, intestines and meconium of calves as early as 20 min after birth (Guzman et al., 2015), which led the authors to suggest that inoculation of the rumen may occur before birth. Studies on human and on mice newborns suggest that colonization of the intestine in these species starts in utero (Ihekweazu and Versalovic, 2018). This has led to investigations in ruminants with contrasting results. Some studies suggest that the colonization of the ruminant intestine may begin before birth (Alipour et al., 2018) but negative results were reported by others (Malmuthuge and Griebel, 2018). Caution should be exerted when interpreting results from humans or other animals as the structure of the placenta and the maternal-foetal interaction differ greatly among mammals, with ruminants having the most complete type of placental barrier (Benirschke et al., 2012). Another caveat is contamination when analysing this kind of samples with low microbial numbers (Malmuthuge and Griebel, 2018). The information available is not enough to confirm or disprove the in utero transfer of microbes into the gut in ruminants.

Whether or not the bacterial colonization of the digestive tract begins before birth, the relative contribution of all the other sources of inoculation is not well known. It is well established that the dam is an important source of microbial inoculation of the newborn rumen. Indeed, in newborns lambs separated from their dams and reared individually, no cellulolytic bacteria, fungi and protozoa established in the rumen (Fonty et al., 1988). Also, similarities between rumen methanogen populations found in newborn twin lambs suggested that the dam was the most important source of inoculation (Skillman et al., 2004). The dam's oral microbiota may be particularly important because of rumination, which would facilitate the oral transfer of rumen microorganisms to the newborn as a result of the natural instinct to lick them after birth. Colostrum and milk are also a source of bacterial colonization of the newborn gut. As for monogastric animals, liquid feeds in suckling ruminants go directly to the true stomach,

the abomasum, through the oesophageal groove. However, the groove is not completely hermetic and small amounts of milk are also found in the rumen where taxa able to utilize milk nutrients such as *Bacteroides* and *Lactobacillus* are dominant in suckling calves fed milk only (Dias et al., 2018). The ability to grow on milk lactose by *F. succinogenes* can explain the active presence of this cellulolytic bacterium in pre-weaning calves (Ghali et al., 2017). The importance of the microbiota from different body sites of the dam to seed the newborn's gastrointestinal tract was explored recently (Alipour et al., 2018; Yeoman et al., 2018). The oral microbiota of the dam most closely resembled the rectal microbiota of the newborn calf in the study of Alipour et al. (2018). Whereas, in the study of Yeoman et al. (2018) the udder skin microbiota of the dam shared the largest number of OTUs with both luminal and mucosal microbiota of the calves from day 1 to 21 as compared with colostrum or vaginal samples. Surprisingly, fibrolytic bacteria and methanogenic archaea sequences were identified in the cow vaginal samples, suggesting a colonization role during birth (Yeoman et al., 2018). This is in addition to the important role that the dam vaginal microbiota has in colonizing the upper respiratory tract of the offspring (Lima et al., 2019).

2.6 Impact of management practices

Assuming that a key turning point in rumen microbial colonization is the introduction of solid feed in the diet, an important issue to address is whether the feeding management of the newborn alters the colonization pattern. Few studies have compared the microbial colonization of the undeveloped rumen in the context of the factors that facilitate (or prevent) the colonization of some microbial groups (i.e. maternal influence, offspring reared in isolation, use of microbial modulators, etc.).

As presented above, the dam has an important influence in early colonization. The maternal influence in relation to management systems has been further addressed in recent studies (Abecia et al., 2014b, 2017, 2018; Belanche et al., 2015; Yeoman et al., 2018). Two main systems exist for rearing offspring in ruminant production. In commercial dairy systems, newborns are typically separated from the dam after birth and fed either milk replacer or whole milk. In contrast, in beef and extensive production systems, the offspring remains with the dam until weaning. These two systems, therefore, imply differences in regards to milk type (whole milk vs. milk replacer) and contact or not with adult conspecifics that in many cases are confounded. Protozoa in artificially reared animals showed a different colonization pattern as compared to those raised by the dams (Abecia et al., 2014a; Belanche et al., 2015). Natural milk feeding via the dam vs. artificial feeding with milk replacer resulted in consistently lower pH in the developing rumen of goat kids that stayed with

the mothers (Abecia et al., 2014a). The authors hypothesized that naturally raised kids would have consumed more concentrate at an earlier stage as a result of social feeding learning as also shown by De Paula Vieira et al. (2012), who observed that the presence of an older companion with pre-weaned calves stimulated feeding behaviour and growth before and after weaning. In addition to learning behaviour, the presence of older conspecifics influences the rumen microbiota as dam-reared goat kits showed substantially greater bacterial diversity throughout the colonization process than those artificially reared (Abecia et al., 2017). Fibrobacteres (one of the main cellulolytic phyla in the rumen) was present in naturally raised kids from day 7 after birth; however, it did not colonize the rumen of kids kept in isolation from dams until day 28, similarly to *Succiniclasticum* which ferment succinate to propionate. Also, greater VFA concentration was observed in the rumen of kids with dams, which may suggest a quicker establishment of microbial fermentation in these animals (Abecia et al., 2014a). However, it remains unknown whether this distinct microbial colonization between animals, reared naturally or artificially, may have effects on the animal digestive performance later in life. The effect of the dam in the colonization after birth deserves further research as it could help to develop new inoculation strategies in livestock farming.

3 Modulating the gastrointestinal microbiota in young ruminants for health and production

As stated in previous sections, a number of factors influence the GIT microbial colonization in young ruminants. Among them, the type of production system, farm management and diet are the most important. Good management practices normally recommended to reduce the onset of diseases and promote a vigorous growth in the first week of life have an obvious impact on the GIT microbiota. For instance, feeding high-quality colostrum allows establishing a beneficial, mucosa-associated bacterial barrier and thus provides protection against enteric infections in young ruminants (Malmuthuge et al., 2015). Colostrum favours the development of beneficial *Bifidobacterium* whereas it reduces potential pathogens and members of *Escherichia* and *Shigella* genera in the colon (Song et al., 2019). Suckling from the dam and contact with conspecific adults is the best way to ensure the natural acquisition of a functional GIT microbiota in early life. However, this otherwise natural practice is not always applicable under current production systems and nutritional interventions in early life may be considered to facilitate (or prevent) the colonization of some microbial groups. In some particular cases, contact with the dam might not be recommended if there is a risk of transmission of non-desirable microbial populations. The risk of transmission of *Mycobacterium avium* subsp. *paratuberculosis*, the causative agent of Johne's disease,

increased with the number of days that newborns remained with affected adults, particularly in the first week of life (Burgess et al., 2018). Inversely, it is reasonable to hypothesize that the contact with some animals should be promoted for facilitating the transmission of microbiotas associated to desired phenotypes such as reduced enteric methane emission or improved feed efficiency (Shabat et al., 2016; Difford et al., 2018).

Diet is a well-known modulator of the GIT microbiota at all ages but it seems to have a particular influencing role in the development of the microbiota and the microbiota-host dialogue in young ruminants (Bach et al., 2017; Dill-McFarland et al., 2019; Dong et al., 2019). Differences in milk processing that can be considered minor from a macronutrient standpoint had large consequences on some gut microbial populations and inflammation parameters. As opposed to heat-treated milk (pasteurized and UHT), raw milk increased *Lactobacillus* numbers, decreased the expression of pro-inflammatory interleukins and improved growth in dairy calves (Bach et al., 2017).

In addition to the management and diet factors cited, two main nutritional interventions in early life may be considered to modulate the GIT microbiota. These are the direct inoculation of specific microorganisms and the use of compounds (i.e. additives) that prevent or facilitate the colonization of some microbial groups. These two strategies are mainly tested for improving phenotypes of interest.

4 Case studies: early-life strategies for improving health and production

Feeding live microorganisms to ruminants is not a novel concept and extensive work has been published on the use of direct-fed microbials (DFM) (Martin and Nisbet, 1992; Jeyanathan et al., 2014). Theodorou et al. (1990) reported that the addition of an anaerobic rumen fungus, *Neocallimastix* spp., increased intake and live weight gain in calves at weaning, while (Ziolecka et al., 1984; Ziolecki et al., 1984) reported that a stabilized rumen extract enhanced live weight gain and stimulated rumen development in calves during weaning. The use of fresh rumen fluid inoculated in early life has gained new attention. Most recent studies showed that inoculation of fresh rumen fluid from adults into the rumen of newborns has a positive effect on productive parameters (Zhong et al., 2014; De Barbieri et al., 2015b). After weaning, average daily gain, intake, digestibility and rumen development improved in treated animals (lambs and kids) as compared to non-treated. Some of these effects may not last, although the composition of ruminal bacterial communities still differed up to 5 months of age, well after the initial inoculation (De Barbieri et al., 2015a,b).

For specific DFM preparations, the administration of lactic acid bacteria to suckling calves was shown to stimulate rumination and ruminal development

(Nakanishi et al., 1993) and the use of lactic acid bacteria or *Bifidobacterium* increased feed conversion efficiency and body weight gain (Abe et al., 1995). In this latter work, the DFMs decreased diarrhoea. The effect of lactic acid bacteria DFM on the prevalence of diarrhoea was confirmed in a meta-analysis (Signorini et al., 2012). Interestingly, the protection was only observed with multi-strains DFM and for calves fed raw milk. All these effects of lactic acid bacteria can be partially ascribed to the positive modulating activities of these bacterial groups on host immunology as described in the subsection interaction host-microbiota (see above). The rumen bacterium *Megasphaera elsdenii* uses lactate to produce butyrate, a beneficial energy compound used by the GIT epithelial mucosa. This bacterium was also used as DFM in young ruminant with contrasting results. Muya et al. (2015) showed improved feed intake and rumen development, suggesting increased epithelium metabolism and improved absorption of digestive end products. Whereas, no effect was observed in another study using the same strain and a similar protocol (Yohe et al., 2018).

Diarrhoea is one of the main health issues in pre-weaned ruminants, particularly in dairy (USDA, 2018). A low faecal microbial diversity, notably a low relative abundance of *Faecalibacterium* spp., a symbiotic bacterium with known anti-inflammatory activity, was associated with an increased incidence of diarrhoea (Oikonomou et al., 2013). Inversely, *Faecalibacterium* spp. is positively associated with weight gain. Substantiating these effects, the use of *Faecalibacterium prausnitzii* as DFM in dairy calves decreased the incidence of severe diarrhoea by half and mortality by two thirds as compared to untreated controls (Foditsch et al., 2015).

Another strategy gaining attention is the use of life yeasts in early life. Although yeasts are widely used in ruminant nutrition (Chaucheyras-Durand et al., 2012), the concept of applying them in the diet of pre-ruminants to alter microbial colonization is more novel. It has been showed that yeasts, particularly as DFM, have positive effects on growth, rumen and small intestine development, immunity and general health of the calf (Kim et al., 2011; Alugongo et al., 2017). Specifically, *Saccharomyces cerevisiae* yeast (SCY) can improve DMI, growth, feed efficiency (Lesmeister and Heinrichs, 2004; Alugongo et al., 2017) and reduce diarrhoea in calves (Galvao et al., 2005; Brewer et al., 2014). Furthermore, subtle improvements were seen in rumen fermentation (increased butyrate production) and rumen papillae growth (Lesmeister and Heinrichs, 2004). These positive results, however, are more pronounced in calves stressed or exposed to significant levels of disease-causing agents. Despite the increasing number of published works studying the use of SCY in early life of ruminants, there is a lack of knowledge about its impact on the microbial colonization. Recently, Terré et al. (2015) reported that supplementing young male calves with live SCY increased the presence of the fibrolytic bacterium *R. albus* in the

rumen and it also increased rumen pH but no effect was observed on other bacteria and protozoa. Supplementation of newborn lambs fed milk replacer by live yeasts was also shown to increase the colonization of the rumen by *F. succinogenes*, fungi and protozoa (Chaucheyras-Durand et al., 2019). Other reported effects of SCY are an increase in *Lactobacillus* in faeces (Fomenky et al., 2017) and greater oxidative burst and phagocytosis activity during weaning stress indicating a stronger innate immune response (Fomenky et al., 2018). Generally, for all DFM and the engraftment of adult rumen microbiota, there is still a need for further research in whole microbiome analysis and areas such as gut immunity and the persistence of effects later in life, especially in dairy systems due to their management of newborns. This information is necessary to draw consistent conclusions on their potential in early life.

5 Case studies: early-life strategies for reducing enteric methane emissions

Modulating the microbial colonization in the developing rumen towards communities that produce less methane is also gaining attention. For this objective, compounds used as feed additives or feed supplements have been tested with two different approaches: one that specifically targets methanogens and the other uses general inhibitors that were described as having various mechanisms of action. For the specific approach, the application of bromochloromethane to young goat kids modified archaeal colonization of the rumen, which was linked to a reduction in methane emission of around 50%. The effects persisted for 3 months after cessation of treatment in kids raised by does that received the same treatment as the kids (Abecia et al., 2013, 2014a). For the generalist modulation approach, different types of lipids and plant extracts with proven efficacy to alter microbial metabolism and methane emissions in adult animals were tested. Debruyne et al. (2018) tested in goats prenatal and postnatal supplementation with coconut oil medium chain fatty acids. The treatment reduced in vitro methane emissions in four-week-old kids by reducing methanogen abundance and activity but at the expense of rumen fermentation and eubacterial abundance. Unfortunately, the treatment also suppressed daily gain of treated kids. Some rumen papillae characteristics differed at 28 weeks old due to postnatal treatment which ended at week 11 of life, indicating rumen papillary development can be affected by the early-life nutritional management. Saro et al. (2018) tested a combination of garlic oil and linseed oil in early life of lambs and the potential impact of re-treating the animals later in life. The archaeal methanogenic community was modified during the treatment but, in accordance with methane emissions, differences with the control group disappeared afterwards. In contrast, bacterial community structure differed

between treated and non-treated lambs during and after the intervention. Rumen and urine metabolomics profiles conducted after the intervention ceased, highlighted interactions between microbes and metabolites, notably that of methylated compounds and Methanomassiliicocceae methanogens. They demonstrated that a long-term early-life intervention induced modifications in the composition of the rumen bacterial community that persisted after the intervention ceased with little or no effect on archaeal and protozoal communities. However, there was no persistence of the early-life intervention on methanogenesis suggesting a notable resilience for this function. Similarly, Lyons et al. (2017) treated lambs with linseed oil and found persistence of differences in the bacterial community structure but not associated changes in phenotypic response.

Methanogens occupy a specific niche in the rumen ecosystem. When methanogens are inhibited, there is a shift in hydrogen flow and other electron-donor metabolites towards alternative electron acceptors such as propionate. This implies the rearrangement of trophic networks and changes in microbial populations that are mainly observed for the bacterial communities in all studies cited above. The abundance of methanogens was not always affected but their diversity was commonly altered although the changes were different probably due to the type of inhibitor used. Linseed and garlic oil decreased the Methanomassiliicoccales relative abundance in lambs (Saro et al., 2018). Bromochloromethane increased the relative abundance of *Methanobrevibacter* and decreased that of *Methanosphaera* in kids (Abecia et al., 2014b). Whereas, linseed increased *Methanosphaera* relative abundance in lambs (Lyons et al., 2017).

6 Conclusion and future trends

From birth, ruminants are constantly exposed to different sources of microbes. The establishment of stable communities depends on the creation of suitable conditions with a close interplay between diet and maturation of the GIT. The sequential evolution of the gut microbiota that is followed by the expansion of metabolic functions is a coordinated process that matches the anatomical and physiological development of the GIT. In the non-ruminant phase (0–3 weeks), the establishment of a diverse microbial community, particularly bacteria in the post-gastric section, is important for health and grow (Oikonomou et al., 2013; Bach et al., 2017), whereas changes in the rumen are predominant during the transition (3–8 weeks) and rumination phases (+8 weeks of age) (Jiao et al., 2015). The periods when these alimentary, anatomical and physiological modifications occur seem to be the more suitable for interventions for long-term modifications of the GIT microbiota (Dill-McFarland et al., 2019).

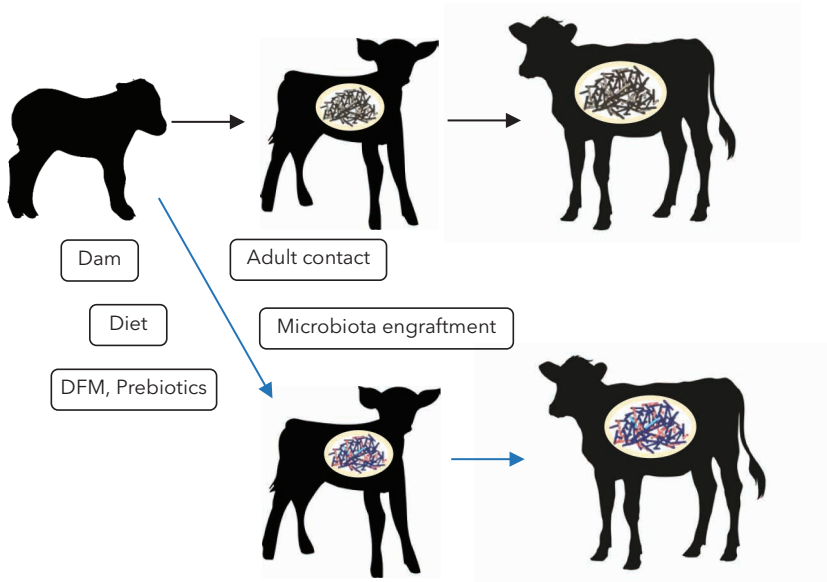

Figure 2 Acquisition of gastrointestinal microbiota in early life could be modulated (blue arrows) through management and targeted interventions to favour health and phenotypes of interest such as increased feed efficiency and decreased methane emission. Modulating options are: allowing contact with dams or mature conspecific harbouring a desirable microbiota, inoculation with gastrointestinal contents (engraftment) obtained from mature conspecific, diet and supplementation with direct-fed microbials (DFM), prebiotics and feed additives.

The GIT microbiota at an early age is influenced and can be modulated by various strategies (Fig. 2). Nevertheless, for the application of these strategies in farms there are still some outstanding questions that need to be addressed. Ongoing research on the role of GIT microbiota on animal phenotypes will bring information on the type of microbial communities that should be favoured in young ruminants. The effect of the prenatal period, including whether there is a prenatal colonization of the GIT and its role in the establishment of the mature microbiota have to be assessed. Similarly, the best window(s) of age when the microbiota could be modulated have to be refined. Establishment of different populations might not be a single isolated event and a continuous exposure seems to be necessary for acquiring adult-like communities (Morgavi et al., 2015) or successfully engraft specific microbes (Yohe et al., 2018). A better definition of the role of early microbial communities on the host-microbiota dialogue and the regulation of the immune system is also important for this subject. Expected advances in all these topics in the coming years have the potential to improve the sustainability of ruminant production through better health in young animals and enhanced efficiency.

7 Where to look for further information

7.1 Key conferences

- Gut Microbiology Symposium; jointly organized by INRA France and the Rowett Institute – Aberdeen University, every two years.
- Congress on Gastrointestinal Function; in Chicago (USA) every two years.
- Annual meetings from animal science societies such as Annual Meeting of the European Federation of Animal Science and American Dairy Science Association.
- Smart Calve rearing Conference (https://smart-calf-rearing.com).

7.2 Major international research projects and networks

- Rumen Microbial Genomics Network, Global Research Alliance-Livestock Group. A large international network fostering collaborations and exchanges between microbiologists working with ruminants (https://globalresearchalliance.org/research/livestock/networks/rumen-microbial-genomics-network/).
- MASTER (Microbiome Applications for Sustainable food systems through Technologies and EnteRprise) a H2020 project focussing on the characterization and modulation of microbiomes from different environments including the GIT of ruminants (http://www.master-h2020.eu/).

8 References

Abe, F., Ishibashi, N. and Shimamura, S. 1995. Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. *J. Dairy Sci.* 78(12), 2838–46. doi:10.3168/jds.S0022-0302(95)76914-4.

Abecia, L., Martín-García, A. I., Martínez, G., Newbold, C. J. and Yáñez-Ruiz, D. R. 2013. Nutritional intervention in early life to manipulate rumen microbial colonization and methane output by kid goats postweaning. *J. Anim. Sci.* 91(10), 4832–40. doi:10.2527/jas.2012-6142.

Abecia, L., Ramos-Morales, E., Martínez-Fernandez, G., Arco, A., Martín-García, A. I., Newbold, C. J. and Yáñez-Ruiz, D. R. 2014a. Feeding management in early life influences microbial colonisation and fermentation in the rumen of newborn goat kids. *Anim. Prod. Sci.* 54(9), 1449–54. doi:10.1071/AN14337.

Abecia, L., Waddams, K. E., Martinez-Fernandez, G., Martin-Garcia, A. I., Ramos-Morales, E., Newbold, C. J. and Yanez-Ruiz, D. R. 2014b. An antimethanogenic nutritional intervention in early life of ruminants modifies ruminal colonization by Archaea. *Archaea* 2014, 841463. doi:10.1155/2014/841463.

Abecia, L., Jiménez, E., Martínez-Fernandez, G., Martín-García, A. I., Ramos-Morales, E., Pinloche, E., Denman, S. E., Newbold, C. J. and Yáñez-Ruiz, D. R. 2017. Natural

and artificial feeding management before weaning promote different rumen microbial colonization but not differences in gene expression levels at the rumen epithelium of newborn goats. *PLoS ONE* 12(8), e0182235. doi:10.1371/journal.pone.0182235.

Abecia, L., Martínez-Fernandez, G., Waddams, K., Martín-García, A. I., Pinloche, E., Creevey, C. J., Denman, S. E., Newbold, C. J. and Yáñez-Ruiz, D. R. 2018. Analysis of the rumen microbiome and metabolome to study the effect of an antimethanogenic treatment applied in early life of kid goats. *Front. Microbiol.* 9, 2227. doi:10.3389/fmicb.2018.02227.

Alipour, M. J., Jalanka, J., Pessa-Morikawa, T., Kokkonen, T., Satokari, R., Hynonen, U., Iivanainen, A. and Niku, M. 2018. The composition of the perinatal intestinal microbiota in cattle. *Sci. Rep.* 8(1), 10437. doi:10.1038/s41598-018-28733-y.

Alugongo, G. M., Xiao, J. X., Wu, Z. H., Li, S. L., Wang, Y. J. and Cao, Z. J. 2017. Review: Utilization of yeast of *Saccharomyces cerevisiae* origin in artificially raised calves. *J. Anim. Sci. Biotechnol.* 8, 34. doi:10.1186/s40104-017-0165-5.

Bach, A., Aris, A., Vidal, M., Fàbregas, F. and Terré, M. 2017. Influence of milk processing temperature on growth performance, nitrogen retention, and hindgut's inflammatory status and bacterial populations in a calf model. *J. Dairy Res.* 84(3), 355–9. doi:10.1017/S0022029917000401.

Belanche, A., de la Fuente, G. and Newbold, C. J. 2015. Effect of progressive inoculation of fauna-free sheep with holotrich protozoa and total-fauna on rumen fermentation, microbial diversity and methane emissions. *FEMS Microbiol. Ecol.* 91(3). doi:10.1093/femsec/fiu026.

Benirschke, K., Burton, G. J. and Baergen, R. N. 2012. *Pathology of the Human Placenta*. Springer Science & Business Media, Heidelberg.

Brewer, M. T., Anderson, K. L., Yoon, I., Scott, M. F. and Carlson, S. A. 2014. Amelioration of salmonellosis in pre-weaned dairy calves fed *Saccharomyces cerevisiae* fermentation products in feed and milk replacer. *Vet. Microbiol.* 172(1–2), 248–55. doi:10.1016/j.vetmic.2014.05.026.

Burgess, T. L., Witte, C. L. and Rideout, B. A. 2018. Early-life exposures and Johne's disease risk in zoo ruminants. *J. Vet. Diagn. Invest.* 30(1), 78–85. doi:10.1177/1040638717735350.

Chaucheyras-Durand, F., Chevaux, E., Martin, C. and Forano, E. 2012. Use of yeast probiotics in ruminants: effects and mechanisms of action on rumen pH, fibre degradation, and microbiota according to the diet. In: Rigobelo, E. (Ed.), *Probiotic in Animals*. Intech Europe, Rijeka, Croatia, pp. 119–52.

Chaucheyras-Durand, F., Ameilbonne, A., Auffret, P., Bernard, M., Mialon, M. M., Duniere, L. and Forano, E. 2019. Supplementation of live yeast based feed additive in early life promotes rumen microbial colonization and fibrolytic potential in lambs. *Sci. Rep.* 9, 19216. doi:10.1038/s41598-019-55825-0.

Chen, Y., Oba, M. and Guan, L. L. 2012. Variation of bacterial communities and expression of toll-like receptor genes in the rumen of steers differing in susceptibility to subacute ruminal acidosis. *Vet. Microbiol.* 159(3–4), 451–9. doi:10.1016/j.vetmic.2012.04.032.

Collado, M. C., Cernada, M., Baüerl, C., Vento, M. and Pérez-Martínez, G. 2012. Microbial ecology and host-microbiota interactions during early life stages. *Gut Microbes* 3(4), 352–65. doi:10.4161/gmic.21215.

Corthésy, B., Gaskins, H. R. and Mercenier, A. 2007. Cross-talk between probiotic bacteria and the host immune system. *J. Nutr.* 137, 781S–90S. doi:10.1093/jn/137.3.781S.

Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J. and Relman, D. A. 2012. The application of ecological theory toward an understanding of the human microbiome. *Science* 336(6086), 1255–62. doi:10.1126/science.1224203.

De Barbieri, I., Hegarty, R. S., Silveira, C., Gulino, L. M., Oddy, V. H., Gilbert, R. A., Klieve, A. V. and Ouwerkerk, D. 2015a. Programming rumen bacterial communities in newborn Merino lambs. *Small Rumin. Res.* 129, 48–59. doi:10.1016/j.smallrumres.2015.05.015.

De Barbieri, I., Hegarty, R. S., Silveira, C. and Oddy, V. H. 2015b. Positive consequences of maternal diet and post-natal rumen inoculation on rumen function and animal performance of Merino lambs. *Small Rumin. Res.* 129, 37–47. doi:10.1016/j.smallrumres.2015.05.017.

Debruyne, S., Ruiz-Gonzalez, A., Artiles-Ortega, E., Ampe, B., Van Den Broeck, W., De Keyser, E., Vandaele, L., Goossens, K. and Fievez, V. 2018. Supplementing goat kids with coconut medium chain fatty acids in early life influences growth and rumen papillae development until 4 months after supplementation but effects on in vitro methane emissions and the rumen microbiota are transient. *J. Anim. Sci.* 96(5), 1978–95. doi:10.1093/jas/sky070.

De Paula Vieira, A., von Keyserlingk, M. A. G. and Weary, D. M. 2012. Presence of an older weaned companion influences feeding behavior and improves performance of dairy calves before and after weaning from milk. *J. Dairy Sci.* 95(6), 3218–24. doi:10.3168/jds.2011-4821.

Dias, J., Marcondes, M. I., Motta de Souza, S., Cardoso da Mata e Silva, B., Fontes Noronha, M., Tassinari Resende, R., Machado, F. S., Cuquetto Mantovani, H., Dill-McFarland, K. A. and Suen, G. 2018. Bacterial community dynamics across the gastrointestinal tracts of dairy calves during preweaning development. *Appl. Environ. Microbiol.* 84(9). doi:10.1128/AEM.02675-17.

Difford, G. F., Plichta, D. R., Lovendahl, P., Lassen, J., Noel, S. J., Hojberg, O., Wright, A. G., Zhu, Z. G., Kristensen, L., Nielsen, H. B., Guldbrandtsen, B. and Sahana, G. 2018. Host genetics and the rumen microbiome jointly associate with methane emissions in dairy cows. *PLoS Genet.* 14(10), e1007580. doi:10.1371/journal.pgen.1007580.

Dill-McFarland, K. A., Breaker, J. D. and Suen, G. 2017. Microbial succession in the gastrointestinal tract of dairy cows from 2 weeks to first lactation. *Sci. Rep.* 7, 40864. doi:10.1038/srep40864.

Dill-McFarland, K. A., Weimer, P. J., Breaker, J. D. and Suen, G. 2019. Diet influences early microbiota development in dairy calves without long-term impacts on milk production. *Appl. Environ. Microbiol.* 85(2), e02141-02118. doi:10.1128/AEM.02141-18.

Donaldson, G. P., Ladinsky, M. S., Yu, K. B., Sanders, J. G., Yoo, B. B., Chou, W. C., Conner, M. E., Earl, A. M., Knight, R., Bjorkman, P. J. and Mazmanian, S. K. 2018. Gut microbiota utilize immunoglobulin A for mucosal colonization. *Science* 360(6390), 795–800. doi:10.1126/science.aaq0926.

Dong, L.-f., Ma, J.-N., Tu, Y. and Diao, Q.-Y. 2019. Weaning methods affect ruminal methanogenic archaea composition and diversity in Holstein calves. *J. Integr. Agric.* 18(5), 1080–92. doi:10.1016/S2095-3119(18)62120-3.

Foditsch, C., Pereira, R. V., Ganda, E. K., Gomez, M. S., Marques, E. C., Santin, T. and Bicalho, R. C. 2015. Oral administration of *Faecalibacterium prausnitzii* decreased the incidence of severe diarrhea and related mortality rate and increased weight

gain in preweaned dairy heifers. *PLoS ONE* 10(12), e0145485. doi:10.1371/journal.pone.0145485.

Fomenky, B. E., Chiquette, J., Bissonnette, N., Talbot, G., Chouinard, P. Y. and Ibeagha-Awemu, E. M. 2017. Impact of *Saccharomyces cerevisiae boulardii* CNCMI-1079 and *Lactobacillus acidophilus* BT1386 on total lactobacilli population in the gastrointestinal tract and colon histomorphology of Holstein dairy calves. *Anim. Feed Sci. Tech.* 234, 151–61. doi:10.1016/j.anifeedsci.2017.08.019.

Fomenky, B. E., Chiquette, J., Lessard, M., Bissonnette, N., Talbot, G., Chouinard, Y. P. and Ibeagha-Awemu, E. M. 2018. *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 and *Lactobacillus acidophilus* BT1386 influence innate immune response and serum levels of acute-phase proteins during weaning in Holstein calves. *Can. J. Anim. Sci.* 98(3), 576–88. doi:10.1139/cjas-2017-0120.

Fonty, G., Gouet, P., Jouany, J. P. and Senaud, J. 1983. Ecological factors determining establishment of cellulolytic bacteria and protozoa in the rumens of meroxenic lambs. *J. Gen. Microbiol.* 129(1), 213–23. doi:10.1099/00221287-129-1-213.

Fonty, G., Gouet, P., Jouany, J.-P. and Senaud, J. 1987. Establishment of the microflora and anaerobic fungi in the rumen of lambs. *J. Gen. Microbiol.* 133(7), 1835–43. doi:10.1099/00221287-133-7-1835.

Fonty, G., Senaud, J., Jouany, J. P. and Gouet, P. 1988. Establishment of ciliate protozoa in the rumen of conventional and conventionalized lambs: influence of diet and management conditions. *Can. J. Microbiol.* 34(3), 235–41. doi:10.1139/m88-044.

Fouhse, J. M., Smiegielski, L., Tuplin, M., Guan, L. L. and Willing, B. P. 2017. Host immune selection of rumen bacteria through salivary secretory IgA. *Front. Microbiol.* 8, 848. doi:10.3389/fmicb.2017.00848.

Friedman, N., Jami, E. and Mizrahi, I. 2017. Compositional and functional dynamics of the bovine rumen methanogenic community across different developmental stages. *Environ. Microbiol.* 19(8), 3365–73. doi:10.1111/1462-2920.13846.

Gagen, E. J., Mosoni, P., Denman, S. E., Jassim, R. Al., McSweeney, C. S. and Forano, E. 2012. Methanogen colonisation does not significantly alter acetogen diversity in lambs isolated 17 h after birth and raised aseptically. *Microb. Ecol.* 64(3), 628–40 doi:10.1007/s00248-012-0024-z.

Galvao, K. N., Santos, J. E. P., Coscioni, A., Villasenor, M., Sischo, W. M. and Berge, A. C. B. 2005. Effect of feeding live yeast products to calves with failure of passive transfer on performance and patterns of antibiotic resistance in fecal *Escherichia coli*. *Reprod. Nutr. Dev.* 45(4), 427–40. doi:10.1051/rnd:2005040.

Ghali, I., Sofyan, A., Ohmori, H., Shinkai, T. and Mitsumori, M. 2017. Diauxic growth of *Fibrobacter succinogenes* S85 on cellobiose and lactose. *FEMS Microbiol. Lett.* 364(15), 9. doi:10.1093/femsle/fnx150.

Guzman, C. E., Bereza-Malcolm, L. T., De Groef, B. and Franks, A. E. 2015. Presence of selected methanogens, fibrolytic bacteria, and proteobacteria in the gastrointestinal tract of neonatal dairy calves from birth to 72 hours. *PLoS ONE* 10(7), e0133048. doi:10.1371/journal.pone.0133048.

Hooper, L. V., Littman, D. R. and Macpherson, A. J. 2012. Interactions between the microbiota and the immune system. *Science* 336(6086), 1268–73. doi:10.1126/science.1223490.

Ihekweazu, F. D. and Versalovic, J. 2018. Development of the pediatric gut microbiome: impact on health and disease. *Am. J. Med. Sci.* 356(5), 413–23. doi:10.1016/j.amjms.2018.08.005.

Jami, E., Israel, A., Kotser, A. and Mizrahi, I. 2013. Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7(6), 1069–79. doi:10.1038/ismej.2013.2.

Janssen, P. H. and Kirs, M. 2008. Structure of the archaeal community of the rumen. *Appl. Environ. Microbiol.* 74(12), 3619–25. doi:10.1128/AEM.02812-07.

Jeyanathan, J., Martin, C. and Morgavi, D. P. 2014. The use of direct-fed microbials for mitigation of ruminant methane emissions: a review. *Animal* 8(2), 250–61. doi:10.1017/S1751731113002085.

Jiao, J., Li, X., Beauchemin, K. A., Tan, Z., Tang, S. and Zhou, C. 2015. Rumen development process in goats as affected by supplemental feeding v. grazing: age-related anatomic development, functional achievement and microbial colonisation. *Br. J. Nutr.* 113(6), 888–900. doi:10.1017/S0007114514004413.

Jiao, J., Zhou, C., Guan, L. L., McSweeney, C. S., Tang, S., Wang, M. and Tan, Z. 2017. Shifts in host mucosal innate immune function are associated with ruminal microbial succession in supplemental feeding and grazing goats at different ages. *Front. Microbiol.* 8, 1655. doi:10.3389/fmicb.2017.01655.

Kim, M. H., Yang, J. Y., Upadhaya, S. D., Lee, H. J., Yun, C. H. and Ha, J. K. 2011. The stress of weaning influences serum levels of acute-phase proteins, iron-binding proteins, inflammatory cytokines, cortisol, and leukocyte subsets in Holstein calves. *J. Vet. Sci.* 12(2), 151–7. doi:10.4142/jvs.2011.12.2.151.

Kuhn, K. A. and Stappenbeck, T. S. 2013. Peripheral education of the immune system by the colonic microbiota. *Semin. Immunol.* 25(5), 364–9. doi:10.1016/j.smim.2013.10.002.

Lei, Y., Zhang, K., Guo, M., Li, G., Li, C., Li, B., Yang, Y., Chen, Y. and Wang, X. 2018. Exploring the spatial-temporal microbiota of compound stomachs in a pre-weaned goat model. *Front. Microbiol.* 9, 1846. doi:10.3389/fmicb.2018.01846.

Lesmeister, K. E. and Heinrichs, A. J. 2004. Effects of corn processing on growth characteristics, rumen development, and rumen parameters in neonatal dairy calves. *J. Dairy Sci.* 87(10), 3439–50. doi:10.3168/jds.S0022-0302(04)73479-7.

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R. and Gordon, J. I. 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat. Rev. Microbiol.* 6(10), 776–88. doi:10.1038/Nrmicro1978.

Li, R. W., Connor, E. E., Li, C., Vi, R. L. B. and Sparks, M. E. 2012. Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. *Environ. Microbiol.* 14(1), 129–39. doi:10.1111/j.1462-2920.2011.02543.x.

Li, W. L., Edwards, A., Riehle, C., Cox, M. S., Raabis, S., Skarlupka, J. H., Steinberger, A. J., Walling, J., Bickhart, D. and Suen, G. 2019. Transcriptomics analysis of host liver and meta-transcriptome analysis of rumen epimural microbial community in young calves treated with artificial dosing of rumen content from adult donor cow. *Sci. Rep.* 9(1), 790. doi:10.1038/s41598-018-37033-4.

Lima, S. F., Bicalho, M. L. S. and Bicalho, R. C. 2019. The *Bos taurus* maternal microbiome: role in determining the progeny early-life upper respiratory tract microbiome and health. *PLoS ONE* 14(3), e0208014. doi:10.1371/journal.pone.0208014.

Lyons, T., Boland, T., Storey, S. and Doyle, E. 2017. Linseed oil supplementation of lambs' diet in early life leads to persistent changes in rumen microbiome structure. *Front. Microbiol.* 8, 1656. doi:10.3389/fmicb.2017.01656.

Malmuthuge, N. and Griebel, P. J. 2018. Fetal environment and fetal intestine are sterile during the third trimester of pregnancy. *Vet. Immunol. Immunopathol.* 204, 59–64. doi:10.1016/j.vetimm.2018.09.005.

Malmuthuge, N., Li, M., Fries, P., Griebel, P. J. and Guan, L. L. 2012. Regional and age dependent changes in gene expression of toll-like receptors and key antimicrobial defence molecules throughout the gastrointestinal tract of dairy calves. *Vet. Immunol. Immunopathol.* 146(1), 18–26. doi:10.1016/j.vetimm.2012.01.010.

Malmuthuge, N., Griebel, P. J. and Guan, L. 2014. Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the gastrointestinal tracts of preweaned calves. *Appl. Environ. Microbiol.* 80(6), 2021–8. doi:10.1128/AEM.03864-13.

Malmuthuge, N., Griebel, P. J. and Guan, L. 2015. The gut microbiome and its potential role in the development and function of newborn calf gastrointestinal tract. *Front. Vet. Sci.* 2, 36. doi:10.3389/fvets.2015.00036.

Martin, S. A. and Nisbet, D. J. 1992. Effect of direct-fed microbials on rumen microbial fermentation. *J. Dairy Sci.* 75(6), 1736–44. doi:10.3168/jds.S0022-0302(92)77932-6.

McGuire, K., Jones, M., Werling, D., Williams, J. L., Glass, E. J. and Jann, O. 2006. Radiation hybrid mapping of all 10 characterized bovine toll-like receptors. *Anim. Genet.* 37(1), 47–50. doi:10.1111/j.1365-2052.2005.01364.x.

Meale, S. J., Li, S., Azevedo, P., Derakhshani, H., Plaizier, J. C., Khafipour, E. and Steele, M. A. 2016. Development of ruminal and fecal microbiomes are affected by weaning but not weaning strategy in dairy calves. *Front. Microbiol.* 7, 582. doi:10.3389/fmicb.2016.00582.

Meale, S. J., Chaucheyras-Durand, F., Berends, H., Guan, L. L. and Steele, M. A. 2017a. From pre- to postweaning: transformation of the young calf's gastrointestinal tract. *J. Dairy Sci.* 100(7), 5984–95. doi:10.3168/jds.2016-12474.

Meale, S. J., Li, S. C., Azevedo, P., Derakhshani, H., DeVries, T. J., Plaizier, J. C., Steele, M. A. and Khafipour, E. 2017b. Weaning age influences the severity of gastrointestinal microbiome shifts in dairy calves. *Sci. Rep.* 7(1), 198. doi:10.1038/s41598-017-00223-7.

Moraïs, S. and Mizrahi, I. 2019. The Road Not Taken: the rumen microbiome, functional groups, and community states. *Trends Microbiol.* 27(6), 538–49. doi:10.1016/j.tim.2018.12.011.

Morgavi, D. P., Rahahao-Paris, E., Popova, M., Boccard, J., Nielsen, K. F. and Boudra, H. 2015. Rumen microbial communities influence metabolic phenotypes in lambs. *Front. Microbiol.* 6, 1060. doi:10.3389/fmicb.2015.01060.

Morvan, B., Dore, J., Rieu-Lesme, F., Foucat, L., Fonty, G. and Gouet, P. 1994. Establishment of hydrogen-utilizing bacteria in the rumen of the newborn lamb. *FEMS Microbiol. Lett.* 117(3), 249–56. doi:10.1016/0378-1097(94)90567-3.

Muya, M. C., Nherera, F. V., Miller, K. A., Aperce, C. C., Moshidi, P. M. and Erasmus, L. J. 2015. Effect of *Megasphaera elsdenii* NCIMB 41125 dosing on rumen development, volatile fatty acid production and blood beta-hydroxybutyrate in neonatal dairy calves. *J. Anim. Physiol. Anim. Nutr. (Berl)* 99(5), 913–8. doi:10.1111/jpn.12306.

Nakanishi, Y., Arave, C. W. and Stewart, P. H. 1993. Effects of feeding *Lactobacillus acidophilus* yogurt on performance and behavior of dairy calves. *J. Dairy Sci.* 76 (Suppl. 1), 244.

Oikonomou, G., Teixeira, A. G. V., Foditsch, C., Bicalho, M. L., Machado, V. S. and Bicalho, R. C. 2013. Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of Faecalibacterium species with health and growth. *PLoS ONE* 8(4), e63157. doi:10.1371/journal.pone.0063157.

Rey, M., Enjalbert, F. and Monteils, V. 2012. Establishment of ruminal enzyme activities and fermentation capacity in dairy calves from birth through weaning. *J. Dairy Sci.* 95(3), 1500–12. doi:10.3168/jds.2011-4902.

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O. and Monteils, V. 2014. Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. *J. Appl. Microbiol.* 116(2), 245–57. doi:10.1111/jam.12405.

Rieu, F., Fonty, G., Gaillard, B. and Gouet, P. 1990. Electron microscopy study of the bacteria adherent to the rumen wall in young conventional lambs. *Can. J. Microbiol.* 36(2), 140–4. doi:10.1139/m90-025.

Saro, C., Hohenester, U. M., Bernard, M., Lagrée, M., Martin, C., Doreau, M., Boudra, H., Popova, M. and Morgavi, D. P. 2018. Effectiveness of interventions to modulate the rumen microbiota composition and function in pre-ruminant and ruminant lambs. *Front. Microbiol.* 9, 1273. doi:10.3389/fmicb.2018.01273.

Savage, D. C. 1977. Microbial ecology of the gastrointestinal tract. *Annu. Rev. Microbiol.* 31, 107–33. doi:10.1146/annurev.mi.31.100177.000543.

Shabat, S. K., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., White, B. A., Shterzer, N. and Mizrahi, I. 2016. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J.* 10(12), 2958–72 doi:10.1038/ismej.2016.62.

Sharpe, M., Latham, M. and Reiter, B. 1975. The immune response of the host animal to bacteria in the rumen and caecum. In: McDonald, I. W. and dan Warner, A. C. I. (Eds), *Digestion and Metabolism in the Ruminant* (1st edn.). The University of New England, Sydney, p. 149.

Signorini, M. L., Soto, L. P., Zbrun, M. V., Sequeira, G. J., Rosmini, M. R. and Frizzo, L. S. 2012. Impact of probiotic administration on the health and fecal microbiota of young calves: a meta-analysis of randomized controlled trials of lactic acid bacteria. *Res. Vet. Sci.* 93(1), 250–8. doi:10.1016/j.rvsc.2011.05.001.

Skillman, L. C., Evans, P. N., Naylor, G. E., Morvan, B., Jarvis, G. N. and Joblin, K. N. 2004. 16S ribosomal DNA-directed PCR primers for ruminal methanogens and identification of methanogens colonising young lambs. *Anaerobe* 10(5), 277–85. doi:10.1016/j.anaerobe.2004.05.003.

Smith, H. W. 1965. The development of the flora of the alimentary tract in young animals. *J. Pathol. Bacteriol.* 90(2), 495–513. doi:10.1002/path.1700900218.

Snoeck, V., Peters, I. R. and Cox, E. 2006. The IgA system: a comparison of structure and function in different species. *Vet. Res.* 37(3), 455–67. doi:10.1051/vetres:2006010.

Song, Y., Malmuthuge, N., Li, F. Y. and Guan, L. L. 2019. Colostrum feeding shapes the hindgut microbiota of dairy calves during the first 12 h of life. *FEMS Microbiol. Ecol.* 95(1), 12. doi:10.1093/femsec/fiy203.

Subharat, S., Shu, D., Zheng, T., Buddle, B. M., Janssen, P. H., Luo, D. and Wedlock, D. N. 2015. Vaccination of cattle with a methanogen protein produces specific antibodies in the saliva which are stable in the rumen. *Vet. Immunol. Immunopathol.* 164(3–4), 201–7. doi:10.1016/j.vetimm.2015.02.008.

Teran, R., Mitre, E., Vaca, M., Erazo, S., Oviedo, G., Hübner, M. P., Chico, M. E., Mattapallil, J. J., Bickle, Q., Rodrigues, L. C. and Cooper, P. J. 2011. Immune system development during early childhood in tropical Latin America: evidence for the age-dependent down regulation of the innate immune response. *Clin. Immunol.* 138(3), 299–310. doi:10.1016/j.clim.2010.12.011.

Terré, M., Maynou, G., Bach, A. and Gauthier, M. 2015. Effect of *Saccharomyces cerevisiae* CNCM I-1077 supplementation on performance and rumen microbiota of dairy calves. *Prof. Anim. Sci.* 31(2), 153–8. doi:10.15232/pas.2014-01384.

Theodorou, M., Beever, D., Haines, M. and Brooks, A. 1990. The effect of a fungal probiotic on intake and performance of early weaned calves. *Anim. Prod.* 50, 577.

Tsuruta, T., Inoue, R., Tsukahara, T., Nakamoto, M., Hara, H., Ushida, K. and Yajima, T. 2012. Commensal bacteria coated by secretory immunoglobulin A and immunoglobulin G in the gastrointestinal tract of pigs and calves. *Anim. Sci. J.* 83(12), 799–804. doi:10.1111/j.1740-0929.2012.01026.x.

USDA. 2018. Dairy 2014, "Health and Management Practices on U.S. Dairy Operations, 2014". Vol. #696.0218. USDA-APHIS-VS-CEAH-NAHMS, Fort Collins, CO.

Vieira-Silva, S., Falony, G., Darzi, Y., Lima-Mendez, G., Garcia Yunta, R., Okuda, S., Vandeputte, D., Valles-Colomer, M., Hildebrand, F., Chaffron, S. and Raes, J. 2016. Species-function relationships shape ecological properties of the human gut microbiome. *Nat. Microbiol.* 1(8), 16088. doi:10.1038/nmicrobiol.2016.88.

Wang, Z., Elekwachi, C., Jiao, J., Wang, M., Tang, S., Zhou, C., Tan, Z. and Forster, R. J. 2017a. Changes in metabolically active bacterial community during rumen development, and their alteration by rhubarb root powder revealed by 16s rRNA amplicon sequencing. *Front. Microbiol.* 8, 159. doi:10.3389/fmicb.2017.00159.

Wang, Z., Elekwachi, C. O., Jiao, J., Wang, M., Tang, S., Zhou, C., Tan, Z. and Forster, R. J. 2017b. Investigation and manipulation of metabolically active methanogen community composition during rumen development in black goats. *Sci. Rep.* 7(1), 422. doi:10.1038/s41598-017-00500-5.

Williams, Y. J., Popovski, S., Rea, S. M., Skillman, L. C., Toovey, A. F., Northwood, K. S. and Wright, A. D. 2009. A vaccine against rumen methanogens can alter the composition of archaeal populations. *Appl. Environ. Microbiol.* 75(7), 1860–6. doi:10.1128/AEM.02453-08.

Wu, H. J. and Wu, E. 2012. The role of gut microbiota in immune homeostasis and autoimmunity. *Gut Microbes* 3(1), 4–14. doi:10.4161/gmic.19320.

Yáñez-Ruiz, D. R., Macías, B., Pinloche, E. and Newbold, C. J. 2010. The persistence of bacterial and methanogenic archaeal communities residing in the rumen of young lambs. *FEMS Microbiol. Ecol.* 72(2), 272–8. doi:10.1111/j.1574-6941.2010.00852.x.

Yáñez-Ruiz, D. R., Abecia, L. and Newbold, C. J. 2015. Manipulating rumen microbiome and fermentation through interventions during early life: a review. *Front. Microbiol.* 6, 1133. doi:10.3389/fmicb.2015.01133.

Yeoman, C. J., Ishaq, S. L., Bichi, E., Olivo, S. K., Lowe, J. and Aldridge, B. M. 2018. Biogeographical differences in the influence of maternal microbial sources on the early successional development of the bovine neonatal gastrointestinal tract. *Sci. Rep.* 8(1), 3197. doi:10.1038/s41598-018-21440-8.

Yohe, T. T., Enger, B. D., Wang, L., Tucker, H. L. M., Ceh, C. A., Parsons, C. L. M., Yu, Z. and Daniels, K. M. 2018. Short communication: does early-life administration of a *Megasphaera elsdenii* probiotic affect long-term establishment of the organism in the rumen and alter rumen metabolism in the dairy calf? *J. Dairy Sci.* 101(2), 1747–51. doi:10.3168/jds.2017-12551.

Zhong, R. Z., Sun, H. X., Li, G. D., Liu, H. W. and Zhou, D. W. 2014. Effects of inoculation with rumen fluid on nutrient digestibility, growth performance and rumen fermentation of early weaned lambs. *Livest. Sci.* 162, 154–8. doi:10.1016/j.livsci.2013.12.021.

Ziolecka, A., Osinska, Z. and Ziolecki, A. 1984. The effect of Stabilized Rumen Extract on growth and development of calves. 1. Liveweight gain and efficiency of feed utilization. *Z. Tierphysiol. Tierernahr. Futtermittelkd.* 51(1–2), 13–20. doi:10.1111/j.1439-0396.1984.tb01406.x.

Ziolecki, A., Kwiatkowska, E. and Laskowska, H. 1984. The effect of Stabilized Rumen Extract on growth and development of calves. 2. Digestive activity in the rumen and development of microflora in the rumen and faeces. *Z. Tierphysiol. Tierernahr. Futtermittelkd.* 51(1–2), 20–31. doi:10.1111/j.1439-0396.1984.tb01407.x.

Part 1

Tools to understand the ruminal microbiome

Chapter 2

A question of culture: bringing the gut microbiome to life in the -omics era

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1 Introduction

The mammalian gut hosts a diverse microbial community (microbiota) that provides a range of ecological, nutritional and immunological functionalities, among others, that are relevant to the health and well-being of the host. For much of the twentieth century our understanding of the mammalian gut microbiota was largely developed using culture-dependent approaches with ruminant livestock providing the deepest mechanistic insights into their functional capacities mainly due to the long-recognised association between the rumen microbiota and animal health and productivity. Many of the seminal works in gut microbiology were originally developed for rumen microbial species and include the description of new media, culturing techniques and the isolation of diverse microbes in a rich scientific literature extending back to the 1940s. However, it was the development of culture-independent metagenomic approaches coupled with advances in DNA-sequencing technologies and computational methods that established the 'gut microbiome' as a discrete field of research. There have been several major metagenomic-based studies on the gut microbiomes of ruminants and other mammals that have provided unprecedented insights into their diversity and functional capacities, and transformed our awareness and appreciation of the extent of microbial 'dark

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matter' in the form of uncultured microbes and their genes that reside in these communities.[1–4]

Despite the insights afforded by these approaches, many studies have also highlighted the limitation of solely applying culture-independent approaches to characterise the microbiome. For instance, cultivation-based studies remain the best way to specifically link microbes with discrete biological activities and thereby provide new opportunities to move beyond the current observational paradigm and towards causation in gut microbiology research.[5–7] In addition, sequencing of individual gut-microbial strains improves mapping and assembly from metagenomic data.[8,9] The human microbiome revolution in particular has led to a renewed interest in culturing approaches and the 'discovery' of culturing approaches and insights that have been well established for generations of rumen microbiologists. Together, these have given new life to culturing efforts and there are now multiple initiatives and related culture collections for mammals including humans (e.g. Culturable Genome Reference,[9] Human Gastrointestinal Bacteria Culture Collection[8]), ruminants (e.g. Hungate1000 collection[10]) and rodents (Mouse Intestinal Bacterial Collection[11]). Notably, several recent studies drawing upon 16S rRNA and metagenomic-based analyses have suggested the perceived unculturability of many gut microbes from mammalian gut environments may have been overstated.[12–16] These claims remain contentious, and, indeed, a recent study suggested that over 70% of human gut-microbial species lack a cultured representative.[17]

The mammalian gut microbiota is highly diverse and typically includes viruses, bacteria, archaea, fungi and protozoa. While it is clear from numerous studies over the years that many fastidious gut microbes grow quite well under laboratory conditions there are others that remain resistant to cultivation as axenic cultures.[18] Our inability to culture these microbes suggests that there are fundamental aspects of their biology that we do not yet understand. There have been many culturing advances that continue to extend the repertoire of gut-microbial isolates[19–21] and meta-omic approaches now also offer unprecedented insights into the metabolic potential of these microbes that could be used to direct and support their growth under laboratory conditions. The integration of these culture-dependent and culture-independent approaches will provide new opportunities to dissect the dynamic host-microbiome relationship.

With that context, this chapter provides an overview of the existing and emerging isolation strategies that can be readily applied in laboratories with access to the types of facilities and equipment routinely used in gut microbiology. In particular, the influence of nutrients on the culturability of fastidious gut bacteria and archaea is discussed, as well as how emerging meta-omic, genetic and antibody-based strategies could be used to bring the gut microbiome to life.

2 Culturing methods and nutrient effects on microbial growth: an overview

This chapter focuses on advances in bacterial and archaeal cultivation but readers interested in the gut mycobiome are referred to the works by Theodorou et al.[22] and Haitjema et al.[23] which provide comprehensive overviews of the approaches used to isolate, cultivate and preserve anaerobic fungi. Despite significant advances in the cultivation of fastidious gut bacteria, archaea and fungi, the propagation of gut-derived phage and protozoa under laboratory conditions continues to remain challenging. Interested readers are referred to Gilbert and Klieve[24] and Newbold et al.[25] respectively, for overviews of our current understanding of ruminal phage and protozoal biology.

The works published by McSweeney et al.[26] and Joblin[27] provide well-structured and comprehensive overviews of the approaches and methods widely used to isolate and propagate fastidious bacteria and archaea from the rumen and other gut environments. The contemporary techniques widely used in anaerobic microbiology are still predominantly based on the approaches developed and described by Hungate, Bryant and their colleagues[28,29] and summarised by Stewart et al.[30] The recipes described by McSweeney et al.[26] and their derivatives are still widely used by the research community and have consistently been shown to support the nutritional requirements of a diverse range of gut bacteria. Most microbiologists will be familiar with the methods used to prepare microbiological media and the use of anaerobic jars. Here, media is typically prepared, inoculated under oxic conditions and then transferred to an anaerobic jar where a reductant is used to generate anoxic conditions. In contrast, the methods and underlying theory used to prepare anoxic media for the more fastidious gut microbes will largely be unfamiliar to the uninitiated but can be quickly mastered with an experienced mentor. Many laboratories that routinely culture anaerobic gut microbes use anaerobic chambers to isolate and propagate anaerobic microbes. However, while anaerobic chambers provide increased flexibility and consistency between experiments it is important that strict work practices are adhered to so as to prevent oxygen contamination of the internal chamber environment. This includes where possible ensuring reagents and consumables are oxygen free before entry into the chamber.

The typical components of anoxic microbiological media include a source of nitrogen and carbon macronutrients (e.g. peptone, glucose), a source of essential micronutrients (e.g. yeast extract, trace metals solution, vitamin solution), a mineral salt solution to buffer the medium against pH changes, an oxygen indicator and a reductant. Media are usually prepared by mixing the individual components in a large autoclavable vessel with the exception of any heat-labile ingredients and the reductant. Since oxygen is poorly soluble in

water, the medium is next rapidly deoxygenated by boiling and then cooled by bubbling with a constant stream of anoxic gas (typically, carbon dioxide or nitrogen), with aluminium foil wrapped around the mouth of the vessel enabling a build-up of positive pressure and preventing entry of atmospheric oxygen into the vessel. Once cooled, the pH is adjusted to the desired range and a reductant is added to titre the effects of any subsequent oxygen contamination. Finally, the medium is aliquoted as required in an anaerobic chamber and autoclaved. Sterile, heat-labile components can be added as required once cooled.

An alternative approach involves the preparation of select medium components separately before combining post-sterilisation in an anaerobic chamber to avoid the formation of Maillard and other toxic products that typically occur between sugars and amino acids during conditions of high heat and temperature.[31] The phosphate salts typically used in buffering salts can also result in the formation of toxic products and inhibition of microbial growth, and media with reduced concentrations can support the recovery of greater microbial diversity including novel isolates.[32–34] Similarly, gelling agents can impact microbial growth and while bacteriological agar is typically used due to its low cost, other alternatives including gellan gum can also improve the recovery of novel microbial diversity.[34–36]

Habitat-simulating media have been widely used to enable isolation of fastidious gut microbes.[33,37] These media support growth of many diverse microbes however a disadvantage is that less abundant or slow-growing subdominant microbes are often out competed by fast-growing microbial 'weeds'.[38] Additional complicating factors are the challenges associated with producing microbial cultures in solid or liquid media. Many microbes, including from gut environments, are auxotrophic and their growth in liquid media is facilitated by cross-feeding with external nutrients that can satisfy their growth requirements.[39–41] In contrast, growth as distinct axenic colonies suggests prototrophy or that the medium can support their nutritional requirements. Taken together, there is much interest in rationally developing new formulations that are permissive for the growth of target microbes but inhibitory for non-target microbes. In the following subsections, the effect of select macro- and micro-nutrients on microbial growth is briefly discussed.

2.1 Nitrogen sources

Much of the organic nitrogen in the rumen is sequestered in ammonium and protein biomass. Ammonia is the principal source of nitrogen for rumen microbes with many bacteria displaying specific amino acid auxotrophies or preferences, and are stimulated by the addition of specific amino acids (e.g. leucine, glycine) or peptides.[42–44] Rumen microbes have thus evolved

specialised strategies to facilitate the release of this nitrogen and its assimilation in the growing cell. The main source of peptide breakdown in the rumen is via the activity of dipeptidyl peptidases which results in the production of dipeptides that are then further catabolised by dipeptidases.[44] It is well-recognised that rumen microbes exhibit specific preferences for nitrogen. For instance Pittman and Bryant[45] demonstrated that *Prevotella ruminicola* (then classified as *Bacteroides ruminicola*) exhibits a preference for peptide and ammonia nitrogen but does not utilise free amino acid nitrogen or nitrogen from a range of other low-molecular weight sources. Similarly, Synergistetes strain MFA1, *Prevotella bryantii* B14, *Selenomonas* and *Streptococcus* spp. exhibit a preference for peptides over amino acids.[46,47] In contrast, other bacteria including *Clostridium aminophilum*, *Peptostreptococcus anaerobius* and *Fibrobacter* spp. exhibit a preference for amino acids.[48,49] As expected, nitrogen sources can impact the growth of specific organisms with peptone sources affecting both the growth rate and cell characteristics of specific bacteria[50–52] while free amino acids can either promote or inhibit[53] the growth of select microbes with implications for their choice in media.

2.2 Carbon sources

Carbohydrates are the predominant source of energy for most gut microbes. Carbohydrates are structurally diverse and as expected gut microbes vary in their ability to utilise them to support growth with many microbes exhibiting specific carbohydrate preferences. McSweeney et al.[26] described a range of media that are selective for cellulolytic, xylanolytic, pectinolytic and amylolytic microbes and these or similar media are still widely used. In the gut, the influence of carbohydrates on microbial growth is influenced by host diet and the extent of host and microbial digestion. Microbes exhibit a hierarchy of carbohydrate utilisation with simpler carbohydrates often preferentially utilised. In the gut, the primary carbohydrate-degrading microbes catabolise the more complex carbohydrates releasing simpler oligosaccharides or monosaccharides to other microbes. These carbohydrate preferences and growth impacts have been widely used to isolate microbes. For instance, rapid transfer of enrichment cultures with complex carbohydrates facilitates enrichment of primary degraders at the expense of secondary degraders that are increasingly diluted. While carbohydrate-based enrichments were historically empirically determined, the advent of whole genome sequencing and custom databases (e.g. dbCAN2[54] and SACCHARIS[55]) has enabled a more selective inclusion of carbohydrates to enrich for and isolate target microbes.

Many gut microbes can switch to asaccharolytic or proteolytic fermentation including clostridia, peptostreptococci and other microbes,[42,56] and acquire

their carbon from non-carbohydrate sources. The gut is also colonised by other microbes that are capable of carbohydrate-independent growth including reported asaccharolyitc microbes (e.g. *Phascolarctobacterium* spp.[57,58] and *Catenibacillus scindens*[59]). Also, acetogens (e.g. *Eubacterium limosum*,[60] tammar wallaby isolate TWA4[61]) and methanogenic archaea can use non-carbohydrate substrates with the latter capable of utilising a diverse array of substrates including carbon dioxide, formate, methanol and methylamines.[62] Thus, microbes can utilise a diverse array of carbon sources and their specific preferences can impact their culturability in the laboratory.

2.3 Micronutrients

Many gut microbes display absolute nutritional requirements for specific micronutrients including vitamins, lipids, transition elements and signalling molecules.[20,63] The importance of vitamins and trace metals as micronutrients is well recognised and where necessary these are typically supplied from standard stock solutions.[26] Many gut microbes exhibit complex micronutrient requirements that may at least partially explain their resistance to growth on laboratory media. In addition, micronutrients are typically provided in trace amounts and it can be difficult to predict the specific requirements of a microbe. Consequently, the inclusion of cell-based extracts (e.g. yeast extract, beef extract) and for many fastidious gut microbes, rumen fluid[26] and faecal waters,[64] act as a source of micro-nutrients and also help simulate a more physiologically relevant environment.

The ability of straight and branched chain-volatile fatty acids to stimulate growth of gut bacteria is also well known.[65,66] Non-volatile fatty acids also stimulate growth with the growth and ability of *Ruminococcus albus* 8 to digest cellulose in a chemically defined medium enhanced by the addition of phenylpropanoic acid to the medium.[67] *R. albus* 8 also utilises phenylacetic acid to synthesise phenylalananine and the rate of cellulose digestion following its addition to defined medium is comparable to that in medium supplemented with rumen fluid.[68] Carbon dioxide also stimulates the growth of important gut bacterial species.[69,70] While methanogens typically use hydrogen for methane production, there is also evidence of adaptations to low-hydrogen environments with *Methanosphaera* sp. WGK6 capable of utilising ethanol as an alternative to hydrogen to fuel methanogenesis.[5] Orthologs of the alcohol and aldehyde dehydrogenase genes suspected of underpinning this capacity are found in other methanogenic species suggesting this may not be a unique phenomenon. Several studies have reported isolation of many environmental isolates in diffusion chambers likely due to the in situ production of essential growth factors. Using a similar approach, Lewis and colleagues[71] screened densely plated bacteria to identify slow-growing colonies whose growth was

dependent on proximally located faster-growing colonies. Using this approach, they identified novel *Faecalibacterium* sp. and *Sutterella* sp. whose growth was dependent on the production of specific quinones by helper bacteria. Purified quinones enabled growth in the absence of helper cells although select strains exhibited quinone specificity. Capitalising on this approach, Lewis and colleagues[72] used a similar approach to identify an isolate that grew in the presence of γ-aminobutyric acid (GABA) producing helper bacteria, or in the absence of the helper bacteria when supplied with GABA. Vartoukian et al.[73] also showed that supplementation of medium with siderophores resulted in the cultivation of novel strains from the oral microbiota.

Micronutrients are typically supplied in trace amounts and Tramontano et al.[53] showed that specific medium components and microbial metabolites could also potentially inhibit growth in a study that examined 90 human gut bacteria. Thus, while ensuring that important nutrients are provided it is also critical that their concentrations do not inhibit growth.

2.4 Reductants

The vast majority of microbes that colonise the gut of ruminants and other mammals are strict anaerobes, and although some can tolerate exposure to atmospheric conditions (e.g. *Bacteroides* spp., *Prevotella* spp.), growth typically occurs only under anoxic conditions. Cysteine-HCl is the reductant of choice for many studies due to ease of use however titanium-based reductants are also widely used due to their greater redox potential.[26,74,75] In a challenge to this paradigm, Raoult and colleagues[76] demonstrated that the anaerobic bacteria *Ruminococcus gnavus* and *Fusobacterium necrophorum* could be grown under atmospheric condition when the medium was supplemented with the reductants ascorbic acid and/or glutathione. In a follow-up study, Dione et al.[77] further extended this work and demonstrated that a diverse set of 623 bacteria including 82 strictly anaerobic and 9 microaerophilic species could be grown in a medium supplemented with ascorbic acid and glutathione.[78] The addition of uric acid, haemin and α-ketoglutarate further improved culturability of the microbes such that only the Mycobacteria failed to grow under the conditions examined. These observations have also been extended to extremely oxygen-sensitive methanogenic archaea.[79,80] While these advancements have not been widely adopted they have been used to culture strict gut anaerobes including *Dysosmobacter welbionis*[81] and they could be used to enable more routine processing of fastidious anaerobes under oxic environments.

In summary, there are a wide range of factors that influence the breadth and depth of microbes that can be cultured. The use of low nutrient medium and prolonged incubation times can support the growth of a broader range of fastidious bacteria potentially by preventing the overgrowth by competing

microbes or reducing the concentration of growth inhibitors.[20,82–85] Culturomics has built on traditional culturing approaches by greatly extending the range of media and growth conditions typically tested resulting in the isolation of many novel gut microbes (Fig. 1a[86,87]). However, the impact of the macro- and micro-nutrients, and other variable growth conditions, on microbial culturability has not been systematically explored although several recent studies have outlined strategies to address this challenge (e.g. Taguchi arrays[88], factorial design), and these will provide both new insights into the metabolic requirements and growth potential of existing isolates,[78,89] and support the growth of fastidious gut microbes ex vivo thus providing opportunities to bring previously uncultured microbes to life.[90,91] These efforts will also be supported by the development

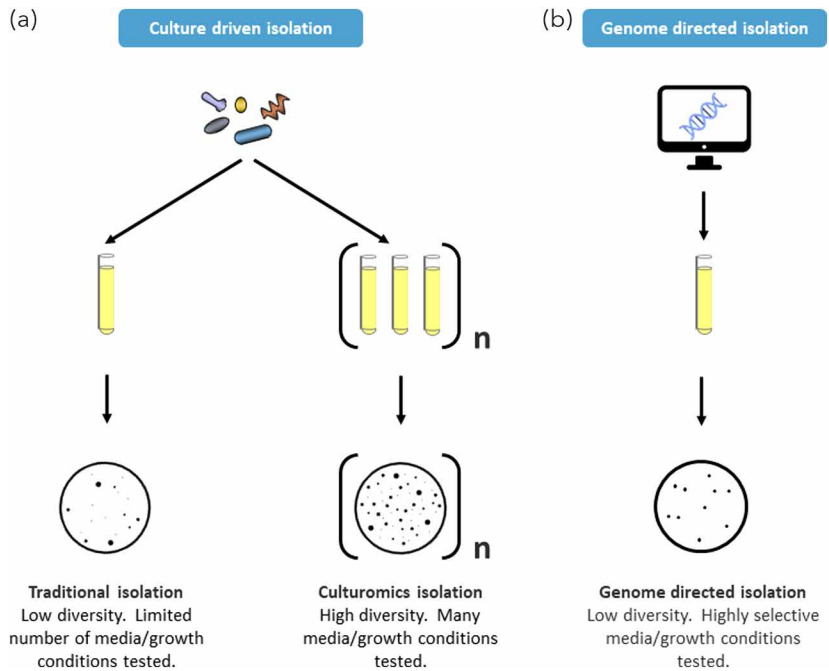

Figure 1 (a) With traditional microbial isolation approaches, a limited number of media and growth conditions are tested based on historical reports, empiric data and the experience of the practitioner. These typically result in limited diversity and are at best moderately selective for target microbes. Culturomics-based isolation builds on these traditional approaches but explores a broader range of media and growth conditions (*n*) which can significantly increase the number of novel microbes recovered. Culturomics captures greater microbial diversity although it is not typically directed towards target microbes. (b) With genome-directed isolation, genomic data from target microbes are analysed to predict their nutritional and growth preferences and then custom media are developed. These media result in low recoverable microbial diversity as they are typically permissive for growth of the target but not non-target microbes.

of small colony-picking robots that have allowed for the first time automated scalable colony picking in anaerobic chambers.

3 Genome-directed isolation of gut microbes

Habitat-simulating media are widely used to maximise growth and recoverable microbial diversity. However, these media tend to favour the isolation of the predominant members of the community and they are less effective in enabling the isolation of subdominant populations. Consequently, there is much interest in rationally developing new medium formulations that are permissive for the growth of target microbes but inhibitory for non-target microbes.

The advent of DNA sequencing revolutionised microbiology and enabled for the first time rational sequenced-based predictions of gene function and by extension the metabolic capacity of the host microbe. This capability was further extended with the publication of the first bacterial genomes, *Haemophilus influenzae* and *Mycoplasma genitalium* in 1995, where numerous predictions were made based on their genome sequences.[92,93] However, it was not until 2005 that Tyson and colleagues[94] first used metagenomic information to rationally direct the isolation of a bacterium from a mixed community. In that study, partially reconstructed genomes from a low-diversity acid mine drainage-associated biofilm revealed the presence of the well-recognised nitrogen fixation *nif* regulon predicted to be encoded by Leptospirillum group III, a lineage in the Nitrospirae phylum with no cultivated representatives. Capitalising on this observation, a combination of growth in nitrogen-free medium with dilution-to-extinction-based enrichment yielded an axenic isolate of *Leptospirillum ferrodiazotrophum* from an inoculum that contained the target microbe at an initial abundance of <10%. This was a major break-through and demonstrated that genome information could be exploited to yield axenic cultures of keystone microbial species. In a more recent study, Leon et al.[95] used metagenomic data coupled with screening of over 2000 microbial colonies and isolated a moderately halophilic bacterium, *Spiribacter salinus*, that comprised as much as 15% of the prokaryotic community in a saltern pond of intermediate salinity. Wurch et al.[96] similarly used genome-informed isolation strategies to produce an enrichment culture of the ectosymbiotic nanoarchaeota *Nanopusillus acidilobi* that comprised 7% of the archaeal population in a mildly acidic geothermal pool, and used cell sorting to produce a co-culture with its crenarchaeote *Acidilobus* host. Genome-informed isolations have also been used to isolate bacteria from animal gut environments. For instance, during analysis of metagenomic data from Tammar wallaby digesta, a taxon affiliated with the Succinivibrionaceae was determined to comprise as much as 9% of the community.[97] Analysis of over 2 Mb of assembled genome data suggested that the bacterium was capable of utilising urea and starch as nitrogen and

carbohydrate sources, respectively, and was resistant to bacitracin. Based on these data, an axenic culture of an isolate termed WG-1 was produced using semi-continuous batch culture methods in a medium containing urea, starch and bacitracin.

Despite these advances, genome-directed isolation of bacteria can be difficult as these genomic data reflect the functional and metabolic capacities rather than the preferences of target microbes. To address this, Bomar et al.[98] applied a metatranscriptomic approach to examine gene expression in the crop microbiome of the medicinal leech *Hirudo verbena* whose community is dominated by *Aeromonas veronii* and a Rikenella-like bacterium. Despite extensive sequence data the Rikenella-like bacterium was resistant for many years to laboratory culture; however, metatranscriptomic analysis of the bacterium, when it was actively proliferating and *Aeromonas veronii* was entering stationary phase, revealed particularly high-level expression of one operon including a gene encoding a member of the GH18 family, endo-β-N-acetylglucosaminidase and propinquitous genes, suggesting that protein-bound glycans are a key energy source for this bacterium. To test this, the glucose in Eggerth-Gagnon medium was replaced with bovine submaxillary glands or porcine gastric mucin and incubated for up to two weeks resulting in the isolation of both *A. veronii* and the Rikenella-like bacterium. Additional clones were isolated using porcine mucin thereby extending the diversity of microbes isolated. A notable advantage of this approach is that it highlights the genes expressed by the microbe under specific conditions thereby providing a deeper insight into its nutritional preferences and metabolic activity during active growth.

In addition to bringing microbes to life, genomic data can be used to refine the culturing conditions for clinically and agriculturally important bacteria. For instance, genome-informed decisions have also been used to develop improved cultivation strategies for important pathogens including *Coxiella burnetii*[99] and the human gut bacterium *Christensenella minuta*.[100] Similarly, a recalcitrant *Frankia* strain affiliated with cluster 2 was cultured following genomic analyses and extensive phenotyping of nodule recovered cells.[101]

Recent efforts have focused on scaling and automating 'genotype-to-phenotype' analyses thereby improving the throughput of isolation strategies. McHardy and colleagues developed Traitar[102] to provide automated prediction of phenotypes from genomic data. Traitar is available for use on a web server or as a download and can predict 67 phenotypes including carbon sources, cell morphology and enzymatic activities. Traitar uses phenotype data from the Global Infectious Disease and Epidemiology Online Network to train phenotype classification models using protein annotation data from sequenced microbial isolates. Traitar was validated using bacterial phenotypes in Bergey's *Manual of Systematics of Archaea and Bacteria* and shown to perform well using incomplete genomes such as those produced from single-cell sequencing or

metagenomic data. Indeed, one of Traitar's most powerful features is the ability to compare phenotypes from multiple genomes which is helpful when working with samples with reduced microbial diversity (e.g. enrichment cultures). Traitar also identifies proteins that may underpin particular phenotypes providing opportunities to better probe the functional capacities of specific microbes. In a parallel development, Feldbauer et al.[103] extended the Python for comparing genotype–phenotype algorithms (PICA) genotype-phenotype association machine learning-based approach[104] to large-scale genome datasets and partial genome sequences although the predictions are limited to 10 phenotypes and are of limited value when isolating gut microbes.

There has also been much interest in genome scale metabolic modelling to elucidate the nutritional requirements of gut microbes. Readers are referred to the comprehensive reviews by Santos et al.[105] and Sen and Orešič[106] describing the theory and different experimental strategies that underpin these models. In brief, these approaches provide genome scale mathematical models of microbial metabolic capacity and thereby predict microbial nutritional preferences of bacteria and the development of tailored media to support their growth. In the largest such study to date, Magnúsdóttir et al., produced the AGORA genome scale metabolic models for 773 sequenced human gut bacteria and provided insights into their metabolic capacity and nutritional preferences.[107] While powerful, genome scale metabolic constructions are susceptible to inaccurate predictions due to the propagation of inaccurate annotations and their inability to identify novel metabolic pathways. For instance, Tramontano et al.[53] showed that only 10 out of 40 AGORA strains could grow on a selection of defined medium highlighting the limited ability of the current genome-led approaches to predict the nutritional requirements of uncultured fastidious gut bacteria. However, the defined medium resource developed by Tramontano et al.[53] enabled further refinement of the AGORA models and supported growth of the remaining 30 strains. Similarly, custom media have been developed for important gut microbes including *Faecalibacterium prausnitzii*[108] and *Akkermansia muciniphila*,[109] although some of these are sub-optimal suggesting the models can be further refined. Nonetheless, the ability of a diverse array of phylogenetically distinct bacteria to grow on defined medium suggests that the nutritional requirements of gut bacteria may not be as extensive as thought.

And so, what can we conclude from these observations? First, in contrast to traditional and culturomics-based approaches, these studies demonstrate that multi-omic-guided isolation strategies can be used to produce highly enriched or axenic cultures of target microbes (Fig. 1b[110]), although examples of isolations from gut environments are limited. Second, this process remains inefficient, and these strategies appear to also work best when applied to samples that already have a high abundance of the target cells or where highly

selective cultivation strategies can be applied. Taken together, there remains a paucity of well-developed tools and *in silico* approaches to exploit the wealth of genomic data and guide the rational production of axenic cultures of select gut microbes although this is increasingly being addressed.[111]

4 Molecular-based isolation of gut microbes

Metagenomic analyses have revealed the gut microbiome of ruminants and humans has been extensively shaped by horizontal gene transfer and several studies have demonstrated the transfer of genetic elements between bacteria in situ in the gut environment. Microbes may experience selective pressure in their natural environments that may make them more receptive to exogenous DNA thus suggesting that molecular approaches could be used to expedite their isolation. This could afford new opportunities to selectively target novel gut microbes and address the paucity of genetically tractable gut microbes.

Based on these observations, Ó Cuív and colleagues developed a novel method termed metaparental mating (MPM) that utilises RP4 (RK2)-mediated conjugative transfer to introduce a recombinant pEHR modular vector bearing an antibiotic resistance marker into recipients in a complex community. The RP4 conjugative transfer system was chosen as it has a wide host range, it can circumvent host restriction modification systems and it is amenable to high-throughput automation. Using the MPM approach and pEHR vector system they demonstrated a molecular approach could be used to selectively isolate fastidious human gut bacteria principally affiliated with *Clostridium* cluster IV, XIVa, XV and XVIII.[112] Critically, the pEHR plasmids were stably maintained in their hosts suggesting they were genetically tractable. The MPM approach could theoretically be used with any suitable RP4 mobilisable vectors and Ó Cuív and colleagues subsequently demonstrated that the narrow host range enterobacterial vector, pJQ200sk(+), could be used to recover *E. coli* transconjugants from a human faecal sample.[113] With the exception of enterobacteria and *Bacteroides* spp., there are currently a paucity of genetically tractable gut microbes that could be used to dissect the functional capacity of the gut microbiome. Isolating gut microbes is a time-consuming process and a key advantage of the MPM approach is that the recovered isolates may be amenable to characterisation using well-established genetic approaches. For instance, Ó Cuív et al. used MPM to isolate a genetically tractable strain of *Enterococcus faecalis* that possesses potent anti-inflammatory activities providing new opportunities to explore the genetic basis for this activity.[114] There are a wide range of RP4 mobilisable vectors that have been described for the dominant taxa in ruminant and human microbes and these could be readily applied to expedite the isolation of genetically tractable strains.[115–117]

Using a similar approach to MPM, Ronda et al.[118] developed modular replicative or integrative vectors and an approach termed Metagenomic Alteration of Gut microbiome by in situ Conjugation (MAGIC), to genetically manipulate microbes in situ in their natural environment. Using this approach, they demonstrated that a diverse range of gut bacteria including *Clostridium* cluster XIVa, *Bacterodies* spp. and *Enterococcus* spp. could be stably modified thereby validating the genetic manipulation of the microbiome in situ. Separately, Brophy et al.[119] developed the more narrow host range XPORT system and demonstrated that it can be used to transfer miniaturised genetic elements based on the integrative and conjugative element from *Bacillus subtilis* (ICE*Bs*1) to donor bacteria. The XPORT system is comprised of a donor strain that carries three cassettes, a mini-ICE*Bs*1 that integrates into recipient bacteria, a type IV secretion system to mediate mini-ICE*Bs*1 transfer and an inducible regulator to control conjugation, that are carried at discrete chromosomal locations. The mini-ICE*Bs*1 typically inserts stably into a conserved leucine tRNA in the most recipient strains but can insert at other sites or remain as an extrachromosomal element where the leucine tRNA is not present. Similar to the MPM approach the XPORT donor strain is counter-selected against through nutritional auxotrophy. Using a biparental mating approach, the mini-ICE*Bs*1 was efficiently transferred to 35 representative human gut and skin bacteria. In addition, application of a MPM approach in situ in soil revealed that the mini-ICE*Bs*1 was also transferred to soil bacteria. All bacteria recovered by Brophy et al.[119] were principally affiliated with the Bacilli and no transfer to *E. coli* was detected confirming the narrow host range of the XPORT system.

These molecular-based approaches to microbial isolation have several advantages over traditional culturing methods. First, the introduction of an antibiotic resistance marker enables recipients to be recovered on nutritionally replete medium supplemented with the appropriate antibiotics. Optimisation of the gene transfer conditions are typically determined empirically and include a pre-screen of the recipient community to assess the extent of resistance to the antibiotic of choice. Second, the strains recovered by molecular methods are likely genetically tractable providing subsequent opportunities to dissect the functional capabilities of target microbes. Unfortunately, it is often not possible to assess in advance the genetic tractability of target microbes, and overcoming host restriction modification systems in undomesticated microbes remains a challenge. Third, these approaches address a renewed interest in using genetic approaches to characterise the functional capacities of gut microbes and have led to the development of an assortment of genetic tools for a diverse range of bacteria and archaea.[112,120,121] It is likely that many of these tools could be customised and applied to isolate new microbes thereby providing a valuable resource of genetically tractable strains to expedite the study of host microbe interactions.

5 Antibody-based isolation of gut microbes

There has also been much interest over several decades in the application of physical methods to isolate/enrich target microbes from mixed communities.[122,123] Immunomagnetic-based cell separation was initially developed to separate mixed populations of eukaryotic cell types (e.g. different blood cell populations), but this technique was then extended to microbial cells. Notably, these approaches were driven by the observation that microbes remain viable when attached to beads and growth can be stimulated by the addition of fresh medium (Fig. 2a[123–125]).

The first practical demonstration of this approach revealed that antibody-mediated immunomagnetic isolation could be used to isolate clinically relevant *E. coli* K88+ strains. Using IgG2 monoclonal antibodies against the *E. coli* K88 pilus antigen immobilised on superparamagnetic beads, Lund et al.[126] demonstrated that bacteria bearing K88 could be isolated and identified using a positive selection test in as little as 30 minutes. In addition, an *E. coli* K88 expressing strain could be specifically recovered in almost pure culture from a mixture of different O serotype *E. coli* strains. Much of the subsequent work in this space was developed to address the detection of clinically relevant pathogens including *Listeria*, *Pseudomonas* and other pathogenic *E. coli* spp. Okrend et al.[127] demonstrated that an *Escherichia coli* O157-specific

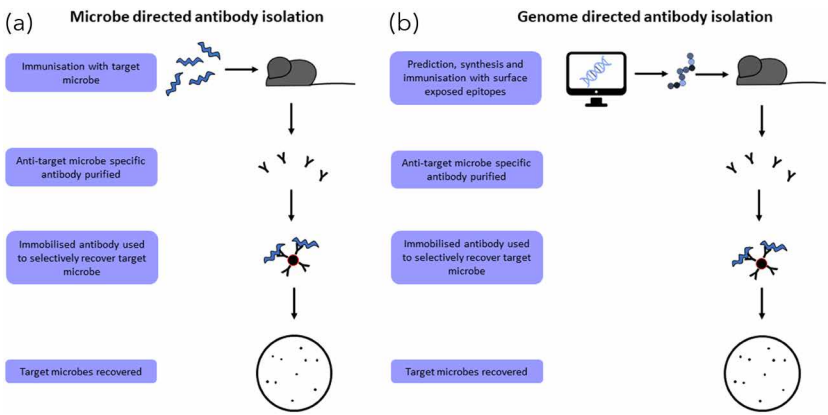

Figure 2 (a) With microbe-directed antibody isolation, a microbe of interest is used to immunise an animal and then anti-target microbe specific antibodies are purified. The antibodies can then be immobilised on magnetic beads and used to physically separate target from non-target microbes in a mixed cell population. By this approach, viable microbes can be isolated and subsequently grown in the appropriate medium. (b) With genome-directed antibody isolation (reverse genomics), genomic data from target microbes are analysed to predict surface exposed and likely immunogenic epitopes. These epitopes are then produced and used to immunise an animal where after the anti-target microbe specific antibodies are purified and used to isolate the target microbe.

IgG antibody could be coated onto magnetic beads and used to selectively enrich the target bacterium from mixed cultures and meat samples, and that the recovered colonies could be identified by plating onto discriminating medium. Notably, Okrend et al.[127] also demonstrated that non-specific binding and isolation of non-target bacteria could be prevented by protamine thereby enabling consistent detection of *E. coli* O157:H7.

Capitalising on these early studies, Close et al.[128] used a naïve scFv library displayed on M13 filamentous phage and identified antibodies that bound to the target bacterium *Lactobacillus acidophilus* ATCC 4356. The antibodies were highly specific for the *L. acidophilus* highly abundant S-layer A protein, and did not bind to a panel of other gut bacteria that included six other *Lactobacillus* spp. Using one of the antibodies, they next demonstrated that it could detect the target bacterium in a mixed community when its abundance was less than 0.2%. Based on this observation they demonstrated that the antibody could be used to sort *L. acidophilus* from a mixed community using fluorescent-activated cell sorting when it comprised <0.2% of the population. A single cell isolated using this approach was sequenced by multiple displacement amplification and provided 63% coverage of the genome while 50 cells yielded 99.8% genome coverage following *de novo* assembly. Finally, the authors demonstrated that a similar approach could be applied to a complex mock microbial community where the target microbes were present at ~10% revealing that species-specific antibodies could be selected without having to culture individual bacteria.

These reports provide key insights into the feasibility of efficiently isolating target microbes from complex microbial communities. However, antibody-based microbial isolation has been rarely used although it has many advantages over traditional culturing approaches including that a single antibody can be used to isolate target microbes from multiple samples and even when the target cells are at low abundance, and that this process is quick and amenable to automation. Furthermore, this technology is also compatible with work in an anaerobic environment and can physically separate cells from other growth inhibitory factors and/or microbes. The production of antigen-specific antibodies has historically been associated with significant time and cost commitments that have outweighed these advantages. In particular, a key limitation has been the requirement of pure populations of target cells to enable antibody production as selection against an antigen is more specific when done in the absence of other antigens.[129] This challenge has now been overcome as the wealth of genomic information enables the identification of candidate antigens that are specific to a microbe of interest. Peptides corresponding to these candidate antigens can then be rapidly produced thereby enabling the rational development of antibodies against select targets and expediting the isolation of target microbes. This approach was elegantly demonstrated by Cross et al.[130] who used genome-informed antibody engineering to isolate

Saccharibacteria and previously uncultured SR1 bacteria. As the technical challenges associated with producing antibodies against rationally selected antigens are addressed it is likely that antibody-based approaches will increasingly be used to target novel uncultured microbes (Fig. 2b).

6 Conclusion and future trends

This chapter provides an overview of the historical challenges associated with culturing fastidious bacteria and archaea from the mammalian gut. In addition, it has provided insights into traditional and new strategies and approaches that have been developed to overcome these challenges. For more detailed information, the reader is referred to the citations listed in the References section and the current peer-reviewed scientific literature for emerging culturing approaches.

The gut microbiome revolution continues to transform efforts to delineate host-microbiome interactions and their influence on the host phenotype. Culture-independent efforts have provided unprecedented insights into the diversity and functional potential of the gut microbiome. However, culture-independent approaches may have reached their apogee with coverage of core gene content and functions increasingly saturated, although most gene products remain functionally uncharacterised. In contrast, culture-dependent approaches have not kept pace with these efforts although interest in cultivating gut microbes has never been higher. It is self-evident that a greater effort should be made to harness the expertise of rumen microbiologists in efforts to better understand the microbiome of humans and laboratory animals. Similarly, the high-throughput approaches widely applied in the human microbiome field could be similarly applied with the rumen microbiome, as ruminant livestock will continue to play an important role in providing high-quality nutritional sources even as efforts to mitigate the impact of intensive farming on the environment through reducing methane and nitrous oxide emissions continue. The development of new cultivation strategies based on improved understanding of nutritional preferences, coupled with more effective exploitation of meta-omic and genetic data, will offer new opportunities to provide deeper mechanistic insights into host-microbiome interactions.

7 References

1. Stewart, R. D., Auffret, M. D., Warr, A., Wiser, A. H., Press, M. O., Langford, K. W., Liachko, I., Snelling, T. J., Dewhurst, R. J., Walker, A. W., Roehe, R. and Watson, M. 2018. Assembly of 913 microbial genomes from metagenomic sequencing of the cow rumen. *Nat. Commun.* 9(1), 870. doi:10.1038/s41467-018-03317-6.
2. Hess, M., Sczyrba, A., Egan, R., Kim, T. W., Chokhawala, H., Schroth, G., Luo, S., Clark, D. S., Chen, F., Zhang, T., Mackie, R. I., Pennacchio, L. A., Tringe, S. G., Visel, A., Woyke,

T., Wang, Z. and Rubin, E. M. 2011. Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. *Science* 331(6016), 463–7. doi:10.1126/science.1200387.

3. Nayfach, S., Shi, Z. J., Seshadri, R., Pollard, K. S. and Kyrpides, N. C. 2019. New insights from uncultivated genomes of the global human gut microbiome. *Nature* 568(7753), 505–10. doi:10.1038/s41586-019-1058-x.
4. Pasolli, E., Asnicar, F., Manara, S., Zolfo, M., Karcher, N., Armanini, F., Beghini, F., Manghi, P., Tett, A., Ghensi, P., Collado, M. C., Rice, B. L., DuLong, C., Morgan, X. C., Golden, C. D., Quince, C., Huttenhower, C. and Segata, N. 2019. Extensive unexplored human microbiome diversity revealed by over 150,000 genomes from metagenomes spanning age, geography, and lifestyle. *Cell* 176(3), 649–662.e20. doi:10.1016/j.cell.2019.01.001.
5. Hoedt, E. C., Cuiv, P. Ó, Evans, P. N., Smith, W. J., McSweeney, C. S., Denman, S. E. and Morrison, M. 2016. Differences down-under: alcohol-fueled methanogenesis by archaea present in Australian macropodids. *ISME J.* 10(10), 2376–88. doi:10.1038/ismej.2016.41.
6. Davis, C. K., Webb, R. I., Sly, L. I., Denman, S. E. and McSweeney, C. S. 2012. Isolation and survey of novel fluoroacetate-degrading bacteria belonging to the phylum Synergistetes. *FEMS Microbiol. Ecol.* 80(3), 671–84. doi:10.1111/j.1574-6941.2012.01338.x.
7. Allison, M. J., Mayberry, W. R., McSweeney, C. S. and Stahl, D. A. 1992. *Synergistes jonesii*, gen. nov., sp.nov.: a rumen bacterium that degrades toxic pyridinediols. *Syst. Appl. Microbiol.* 15(4), 522–9. doi:10.1016/S0723-2020(11)80111-6.
8. Forster, S. C., Kumar, N., Anonye, B. O., Almeida, A., Viciani, E., Stares, M. D., Dunn, M., Mkandawire, T. T., Zhu, A., Shao, Y., Pike, L. J., Louie, T., Browne, H. P., Mitchell, A. L., Neville, B. A., Finn, R. D. and Lawley, T. D. 2019. A human gut bacterial genome and culture collection for improved metagenomic analyses. *Nat. Biotechnol.* 37(2), 186–92. doi:10.1038/s41587-018-0009-7.
9. Zou, Y., Xue, W., Luo, G., Deng, Z., Qin, P., Guo, R., Sun, H., Xia, Y., Liang, S., Dai, Y., Wan, D., Jiang, R., Su, L., Feng, Q., Jie, Z., Guo, T., Xia, Z., Liu, C., Yu, J., Lin, Y., Tang, S., Huo, G., Xu, X., Hou, Y., Liu, X., Wang, J., Yang, H., Kristiansen, K., Li, J., Jia, H. and Xiao, L. 2019. 1,520 reference genomes from cultivated human gut bacteria enable functional microbiome analyses. *Nat. Biotechnol.* 37(2), 179–85. doi:10.1038/s41587-018-0008-8.
10. Seshadri, R., Leahy, S. C., Attwood, G. T., Teh, K. H., Lambie, S. C., Cookson, A. L., Eloe-Fadrosh, E. A., Pavlopoulos, G. A., Hadjithomas, M., Varghese, N. J., Paez-Espino, D., Hungate1000 project collaborators, Perry, R., Henderson, G., Creevey, C. J., Terrapon, N., Lapebie, P., Drula, E., Lombard, V., Rubin, E., Kyrpides, N. C., Henrissat, B., Woyke, T., Ivanova, N. N. and Kelly, W. J. 2018. Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nat. Biotechnol.* 36(4), 359–67. doi:10.1038/nbt.4110.
11. Lagkouvardos, I., Pukall, R., Abt, B., Foesel, B. U., Meier-Kolthoff, J. P., Kumar, N., Bresciani, A., Martínez, I., Just, S., Ziegler, C., Brugiroux, S., Garzetti, D., Wenning, M., Bui, T. P., Wang, J., Hugenholtz, F., Plugge, C. M., Peterson, D. A., Hornef, M. W., Baines, J. F., Smidt, H., Walter, J., Kristiansen, K., Nielsen, H. B., Haller, D., Overmann, J., Stecher, B. and Clavel, T. 2016. The Mouse Intestinal Bacterial Collection (miBC) provides host-specific insight into cultured diversity and functional potential of the gut microbiota. *Nat. Microbiol.* 1(10), 16131. doi:10.1038/nmicrobiol.2016.131.

12. Martiny, A. C. 2019. High proportions of bacteria are culturable across major biomes. *ISME J.* 13(8), 2125–8. doi:10.1038/s41396-019-0410-3.
13. Lagkouvardos, I., Overmann, J. and Clavel, T. 2017. Cultured microbes represent a substantial fraction of the human and mouse gut microbiota. *Gut Microbes* 8(5), 493–503. doi:10.1080/19490976.2017.1320468.
14. Lloyd, K. G., Steen, A. D., Ladau, J., Yin, J. and Crosby, L. 2018. Phylogenetically novel uncultured microbial cells dominate earth microbiomes. *mSystems* 3(5), e00055-18. doi:10.1128/mSystems.00055-18.
15. Zehavi, T., Probst, M. and Mizrahi, I. 2018. Insights into culturomics of the rumen microbiome. *Front. Microbiol.* 9, 1999. doi:10.3389/fmicb.2018.01999.
16. Lau, J. T., Whelan, F. J., Herath, I., Lee, C. H., Collins, S. M., Bercik, P. and Surette, M. G. 2016. Capturing the diversity of the human gut microbiota through culture-enriched molecular profiling. *Genome Med.* 8(1), 72–. doi:10.1186/s13073-016-0327-7.
17. Almeida, A., Nayfach, S., Boland, M., Strozzi, F., Beracochea, M., Shi, Z. J., Pollard, K. S., Parks, D. H., Hugenholtz, P., Segata, N., Kyrpides, N. C. and Finn, R. D. 2019. A unified sequence catalogue of over 280,000 genomes obtained from the human gut microbiome. *bioRxiv*, 762682.
18. He, X., McLean, J. S., Edlund, A., Yooseph, S., Hall, A. P., Liu, S. Y., Dorrestein, P. C., Esquenazi, E., Hunter, R. C., Cheng, G., Nelson, K. E., Lux, R. and Shi, W. 2015. Cultivation of a human-associated TM7 phylotype reveals a reduced genome and epibiotic parasitic lifestyle. *Proc. Natl. Acad. Sci. U. S. A.* 112(1), 244–9. doi:10.1073/pnas.1419038112.
19. Stewart, E. J. 2012. Growing unculturable bacteria. *J. Bacteriol.* 194(16), 4151–60. doi:10.1128/JB.00345-12.
20. Vartoukian, S. R., Palmer, R. M. and Wade, W. G. 2010. Strategies for culture of 'unculturable' bacteria. *FEMS Microbiol. Lett.* 309(1), 1–7. doi:10.1111/j.1574-6968.2010.02000.x.
21. Clavel, T., Lagkouvardos, I. and Stecher, B. 2017. From complex gut communities to minimal microbiomes via cultivation. *Curr. Opin. Microbiol.* 38, 148–55. doi:10.1016/j.mib.2017.05.013.
22. Theodorou, M. K., Trinci, A. P. J. and Brookman, J. 2005. Anaerobic fungi. In: Makkar, H. P. S. and McSweeney, C. S. (Eds), *Methods in Gut Microbial Ecology for Ruminants*. Springer Netherlands, Dordrecht, pp. 55–66.
23. Haitjema, C. H., Solomon, K. V., Henske, J. K., Theodorou, M. K. and O'Malley, M. A. 2014. Anaerobic gut fungi: advances in isolation, culture, and cellulolytic enzyme discovery for biofuel production. *Biotechnol. Bioeng.* 111(8), 1471–82. doi:10.1002/bit.25264.
24. Gilbert, R. A. and Klieve, A. V. 2015. Ruminal viruses (Bacteriophages, Archaeaphages). In: Puniya, A. K., Singh, R. and Kamra, D. N. (Eds), *Rumen Microbiology: From Evolution to Revolution*. Springer, New Delhi India, pp. 121–41.
25. Newbold, C. J., de la Fuente, G., Belanche, A., Ramos-Morales, E. and McEwan, N. R. 2015. The role of ciliate protozoa in the rumen. *Front. Microbiol.* 6(1313), 1313. doi:10.3389/fmicb.2015.01313.
26. McSweeney, C. S., Denman, S. E. and Mackie, R. I. 2005. Rumen bacteria. In: Makkar, H. P. S. and McSweeney, C. S. (Eds), *Methods in Gut Microbial Ecology for Ruminants*. Springer, Dordrecht, pp. 23–37.
27. Joblin, K. N. 2005. Methanogenic archaea. In: Makkar, H. P. S. and McSweeney, C. S. (Eds), *Methods in Gut Microbial Ecology for Ruminants*. Springer Netherlands, Dordrecht, pp. 47–53.

28. Eller, C., Crabill, M. R. and Bryant, M. P. 1971. Anaerobic roll tube media for nonselective enumeration and isolation of bacteria in human feces. *Appl. Microbiol.* 22(4), 522–9. doi:10.1128/AEM.22.4.522-529.1971.
29. Hungate, R. E. 1969. A roll tube method for cultivation of strict anaerobes. *Methods in Microbiology* 3(Part B), 117–32.
30. Stewart, C. S., Flint, H. J. and Bryant, M. P. 1997. The rumen bacteria. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem*. Springer Netherlands, Dordrecht, pp. 10–72.
31. Einarsson, H., Snygg, B. G. and Eriksson, C. 1983. Inhibition of bacterial growth by Maillard reaction products. *J. Agric. Food Chem.* 31(5), 1043–7. doi:10.1021/jf00119a031.
32. Nyonyo, T., Shinkai, T., Tajima, A. and Mitsumori, M. 2013. Effect of media composition, including gelling agents, on isolation of previously uncultured rumen bacteria. *Lett. Appl. Microbiol.* 56(1), 63–70. doi:10.1111/lam.12019.
33. Kenters, N., Henderson, G., Jeyanathan, J., Kittelmann, S. and Janssen, P. H. 2011. Isolation of previously uncultured rumen bacteria by dilution to extinction using a new liquid culture medium. *J. Microbiol. Methods* 84(1), 52–60. doi:10.1016/j.mimet.2010.10.011.
34. Tanaka, T., Kawasaki, K., Daimon, S., Kitagawa, W., Yamamoto, K., Tamaki, H., Tanaka, M., Nakatsu, C. H. and Kamagata, Y. 2014. A hidden pitfall in the preparation of agar media undermines microorganism cultivability. *Appl. Environ. Microbiol.* 80(24), 7659–66. doi:10.1128/AEM.02741-14.
35. Kawasaki, K. and Kamagata, Y. 2017. Phosphate-catalyzed hydrogen peroxide formation from agar, gellan, and kappa-carrageenan and recovery of microbial cultivability via catalase and pyruvate. *Appl. Environ. Microbiol.* 83(21). doi:10.1128/AEM.01366-17.
36. Das, N., Tripathi, N., Basu, S., Bose, C., Maitra, S. and Khurana, S. 2015. Progress in the development of gelling agents for improved culturability of microorganisms. *Front. Microbiol.* 6, 698. doi:10.3389/fmicb.2015.00698.
37. Rettedal, E. A., Gumpert, H. and Sommer, M. O. A. 2014. Cultivation-based multiplex phenotyping of human gut microbiota allows targeted recovery of previously uncultured bacteria. *Nat. Commun.* 5, 4714. doi:10.1038/ncomms5714.
38. Cray, J. A., Bell, A. N., Bhaganna, P., Mswaka, A. Y., Timson, D. J. and Hallsworth, J. E. 2013. The biology of habitat dominance; can microbes behave as weeds? *Microb. Biotechnol.* 6(5), 453–92. doi:10.1111/1751-7915.12027.
39. Zengler, K. and Zaramela, L. S. 2018. The social network of microorganisms – how auxotrophies shape complex communities. *Nat. Rev. Microbiol.* 16(6), 383–90. doi:10.1038/s41579-018-0004-5.
40. Mee, M. T., Collins, J. J., Church, G. M. and Wang, H. H. 2014. Syntrophic exchange in synthetic microbial communities. *Proc. Natl. Acad. Sci. U. S. A.* 111(20), E2149–56. doi:10.1073/pnas.1405641111.
41. Sharma, V., Rodionov, D. A., Leyn, S. A., Tran, D., Iablokov, S. N., Ding, H., Peterson, D. A., Osterman, A. L. and Peterson, S. N. 2019. B-vitamin sharing promotes stability of gut microbial communities. *Front. Microbiol.* 10(1485), 1485. doi:10.3389/fmicb.2019.01485.
42. Richardson, A. J., McKain, N. and Wallace, R. J. 2013. Ammonia production by human faecal bacteria, and the enumeration, isolation and characterization of

bacteria capable of growth on peptides and amino acids. *BMC Microbiol.* 13, 6. doi:10.1186/1471-2180-13-6.
43. Oliphant, K. and Allen-Vercoe, E. 2019. Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. *Microbiome* 7(1), 91–. doi:10.1186/s40168-019-0704-8.
44. Wallace, R. J. 1996. Ruminal microbial metabolism of peptides and amino acids. *J. Nutr.* 126(4 Suppl.), 1326S–34S. doi:10.1093/jn/126.suppl_4.1326S.
45. Pittman, K. A. and Bryant, M. P. 1964. Peptides and other nitrogen sources for growth of *Bacteroides ruminicola*. *J. Bacteriol.* 88, 401–10. doi:10.1128/JB.88.2.401-410.1964.
46. Atasoglu, C., Valdés, C., Walker, N. D., Newbold, C. J. and Wallace, R. J. 1998. *De novo* synthesis of amino acids by the ruminal bacteria *Prevotella bryantii* B14, *Selenomonas ruminantium* HD4, and *Streptococcus bovis* ES1. *Appl. Environ. Microbiol.* 64(8), 2836–43. doi:10.1128/AEM.64.8.2836-2843.1998.
47. Leong, L. E., Denman, S. E., Hugenholtz, P. and McSweeney, C. S. 2016. Amino acid and peptide utilization profiles of the fluoroacetate-degrading bacterium synergistetes strain MFA1 under varying conditions. *Microb. Ecol.* 71(2), 494–504. doi:10.1007/s00248-015-0641-4.
48. Chen, G. and Russell, J. B. 1989. More monensin-sensitive, ammonia-producing bacteria from the rumen. *Appl. Environ. Microbiol.* 55(5), 1052–7. doi:10.1128/AEM.55.5.1052-1057.1989.
49. Ling, J. R. and Armstead, I. P. 1995. The *in vitro* uptake and metabolism of peptides and amino acids by five species of rumen bacteria. *J. Appl. Bacteriol.* 78(2), 116–24. doi:10.1111/j.1365-2672.1995.tb02831.x.
50. Gray, V. L., Muller, C. T., Watkins, I. D. and Lloyd, D. 2008. Peptones from diverse sources: pivotal determinants of bacterial growth dynamics. *J. Appl. Microbiol.* 104(2), 554–65. doi:10.1111/j.1365-2672.2007.03577.x.
51. Hinton, A. 2016. Growth of *Campylobacter* incubated aerobically in fumarate-pyruvate media or media supplemented with dairy, meat, or soy extracts and peptones. *Food Microbiol.* 58, 23–8. doi:10.1016/j.fm.2016.03.010.
52. Schär-Zammaretti, P., Dillmann, M. L., D'Amico, N., Affolter, M. and Ubbink, J. 2005. Influence of fermentation medium composition on physicochemical surface properties of *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.* 71(12), 8165–73. doi:10.1128/AEM.71.12.8165-8173.2005.
53. Tramontano, M., Andrejev, S., Pruteanu, M., Klünemann, M., Kuhn, M., Galardini, M., Jouhten, P., Zelezniak, A., Zeller, G., Bork, P., Typas, A. and Patil, K. R. 2018. Nutritional preferences of human gut bacteria reveal their metabolic idiosyncrasies. *Nat. Microbiol.* 3(4), 514–22. doi:10.1038/s41564-018-0123-9.
54. Zhang, H., Yohe, T., Huang, L., Entwistle, S., Wu, P., Yang, Z., Busk, P. K., Xu, Y. and Yin, Y. 2018. dbCAN2: a meta server for automated carbohydrate-active enzyme annotation. *Nucleic Acids Res.* 46(W1), W95–W101. doi:10.1093/nar/gky418.
55. Jones, D. R., Thomas, D., Alger, N., Ghavidel, A., Inglis, G. D. and Abbott, D. W. 2018. SACCHARIS: an automated pipeline to streamline discovery of carbohydrate active enzyme activities within polyspecific families and de novo sequence datasets. *Biotechnol. Biofuels* 11(1), 27. doi:10.1186/s13068-018-1027-x.
56. Smith, E. A. and Macfarlane, G. T. 1998. Enumeration of amino acid fermenting bacteria in the human large intestine: effects of pH and starch on peptide metabolism and dissimilation of amino acids. *FEMS Microbiol. Ecol.* 25(4), 355–68. doi:10.1111/j.1574-6941.1998.tb00487.x.

57. Watanabe, Y., Nagai, F. and Morotomi, M. 2012. Characterization of *Phascolarctobacterium succinatutens* sp. nov., an asaccharolytic, succinate-utilizing bacterium isolated from human feces. *Appl. Environ. Microbiol.* 78(2), 511–8. doi:10.1128/AEM.06035-11.
58. Shigeno, Y., Kitahara, M., Shime, M. and Benno, Y. 2019. *Phascolarctobacterium wakonense* sp. nov., isolated from common marmoset (*Callithrix jacchus*) faeces. *Int. J. Syst. Evol. Microbiol.* 69(7), 1941–6. doi:10.1099/ijsem.0.003407.
59. Braune, A. and Blaut, M. 2018. *Catenibacillus scindens* gen. nov., sp. nov., a C-deglycosylating human intestinal representative of the *Lachnospiraceae*. *Int. J. Syst. Evol. Microbiol.* 68(10), 3356–61. doi:10.1099/ijsem.0.003001.
60. Kelly, W. J., Henderson, G., Pacheco, D. M., Li, D., Reilly, K., Naylor, G. E., Janssen, P. H., Attwood, G. T., Altermann, E. and Leahy, S. C. 2016. The complete genome sequence of *Eubacterium limosum* SA11, a metabolically versatile rumen acetogen. *Stand. Genom. Sci.* 11, 26. doi:10.1186/s40793-016-0147-9.
61. Gagen, E. J., Wang, J., Padmanabha, J., Liu, J., de Carvalho, I. P., Liu, J., Webb, R. I., Al Jassim, R., Morrison, M., Denman, S. E. and McSweeney, C. S. 2014. Investigation of a new acetogen isolated from an enrichment of the tammar wallaby forestomach. *BMC Microbiol.* 14, 314. doi:10.1186/s12866-014-0314-3.
62. Gaci, N., Borrel, G., Tottey, W., O'Toole, P. W. and Brugère, J. F. 2014. Archaea and the human gut: new beginning of an old story. *World J. Gastroenterol.* 20(43), 16062–78. doi:10.3748/wjg.v20.i43.16062.
63. Xavier, J. C., Patil, K. R. and Rocha, I. 2017. Integration of biomass formulations of genome-scale metabolic models with experimental data reveals universally essential cofactors in prokaryotes. *Metab. Eng.* 39, 200–8. doi:10.1016/j.ymben.2016.12.002.
64. Ternan, N. G., Moore, N. D., Smyth, D., McDougall, G. J., Allwood, J. W., Verrall, S., Gill, C. I. R., Dooley, J. S. G. and McMullan, G. 2018. Increased sporulation underpins adaptation of *Clostridium difficile* strain 630 to a biologically–relevant faecal environment, with implications for pathogenicity. *Sci. Rep.* 8(1), 16691. doi:10.1038/s41598-018-35050-x.
65. Allison, M. J., Bryant, M. P. and Doetsch, R. N. 1958. Volatile fatty acid growth factor for cellulolytic cocci of bovine rumen. *Science* 128(3322), 474–5. doi:10.1126/science.128.3322.474.
66. Bryant, M. P. and Robinson, I. M. 1962. Some nutritional characteristics of predominant culturable ruminal bacteria. *J. Bacteriol.* 84, 605–14. doi:10.1128/JB.84.4.605-614.1962.
67. Hungate, R. E. and Stack, R. J. 1982. Phenylpropanoic acid: growth factor for *Ruminococcus albus*. *Appl. Environ. Microbiol.* 44(1), 79–83. doi:10.1128/AEM.44.1.79-83.1982.
68. Stack, R. J., Hungate, R. E. and Opsahl, W. P. 1983. Phenylacetic acid stimulation of cellulose digestion by *Ruminococcus albus* 8. *Appl. Environ. Microbiol.* 46(3), 539–44. doi:10.1128/AEM.46.3.539-544.1983.
69. Dehority, B. A. 1971. Carbon dioxide requirement of various species of rumen bacteria. *J. Bacteriol.* 105(1), 70–6. doi:10.1128/JB.105.1.70-76.1971.
70. Reilly, S. 1980. The carbon dioxide requirements of anaerobic bacteria. *J. Med. Microbiol.* 13(4), 573–9. doi:10.1099/00222615-13-4-573.
71. Fenn, K., Strandwitz, P., Stewart, E. J., Dimise, E., Rubin, S., Gurubacharya, S., Clardy, J. and Lewis, K. 2017. Quinones are growth factors for the human gut microbiota. *Microbiome* 5(1), 161. doi:10.1186/s40168-017-0380-5.

72. Strandwitz, P., Kim, K. H., Terekhova, D., Liu, J. K., Sharma, A., Levering, J., McDonald, D., Dietrich, D., Ramadhar, T. R., Lekbua, A., Mroue, N., Liston, C., Stewart, E. J., Dubin, M. J., Zengler, K., Knight, R., Gilbert, J. A., Clardy, J. and Lewis, K. 2019. GABA-modulating bacteria of the human gut microbiota. *Nat. Microbiol.* 4(3), 396–403. doi:10.1038/s41564-018-0307-3.
73. Vartoukian, S. R., Adamowska, A., Lawlor, M., Moazzez, R., Dewhirst, F. E. and Wade, W. G. 2016. *In vitro* cultivation of 'Unculturable' oral bacteria, facilitated by community culture and media supplementation with siderophores. *PLoS ONE* 11(1), e0146926. doi:10.1371/journal.pone.0146926.
74. Jones, G. A. and Pickard, M. D. 1980. Effect of titanium (III) citrate as reducing agent on growth of rumen bacteria. *Appl. Environ. Microbiol.* 39(6), 1144–7. doi:10.1128/AEM.39.6.1144-1147.1980.
75. Zehnder, A. J. and Wuhrmann, K. 1976. Titanium (III) citrate as a nontoxic oxidation-reduction buffering system for the culture of obligate anaerobes. *Science* 194(4270), 1165–6. doi:10.1126/science.793008.
76. La Scola, B., Khelaifia, S., Lagier, J. C. and Raoult, D. 2014. Aerobic culture of anaerobic bacteria using antioxidants: a preliminary report. *Eur. J. Clin. Microbiol. Infect. Dis.* 33(10), 1781–3. doi:10.1007/s10096-014-2137-4.
77. Dione, N., Khelaifia, S., La Scola, B., Lagier, J. C. and Raoult, D. 2016. A quasi-universal medium to break the aerobic/anaerobic bacterial culture dichotomy in clinical microbiology. *Clin. Microbiol. Infect.* 22(1), 53–8. doi:10.1016/j.cmi.2015.10.032.
78. Hwang, C.-F., Chang, J.-H., Houng, J.-Y., Tsai, C.-C., Lin, C.-K. and Tsen, H.-Y. 2012. Optimization of medium composition for improving biomass production of *Lactobacillus plantarum* Pi06 using the Taguchi array design and the Box-Behnken method. *Biotechnol. Bioprocess Eng.* 17(4), 827–34. doi:10.1007/s12257-012-0007-4.
79. Khelaifia, S., Lagier, J. C., Nkamga, V. D., Guilhot, E., Drancourt, M. and Raoult, D. 2016. Aerobic culture of methanogenic archaea without an external source of hydrogen. *Eur. J. Clin. Microbiol. Infect. Dis.* 35(6), 985–91. doi:10.1007/s10096-016-2627-7.
80. Khelaifia, S., Raoult, D. and Drancourt, M. 2013. A versatile medium for cultivating methanogenic archaea. *PLoS ONE* 8(4), e61563. doi:10.1371/journal.pone.0061563.
81. Le Roy, T., Van der Smissen, P., Paquot, A., Delzenne, N., Muccioli, G. G., Collet, J. F. and Cani, P. D. 2019. *Dysosmobacter welbionis* gen. nov., sp. nov., isolated from human faeces and emended description of the genus *Oscillibacter*. *Int. J. Syst. Evol. Microbiol.*. doi:10.1099/ijsem.0.003547.
82. Pulschen, A. A., Bendia, A. G., Fricker, A. D., Pellizari, V. H., Galante, D. and Rodrigues, F. 2017. Isolation of uncultured bacteria from Antarctica using long incubation periods and low nutritional media. *Front. Microbiol.* 8, 1346. doi:10.3389/fmicb.2017.01346.
83. Kato, S., Yamagishi, A., Daimon, S., Kawasaki, K., Tamaki, H., Kitagawa, W., Abe, A., Tanaka, M., Sone, T., Asano, K. and Kamagata, Y. 2018. Isolation of previously uncultured slow-growing bacteria by using a simple modification in the preparation of agar media. *Appl. Environ. Microbiol.* 84(19), e00807-18. doi:10.1128/AEM.00807-18.
84. Bartelme, R. P., Custer, J. M., Dupont, C. L., Espinoza, J. L., Torralba, M., Khalili, B. and Carini, P. 2019. The concentration of heterotrophic growth substrates in culture

medium is a crucial determinant of the culturability of subsurface soil microbes. *bioRxiv*:726661.

85. Braun, P. R., Al-Younes, H., Gussmann, J., Klein, J., Schneider, E. and Meyer, T. F. 2008. Competitive inhibition of amino acid uptake suppresses chlamydial growth: involvement of the chlamydial amino acid transporter BrnQ. *J. Bacteriol.* 190(5), 1822–30. doi:10.1128/JB.01240-07.
86. Ito, T., Sekizuka, T., Kishi, N., Yamashita, A. and Kuroda, M. 2019. Conventional culture methods with commercially available media unveil the presence of novel culturable bacteria. *Gut Microbes* 10(1), 77–91. doi:10.1080/19490976.2018.1491265.
87. Lagier, J. C., Khelaifia, S., Alou, M. T., Ndongo, S., Dione, N., Hugon, P., Caputo, A., Cadoret, F., Traore, S. I., Seck, E. H., Dubourg, G., Durand, G., Mourembou, G., Guilhot, E., Togo, A., Bellali, S., Bachar, D., Cassir, N., Bittar, F., Delerce, J., Mailhe, M., Ricaboni, D., Bilen, M., Dangui Nieko, N. P., Dia Badiane, N. M., Valles, C., Mouelhi, D., Diop, K., Million, M., Musso, D., Abrahão, J., Azhar, E. I., Bibi, F., Yasir, M., Diallo, A., Sokhna, C., Djossou, F., Vitton, V., Robert, C., Rolain, J. M., La Scola, B., Fournier, P. E., Levasseur, A. and Raoult, D. 2016. Culture of previously uncultured members of the human gut microbiota by culturomics. *Nat. Microbiol.* 1, 16203. doi:10.1038/nmicrobiol.2016.203.
88. Rao, R. S., Kumar, C. G., Prakasham, R. S. and Hobbs, P. J. 2008. The Taguchi methodology as a statistical tool for biotechnological applications: a critical appraisal. *Biotechnol. J.* 3(4), 510–23. doi:10.1002/biot.200700201.
89. Manzoor, A., Qazi, J. I., Haq, I. U., Mukhtar, H. and Rasool, A. 2017. Significantly enhanced biomass production of a novel bio-therapeutic strain Lactobacillus plantarum (AS-14) by developing low cost media cultivation strategy. *J. Biol. Eng.* 11(1), 17. doi:10.1186/s13036-017-0059-2.
90. Li, L., Zhang, X., Ning, Z., Mayne, J., Moore, J. I., Butcher, J., Chiang, C. K., Mack, D., Stintzi, A. and Figeys, D. 2018. Evaluating *in vitro* culture medium of gut microbiome with orthogonal experimental design and a metaproteomics approach. *J. Proteome Res.* 17(1), 154–63. doi:10.1021/acs.jproteome.7b00461.
91. Poeker, S. A., Lacroix, C., de Wouters, T., Spalinger, M. R., Scharl, M. and Geirnaert, A. 2019. Stepwise development of an *in vitro* continuous fermentation model for the murine caecal microbiota. *Front. Microbiol.* 10(1166), 1166. doi:10.3389/fmicb.2019.01166.
92. Fraser, C. M., Gocayne, J. D., White, O., Adams, M. D., Clayton, R. A., Fleischmann, R. D., Bult, C. J., Kerlavage, A. R., Sutton, G., Kelley, J. M., Fritchman, R. D., Weidman, J. F., Small, K. V., Sandusky, M., Fuhrmann, J., Nguyen, D., Utterback, T. R., Saudek, D. M., Phillips, C. A., Merrick, J. M., Tomb, J. F., Dougherty, B. A., Bott, K. F., Hu, P. C., Lucier, T. S., Peterson, S. N., Smith, H. O., Hutchison, C. A. and Venter, J. C. 1995. The minimal gene complement of *Mycoplasma genitalium*. *Science* 270(5235), 397–403. doi:10.1126/science.270.5235.397.
93. Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A. and Merrick, J. M., McKenney, K., Granger, S., FitzHugh, W., Fields, C., Gocyne, J. D., Scott, J., Shirley, R., Li-Ing, L., Glodek, A., Kelley, J. M., Weidman, J. F., Phillips, C. A., Spriggs, T., Hedblom, E., Cotton, M. D., Utterback, T. R., Hanna, M. C., Nguyen, D. T., Saudek, D. M., Brandon, R. C., Fine, L. D., Fritchman, J. L., Fuhrmann, J. L., Geoghagen, N. S. M., Gnehm, C. L., McDonald, L. A., Small, K. V., Fraser, C. M., Smith, H. O. and Venter, J. C. 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* 269(5223), 496–512. doi:10.1126/science.7542800.

94. Tyson, G. W., Lo, I., Baker, B. J., Allen, E. E., Hugenholtz, P. and Banfield, J. F. 2005. Genome-directed isolation of the key nitrogen fixer *Leptospirillum ferrodiazotrophum* sp. nov. from an acidophilic microbial community. *Appl. Environ. Microbiol.* 71(10), 6319–24. doi:10.1128/AEM.71.10.6319-6324.2005.
95. León, M. J., Fernández, A. B., Ghai, R., Sánchez-Porro, C., Rodriguez-Valera, F. and Ventosa, A. 2014. From metagenomics to pure culture: isolation and characterization of the moderately halophilic bacterium *Spiribacter salinus* gen. nov., sp. nov. *Appl. Environ. Microbiol.* 80(13), 3850–7. doi:10.1128/AEM.00430-14.
96. Wurch, L., Giannone, R. J., Belisle, B. S., Swift, C., Utturkar, S., Hettich, R. L., Reysenbach, A. L. and Podar, M. 2016. Genomics-informed isolation and characterization of a symbiotic Nanoarchaeota system from a terrestrial geothermal environment. *Nat. Commun.* 7, 12115–. doi:10.1038/ncomms12115.
97. Pope, P. B., Smith, W., Denman, S. E., Tringe, S. G., Barry, K., Hugenholtz, P., McSweeney, C. S., McHardy, A. C. and Morrison, M. 2011. Isolation of *Succinivibrionaceae* implicated in low methane emissions from *Tammar wallabies*. *Science* 333(6042), 646–8. doi:10.1126/science.1205760.
98. Bomar, L., Maltz, M., Colston, S. and Graf, J. 2011. Directed culturing of microorganisms using metatranscriptomics. *mBio* 2(2), e00012–11. doi:10.1128/mBio.00012-11.
99. Omsland, A., Cockrell, D. C., Howe, D., Fischer, E. R., Virtaneva, K., Sturdevant, D. E., Porcella, S. F. and Heinzen, R. A. 2009. Host cell-free growth of the Q fever bacterium *Coxiella burnetii*. *Proc. Natl Acad. Sci. U. S. A.* 106(11), 4430–4. doi:10.1073/pnas.0812074106.
100. Xiao, H., Liu, B., Yong, J. and Zhou, H. 2019. Quantitative analysis and medium components optimizing for culturing a fastidious bacterium *Christensenella minuta*. *bioRxiv*:632836.
101. Gtari, M., Ghodhbane-Gtari, F., Nouioui, I., Ktari, A., Hezbri, K., Mimouni, W., Sbissi, I., Ayari, A., Yamanaka, T., Normand, P., Tisa, L. S. and Boudabous, A. 2015. Cultivating the uncultured: growing the recalcitrant cluster-2 Frankia strains. *Sci. Rep.* 5, 13112. doi:10.1038/srep13112.
102. Weimann, A., Mooren, K., Frank, J., Pope, P. B., Bremges, A. and McHardy, A. C. 2016. From genomes to phenotypes: traitar, the microbial trait analyzer. *mSystems* 1(6). doi:10.1128/mSystems.00101-16.
103. Feldbauer, R., Schulz, F., Horn, M. and Rattei, T. 2015. Prediction of microbial phenotypes based on comparative genomics. *BMC Bioinformatics* 16(S1). doi:10.1186/1471-2105-16-S14-S1.
104. MacDonald, N. J. and Beiko, R. G. 2010. Efficient learning of microbial genotype-phenotype association rules. *Bioinformatics* 26(15), 1834–40. doi:10.1093/bioinformatics/btq305.
105. van der Ark, K. C. H., van Heck, R. G. A., Martins Dos Santos, V. A. P., Belzer, C. and de Vos, W. M. 2017. More than just a gut feeling: constraint-based genome-scale metabolic models for predicting functions of human intestinal microbes. *Microbiome* 5(1), 78. doi:10.1186/s40168-017-0299-x.
106. Sen, P. and Orešič, M. 2019. Metabolic modeling of human gut microbiota on a genome scale: an overview. *Metabolites* 9(2), 22. doi:10.3390/metabo9020022.
107. Magnúsdóttir, S., Heinken, A., Kutt, L., Ravcheev, D. A., Bauer, E., Noronha, A., Greenhalgh, K., Jäger, C., Baginska, J., Wilmes, P., Fleming, R. M. and Thiele, I. 2017.

Generation of genome-scale metabolic reconstructions for 773 members of the human gut microbiota. *Nat. Biotechnol.* 35(1), 81–9. doi:10.1038/nbt.3703.

108. Heinken, A., Khan, M. T., Paglia, G., Rodionov, D. A., Harmsen, H. J. M. and Thiele, I. 2014. Functional metabolic map of *Faecalibacterium prausnitzii*, a beneficial human gut microbe. *J. Bacteriol.* 196(18), 3289–302. doi:10.1128/JB.01780-14.
109. Ottman, N., Davids, M., Suarez-Diez, M., Boeren, S., Schaap, P. J., Martins Dos Santos, V. A. P., Smidt, H., Belzer, C. and de Vos, W. M. 2017. Genome-scale model and omics analysis of metabolic capacities of *Akkermansia muciniphila* reveal a preferential mucin-degrading lifestyle. *Appl. Environ. Microbiol.* 83(18). doi:10.1128/AEM.01014-17.
110. Gutleben, J., Chaib De Mares, M., van Elsas, J. D., Smidt, H., Overmann, J. and Sipkema, D. 2018. The multi-omics promise in context: from sequence to microbial isolate. *Crit. Rev. Microbiol.* 44(2), 212–29. doi:10.1080/1040841X.2017.1332003.
111. Zhang, A. N., Mao, Y., Wang, Y. and Zhang, T. 2019. Mining traits for the enrichment and isolation of not-yet-cultured populations. *Microbiome* 7(1), 96. doi:10.1186/s40168-019-0708-4.
112. Ó Cuív, P. Ó, Smith, W. J., Pottenger, S., Burman, S., Shanahan, E. R. and Morrison, M. 2015. Isolation of genetically tractable most-wanted bacteria by metaparental mating. *Sci. Rep.* 5, 13282. doi:10.1038/srep13282.
113. Ó Cuív, P., Burman, S., Pottenger, S. and Morrison, M. 2016. Exploring the bioactive landscape of the gut microbiota to identify metabolites underpinning human health. In: Beale, D. J., Kouremenos, K. A. and Palombo, E. A. (Eds), *Microbial Metabolomics: Applications in Clinical, Environmental, and Industrial Microbiology*. Springer International Publishing, Cham, pp. 49–82.
114. Ó Cuív, P., Giri, R., Hoedt, E. C., McGuckin, M. A., Begun, J. and Morrison, M. 2018. *Enterococcus faecalis* AHG0090 is a genetically tractable bacterium and produces a secreted peptidic bioactive that suppresses nuclear factor kappa B activation in human gut epithelial cells. *Front. Immunol.* 9(790), 790. doi:10.3389/fimmu.2018.00790.
115. Heap, J. T., Kuehne, S. A., Ehsaan, M., Cartman, S. T., Cooksley, C. M., Scott, J. C. and Minton, N. P. 2010. The ClosTron: mutagenesis in *Clostridium* refined and streamlined. *J. Microbiol. Methods* 80(1), 49–55. doi:10.1016/j.mimet.2009.10.018.
116. Horn, N., Carvalho, A. L., Overweg, K., Wegmann, U., Carding, S. R. and Stentz, R. 2016. A novel tightly regulated gene expression system for the human intestinal symbiont *Bacteroides thetaiotaomicron*. *Front. Microbiol.* 7(1080), 1080. doi:10.3389/fmicb.2016.01080.
117. Mimee, M., Tucker, A., Voigt, C. and Lu, T. 2015. Programming a human commensal bacterium, *Bacteroides thetaiotaomicron*, to sense and respond to stimuli in the murine gut microbiota. *Cell Syst.* 1(1), 62–71. doi:10.1016/j.cels.2015.06.001.
118. Ronda, C., Chen, S. P., Cabral, V., Yaung, S. J. and Wang, H. H. 2019. Metagenomic engineering of the mammalian gut microbiome *in situ*. *Nat. Methods* 16(2), 167–70. doi:10.1038/s41592-018-0301-y.
119. Brophy, J. A. N., Triassi, A. J., Adams, B. L., Renberg, R. L., Stratis-Cullum, D. N., Grossman, A. D. and Voigt, C. A. 2018. Engineered integrative and conjugative elements for efficient and inducible DNA transfer to undomesticated bacteria. *Nat. Microbiol.* 3(9), 1043–53. doi:10.1038/s41564-018-0216-5.

120. García-Bayona, L. and Comstock, L. E. 2019. Streamlined genetic manipulation of diverse bacteroides and parabacteroides isolates from the human gut microbiota. *mBio* 10(4), e01762-19. doi:10.1128/mBio.01762-19.
121. Enzmann, F., Mayer, F., Rother, M. and Holtmann, D. 2018. Methanogens: biochemical background and biotechnological applications. *AMB Express* 8(1), 1–. doi:10.1186/s13568-017-0531-x.
122. Olsvik, O., Popovic, T., Skjerve, E., Cudjoe, K. S., Hornes, E., Ugelstad, J. and Uhlén, M. 1994. Magnetic separation techniques in diagnostic microbiology. *Clin. Microbiol. Rev.* 7(1), 43–54. doi:10.1128/cmr.7.1.43.
123. Palm, N. W., de Zoete, M. R., Cullen, T. W., Barry, N. A., Stefanowski, J., Hao, L., Degnan, P. H., Hu, J., Peter, I., Zhang, W., Ruggiero, E., Cho, J. H., Goodman, A. L. and Flavell, R. A. 2014. Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell* 158(5), 1000–10. doi:10.1016/j.cell.2014.08.006.
124. Torensma, R., Visser, M. J., Aarsman, C. J., Poppelier, M. J., van Beurden, R., Fluit, A. C. and Verhoef, J. 1992. Monoclonal antibodies that detect live salmonellae. *Appl. Environ. Microbiol.* 58(12), 3868–72. doi:10.1128/AEM.58.12.3868-3872.1992.
125. Armstrong, H., Alipour, M., Valcheva, R., Bording-Jorgensen, M., Jovel, J., Zaidi, D., Shah, P., Lou, Y., Ebeling, C., Mason, A. L., Lafleur, D., Jerasi, J., Wong, G. K., Madsen, K., Carroll, M. W., Huynh, H. Q., Dieleman, L. A. and Wine, E. 2019. Host immunoglobulin G selectively identifies pathobionts in pediatric inflammatory bowel diseases. *Microbiome* 7(1), 1. doi:10.1186/s40168-018-0604-3.
126. Lund, A., Hellemann, A. L. and Vartdal, F. 1988. Rapid isolation of K88+ *Escherichia coli* by using immunomagnetic particles. *J. Clin. Microbiol.* 26(12), 2572–5. doi:10.1128/JCM.26.12.2572-2575.1988.
127. Okrend, A. J. G., Rose, B. E. and Lattuada, C. P. 1992. Isolation of *Escherichia coli* 0157:H7 using 0157 specific antibody coated magnetic beads. *J. Food Prot.* 55(3), 214–7. doi:10.4315/0362-028X-55.3.214.
128. Close, D. W., Ferrara, F., Dichosa, A. E. K., Kumar, S., Daughton, A. R., Daligault, H. E., Reitenga, K. G., Velappan, N., Sanchez, T. C., Iyer, S., Kiss, C., Han, C. S. and Bradbury, A. R. 2013. Using phage display selected antibodies to dissect microbiomes for complete de novo genome sequencing of low abundance microbes. *BMC Microbiol.* 13(1), 270. doi:10.1186/1471-2180-13-270.
129. Lou, J., Marzari, R., Verzillo, V., Ferrero, F., Pak, D., Sheng, M., Yang, C., Sblattero, D. and Bradbury, A. 2001. Antibodies in haystacks: how selection strategy influences the outcome of selection from molecular diversity libraries. *J. Immunol. Methods* 253(1–2), 233–42. doi:10.1016/s0022-1759(01)00385-4.
130. Cross, K. L., Campbell, J. H., Balachandran, M., Campbell, A. G., Cooper, S. J., Griffen, A., Heaton, M., Joshi, S., Klingeman, D., Leys, E., Yang, Z., Parks, J. M. and Podar, M. 2019. Targeted isolation and cultivation of uncultivated bacteria by reverse genomics. *Nat. Biotechnol.* 37(11), 1314–21. doi:10.1038/s41587-019-0260-6.

Chapter 3

Rumen metabolomics – a powerful tool for discovery and understanding of rumen functionality and health

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1 Introduction

The rumen is a complex ecosystem comprised of microorganisms including bacteria, archaea, fungi and protozoa (de Almeida et al., 2018). In recent years our understanding of the immense impact the gut microbiome has on host performance, health and well-being has come to light. While the majority of studies in the field of microbiome science to date have been conducted in a human context, much of the observed impacts are transferable to animals. Indeed, the gut microbiome has been described as a virtual endocrine organ in the context of both humans (O'Hara and Shanahan, 2006) and domesticated animals (O'Callaghan et al., 2016). This is as a result of its ability to impact the functioning of both local (within the gut) and distal organs and systems throughout the body, such as host metabolism, brain and behaviour, liver function, cardiovascular system, enteric nervous system and immune system (O'Callaghan et al., 2016).

Currently at ~7.5 billion people, the world population is increasing, with that so too does the global demand for food. The rumen provides ruminants with the ability to digest materials that are indigestible for humans, such as cellulose-rich feedstuffs. This digestion produces metabolite substrates for rumen microbes and nutrients for the production of highly nutritious food items such as milk and meat (Saleem et al., 2013). It is therefore apparent that greater

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understanding of the rumen microbiome and its functionality will be key in maximising efficiency, health, sustainability and productivity of domesticated production animals in the future.

The metabolome is formally defined as the collection of all small-molecule metabolites (endogenous or exogenous) that can be found in a living cell or living organism (Wishart, 2005). Metabolomics is an emerging field of study for the analysis, characterisation and quantification of small molecules and metabolites, using technologies such as nuclear magnetic resonance (NMR), liquid/gas chromatography (LC/GC) coupled with mass spectrometry (MS) (Wishart, 2008). The application of metabolomics has traditionally been used in biomedical, nutritional and crop research. However, in recent years, metabolomics has been gaining prominence in the fields of livestock research and livestock monitoring – for animal health assessment, disease diagnosis and bio product characterisation (Goldansaz et al., 2017). There is also an increased interest in the application of metabolomics for identification of prospective biomarkers of production traits such as weight gain, milk quality and health (do Prado et al., 2018). Biomarkers can also be extremely useful in confirming food authenticity and combatting food fraud increasing food security. Metabolomics offers a variety of benefits over traditional wet chemistry type analysis of samples in that it offers high throughput analysis of a large variety of metabolites at one time. In addition, it can be non-invasive and is often less time-consuming with more cost-effective sample preparation than traditional approaches. However, the instrumentation and expertise required for metabolomic analysis are not yet widely available, are expensive to purchase and maintain, and require large data resources to facilitate and interpret results. Primarily focussing on bovine animals, the objective of this chapter is to provide an overview of the rumen metabolome as we know it today and highlight factors that can affect its composition and resulting functionality.

1.1 Targetted versus untargeted metabolomics

Metabolomic analysis can be carried out using a targeted or non-targeted approach, each with its own advantages and disadvantages. Targeted metabolomics involves analysing samples for a pre-defined list of metabolites with quantitative measurement. As such, prior knowledge of the samples and molecules of interest is required with typical applications including biomarker identification and validation, and analysis of metabolic pathways. Targeted metabolomics can be beneficial in terms of reduced data analysis and spectra interpretation, which can often be very time-consuming. However, by its nature the targeted approach is limiting, as it does not examine global coverage of the metabolome. Untargeted metabolomics, on the other hand, does not work off a predefined list of metabolites but instead aims to identify whatever metabolites

are detectable within the sample, restricted only by instrumentation sensitivity and coverage, and extraction methodology. While untargeted metabolomics offers increased coverage and characterisation of the sample metabolome, offering the opportunity for discovery of novel compounds and biomarkers (Cajka and Fiehn, 2016), it can often require more time-consuming data analysis for spectral peak identification and interpretation, and is not as quantitative as the targeted approach.

2 The rumen metabolome: technologies for analysis and extraction techniques

Efforts to characterise the composition of the rumen metabolome have been carried out in the past. Pioneering work carried out by Saleem et al. (2013) aimed to characterise the rumen metabolome using a range of technologies. The product of this research was the development of the rumen metabolome database (www.rumendb.ca) which is a comprehensive web accessible resource containing >200 positively identified and quantified rumen metabolites, their structures and respective concentrations.

An example process for rumen metabolomics analysis is shown in Fig. 1. The two leading analytical approaches for metabolomic analysis are nuclear magnetic resonance (NMR) and mass spectrometry (MS). Within these a variety of different technologies exist for analysis including ^{1}H-NMR, ^{13}C-NMR, ^{15}N-NMR and ^{31}P-NMR. Although NMR is a broadly used technique, others including gas chromatography with mass spectrometry (GC-MS), liquid chromatography with mass spectrometry (LC-MS), capillary electrophoresis with mass spectrometry (CE-MS) are also used, each with their own advantages and limitations (Markley et al., 2017). While MS techniques offer higher sensitivity, and with that, increased numbers of detectable compounds, NMR is more suited for analysis of more abundant compounds present. NMR has been demonstrated to be highly reproducible and quantitative, often with reduced sample preparation steps involved (Markley et al., 2017). Wishart (2008) reviewed the topic and highlighted the advantages and disadvantages of NMR, GC-MS and LC-MS spectroscopy for metabolomic analysis. As each has its own level of specificity for metabolite detection, a variety of approaches is advocated when trying to comprehensively characterise the metabolome of a sample.

Saleem et al. (2013) examined the rumen metabolome using a combination of NMR spectroscopy, inductively coupled plasma mass-spectroscopy (ICP-MS), GC-MS, direct flow injection mass spectrometry (DFI-MS) and lipidomics. In total 246 metabolites were identified across the various instruments. Using NMR, Saleem et al. (2013) identified and quantified 50 compounds in rumen fluid, and 98% of all visible peaks were assigned to a compound. Using a GC-MS approach 28 polar metabolites were identified and quantified, 8 of

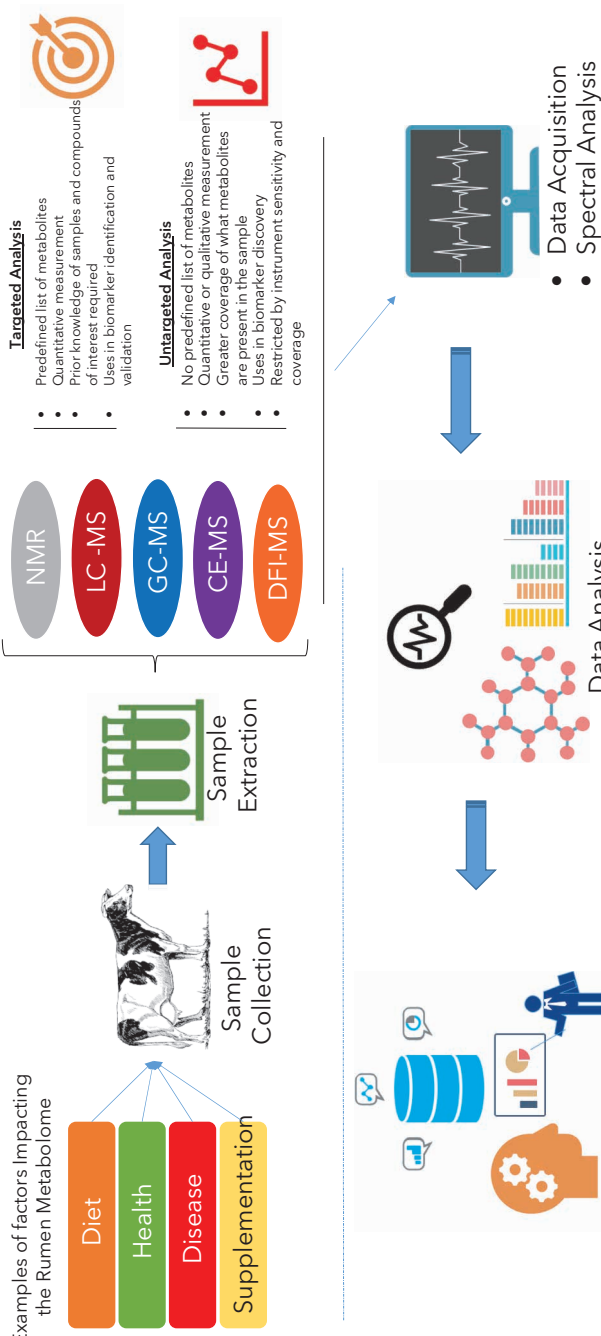

Figure 1 Illustration of methodology for rumen metabolomic analysis.

these compounds could not be detected by NMR; however, NMR detected 30 compounds that GC-MS could not. With that only 60% of visible GC-MS peaks could be positively identified. In the same study, DFI MS-MS was demonstrated to quantify 116 metabolites. In total DFI MS-MS detected 98 compounds or compound species that were undetectable using GC-MS or NMR. Gas chromatography with time of flight MS has also been successfully used to examine the rumen metabolome, identifying 165 metabolites (Sun et al., 2015). The results from the study strengthen the viewpoint that for a more global view of a sample metabolome a multi-technology analysis approach is beneficial.

A study by de Almeida et al. (2018), using liquid chromatography-high resolution MS examined the impact of sample extraction technique on resulting compound identification; including liquid-liquid extraction, solid phase extraction, original QuEChERS, buffered QuEChERS and an acid base QuEChERS technique. In total the study identified 1882 molecular features, only 3.56% of which had a positive match using the Global Natural Product Social Molecular Networking database. Between methods the liquid-liquid extraction resulted in the greatest abundance of molecular features, extracting compounds with moderate polarity, and nonpolar and hydrophobic characteristics. The authors went on to demonstrate the impact of pH on extraction characteristics. Whereas the solid phase extraction technique extracted primarily low to medium polarity compounds, dependent on the choice of solvent, the QuEChERS method was successful for extraction of compounds of low to medium polarity.

2.1 Tools for the interpretation of metabolomic data

The collected data then needs to be analysed and interpreted; a powerful tool for the analysis and interpretation of metabolomic data is MetaboAnalyst (http://www.metaboanalyst.ca). Handling the most common metabolomics data types including MS and NMR, this comprehensive web application also supports a number of data analysis and data visualisation tasks using a range of univariate and multivariate methods such as PCA (principal component analysis), PLSDA (partial least squares discriminant analysis), heatmap clustering and machine-learning methods as well as tools for interpretation analysis (Xia and Wishart, 2016; Chong et al., 2018). Other tools also include SIMCA for multivariate data analysis, Chenomx, which is an NMR analysis software, works with comprehensive Metabolite Reference Libraries to both identify and measure concentrations of compounds visible in the NMR spectra. Bioconductor (https://www.bioconductor.org/) is an open source software for bioinformatics and a variety of packages are available in R such as 'Metab', which is a package for high-throughput processing of metabolomic data with subsequent data analysis from GC-MS (Aggio et al., 2011). There are a variety of tools for the examination of enriched pathways of different metabolites including the Kyoto

Encyclopaedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/) and the Small Molecular Pathway Database (SMPDB, http://smpdb.ca/). There are also a number of freely available databases to aid in the identification and interpretation of metabolomics, including, but not restricted to, The Bovine Rumen Metabolome Database (http://www.rumendb.ca), The Bovine Metabolome Database (http://www.cowmetdb.ca), The Milk Composition Database (Foroutan et al., 2019), The Human Metabolome Database (Wishart et al., 2013), The Exposome-Explorer (Neveu et al., 2017), Phenol-Explorer (Rothwell et al., 2013), Food DB (http://foodb.ca/), Small Molecule Pathway Database (Jewison et al., 2014), The Toxic Exposome Database (Wishart et al., 2015), The Yeast Metabolome Database (Jewison et al., 2012), The Human Urine Metabolome (Bouatra et al., 2013), The Human Serum Metabolome (Psychogios et al., 2011) and others.

3 Factors impacting the rumen metabolome

The majority of rumen metabolome-based studies, currently available, have examined the impact of animal diet on the composition of the rumen metabolome. It has been reported that the chemical composition of the ruminal fluid is reflective of the interaction between the rumen microbiome and diet (Saleem et al., 2013). There are a variety of feeding systems practised throughout the world, often dictated by the region's climate, environment and land availability, and by the nutrient and energy requirements of cows.

The majority of dairy systems in the world practice an indoor style total mixed ration (TMR) feeding system while a small portion of regions with temperate climate, plentiful rainfall and good land availability practice pasture or 'Grass-Fed' dairy systems such as in Ireland and New Zealand. Saleem et al. (2013) examined the impact of increased proportions of barley grain in the cow diet (0%, 15%, 30% and 45% barley grain) on the rumen metabolome. Diet was demonstrated to have a significant effect on the composition of the rumen metabolome with compounds such as glucose, propionate, phenylacetate, butyrate, hypoxanthine, cadaverine, methylamine, putrescine, arginine, valine, serine and L-alanine most affected, increasing in line with the proportion of barley grain. Such results highlight that starch-rich diets increase the availability of free glucose to act as a substrate for increased growth of bacteria with subsequent increased production of volatile fatty acids, which would theoretically be of benefit from an animal production perspective. Accordingly, Zhang et al. (2017) reported that higher percentages of ruminal propionate and butyrate in groups fed higher concentrate diets could be beneficial to the energy metabolism and development of the heifer rumen. However Ametaj et al. (2010) also examined the impact of increased proportion of cereal grain in the cow diet and reported a response to a variety of compounds in the rumen,

to the increase in grain content of the diet. They discussed that the rumen of cows fed barley grain at concentrations >30% had unhealthy levels of a variety of potentially toxic metabolites. Saleem et al. (2012) confirmed these findings and reported that diets containing >30% barley grain resulted in rumens with increased concentrations of several toxic, inflammatory and unnatural compounds. The negative effects of high grain diets have also been reported in the goat rumen metabolome (Hua et al., 2017).

Also looking at the diet type, Zhang et al. (2017) demonstrated that altering the dietary forage to concentrate ratio significantly altered concentrations of amino acids, lipids, organic acids and carbohydrates in the rumen. The type of roughage fed has also been demonstrated to impact the rumen metabolome, whereby levels of organic acids, amines and amino acids are affected (Zhao et al., 2014). Similarly Sun et al. (2015) reported significant differences in the rumen metabolome between cows fed alfalfa hay versus corn stover whereby the higher quality alfalfa hay diet resulted in higher concentrations of amino acids, peptides and carbohydrates.

In a comprehensive dietary system's comparison study, O'Callaghan et al. (2018) examined the impact of pasture and indoor TMR dietary systems on the rumen and milk metabolome using NMR. Pasture-based diets resulted in increased concentrations of isoacids, amino acids and p-cresol, whereas the TMR feeding system resulted in increased concentrations of sugars, choline and 3-phenylpropionate. This study also highlighted the link and passage of rumen metabolites into raw milk whereby a variety of metabolites were shared across both rumen fluid and milk. P-cresol is a metabolite produced in the rumen (Martin, 1982; Carlson and Breeze, 1984) that has been receiving much attention in recent years in terms of the sensory quality of pasture-derived milk and milk products. P-cresol is a potent odorant and if present at low levels it provides barny or animal-like flavours to dairy products (Lopez and Lindsay, 1993). These flavours have been described as undesirable traits to palates that are not traditionally accustomed to pasture-derived milk and dairy products. Similarly, Sun et al. (2015, 2017) reported a variety of metabolites that are mutual across a variety of bio fluids including rumen fluid, serum, milk and urine. Sun et al. (2017) combined metabolomic analysis of the rumen with that of serum, milk, urine and the mammary gland metabolomic profiles to examine metabolic differences between lactating and non-lactating animals. Discussing that 33 metabolites were shared across the different samples the authors observed that 5 metabolic pathways demonstrated functional enrichment, including gluconeogenesis, pyruvate metabolism, the tricarboxylic acid cycle (TCA), glycerolipid metabolism and aspartate metabolism between lactating and non-lactating cows. O'Callaghan et al. (2018) highlighted a number of metabolites that show promise as biomarkers of 'Grass-Fed' dairy products including dimethyl sulfone and hippuric acid in milk, and demonstrated that NMR

metabolomics coupled with multivariate analysis is capable of distinguishing between the pasture and TMR rumen and milk metabolomes, highlighting NMR metabolomics potential as a verification tool for the future, in the context of increasing awareness around food authenticity and food fraud. Interestingly the same study also examined the rumen microbiome using 16S rRNA gene sequencing and while no significant difference in the rumen microbiome between diets was observed, likely as a result of the shortened adaptation period, differences in the rumen metabolome would suggest that short-term changes in the diet do not alter the overall structure of the microbiome but rather change the functionality of the rumen microbial community resulting in changes to the rumen metabolome.

Rumen metabolomics has shown promise in determining factors that impact the average daily weight gain in animals. Artegoitia et al. (2017) identified 33 metabolites that were associated with differences in high and low average daily weight gain of steers. The authors demonstrated how rumen metabolomic analysis of steers highlighted that alteration of the functional pathways for linoleic and alpha-linolenic acid metabolism, and biosynthesis of aromatic amino acids, was associated with average daily gain.

Live yeast supplementation to ruminants has been examined in the past with reported benefits to animal feed efficiency and performance. Such responses have been associated with alteration of the rumen microbiome, promoting cellulolytic fibre-degrading bacteria, increased oxygen scavenging function (Calsamiglia et al., 2005) and decreased ruminal lactate concentration (Dias et al., 2018; Robinson, 2010). Rumen metabolomics was carried out using LC-MS to better understand the underlying mechanisms of these benefits. Ogunade et al. (2019) reported that live yeast supplementation increased the concentrations of 4-cyclohexanedione and glucopyranoside and decreased the concentrations of threonic acid, xanthosine, deoxycholic acid, lauroylcarnitine, methoxybenzoic acid and pentadecylbenzoic acid. Furthermore, the authors reported that alterations in the rumen microbiome were positively correlated with several metabolites involved in pathways for metabolism of amino acids and energy, contributing to improved feed efficiency and performance attributes previously associated with yeast supplementation.

Sub-acute ruminal acidosis (SARA) is a common metabolic disease in high-concentrate dietary systems. The effects of SARA on cattle can include decreased dry matter intake, reduced milk yield, reduced milk fat concentration, decreased ruminal pH, accumulation of biogenic amines and volatile fatty acids, and laminitis. A variety of compounds in the rumen related to SARA have previously been reported including methylamine, nitrosadime, thylamine and ethanol (Ametaj et al., 2010). Thiamine supplementation as a mitigation strategy against SARA and its impact on the rumen metabolome was investigated by Xue et al. (2018). Feeding a SARA-inducing diet was demonstrated to increase

ruminal concentrations of VFAs, pyruvate, lactic acid and biogenic amines. In contrast, the inclusion of thiamine in the SARA-inducing diet was demonstrated to attenuate the SARA with reduced content of biogenic amines, increasing the pyruvate formate-lyase activity inhibiting lactate generation.

4 Future trends in research

Although still an emerging field of research, the power of rumen metabolomics for the detection of compounds/metabolites that have impacted our understanding of ruminant metabolism, disease and the mechanism of dietary intervention effects is profound. A greater understanding of the factors affecting the rumen metabolome offers the potential for innovative intervention strategies to be put in place proactively, preventing disadvantageous changes to rumen function and loss of productivity.

While the choice of instrumentation and extraction methodology impacts the detectable metabolites present, it is often the case, in untargeted metabolomics, that the majority of spectral peaks detected remain unidentified. In certain cases studies have reported that of the >1800 peaks observed, only ~3.5% could be positively identified (de Almeida et al., 2018). Future collaborative innovations that enhance and increase the efficiency of peak identification and characterisation in untargeted metabolomics with publicly available interactive webtools for data integration will be very beneficial and will further our understanding of rumen metabolism and identify novel biomarkers of important physiological conditions. Such results could potentially enable the development of proactive intervention strategies to increase the productivity and sustainability of ruminants and their milk and meat products, and increase animal health and welfare. The majority of existing studies to date have examined how diet, dietary interventions or health status affect the rumen metabolome. Given the impact the rumen can have on other organs throughout the body, future research on the impact of other factors such as breed, genetic heterogeneity and lactation on the rumen metabolome and function would be of interest.

One of the major challenges facing the global agricultural industry today is its contribution to global warming. Methane emissions from ruminants have been reported as a significant contributor to the environmental footprint of agriculture. While methane represents 16% of the total greenhouse gases (Wallace et al., 2015), ruminants are estimated to contribute up to 37% of this in some regions (Cottle et al., 2011). Furthermore, the production of methane from ruminants represents a loss of energy that could potentially be otherwise used for milk production and growth (Tapio et al., 2017). Any efforts to reduce the emission of greenhouse gases from the agricultural industry would be very beneficial for the environment and consumer perception of the industry. To date

the majority of the work for understanding and reducing methane emissions in ruminants has focussed on the rumen microbiome. Such studies have identified members of the microbiome that are associated with methane production including the archaea, ciliate protozoa, the anaerobic fungi, Succinovibrionaceae and Prevotella (Wallace et al., 2015; Tapio et al., 2017). Furthermore alteration to metabolic pathways has been associated with methane emissions such as formate metabolism, H_2 production and methanogenesis which are inherently linked to methane emissions (Tapio et al., 2017). Martinez-Fernandez et al. (2016) demonstrated the effect of chloroform for reduction of CH_4 production in cattle. The authors demonstrated that a 30–35% reduction in methane formation was possible, resulting from a redirection of [H] into more reduced microbial end-products without any adverse effects on general rumen function. However, as chloroform cannot be used under farming conditions due to its toxicity and environmental impacts, Martinez-Fernandez et al. (2018) examined the impact of 3-nitrooxypropanol (3-NOP) and chloroform on methane (CH_4) and H_2 production, ruminal metabolites and microbial community structure in cattle fed a tropical forage diet. The authors demonstrated that 3-NOP had a similar effect as chloroform for inhibition of methanogenesis. A variety of metabolites and methanogens in the rumen were altered by feeding of the compounds and it is hypothesised that the response to inhibition of methanogenesis is resultant from a redirection of [H] away from CH_4 formation to other reduced end-products that may benefit the host animals. While reduced methane emissions are desirable it is important to note the role methane production plays in avoiding build-up of H_2 in the rumen, which would otherwise alter the pH of the rumen by greatly impacting the ability of the rumen to digest fibre (Matthews et al., 2019). This considered, a holistic approach in tackling the issue is needed. To date, the application of metabolomics in further understanding the dynamics of methane production in the rumen has been limited but offers a unique opportunity to understand the link between diet, microbiome, rumen metabolism and production of metabolites and the production of methane in cattle. A greater understanding of the interplay of these factors would be highly beneficial for the mitigation of methane through the development of therapies that could reduce the overall carbon footprint of the agricultural industry.

5 Conclusion

There are a variety of technologies available to conduct metabolomic analysis each with its own advantages and limitations; the choice of extraction methodology can impact the detectable metabolites. For a more complete view of the metabolome a multi-technology approach is advocated.

A huge array of work has been carried out to date to better understand the composition and function of the rumen; however, there is still a

considerable level unknown about this virtual organ. Examination of the rumen metabolome can identify effects of diets on ruminant metabolism, feed efficiency, subsequent milk composition and quality, and animal health. Future innovation in technologies with improved sensitivities will be highly beneficial for identification and characterisation of the metabolome and will yield novel metabolites that may have an important role in our understanding of metabolism and production of ruminants.

6 References

Aggio, R., Villas-Bôas, S. G. and Ruggiero, K. 2011. Metab: an R package for high-throughput analysis of metabolomics data generated by GC-MS. *Bioinformatics* 27(16), 2316–8. doi:10.1093/bioinformatics/btr379.

Ametaj, B. N., Zebeli, Q., Saleem, F., Psychogios, N., Lewis, M. J., Dunn, S. M., Xia, J. and Wishart, D. S. 2010. Metabolomics reveals unhealthy alterations in rumen metabolism with increased proportion of cereal grain in the diet of dairy cows. *Metabolomics* 6(4), 583–94. doi:10.1007/s11306-010-0227-6.

Artegoitia, V. M., Foote, A. P., Lewis, R. M. and Freetly, H. C. 2017. Rumen fluid metabolomics analysis associated with feed efficiency on crossbred steers. *Scientific Reports* 7(1), 2864. doi:10.1038/s41598-017-02856-0.

Bouatra, S., Aziat, F., Mandal, R., Guo, A. C., Wilson, M. R., Knox, C., Bjorndahl, T. C., Krishnamurthy, R., Saleem, F., Liu, P., Dame, Z. T., Poelzer, J., Huynh, J., Yallou, F. S., Psychogios, N., Dong, E., Bogumil, R., Roehring, C. and Wishart, D. S. 2013. The human urine metabolome. *PLoS ONE* 8(9), e73076. doi:10.1371/journal.pone.0073076.

Cajka, T. and Fiehn, O. 2016. Toward merging untargeted and targeted methods in mass spectrometry-based metabolomics and lipidomics. *Analytical Chemistry* 88(1), 524–45. doi:10.1021/acs.analchem.5b04491.

Calsamiglia, S., Castillejos, L. and Busquet, M. 2005. Alternatives to antimicrobial growth promoters in cattle. In: Smith, P. and Wiseman, J. (Eds), *Recent Advances in Animal Nutrition*. Nottingham University Press, Nottingham, UK, 129–67. doi:10.5661/recadv-05-129.

Carlson, J. R. and Breeze, R. G. 1984. Ruminal metabolism of plant toxins with emphasis on indolic compounds. *Journal of Animal Science* 58(4), 1040–9. doi:10.2527/jas1984.5841040x.

Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., Wishart, D. S. and Xia, J. 2018. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Research* 46(W1), W486–94. doi:10.1093/nar/gky310.

Cottle, D. J., Nolan, J. V. and Wiedemann, S. G. 2011. Ruminant enteric methane mitigation: a review. *Animal Production Science* 51(6). doi:10.1071/AN10163.

De Almeida, R. T. R., Do Prado, R. M., Porto, C., Dos Santos, G. T., Huws, S. A. and Pilau, E. J. 2018. Exploring the rumen fluid metabolome using liquid chromatography-high-resolution mass spectrometry and Molecular Networking. *Scientific Reports* 8(1), 17971. doi:10.1038/s41598-018-36196-4.

Dias, A. L. G., Freitas, J. A., Micai, B., Azevedo, R. A., Greco, L. F. and Santos, J. E. P. 2018. Effect of supplemental yeast culture and dietary starch content on rumen

fermentation and digestion in dairy cows. *Journal of Dairy Science* 101(1), 201–21. doi:10.3168/jds.2017-13241.

Do Prado, R. M., Porto, C., Nunes, E., De Aguiar, C. L. and Pilau, E. J. 2018. Metabolomics and agriculture: what can be done? *mSystems* 3(2), e00156-17. doi:10.1128/mSystems.00156-17.

Foroutan, A., Guo, A. C., Vazquez-Fresno, R., Lipfert, M., Zhang, L., Zheng, J., Badran, H., Budinski, Z., Mandal, R., Ametaj, B. N. and Wishart, D. S. 2019. Chemical composition of commercial cow's milk. *Journal of Agricultural and Food Chemistry* 67(17), 4897–914. doi:10.1021/acs.jafc.9b00204.

Goldansaz, S. A., Guo, A. C., Sajed, T., Steele, M. A., Plastow, G. S. and Wishart, D. S. 2017. Livestock metabolomics and the livestock metabolome: a systematic review. *PLoS ONE* 12(5), e0177675. doi:10.1371/journal.pone.0177675.

Hua, C. F., Tian, J., Tian, P., Cong, R. H., Luo, Y. W., Geng, Y. L., Tao, S. Y., Ni, Y. D. and Zhao, R. Q. 2017. Feeding a high concentration diet induces unhealthy alterations in the composition and metabolism of ruminal microbiota and host response in a goat model. *Frontiers in Microbiology* 8, 138. doi:10.3389/fmicb.2017.00138.

Jewison, T., Knox, C., Neveu, V., Djoumbou, Y., Guo, A. C., Lee, J., Liu, P., Mandal, R., Krishnamurthy, R., Sinelnikov, I., Wilson, M. and Wishart, D. S. 2012. YMDB: the yeast metabolome database. *Nucleic Acids Research* 40(Database issue), D815–20. doi:10.1093/nar/gkr916.

Jewison, T., Su, Y., Disfany, F. M., Liang, Y., Knox, C., Maciejewski, A., Poelzer, J., Huynh, J., Zhou, Y., Arndt, D., Djoumbou, Y., Liu, Y., Deng, L., Guo, A. C., Han, B., Pon, A., Wilson, M., Rafatnia, S., Liu, P. and Wishart, D. S. 2014. Smpdb 2.0: big improvements to the small molecule pathway database. *Nucleic Acids Research* 42(Database issue), D478–84. doi:10.1093/nar/gkt1067.

Lopez, V. and Lindsay, R. C. 1993. Metabolic conjugates as precursors for characterizing flavor compounds in ruminant milks. *Journal of Agricultural and Food Chemistry* 41(3), 446–54. doi:10.1021/jf00027a019.

Markley, J. L., Brüschweiler, R., Edison, A. S., Eghbalnia, H. R., Powers, R., Raftery, D. and Wishart, D. S. 2017. The future of NMR-based metabolomics. *Current Opinion in Biotechnology* 43, 34–40. doi:10.1016/j.copbio.2016.08.001.

Martin, A. K. 1982. The origin of urinary aromatic compounds excreted by ruminants: 3. The metabolism of phenolic compounds to simple phenols. *British Journal of Nutrition* 48(3), 497–507. doi:10.1079/bjn19820135.

Martinez-Fernandez, G., Denman, S. E., Yang, C., Cheung, J., Mitsumori, M. and McSweeney, C. S. 2016. Methane inhibition alters the microbial community, hydrogen flow, and fermentation response in the rumen of cattle. *Frontiers in Microbiology* 7, 1122. doi:10.3389/fmicb.2016.01122.

Martinez-Fernandez, G., Duval, S., Kindermann, M., Schirra, H. J., Denman, S. E. and McSweeney, C. S. 2018. 3-NOP vs. Halogenated compound: methane production, ruminal fermentation and microbial community response in forage fed cattle. *Frontiers in Microbiology* 9, 1582. doi:10.3389/fmicb.2018.01582.

Matthews, C., Crispie, F., Lewis, E., Reid, M., O'Toole, P. W. and Cotter, P. D. 2019. The rumen microbiome: a crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. *Gut Microbes* 10(2), 115–32. doi:10.1080/19490976.2018.1505176.

Neveu, V., Moussy, A., Rouaix, H., Wedekind, R., Pon, A., Knox, C., Wishart, D. S. and Scalbert, A. 2017. Exposome-Explorer: a manually-curated database on biomarkers

of exposure to dietary and environmental factors. *Nucleic Acids Research* 45(D1), D979–84. doi:10.1093/nar/gkw980.

O'Callaghan, T. F., Ross, R. P., Stanton, C. and Clarke, G. 2016. The gut microbiome as a virtual endocrine organ with implications for farm and domestic animal endocrinology. *Domestic Animal Endocrinology* 56 (Suppl.), S44–55. doi:10.1016/j.domaniend.2016.05.003.

O'Callaghan, T. F., Vázquez-Fresno, R., Serra-Cayuela, A., Dong, E., Mandal, R., Hennessy, D., McAuliffe, S., Dillon, P., Wishart, D. S., Stanton, C. and Ross, R. P. 2018. Pasture feeding changes the bovine rumen and milk metabolome. *Metabolites* 8(2), 27. doi:10.3390/metabo8020027.

Ogunade, I., Schweickart, H., McCoun, M., Cannon, K. and McManus, C. 2019. Integrating 16S rRNA sequencing and LC-MS-based metabolomics to evaluate the effects of live yeast on rumen function in beef cattle. *Animals* 9(1), 28. doi:10.3390/ani9010028.

O'Hara, A. M. and Shanahan, F. 2006. The gut flora as a forgotten organ. *EMBO Reports* 7(7), 688–93. doi:10.1038/sj.embor.7400731.

Psychogios, N., Hau, D. D., Peng, J., Guo, A. C., Mandal, R., Bouatra, S., Sinelnikov, I., Krishnamurthy, R., Eisner, R., Gautam, B., Young, N., Xia, J., Knox, C., Dong, E., Huang, P., Hollander, Z., Pedersen, T. L., Smith, S. R., Bamforth, F., Greiner, R., McManus, B., Newman, J. W., Goodfriend, T. and Wishart, D. S. 2011. The human serum metabolome. *PLoS ONE* 6(2), e16957. doi:10.1371/journal.pone.0016957.

Robinson, P. 2010. Yeast products for growing and lactating ruminants: a literature summary of impacts on rumen fermentation and performance. Cooperative Extension Specialist Department of Animal Science, University of California, Davis, CA, p. 95616.

Rothwell, J. A., Perez-Jimenez, J., Neveu, V., Medina-Remón, A., M'hiri, N., García-Lobato, P., Manach, C., Knox, C., Eisner, R., Wishart, D. S. and Scalbert, A. 2013. Phenol-Explorer 3.0: a major update of the Phenol-Explorer database to incorporate data on the effects of food processing on polyphenol content. *Database: the Journal of Biological Databases and Curation* 2013, bat070. doi:10.1093/database/bat070.

Saleem, F., Ametaj, B. N., Bouatra, S., Mandal, R., Zebeli, Q., Dunn, S. M. and Wishart, D. S. 2012. A metabolomics approach to uncover the effects of grain diets on rumen health in dairy cows. *Journal of Dairy Science* 95(11), 6606–23. doi:10.3168/jds.2012-5403.

Saleem, F., Bouatra, S., Guo, A. C., Psychogios, N., Mandal, R., Dunn, S. M., Ametaj, B. N. and Wishart, D. S. 2013. The bovine ruminal fluid metabolome. *Metabolomics* 9(2), 360–78. doi:10.1007/s11306-012-0458-9.

Sun, H. Z., Wang, D. M., Wang, B., Wang, J. K., Liu, H. Y., Guan, le L. and Liu, J. X. 2015. Metabolomics of four biofluids from dairy cows: potential biomarkers for milk production and quality. *Journal of Proteome Research* 14(2), 1287–98. doi:10.1021/pr501305g.

Sun, H. Z., Shi, K., Wu, X. H., Xue, M. Y., Wei, Z. H., Liu, J. X. and Liu, H. Y. 2017. Lactation-related metabolic mechanism investigated based on mammary gland metabolomics and 4 biofluids' metabolomics relationships in dairy cows. *BMC Genomics* 18(1), 936. doi:10.1186/s12864-017-4314-1.

Tapio, I., Snelling, T. J., Strozzi, F. and Wallace, R. J. 2017. The ruminal microbiome associated with methane emissions from ruminant livestock. *Journal of Animal Science and Biotechnology* 8, 7. doi:10.1186/s40104-017-0141-0.

Wallace, R. J., Rooke, J. A., Mckain, N., Duthie, C. A., Hyslop, J. J., Ross, D. W., Waterhouse, A., Watson, M. and Roehe, R. 2015. The rumen microbial metagenome associated with high methane production in cattle. *BMC Genomics* 16, 839. doi:10.1186/s12864-015-2032-0.

Wishart, D. S. 2005. Metabolomics: the principles and potential applications to transplantation. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 5(12), 2814–20. doi:10.1111/j.1600-6143.2005.01119.x.

Wishart, D. S. 2008. Metabolomics: applications to food science and nutrition research. *Trends in Food Science and Technology* 19(9), 482–93. doi:10.1016/j.tifs.2008.03.003.

Wishart, D. S., Jewison, T., Guo, A. C., Wilson, M., Knox, C., Liu, Y., Djoumbou, Y., Mandal, R., Aziat, F., Dong, E., Bouatra, S., Sinelnikov, I., Arndt, D., Xia, J., Liu, P., Yallou, F., Bjorndahl, T., Perez-Pineiro, R., Eisner, R., Allen, F., Neveu, V., Greiner, R. and Scalbert, A. 2013. HMDB 3.0—the human metabolome database in 2013. *Nucleic Acids Research* 41(Database issue), D801–7. doi:10.1093/nar/gks1065.

Wishart, D., Arndt, D., Pon, A., Sajed, T., Guo, A. C., Djoumbou, Y., Knox, C., Wilson, M., Liang, Y., Grant, J., Liu, Y., Goldansaz, S. A. and Rappaport, S. M. 2015. T3DB: the toxic exposome database. *Nucleic Acids Research* 43(Database issue), D928–34. doi:10.1093/nar/gku1004.

Xia, J. and Wishart, D. S. 2016. Using MetaboAnalyst 3.0 for comprehensive metabolomics data analysis. *Current Protocols in Bioinformatics* 55(14), 14.10.1–14.10.91. doi:10.1002/cpbi.11.

Xue, F., Pan, X., Jiang, L., Guo, Y. and Xiong, B. 2018. GC–MS analysis of the ruminal metabolome response to thiamine supplementation during high grain feeding in dairy cows. *Metabolomics: Official Journal of the Metabolomic Society* 14(5), 67. doi:10.1007/s11306-018-1362-8.

Zhang, J., Shi, H. T., Wang, Y. J., Li, S. L., Cao, Z. J., Ji, S. K., He, Y. and Zhang, H. T. 2017. Effect of dietary forage to concentrate ratios on dynamic profile changes and interactions of ruminal microbiota and metabolites in holstein heifers. *Frontiers in Microbiology* 8, 2206. doi:10.3389/fmicb.2017.02206.

Zhao, S., Zhao, J., Bu, D., Sun, P., Wang, J. and Dong, Z. 2014. Metabolomics analysis reveals large effect of roughage types on rumen microbial metabolic profile in dairy cows. *Letters in Applied Microbiology* 59(1), 79–85. doi:10.1111/lam.12247.

Chapter 4

A conceptual approach to the mathematical modelling of microbial functionality in the rumen

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1 Introduction

The rumen as a digestive organ plays a very important role in feed digestion in ruminants because it hosts a vast microbial population that enables the host to ferment fibrous (as well as non-fibrous) feed and benefit from the end-products produced. Because of this, ruminants can thrive on feeds of low nutritional value or even on those feeds deemed inedible by many other animals. The digestive role of the rumen is mainly fermentative and is attributed to the activity of the microbial population it harbours. This population includes bacteria, protozoa and fungi, but due to the rather anoxic intra-ruminal conditions it also includes Archaea (methanogens). Although there is enormous complexity and functional diversity of this microbial population across species, a core microbiome always appears to be present across ruminant species and across regions and management types (Henderson et al., 2015). Variation around this core microbiome was established to be related most to diet, which is an outcome fully comprehensible as the diet determines the amount, the type and the matrix in which substrates become available for the rumen microbiota. Furthermore, in interaction with the ruminant host (rumination and particle size reduction, rumen contractions and movement of rumen contents, rumen outflow) the diet strongly impacts on the dynamics of intra-ruminal flow of digesta and on the physical and chemical conditions in

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rumen digesta, both being main drivers of rumen microbial activity (Zebeli et al., 2012; Bannink et al., 2016). Relevant physical and chemical conditional factors are particle size distribution of rumen contents, the different physical phases in the rumen including a fibrous mat, fluid volume, water and particle flow rate, fluid osmolality, acidity and redox potential, and the gas pressures of carbon dioxide, methane and hydrogen. Most of these factors have received attention from researchers for more than half a century now in modelling efforts, with rumen redox potential (Dijkstra et al., 2020), the effect of rumen gas pressures (Van Lingen et al., 2017) and effects of osmolality (López et al., 2003) having received least attention. The current technological advancement made in molecular biology and biotechnology enables researchers to study the rumen environment with increasing detail. The recent e-book of Ungerfeld and Newbold (2018) contains various excellent reviews demonstrating the recent developments in this field of research. In their editorial, they foresee future developments resulting from the integration of microbial ecology 'multi-omics' techniques, in particular regarding the expression of functional genes as well as the application of physical-chemical principles and the refinement of thermodynamic and kinetic measurements in the rumen environment.

To benefit from all the data gathered, with new techniques and new data types being introduced continuously, mathematical models need to be constructed that attempt to capture the biological evidence gathered and predict functionality at the level of the whole rumen. There have been many modelling efforts at the level of the whole rumen already; however, they vary substantially in the levels of organization represented and in modelling concepts adopted. Figure 1 lists the various levels of organization involved with extant and most complex dynamic, mechanistic approaches available to date. The most detailed approaches so far are the dynamic models which are successors of the models developed by Baldwin et al. (1987) and Dijkstra et al. (1992), and many descendants from these two early models have been published meanwhile by various groups. Other model approaches, as reviewed by Tedeschi et al. (2014), followed a more simple approach (which may be for very good reasons, depending on the actual aim of the modelling effort) adopting either static or more empirically based, less mechanistic representations (reviewed by Bannink et al., 2016). Recently, the effect of thermodynamic driving forces on rumen microbiota (Van Lingen et al., 2016) and the effect of rumen hydrogen dynamics on rumen microbial activity (Wang et al., 2016; Van Lingen et al., 2019) were implemented to represent thermodynamic control of rumen hydrogen pressure on the formation of end products of fermentation. However, none of these rumen modelling efforts involves the aforementioned integration with results from multi-omics techniques. Quantification of microbial functionality by models that aim to predict whole rumen function still seems to go parallel instead of being integrated with quantification from multi-omics approaches.

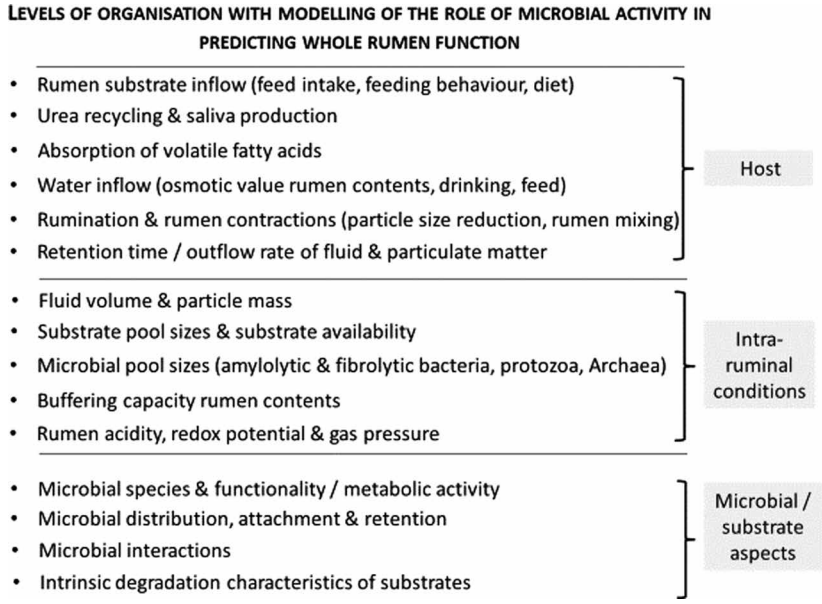


Figure 1 Schematic representation of the various levels of organization related to rumen function, ranging from the whole rumen level to that of multi-omics data collection.

This chapter discusses the concepts and the approaches taken with the quantification or mathematical modelling of rumen microbiota. The consequences from the perspective of mathematical modelling of microbial functionality at the whole rumen level are discussed as it is thought that models need to cover this level to predict fermentative and digestive aspects of the rumen as an organ.

2 Conceptual approaches in modelling whole rumen function

2.1 Interactions between ruminant host and rumen content

There are extensive interactions between rumen content and the host ruminant (Fig. 1). Genetic as well as environmental factors underlie the variation among individuals in feeding and rumination behaviour, in the morphological and physiological aspects of the rumen wall, and in the regulation of rumen contractions and passage through the reticulum towards the omasum and abomasum. The regulatory mechanisms in place for the host to control retention time of fluid and particles are of high importance for the adaptive capacity of the host to a wide range of diet qualities. By these mechanisms, more slowly degradable particles may reside longer in the rumen, leading to a larger rumen volume with increased content of slowly degradable particles,

enhancing the available time for micro-organisms to degrade them before they flow out of the rumen. Another important determinant of rumen microbial activity is the buffer mechanisms of saliva production and absorption of volatile fatty acids (VFA) as end products of that microbial activity. The rumen wall appears to be highly adaptive to the acid load it receives from the rumen environment, demonstrating the regulatory role the rumen wall tissue has in ensuring rumen fermentation. Without these buffer mechanisms the rumen would become acidic and diminish the activity of micro-organisms that ferment the feed ingested by the ruminant and reduce faecal digestibility and nutritive value of the feed. A further important interaction between host and the rumen environment is the process of nitrogen (N) and phosphorus recycling from blood to the rumen through the rumen wall and through the saliva produced.

Most of the aforementioned interactions between ruminant host and rumen microbial activity are standard elements of the more complex rumen models that are available (Bannink et al., 2016). These models aim to quantify whole rumen function and to predict feed degradation and the formation of end products of fermentation (microbial protein synthesis, formation of VFA, ammonia and methane). Whereas the more detailed rumen models adopt a highly mechanistic representation, more applied models such as the protein evaluation systems applied in current practice, are far less complex. These applied models take general assumptions on, for example, fractional rumen outflow rates and intra-ruminal conditions (reviewed by Tedeschi et al., 2014). They also consider the concept of calculated rumen N balance which should not become below a threshold in order to prevent a potential N shortage in the rumen. In contrast, more mechanistic models attempt to describe the process of N recycling in itself, and as a result have predicted rumen fermentation also as a function of modelled N recycling. Many of the interactions between the ruminant host and rumen content (Fig. 1) are basically ignored in applied rumen models. Although these interactions may have been represented in the more complex and mechanistic rumen models, these still describe the average ruminant host because for many of the model parameters no phenotypic data are available which hampers the parameterization of individuals. For a further discussion of interactions and how these are adopted in rumen models, the reader is referred to a review by Bannink et al. (2016). Many rumen models have a position in between the extremes discussed above, and for an overview the reader is referred to the review by Tedeschi et al. (2014).

2.2 Intra-ruminal conditions

Also at the intra-ruminal level various physical-chemical parameters that affect microbial activity have been the subject of modelling. These include, among others, the effect of rumen hydrogen as a thermodynamic driving force for

microbial metabolism and on activity of methanogens (Wang et al., 2016; Van Lingen et al., 2016, 2019), regulation of rumen acidity by the host, including the activity of epithelial tissue in the rumen wall (Bannink et al., 2012) and the dynamics of particle distribution, particle size reduction and rumen fluid outflow (Gregorini et al., 2015). Through their direct effect on microbial metabolism, quantification of these parameters is important for accurate prediction of the consequences of nutritional strategies on rumen function, or of any host-specificity of rumen function. Despite their importance, intra-ruminal conditions as driving factors will not be discussed in-depth in this chapter, rather we focus on the diversity in composition and functionality of rumen microbiota along with its consequences for rumen function in terms of the microbial fermentation process in the entire rumen digesta volume.

2.3 Types of microbial functionality

Two important elements of microbial activity are distinguished in extant whole rumen models. Amylolytic activity is associated with the utilization of readily degradable carbohydrates such as sugars and starch, which are generally of small particle size or present in the fluid phase and for which a short retention time in the rumen is assumed. Fibrolytic activity is associated with the utilization of structural carbohydrates with generally a larger particle size and a much longer rumen retention time. Different microbial populations thrive on both types of substrate, although the distinction is not absolute in reality and many microbial species may use multiple sources of substrate. This makes the distinction between amylolytic and fibrolytic activity somewhat arbitrary and not so clearly associated to species level. Next to bacterial activity, there is a large population of protozoa in the rumen which prey on bacteria, thereby contributing to intra-ruminal recycling, but also utilizing feed substrate (review Newbold et al., 2015). Protozoa have a much longer retention time in the rumen than bacteria because they are more able to resist passage or sequester. Despite the potentially prominent role of protozoa, almost none of rumen models developed so far distinguishes protozoal activity, next to amylolytic and fibrolytic bacterial activity (Tedeschi et al., 2014). Exceptions are the modelling work of Dijkstra (1994) and Hook et al. (2017) in which rumen protozoa dynamics and the sequestration of protozoa in the rumen have been quantified. A more detailed representation of functional microbial classes was adopted by Nagorcka et al. (2000) who used a series of consensus stoichiometries for VFA production for individual microbial classes and weighted these according to the proportion that an individual class contributes to the overall pool utilizing a specific substrate type. This did not lead to a further diversification of functional microbial pools that are represented in the model, but to an altered VFA stoichiometry. Recently, this approach was introduced in an Australian rumen model for beef production by Dougherty

et al. (2017) and an additional class of lactate-utilizing bacteria was introduced as a pool size in the model. Unfortunately, model equations or assumptions have not been reported, hampering comparison with the representation in other rumen models, but the approach generally compares with that used in previous efforts to develop dynamic, mechanistic rumen models (reviews by Tedeschi et al., 2014; Bannink et al., 2016).

A more detailed and dynamic approach to study lactate (as end product of microbial activity) metabolism in the rumen in response to nutrition was undertaken by Mills et al. (2014). They introduced the dynamics of lactate-producing and lactate-utilizing bacteria (the latter replacing the amylolytic bacteria pool) next to fibrolytic bacteria and protozoa in the model of Dijkstra (1994). The model allowed them to introduce specific parameterizations in the model required to investigate the competition between bacteria and protozoa for rumen lactate, and the dynamic interactions between microbial pools. With the interest to quantify enteric methane in ruminants, there has been a lot of interest in quantifying methanogenesis. In almost all models methane is calculated by the complete conversion of net hydrogen production as an outcome of rumen hydrogen balance to methane. Only recently, methanogens have been represented as a functional microbial class in the rumen model of Van Lingen et al. (2019).

Summarizing, the functional classes of micro-organisms generally represented in mathematical rumen models are amylolytic bacteria and fibrolytic bacteria. More specialized models may distinguish lactate-producing and lactate-utilizing bacteria, protozoa and methanogens. None of the rumen models include a further refinement into specific classes representing individual genera, or a more specific functionality linking to data that becomes available from multi-omics techniques. Extant rumen models typically distinguish substrate types as reported of feeding trials and associated microbial activity instead of specific genera in the microbial community. The pragmatic reason is that empirical information is available on the rumen degradation characteristics of these substrates (e.g. Li et al., 2018; Dijkstra et al., 1992).

2.4 Microbial interactions

A key aspect of the functionality of the rumen microbial population is the interaction between the various classes of micro-organisms. In principle, a modeller desires to distinguish these classes and their interactions in the model representation, because these are linked closely to observed functionality in terms of capacity to degrade and ferment various substrates, and to the formation of end products of this process including microbial mass, VFA, ammonia, hydrogen and methane. Modelling a microbial interaction implies that the modelled activity of one microbial class interacts with another. From

studies of the rumen microbiome it is noted that a large diversity of microbial species is present and consortia of species appear to work together to perform complex tasks such as pasture degradation (Belanche et al., 2019). Simultaneously, the microbial community, and its diversity, is observed to be highly adaptable and depends strongly on diet (Henderson et al., 2015). However, for modelling perspectives it is important to note that the rumen microbial community has a remarkable functional redundancy, that is, an overlap of function among multiple species. Within studies, considerable changes in microbial community composition and its diversity often do not translate into changes in crucial fermentation metrics including pH and molar proportions of VFA, clearly suggesting functional redundancy (Weimer, 2015). Also in the study of Belanche et al. (2019) large changes in microbiome were observed by changing the feeding of sheep from hay-concentrate towards ryegrass pasture. In dairy cattle, bacterial community richness was lower during lactation than during the dry period, probably related to the greater feed intake and concentrate proportion in the diet of lactating cows compared with dry cows (Dieho et al., 2017) (Fig. 2). A pronounced, short-term decrease in bacterial community richness was observed with the onset of increase in concentrate allowance post-calving. The decline in richness was much more severe with a more rapid increase in concentrate allowance post-partum, but the decline remained to be a temporal phenomenon. However, Dieho et al. (2017) showed that these marked changes in microbial composition were not associated with changes in rumen degradation kinetics of feed. Although such very detailed observations on the composition of the microbial community in rumen samples give an interesting insight in how the microbiota can adapt to various rumen conditions and the diet, they do not yet deliver insight in microbial interactions nor do they give an indication of how they might be quantified in functional terms with a rumen modelling effort. For this, quantitative insight in specific functionalities of the microbiota is needed next to that of abundance of genera. Shotgun metagenome sequencing of the rumen microbiome can facilitate the gathering of knowledge on specific functionalities of the microbiota. However, there is dearth of studies using shotgun metagenomics compared to the 16S/18S/ITS-based amplicon sequencing of rumen microbiome.

Following the approach taken with extant rumen models, the most prevalent microbial functionalities to distinguish are that of amylolytic activity (utilization of solubilized or readily degradable carbohydrates) and that of fibrolytic activity (utilization of structural carbohydrates). This simplification of microbial functionality adopts the concept that energy substrates in general determine microbial growth most and these substrate types deliver the majority of energy available. However, other specific modelling aims are thinkable as well: lactic acid production and utilization may be considered in view of modelling rumen acidosis, a proteolytic functional group and protozoa may be included in view

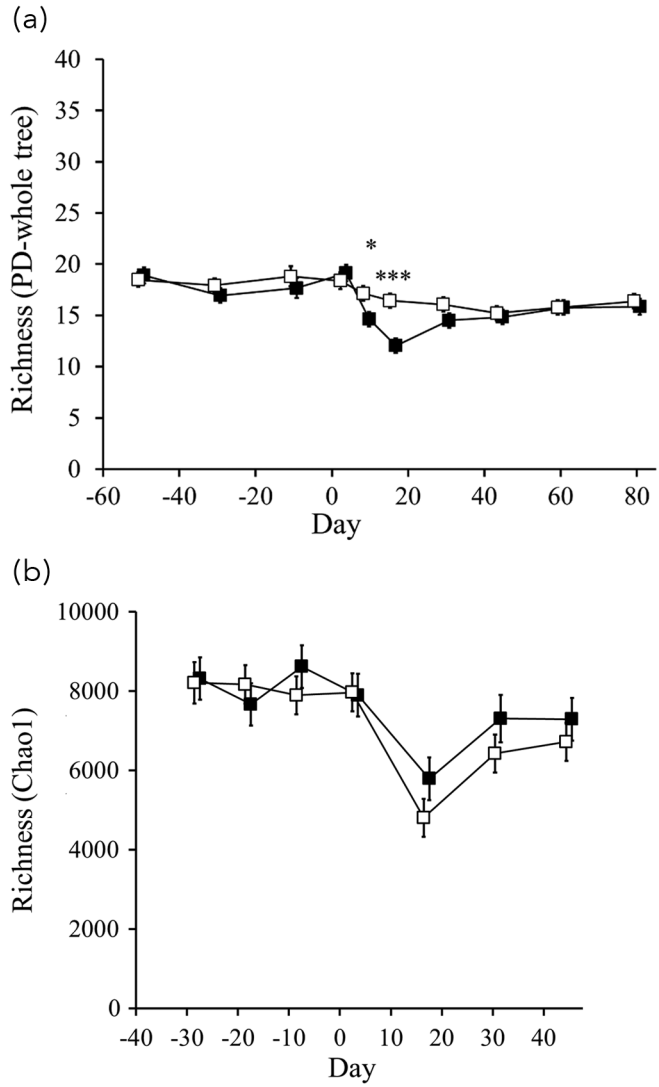


Figure 2 (a) Bacterial community richness (■□; based on 'phylogenetic diversity whole tree' richness metric; Faith, 1992) in the rumen of dairy cattle during the pretreatment and treatment period with a rapid (1.0 kg of DM/d; ■, n = 6) and gradual (0.25 kg of DM/d; □, n = 6) increment of concentrate allowance postpartum. Values represent least squares means ± standard error ($P < 0.001$ with *** and $P < 0.05$ with * for the significance of difference between rapid and gradual increment (reproduced from Dieho et al., 2017). (b) Bacterial community richness (■□; based on 'Chao1' richness metric; Chao, 1984) for a control dry period ration (0.0 kg DM/d concentrate, □; n = 5) and a dry period ration with supplemental concentrate (3.0 kg DM/d concentrate, ■; n = 4) in the rumen of dairy cattle during the dry period and the subsequent lactation (no difference in diet composition between both groups in lactation period). Values represent least squares means ± standard error. Unpublished results from Dieho et al.

of modelling efficiency of N utilization in the rumen, or rumen wall-associated micro-organisms may be included to represent their role in rumen function.

A functionally important aspect considered is whether the micro-organisms exhibit a liquid- or particle-associated retention in the rumen. In general, mechanistic rumen models represent these two functionality aspects (Tedeschi et al., 2014; Bannink et al., 2016). Rumen models that employ more detailed aspects of microbial activity further distinguish between microbial classes, for example, between bacterial and protozoal functionality (Dijkstra, 1994), between lactate producers and lactate utilizers (e.g. Mills et al., 2014; Dougherty et al., 2017), between a hydrogen-sensitive (with fast substrate degradation and microbial growth) and hydrogen-insensitive metabolism (with slow degradation and growth) (e.g. Van Lingen et al., 2017), or the capacity for synthesis of storage polysaccharides (e.g. Dijkstra et al., 1992). Most extant rumen models treat the distinct microbial functionalities as independent processes. For example, amylolytic activity is usually modelled as a process being independent from that of fibrolytic activity. Only through 'literally' representing some kind of exchange between microbial classes, or a common substrate pool that is utilized by multiple microbial classes, a dependency is introduced. It may be that microbial growth of the multiple classes is made dependent on a common pool of nitrogenous substrate; in this case an interaction between classes is modelled through substrate availability (Li et al., 2019). Another substrate-related interaction was modelled by Dijkstra et al. (1992) and Dijkstra (1994) with protozoa and amylolytic bacteria scavenging readily available substrates such as sugars and starch, and storing these inside their cells, preventing other microbes from rapidly fermenting these carbohydrates and thus alleviating rumen acidification and preventing build-up of rumen hydrogen pressure. An interaction may also be modelled through a direct interaction between the mass of microbial classes. An example of this is protozoa preying on bacteria (Dijkstra, 1994), or methanogen activity depending on rumen hydrogen which is produced by protozoa and bacteria and affecting the fermentation profile of bacteria (and perhaps protozoa) and in turn altering this hydrogen pressure again (Van Lingen et al., 2019). If these kinds of interactions have not been presented (i.e. no common substrate pools, nor a direct interaction between microbial classes), modellers implicitly or explicitly assumed that activity of these microbial classes is independent (Russell et al., 1992).

Often, microbial functionalities have not been incorporated in a whole rumen model at the level of genera or even species. An exception is the linear programming model of Reichl and Baldwin (1976), in which more than 10 prominent rumen microbial species were assigned to one of eight microbial groups defined on the basis of substrate specificity and substrate fermentation rate and on nutrient requirements for growth. Another exception is the representation of *Streptococcus bovis* and *Megasphaera elsdenii* as the

main rumen lactate utilizer and producer, respectively, in the rumen in order to study rumen lactate dynamics and acidity (Mills et al., 2014; Dougherty et al., 2017). No rumen modelling attempts are known where multiple microbial functionalities (represented as separate rumen pool sizes in the model) depend on the same carbohydrate source (sugars, starch, hemi-cellulose, cellulose), apart from the model of Dijkstra (1994; a more detailed version of Dijkstra et al., 1992) where protozoa and bacteria utilize the same carbohydrates types. Although there is lot of attention and search for consortia of micro-organisms being responsible for certain functionality by use of omics techniques, this has so far not led to the necessary knowledge that could be used for the representation of microbial activity in mathematical prediction models of whole rumen function. Nevertheless, these omics-driven techniques do lead to a wealth of new data and diverse types of data (Fig. 3) which lead to some new insights on changes in the composition and (putative) functionality (although towards this, relevant data is still limited) of the rumen microbial population under various rumen conditions (Denman et al., 2018; Stewart et al., 2018, 2019). Appropriate and proper interpretation of such data may lead to new concepts that may be introduced in models of whole rumen function. If these concepts are incorporated in current model representations of rumen function, model applicability may extend substantially and move in to multi-omics research approaches. Some concepts reported in literature will be discussed in the following section.

2.5 Concepts of microbial community dynamics

Related to the functionality of the rumen microbiota, terms such as functional redundancy and resilience have been introduced and discussed for complex ecosystems. Weimer (2015) reflected on the possibility to engineer the rumen ecosystems, characterized by enormous diversity of niches and micro-organisms. One of his key points was the presence of a strong functional redundancy in rumen microbiota, meaning that even with a reduced value for richness or diversity of the microbiota the functionality in terms of rumen fermentation capacity and digestion may remain rather unaffected due to overlap of function among multiple species (Weimer, 2015). Resilience is a key feature attributed to the rumen microbial population because of its ability to recover from strong perturbations (Weimer, 2015; Scheffer et al., 2015); such perturbations in the case of the rumen are changes in feed and water intake, and diet composition. The effect of these perturbations through diet was demonstrated by Dieho et al. (2017) in dairy cows, where an increase in dry matter and concentrate intake post-partum was observed to reduce richness of the bacterial community to some extent (Fig. 2a; open symbols; richness based on the 'phylogenetic diversity whole tree' richness metric;

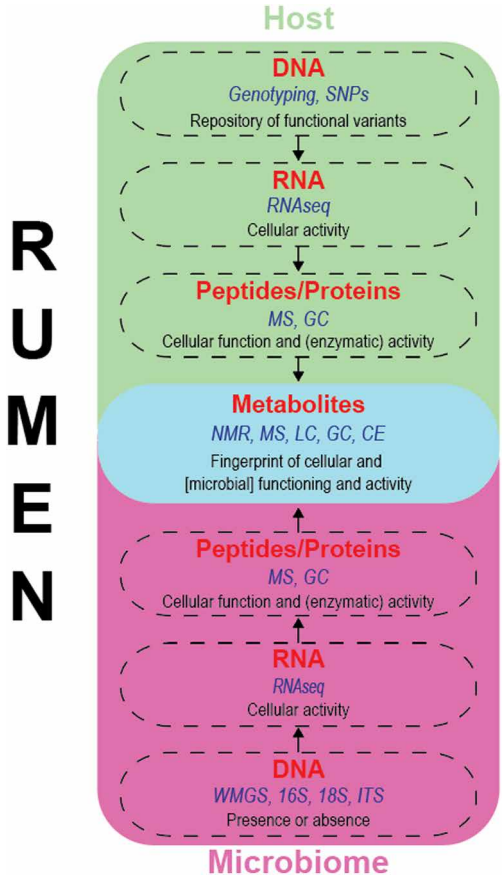


Figure 3 Schematic overview of the host and microbiome and associated biological levels and functions regarding the rumen. Both the host (green and blue) and microbiome (magenta and blue) can influence the rumen functioning. For the host and microbiome several associated biological levels are depicted, that is, DNA, RNA, peptides/proteins, and metabolites. Below all these different biological levels, the molecular techniques and resulting activity/outcome are depicted. The metabolites are placed centrally, because this is the integration of the interaction between the host and the microbiome. SNPs, single nucleotide polymorphisms; MS, mass spectrometry; GC, gas chromatography; NMR, nuclear magnetic resonance; LC, liquid chromatography; CE, capillary electrophorese, WMGS, whole metagenome sequencing; ITS, Internal transcribed spacer.

Faith, 1992). The decrease in richness was much stronger with a more rapid increase of concentrate intake post-partum from 1 to 11 kg concentrate DM/d (Fig. 2a; closed symbols). This strong effect disappeared largely after a prolonged period where concentrate intake did not differ anymore between the groups, and bacterial richness did not differ between the cows despite initial differences in concentrate intake, indicating resilience of the microbial

population. Meanwhile, in situ fractional degradation rate of incubated feed appeared to remain unaffected irrespective of the changes in richness, which indicates functional redundancy. In a follow-up trial (Dieho et al., unpublished) the effect of 3 kg concentrate DM/d during the final 28 days of the dry period was tested, without a post-calving difference in concentrate build-up from 1 to 9 kg concentrate DM/d. Richness for concentrate-fed cows did not differ at the end of the dry period, probably because total feed intake remained low (12 kg DM/d). During early lactation when all cows received the same lactation diet, the numerically higher bacterial community richness observed in cows that received concentrate during the dry period did not differ from that of cows that received no concentrate in the dry period (Fig. 2b; based on the 'Chao1' richness metric; Chao, 1984). This may be an indication that a microbial population at the end of the dry period can be better prepared for the rapid increase in concentrate intake after calving; however, this would have to be confirmed with more conclusive results. These results indicate that transient changes in richness of the microbial community occur with changes in feeding, which includes the level of feeding but in particular rapid and substantial changes in fermentable organic matter intake. Eventually, the community appears to bounce back close to the richness that existed during the unperturbed state (Fig. 2a and b), although the general change in feed intake level and diet composition (viz., a low intake level and low concentrate proportion during dry period vs. high intake level and concentrate proportion during lactation) did result in a smaller permanent change in bacterial diversity. The rumen microbial population hence appeared rather resilient in these two studies with non-significant changes in in situ degradation characteristics, indicating that despite changes in bacterial diversity rumen degradation rate of feed was maintained. Using a systems biology approach, Belanche et al. (2019) reported more permanent changes in community structure and abundance of key microbial genera with a dietary change from hay/concentrate towards ryegrass pasture. They refer to this with the term plasticity as an indication of the capacity of the rumen microbiota to adapt to strong changes in the dietary and rumen fermentation conditions without losing its functionality. Generally, the rumen microbiota seems to be complex and stable over time in terms of composition in response to permanent changes in diet composition, although on the short run functional and metabolic activities of rumen microbiome do change (O'Callaghan et al. 2018).

None of the aforementioned functional qualifications of observed dynamics and behaviour of the rumen microbial population are covered by extant mathematical models that aim to predict whole rumen function; not even conceptual-wise (Dougherty et al., 2017; Li et al., 2018; Van Lingen et al., 2019). Nevertheless, it seems relevant to enable rumen models to represent these. Although genome-scale metabolic models (GEMs) are being developed and

have already been described for specific species and for pairwise interactions, what is still underexposed is this dynamic behaviour in entire living microbial communities such as that in the rumen. This complexity becomes prevalent from the functional complementarity and functional redundancy in the rumen microbiota. In a theoretical approach, Scheffer et al. (2015) state how functional complementarity can be seen to promote the overall ecosystem processes, whereas functional redundancy promotes the resiliency of these ecosystem processes against perturbations by the insurance effect of biodiversity. Scheffer et al. (2015) further state that while redundancy may be the rule in smaller creatures, resilience is thought to be less prone to occur with the larger species. Although many species may have the same functional role (and utilize the same substrates) they may differ in how they respond to stressors. The more unique function of the larger species community in the ecosystem, the lower richness of this community and their functional redundancy being more rare, makes that their loss from the ecosystem may lead to drastic shifts in ecological functioning. Although Scheffer et al. (2015) demonstrate the concept for the world's over 4000 species of diving beetles, the theoretical concept may apply just as well to the rumen as a complex ecosystem with a similar large diversity of microbial species coexisting, including the smaller bacteria and the larger protozoa. In this respect, it could be interesting to study the role and change of the contribution of protozoa in the rumen ecosystem (Newbold et al., 2015) which are of a much larger size than the bacterial community and may differ in cell size by a factor from 20 to 100 and can make up half of total rumen microbial biomass (Bainbridge et al., 2018). Functional redundancy and resilience of the rumen ecosystem seems well established for the bacterial population (Weimer, 2015). However, it may apply less to the much larger protozoa whose functional role in the rumen could be more vulnerable to perturbations as their maximal substrate utilization rate is low (modelled by Dijkstra, 1994). Perturbations could (temporarily) diminish their role in the rumen (despite their capacity to resist passage and sequester, and establish themselves after inoculation by companion ruminants), and hence lead to a new equilibrium in the rumen and a shift in functioning of the microbial ecosystem as a whole. The same may hold for the Archaea as these have a lower and delayed activity after feeding than bacteria (Van Lingen et al., 2017). Understanding their vulnerability to perturbations is important as protozoa and Archaea play key roles in the rumen ecosystem, regulating substrate fermentation rate and hydrogen dynamics, and thereby the overall rumen fermentation pattern (Newbold et al., 2015). This pattern is an important response variable to predict by models of whole rumen function, as it determines the ratio of glucogenic:ketogenic nutrients that the host absorbs from the rumen, as well as methane emissions (Dijkstra, 1994; Huws et al., 2018; Van Lingen et al., 2019). It would be highly interesting to study whether the concepts of functional redundancy and resilience generally

apply to protozoa and Archaea, or whether sudden functional changes of the rumen ecosystem may occur due to sudden perturbations. These changes may involve dietary changes or the feeding of specific supplements. Evaluating vulnerability when selecting for different rumen fermentation profiles with breeding may also be of large interest. For example, ewes selected for low methane yield did have smaller rumens and shorter rumen retention times of particulate and liquid digesta (Goopy et al., 2014). If rumen functional changes can be demonstrated, understanding them would become a key to implement them in models of whole rumen function. Experimental rumen studies and analysis of behaviour of the rumen microbiota would probably have to be designed differently. Current studies often report differences among rumen states in an empirical, descriptive manner but without much understanding of the consequence for the ecosystem's functionality and how to quantify this in models of whole rumen function (i.e. in terms of digestibility, acidity, microbial synthesis rate, intra-ruminal recycling, absorption of VFA as end products of fermentation).

3 Quantifying rumen microbial functionality

In a review by Denman et al. (2018) discussing the characterization of rumen microbiota function when using multiple omics techniques, they conclude that integration of ~omics derived data, when strengthened by (computational) modelling, will allow for the construction of rumen-specific microbial (metabolic) models. An example is the use of GEMs, and these models focus on translating an organism's metabolic capacity into mathematical representations. This method has been applied to bacterial systems (Oberhardt et al., 2009), and can be used to characterize phenotypes, to perform metabolic engineering, and to study (inter)species interactions (Freilich et al., 2011). Each individual microbial species has its own activity which is associated to its behavioural characteristics, however, and this introduces a new layer of complexity (Wintermute and Silver, 2010). Moreover, in biology, the capacity of microbial species to adapt to a 'mixed' culture is central. Application of integrative biology has the promise to make it possible to steer away from laboratory microbes (artificial) and to start focussing on understanding the more natural course of microbial behaviour (biological). Integrative biology refers to transdisciplinary research, that is, integrating (heterogenous) data from different biological levels and gathered within different disciplines, as in the first section of this chapter has been discussed for the case of whole rumen function (Fig. 1). These levels may hence include the level of the ruminant host, for example, performance, body weight, feed intake, feed digestion, host behaviour (rumination, feed intake behaviour, salivation), and host DNA and gene expression in specific tissues. In principle it also may cover the lowest level at which data are gathered for functioning

of the rumen microbial ecosystem, which is the composition of the microbial population (DNA), as well as its gene expression (RNA, proteins), and enzyme capacity (proteins), enzyme activity measurements (conversions, fluxes) and the biological fingerprinting of metabolism (i.e. metabolites). Extant rumen models have traditionally been developed 'top-down' based on principles of digestive physiology (i.e. working from the level of whole rumen downwards by including additional details and complexity) instead of 'bottom-up' (i.e. working from the most detailed molecular level though multi-omics techniques upwards by analysing, integrating and associating to attempt to grasp whole rumen function). But, approaching 'complex' biological behaviour from both angles simultaneously may result in innovative ways to pursue the most relevant underlying biological mechanisms and their quantification. Prediction capacity of extant whole rumen mathematical models probably can benefit from such understanding, and they may serve to generate putative novel application strategies, as already were discussed for microbial population dynamics in the previous section. At the moment it is unclear, however, of how to grasp this understanding for the rumen ecosystem under *in vivo* conditions under a wide range of nutritional conditions and states, into mathematical models at the whole rumen level. These models aim to predict function at the whole rumen level (Bannink et al., 2016) and certainly do not represent the level of detail that multi-omics analysis normally delivers (Chong and Xia, 2017). This represents a gap to be covered in order to bring both approaches together. The scheme in Fig. 3 indicates the various biological levels and techniques that can be used to gather data for the host and for the microbiome to measure rumen functionality. These data can be related to whole rumen observations at the rumen level (fermentation rate, rumen outflow rate, microbial synthesis rate) or at the host level (faecal digestion, production, feed efficiency), which is normally referred to as phenotypic data (Huws et al., 2018). Datasets on observed characteristics of the rumen microbial population or the multi-omics associated with it do not yet seem to address the consequences for microbial functionality at the level represented in the extant models of whole rumen function. A translation of results towards the latter (e.g. by improved and more detailed phenotyping; Huws et al., 2018) is prerequisite for these multi-omics data to become applied in extant predictive models, and this should receive more attention in future research. Changes in and associations between consortia of rumen micro-organisms and rumen metabolites can be studied, delivering further insight in the consequences of events. Events may include weaning and the effect of the dam on the primal bacterial community (Abecia et al., 2018), or altered presence of rumen protozoa harbouring bacterial communities that are compositionally different from their surroundings which may aid to further explain interactions between both (Levy & Jami, 2018). Several challenges lie ahead however for modelling the functionality of the rumen microbiome with

the help of these kinds of results. In reviewing these challenges, Huws et al. (2018) conclude that often the phenotype of the ruminant host is less well described, hampering progress to quantify and predict (it is noted that this in particular holds for the rumen as a functional organ). They further concluded that possibilities to aid the further development of mathematical models and rumen proxies are foreseen, and may revolutionize our understanding of rumen microbiome.

Furthermore, currently available mathematical models of whole rumen function cannot handle, yet, the more transient effects or changes in microbial function that may observed in the rumen. These models adopt a rather deterministic approach which does not leave room for the rumen microbiota to have a metabolic 'memory' as a consequence of stressors, incidental perturbations or longer history of the rumen state. In contrast, these models adopt the concept of steady-state which basically ignores such temporal changes in microbial function. Typically, a 2-week period is taken as a minimum to let the rumen microbiota adapt to a new diet and achieve such steady-state with experimental work (Hristov et al., 2019). This indicates that short-term dynamics in principle are relevant to consider with rumen function and a changing diet or host. Short-run functional and metabolic activity of the rumen microbiota does change (O'Callaghan et al., 2018) and it may prove to be relevant when trying to understand and predict rumen dynamics and functionality, or to implement limits to functional redundancy and complementarity and resilience to rumen microbiota, as a function of dietary and host-related factors.

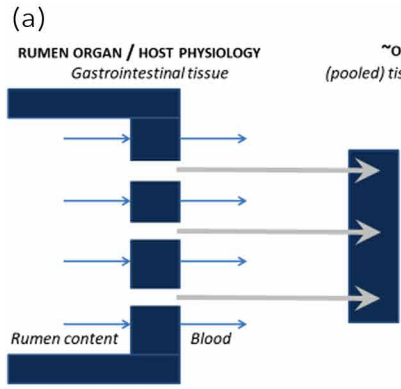
Associations may be explored (Chong and Xia, 2017; Huws et al., 2018; Denman et al., 2018) and perhaps become applied for practical purposes in the near future. It is important, however, to note that results only can be used to improve current mathematical prediction models of whole rumen function (as reviewed by Tedeschi et al., 2014, and Bannink et al., 2016) if results, or their associations, can be translated into terms that address the levels of functionality represented in these models. The descriptive and technology-driven nature of multi-omics studies delivers an unprecedented amount of information and level of detail. The descriptive nature of these studies in itself does not have to be problematic because extant whole rumen prediction models were also developed mostly from descriptive knowledge. However, to let the analysis of these results serve extant rumen prediction models is a real challenge, and a more fundamental one than the need for good and more detailed phenotyping. The units that apply to the multi-omics observations are key aspects of this challenge and will be discussed in the next section. The level of aggregation or of functionality that is addressed by data analysis is another challenge as whole rumen modelling requires quantification of substrate degradation rate based on the presence of that substrate and microbiota in the rumen (Dijkstra et al., 1992; Li et al., 2018), or of (net) total rumen microbial protein synthesis rate

(Dijkstra et al., 1998). Such quantifications are not *per se* delivered by analyses of multi-omics data.

4 Units and sampling techniques

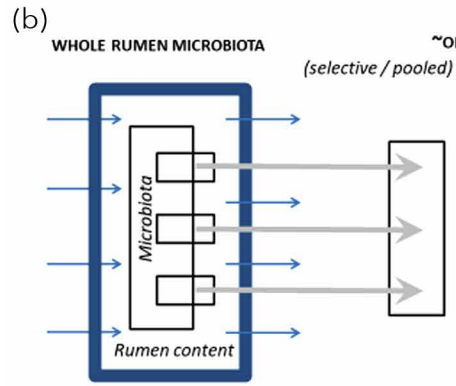
Multi-omics techniques are used to analyse material from samples taken from the target organ (rumen wall tissues, rumen digesta or rumen fluid, rumen microbiome). In this respect they have the promise to give insight and further quantitative understanding of the functionality of rumen microbiota by a 'bottom-up' approach (Huws et al., 2018; Denman et al., 2018). Although mathematical prediction models of whole rumen function represent the same biological entities, generally a disparity remains in the units adopted in mathematical prediction models of whole rumen function and those adopted in multi-omics analysis. Figure 4 attempts to clarify this disparity when taking samples from rumen tissues (Fig. 4a), from the rumen microbiota (Fig. 4b) or from rumen fluid (Fig. 4c). To make ~omics-based data and their analysis directly applicable for modelling of whole rumen function, this unit difference needs to be overcome by upscaling of results from samples (which in essence only deliver a concentration) to the level of response of the whole rumen wall tissue, the whole rumen microbial population or the whole rumen fluid volume (quantity), respectively. A further essential element lacking with multi-omics techniques is that of 'the fluxes', that is, the flux in and out of rumen wall tissue (at the mucosal as well as the serosal side) and the flux in and out of the rumen (through the oesophagus and the reticulum). The prime interest of predictive rumen models is to quantify fluxes, and how these vary with rumen microbial functionality and dietary intake, not concentrations. To arrive at the level of rumen flux predictions the dynamic, mechanistic models of whole rumen function describe enzymatic degradation and microbial synthetic capacity as functions of the available substrate and the microbial population present in the entire rumen. This means that the proteins or enzymatic capacity, which is concentration dependent in essence, needs to be quantified (Bannink et al., 2016). Other observations remain indirect measurements of this capacity, such as those on DNA, RNA and metabolites in rumen samples. Matching 'bottom-up' and 'top-down' approaches with quantification of rumen function means that common-ground needs to be found at the level of proteins and enzymatic capacity; in the rumen contents of the microbial population present, in the host of the enzymatic activity and the response of the rumen wall (epithelia and muscular tissue) and in the rumen organ the inflow and outflow of material and fluid including buffering of rumen contents and recycling of N and phosphorus from blood (Figs. 1 and 3).

Apart from the need to bridge the disparity in units delivered and units required, the sampling procedure of rumen contents in itself is already a critical methodological aspect to consider (Fig. 4b and c). It has also been established



UNITS	
Tissue volume, mass	l, kg
Fluid volume	l
Tissue area	m^2
Tissue concentration	mol/l
External conc.	mol/l
Blood flow	l/h
Tissue fluxes	mol/h
Protein function	mol/h
Tissue functions	mol/h

UNITS	
Tissue activity	mol/(h . l tissue sample)
Protein function	mol/(h . l tissue sample)
Gene expression	fold-difference/(l tissue sample)
Metabolites	mol/l tissue sample



UNITS that apply to rumen function	
Rumen contents	l, kg
Fluid volume	l
Particle mass	kg
Particle surface area	m^2
Free / adherent fluid	kg
Particle outflow	/h
Fluid outflow	/h
Microbial activity	mol/(h . kg microbial mass . mol substrate)
Microbial presence	kg/kg content
Substrate presence	mol/kg content

UNITS that apply to a rumen sample	
DNA	g/(kg microbial mass . l sample)
RNA	g/(kg microbial mass . l sample)
Proteins	mol/(kg microbial mass . l sample)

Figure 4 ▶ (Continued)

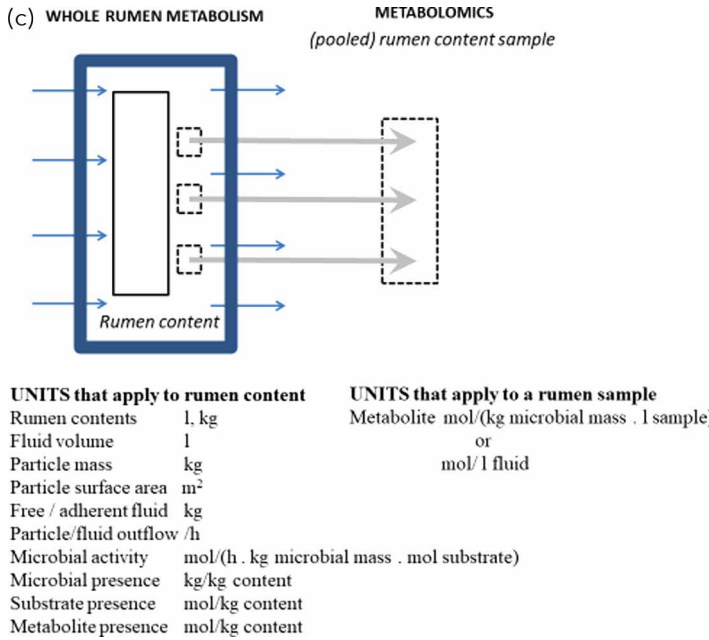


Figure 4 Schematic representation of the difference in units that apply to the whole rumen (at the left) and samples from the rumen (at the right), for the case of (a) rumen wall tissue, (b) the rumen microbial population, and (c) rumen fluid.

that many other biological factors can affect the outcome of microbial community analysis (Weimer, 2015). These factors include, genetics (Li et al., 2019), age (Jami et al., 2013), diet, phase of ruminal contents sampled (Mohammed et al., 2012), plane of nutrition of the host, sampling moment during the day, existing ruminal pH and animal individuality (Weimer et al., 2010). The latter probably being most related to animal behaviour and rumen morphological and physiological aspects. All these factors may have important implications for the study design and the selection of animals. Another set of factors related to the study design is the sample collection procedure; the site of sampling of digesta phase, the processing of the sample, the isolation methods used and the method of analysis. Variation in all could jeopardise the comparison of data from multiple studies as done with meta-analysis. Sampling of rumen content (e.g. preceding feeding) does not necessarily reflect daily average rumen function. In a study with frequent rumen measurement during the day and twice daily feeding, Van Lingen et al. (2017) established that time after feeding actually explained more of the observed variation in rumen metabolite concentration and composition of rumen microbiota than the dietary inclusion of linseed oil. It is hence evident that a single sampling moment probably will not reflect daily average rumen function. Furthermore, since long (Martin and

Michalet-Doreau 1995; Huhtanen et al. 1992), it is known that substantial diurnal variation exists in the enzymatic activity of rumen microbiota, with a potentially long delay after feeding for particulate associated bacteria to develop their maximum enzymatic degradation capacity. Diurnal variation of this size cannot be ignored when results are based on samples taken at a single time point, and have to be accounted when deriving conclusions and generalizing results.

5 Conclusion and future trends

Both the classical approach to quantify whole rumen function ('top-down') and the 'next-generation' approach by using multi-omics technologies ('bottom-up') have their own benefits and limitations for studying rumen biology. With the classical modelling approach rumen function is predicted by representing the basic chemical and physical principles of rumen processes, adopting a dynamic approach to quantify the interactions between substrate and the microbial population, at least with the most complex models (Fig. 1). Typically, the higher levels of organization are also represented including the influence of host physiological processes and intra-ruminal conditions. Furthermore, the chemical classification of substrates based on nutritional studies describes the basis to distinguish between the microbial functionalities and to quantify the substrate availability for microbial use (Bannink et al., 2016). In contrast, the 'next-generation' approach focusses on molecular characteristics by gathering multi-omics data from rumen samples. This approach is rather technology-driven and descriptive but benefits from the level of detail and the vast amount of ~omics-derived data that can be gathered. By metagenomic sequencing of the rumen microbiome, or by characterization of rumen metabolites, associations can be derived with the phenotype or the response of interest. We identified a mismatch between these two approaches in the previous sections, as they both focus on different 'parts' of the rumen. There is a disparity between the units used and functional aspects quantified, and there is a disparity between the aim of the quantification method (either finding associations or predictions based on a mechanism). Figure 5 attempts to illustrate these differences and disparities. It is envisaged that both approaches may benefit from each other in future research if the multi-omics methods strive to deliver functional characteristics that directly relate (or can be related) to the elements used in extant mathematical models of whole rumen function. The latter may benefit from multi-omics methodologies because of the far more detailed insight that can be gained in microbial population functionality than the current distinction based on substrate type. An important first step of improvement would have to be the standardization of techniques to make studies inter-comparable, so that centralized databases can be created that contain data from both 'parts' of the rumen. Such a database not only needs to harbour information about the

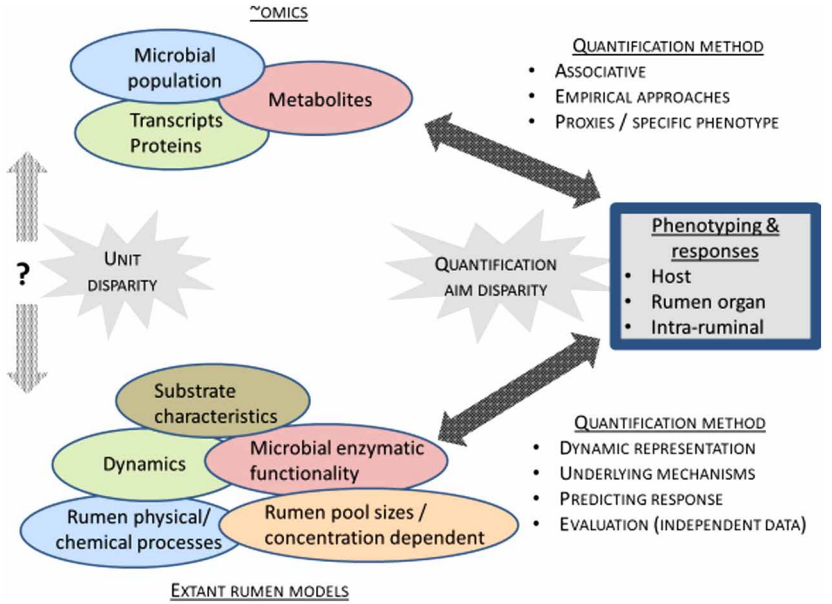


Figure 5 Schematic representation of the multi-omics approach (bottom-up) and the extant whole rumen modelling approach (top-down), illustrating the disparity in the units and levels of organization involved as well as the disparity in the aim of the quantification method.

experimental setup and host characteristics, but should also include detailed aspects of rumen function.

In conclusion, the exchange of knowledge would be enhanced if multi-omics results (and the knowledge derived) are more related to processes and elements present in extant whole rumen models. These models have been demonstrated to be important for explanation of observed variation in rumen function and microbial activity, that is, degradation and fermentation, rumen outflow and absorption, and synthesis of microbial mass. Quantifying such relationships would allow modellers to incorporate new elements or concepts and let extant prediction models of whole rumen function benefit from the increased insight and level of detail of knowledge on rumen microbial functionality. With further integration of the ~omics and whole rumen modelling approach the problem of disparity of the units used must be resolved, as well as the disparity in prediction aims of quantitative approaches.

6 Where to look for further information

There are several research activities ongoing that make attempts to quantify rumen microbiome functionality by the use of ~omics techniques. Furthermore,

the global research community is organized through the Global Research Alliance (GRA) (https://globalresearchalliance.org) and its associated *Rumen Microbial and Genomics Network* (https://globalresearchalliance.org/research/livestock/networks/rumen-microbial-genomics-network) and *Feed and Nutrition Network* (https://globalresearchalliance.org/research/livestock/networks/feed-nutrition-network).

The reader is referred to the following information:

- A position paper on the topic of how to use ~omics data to quantify rumen functionality by Huws et al. (2018), in close association with the *Rumen Microbial and Genomics Network* https://www.frontiersin.org/articles/10.3389/fmicb.2018.02161/full,
- A special issue in Frontiers of Microbiology by Ungerfeld & Newbold as guest editors on how to engineer rumen metabolic pathways https://www.frontiersin.org/articles/10.3389/fmicb.2017.02627/full,
- Results of the Global Rumen Census by Henderson et al. (2015) https://www.nature.com/articles/srep14567,
- Results of the CEDERS project with a focus on capturing dietary effects on on-farm GHG emissions with inventory and farm accounting methodology, as well as results from other ERAGAS projects. https://www.eragas.eu/en/eragas/Research-projects,
- Various communications through the Livestock Research Group of the GRA which includes report of activities and achievements of the *Rumen Microbial and Genomics Network* and *Feed and Nutrition Network* https://globalresearchalliance.org/research/livestock.

7 References

Abecia, L., Martínez-Fernandez, G., Waddams, K., Martín-García, A. I., Pinloche, E., Creevey, C. J., Denman, S. E., Newbold, C. J. and Yáñez-Ruiz, D. R. 2018. Analysis of the rumen microbiome and metabolome to study the effect of an antimethanogenic treatment applied in early life of kid goats. *Frontiers in Microbiology* 9, 2227. doi:10.3389/fmicb.2018.02227.

Bainbridge, M. L., Saldinger, L. K., Barlow, J. W., Alvez, J. P., Roman, J. and Kraft, J. 2018. Alteration of rumen bacteria and protozoa through grazing regime as a tool to enhance the bioactive fatty acid content of bovine milk. *Frontiers in Microbiology* 9, 904. doi:10.3389/fmicb.2018.00904.

Baldwin, R. L., Thornley, J. H. M. and Beever, D. E. 1987. Metabolism of the lactating cow. II. Digestive elements of a mechanistic model. *Journal of Dairy Research* 54(1), 107–31. doi:10.1017/s0022029900025231.

Bannink, A., Gerrits, W. J. J., France, J. and Dijkstra, J. 2012. Variation in rumen fermentation and the rumen wall during the transition period in dairy cows. *Animal Feed Science and Technology* 172(1–2), 80–94. doi:10.1016/j.anifeedsci.2011.12.010.

Bannink, A., van Lingen, H. J., Ellis, J. L., France, J. and Dijkstra, J. 2016. The contribution of mathematical modeling to understanding dynamic aspects of rumen metabolism. *Frontiers in Microbiology* 7, 1820. doi:10.3389/fmicb.2016.01820.

Belanche, A., Kingston-Smith, A. H., Griffith, G. W. and Newbold, C. J. 2019. A multi-kingdom study reveals the plasticity of the rumen microbiota in response to a shift from non-grazing to grazing diets in sheep. *Frontiers in Microbiology* 10, 122. doi:10.3389/fmicb.2019.00122.

Chao, A. 1984. Non-parametric estimation of the number of classes in a population. *Scandinavian Journal of Statistics* 11, 265–70.

Chong, J. and Xia, J. 2017. Computational approaches for integrative analysis of the metabolome and microbiome. *Metabolites* 7(4), 62. doi:10.3390/metabo7040062.

Denman, S. E., Morgavi, D. P. and McSweeney, C. S. 2018. Review: The application of omics to rumen microbiota function. *Animal: an International Journal of Animal Bioscience* 12(s2), s233–45. doi:10.1017/S175173111800229X.

Dieho, K., van den Bogert, B., Henderson, G., Bannink, A., Ramiro-Garcia, J., Smidt, H. and Dijkstra, J. 2017. Changes in rumen microbiota composition and *in situ* degradation kinetics during the dry period and early lactation as affected by rate of increase of concentrate allowance. *Journal of Dairy Science* 100(4), 2695–710. doi:10.3168/jds.2016-11982.

Dijkstra, J. 1994. Simulation of the dynamics of protozoa in the rumen. *British Journal of Nutrition* 72(5), 679–99. doi:10.1079/bjn19940071.

Dijkstra, J., Neal, H. D., Beever, D. E. and France, J. 1992. Simulation of nutrient digestion, absorption and outflow in the rumen: model description. *Journal of Nutrition* 122, 2239–56.

Dijkstra, J., France, J. and Davies, D. R. 1998. Different mathematical approaches to estimating microbial protein supply in ruminants. *Journal of Dairy Science* 81(12), 3370–84. doi:10.3168/jds.S0022-0302(98)75902-8.

Dijkstra, J., van Gastelen, S., Dieho, K., Nichols, K. and Bannink, A. 2020. Rumen pH and redox potential measured by rumen sensors: data and interpretation for key rumen metabolic processes. *Animal: an International Journal of Animal Bioscience* 14(s1), s176–86. doi:10.1017/S1751731119003112.

Dougherty, H. C., Kebreab, E., Evered, M., Little, B. A., Ingham, A. B., Hegarty, R. S., Pacheco, D. and McPhee, M. J. 2017. The AusBeef model for beef production: I. Description and evaluation. *Journal of Agricultural Science* 155(9), 1442–58. doi:10.1017/S0021859617000429.

Faith, D. P. 1992. Conservation evaluation and phylogenetic diversity. *Biological Conservation* 61(1), 1–10. doi:10.1016/0006-3207(92)91201-3.

Freilich, S., Zarecki, R., Eilam, O., Segal, E. S., Henry, C. S., Kupiec, M., Gophna, U., Sharan, R. and Ruppin, E. 2011. Competitive and cooperative metabolic interactions in bacterial communities. *Nature Communications* 2, 589. doi:10.1038/ncomms1597.

Goopy, J. P., Donaldson, A., Hegarty, R., Vercoe, P. E., Haynes, F., Barnett, M. and Oddy, V. H. 2014. Low-methane yield sheep have smaller rumens and shorter rumen retention time. *British Journal of Nutrition* 111(4), 578–85. doi:10.1017/S0007114513002936.

Gregorini, P., Beukes, P., Waghorn, G., Pacheco, D. and Hanigan, M. 2015. Development of an improved representation of rumen digesta outflow in a mechanistic and dynamic model of a dairy cow, Molly. *Ecological Modelling* 313, 293–306. doi:10.1016/j.ecolmodel.2015.06.042.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Global Rumen Census Collaborators and Janssen, P. H. 2015 Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific Reports* 5, 14567. doi:10.1038/srep14567.

Hook, S. E., France, J. and Dijkstra, J. 2017. Further assessment of the protozoal contribution to the nutrition of the ruminant animal. *Journal of Theoretical Biology* 416, 8–15. doi:10.1016/j.jtbi.2016.12.007.

Hristov, A. N., Bannink, A., Crompton, L. A., Huhtanen, P., Kreuzer, M., McGee, M., Nozière, P., Reynolds, C. K., Bayat, A. R., Yáñez-Ruiz, D. R., Dijkstra, J., Kebreab, E., Schwarm, A., Shingfield, K. J. and Yu, Z. 2019. Invited review: Nitrogen in ruminant nutrition: a review of measurement techniques. *Journal of Dairy Science* 102(7), 5811–52. doi:10.3168/jds.2018-15829.

Huhtanen, P., Dakowski, P. and Vanhatalo, A. 1992. Composition, digestibility and particle-associated enzyme activities in rumen digesta as influenced by particle size and time after feeding. *Journal of Animal and Feed Sciences* 1(3–4), 223–35. doi:10.22358/jafs/69913/1992.

Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., Muñoz-Tamayo, R., Forano, E., Waters, S. M., Hess, M., Tapio, I., Smidt, H., Krizsan, S. J., Yáñez-Ruiz, D. R., Belanche, A., Guan, L., Gruninger, R. J., McAllister, T. A., Newbold, C. J., Roehe, R., Dewhurst, R. J., Snelling, T. J., Watson, M., Suen, G., Hart, E. H., Kingston-Smith, A. H., Scollan, N. D., do Prado, R. M., Pilau, E. J., Mantovani, H. C., Attwood, G. T., Edwards, J. E., McEwan, N. R., Morrisson, S., Mayorga, O. L., Elliott, C. and Morgavi, D. P. 2018. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Frontiers in Microbiology* 9, 2161. doi:10.3389/fmicb.2018.02161.

Jami, E., Israel, A., Kotser, A. and Mizrahi, I. 2013. Exploring the bovine rumen bacterial community from birth to adulthood. *ISME Journal* 7(6), 1069–79. doi:10.1038/ismej.2013.2.

Levy, B. and Jami, E. 2018. Exploring the prokaryotic community associated with the rumen ciliate protozoa population. *Frontiers in Microbiology* 9, 2526. doi:10.3389/fmicb.2018.02526.

Li, M. M., White, R. R. and Hanigan, M. D. 2018. An evaluation of Molly cow model predictions of ruminal metabolism and nutrient digestion for dairy and beef diets. *Journal of Dairy Science* 101(11), 9747–67. doi:10.3168/jds.2017-14182.

Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., Fitzsimmons, C., Plastow, G. and Guan, L. L. 2019. Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. *Microbiome* 7(1), 92. doi:10.1186/s40168-019-0699-1.

López, S., Hovell, F. D. D., Dijkstra, J. and France, J. 2003. Effects of volatile fatty acid supply on their absorption and on water kinetics in the rumen of sheep sustained by intragastric infusions. *Journal of Animal Science* 81(10), 2609–16. doi:10.2527/2003.81102609x.

Martin, C. and Michalet-Doreau, B. 1995. Variations in mass and enzyme activity of microorganisms: effect of barley and buffer supplements. *Journal of the Science of Food and Agriculture* 67(3), 407–13. doi:10.1002/jsfa.2740670319.

Mills, J. A. N., Crompton, L. A., Ellis, J. L., Dijkstra, J., Bannink, A., Hook, S., Benchaar, C. and France, J. 2014. A dynamic mechanistic model of lactic acid metabolism in the rumen. *Journal of Dairy Science* 97(4), 2398–414. doi:10.3168/jds.2013-7582.

Mohammed, R., Stevenson, D. M., Weimer, P. J., Penner, G. B. and Beauchemin, K. A. 2012. Individual animal variability in ruminal bacterial communities and ruminal acidosis in primiparous Holstein cows during the periparturient period. *Journal of Dairy Science* 95(11), 6716–30. doi:10.3168/jds.2012-5772.

Nagorcka, B. N., Gordon, G. L. R. and Dynes, R. A. 2000. Towards a more accurate representation of fermentation in mathematical models of the rumen. In: McNamara, J. P., France, J. and Beever, D. E. (Eds), *Modelling Nutrient Utilization in Farm Animals*. CAB International, Wallingford, UK, pp. 37–48.

Newbold, C. J., de la Fuente, G., Belanche, A., Ramos-Morales, E. and McEwan, N. R. 2015. The role of ciliate protozoa in the rumen. *Frontiers in Microbiology* 6, 1313. doi:10.3389/fmicb.2015.01313.

Oberhardt, M. A., Palsson, B. Ø and Papin, J. A. 2009. Applications of genome-scale metabolic reconstructions. *Molecular Systems Biology* 5, 320. doi:10.1038/msb.2009.77.

O'Callaghan, T. F., Vazquez-Fresno, R., Serra-Cayuela, A., Dong, E., Mandal, R., Hennessy, D., McAuliffe, S., Dillon, P., Wishart, D. S., Stanton, C. and Ross, R. P. 2018. Pasture feeding changes the bovine rumen and milk metabolome. *Metabolites* 8(2), 27. doi:10.3390/metabo8020027.

Reichl, J. R. and Baldwin, R. L. 1976. A rumen linear programming model for evaluation of concepts of rumen microbial function. *Journal of Dairy Science* 59(3), 439–54. doi:10.3168/jds.S0022-0302(76)84225-7.

Russell, J. B., O'Connor, J. D., Fox, D. G., Van Soest, P. J. and Sniffen, C. J. 1992. A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. *Journal of Animal Science* 70(11), 3551–61. doi:10.2527/1992.70113551x.

Scheffer, M., Vergnon, R., van Nes, E. H., Cuppen, J. G. M., Peeters, E. T. H. M., Leijs, R. and Nilsson, A. N. 2015. The evolution of functionally redundant species; evidence from beetles. *PLoS ONE* 10(10), e0137974. doi:10.1371/journal.pone.0137974.

Stewart, R. D., Auffret, M. D., Warr, A., Wiser, A. H., Press, M. O., Langford, K. W., Liachko, I., Snelling, T. J., Dewhurst, R. J., Walker, A. W., Roehe, R. and Watson, M. 2018. Assembly of 913 microbial genomes from metagenomic sequencing of the cow rumen. *Nature Communications* 9(1), 870. doi:10.1038/s41467-018-03317-6.

Stewart, R. D., Auffret, M. D., Warr, A., Walker, A. W., Roehe, R. and Watson, M. 2019. Compendium of 4,941 rumen metagenome-assembled genomes for rumen microbiome biology and enzyme discovery. *Nature Biotechnology* 37(8), 953–61. doi:10.1038/s41587-019-0202-3.

Tedeschi, L. O., Cavalcanti, L. F. L., Fonseca, M. A., Herrero, M. and Thornton, P. K. 2014. The evolution and evaluation of dairy cattle models for predicting milk production: an agricultural model intercomparison and improvement project (AgMIP) for livestock. *Animal Production Science* 54(12), 2052–67. doi:10.1071/AN14620.

Ungerfeld, E. M. and Newbold, C. J. 2018. Editorial: Engineering rumen metabolic pathways: where we are, and where are we heading. *Frontiers in Microbiology* 8, 2627. doi:10.3389/fmicb.2017.02627.

Van Lingen, H. J., Plugge, C. M., Fadel, J. G., Kebreab, E., Bannink, A. and Dijkstra, J. 2016. Thermodynamic driving force of hydrogen on rumen microbial metabolism: a theoretical investigation. *PLoS ONE* 11(10), e0161362. doi:10.1371/journal.pone.0161362.

Van Lingen, H. J., Edwards, J. E., Vaidya, J. D., van Gastelen, S., Saccenti, E., van den Bogert, B., Bannink, A., Smidt, H., Plugge, C. M. and Dijkstra, J. 2017. Diurnal dynamics of

gaseous and dissolved metabolites and microbiota composition in the bovine rumen. *Frontiers in Microbiology* 8, 425. doi:10.3389/fmicb.2017.00425.

Van Lingen, H. J., Fadel, J. G., Moraes, L. E., Bannink, A. and Dijkstra, J. 2019. Bayesian mechanistic modeling of thermodynamically controlled volatile fatty acid, hydrogen and methane production in the bovine rumen. *Journal of Theoretical Biology* 480, 150–65. doi:10.1016/j.jtbi.2019.08.008.

Wang, Y., Janssen, P. H., Lynch, T. A., van Brunt, Bv and Pacheco, D. 2016. A mechanistic model of hydrogen–methanogen dynamics in the rumen. *Journal of Theoretical Biology* 393, 75–81. doi:10.1016/j.jtbi.2015.12.026.

Weimer, P. J. 2015. Redundancy, resilience and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations. *Frontiers in Microbiology* 6, 296. doi:10.3389/fmicb.2015.00296.

Weimer, P. J., Stevenson, D. M., Mantovani, H. C. and Man, S. L. C. 2010. Host specificity of the ruminal bacterial community in the dairy cow following near-total exchange of ruminal contents. *Journal of Dairy Science* 93(12), 5902–12. doi:10.3168/jds.2010-3500.

Wintermute, E. H. and Silver, P. A. 2010. Dynamics in the mixed microbial concourse. *Genes and Development* 24(23), 2603–14. doi:10.1101/gad.1985210.

Zebeli, Q., Aschenbach, J. R., Tafaj, M., Boguhn, J., Ametaj, B. N. and Drochner, W. 2012. Invited review: Role of physically effective fiber and estimation of dietary fiber adequacy in high-producing dairy cattle. *Journal of Dairy Science* 95(3), 1041–56. doi:10.3168/jds.2011-4421.

Part 2

The rumen microbiota

Chapter 5

Genome sequencing and the rumen microbiome

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1 Introduction

The advent of genome sequencing has had an impact on every aspect of biology, furthering our understanding of classification, taxonomy and evolutionary relationships through to predicting structure and function of proteins. Rumen microbiology has advanced through genome sequencing of its resident microbes, allowing comparison of taxa, function and metabolism not only within an animal, but also in varying conditions such as changing diets and different ruminant hosts across the world.

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Before whole genome sequencing became available, characterisation of function was achieved using morphology, biochemistry and additive or subtractive experimentation to determine function or capability (Bryant, 1959; Leahy et al., 2013). Small-scale sequencing allowed bacterial and archaeal communities to be connected to important rumen functions using the 16S rRNA gene to assign taxonomic groups and later linking this to function by association to available sequenced genomes (Wilkinson et al., 2018).

Although this approach was informative, these results rely heavily on information from sparsely populated databases with limited representatives for rumen microbes, and did not account for smaller communities that also contribute to some functions, or those that play a role in multiple different functions (Delgado et al., 2019). The advancement of '-omics' technologies has enriched the understanding of the rumen microbiome in terms of knowledge of the diversity, structure and function of the rumen microbial community (Kingston-Smith et al., 2013; Morgavi et al., 2013; Creevey et al., 2014), an understanding that is essential for the improvement of ruminant production while mitigating negative environmental consequences.

This chapter aims to highlight some of the key studies that have utilised knowledge developed from genome sequencing to contribute to our understanding of rumen microorganisms, their function and role in the ruminal ecosystem, and what implications these have for nutritional strategies to optimise microbial processes. A summary of the key discussion points can be seen in Fig. 1.

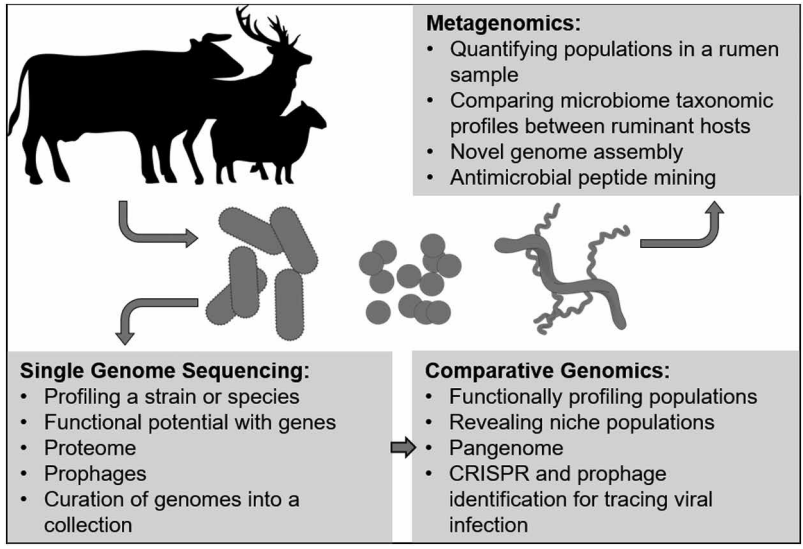

Figure 1 A summary of the key discussion points of this chapter.

2 The first rumen microbial genome

The first genome belonging to a rumen microbe that was sequenced and analysed was *Wolinella (Vibrio) succinogenes* DSMZ1740, published in 2003 (Baar et al., 2003). This occurred eight years after the first bacterial genomes were sequenced, the human pathogens *Haemophilus influenzae* Rd (Fleischmann et al., 1995) and *Mycoplasma genitalium* (Fraser et al., 1995). Although *W. succinogenes* was first isolated from the rumen of a cow, the 16S rRNA gene and its predicted protein complement revealed similarities to the human pathogens *Helicobacter pylori* and *Campylobacter jejuni,* despite *W. succinogenes* not having shown pathogenicity in either its ruminant host or humans (Baar et al., 2003).

The presence of this species in humans provided the main motivation for sequencing the genome of this ruminal bacterium in particular. Analysis of the genes and enzymes encoded by this genome confirmed that during growth, *W. succinogenes* grows by anaerobic respiration using H_2 to reduce fumarate to succinate, but cannot ferment glucose due to the lack of a glucokinase-encoding gene, and glucose or alternative carbohydrate transport systems. The presence of other carbohydrate metabolism enzymes led to the hypothesis that this bacterium likely uses glycolytic enzymes for gluconeogenesis instead (Baar et al., 2003).

The identification of genes in *W. succinogenes* homologous to distantly related microorganisms was highlighted as evidence of horizontal gene transfer. Furthermore, it was concluded, based on the complement of pathways possessed by this bacterium and the large number of genes present that were uncommon to other genomes in the same taxonomic class, that *W. succinogenes* is not adapted solely to its role as a rumen bacterium, a surprising revelation that disagreed with the hypotheses of the time (Baar et al., 2003).

From 2003 onwards, there was a slow but steady increase in completed genomes from cultured rumen microbes (though rarely more than 10 in any year). This continued until 2018 when the largest increase occurred with the publication of nearly 500 genomes from the Hungate collection (Seshadri et al., 2018) (Fig. 2). Parallel to this, genomes from uncultured rumen organisms began to appear in 2011 (Hess et al., 2011), rapidly increasing in number so that they now outnumber those from cultured organisms by over a factor of 10 (Fig. 2).

3 The power of sequencing a single genome

Although studying the phenotype of a microorganism is an important part of characterisation, sequencing a single genome from a bacterial culture

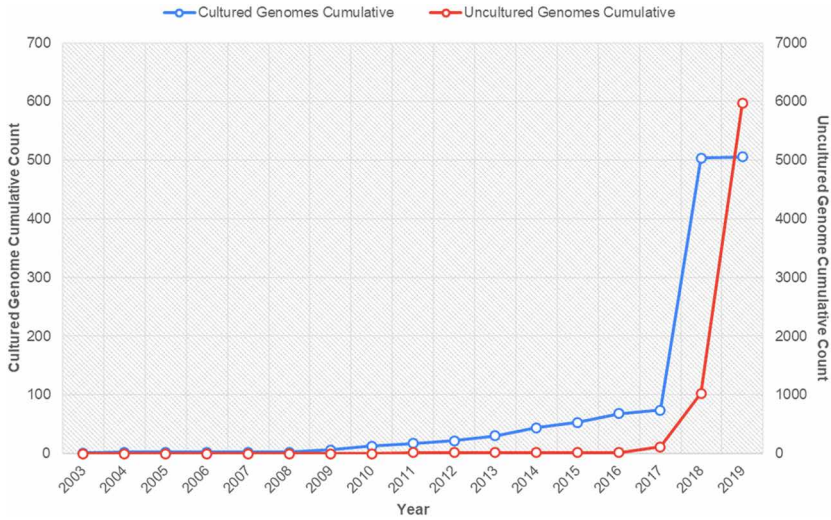

Figure 2 Frequency of rumen microbial genomes published over time.

reveals more about the genotypic potential, and the underpinnings of the phenotype. Investigating the genomes of isolates in culture where phenotypic information has been recorded and its functional capabilities tested, provides an immediate set of hypotheses to investigate, and allows the unravelling of the genomic elements behind the observed phenotypic responses (Creevey et al., 2014).

The predominant cellulolytic bacterium *Fibrobacter succinogenes* is an example of using genomic studies to elucidate the genomic mechanisms behind observed phenotypes in a rumen microbe. To determine the cellulolytic activity of this microbe, a culture of *F. succinogenes* S85 was exposed to a variety of conditions and variables including pH and population size, to achieve the best simulations of the rumen environment (Weimer, 1993). These culture-confined experiments determined the reliance of this bacterium on cellulose, with the ability to rapidly degrade crystalline cellulose, at a rate notably faster than other rumen bacteria in culture (Weimer, 1993). Further assays to determine polysaccharide hydrolysis and usage revealed that only products of cellulose degradation were used, whereas a number of other polysaccharides were degraded but not used. Genome sequencing of this organism many years later revealed the lack of enzymes required to further break down or utilise degradation products of any of the tested polysaccharides other than cellulose (Suen et al., 2011).

Analysis of the first complete methanogen genome (*Methanobrevibacter ruminantium* M1*)* revealed important biological insights to this important group. This organism possesses a larger genome size than other species in the same family from other environments, due to a myriad of genes unique to

this genome, as well as an integrated prophage and large numbers of surface adhesin-related genes (Leahy et al., 2010). Furthermore, this genome provided an insight into those genes involved in methanogenesis. As a pathway which culminates in the production of methane, resolving the genes involved in this process provides important information on the role of these microbes in methane emissions from ruminants. Finally, the genome allowed archaeal cell structures to be elucidated, presenting an opportunity for antigen discovery and novel antibody design for vaccine production as a methane mitigation strategy (Leahy et al., 2010, 2013).

This demonstrates the information that can be gleaned from individual genomes, but with large numbers of sequenced genomes, further insights through comparative genomics becomes a possibility.

4 Curation of a reference genome catalogue for the rumen microbiome

With an increasing availability of genome sequencing, there was a need for rumen-specific representatives to populate growing sequence databases and resources and to allow for more functional annotations to be allocated to novel rumen microbes, feeding into a loop of increasing information and understanding. For those microbes that could be isolated and purified from the rumen using culturing methods, DNA could be extracted and their genomes sequenced and gathered, forming a reliable resource specifically for the rumen microbiome. This was the primary aim of the Hungate 1000 project, a collaboration of multiple efforts across the globe to isolate and sequence 1000 unique microbes from the rumen to create a high-quality reference microbial genome catalogue (Seshadri et al., 2018).

It combined 91 already publicly available genomes with a further 410 genomes sequenced using Illumina and PacBio sequencing technologies, capturing isolates representing a variety of cultivable microbes, with multiple genomes from the predominant species also populating the collection. This project concluded in 2018, with input from across 21 countries and a final total of 501 genomes sequenced and made readily available as the Hungate Collection (Seshadri et al., 2018). Although most samples originated from cattle, sheep and other ruminants such as calves, goats and deer, genomes were also included from bacteria isolated from non-ruminants, such as horse, goose and pig, as these isolates are also known to reside in the rumen but no rumen isolates were available to add to the collection (Seshadri et al., 2018).

Advantages of a collaborative reference genome catalogue include offering the knowledge of traceability of the genome back to a cultured isolate, resulting in high-quality genomes with little concern for genome contamination or integration of DNA fragments from other species. As these microbes

represent existing cultures, once predictions are carried out on a genome, they can be corroborated by testing in culture (Creevey et al., 2014). Large-scale collaborations across the globe like this increase the variety and coverage of different ruminants sampled, giving a better insight to the rumen microbiome. Finally, having such a reliable catalogue can allow for novel microbes in any new metagenomic data to be enriched by removing sequences belonging to known microorganisms (Wilkinson et al., 2018).

The primary disadvantage to such a catalogue is the reliance on cultivation before sequencing, which brings other issues such as laboratory culturing and maintenance which can often influence or even change some microorganisms, let alone the costs associated with maintaining those cultures. Although those microbes included in the catalogue should be representative, it is difficult to determine how widely distributed they are (Attwood et al., 2008b). Because of this, these reference genomes should instead be considered a snapshot of the isolate at that time and limitations be kept in mind when doing analyses, although there are some steps that can be taken to overcome these, such as by relaxing thresholds for an acceptable hit when doing sequence similarity-based searches against the reference sequence set.

Despite this, the release of the Hungate collection (Seshadri et al., 2018) has had enormous impact on rumen microbiological research, including facilitating novel tool development for the rumen microbiome (Wilkinson et al., 2018), generating insights into alternative hydrogen uptake pathways in the rumen (Greening et al., 2019), identifying organisms capable of methyl-compound production (Kelly et al., 2019), increasing characterisation of antibiotic resistance in rumen microbiota (Sabino et al., 2019), revealing polysaccharide-degrading capabilities of groups such as the Butyrivibrio (Palevich et al., 2019) and identifying inter-bacterial communication in the rumen microbiome (Won et al., 2020).

Identifying genes in the genomes does not indicate usage or whether they are indeed usable, instead should be considered to be the metabolic potential of the microorganism (Creevey et al., 2014). Yet the greatest limitation is that the microorganism first requires isolating and culturing *ex vivo*, a feat not always possible. It is currently thought that around 23% of the rumen microbes are cultivable, with a positive correlation between population size and cultivability (Zehavi et al., 2018). However, renewed interest in applying 'culturomics' to the rumen will likely overcome this hurdle (Huws et al., 2018; Zehavi et al., 2018).

5 Application of metagenomic data for novel genome construction

Along with higher-throughput and cheaper sequencing methods came the intriguing possibility of assembling genomes from metagenomic data. From some of the earliest environmental metagenomic sequencing projects, it

became apparent that the assembly of complete microbial genomes was possible from metagenomic data (Tyson et al., 2004). While these initial successes were from very simple microbial communities, this seeded the development of sophisticated computational algorithms capable of reconstructing genomes from metagenomic data (Alneberg et al., 2014; Kang et al., 2015; Gregor et al., 2016) even from highly complex communities like the rumen (Hess et al., 2011; Pope et al., 2012; Svartström et al., 2017; Stewart et al., 2018).

In general, this is achieved by first carrying out an assembly of the metagenomic data followed by alignment of the original reads to the assembly, estimating the 'coverage' (i.e. how many time each assembled DNA sequence or 'contig' is represented in the original data) and then calculating the frequency of strings of nucleotide sequences of a given length (usually referred to as length 'k' and resulting in the general term for these nucleotide strings as 'k-mers'). The importance of the 'k-mers' stems from previous research that showed it was possible to identify k-mer profiles specific to individual species (Karlin et al., 1998). Therefore, with metagenomic data it is possible to calculate k-mer frequencies of all the contigs in the assembly and sort them into groups based on the species from which they came.

This process, termed 'binning', is one of the most important steps in all approaches that reconstruct genomes from metagenomic data. All the assembled contigs that are 'binned' together are therefore treated as if from a single organism and represent a 'Metagenomically Assembled Genome' (MAG), sometimes referred to earlier in the literature as 'taxonomic binning'. Further enhancements to this approach have included calculating where contigs in bins have correlated abundances across multiple samples (with the reasoning that if they all come from the same organism, they must all have correlated abundances) (Alneberg et al., 2014).

The advantage in utilizing an approach which assembles genomes from metagenomic data is that it has the potential of capturing sequence information from organisms which are difficult to culture in vitro (Creevey et al., 2014), potentially revealing novel functions and providing a greater understanding of the contribution of each organism to the overall rumen function.

The approach of binning metagenomic data from the rumen to provide a genome-scale overview of function has gathered a lot of pace in recent years (Hess et al., 2011; Pope et al., 2012; Svartström et al., 2017; Stewart et al., 2018; Stewart et al., 2019b), resulting in a total of over 5000 genomes across all these data sets, dwarfing the numbers sequenced from single isolated cultures (Fig. 2). It is difficult to assess the extent to which these MAGS have increased our sampling of the known poorly sampled clades of bacteria (Creevey et al., 2014), as with cultured genomes this knowledge has been obtained by comparison of the 16S rRNA gene found in the genomes to large-scale meta-taxonomic studies using the same marker (such as the Global Rumen Census (Henderson et al., 2015)).

The variable and repetitive nature of the 16S gene however, makes this a notoriously difficult gene to reconstruct from metagenomic data and so it is missing from the vast majority of rumen MAGs constructed to date. In the absence of a comparison to meta-taxonomic data, we must instead rely on other more easily reconstructed, universal genes for comparisons (Ciccarelli et al., 2006; Creevey et al., 2011), but this only allows comparisons of these two types of genomes from the rumen (cultured and MAGs), identifying where MAGs are closely related to cultured genomes and allowing taxonomic classification, but making inferences beyond this difficult.

We can however compare the relative abundance of the different taxonomic families identified by the three different methods (cultured genomes, MAGs and 16S amplicon sequencing) to understand where gaps in our knowledge may still exist (Table 1). While the top three taxonomic families in the Hungate collection (Seshadri et al., 2018), MAGs (Stewart et al., 2019b) and the global

Table 1 Relative abundance of genomes (number of genomes in brackets) from the Hungate collection (Seshadri et al., 2018) and metagenomically assembled genomes (MAGs) (Stewart et al., 2019b) of the average top 20 most abundant bacterial families identified across multiple ruminant species from the Global Rumen Census (Henderson et al., 2015)

Family	Hungate %	MAGs %	Global Rumen Census %
Prevotellaceae	7 (36)	16 (521)	35.29
Ruminococcaceae	8 (38)	35 (1111)	17.53
Lachnospiraceae	32 (162)	20 (640)	16.51
Paraprevotellaceae	0 (0)	0 (0)	5.13
Veillonellaceae	6 (30)	1 (25)	3.34
Fibrobacteraceae	0 (2)	1 (42)	3.10
Succinivibrionaceae	1 (7)	1 (16)	2.73
Mogibacteriaceae	0 (0)	0 (0)	2.04
Christensenellaceae	0 (0)	0 (0)	1.37
Clostridiaceae	3 (13)	1 (35)	1.29
Erysipelotrichaceae	3 (13)	9 (291)	1.28
Acidaminococcaceae	1 (4)	1 (43)	1.14
Bacteroidaceae	4 (18)	0 (0)	< 1
Spirochaetaceae	1 (6)	2 (52)	< 1
Anaerolinaceae	0 (0)	0 (0)	< 1
Corynebacteriaceae	0 (2)	0 (2)	< 1
Dethiosulfovibrionaceae	0 (0)	0 (0)	< 1
Lactobacillaceae	2 (10)	1 (22)	< 1
Streptococcaceae	8 (39)	0 (6)	< 1
Anaeroplasmataceae	0 (0)	0 (0)	< 1

For each of the data sets only those genomes which were identified at the family level were included.

rumen census (16S sequencing) (Henderson et al., 2015) are represented by *Prevotella, Ruminococcaceae* and *Lachnospiraceae*, they all disagree on their rank order (Table 1). This disagreement increases further down the list. These differences may represent diverse diets, breeds or geographical locations of the host animals from which the samples were taken and highlights where further research efforts may still be required.

Regardless of this issue, it is difficult to ignore the vast resource of genomic data from the rumen that MAGs represent to date and the genetic novelty that they are uncovering. For instance, the proposition of novel groups such as the Proteobacteria to those that could be considered 'core' to rumen microbiomes globally (Stewart et al., 2019b), although this may be skewed by the predominantly bovine origin of the data sets analysed to date. Perhaps the most startling insight has been the continuously increasing list of novel polysaccharide utilization loci, CAZymes and genes involved in cellulosomes, accumulating in recent studies which have identified hundreds of thousands of novel genes involved in carbohydrate metabolism (Stewart et al., 2019b).

There is no doubt that MAGs will contribute to our further understanding of the functional potential of the rumen microbial community and offer huge benefits to future developments of enzyme technologies in biorefineries (Svartström et al., 2017). Furthermore, the demonstrated classification rates of 50–70% of novel metagenomic data against this increasing genomic reference resource suggest that future rumen metagenomic studies may not have to carry out the difficult *de novo* assembly and annotation steps, perhaps allowing more shallow but higher replicated rumen microbiome sampling studies to be carried out. A natural extension to this is the generation of reference data sets of rumen-specific protein sequences which has emerged from these data sets (Stewart et al., 2019b).

It is the advent of long-read sequencing where the future generation of MAGs may best benefit. In existing rumen microbiome studies where this type of technology has been applied, many complete genome sequences have been reconstructed (Stewart et al., 2019b), capturing the 16S gene in its entirety. This approach will finally allow the comparison of uncultured microbial genomes to previously generated meta-taxonomic studies. This will be particularly useful for those meta-taxonomic studies where detailed phenotypic measurements of the rumen microbiome and host have been carried out, allowing more precise inference of the functional potential (Wilkinson et al., 2018) of the communities found to be associated with efficiency, health and environmental impact.

6 Comparative genomics and key functions in the rumen

Comparative analysis of the genomes sequenced in the Hungate collection (Seshadri et al., 2018) revealed that just over 2% of all genes identified in

the 410 sequenced genomes were involved in polysaccharide degradation, the majority belonging to the hyper diverse family of glycosyl hydrolases. Comparative analysis of the distribution of all genes involved in degradation and metabolism of plant structural carbohydrates allowed prediction of specialised roles within the community for different fermentation pathways. As these genomes represent isolates in culture these predictions can be tested in vitro, leading to possible in vivo strategies for optimising rumen function. Interestingly, this analysis also identified functions not previously thought relevant to this environment: A feature identified in Bacteroidetes genomes was the presence of glycosyl hydrolases involved in the degradation of host glycans. Seshadri et al. (2018) speculate that it may hint at a use of N-linked glycoproteins from the host salivary secretions.

Fewer of the nitrogen metabolism pathways are as well established as those for carbohydrates, with past studies tending towards concentrating on the cellulose and plant cell-wall degradation more so than protein breakdown. However, nitrogen metabolism by the microbial component of the rumen directly links to the host's nutrition. By allowing protein synthesis in microbes of the rumen from a variety of nitrogen sources and then absorbing microbial protein in the intestines, ruminants are able to efficiently gain access to a protein source (Wallace et al., 1997). Taking steps towards understanding protein metabolism and catabolism in the rumen therefore is important for host feed efficiency.

By combining protein and transcription studies with the available genome sequence, the nitrogen metabolism pathways available to *Prevotella ruminicola* 23 were elucidated (Kim et al., 2017). As a predominant species in the rumen microbial community, as well as a resident of human gut and oral microbiomes, studying the nitrogen pathways of *Prevotella* species is therefore a worthy endeavour. Evidence for adaptation to a fluctuating and complex environment was seen through the upregulation of ammonium transporters when ample ammonium is available to cells, but once this level drops peptide and polyamine transporters are upregulated instead, showing an adaptability to different nitrogen sources (Kim et al., 2017).

Another area of interest is the amino acid and peptide degraders. While they only form a small proportion in the rumen microbiome, the production of ammonia from the degradation process is detrimental to the environment as excess ammonia is excreted from the host as urea also representing a loss of nitrogen to the host, as this removal is a loss of nitrogen. The hyper ammonia-producing bacteria are particularly important to these processes, and continuing research aims to use their genomes and transcriptomes to better understand their functions, with a view to identify the genotype associated with this such that more microbes with this phenotype can be found (Friedersdorff et al., 2019).

Bacterial genomes are inherently dynamic, especially in species that are part of a diverse microbiome where competition is rife. In this situation genes are always being transferred, uptaken or undergoing mutations and selection pressures of some kind. As a result of this, genetic diversity and fluidity within the bacterial gene pool is to be expected (Andreani et al., 2017). This has become particularly apparent as an ever-expanding mass of bacterial genomic data has become available for analysis, and the development of new techniques for these analyses have become necessary (Kislyuk et al., 2011). One such advancement has been the investigation of 'pangenomes' (Tettelin et al., 2005) in response to the realisation that any single genome is a poor representative of the genetic variability in a species. In this approach, the set of genes that are shared between individuals of a species is termed the 'core genome' whereas the 'accessory genome' is formed of any other genes. Collectively, across a population, they form the pangenome.

Using the genome sequences to define the pangenome of a species facilitates our understanding of its biology and functional role in vivo. This makes it invaluable for understanding a species or population as a whole. For example, understanding the repertoire of genes that a strain may have access to could prove useful for predicting the transfer of antibiotic resistance genes (Subedi et al., 2019). Rumen bacteria have been found to possess extensive isoforms for certain metabolic enzymes, which would suggest a larger accessory genome, allowing the degradation and metabolism of a wider variety of substrates (Rubino et al., 2017).

Practical applications of this could potentially involve the improvement of biofuel production efficiency, though a more detailed understanding of the organisms that produce enzymes may be required, such as cellulases and hemicellulases to break down plant structural carbohydrates. Additionally, many organisms within the rumen work collaboratively, with various organisms playing a role at different stages of a metabolic reaction. One example of this within the rumen is biohydrogenation, that is, the conversion of poly-unsaturated fatty acids to a more saturated form by microbial action (Mosley et al., 2002). *Butyrivibrio fibrisolvens* is capable of biohydrogenating linoleic acid (18:2) to cis9, trans-11 conjugated linoleic acid. This is then reduced to stearic acid (18:0) by *B. proteoclasticus* (Ramos-Morales et al., 2013).

Generally, as new strains are added to the population, new genes continue to be added to the gene pool. If the addition of each new strain adds a multitude of genes to the pool, the pangenome can be called 'open'. Conversely, if the number of new genes added is limited, the pangenome is 'closed' (Tettelin et al., 2008). As such, the *Butyrivibrio* genome is incredibly open, with some species of the genus having less than 3% of all its genes being core. While much of the core genome can be attributed to housekeeping genes (that is, those necessary for the survival of the cell), the accessory genome tends to have genes involved

in a wider variety of functions. For example, the core genome of *B. fibrisolvens* is predominantly composed of genes involved with translation, ribosomal structure and biogenesis (70%), while accessory genome function is much more evenly distributed across a range of functions ranging from carbohydrate metabolism to replication, recombination and repair (Pidcock et al., 2019).

Given that strains belonging to the same species are assumed to be genomically similar, evidence of such extensive genomic variation within a species calls into question the very nature of this taxonomic relationship between these organisms, and further the entire species concept (Bentley, 2009). The term species therefore tends to carry connotations and functional implications that members of that species should possess, but that may not be entirely reliable (Papke, 2009). It is important to remember, however, that not all strains of these species will have the same set of genes, and will therefore not be capable of catalysing exactly the same reactions (Hussain et al., 2016). This demonstrates that a more thorough understanding of the pangenome may also be important for agricultural optimisation, such as ruminant feed efficiency.

7 The genome as a blueprint of the proteome

The term proteome was coined in the 1990s to describe the protein complement coded for by the genome (Wilkins et al., 1996). Proteomics is a complementary technique to other 'omics platforms providing a more comprehensive understanding of functionality within living organisms. Genomics provides the 'blueprint' of possible gene products for proteomics (Tyers and Mann, 2003). However, proteomics is far more challenging than genome sequencing due to the nature of the effort required to identify and quantify all proteins of a proteome. For example, there are possibly 100 000 protein forms encoded by approximately 20 235 genes in the human genome (Zhang et al., 2013).

The advancement of DNA sequencing has provided a sequence infrastructure for protein analysis and proteins can be identified by using mass spectrometry data to search protein and nucleotide sequence databases (Yates, 2000). Shotgun proteomics is the most commonly applied method of proteomics and involves the proteolytic digestion of proteins into their subsequent peptides that are then identified by mass spectrometry. Proteins are then determined by searching sequence databases with peptide spectrum-matching algorithms acquiring data from existing genome projects (Carvalho et al., 2016). The term shotgun is applied due to its analogy to shotgun genome sequencing (Yates, 1998; Zhang et al., 2013) where DNA is sheared into smaller contigs and sequenced.

Recently, proteomics has been applied to enhance the understanding of the rumen microbiome. This has been undertaken using a meta-proteomic

approach, which given the complexity of the rumen system aims to determine the protein complement of microbiota at a given point in time (Wilmes and Bond, 2004). The metaproteomic complement of the rumen system, although still a developing technique, has been addressed during recent years with the identification of proteins using shotgun proteomics (Deusch and Seifert, 2015; Snelling and Wallace, 2017; Hart et al., 2018). By applying the metaproteomics technique in reference to published metagenomes, trends of protein diversity of the rumen by their predicted species of origin was found to align with taxonomic profiles from metagenomic and 16S rRNA profiles (Hart et al., 2018). Proteomics is a considerably challenging technique, however, the continued development of current methodologies will allow for a more detailed annotation of functionality within the rumen microbiome. The application of the combination of the various 'omics platforms will improve the understanding within the rumen ecosystem for future potential beneficial rumen manipulation.

8 Genome sequencing and interactions across the microbiome

The genomes of bacteria not only reveal their functions, abilities and roles in the rumen microbiome, but can also highlight the interactions with other microorganisms. Always under competitive pressure, microbes have developed niche specialisation and defence mechanisms to protect their niche. Niche specialisation in the rumen has been linked to the possession of an exclusive repertoire of carbohydrate-active enzymes, with one study by Rubino et al. (Rubino et al., 2017) that examined the genetic variants of proteins ('isoforms' as defined by (Schlüter et al., 2009)) in their population. The study examined how populations of the *Clostridium* and *Prevotella* genera, while sharing the same biochemical capabilities, presented stark differences in isoform diversity for specific classes of proteins related to the degradation and use of different types of plant cell-wall carbohydrates. Moreover, such adaptations could be part of the temporal dynamics of plant material colonisation in the rumen (Huws et al., 2016), suggesting that in the rumen standing variation is one of the driving forces for adaptation to the changing conditions in the rumen (Barrett and Schluter, 2008).

The production and secretion of antimicrobial compounds as a defence mechanism is of particular interest due to the potential they hold for application as therapeutics (Williams, 2009). This is another result of the intra-microbial competition that is present in the rumen, with antimicrobial compounds forming one of the many secondary microbial metabolites that indicate a history of function. Other inter-microbial interactions that the bacteria and archaea in particular face are those with parasitic viruses. The rumen is well

populated with a diverse community of viruses, some of which are predatory on microbes and although they form a considerable portion of the rumen microbiome, research effort into this community has seen less focus than other ruminal microorganisms (Gilbert et al., 2020). Bacteriophages are viruses that prey on bacteria, and these alone have been found at concentrations of 10^7–10^8 per millilitre of rumen fluid (Klieve and Bauchop, 1988).

Bacteriophages are predatory viruses of bacteria (this term may sometimes be applied to archaeal viruses as well), requiring a specific species or even strain of bacteria to replicate itself through one of two ways. Once bound to the cell surface, the phage inserts its genome, generally stored in the capsid, into the host cell, where it undergoes one of the two cycles: lytic or lysogenic. Replication occurs using bacterial machinery and the phage genome to produce more virions, eventually producing cell-bursting lytic enzymes, causing cell death and expulsion of virions to infect more cells, completing the lytic cycle. This cycle is detrimental to the host bacterial cell, leading to evolution of various defence mechanisms, most notably the CRISPR-Cas system. Alternatively the phage genome is integrated into the bacterial cell genome, where it remains as a prophage and is passed on to daughter cells until such a time when the bacterial cell is disrupted in some way, and the prophage excises itself and enters the lytic cycle. This prophage can be identified in part or even complete when analysing bacterial genomes, which allows tracking of phage infection.

8.1 Viral sequences in the genome and prophage infections

Even before the availability of bacterial genomes, it was possible to determine the presence of integrated phages through induction. Cultures of rumen bacteria were treated with the mutagen mitomycin C to disrupt the bacterial cells and induce any prophages present to undergo the lytic cycle and form virions, which were then observed using microscopy (Klieve et al., 1989). With the genome sequence to hand, culturing is not always necessary, as the presence of prophages or proviruses can be determined by searching for genes homologous to known viral proteins, indicating possible past infection with a bacteriophage or archaeal virus, or in the case where a number of these genes are found together as a complete prophage, be evidence of where the phage has integrated within the host. Analysis of the genomes sequenced from individual species of methanogens and acetogens have identified provirus regions. For example, a 55 kb provirus was found in *Eubacterium limosum* SA11 (Kelly et al., 2016), one of length 49 kb in *Methanobrevibacter millerae* SM9 and one of 37 kb in *Methanobacterium formicicum* BRM9.

Although the identification of these proviruses in these microbes indicates the pervasiveness of virus and phage infection, for the vast majority we still do not know whether they are functional. One of the few with functional evidence

is the provirus ϕmru (Attwood et al., 2008a). While assembling the genome of the methanogen *Methanobrevibacter ruminantium* M1, a region with high GC content and coverage of around 40 kb in length was revealed to be a complete and intact provirus. Open reading frames were predicted and found to correspond to genes encoding important viral functions such as integration, structural proteins and DNA replication, and this complete provirus was named ϕmru (Attwood et al., 2008a). ϕmru also encodes a unique endoisopeptidase PeiR, an enzyme that when added to a culture of *M. ruminantium* M1 (and other organisms) lysed the host cells and reduced methane production. This demonstrated the potential applications of recombinant viral enzymes for biocontrol of methanogens (Leahy et al., 2010; Altermann et al., 2018) and how searching for integrated prophages in the sequenced genomes of microorganisms resident in the rumen may lead to the identification of novel approaches for methane mitigation through population control.

8.2 Using CRISPR sequences to reveal phage:host interactions

Clustered regularly interspaced short palindromic repeats (CRISPR) along with a Cas enzyme form the bacterial and archaeal defence mechanism against incoming viral infections. By integrating short regions of foreign viral nucleic acids into the host genome within a CRISPR array, this small RNA sequence can be recruited along with an enzyme complex to target and bind to invading foreign nucleic acids (Karginov and Hannon, 2010). The identification of CRISPR arrays and phage spacers in available genome sequences indicates recent interactions between host bacterial or archaeal populations and bacteriophages or archaeal viruses, respectively. Such CRISPR arrays were identified in the methanogens *Methanobrevibacter ruminantium* M1 (Attwood et al., 2008a), *M. millerae* SM9 (Kelly et al., 2016), *Methanobacterium formicium* BRM9 (Kelly et al., 2014) and acetogen *Eubacterium limosum* SA11 (Kelly et al., 2016). These were only identified as properties of the genomes and no further investigation of their implications for viral infections was carried out.

In the genomes from the Hungate collection, 241 have been identified as containing CRISPR regions. However only 2.7% of all the spacers identified had a match in the viral databases (Seshadri et al., 2018) revealing novel relationships between 31 of the Hungate microorganisms and 83 viruses. Only 26% of the spacers were similar to other sequences isolated from the rumen, compared to ~61% that were similar in human intestinal samples, highlighting the bias of the source of viral sequences currently deposited in public databases. To date only five lytic phages isolated from the rumen have been sequenced (Gilbert et al., 2017) and thus the future genome sequencing of bacteriophage and viral populations will be important to increase the understanding of inter-microbial interactions of viruses and host microbes resident in the rumen.

8.3 Competition resources in a microbiome

The existence of natural antimicrobials is predicated on the fact that to survive, or maintain an ecological niche, nearly all forms of life develop systems of antimicrobial defence against competing species or infectious agents, for example, bacterial, fungal, parasitic or viral. These antimicrobials either constitute a form of competitive advantage for smaller organisms in a microbiome or act directly as part of the innate immune response against invasive pathogens in the case of larger multi-celled creatures (Aminov, 2010). A critical component of both these types of protective system is a group of secondary metabolites known as antimicrobial peptides (AMPs), also often referred to as innate immunity peptides or host defence peptides. AMPs are arguably the most widespread system of natural antimicrobial defence, present in virtually every class of life, from unicellular to multicellular organisms including bacteria, archaea, fungi, plants, insects, amphibians, fish, reptiles and mammals (Thomas, 2019).

Bacteriocins are bacterial AMPs ribosomally encoded in bacterial genomes and primarily biocidal against closely related strains. Unsurprisingly, examples of class I (lantibiotic) bacteriocin encoding gene clusters have been found in ruminal bacteria, such as the broad spectrum antibiotic Butyrivibriocin OR79A (Kalmokoff et al., 1999) and Bovicin HC5, from *Streptococcus bovis* HC5 (Mantovani et al., 2002). Lantibiotics often undergo significant post-translational modification while class II bacteriocins form a larger group with higher diversity with respect to structure and antimicrobial activity (Ditu et al., 2014) and do not require extensive post-translational modification (Yang et al., 2014). Class II bacteriocins have also been detected in ruminal bacteria, for example, *Streptococcus* spp. (Bovicin 255) (Whitford et al., 2001) and Albusin B from *Ruminococcus albus* 7 inhibits growth of *R. flavefaciens* FD-1 (Chen et al., 2004).

Because of the growing number of microbial genome sequences from cultivated ruminal bacteria, *in silico* strategies to identify and characterise gene clusters encoding bacteriocins and defensive metabolites have rapidly evolved for the rumen microbes in both the Hungate 1000 collection and through environmental genomic data set mining (Oyama et al., 2017a). While the Hungate project enhanced the data available for analysis, the ruminal prokaryotome remains a potentially valuable under-explored resource of antimicrobials (Oyama et al., 2017b). A 2015 study employed bioinformatic analysis of the then available Hungate collection, examining 224 ruminal bacteria and 5 ruminal archaea and identified 46 bacteriocin gene clusters across multiple classes among 33 of the tested strains (Azevedo et al., 2015).

The level of bacterial and archaeal warfare in the rumen should be measured alongside the presence of antibiotic producing eukaryotic organisms (such as anaerobic fungi and ciliates) and plant material undergoing digestion (Oyama

et al., 2017b), as the degrading plant material itself is a critical component of the interplay between various microbial species fighting for shared resources and against one another. There are also ruminant host defence and other bovine or ovine secondary metabolites present within the environment (Agarwal et al., 2015) which contribute to a complex biological warfare ecosystem. There is considerable interest in using compounds identified in the rumen as probiotics or treatment regimes by manipulating the rumen microbiome of agricultural animals directly through the use of AMPs as food additives (Wang et al., 2016; Liu et al., 2017).

8.4 The effect of antimicrobials in the rumen

Antibiotic resistance genes are present in complex microbial communities where the production of antibiotics confers an evolutionary advantage in terms of survival (Huttner et al., 2013; Blair et al., 2015; Brown and Wright, 2016). The presence of such compounds can in turn put evolutionary pressure on competing organisms to evolve resistance to these antibiotics in order to ensure their own survival (Manges et al., 2001; Aminov and Mackie, 2007; Denning et al., 2017). The development of resistance can be further exacerbated by anthropogenic intervention, such as the overuse of antibiotics or their use in agriculture (Shea, 2003; Fischbach and Walsh, 2009; Moran, 2017). Analysis of resistance in varied environments can facilitate the prediction of future mechanisms of antibiotic and antimicrobial resistance that arise through transfer to clinically important microorganisms (Huttner et al., 2013; O'Neill, 2016; Martens and Demain, 2017).

Due to the close contact that occurs between humans and ruminants both on farms and in abattoirs, the rumen resistome is regarded as an important source of clinically relevant antibiotic resistance genes (ARGs) with opportunities to transfer to human pathogens. The presence and abundance of specific ARGs within the bovine digestive tract are known to change during treatment with antibiotics (Kanwar et al., 2014), and ARGs have previously been identified within the rumen as well as in ruminant faeces (Flint and Stewart, 1987). As the microbial ecosystem in the rumen is so diverse (Choudhury et al., 2015; Henderson et al., 2015) the likelihood of novel antimicrobial compounds being produced is high, as is the resulting development of resistance to these compounds. Indeed, deep sequence analysis has revealed the ovine rumen as a reservoir of ARGs (Hitch et al., 2018) which can be treated as a tangible environmental pollutant. This was subsequently supported by a large-scale study of antimicrobial resistance genes in 435 rumen microbial genomes, identifying high abundance of genes encoding tetracycline resistance and evidence that the tet(W) gene is under positive selective pressure (Sabino et al., 2019).

9 Conclusion

Insights into genomes and metagenomes of rumen organisms have been made possible by a wealth of tools developed by the scientific community. Table 2 lists some of the tools used to date to analyse rumen-associated data sets, particularly those that pertain to microbial genomes. In general the main steps in analysing this type of data can be summarised into data quality assessment; genome/metagenome assembly; assembly validation and statistics; genome binning (for metagenomic data); gene calling or open reading frame (ORF) prediction; functional and genome annotation; genome comparisons, taxonomy and phylogenetics; differential analysis and viral interactions (CRISPR and Prophages).

While Table 2 provides an overview of tools utilised previously that should be implementable on similar rumen-associated datasets, with the evolving bioinformatics environment, newer (and possibly better) tools are likely to become available over time. Furthermore, there are often multiple tools for the same analysis or process, each with its own advantages, disadvantages or specific use cases. We advise the reader to examine carefully the pros and cons of each tool before embarking on a large-scale bioinformatic analysis and to make use of the many online (and offline) courses available for the analysis of this type of data (such as the EMBL-EBI metagenomics training portal: https://www.ebi.ac.uk/training/online/topic/metagenomics).

Often such tools are linked together so that several (or all) of the steps outlined above are carried out automatically into a 'pipeline' or 'workflow'. Researchers with limited previous bioinformatics experience often prefer pipelines and/or graphical interfaces for these instead of command-line software tools. Galaxy (Afgan et al., 2018) is an excellent example of a platform that provides both a graphical interface and allows for the construction of automated workflows. Many institutions now implement local copies of Galaxy for the use of their researchers, but open online copies also exist free for anyone to use (such as https://usegalaxy.org/).

The disadvantage of using pipelines is that the user may end up treating the analysis as a 'black box', where data goes in and answers come out. This can leave researchers sometimes with little control or understanding of the analysis performed. Furthermore, regular updates of popular tools, or creation of novel tools that supersede older ones can result in a pipeline quickly becoming out of date. Some examples of those pipelines used for the analysis of rumen-associated data are included in Table 2.

10 Future trends

As sequencing becomes cheaper and more widely available, genomics as a whole should see an increase not only in the amount of data, but also in the quality

Table 2 A list of tools utilised to assess and analyse rumen-associated datasets, with the reference of the tool, and reference to their use

Method	Tool	Tool reference and purpose	Use on rumen-associated data
Read Quality Control	Trimmomatic	Bolger et al. (2014); a flexible tool that trims and filters reads for quality, as well as remove sequencing adapters.	Stewart et al. (2018)
	Sickle	Joshi and Fass (2011); uses sliding window approach to trim reads and adapters based on quality and read length.	Svartström et al. (2017)
	FLASH	Magoč and Salzberg (2011); a method to join short reads in paired end sequencing data before assembly to improve downstream processes.	Shi et al. (2014)
	BBDuK (part of the BBTools suite)	Bushnell, B. (Available at: sourceforge.net/projects/bbmap/); quality trimming and filtering, and adapter removal using K-mers.	Stewart et al. (2018)
	poRe	Watson et al. (2015); an R package to handle MinION Nanopore sequencing data.	Stewart et al. (2019b)
Assembly	Velvet	Zerbino and Birney (2008); uses K-mers and de Bruijn graphs for long contig and genome assembly.	Seshadri et al. (2018), Hess et al. (2011)
	ALLPATHS	Butler et al. (2008); genome assembly using whole-genome shotgun microreads (25-50bp).	Seshadri et al. (2018)
	HGAP	Chin et al. (2013); assembling a genome from a long-insert shotgun single sample DNA library along with Single Molecule, Real-Time (SMRT) data.	Seshadri et al. (2018)
	MEGAHIT	Li et al. (2015); an assembler suitable for large and complex metagenomic data.	Stewart et al. (2018, 2019b)
	MetaSPAdes	Nurk et al. (2017); metagenomic assembler.	Stewart et al. (2018)
	Ray	Boisvert et al. (2010); short-read assembler that takes reads from a variety and combination of sequencing platforms.	Svartström et al. (2017)
	Newbler	Margulies et al. (2005); shotgun sequence assembler	Svartström et al. (2017), Pope et al. (2012)
	Canu	Koren et al. (2017); assembles long-read sequence data such as from PacBio or nanopore.	Stewart et al. (2019b)

(*Continued*)

Table 2 (*Continued*)

Method	Tool	Tool reference and purpose	Use on rumen-associated data
Assembly Validation and Statistics	Bowtie	Langmead et al. (2009); aligns short reads (25–50 bp) to an assembled genome. For longer read length, use Bowtie2 (Langmead and Salzberg, 2012).	Hess et al. (2011)
	Burrows-Wheeler Aligner (BWA)	Li (2013); aligns reads or query sequences to contigs, scaffolds or genomes.	Stewart et al. (2018, 2019b), Seshadri et al. (2018)
	SamTools	Li et al. (2009); a suite of tools for handling the sequence alignment/MAP (SAM) file formats.	Stewart et al. (2018, 2019b) used to convert SAM files to BAM files
	CheckM	Parks et al. (2015); a tool to assess the quality of a genome by estimating completion and contamination.	Stewart et al. (2018, 2019b), Svartström et al. (2017), Seshadri et al. (2018)
	nanopolish	Simpson, J (available at https://github.com/jts/nanopolish); does signal level analysis to improve quality of long-read nanopore data.	Stewart et al. (2019b)
	Racon	Vaser et al. (2017); rapid consensus module to help assemble high-quality genomes from nanopore long-read sequencing.	Stewart et al. (2019b)
Genome Binning	MetaBAT2	Kang et al. (2019); a genome binning tool from metagenomic data	Stewart et al. (2018, 2019b) Used to bin metagenomes and calculate coverage from BAM files.
	dRep	Olm et al. (2017); a dereplication tool that uses pairwise genome comparisons within a bin to choose the highest quality genome.	Stewart et al. (2018, 2019b)
	CONCOCT	Alneberg et al. (2014); a tool to bin metagenomic contigs using sequence coverage and composition.	Svartström et al. (2017)
	PhyloPythiaS	Patil et al. (2011); a sequence compositional classifier to assign taxonomy to metagenomic data.	Pope et al. (2012) used for taxonomic binning.
Gene Calling or ORF Prediction	Prodigal	Hyatt et al. (2010); gene caller that claims to have better gene structure prediction, translation initiation site recognition and reduced false positives compared to other gene callers.	Seshadri et al. (2018), Stewart et al. (2019b), Pope et al. (2012)
	MetaGeneMark	Zhu et al. (2010); predicts genes in assembled genomes from shotgun sequencing of microbes.	Hess et al. (2011)

	GeneMark.hmm	Borodovsky et al. (2003); uses hidden Markov models to predict genes in prokaryotes.	Pope et al. (2012)
	MetaGene	Noguchi et al. (2006); using codon frequency and GC content, this tool predicts genes in environmental genome shotgun sequences.	Pope et al. (2012)
	GenePRIMP	Pati et al. (2010); a pipeline to improve gene prediction in prokaryotic genomes.	Seshadri et al. (2018)
	Microbial Genome Annotation Pipeline (MGAP)	Huntemann et al. (2015); a comprehensive pipeline that is the standard method used by the DOE's Joint Genome Institute (JGI). It combines tools to carry out quality control, structural annotation and functional annotation, including Prodigal and GenePrimp for gene calling, tRNAscan to find tRNAs and so on.	Seshadri et al. (2018) used for gene calling.
Functional and Genome Annotation	Prokka	Seemann (2014); a pipeline to annotate full prokaryotic genomes. Also integrates other programmes and algorithms, such as Prodigal for gene calling and HMMER for searching protein family profiles.	Stewart et al. (2019b), Svartström et al. (2017)
	IMG/M	Chen et al. (2019); integrated microbial genomes and microbiomes (IMG/M) is an online pipeline that analyses and annotates genomic and metagenomic data. It combines data from the JGI and user uploads and uses a myriad of tools such as HMMER and BLAST to search a variety of gene and protein databases.	Seshadri et al. (2018) used for annotation. Pope et al. (2012)
	USEARCH	Edgar (2010); a tool for global and local searches of sequence databases.	Shi et al. (2014) Used in conjunction with the KEGG database.
	Diamond	Buchfink et al. (2015); fast alignment of a large number of protein sequences to a reference database.	Stewart et al. (2018, 2019b) used to compare proteomes of genome bins to the UniProt TrEMBL database. Svartström et al. (2017) used to determine taxonomy for contigs by searching against the NCBI non-redundant protein database.

(*Continued*)

Table 2 (*Continued*)

Method	Tool	Tool reference and purpose	Use on rumen-associated data
	dbCAN	Yin et al. (2012); an online tool to annotate protein sequences with carbohydrate active enzyme (CAZymes) domains.	Stewart et al. (2018, 2019b) used dbCAN2.
	Profile Hidden Markov Model methods	Eddy (1998), Mistry et al. (2013) using a tool such as HMMER or Pfam_scan to search profile hidden Markov models of databases such as CAZy or pfam.	Seshadri et al. (2018), Hess et al. (2011), Stewart et al. (2018, 2019b), Svartström et al. (2017), Pope et al. (2012)
	MAGpy	Stewart et al. (2019a); a pipeline for annotating and characterising MAGS, comprised of CheckM, Prodigal, BLASTP in Diamond, Pfam-Scan, PhyloPhlAn and sourmash.	Stewart et al. (2018, 2019b)
	tRNAscan-SE	Lowe and Eddy (1997); predicts tRNA genes.	Stewart et al. (2019b)
	barrnap	Seeman, T - available at https://github.com/tseemann/barrnap; predicts rRNA genes in genomes.	Stewart et al. (2019b)
	RNAmmer	Lagesen et al. (2007); uses hidden markov models to predict rRNA genes.	Svartström et al. (2017)
	SINA	Pruesse et al. (2012); SILVA Incremental Aligner – multiple sequence alignments of rRNA genes according to global SILVA alignment, as well as using last common ancestor information for classification.	Svartström et al. (2017)
	PULpy	Stewart et al. (2018) finds polysaccharide utilisation loci.	Stewart et al. (2019b)
Genome Comparisons, Taxonomy and Phylogenetics	sourmash	Titus Brown and Irber (2016); uses MinHash sketches of DNA sequences to search and compare to others.	Stewart et al. (2018) used to compare genome bins to many published genomes.
	MEGAN4	Huson et al. (2011) tool for taxonomic and functional analysis of metagenomic, metatranscriptomic, metaproteomic and metataxonomic (rRNA) data.	Svartström et al. (2017)
	MUMer	Kurtz et al. (2004) rapidly aligns complete or draft genomes.	Stewart et al. (2019b)
	Minimap2	Li (2018) used to map long DNA or mRNA sequences to a reference genome or sequence.	Stewart et al. (2019b) used to map short Illumina reads to nanopore assemblies.

	FastANI	Jain et al. (2018) calculates average nucleotide identity as a metric of similarity across complete or draft genomes.	Stewart et al. (2019b)
	PhyloPhlAn	Segata et al. (2013) tool to assign phylogeny and taxonomy to microbes.	Stewart et al. (2018), Svartström et al. (2017)
	RAxML	Stamatakis (2014); phylogenetic analysis and tree building software.	Seshadri et al. (2018)
	Interactive Tree of Life (iTOL)	Letunic and Bork (2007); online interactive tool to graphically display and annotate trees.	Seshadri et al. (2018, , 2019b)
	FigTree	Rambaut, A (available at: http://tree.bio.ed.ac.uk/software/figtree/); graphical viewing software designed to display output from the BEAST phylogenetic tool.	Svartström et al. (2017), Stewart et al. (2018, 2019b)
	GraPhlAn	Asnicar et al. (2015); compact visualisation of large data sets of microbial genomes and metagenomes.	Stewart et al. (2018, 2019b)
	Kraken	Wood and Salzberg (2014) gives taxonomical information about metagenomic sequences quickly using K-mer based searches.	Stewart et al. (2018)
	CD-HIT	Fu et al. (2012); a tool to cluster sequences and reduce replication and redundancy.	Stewart et al. (2019b)
Differential Analysis	DESeq2	Love et al. (2014); an R package for differential analysis of count data, predominantly for RNA-seq and expression data.	Stewart et al. (2019b) used to compare to low and high methane data produced previously by Shi et al (2014).
	Metastats	White et al. (2009) a tool to detect differences in abundances in count data from metagenomic samples with two different treatments.	Seshadri et al. (2018) used to compare counts of genes that aligned to different protein families.
Viral Interactions; CRISPR and Prophages	IMG/M and IMG/VR database	Chen et al. (2019); detects CRISPR elements which can then be searched in the virus-specific database (Paez-Espino et al., 2017).	Seshadri et al. (2018)

and resolution, possibly at an even greater rate than seen over the last few years. Predictions about the future of genomics have a tendency to revolve around human genetics and therapeutics (Cheifet, 2019); however, there is something to be added about the implications of genome sequencing in environmental and microbiome samples. For instance, we may see a focus away from sequencing isolates to relying more on genomes from metagenomic sequencing. However as outlined earlier, this currently comes at the cost of not being able to directly compare to historical meta-taxonomic studies and will always lack the confirmatory evidence that can only come from having isolates in culture. Approaches for predicting culture conditions from the gene content of MAGs (Song et al., 2008) may eventually fill this gap, but for the moment the most promise comes from approaches that experiment with culture conditions to isolate the 'unculturable' (i.e. culturomics) or use single-cell sorting to isolate individual cells of microorganisms for sequencing (Huws et al., 2018). The technologies behind this latter approach have been maturing rapidly and will likely be the source of the next wave of genomic information from the rumen microbiome.

With an ever-increasing global population, ruminant agriculture will play an important role in sustaining adequate nutrient-rich food supplies and livelihoods. However, the implications of an increasing ruminant population on the environment and global warming cannot be ignored. What influences and effects farming practices have on ruminant host health and feed efficiency, and the subsequent emissions of enteric methane and other waste products, is something that needs to be better understood, and to do so requires a sound understanding of the rumen microbiome (Huws et al., 2018). The generation of the genome datasets outlined previously go a long way towards providing the information needed to answer these questions. However, in order to be able to put this information into the hands of the researchers addressing these questions, comprehensive rumen microbial genome databases that can be searched and queried need to be constructed. In an ideal world, such databases would contain complete and detailed metabolic pathways linked to functional annotations, making it possible to answer questions such as 'which species contribute to this function', 'how do these pathways contribute to the overall function of the rumen?' or 'what is the ideal rumen microbial community that is enhanced for this function?'. These types of questions are currently very difficult to answer without specialist computational skills (Friedersdorff et al., 2019) and so there is a case to be made that the development of such resources, made publicly available without cost, should be a priority for future rumen microbial genome research globally.

11 Where to look for further information

The Joint Genome Institute (JGI) Integrated Microbial Genomes and Microbiomes (IMG/M) resource (https://img.jgi.doe.gov/) provides annotation,

analysis and distribution of microbial genomes and microbiome datasets sequenced at the JGI and hosts all the novel genomes sequenced as part of the Hungate collection of rumen microbiome genomes.

Centres of expertise include:

The Rumen Microbial Genomics (RMG) network (http://www.rmgnetwork.org/).

The livestock research group of the Global Research Alliance (GRA) on agricultural greenhouse gases (https://globalresearchalliance.org/research/livestock/).

12 References

Afgan, E., Baker, D., Batut, B., van den Beek, M., Bouvier, D., Cech, M., Chilton, J., Clements, D., Coraor, N., Grüning, B. A., Guerler, A., Hillman-Jackson, J., Hiltemann, S., Jalili, V., Rasche, H., Soranzo, N., Goecks, J., Taylor, J., Nekrutenko, A. and Blankenberg, D. 2018. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Research* 46(W1), W537–44. doi:10.1093/nar/gky379.

Agarwal, N., Kamra, D. N. and Chaudhary, L. C. 2015. Rumen microbial ecosystem of domesticated ruminants. In: Puniya, A. K., Singh, R. and Kamra, D. N. (Eds), *Rumen Microbiology: from Evolution to Revolution*. Springer, New Delhi, India, pp. 17–30. doi:10.1007/978-81-322-2401-3_2.

Alneberg, J., Bjarnason, B. S., de Bruijn, I., Schirmer, M., Quick, J., Ijaz, U. Z., Lahti, L., Loman, N. J., Andersson, A. F. and Quince, C. 2014. Binning metagenomic contigs by coverage and composition. *Nature Methods* 11(11), 1144–6. doi:10.1038/nmeth.3103.

Altermann, E., Schofield, L. R., Ronimus, R. S., Beatty, A. K. and Reilly, K. 2018. Inhibition of rumen methanogens by a novel archaeal lytic enzyme displayed on tailored bionanoparticles. *Frontiers in Microbiology* 9, 2378. doi:10.3389/fmicb.2018.02378.

Aminov, R. I. 2010. A brief history of the antibiotic era: lessons learned and challenges for the future. *Frontiers in Microbiology* 1, 134. doi:10.3389/fmicb.2010.00134.

Aminov, R. I. and Mackie, R. I. 2007. Evolution and ecology of antibiotic resistance genes. *FEMS Microbiology Letters* 271(2), 147–61. doi:10.1111/j.1574-6968.2007.00757.x.

Andreani, N. A., Hesse, E. and Vos, M. 2017. Prokaryote genome fluidity is dependent on effective population size. *The ISME Journal* 11(7), 1719–21. doi:10.1038/ismej.2017.36.

Asnicar, F., Weingart, G., Tickle, T. L., Huttenhower, C. and Segata, N. 2015. Compact graphical representation of phylogenetic data and metadata with GraPhlAn. *PeerJ* 3, e1029. doi:10.7717/peerj.1029.

Attwood, G. T., Kelly, W. J., Altermann, E. H. and Leahy, S. C. 2008a. Analysis of the Methanobrevibacter ruminantium draft genome: understanding methanogen biology to inhibit their action in the rumen. *Australian Journal of Experimental Agriculture* 48(2), 83. doi:10.1071/EA07269.

Attwood, G. T., Kelly, W. J., Altermann, E. H., Moon, C. D., Leahy, S. and Cookson, A. L. 2008b. Application of rumen microbial genome information to livestock systems

in the postgenomic era. *Australian Journal of Experimental Agriculture* 48(7), 695. doi:10.1071/EA07408.

Azevedo, A. C., Bento, C. B. P., Ruiz, J. C., Queiroz, M. V. and Mantovani, H. C. 2015. Distribution and genetic diversity of bacteriocin gene clusters in rumen microbial genomes. *Applied and Environmental Microbiology* 81(20), 7290–304. doi:10.1128/AEM.01223-15.

Baar, C., Eppinger, M., Raddatz, G., Simon, J., Lanz, C., Klimmek, O., Nandakumar, R., Gross, R., Rosinus, A., Keller, H., Jagtap, P., Linke, B., Meyer, F., Lederer, H. and Schuster, S. C. 2003. Complete genome sequence and analysis of Wolinella succinogenes. *Proceedings of the National Academy of Sciences of the United States of America* 100(20), 11690–5. doi:10.1073/pnas.1932838100.

Barrett, R. D. H. and Schluter, D. 2008. Adaptation from standing genetic variation. *Trends in Ecology and Evolution* 23(1), 38–44. doi:10.1016/j.tree.2007.09.008.

Bentley, S. 2009. Sequencing the species pan-genome. *Nature Reviews. Microbiology* 7(4), 258–9. doi:10.1038/nrmicro2123.

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O. and Piddock, L. J. V. 2015. Molecular mechanisms of antibiotic resistance. *Nature Reviews. Microbiology* 13(1), 42–51. doi:10.1038/nrmicro3380.

Boisvert, S., Laviolette, F. and Corbeil, J. 2010. Ray: simultaneous assembly of reads from a mix of high-throughput sequencing technologies. *Journal of Computational Biology* 17(11), 1519–33. doi:10.1089/cmb.2009.0238.

Bolger, A. M., Lohse, M. and Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30(15), 2114–20. doi:10.1093/bioinformatics/btu170.

Borodovsky, M., Mills, R., Besemer, J. and Lomsadze, A. 2003. Prokaryotic gene prediction using GeneMark and GeneMark.hmm. *Current Protocols in Bioinformatics* 1(1), 4.5.1–16. doi:10.1002/0471250953.bi0405s01.

Brown, E. D. and Wright, G. D. 2016. Antibacterial drug discovery in the resistance era. *Nature* 529(7586), 336–43. doi:10.1038/nature17042.

Bryant, M. P. 1959. Bacterial species of the rumen. *Bacteriological Reviews* 23(3), 125–53. doi:10.1128/MMBR.23.3.125-153.1959.

Buchfink, B., Xie, C. and Huson, D. H. 2015. Fast and sensitive protein alignment using DIAMOND. *Nature Methods* 12(1), 59–60. doi:10.1038/nmeth.3176.

Butler, J., MacCallum, I., Kleber, M., Shlyakhter, I. A., Belmonte, M. K., Lander, E. S., Nusbaum, C. and Jaffe, D. B. 2008. ALLPATHS: de novo assembly of whole-genome shotgun microreads. *Genome Research* 18(5), 810–20. doi:10.1101/gr.7337908.

Carvalho, P. C., Lima, D. B., Leprevost, F. V., Santos, M. D. M., Fischer, J. S. G., Aquino, P. F., Moresco, J. J., Yates, J. R. and Barbosa, V. C. 2016. Integrated analysis of shotgun proteomic data with PatternLab for proteomics 4.0. *Nature Protocols* 11(1), 102–17. doi:10.1038/nprot.2015.133.

Cheifet, B. 2019. Where is genomics going next? *Genome Biology* 20(1), 17. doi:10.1186/s13059-019-1626-2.

Chen, J., Stevenson, D. M. and Weimer, P. J. 2004. Albusin B, a bacteriocin from the ruminal bacterium *Ruminococcus albus* 7 that inhibits growth of *Ruminococcus flavefaciens*. *Applied and Environmental Microbiology* 70(5), 3167–70. doi:10.1128/aem.70.5.3167-3170.2004.

Chen, I.-M. A., Chu, K., Palaniappan, K., Pillay, M., Ratner, A., Huang, J., Huntemann, M., Varghese, N., White, J. R., Seshadri, R., Smirnova, T., Kirton, E., Jungbluth, S. P., Woyke,

T., Eloe-Fadrosh, E. A., Ivanova, N. N. and Kyrpides, N. C. 2019. IMG/M v.5.0: an integrated data management and comparative analysis system for microbial genomes and microbiomes. *Nucleic Acids Research* 47(D1), D666–77. doi:10.1093/nar/gky901.

Chin, C. S., Alexander, D. H., Marks, P., Klammer, A. A., Drake, J., Heiner, C., Clum, A., Copeland, A., Huddleston, J., Eichler, E. E., Turner, S. W. and Korlach, J. 2013. Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. *Nature Methods* 10(6), 563–9. doi:10.1038/nmeth.2474.

Choudhury, P. K., Salem, A. Z. M., Jena, R., Kumar, S., Singh, R. and Puniya, A. K. 2015. Rumen microbiology: an overview. In: Puniya, A. K., Singh, R. and Kamra, D. N. (Eds), *Rumen Microbiology: from Evolution to Revolution*. Springer, New Delhi, India, pp. 3–16. doi:10.1007/978-81-322-2401-3_1.

Ciccarelli, F. D., Doerks, T., von Mering, C., Creevey, C. J., Snel, B. and Bork, P. 2006. Toward automatic reconstruction of a highly resolved tree of life. *Science* 311(5765), 1283–7. doi:10.1126/science.1123061.

Creevey, C. J., Doerks, T., Fitzpatrick, D. A., Raes, J. and Bork, P. 2011. Universally distributed single-copy genes indicate a constant rate of horizontal transfer. *PLoS ONE* 6(8), e22099. doi:10.1371/journal.pone.0022099.

Creevey, C. J., Kelly, W. J., Henderson, G. and Leahy, S. C. 2014. Determining the culturability of the rumen bacterial microbiome. *Microbial Biotechnology* 7(5), 467–79. doi:10.1111/1751-7915.12141.

Delgado, B., Bach, A., Guasch, I., González, C., Elcoso, G., Pryce, J. E. and Gonzalez-Recio, O. 2019. Whole rumen metagenome sequencing allows classifying and predicting feed efficiency and intake levels in cattle. *Scientific Reports* 9(1), 11. doi:10.1038/s41598-018-36673-w.

Denning, D. W., Perlin, D. S., Muldoon, E. G., Colombo, A. L., Chakrabarti, A., Richardson, M. D. and Sorrell, T. C. 2017. Delivering on antimicrobial resistance agenda not possible without improving fungal diagnostic capabilities. *Emerging Infectious Diseases* 23(2), 177–83. doi:10.3201/eid2302.152042.

Deusch, S. and Seifert, J. 2015. Catching the tip of the iceberg - evaluation of sample preparation protocols for metaproteomic studies of the rumen microbiota. *Proteomics* 15(20), 3590–5. doi:10.1002/pmic.201400556.

Ditu, L.-M., Chifiriuc, M., Pelinescu, D., Avram, I., Pircalabioru, G. and Mihaescu, G. 2014. Class I and II bacteriocins: structure, biosynthesis and drug delivery systems for the improvement of their antimicrobial activity. *International Journal of Clinical Pharmacology and Therapeutics* 11(2), 121–7. doi:10.2174/15701646110214091712242l.

Eddy, S. R. 1998. Profile hidden Markov models. *Bioinformatics* 14(9), 755–63. doi:10.1093/bioinformatics/14.9.755.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26(19), 2460–1. doi:10.1093/bioinformatics/btq461.

Fischbach, M. A. and Walsh, C. T. 2009. Antibiotics for emerging pathogens. *Science* 325(5944), 1089–93. doi:10.1126/science.1176667.

Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A. and Merrick, J. M. 1995. Whole-genome random sequencing and assembly of Haemophilus influenzae Rd. *Science* 269(5223), 496–512. doi:10.1126/science.7542800.

Flint, H. J. and Stewart, C. S. 1987. Antibiotic resistance patterns and plasmids of ruminal strains of *Bacteroides ruminicola* and *Bacteroides multiacidus*. *Applied Microbiology and Biotechnology* 26(5), 450–5. doi:10.1007/BF00253531.

Fraser, C. M., Gocayne, J. D., White, O., Adams, M. D., Clayton, R. A., Fleischmann, R. D., Bult, C. J., Kerlavage, A. R., Sutton, G., Kelley, J. M., Fritchman, R. D., Weidman, J. F., Small, K. V., Sandusky, M., Fuhrmann, J., Nguyen, D., Utterback, T. R., Saudek, D. M., Phillips, C. A., Merrick, J. M., Tomb, J. F., Dougherty, B. A., Bott, K. F., Hu, P. C., Lucier, T. S., Peterson, S. N., Smith, H. O., Hutchison, C. A. and Venter, J. C. 1995. The minimal gene complement of *Mycoplasma genitalium*. *Science* 270(5235), 397–403. doi:10.1126/science.270.5235.397.

Friedersdorff, J., Creevey, C. and Kingston-Smith, A. 2019. Characterising the genomes and transcriptomes of hyper ammonia producing bacteria from the rumen. *Access Microbiology* 1(1A). doi:10.1099/acmi.ac2019.po0523.

Fu, L., Niu, B., Zhu, Z., Wu, S. and Li, W. 2012. CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* 28(23), 3150–2. doi:10.1093/bioinformatics/bts565.

Gilbert, R. A., Kelly, W. J., Altermann, E., Leahy, S. C., Minchin, C., Ouwerkerk, D. and Klieve, A. V. 2017. Toward understanding phage:host interactions in the rumen; complete genome sequences of lytic phages infecting rumen bacteria. *Frontiers in Microbiology* 8, 2340. doi:10.3389/fmicb.2017.02340.

Gilbert, R. A., Townsend, E. M., Crew, K. S., Hitch, T. C. A., Friedersdorff, J. C. A., Creevey, C. J., Pope, P. B., Ouwerkerk, D. and Jameson, E. 2020. Rumen virus populations: technological advances enhancing current understanding. *Frontiers in Microbiology* 11, 450. doi:10.3389/fmicb.2020.00450.

Greening, C., Geier, R., Wang, C., Woods, L. C., Morales, S. E., McDonald, M. J., Rushton-Green, R., Morgan, X. C., Koike, S., Leahy, S. C., Kelly, W. J., Cann, I., Attwood, G. T., Cook, G. M. and Mackie, R. I. 2019. Diverse hydrogen production and consumption pathways influence methane production in ruminants. *The ISME Journal* 13(10), 2617–32. doi:10.1038/s41396-019-0464-2.

Gregor, I., Dröge, J., Schirmer, M., Quince, C. and McHardy, A. C. 2016. PhyloPythiaS+: a self-training method for the rapid reconstruction of low-ranking taxonomic bins from metagenomes. *PeerJ* 4, e1603. doi:10.7717/peerj.1603.

Hart, E. H., Creevey, C. J., Hitch, T. and Kingston-Smith, A. H. 2018. Meta-proteomics of rumen microbiota indicates niche compartmentalisation and functional dominance in a limited number of metabolic pathways between abundant bacteria. *Scientific Reports* 8(1), 10504. doi:10.1038/s41598-018-28827-7.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Global Rumen Census Collaborators and Janssen, P. H. 2015 Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific Reports* 5, 14567. doi:10.1038/srep14567.

Hess, M., Sczyrba, A., Egan, R., Kim, T. W., Chokhawala, H., Schroth, G., Luo, S., Clark, D. S., Chen, F., Zhang, T., Mackie, R. I., Pennacchio, L. A., Tringe, S. G., Visel, A., Woyke, T., Wang, Z. and Rubin, E. M. 2011. Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. *Science* 331(6016), 463–7. doi:10.1126/science.1200387.

Hitch, T. C. A., Thomas, B. J., Friedersdorff, J. C. A., Ougham, H. and Creevey, C. J. 2018. Deep sequence analysis reveals the ovine rumen as a reservoir of antibiotic resistance genes. *Environmental Pollution* 235, 571–5. doi:10.1016/j.envpol.2017.12.067.

Huntemann, M., Ivanova, N. N., Mavromatis, K., Tripp, H. J., Paez-Espino, D., Palaniappan, K., Szeto, E., Pillay, M., Chen, I. M., Pati, A., Nielsen, T., Markowitz, V. M. and Kyrpides, N. C. 2015. The standard operating procedure of the DOE-JGI Microbial Genome

Annotation Pipeline (MGAP v.4). *Standards in Genomic Sciences* 10, 86. doi:10.1186/s40793-015-0077-y.

Huson, D. H., Mitra, S., Ruscheweyh, H. J., Weber, N. and Schuster, S. C. 2011. Integrative analysis of environmental sequences using MEGAN4. *Genome Research* 21(9), 1552–60. doi:10.1101/gr.120618.111.

Hussain, M. H., Zainol, S., Ming Chong, N. F. and Awang Husaini, A. A. S. 2016 Characterisation of *Klebsiella pneumoniae* Xylanase and increment of its activity in heterologous expression system. *Borneo Journal of Resource Science and Technology* 6(1), 1–9. doi:10.33736/bjrst.209.2016.

Huttner, A., Harbarth, S., Carlet, J., Cosgrove, S., Goossens, H., Holmes, A., Jarlier, V., Voss, A. and Pittet, D. 2013. Antimicrobial resistance: a global view from the 2013 World Healthcare-Associated Infections Forum. *Antimicrobial Resistance and Infection Control* 2, 31. doi:10.1186/2047-2994-2-31.

Huws, S. A., Edwards, J. E., Creevey, C. J., Rees Stevens, P., Lin, W., Girdwood, S. E., Pachebat, J. A. and Kingston-Smith, A. H. 2016. Temporal dynamics of the metabolically active rumen bacteria colonizing fresh perennial ryegrass. *FEMS Microbiology Ecology* 92(1). doi:10.1093/femsec/fiv137.

Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., Muñoz-Tamayo, R., Forano, E., Waters, S. M., Hess, M., Tapio, I., Smidt, H., Krizsan, S. J., Yáñez-Ruiz, D. R., Belanche, A., Guan, L., Gruninger, R. J., McAllister, T. A., Newbold, C. J., Roehe, R., Dewhurst, R. J., Snelling, T. J., Watson, M., Suen, G., Hart, E. H., Kingston-Smith, A. H., Scollan, N. D., do Prado, R. M., Pilau, E. J., Mantovani, H. C., Attwood, G. T., Edwards, J. E., McEwan, N. R., Morrisson, S., Mayorga, O. L., Elliott, C. and Morgavi, D. P. 2018. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Frontiers in Microbiology* 9, 2161. doi:10.3389/fmicb.2018.02161.

Hyatt, D., Chen, G. L., Locascio, P. F., Land, M. L., Larimer, F. W. and Hauser, L. J. 2010. Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* 11, 119. doi:10.1186/1471-2105-11-119.

Jain, C., Rodriguez-R, L. M., Phillippy, A. M., Konstantinidis, K. T. and Aluru, S. 2018. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nature Communications* 9(1), 5114. doi:10.1038/s41467-018-07641-9.

Joshi, N. A. and Fass, J. N. 2011. Sickle: a sliding-window, adaptive, quality-based trimming tool for FastQ files (Version 1.33) [Software]. Available at https://github.com/najoshi/sickle.

Kalmokoff, M. L., Lu, D., Whitford, M. F. and Teather, R. M. 1999. Evidence for production of a new lantibiotic (butyrivibriocin OR79A) by the ruminal anaerobe *Butyrivibrio fibrisolvens* OR79: characterization of the structural gene encoding butyrivibriocin OR79A. *Applied and Environmental Microbiology* 65(5), 2128–35. doi:10.1128/AEM.65.5.2128-2135.1999.

Kang, D. D., Froula, J., Egan, R. and Wang, Z. 2015. MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. *PeerJ* 3, e1165. doi:10.7717/peerj.1165.

Kang, D. D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H. and Wang, Z. 2019. MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *PeerJ* 7, e7359. doi:10.7717/peerj.7359.

Kanwar, N., Scott, H. M., Norby, B., Loneragan, G. H., Vinasco, J., Cottell, J. L., Chalmers, G., Chengappa, M. M., Bai, J. and Boerlin, P. 2014. Impact of treatment strategies

on cephalosporin and tetracycline resistance gene quantities in the bovine fecal metagenome. *Scientific Reports* 4, 5100. doi:10.1038/srep05100.

Karginov, F. V. and Hannon, G. J. 2010. The CRISPR system: small RNA-guided defense in bacteria and archaea. Molecular Cell 37(1), 7–19. doi:10.1016/j.molcel.2009.12.033.

Karlin, S., Campbell, A. M. and Mrázek, J. 1998. Comparative DNA analysis across diverse genomes. *Annual Review of Genetics* 32, 185–225. doi:10.1146/annurev.genet.32.1.185.

Kelly, W. J., Leahy, S. C., Li, D., Perry, R., Lambie, S. C., Attwood, G. T. and Altermann, E. 2014. The complete genome sequence of the rumen methanogen Methanobacterium formicicum BRM9. *Standards in Genomic Sciences* 9, 15. doi:10.1186/1944-3277-9-15.

Kelly, W. J., Henderson, G., Pacheco, D. M., Li, D., Reilly, K., Naylor, G. E., Janssen, P. H., Attwood, G. T., Altermann, E. and Leahy, S. C. 2016. The complete genome sequence of *Eubacterium limosum* SA11, a metabolically versatile rumen acetogen. *Standards in Genomic Sciences* 11, 26. doi:10.1186/s40793-016-0147-9.

Kelly, W. J., Leahy, S. C., Kamke, J., Soni, P., Koike, S., Mackie, R., Seshadri, R., Cook, G. M., Morales, S. E., Greening, C. and Attwood, G. T. 2019. Occurrence and expression of genes encoding methyl-compound production in rumen bacteria. *Animal Microbiome* 1(1), 15. doi:10.1186/s42523-019-0016-0.

Kim, J. N., Méndez-García, C., Geier, R. R., Iakiviak, M., Chang, J., Cann, I. and Mackie, R. I. 2017. Metabolic networks for nitrogen utilization in *Prevotella ruminicola* 23. *Scientific Reports* 7(1), 7851. doi:10.1038/s41598-017-08463-3.

Kingston-Smith, A. H., Davies, T. E., Rees Stevens, P. and Mur, L. A. J. 2013. Comparative metabolite fingerprinting of the rumen system during colonisation of three forage grass (*Lolium perenne* L.) varieties. *PLoS ONE* 8(11), e82801. doi:10.1371/journal.pone.0082801.

Kislyuk, A. O., Haegeman, B., Bergman, N. H. and Weitz, J. S. 2011. Genomic fluidity: an integrative view of gene diversity within microbial populations. *BMC Genomics* 12, 32. doi:10.1186/1471-2164-12-32.

Klieve, A. V. and Bauchop, T. 1988. Morphological diversity of ruminal bacteriophages from sheep and cattle. *Applied and Environmental Microbiology* 54(6), 1637–41. doi:10.1128/AEM.54.6.1637-1641.1988.

Klieve, A. V., Hudman, J. F. and Bauchop, T. 1989. Inducible bacteriophages from ruminal bacteria. *Applied and Environmental Microbiology* 55(6), 1630–4. doi:10.1128/AEM.55.6.1630-1634.1989.

Koren, S., Walenz, B. P., Berlin, K., Miller, J. R., Bergman, N. H. and Phillippy, A. M. 2017. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome Research* 27(5), 722–36. doi:10.1101/gr.215087.116.

Kurtz, S., Phillippy, A., Delcher, A. L., Smoot, M., Shumway, M., Antonescu, C. and Salzberg, S. L. 2004. Versatile and open software for comparing large genomes. *Genome Biology* 5(2), R12. doi:10.1186/gb-2004-5-2-r12.

Lagesen, K., Hallin, P., Rødland, E. A., Staerfeldt, H. H., Rognes, T. and Ussery, D. W. 2007. RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Research* 35(9), 3100–8. doi:10.1093/nar/gkm160.

Langmead, B. and Salzberg, S. L. 2012. Fast gapped-read alignment with Bowtie 2. *Nature Methods* 9(4), 357–9. doi:10.1038/nmeth.1923.

Langmead, B., Trapnell, C., Pop, M. and Salzberg, S. L. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology* 10(3), R25. doi:10.1186/gb-2009-10-3-r25.

Leahy, S. C., Kelly, W. J., Altermann, E., Ronimus, R. S., Yeoman, C. J., Pacheco, D. M., Li, D., Kong, Z., McTavish, S., Sang, C., Lambie, S. C., Janssen, P. H., Dey, D. and Attwood, G. T. 2010. The genome sequence of the rumen methanogen *Methanobrevibacter ruminantium* reveals new possibilities for controlling ruminant methane emissions. *PLoS ONE* 5(1), e8926. doi:10.1371/journal.pone.0008926.

Leahy, S. C., Kelly, W. J., Ronimus, R. S., Wedlock, N., Altermann, E. and Attwood, G. T. 2013. Genome sequencing of rumen bacteria and archaea and its application to methane mitigation strategies. *Animal* 7(Suppl. 2), 235–43. doi:10.1017/S1751731113000700.

Letunic, I. and Bork, P. 2007. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics* 23(1), 127–8. doi:10.1093/bioinformatics/btl529.

Li, H. 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. Available at: http://arxiv.org/abs/1303.3997 (accessed on 12 September 2019).

Li, H. 2018. Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics* 34(18), 3094–100. doi:10.1093/bioinformatics/bty191.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R. and 1000 Genome Project Data Processing Subgroup. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25(16), 2078–9. doi:10.1093/bioinformatics/btp352.

Li, D., Liu, C. M., Luo, R., Sadakane, K. and Lam, T. W. 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* 31(10), 1674–6. doi:10.1093/bioinformatics/btv033.

Liu, Q., Yao, S., Chen, Y., Gao, S., Yang, Y., Deng, J., Ren, Z., Shen, L., Cui, H., Hu, Y., Ma, X. and Yu, S. 2017. Use of antimicrobial peptides as a feed additive for juvenile goats. *Scientific Reports* 7(1), 12254. doi:10.1038/s41598-017-12394-4.

Love, M. I., Huber, W. and Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* 15(12), 550. doi:10.1186/s13059-014-0550-8.

Lowe, T. M. and Eddy, S. R. 1997. TRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Research* 25(5), 955–64. doi:10.1093/nar/25.5.955.

Magoč, T. and Salzberg, S. L. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27(21), 2957–63. doi:10.1093/bioinformatics/btr507.

Manges, A. R., Johnson, J. R., Foxman, B., O'Bryan, T. T., Fullerton, K. E. and Riley, L. W. 2001. Widespread distribution of urinary tract infections caused by a multidrug-resistant *Escherichia coli* clonal group. *The New England Journal of Medicine* 345(14), 1007–13. doi:10.1056/NEJMoa011265.

Mantovani, H. C., Hu, H., Worobo, R. W. and Russell, J. B. 2002. Bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5. *Microbiology* 148(11), 3347–52. doi:10.1099/00221287-148-11-3347.

Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., Berka, J., Braverman, M. S., Chen, Y. J., Chen, Z., Dewell, S. B., Du, L., Fierro, J. M., Gomes, X. V.,

Godwin, B. C., He, W., Helgesen, S., Ho, C. H., Irzyk, G. P., Jando, S. C., Alenquer, M. L., Jarvie, T. P., Jirage, K. B., Kim, J. B., Knight, J. R., Lanza, J. R., Leamon, J. H., Lefkowitz, S. M., Lei, M., Li, J., Lohman, K. L., Lu, H., Makhijani, V. B., McDade, K. E., McKenna, M. P., Myers, E. W., Nickerson, E., Nobile, J. R., Plant, R., Puc, B. P., Ronan, M. T., Roth, G. T., Sarkis, G. J., Simons, J. F., Simpson, J. W., Srinivasan, M., Tartaro, K. R., Tomasz, A., Vogt, K. A., Volkmer, G. A., Wang, S. H., Wang, Y., Weiner, M. P., Yu, P., Begley, R. F. and Rothberg, J. M. 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437(7057), 376–80. doi:10.1038/nature03959.

Martens, E. and Demain, A. L. 2017. The antibiotic resistance crisis, with a focus on the United States. *The Journal of Antibiotics* 70(5), 520–6. doi:10.1038/ja.2017.30.

Mistry, J., Finn, R. D., Eddy, S. R., Bateman, A. and Punta, M. 2013. Challenges in homology search: HMMER3 and convergent evolution of coiled-coil regions. *Nucleic Acids Research* 41(12), e121. doi:10.1093/nar/gkt263.

Moran, D. 2017. Antimicrobial resistance in animal agriculture: understanding user attitudes and behaviours. *The Veterinary Record* 181(19), 508–9. doi:10.1136/vr.j5142.

Morgavi, D. P., Kelly, W. J., Janssen, P. H. and Attwood, G. T. 2013. Rumen microbial (meta) genomics and its application to ruminant production. *Animal* 7 (Suppl. 1), 184–201. doi:10.1017/S1751731112000419.

Mosley, E. E., Powell, G. L., Riley, M. B. and Jenkins, T. C. 2002. Microbial biohydrogenation of oleic acid to trans isomers in vitro. *Journal of Lipid Research* 43(2), 290–6.

Noguchi, H., Park, J. and Takagi, T. 2006. MetaGene: prokaryotic gene finding from environmental genome shotgun sequences. *Nucleic Acids Research* 34(19), 5623–30. doi:10.1093/nar/gkl723.

Nurk, S., Meleshko, D., Korobeynikov, A. and Pevzner, P. A. 2017. metaSPAdes: a new versatile metagenomic assembler. *Genome Research* 27(5), 824–34. doi:10.1101/gr.213959.116.

Olm, M. R., Brown, C. T., Brooks, B. and Banfield, J. F. 2017. dRep: a tool for fast and accurate genomic comparisons that enables improved genome recovery from metagenomes through de-replication. *The ISME Journal* 11(12), 2864–8. doi:10.1038/ismej.2017.126.

Oyama, L. B., Crochet, J. A., Edwards, J. E., Girdwood, S. E., Cookson, A. R., Fernandez-Fuentes, N., Hilpert, K., Golyshin, P. N., Golyshina, O. V., Privé, F., Hess, M., Mantovani, H. C., Creevey, C. J. and Huws, S. A. 2017a. Buwchitin: a ruminal peptide with antimicrobial potential against Enterococcus faecalis. *Frontiers in Chemistry* 5, 51. doi:10.3389/fchem.2017.00051.

Oyama, L. B., Girdwood, S. E., Cookson, A. R., Fernandez-Fuentes, N., Privé, F., Vallin, H. E., Wilkinson, T. J., Golyshin, P. N., Golyshina, O. V., Mikut, R., Hilpert, K., Richards, J., Wootton, M., Edwards, J. E., Maresca, M., Perrier, J., Lundy, F. T., Luo, Y., Zhou, M., Hess, M., Mantovani, H. C., Creevey, C. J. and Huws, S. A. 2017b. The rumen microbiome: an underexplored resource for novel antimicrobial discovery. *NPJ Biofilms and Microbiomes* 3, 33. doi:10.1038/s41522-017-0042-1.

O'Neill, J. 2016. *Tackling Drug-Resistant Infections Globally: Final Report and Recommendations. Review of Antimicrobial Resistance*. HM Government and Wellcome trust, London, UK.

Paez-Espino, D., Chen, I. A., Palaniappan, K., Ratner, A., Chu, K., Szeto, E., Pillay, M., Huang, J., Markowitz, V. M., Nielsen, T., Huntemann, M., K Reddy, T. B., Pavlopoulos, G. A., Sullivan, M. B., Campbell, B. J., Chen, F., McMahon, K., Hallam, S. J., Denef, V., Cavicchioli, R., Caffrey, S. M., Streit, W. R., Webster, J., Handley, K. M., Salekdeh,

G. H., Tsesmetzis, N., Setubal, J. C., Pope, P. B., Liu, W. T., Rivers, A. R., Ivanova, N. N. and Kyrpides, N. C. 2017. IMG/VR: a database of cultured and uncultured DNA Viruses and retroviruses. *Nucleic Acids Research* 45(D1), D457–65. doi:10.1093/nar/gkw1030.

Palevich, N., Kelly, W. J., Leahy, S. C., Denman, S., Altermann, E., Rakonjac, J. and Attwood, G. T. 2019. Comparative genomics of rumen Butyrivibrio uncovers a continuum of polysaccharide-degrading capabilities. *Applied and Environmental Microbiology* 86(1). doi:10.1128/AEM.01993-19.

Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P. and Tyson, G. W. 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Research* 25(7), 1043–55. doi:10.1101/gr.186072.114.

Pati, A., Ivanova, N. N., Mikhailova, N., Ovchinnikova, G., Hooper, S. D., Lykidis, A. and Kyrpides, N. C. 2010. GenePRIMP: a gene prediction improvement pipeline for prokaryotic genomes. *Nature Methods* 7(6), 455–7. doi:10.1038/nmeth.1457.

Patil, K. R., Haider, P., Pope, P. B., Turnbaugh, P. J., Morrison, M., Scheffer, T. and McHardy, A. C. 2011. Taxonomic metagenome sequence assignment with structured output models. *Nature Methods* 8(3), 191–2. doi:10.1038/nmeth0311-191.

Pidcock, S., Skvortsov, T., Santos, F., Ting, K. S., Creevey, C. and Huws, S. 2019. Understanding the evolution and metabolic capabilities of the Butyrivibrio group. *Access Microbiology* 1(1A), (1A). doi:10.1099/acmi.ac2019.po0430.

Pope, P. B., Mackenzie, A. K., Gregor, I., Smith, W., Sundset, M. A., McHardy, A. C., Morrison, M. and Eijsink, V. G. H. 2012. Metagenomics of the Svalbard reindeer rumen microbiome reveals abundance of polysaccharide utilization loci. *PLoS ONE* 7(6), e38571. doi:10.1371/journal.pone.0038571.

Pruesse, E., Peplies, J. and Glöckner, F. O. 2012. SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* 28(14), 1823–9. doi:10.1093/bioinformatics/bts252.

Ramos-Morales, E., Martínez-Fernández, G., Abecia, L., Martin-García, A. I., Molina-Alcaide, E. and Yáñez-Ruiz, D. R. 2013. Garlic derived compounds modify ruminal fatty acid biohydrogenation and induce shifts in the Butyrivibrio community in continuous-culture fermenters. *Animal Feed Science and Technology* 184(1–4), 38–48. doi:10.1016/j.anifeedsci.2013.05.017.

Rubino, F., Carberry, C., Waters, S. M., Kenny, D., McCabe, M. S. and Creevey, C. J. 2017. Divergent functional isoforms drive niche specialisation for nutrient acquisition and use in rumen microbiome. *The ISME Journal* 11(6), 1510. doi:10.1038/ismej.2017.34.

Sabino, Y. N. V., Santana, M. F., Oyama, L. B., Santos, F. G., Moreira, A. J. S., Huws, S. A. and Mantovani, H. C. 2019. Characterization of antibiotic resistance genes in the species of the rumen microbiota. *Nature Communications* 10(1), 5252. doi:10.1038/s41467-019-13118-0.

Schlüter, H., Apweiler, R., Holzhütter, H. G. and Jungblut, P. R. 2009. Finding one's way in proteomics: a protein species nomenclature. *Chemistry Central Journal* 3, 11. doi:10.1186/1752-153X-3-11.

Seemann, T. 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30(14), 2068–9. doi:10.1093/bioinformatics/btu153.

Segata, N., Börnigen, D., Morgan, X. C. and Huttenhower, C. 2013. PhyloPhlAn is a new method for improved phylogenetic and taxonomic placement of microbes. *Nature Communications* 4, 2304. doi:10.1038/ncomms3304.

Seshadri, R., Leahy, S. C., Attwood, G. T., Teh, K. H., Lambie, S. C., Cookson, A. L., Eloe-Fadrosh, E. A., Pavlopoulos, G. A., Hadjithomas, M., Varghese, N. J., Paez-Espino, D., Hungate1000 Project Collaborators, Perry, R., Henderson, G., Creevey, C. J., Terrapon, N., Lapebie, P., Drula, E., Lombard, V., Rubin, E. and Kelly, W. J. 2018. Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nature Biotechnology* 36(4), 359–67. doi:10.1038/nbt.4110.

Shea, K. M. 2003. Antibiotic resistance: what is the impact of agricultural uses of antibiotics on children's health? *Pediatrics* 112(1 Pt 2), 253–8.

Shi, W., Moon, C. D., Leahy, S. C., Kang, D., Froula, J., Kittelmann, S., Fan, C., Deutsch, S., Gagic, D., Seedorf, H., Kelly, W. J., Atua, R., Sang, C., Soni, P., Li, D., Pinares-Patiño, C. S., McEwan, J. C., Janssen, P. H., Chen, F., Visel, A., Wang, Z., Attwood, G. T. and Rubin, E. M. 2014. Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. *Genome Research* 24(9), 1517–25. doi:10.1101/gr.168245.113.

Snelling, T. J. and Wallace, R. J. 2017. The rumen microbial metaproteome as revealed by SDS-PAGE. *BMC Microbiology* 17(1), 9. doi:10.1186/s12866-016-0917-y.

Song, H., Kim, T. Y., Choi, B. K., Choi, S. J., Nielsen, L. K., Chang, H. N. and Lee, S. Y. 2008. Development of chemically defined medium for Mannheimia succiniciproducens based on its genome sequence. *Applied Microbiology and Biotechnology* 79(2), 263–72. doi:10.1007/s00253-008-1425-2.

Stamatakis, A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30(9), 1312–3. doi:10.1093/bioinformatics/btu033.

Stewart, R. D., Auffret, M. D., Warr, A., Wiser, A. H., Press, M. O., Langford, K. W., Liachko, I., Snelling, T. J., Dewhurst, R. J., Walker, A. W., Roehe, R.. and Watson, M. 2018. Assembly of 913 microbial genomes from metagenomic sequencing of the cow rumen. *Nature Communications* 9(1), 870. doi:10.1038/s41467-018-03317-6.

Stewart, R. D., Auffret, M. D., Snelling, T. J., Roehe, R. and Watson, M. 2019a. MAGpy: a reproducible pipeline for the downstream analysis of metagenome-assembled genomes (MAGs). *Bioinformatics* 35(12), 2150–2. doi:10.1093/bioinformatics/bty905.

Stewart, R. D., Auffret, M. D., Warr, A., Walker, A. W., Roehe, R. and Watson, M. 2019b. Compendium of 4,941 rumen metagenome-assembled genomes for rumen microbiome biology and enzyme discovery. *Nature Biotechnology* 37(8), 953–61. doi:10.1038/s41587-019-0202-3.

Subedi, D., Kohli, G. S., Vijay, A. K., Willcox, M. and Rice, S. A. 2019. Accessory genome of the multi-drug resistant ocular isolate of *Pseudomonas aeruginosa* PA34. *PLoS ONE* 14(4), e0215038. doi:10.1371/journal.pone.0215038.

Suen, G., Weimer, P. J., Stevenson, D. M., Aylward, F. O., Boyum, J., Deneke, J., Drinkwater, C., Ivanova, N. N., Mikhailova, N., Chertkov, O., Goodwin, L. A., Currie, C. R., Mead, D. and Brumm, P. J. 2011. The complete genome sequence of Fibrobacter succinogenes S85 reveals a cellulolytic and metabolic specialist. *PLoS ONE* 6(4), e18814. doi:10.1371/journal.pone.0018814.

Svartström, O., Alneberg, J., Terrapon, N., Lombard, V., de Bruijn, I., Malmsten, J., Dalin, A. M., El Muller, E., Shah, P., Wilmes, P., Henrissat, B., Aspeborg, H. and Andersson, A. F. 2017. Ninety-nine de novo assembled genomes from the moose (*Alces alces*) rumen microbiome provide new insights into microbial plant biomass degradation. *The ISME Journal* 11(11), 2538–51. doi:10.1038/ismej.2017.108.

Tettelin, H., Masignani, V., Cieslewicz, M. J., Donati, C., Medini, D., Ward, N. L., Angiuoli, S. V., Crabtree, J., Jones, A. L., Durkin, A. S., Deboy, R. T., Davidsen, T. M., Mora, M., Scarselli, M., Margarit y Ros, I., Peterson, J. D., Hauser, C. R., Sundaram, J. P., Nelson, W. C., Madupu, R. and Fraser, C. M. 2005. Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial 'pan-genome'. *Proceedings of the National Academy of Sciences of the United States of America* 102(39), 13950–5. doi:10.1073/pnas.0506758102.

Tettelin, H., Riley, D., Cattuto, C. and Medini, D. 2008. Comparative genomics: the bacterial pan-genome. *Current Opinion in Microbiology* 11(5), 472–7. doi:10.1016/j.mib.2008.09.006.

Papke, R. T. 2009. A critique of prokaryotic species concepts. *Methods in Molecular Biology* 532, 379–95. doi:10.1007/978-1-60327-853-9_22.

Thomas, B. J. 2019. Eukaryotic and bacterial antimicrobial peptides. In: John Wiley & Sons Ltd (Ed.), *eLS*. John Wiley & Sons, Ltd, Chichester, UK. doi:10.1002/9780470015902.a0028360.

Titus Brown, C. and Irber, L. 2016. sourmash: a library for MinHash sketching of DNA. *The Journal of Open Source Software* 1(5). doi:10.21105/joss.00027.

Tyers, M. and Mann, M. 2003. From genomics to proteomics. *Nature* 422(6928), 193–7. doi:10.1038/nature01510.

Tyson, G. W., Chapman, J., Hugenholtz, P., Allen, E. E., Ram, R. J., Richardson, P. M., Solovyev, V. V., Rubin, E. M., Rokhsar, D. S. and Banfield, J. F. 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* 428(6978), 37–43. doi:10.1038/nature02340.

Vaser, R., Sović, I., Nagarajan, N. and Šikić, M. 2017. Fast and accurate de novo genome assembly from long uncorrected reads. *Genome Research* 27(5), 737–46. doi:10.1101/gr.214270.116.

Wallace, R. J., Onodera, R. and Cotta, M. A. 1997. Metabolism of nitrogen-containing compounds. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem* (2nd edn.). Blackie Academic & Professional, London, UK, pp. 283–328.

Wang, S., Zeng, X., Yang, Q. and Qiao, S. 2016. Antimicrobial peptides as potential alternatives to antibiotics in food animal industry. *International Journal of Molecular Sciences* 17(5). doi:10.3390/ijms17050603.

Watson, M., Thomson, M., Risse, J., Talbot, R., Santoyo-Lopez, J., Gharbi, K. and Blaxter, M. 2015. poRe: an R package for the visualization and analysis of nanopore sequencing data. *Bioinformatics* 31(1), 114–5. doi:10.1093/bioinformatics/btu590.

Weimer, P. J. 1993. Effects of dilution rate and pH on the ruminal cellulolytic bacterium *Fibrobacter succinogenes* S85 in cellulose-fed continuous culture. *Archives of Microbiology* 160(4), 288–94. doi:10.1007/bf00292079.

White, J. R., Nagarajan, N. and Pop, M. 2009. Statistical methods for detecting differentially abundant features in clinical metagenomic samples. *PLoS Computational Biology* 5(4), e1000352. doi:10.1371/journal.pcbi.1000352.

Whitford, M. F., McPherson, M. A., Forster, R. J. and Teather, R. M. 2001. Identification of bacteriocin-like inhibitors from rumen *Streptococcus* spp. and isolation and characterization of bovicin 255. *Applied and Environmental Microbiology* 67(2), 569–74. doi:10.1128/AEM.67.2.569-574.2001.

Wilkins, M. R., Pasquali, C., Appel, R. D., Ou, K., Golaz, O., Sanchez, J.-C., Yan, J. X., Gooley, A. A., Hughes, G., Humphery-Smith, I., Williams, K. L. and Hochstrasser, D. F. 1996. From proteins to proteomes: large scale protein identification by two-dimensional

electrophoresis and amino acid analysis. *Nature Biotechnology* 14(1), 61–5. doi:10.1038/nbt0196-61.

Wilkinson, T. J., Huws, S. A., Edwards, J. E., Kingston-Smith, A. H., Siu-Ting, K., Hughes, M., Rubino, F., Friedersdorff, M. and Creevey, C. J. 2018. Cowpi: a rumen microbiome focussed version of the picrust functional inference software. *Frontiers in Microbiology* 9, 1095. doi:10.3389/fmicb.2018.01095.

Williams, P. G. 2009. Panning for chemical gold: marine bacteria as a source of new therapeutics. *Trends in Biotechnology* 27(1), 45–52. doi:10.1016/j.tibtech.2008.10.005.

Wilmes, P. and Bond, P. L. 2004. The application of two-dimensional polyacrylamide gel electrophoresis and downstream analyses to a mixed community of prokaryotic microorganisms. *Environmental Microbiology* 6(9), 911–20. doi:10.1111/j.1462-2920.2004.00687.x.

Won, M. Y., Oyama, L. B., Courtney, S. J., Creevey, C. J. and Huws, S. A. 2020. Can rumen bacteria communicate to each other? *Microbiome* 8(1), 23. doi:10.1186/s40168-020-00796-y.

Wood, D. E. and Salzberg, S. L. 2014. Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biology* 15(3), R46. doi:10.1186/gb-2014-15-3-r46.

Yang, S. C., Lin, C. H., Sung, C. T. and Fang, J. Y. 2014. Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Frontiers in Microbiology* 5, 241. doi:10.3389/fmicb.2014.00241.

Yates, J. R. 1998. Database searching using mass spectrometry data. *Electrophoresis* 19(6), 893–900. doi:10.1002/elps.1150190604.

Yates, J. R. 2000. Mass spectrometry. *Trends in Genetics* 16(1), 5–8. doi:10.1016/S0168-9525(99)01879-X.

Yin, Y., Mao, X., Yang, J., Chen, X., Mao, F. and Xu, Y. 2012. dbCAN: a web resource for automated carbohydrate-active enzyme annotation. *Nucleic Acids Research* 40(Web Server issue), W445–51. doi:10.1093/nar/gks479.

Zehavi, T., Probst, M. and Mizrahi, I. 2018. Insights into culturomics of the rumen microbiome. *Frontiers in Microbiology* 9, 1999. doi:10.3389/fmicb.2018.01999.

Zerbino, D. R. and Birney, E. 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Research* 18(5), 821–9. doi:10.1101/gr.074492.107.

Zhang, Y., Fonslow, B. R., Shan, B., Baek, M. C. and Yates, J. R. 2013. Protein analysis by shotgun/bottom-up proteomics. *Chemical Reviews* 113(4), 2343–94. doi:10.1021/cr3003533.

Zhu, W., Lomsadze, A. and Borodovsky, M. 2010. Ab initio gene identification in metagenomic sequences. *Nucleic Acids Research* 38(12), e132. doi:10.1093/nar/gkq275.

Chapter 6

The Rumen *Archaea*

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1 Introduction

Farming of ruminant animals for milk, meat and fiber products is an important agricultural activity, contributing significantly to the economies of many countries around the world. Ruminant productivity is driven by a wide range of plant-based feed materials, which are broken down in the ruminant forestomach (reticulo-rumen) and fermented to volatile fatty acids (VFAs), carbon dioxide (CO_2) and methane (CH_4). CH_4 is produced by a group of microorganisms called methanogenic *Archaea*, using a limited range of substrates via relatively simple metabolisms. CH_4 is mainly produced in the rumen and released into the atmosphere via eructation, with only ~10% of CH_4 emissions being produced from fermentation in the hindgut (Murray et al., 1978). The emission of CH_4 from farmed animals is a significant contributor to anthropogenic greenhouse gases (GHGs), making up around 14% of the world's total GHG emissions. Ruminant CH_4 emissions have been linked to global warming and climate change and there is increased interest in finding ways to reduce such emissions to reduce energy loss from the animal and to decrease the environmental impacts of farming ruminants. In this chapter, the types of *Archaea* found in the rumen

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will be reviewed through a summary of the rumen cultivation studies and molecular surveys of rumen archaeal community composition. The chapter will also assess methanogen function via assessment of genes encoded in the genomic information retrieved from pure cultures of methanogens, as well as from re-assemblies of genomes derived from metagenome data sets from mixed enrichment cultures and directly from rumen samples.

2 *Archaea* in the rumen

Methanogens belong primarily to the phylum *Euryarchaeota*, within the domain *Archaea*, an ancient lineage of microorganisms that have the appearance of bacteria, but which are phylogenetically distinct. The *Euryarchaeota* are currently divided into 4 classes (*Methanobacteria*, *Methanococci*, *Methanomicrobia* and *Methanopyri*) and 7 orders (*Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanocellales*, *Methanosarcinales, Methanopyrales* and *Methanomassiliicoccales*). Recently, the discovery of putative CH_4 metabolism pathways in the *Bathyarchaeota* (Evans et al., 2015), the newly described *Candidatus* '*Methanofastidiosa*' (Nobu et al., 2016) and the phylum *Verstraetearchaeota* (Vanwonterghem et al., 2016), indicate that the ability to produce CH_4 is distributed beyond the *Euryarchaeota* (Evans et al., 2019). However, a global survey of ruminants, discussed in detail later, indicates that ruminal CH_4 formation is restricted to methanogens belonging to the phylum *Euryarchaeota* (Henderson et al., 2015).

Methanogenic *Archaea* are usually found in carbon-rich, anaerobic environments where nitrate, sulfate and oxidised iron concentrations are low. In these environments, methanogens act as terminal reducers of carbon, using a small range of simple compounds as energy sources for growth. Methanogens cannot compete in environments where nitrate, sulfate, oxygen, or oxidised iron are present, as microbes that use these terminal electron acceptors have greater free-energy changes available from their metabolisms, thus allowing greater rates of ATP production and biomass formation compared to methanogenesis.

Ruminants are fed on a wide range of plant-based materials, including grasses and legumes (either fed fresh via grazing, or conserved by ensiling or drying), grains, (corn, barley, oats, wheat), fodder crops (brassicas, tree lucerne, willow, lucaena), waste products from horticultural activities (palm kernel expeller, grape marc, bagasse, rice straw) or combinations of the above. These plant-based diets are carbon-rich, while sulfate, iron and nitrate concentrations (Kennedy and Milligan, 1978; Hungate, 1966; Jamieson, 1959) are usually low. Therefore, in the rumen the metabolism of one-carbon compounds to CH_4 dominates the terminal reduction process. Once feed materials enter the rumen, they become colonised, principally by rumen bacteria, which secrete enzymes that attack and breakdown the various plant components. Structural

polysaccharides (cellulose, hemicelluloses, pectin) are depolymerised to oligosaccharides and simple sugars, which are then converted via several different fermentation schemes to volatile fatty acids (VFAs, mainly acetate, propionate and butyrate, and smaller amounts of formate, valerate, succinate, lactate and branched chain VFAs) CO_2 and hydrogen (H_2). Non-structural polysaccharides (starch, fructans) and soluble sugars are also rapidly fermented to VFAs. Methylated compounds, such as methylamines, methylsulfides and methanol, may also be released from degradation of plant lipids, sulfur amino acids or pectins, respectively, in the rumen (Patterson and Hespell, 1979; Pol and Demeyer, 1988). Unlike other carbon-rich anaerobic environments, the VFAs produced are not further oxidised via syntrophic microbial reactions in the rumen, as organisms that degrade VFAs to smaller compounds and H_2 are slow growing, and the turnover rate of material through the rumen is too high to allow their establishment (Janssen, 2010). Instead, the VFAs are absorbed through the rumen epithelium and are metabolised by the ruminant for energy and growth.

A small range of these fermentation end products can serve as energy sources for methanogen growth, including H_2, formate, acetate, short-chain alcohols, methylamines and methylsulfides (Zinder, 1993). The carbon serving as the terminal electron acceptor in the formation of CH_4 may come from CO_2, formate, methanol, methyl-compounds, or the carbonyl group of acetate. Because these substrates are fermentation end products of other organisms that degrade more complex organic compounds, methanogens often form stable associations with these organisms to facilitate substrate transfers. There are four modes of CH_4 formation recognised in methanogens (Thauer et al., 2008): (i) the hydrogenotrophic (H_2-consuming) pathway where H_2 is used to reduce CO_2 to CH_4 in 7 enzymatic steps, (ii) the H_2-dependent methylotrophic (methyl-compound-consuming) where H_2 is used to reduce methyl-compounds to CH_4, (iii) the H_2-independent methylotrophic pathway in which methyl-compounds can be disproportionated to CH_4 and CO_2 and (iv) the acetoclastic (acetate-cleaving) pathway where acetate is metabolised to produce CH_4 and CO_2 (Fig. 1). Each of these pathways differ in the amounts of free energy available (Table 1). Quantitative analyses indicate that H_2 is the principal energy source for methanogenesis (Hungate et al., 1970; Hungate, 1975), although formate may contribute up to 18% of the CH_4 formed in the rumen (Hungate et al., 1970). Methylamines and methanol can also be used by methylotrophic methanogens from the order *Methanosarcinales*, by *Methanosphaera* spp. from the order *Methanobacteriales* (Liu and Whitman, 2008) and also by members of the newly described 7th order of methanogens, the *Methanomassiliicoccales*. The contribution that methyl-compounds make to ruminal methanogenesis has not been measured directly, but it has been observed that methanol infused into the rumen of sheep was rapidly and completely converted to CH_4 (Pol

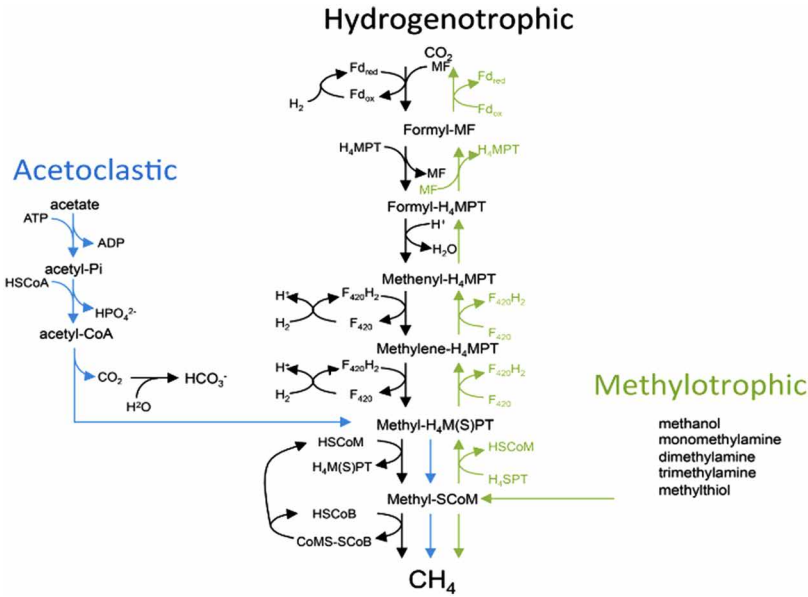


Figure 1 Methanogenesis pathways in methanogens. MF, methanofuran; Formyl-MF (formylmethanofuran), H_4MPT, tetrahydromethanopterin; F420, 8-hydroxy-f-deazaflavin; CoM, coenzyme M (2-sulfanylethanesulfonate), CoB, coenzyme B (7-mercaptoheptanoyl-threoninephosphate) (Thauer, 1998).

Table 1 Free energies of reaction associated with ruminal methanogenesis and competing H_2 utilization pathways*

Methanogenic pathway	Reaction	$\Delta G^{0\prime}$ (kJ/mol)*
Hydrogenotrophic	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	−135
Methylotrophic		
Methanol H_2-independent	$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	−105
Methanol H_2-dependent	$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	−113
Methylamine	$4CH_3\text{-}NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_3$	−75
Dimethylamine	$2(CH_3)_2\text{-}NH + 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_3$	−73
Trimethylamine	$4(CH_3)_3\text{-}N + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_3$	−74
Dimethylsulfide	$2(CH_3)_2\text{-}S + 2H_2O \rightarrow 3CH_4 + CO_2 + 2H_2S$	−49
Acetoclastic	$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	−33

*Adapted from Liu and Whitman (2008).

and Demeyer, 1988), indicating that the availability of methyl-compounds may limit methylotrophy in the rumen. Estimates of the abundance of methanogens capable of reducing the methyl groups derived from methanol or methylamines indicate that ~22% of rumen methanogens detected in a global census are likely to be methylotrophs (Henderson et al., 2015). While acetate is present

in high concentration in the rumen (~ 60 mM), CH_4 formation from acetate is thought to be negligible, as acetoclastic methanogens have doubling times of 1–2 days and are not able to grow quickly enough to overcome being washed out of the rumen where the liquid retention time is usually less than 1 day. In the global rumen census mentioned above, methanogens capable of forming CH_4 from acetate (*Methanosarcina* spp. and *Methanosaeta* spp.) were extremely rare, representing less than 0.015% of all *Archaea* (Henderson et al., 2015). Nevertheless, acetate-utilizing methanogens, such as *Methanosarcina*, have been isolated from the rumen but usually when alternative substrates such as methanol or methylamines are present (Patterson and Hespell, 1979). *Methanosarcina* spp. are known to grow more quickly on methanol or methylamines than on H_2 (Hutten et al., 1980; Sowers et al., 1984) and presumably sustain their populations in the rumen by growing more quickly when these substrates are available. *Methanosarcina* also appear in significant numbers when the rate of rumen turnover has been slowed dramatically (Rowe et al., 1979), probably allowing them to grow primarily by using acetate.

Although H_2 is the main energy source for methanogenesis in the rumen, other hydrogenotrophic microorganisms can compete for H_2 under certain ruminal conditions (Table 2). Homoacetogens, which use H_2 to reduce CO_2 to acetate via an acetogenesis pathway, have been isolated from rumen contents (Genthner et al., 1981; Rieu-Lesme et al., 1996; Greening and Leedle, 1989) and have been detected at low levels in molecular surveys (Morvan et al., 1994, 1996; Henderson et al., 2010). However, homoacetogens have H_2 thresholds of ~350–700 nM, whereas hydrogenotrophic methanogens are much lower, between 20 and 75 nM (Breznak and Kane, 1990; Mackie and Bryant, 1994; Cord-Ruwisch et al., 1988; Kim, 2012). The dissolved H_2 concentration in the rumen fluctuates widely depending on diet, time of feeding and rumen turnover rates, but is usually between 400 and 3400 nM (Janssen, 2010). Typically, these H_2 concentrations are above the threshold required for methanogens

Table 2 Gibbs free energies and thresholds of H_2-consuming reactions competing with methanogenesis in the rumen

Reaction	Reaction	ΔG (kJ/ mol H_2)*	H_2 threshold (ppm)**
Acetogenesis	$4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O$	−2.2	520–700
Hydrogenotrophic methanogenesis	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	−16.9	28–100
Sulfate reduction	$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	−21.1	9
Fumarate reduction	H_2 + fumarate → succinate	−84	0.02
Nitrate reduction	$4H_2 + NO_3^- + 2H^+ \rightarrow NH_4^+ + 3H_2O$	−125.5	0.02

Data from *Ungerfeld and Kohn (2006), **Cord-Ruwisch et al. (1988).

but often below those for acetogens. Also, the free energy available from hydrogenotrophic methanogenesis (-135 kJ/mol) is greater than that from reductive acetogenesis (-71.6 kJ/mol), ensuring that methanogens dominate the H_2-utilising niche in the rumen (Cord-Ruwisch et al., 1988; Ungerfeld and Kohn, 2006; Janssen, 2010). Sulfate reducers, (e.g. *Desulfovibrio desulfuricans*; Huisingh et al., 1974; Howard and Hungate, 1976) and nitrate reducers (e.g. *Selenomonas ruminantium*, *Wolinella succinogenes*; Henderson, 1980; Martin and Park, 1996; Wolin et al., 1961; Tanner et al., 1981; Unden et al., 1980; Jones, 1972; Iwamoto et al., 2002; Bokranz et al., 1983) are also potential competitors for H_2. However, as indicated above, the concentrations of sulfate and nitrate are usually too low for these processes to be significant in the rumen. Fumarate reduction to succinate by organisms such as *W. succinogenes* is possible in the rumen (Wolin et al., 1961), but fumarate is mainly an intracellular intermediate and is typically not found at concentrations sufficient to account for significant H_2 use. Similarly, the reduction of nitro-compounds sometimes encountered in forage material (e.g. 3-nitropropanol, 3-nitropropionate) by organisms such as *Denitrobacterium detoxificans* is only likely to play a minor role (Anderson et al., 1993, 1996, 2000).

3 Cultivation of methanogenic *Archaea* from the rumen

Methanogens are strict anaerobes with very specific growth requirements (McAllister et al., 1996), and early attempts to cultivate methanogens from any source were hampered by difficulties attaining and maintaining anaerobiosis at sufficiently low redox potential (-340 mV) to allow methanogens to grow *in vitro*. It was not until 1940, when an anaerobic agar shake method was developed, that colonies of methanogens could be obtained in culture (Barker, 1940). Robert Hungate perfected the preparation of pre-reduced media (Bryant, 1972; Hungate, 1950, 1969) and developed methods for the exclusion of oxygen in the preparation and sterilization of anoxic media as well as methods for the aseptic inoculation and transfer of anaerobic microbes in media where an oxidation/reduction potential below -330 mV was maintained. This involved using a carbon dioxide–bicarbonate–carbonate buffer to maintain a pH near neutrality, the use of cysteine and sodium sulfide as reducing agents and employing resazurin as an oxidation/reduction indicator. To aseptically remove a stopper from a culture tube in which a methanogen had generated a negative pressure required exceptional skill for the operator to prevent contamination or the entrance of oxygen into the tube. The demands for perfection in use of these techniques were so high that only a few methanogens had been isolated in pure culture prior to 1974. The development of procedures where methanogens could be cultured in a pressurized atmosphere was a major advance in the isolation and culture of these organisms whereby the chances

for contamination or loss of reducing potential were essentially eliminated (Balch and Wolfe, 1976; Balch et al., 1979). Culture purity was also an issue; one of the first methanogen cultures obtained from a sediment sample, *Methanobacillus omelianskii*, (Barker, 1936a,b) and studied in detail by several workers (Barker, 1940, 1941; Barker et al., 1940; Johns and Barker, 1960; Wolin et al., 1963, 1964a,b; Wood et al., 1965; Knight et al., 1966), was later found to be a mixed culture. This mixed culture was eventually purified and provided cultures of a methanogen (described as *Methanobacterium* M.o.H and renamed *Methanobacterium bryantii*, (Bryant et al., 1967) and a fatty acid-oxidising synthroph, *Syntrophomonas wolfei* (McInerney et al., 1979, 1981a). Although the cause of some frustration, the tendency of methanogen cultures to contain other organisms is a common theme encountered throughout the history of methanogen cultivation and reflects their need to grow in close association with organisms supplying them with substrates and/or growth factors.

Knowledge of ruminal *Archaea* was derived initially from cultivation of methanogens from a variety of ruminant species (Table 3). Beijer (1952) first described the isolation of a *Methanosarcina* and a *Methanobacterium* from rumen fluid of a goat. Oppermann et al. (1957) described the enrichment of bovine rumen fluid samples on formate producing a mixed culture that contained a methanogen identified as *Methanobacterium formicicum* while enrichment on acetate produced a short plump rod which was thought to be a morphological variant of *Methanobacterium sohngenii*. Subsequently, another culture with characteristics similar to this *Mb. sohngenii* was isolated and described as *Methanothrix soehngenii* (Huser et al., 1982). Nelson et al. (1958) also described stable enrichment cultures which produced CH_4 from butyric and valeric acids from bovine rumen fluid. However, the first rumen methanogen isolated, purified and described in detail was *Methanobacterium ruminantium* (Smith and Hungate, 1958). The genus designation later changed to *Methanobrevibacter* (Balch et al., 1979) and the current species type strain *Methanobrevibacter ruminantium* $M1^T$ (DSM 1093) was re-isolated from bovine rumen contents by Bryant (Bryant, 1965) because the original strain failed to survive in storage. The survival of purified methanogen cultures and their long-term maintenance in culture collections is an on-going problem in rumen microbiology laboratories around the world.

Since these early cultivation experiments, a number of methanogens have been isolated and, in some circumstances characterised, from rumen contents of cattle and sheep in various locations around the world, often reflecting the local importance of particular ruminant species (Table 3). For example, in the United States where dairying and raising beef cattle dominated ruminant farming operations, there was a particular interest in characterising CH_4 formation in bovines. Paynter and Hungate (1968) isolated a methanogen culture from the

Table 3 Methanogen cultures isolated from ruminants

Culture	Strain designation (Culture Collection Accession)	Substrates used*	Origin	Reference
Hydrogenotrophic methanogens				
Methanobrevibacter olleyae	KM1H5-1P^T (DSM 16632)	H_2 + CO_2, HCOOH	Sheep rumen Australia	Rea et al. (2007)
Methanobrevibacter olleyae	OCP	H_2 + CO_2	Cattle rumen Australia	Rea et al. (2007)
Methanobrevibacter olleyae	AK-87	H_2 + CO_2	Cattle rumen Australia	Rea et al. (2007)
Methanobrevibacter boviskoreani	JH1	H_2 + CO_2, HCOOH, EtOH*	Cattle (Hanwoo) rumen Korea	Lee et al. (2013a)
Methanobrevibacter millerae	ZA-10^T (DSM 16643)	H_2 + CO_2, HCOOH poorly	Cattle rumen USA	Miller et al. (1986), Rea et al. (2007)
Methanobrevibacter olleyae	YLM1	H_2 + CO_2*, HCOOH *	Sheep rumen NZ	Skillman et al. (2004)
Methanobrevibacter olleyae	BU1	Enriched on H_2 + CO_2	Buffalo rumen India	Joshi et al. (2018)
Methanobrevibacter olleyae	GO1	Enriched on H_2 + CO_2	Goat rumen India	Joshi et al. (2018)
Methanobrevibacter ruminantium	M1^T (DSM 1093)	H_2 + CO_2, HCOOH	Cattle rumen NZ	Bryant (1965)
Methanobrevibacter ruminantium	0-16B, RMB-1,-2,-3,-4	H_2 + CO_2, HCOOH	Cattle rumen USA	Lovely et al. (1984)
Methanobrevibacter ruminantium	MF_2	H_2 + CO_2	Sheep rumen France	Chaucheyras et al. (1995)
Methanobrevibacter ruminantium	YE286	H_2 + CO_2*, HCOOH *	Cattle rumen Australia	GOLD project ID Gp0035230

Methanobrevibacter smithii	GMS-01	ND	Goat rumen India	Gupta and Chaudhary (2010)
Methanobrevibacter smithii (*millerae*)	SM9	$H_2 + CO_2$*, HCOOH *	Sheep rumen NZ	Unpublished
Methanobrevibacter sp.	AbM4	$H_2 + CO_2$*, HCOOH *, EtOH + CO_2*	Sheep abomasum NZ	Unpublished
Methanobrevibacter sp.	YE 301	$H_2 + CO_2$	Cattle rumen Australia	Gilbert et al. (2010)
Methanobrevibacter sp.	YE 304	$H_2 + CO_2$	Cattle rumen Australia	Gilbert et al. (2010)
Methanobrevibacter sp.	YE315	$H_2 + CO_2$*, HCOOH *	Cattle rumen Australia	GOLD project ID Gp0118019
Methanobrevibacter sp.	RA-1	$H_2 + CO_2$, HCOOH used poorly	Sheep rumen NZ	Bauchop and Mountfort (1981)
Methanobrevibacter sp.	MB-9	$H_2 + CO_2$, HCOOH, 2PrOH weak	Sheep rumen Japan	Tokura et al. (1999)
Methanobrevibacter spp.	Z3, Z4, Z6, Z8, ZA-4	$H_2 + CO_2$, HCOOH poorly	Cattle rumen USA	Miller et al. (1986)
Methanobrevibacter thaueri	ISO4-G16	ND	Sheep rumen NZ	Jeyanathan (2010)
Methanoculleus bourgensis	KOR-2	$H_2 + CO_2$, HCOOH	Cattle rumen Korea	Battumur et al. (2019)
Methanomicrobium mobile	Strain 1, BP^T (DSM 1539)	$H_2 + CO_2$, HCOOH	Cattle rumen USA	Paynter and Hungate (1968)
Methanomicrobium mobile	BRM16	$H_2 + CO_2$, HCOOH	Cattle rumen NZ	Jarvis et al. (2000)
Methanobacterium sp.	ND	$H_2 + CO_2$, HCOOH	Goat USA	Beijer (1952)

(*Continued*)

Table 3 (*Continued*)

Culture	Strain designation (Culture Collection Accession)	Substrates used*	Origin	Reference
Methanobacterium bryantii	YE 299	$H_2 + CO_2$	Cattle rumen Australia	Gilbert et al. (2010)
Methanobacterium formicicum	BRM9	$H_2 + CO_2$, HCOOH	Cattle rumen NZ	Jarvis et al. (2000)
Methanobacterium formicicum	SM1	Enriched on $H_2 + CO_2$	Ovine rumen India	Joshi et al. (2018)
Methanobacterium formicicum	ND	Enriched on HCOOH	Cattle rumen USA	Oppermann et al. (1957)
Methanocorpusculum aggregans	BU5	Enriched on $H_2 + CO_2$	Buffalo rumen India	Joshi et al. (2018)
H_2-dependent methylotrophs				
Methanosphaera sp.	ISO3-F5	$H_2 + CO_2$ + MeOH + CH_3COOH	Sheep rumen NZ	Jeyanathan (2010)
Methanosphaera sp.	BMS	H_2 + MeOH	Cattle (Brahman) Australia	Hoedt (2017)
Methanomassilicoccales sp.	ISO4-H5	H_2 + MeOH*, MMA*, DMA*, TMA*	Sheep rumen NZ	Jeyanathan (2010)
Methanomassilicoccales sp.	ISO4-G1	H_2 + MeOH*, MMA*, DMA*, TMA*	Sheep rumen NZ	Jeyanathan (2010)
Methanomassilicoccales sp.	ISO4-G11	ND	Sheep rumen NZ	Jeyanathan (2010)
Thermoplasmatales-associated lineage C (TALC)	BRNA1	H_2 + MeOH*, MMA*, DMA*, TMA*	Cattle rumen Australia	Genbank CP002916.1, 2011
Rumen Cluster C (RCC)	LGM-AF04	ND	Goat rumen China	Jin et al. (2014)

Acetoclastic methanogens				
Methanosarcina	ND	Acetate	Goat USA	Beijer (1952)
Methanosarcina barkeri	CM1	H_2 + CO_2, MeOH, CH_3COOH, MMA, TMA	Cattle rumen NZ	Jarvis et al. (2000)
Methanosarcina sp.	ND	MeOH, MMA, TMA, CH_3COOH, H_2 + CO_2	Cattle rumen USA	McInerney et al. (1981b)
Methanothrix (Methanobacterium) sohngenii	No strain designation reported	Enriched on CH_3COOH	Cattle rumen USA	Oppermann et al. (1957)
Co- or enrichment cultures of rumen origin				
Methanobrevibacter ruminantium coculture with *Neocallimastix*	Yaktz 1-7	ND	Yak rumen Tibet	Wei et al. (2016)
Methanobrevibacter ruminantium coculture with *Piromyces*	Yak G18	ND	Yak rumen Tibet	Wei et al. (2017)
Methanobrevibacter sp. Z8 in coculture with *Piromyces*	F2	ND	Goat rumen China	Jin et al. (2011)
Methanobrevibacter thaueri CW in coculture with *Piromyces*	F1	ND	Goat rumen China	Jin et al. (2011)
Methanomassiliicoccales enrichment	RumEn M1 Environ clade	Enriched on CH_3COOH, HCOOH, TMA; MeOH* MMA*	Cattle rumen Austria	Söllinger et al. (2016)
Methanomassiliicoccales enrichment	RumEn M2 GIT clade	Enriched on CH_3COOH, HCOOH, TMA	Cattle rumen Austria	Söllinger et al. (2016)
Methanomassiliicoccales enrichment	1R26 GIT clade	Enriched on CH_3COOH, HCOOH, TMA*, MeOH*	Cattle rumen Denmark	Noel et al. (2016)
Rumen Cluster C phylotype in coculture with a *Piromyces* sp.	LGM-AFM04	ND	Caprine rumen China	Jin et al. (2011)

(*Continued*)

Table 3 (*Continued*)

Culture	Strain designation (Culture Collection Accession)	Substrates used*	Origin	Reference
Methanogen cultures of organisms from non-rumen sources but known to inhabit the rumen				
Methanosphaera sp.	WGK6	H_2 + MeOH, EtOH + MeOH	Western Grey kangaroo Australia	Hoedt et al. (2016)
Methanobrevibacter sp. 1Y co-culture with *Piromyces* sp.	1Y	ND	Buffalo faeces China	Jin et al. (2011)
Methanobrevibacter gottschalkii	HO^T	H_2 + CO_2	Horse faeces	Miller and Lin (2002)
Methanobrevibacter thaueri	CW^T	H_2 + CO_2	Cow faeces	Miller and Lin (2002)
Methanobrevibacter wolinii	SH^T (DSM 11976)	H_2 + CO_2	Ovine faeces USA	Miller and Lin (2002)
Methanobrevibacter woesei	GS^T	H_2 + CO_2, HCOOH	Goose faeces	Miller and Lin (2002)

*inferred from genome sequence.
ND; not determined.

bovine rumen and called it *Methanobacterium mobilis*, as it had a single polar flagellum allowing the cells to be motile. This organism was later reclassified as *Methanomicrobium mobile* (Balch et al., 1979). Patterson and Hespell (1979) enumerated *Methanosarcina* from rumen contents at between 10^5 and 10^6 ml^{-1} and demonstrated that *Methanosarcina barkeri* MS, originally isolated from sediments, could use trimethylamine and methylamine (breakdown products of choline metabolism) for growth, but the rumen-derived *Mbb. ruminantium* M1 could not. They concluded that *Methanosarcina* were likely the main organisms using methylamines in the rumen. McInerney et al. (1981b) retrieved an anaerobic butyrate-degrading bacterium, morphologically similar to *Syntrophomonas wolfei*, from an enrichment of bovine rumen fluid in a mixed culture with *Desulfovibrio* strain G11 and a *Methanosarcina* species. This explained the apparent use of butyrate for CH_4 formation (Nelson et al., 1958), but the enrichment of a *Methanosarcina* sp. as the main H_2-using organism was considered unusual as *Methanospirillum hungatei* had been more typically found in association with *S. wolfei*, and *Methanobrevibacter* were usually more abundant in rumen fluid. Lovely et al. (1984) isolated *Mbb. ruminantium*-like strains, 10-16B and RMB-1, which were able to synthesize their own coenzyme M (CoM), while strains RMB-2, RMB-3 and RMB-4 required CoM addition for growth. The CoM-requiring strains had slower maximal growth rates and more complex nutritional requirements than the strains that could synthesize their own CoM and were therefore thought to occupy different niches in the rumen. A further six methanogen strains (Z3, Z4, Z6, Z8, ZA-4, ZA-10) were retrieved from high dilutions of bovine rumen contents with morphologies and physiologies characteristic of *Methanobrevibacter* spp. (Miller et al., 1986). Four of the strains required CoM, two did not, and growth of all strains either depended on, or were stimulated by, a mixture of *iso*-butyric, *iso*-valeric, 2-methylbutyric and valeric acids, indicating a level of dependence on other rumen organisms for provision of essential growth factors. However, none of the strains reacted with antisera raised against *Mbb. ruminantium* M1, suggesting they represented a wider diversity of immunologically distinct *Methanobrevibacter* strains.

Around the same time in New Zealand (NZ), work had begun on characterisation of methanogens found associated with a group of rumen microorganisms newly described by Colin Orpin, the anaerobic rumen fungi (Orpin, 1975). Bauchop and Mountfort (1981) isolated colonies of a *Methanobrevibacter*-like methanogen (designated RA-1) as a co-culture partner with an anaerobic fungus, *Neocallimastix frontalis* PN-1. When grown with RA-1, the fungus showed a greater extent and rate of cellulose degradation, due to removal of H_2 and formate by the methanogen. In later cultivation studies, the estimates of methanogen populations ranged from 10^7 to 10^9/g of rumen contents in ruminants fed concentrate diets, while in pasture-fed ruminants the estimates of methanogens ranged from 10^9 to 10^{10}/g of rumen contents

(Joblin, 2005). Sheep maintained on a fresh forage diet were the source of a strain of *Mbb. smithii* SM9 (Joblin, 1999), and a *Methanobrevibacter* sp. strain, designated AbM4, was obtained from a sheep abomasum as part of a study into effects of the nematode *Ostertagia circumcincta* on the abomasal environment (Simcock et al., 1999). As part of renewed investigations into methanogenesis in NZ ruminants, Jarvis et al. (2000) isolated three methanogen strains from cattle grazing a ryegrass-white clover pasture in NZ and assigned them to the methanogenic species *Mb. formicicum* (BRM9), *Mm. mobile* (BRM16) and *Ms. barkeri* (CM1), while Skillman et al. (2004) isolated a strain of *Mbb. olleyae*, YLM1, from the rumen of a lamb. A later series of experiments targeting the isolation of previously uncultured rumen methanogens from sheep (Jeyanathan, 2010) succeeded in isolating a rumen *Methanosphaera* sp. ISO3-F5, and four mixed cultures each containing a single methanogen type. One mixed culture, designated ISO4-G16, contained a methanogen which was closely related to *Mbb. thaueri*, while the remaining three cultures (ISO4-G1, ISO4-G11, ISO4-H5) had methanogens which were closely affiliated with the new methanogen order, *Methanomassiliicoccales*. The *Methanomassiliicoccales* strain within the ISO4-H5 culture was subsequently purified (Li, 2016) from its sole co-culture partner, a *Succinivibrio dextrinisolvens* strain, designated H5.

Isolations of methanogens from ruminant livestock in Australia have produced three formate-utilizing methanogens from bovine and ovine sources which were assigned to *Mbb. olleyae* (strain KM1H5-1P^T from a sheep, OCP and AK-87 from cattle), and characterised the strain ZA-10^T previously isolated from a cow by Miller et al. (1986) as a new species*, Mbb. millerae*, (Rea et al., 2007). *Methanobrevibacter* isolates of bovine origin, designated YE301 (GQ906575, 98% similar to *Mbb. smithii* ATCC 35061) and YE304 (GQ906576, 98% similar to *Mbb. smithii*) and a bovine isolate of *Mb. bryantii*, designated YE299 (GQ906568, 99% similar to *Mb. bryantii* DSM 863) were also isolated in Brisbane and used for screening organisms for inhibitory activity against methanogens (Gilbert et al., 2010). A *Methanobrevibacter* sp. YE315 strain was also isolated from pooled rumen fluid obtained from four *Bos indicus* cross finisher cattle fed low-quality pangola (*Digitaria eriantha*) hay for 28 days (Dianne Ouwerkerk, unpublished results; GOLD project ID Gp0118019). A strain of the *Thermoplasmatales*-associated lineage C (TALC) group BRNA1 was retrieved as part of a multispecies enrichment culture from rumen contents of a bovine in Australia (Genbank CP002916.1, 2011; Denman et al., unpublished results) and a similar organism was isolated from the faeces of a chicken (Padmanabha et al., 2013). Although not ruminants, kangaroos have a fermentative-type forestomach and have been the source of methanogen cultures. The forestomach contents of a Western Grey kangaroo yielded a *Methanosphaera* isolate (*Methanosphaera* sp. WGK6) which had the unusual capability of using ethanol as the source of reducing equivalents to reduce CO_2 to CH_4 (Hoedt et al., 2016, 2018). Another

strain of *Methanosphaera* (*Methanosphaera* sp. BMS) was also isolated from a Brahman steer in Gatton, Queensland (Hoedt, 2017; unpublished genome sequence; JGI IMG Genome ID 2651869595).

A variety of methanogen isolates have also been reported from rumen ecology studies in other countries, including a *Mbb. ruminantium* strain (MF2), isolated from the rumen of a sheep in the INRA laboratory at Clermont Ferrand-Theix, France (Chaucheyras et al., 1995), a *Methanobrevibacter* sp. (strain MB-9; 97% similarity with *Mbb. ruminantium* M1) from a methanogen enrichment of a rumen ciliate preparation from a sheep in Japan (Tokura et al., 1999), and a *Mbb. smithii* strain (GMS-01) from the rumen of a goat in India (Gupta and Chaudhary, 2010). More recently, several new strains of methanogens closely related to *Mbb. olleyae*, *Mb. subterraneum*, *Methanocorpusculum aggregans* and *Mb. formicicum* have been isolated from water buffalo, goats and sheep (Joshi et al., 2018). Similar to the earlier work described with NZ sheep, *Mbb. ruminantium*-like strains (Yak-G18, Yaktz 1-7) have been retrieved as part of a co-culture with the anaerobic fungi *Piromyces* and *Neocallimastix frontalis* sourced from the rumen fluid of Tibetan yaks (Wei et al., 2016, 2017). A variety of herbivore species in China have also been the source of methanogens found in natural association with various rumen fungi (Jin et al., 2011). A further study of fungal enrichment cultures from rumen samples of Haimen goats (Jin et al., 2014) found that a novel species belonging to Rumen Cluster C (RCC) grew in subcultures of the anaerobic fungus *Piromyces* sp. In South Korea, a methanogen very closely related to *Methanobrevibacter* sp. AbM4 was isolated from the rumen of Korean native cattle (HanWoo; *Bos taurus coreanae*) and was described as a new *Methanobrevibacter* species, *Mbb. boviskoreani* JH1 (Lee et al., 2013a). Holstein steers in South Korea were the source of a rarely cultivated rumen methanogen, *Methanoculleus bourgensis* strain KOR-2 (Battumur et al., 2019).

Complimenting the description of the TALC BRNA1 strain and the ISO4-G1, ISO4-G11, ISO4-H5 *Methanomassilicoccales* enrichment cultures described above, methanogenic organisms affiliated with *Methanomassiliicoccales* clades were enriched using trimethylamine-containing medium from bovine rumen fluid collected from Brown Swiss cattle in Austria (Söllinger et al., 2016). The analysis of 16S rRNA gene sequences indicated that the enrichments contained one organism 96% identical to that of *Mms. luminyensis* and *Ca. M. intestinalis*, and another organism which was 95% identical to *Ca. Mms. termitum* and 91% to *Ca. Mms. alvus*. Another enrichment culture from bovine rumen contents which produced CH_4 from trimethylamine and methanol contained a "*Candidatus Methanomethylophilus*" sp. 1R26, another member of the *Methanomassiliicoccales* order (Noel et al., 2016).

Although *Mbb. gottschalkii* HO^T (horse faeces), *Mbb. thaueri* CW^T (cow faeces), *Mbb. woesei* GS^T (goose faeces) and *Mbb. wolinii* SH^T (sheep faeces)

are known to be rumen-inhabiting methanogens, the available cultures have been isolated from non-rumen sources (Rea et al., 2007; Miller and Lin, 2002).

Despite the numerous reports of methanogen isolations and enrichments, only a small number of rumen methanogen species have been validly described (Smith and Hungate, 1958; Bryant, 1965; Jarvis et al., 2000; Rea et al., 2007; Lee et al., 2013a) and are available in commercial culture collections (Table 3). As indicated above, this is partially due to many methanogen cultures being difficult to purify, but also to their tendency to lose viability during long-term storage. Several commonly used methods for storage of rumen methanogens include freezing at −80 °C with 10% glycerol or in 5% dimethyl sulfoxide, or freeze-drying (Tindall, 2007), however, the reasons for their poor survival during storage are not fully understood.

4 The use of molecular techniques to identify and quantify rumen methanogens

The early cultivation-based studies summarised above relied heavily on determination of a small number of phenotypic characteristics to identify methanogens, and it was not until the emergence of molecular biology approaches and the analysis of DNA, that methanogens could be identified unequivocally via hybridization and sequencing techniques. Early molecular detection work carried out in the laboratory of David Stahl, used RNAs extracted from rumen samples and blotted onto filters which were then probed with labelled oligonucleotide hybridization probes specific to the 16S rRNA of methanogens (Lin et al., 1997). For the first time, this approach provided quantitative data on total *Archaea* in rumen samples and was used to characterise the types of methanogens found in a variety of animals. Archaeal small subunit ribosomal RNAs (SSU rRNAs) accounted for 0.3–3.3% of the ruminal sample depending on ruminant species (Lin et al., 1997) and 1.46% of the SSU rRNAs in the protozoal fraction when separated from bacteria (Sharp et al., 1998). Representatives of the family *Methanobacteriaceae* were the most abundant methanogen population in cattle and goats, while the *Methanomicrobiales* were most abundant in sheep (Lin et al., 1997). The *Methanobacteriaceae* were also found to constitute 99.2% of the *Archaea* in the protozoal fraction, which suggested a particular importance of this methanogen group as symbionts of rumen protozoa (Sharp et al., 1998). The order *Methanosarcinales*, previously considered second in abundance after the *Methanobacteriaceae*, accounted for very low levels of total archaeal SSU rRNA.

Another use of labelled probes was found in fluorescence in situ hybridization (FISH), where the probes are used to hybridize with ribosomal RNAs, enabling the visualisation of a target population within a microbial community. Soliva et al. (2003) utilized FISH to study the effects of a mixture

of lauric and myristic acid on rumen methanogens, and successfully identified methanogens of the order *Methanococcales*, which were previously not reported in PCR-based analyses. Valle et al. (2015) used co-factor F_{420} (F_{420}) autofluorescence and confocal laser scanning microscopy to identify rumen methanogens and define their spatial distribution in free-living, biofilm-, and protozoa-associated microenvironments. 16S rRNA methanogen-specific probes, including Arch915, bound to some cells that lacked F_{420}, possibly identifying uncharacterized *Methanomassiliicoccales* or reflecting non-specific binding to other members of the rumen bacterial community. A probe targeting RNA from the methanogenesis-specific methyl coenzyme M reductase (*mcr*) gene was shown to detect cultured *Methanosarcina* cells with signal intensities comparable to those of 16S rRNA probes. However, the probe failed to hybridize with the majority of F_{420}-emitting rumen methanogens, possibly because of differences in cell wall permeability among methanogen species. Methanogens were shown to integrate into microbial biofilms and to exist as ecto- and endosymbionts with rumen protozoa.

Following the development of the polymerase chain reaction (PCR) and commercial thermal cyclers, 16S rRNA gene amplification, cloning and sequencing became possible (Böttger, 1989) which greatly facilitated the identification and quantification of methanogens and led to the reporting of previously uncultured methanogens. For example, Whitford et al. (2001) detected previously uncultured species from the bovine rumen belonging to the *Methanobacteriaceae* and *Methanosarcinaceae* families. The metabolic marker gene *mcrA*, mentioned above, has also been targeted via PCR amplification and sequencing (Tatsuoka et al., 2004; Ozutsumi et al., 2012; Poulsen et al., 2013; Shi et al., 2014; Denman et al., 2007; Popova et al., 2011).

Some methanogens are known to associate closely with protozoa in the rumen, either as intracellular or extracellular symbionts (Vogels et al., 1980; Finlay et al., 1994) and PCR-based methods have also allowed their investigation. Methanogen symbionts have been identified by isolating single protozoal cells from rumen contents and PCR amplification of associated methanogen 16S rRNA gene sequences (Irbis and Ushida, 2004; Regensbogenova et al., 2004) or via separation of entire protozoal communities followed by 16S rRNA or mcrA gene sequencing (Chagan et al., 1999; Tokura et al., 1999; Tymensen et al., 2012; Xia et al., 2014; Valle et al., 2015). Methanogens benefit from this association by having access to H_2, produced as a result of hydrogenosome metabolism by protozoa, thereby producing more CH_4, while the protozoa benefit by the lowered H_2 partial pressure, allowing energetically more favourable end products (Müller, 1993; Finlay et al., 1994; Akhmanova et al., 1998). *Methanobrevibacter* species were found to dominate the methanogens associated with protozoa, although species from the order *Methanomassiliicoccales* and the genera *Methanosphaera* and

Methanomicrobium also contributed minor populations (Janssen and Kirs, 2008; Paul et al., 2012; Iino et al., 2013). The types of methanogens associated with particular rumen protozoa varies (Finlay et al., 1994; Lloyd et al., 1996), but no clear difference in methanogen diversity associated with different protozoal species was found (Belanche et al., 2014).

The advent of PCR also spawned several new microbial 'finger-printing' techniques targeting the 16S rRNA genes of methanogens. Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE) have been used to electrophoretically separate 16S rRNA gene amplicons varying in sequence in a gradient of denaturant (DGGE) or temperature (TGGE). Nicholson et al. (2007) used the PCR-TTGE technique to examine rumen methanogens of cattle and sheep, while Yu et al. (2008) found that fat supplements altered PCR-DGGE profiles of methanogens, increasing the presence of *Ms. stadtmanae* and decreasing that of *Methanobrevibacter* sp. AbM4. Zhou et al. (2010) analysed PCR-DGGE profiles of rumen methanogens in cattle fed a growing diet compared to a finishing diet and saw strong transition from a methanogen community dominated by *Mbb. ruminantium* to a mixture of different methanogen species. Terminal Fragment Length Polymorphism (TRFLP), in which the PCR-amplified 16S rRNA gene amplicon is digested using a restriction endonuclease and the size of the labelled terminal fragment is analysed, has also been applied to characterise methanogens from different gut compartments in dairy cows (Frey et al., 2010) and associated with rumen protozoa (Belanche et al., 2014). While these techniques were useful for showing broad changes in methanogen communities, they had several limitations. These included difficulties interpreting multiple bands and whether they arise from the same or different genomes, a lack of quantitative information of each phylotype and the need to sequence excised bands or cloned fragments to gain more definitive identification of organisms, and now these techniques have been superseded.

16S rRNA gene clone libraries therefore gained greater use, and have been used extensively to characterise rumen archaeal communities under a range of conditions (Ohene-Adjei et al., 2007; Regensbogenova et al., 2004; Shin et al., 2004; Skillman et al., 2006;Tajima et al., 2001; Tokura et al., 1999; Wright et al., 2004, 2006, 2007, 2008). These studies were summarised in a meta-analysis by Janssen and Kirs (2008) which showed that the majority of rumen methanogens fell into two known genus-level groups; the *Methanobrevibacter* spp., the *Methanomicrobium* spp., and importantly showed the presence of a large uncharacterised clade, referred to variously as Rumen Cluster C (RCC) or *Thermoplasmatales*-associated lineage C (TALC). The 16S rRNA gene evidence for this uncultivated group spurred new attempts to retrieve these organisms in pure cultures (Jeyanathan, 2010; Denman et al., 2011 unpublished results Genbank CP002916.1; Li et al., 2016a). These rumen organisms have

recently been recognised, along with similar organisms detected in the human gut and environmental samples, as a new order of methanogens, the *Methanomassiliicoccales*.

A disadvantage of using clone libraries is that the PCR reaction amplifies DNA exponentially and therefore a particular microbe's abundance can only be expressed relative to that of other organisms amplified by the same primer set, and an absolute number of methanogens is not produced that can be related to a microbial density observed in the rumen. Quantitative real-time PCR (qRT-PCR) was developed to allow quantitation of a target from the early phase of PCR amplification. When compared to a reference standard, the absolute quantity of the target can be determined. The method has found use in quantifying the absolute abundance of archaeal communities (Hook et al., 2009; Ohene-Adjei et al., 2008; Zhou et al., 2009, 2010) and to compare microbes in animals with differing feed efficiencies (Zhou et al., 2009). A particular advantage of the technique is its ability to detect low numbers of *Archaea* in digesta samples (Frey et al., 2010), but its high cost and low throughput have limited its use for microbial ecology applications.

The low throughput of the 16S rRNA gene cloning and sequencing technique also limited the number of clones that could be analysed. However, rapid advances in DNA sequencing technologies have subsequently allowed the high throughput sequencing of amplicon libraries which has greatly increased the number of sequences which can be analysed in each sample. Barcoding the primers used in generating the 16S rRNA gene amplicons has also allowed the multiplexing of samples within a single sequencing run, and barcoded *Archaea*-specific primers are now used routinely for rumen microbial community amplification and sequencing via a variety of sequencing platforms (Kittelmann et al., 2013, 2014; Shi et al., 2014; Henderson et al., 2015; Seedorf et al., 2015; Myer et al., 2016; Lee et al., 2012; Li et al., 2016a; de la Fuente et al., 2014; Snelling et al., 2014; McCabe et al., 2015; Fouts et al., 2012; Indugu et al., 2016). These high-throughput techniques have been compared between themselves and with other microbial quantitation approaches (Myer et al., 2016; Snelling et al., 2014; de la Fuente et al., 2014; Li et al., 2016a; Indugu et al., 2016), used to characterise the archaeal communities associated with cattle and sheep (Seedorf et al., 2015; Fouts et al., 2012) and between high and low CH_4 yield sheep (Kittelmann et al., 2013, 2014; Shi et al., 2014), correlated with rumen metabolites (Lee et al., 2012) and used to examine bacterial and archaeal communities in diet-restricted cattle (McCabe et al., 2015). The landmark, global rumen census study by Henderson et al. (2015) used this approach and found that methanogens are similar in ruminants farmed around the world and are influenced mainly by the diet of the host animal. The most abundant methanogen species were hydrogenotrophs belonging to the genus *Methanobrevibacter*: principally the species *Mbb.*

ruminantium and its relatives (called the RO clade), and *Mbb. gottschalkii* and its relatives (called the SGMT clade). These two methanogen clades on average made up 74% of all rumen *Archaea*. A species of the methylotrophic *Methanosphaera* and two other, more distantly related, methylotrophic methanogens affiliated with the newly identified methanogen family *Methanomassiliicoccaceae*, comprised almost 16% of the *Archaea* found in the rumen. Some of the methanogens identified by marker gene analyses are not yet represented in culture collections, so their functions remain unknown. By using the marker gene abundance information and assigning physiologies to each methanogen group based on knowledge of the metabolism of closely related methanogens that have been cultivated, it can be deduced that the majority (~78%) of *Archaea* in the rumen are hydrogenotrophic methanogens, while others (~22%) are likely to grow via methylotrophy using H_2 to reduce methyl groups derived from methanol or methylamines (Henderson et al., 2015). The study also confirmed that the rumen archaeal communities worldwide are almost exclusively made up of methanogenic *Archaea*. Two groups of uncultured *Archaea* have been detected in 16S rRNA gene libraries, the Qld26 group (Wright et al., 2006, 2007) and a clade of *Crenarchaeota* (Shin et al., 2004). The phylogenetic affiliation of the Qld26 group suggests that it has an hydrogenotrophic mode of methanogenesis, but the physiologies and functions of the *Crenarchaeota* group are not known. The Global Rumen Census study also found some sequences defined as 'Other' or with no BLAST matches, on average at less than 0.04% abundance in any single sample and at less than 1% maximum relative abundance (Henderson et al., 2015); therefore, these groups are unlikely to contribute significantly to the rumen methanogen community.

The analysis of 16S rRNA gene amplicon data requires comprehensive and well-curated reference sequences to make useful inferences regarding the community relationships between organisms detected in the analyses. In this regard, a database designed for species-level taxonomic assignment of 16S rRNA gene amplicon data targeting methanogenic *Archaea* from the rumen and from animal and human intestinal tracts has been developed (Seedorf et al., 2014). The database is called RIM-DB (Rumen and Intestinal Methanogen-DB) and it contains 2379 almost full-length chimera-checked 16S rRNA gene sequences. The database is compatible with the Quantitative Insights Into Microbial Ecology (QIIME) analysis pipeline and can be used in conjunction with the SILVA database.

The next-generation sequencing (NGS) technologies used in the high throughput analysis of 16S rRNA gene amplicons have developed rapidly and these massively parallel sequencing technologies have also enabled the deep shotgun sequencing of metagenomic DNAs from the rumen. Their first application to the rumen was the study by Hess et al. (2011) which characterized

the biomass-degrading genes and genomes from microbes adherent to plant fiber incubated in the rumen of a cow. The study sequenced and analyzed 268 gigabases (Gb) of metagenomic DNA, identifying 27 755 putative carbohydrate-active genes, and importantly, demonstrated the feasibility of re-assembling uncultured microbial genomes from the rumen metagenome data. The increased capacity and throughput of NGS technologies has also enabled the retrieval of less abundant sequences, including those of the ruminal *Archaea*. This high throughput, deep sequencing approach also recovered methanogen genes in sufficient numbers for robust reconstruction of their metabolic pathways and estimation of gene expression activities from transcript abundances in rumen samples from NZ sheep selected for their CH_4 yield phenotypes as being either high or low CH_4 emitters (Shi et al., 2014). This study demonstrated a similar abundance of methanogens and methanogenesis pathway genes in the high and low CH_4 yield animals, but transcription of the hydrogenotrophic methanogenesis pathway genes was substantially increased in sheep with high CH_4. These results indicated a response of methanogenesis functions of the resident methanogens to the supply of H_2, and it was hypothesised that the changes in methanogen gene expression were controlled indirectly by the rumen size, the particle retention time and/or digesta passage rate in sheep (Shi et al., 2014) and related to a lactate formation and utilisation metabolism leading to less H_2 and less CH_4 (Kamke et al., 2016). Wirth et al. (2018) have also examined the rumen fluid fraction of 10 Holstein dairy cows using whole metagenome and transcriptome sequencing by Ion Torrent sequencing technology. Although the number of sequence reads retrieved was low, they reported a core microbiome consisting of 47 bacterial genera and a single archaeal genus, *Methanobrevibacter*.

Recently, 913 draft bacterial and archaeal genomes were re-assembled from 800 Gb of rumen metagenomic sequence data generated from 43 Scottish cattle (Stewart et al., 2018). The study used a combination of metagenomic binning and a DNA crosslinking method that physically connected DNA molecules in close proximity thereby capturing long-range genomic contiguity. Many of these metagenome re-assembled genomes (MAGs), (designated as Rumen Uncultured Genomes (RUGs)) represented previously unsequenced strains and species, and collectively contained more than twice the number of CAZYme genes than found in the Hess et al. (2011) study. The re-assembled genomes also included 28 rumen uncultured methanogen sequences, of which 17 clustered with the *Mbb. gottschalkii*-related SGMT clade, 8 clustered with the *Mbb. ruminantium*-related RO clade, 1 was affiliated with *Methanosphaera* strain WGK6 isolated from a kangaroo and 2 were closely related to *Candidatus Methanomethylophilus*. This publication highlights the emergence of MAGs as a powerful new approach for improving the coverage of rumen microbial genomes in the public databases and their tremendous

Table 4 Genome sequences of rumen methanogens and metagenome-assembled genomes

Genome/Sample Name (Culture collection)	Sequencing project	IMG Genome ID	Genome Size*	Gene Count*	Origin	Sequence Status	Reference
Genomes from rumen-derived cultures							
Methanobacterium formicicum BRM9	AgR[1] (PGGRC[2])	2630968343	2449987	2452	Bovine	Draft	Kelly et al. (2014)
Methanobrevibacter boviskoreani JH1	KRIBB[3]	2551306657	2045801	1799	Bovine	Permanent Draft	Lee et al. (2013b)
Methanobrevibacter millerae ZA-10 (DSM 16643)	JGI[4] (Hungate1000[5])	2593339167	2725667	2467	Bovine	Permanent Draft	Seshadri et al. (2018)
Methanobrevibacter millerae SM9	AgR (PGGRC)	2654587756	2543538	2365	Ovine	Draft	Kelly et al. (2016b)
Methanobrevibacter olleyae 1H5-1P (DSM 16632)	JGI (Hungate1000)	2593339150	2122444	1854	Bovine	Permanent Draft	Seshadri et al. (2018)
Methanobrevibacter olleyae YLM1	AgR (PGGRC)	2681812966	2201192	1882	Ovine	Draft	Kelly et al. (2016a)
Methanobrevibacter ruminantium M1 (DSM 1093)	AgR (PGGRC)	646311943	2937203	2283	Bovine	Finished	Leahy et al. (2010)
Methanobrevibacter ruminantium YE286	Macrogen[6]	2524023089	1816712	1728	Bovine	Draft	
Methanobrevibacter sp. AbM4	AgR (NZAGRC)	2540341183	1998189	1716	Ovine	Finished	Leahy et al. (2013)
Methanobrevibacter sp. YE315	Queensland govt[7]	2667527400	2273296	1982	Bovine	Draft	
Methanomicrobium mobile BP (DSM 1539)	JGI (Hungate1000)	2571042922	1711791	1686	Bovine	Permanent Draft	Seshadri et al. (2018)
Methanosphaera sp. BMS	U. Queensland[8]	2651869595	2868093	2258	Bovine	Draft	

Methanogenic archaeon ISO4-H5	AgR (NZAGRC[9])	2660238307	1937883	1890	Ovine	Draft	Li et al. (2016b)
Unclassified archaeon ISO4-G1	AgR (NZAGRC)	2667527398	1593503	1549	Ovine	Draft	Kelly et al. (2016c)
Thermoplasmatales archaeon BRNA1	CSIRO[10]	2565956561	1461105	1577	Bovine	Finished	
Methanosarcina barkeri CM1	AgR (PGGRC)	2634166439	4501171	3878	Bovine	Draft	Lambie et al. (2015)
Methanosarcina sp. DSM 11855	JGI (Hungate1000)	2595698250	3100602	2732	Ovine	Permanent Draft	Seshadri et al. (2018)
Genomes sequenced from cultures of non-rumen origin							
Methanobrevibacter gottschalkii HO^T (DSM 11977)	JGI (KMG-IV[11])	2786546169	1878029	1898	Equine faeces	Draft	
Methanobrevibacter gottschalkii HO^T (DSM 11977)	CSIRO	2622736425	1879371	1889	Equine faeces	Draft	
Methanobrevibacter gottschalkii PG (DSM 11978)	JGI (Hungate1000)	2654588138	1864477	1942	Pig faeces	Draft	Seshadri et al. (2018)
Methanobrevibacter thaueri CW^T (DSM 11995)	CSIRO	2622736427	2244956	2184	Bovine faeces	Draft	
Methanobrevibacter woesei GS^T (DSM 11979)	CSIRO	2622736428	1544134	1628	Goose faeces	Draft	
Methanosphaera stadtmanae MCB-3 (DSM 3091)	Max Planck Institute[12]	637000163	1767403	1592	Human faeces	Finished	Fricke et al. (2006)
Methanosphaera stadtmanae WGK6	454 Life Sciences[13]	2595698213	1729155	1643	Kangaroo gut	Draft	Hoedt et al. (2016)
Methanobrevibacter wolinii SH^T (DSM 11976)	JGI (Hungate1000)	2558860120	2041814	1747	Ovine faeces	Permanent Draft	Seshadri et al. (2018)

(*Continued*)

Table 4 (*Continued*)

Genome/Sample Name (Culture collection)	Sequencing project	IMG Genome ID	Genome Size*	Gene Count*	Origin	Sequence Status	Reference
Metagenome assembled genomes from rumen samples or enrichments							
Methanomassiliicoccales archaeon RumEn M1	U. Vienna[14]	2667527221	2121026	1926	Bovine	Draft	Söllinger et al. (2016)
Methanomassiliicoccales archaeon RumEn M2	U. Vienna	2667527222	1280797	1225	Bovine	Draft	Söllinger et al. (2016)
Candidatus Methanomethylophilus sp. hRUG898	Roslin Institute[15]	2799112866	1388681	1459	Bovine	Draft	Stewart et al. (2018)
Candidatus Methanomethylophilus sp. RUG779	Roslin Institute	2799112865	1262884	1400	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG011	Roslin Institute	2799112914	2484127	2252	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG012	Roslin Institute	2799112907	2366395	2113	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG018	Roslin Institute	2799112916	2431573	2363	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG020	Roslin Institute	2799112915	2285716	2036	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG031	Roslin Institute	2799112917	2324332	2214	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG076	Roslin Institute	2799112919	1863219	1669	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG092	Roslin Institute	2799112922	2324420	2154	Bovine	Draft	Stewart et al. (2018)

Methanobrevibacter sp. RUG120	Roslin Institute	2799112903	2575445	2281	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG121	Roslin Institute	2799112904	2386750	2220	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG186	Roslin Institute	2799112911	2487973	2342	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG201	Roslin Institute	2799112913	1920279	1753	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG236	Roslin Institute	2799112912	1908294	2016	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG256	Roslin Institute	2799112920	1806492	1825	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG338	Roslin Institute	2799112921	2281773	2175	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG492	Roslin Institute	2799112909	2038501	2127	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG526	Roslin Institute	2799112910	1740466	2013	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG545	Roslin Institute	2799112908	2220727	2146	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG648	Roslin Institute	2799112924	2291898	2360	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG780	Roslin Institute	2799112905	1767289	1913	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG787	Roslin Institute	2799112906	1597431	1832	Bovine	Draft	Stewart et al. (2018)
Methanobrevibacter sp. RUG823	Roslin Institute	2799112918	1424219	1638	Bovine	Draft	Stewart et al. (2018)

(*Continued*)

Table 4 (*Continued*)

Genome/Sample Name (Culture collection)	Sequencing project	IMG Genome ID	Genome Size*	Gene Count*	Origin	Sequence Status	Reference
Methanobrevibacter sp. RUG833	Roslin Institute	2799112923	2477776	2872	Bovine	Draft	Stewart et al. (2018)
Methanosphaera sp. RUG761	Roslin Institute	2799112880	1638250	1752	Bovine	Draft	Stewart et al. (2018)

*assembled.

[1]AgResearch Ltd, New Zealand; [2]Pastoral Greenhouse Gas Research Consortium, New Zealand; [3]Korea Research Institute of Bioscience and Biotechnology; [4]US Department of Energy Joint Genome Institute; [5]The Hungate 1000. A catalogue of reference genomes from the rumen microbiome; [6]Macrogen; [7]Queensland Government; [8]University of Queensland; [9]New Zealand Agricultural Greenhouse Gas Research Centre; [10]Commonwealth Scientific and Industrial Research Organisation; [11]The One Thousand Microbial Genomes Phase 4 Project; [12]Max Planck Institute; Germany; [13]454 Life Sciences; [14]University of Vienna, Austria; [15]Beef and Sheep Research Centre of Scotland's Rural College, The Roslin Institute, Scotland, UK.

potential for enhancing the identification and classification metagenomic reads from rumen ecology studies. The NGS technologies have dramatically decreased the costs of DNA sequencing and have allowed more methanogen genomes to be sequenced. Currently in the JGI GOLD database, there are 17 genome sequences of rumen-derived methanogen cultures, 8 genomes from methanogen cultures isolated from non-rumen sources but for which there are 16S rRNA sequences retrieved from the rumen, and 25 methanogen MAGs derived from rumen metagenome sequence data (Table 4). The genomes from rumen methanogen cultures are dominated by representatives of the genus *Methanobrevibacter* reflecting their numbers among the cultivated isolates, and their abundance in the rumen. However, there are also genomes available from the less numerous methanogen groups, including additional genera of the family *Methanobacteriaceae* (*Methanosphaera, Methanomicrobium, Methanobacterium*), the order *Methanosarcinales* (*Methanosarcina*) and three representatives of the new methanogen order *Methanomassiliicoccales*. The following sections describe the main features of rumen methanogens derived from analyses of their genome sequences, first dealing with hydrogenotrophic methanogens using *Mbb. ruminantium* M1 as the model hydrogenotroph, followed by a genomic analysis of the methylotrophic methanogens using *Methanosphaera* and *Methanomassiliicoccales* sp. ISO4-H5 as examples.

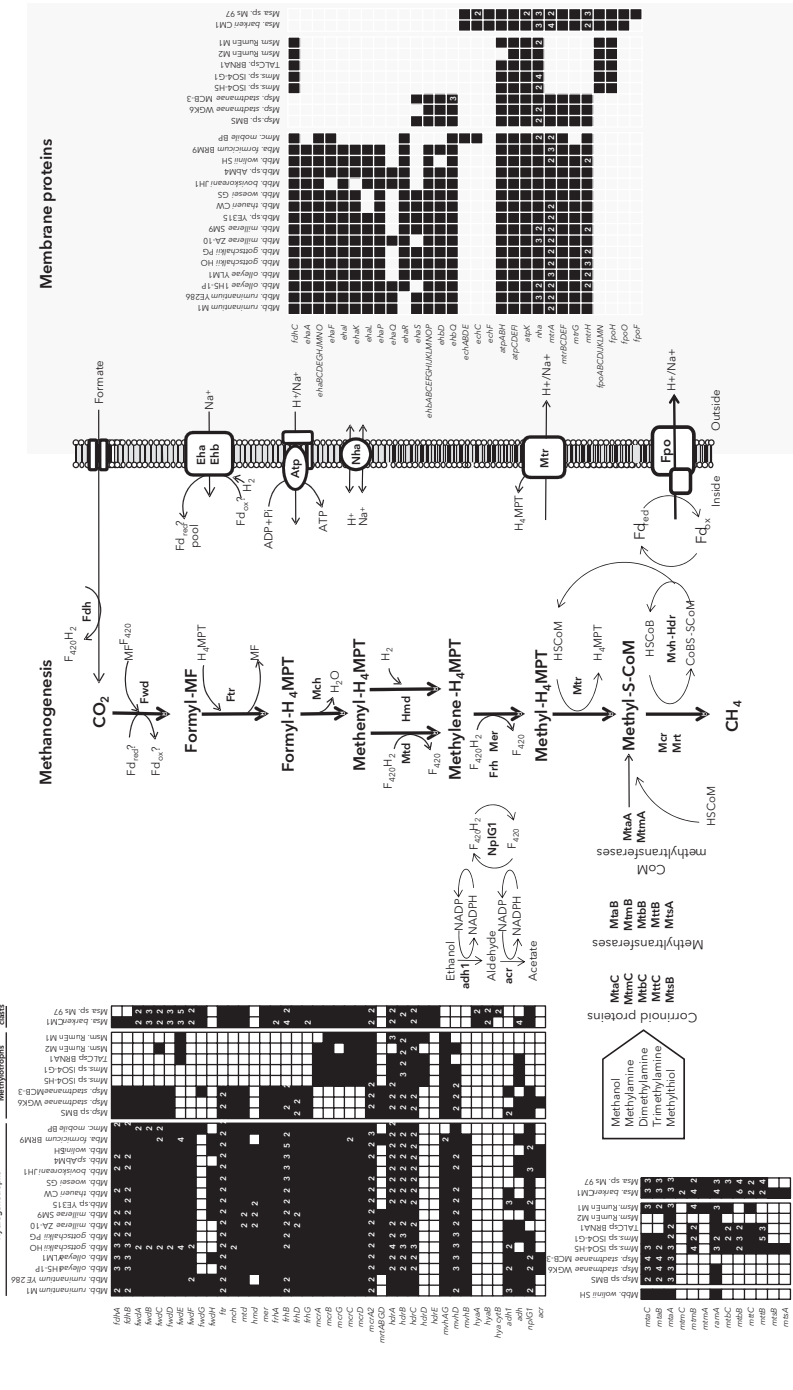
5 Hydrogenotrophic methanogens: *Methanobrevibacter ruminantium* M1

Hydrogenotrophic methanogens make up the majority of *Archaea* found in the rumen and as indicated previously they are adapted to grow using the fluctuating H_2 concentrations found in the rumen. *Methanobrevibacter ruminantium* $M1^T$ (DSM 1093) is an hydrogenotrophic methanogen, representative of the RO clade of *Methanobrevibacter* strains found in a range of ruminant species under a variety of dietary conditions (Henderson et al., 2015). On average, 16S rRNA gene sequences attributed to *Mbb. ruminantium* accounted for 27% of rumen *Archaea* across samples analysed in a global rumen census. *Mbb. ruminantium* $M1^T$ is one of the best studied rumen methanogen strains and because M1 cultures were amenable to routine growth in the laboratory (Smith and Hungate, 1958; Bryant, 1965), this organism was selected as the first rumen methanogen for genome sequencing (Leahy et al., 2010). The *Mbb. ruminantium* genome confirmed that this methanogen has a hydrogenotrophic mode of methanogenesis, encoding all of the enzymes, and most of the cofactors, required for conversion of H_2 + CO_2 and formate through to CH_4 (Fig. 1). M1 did not encode a methyl coenzyme reductase II (*mcrII* or *mrt*), an isoenzyme of the methyl CoM reductase I (*mcr*) enzyme which is differentially regulated during growth (Reeve et al., 1997) to mediate CH_4 formation at high

partial pressures of H_2, suggesting it is adapted to operate at low H_2 partial pressures in the rumen.

The genes encoding the hydrogenotrophic methanogenesis pathway in M1 are strongly conserved (Fig. 2), reflecting the importance of this pathway in the generation of energy in rumen methanogens. The methanogenesis pathway deduced from the M1 gene compliment proceeds via carrier-bound intermediates and unique cofactors, and reducing equivalents are provided from H_2 by the action of hydrogenases or from formate through formate dehydrogenase. M1 contains five hydrogenase genes, two membrane-bound energy converting [Ni-Fe] hydrogenases, (*ehaABCDEFGHIJKLMNOPQR*, *ehbABCDEFGHIJKLMNOPQ*), a F_{420}-non-reducing hydrogenase (*mvhABDG*), a F_{420}-reducing hydrogenase (*frhABB2DDG*) and a H_2-forming methylene-H_4MPT dehydrogenase (*hmd*), the encoded enzymes of which are all predicted to be located in the cell cytoplasm. The pathway begins with the binding of CO_2 to methanofuran (MF) and its reduction to formyl-MF, catalysed by tungsten (W)-containing formylmethanofuran dehydrogenase, (*fwdABCDEFGH*). Formyl transferase (*ftr*) then transfers the formyl moiety

Figure 2 Methanogenesis gene conservation in rumen methanogens. The presence of a black square indicates the presence of the gene in the corresponding genome, a number within a black square indicates the number of genes of a particular type present, while a white square indicates the absence of the gene. Gene abbreviations: *fdh*, formate dehydrogenase (F_{420}) (EC 1.2.99.-); *fdhC*, formate/nitrite transporter; *fwd*, tungsten formylmethanofuran dehydrogenase; *ftr*, formylmethanofuran-tetrahydrom ethanopterin formyltransferase (EC 2.3.1.101); *mch*, methenyltetrahydromethanopterin cyclohydrolase (EC 3.5.4.27); *mtd*, methylenetetrahydromethanopterin dehydrogenase (EC 1.5.99.9); *hmd*, H_2-forming methylenetetrahydromethanopterin dehydrogenase (EC 1.12.98.2); *mer*, methylenetetrahydromethanopterin reductase (EC 1.5.99.11); *frh*, coenzyme F_{420}-reducing hydrogenase; *fno*, reduced coenzyme F_{420}:NADP oxidoreductase; *mcr*, methyl-coenzyme M reductase; *hdr*, CoB-CoM heterodisulfide reductase; *mvh*, methyl viologen-reducing hydrogenase; *adh1*, NADP-dependent alcohol dehydrogenase; *adh*, alcohol dehydrogenase; *nplG1*, reduced coenzyme F_{420}:NADP oxidoreductase (EC 1.6.99.-); *acr*, acyl-CoA reductase; *mtaC*, methanol corrinoid protein; *mtaA*, methyl:coenzyme M methyltransferase; *mtaB*, methanol:corrinoid methyltransferase; *mtmB*, monomethylamine:corrinoid methyltransferase; *mtmC*, monomethylamine corrinoid protein; *mtbB*, dimethylamine:corrinoid methyltransferase; *mtmB*, methylamine-specific methylcobalamin:coenzyme M methyltransferase, *mtmB*, monomethylamine:corrinoid methyltransferase; *mtmC*, methylamine methyltransferase corrinoid activation protein; *mtbC*, dimethylamine corrinoid protein; *mttB*, trimethylamine:corrinoid methyltransferase; *mttC*, trimethylamine corrinoid protein; *mtsA*, bifunctional methylthiol:corrinoid methyltransferase; *mtsB*, methylthiol corrinoid protein; *eha*, membrane-bound hydrogenase; *ehb*, membrane-bound hydrogenase; *ech*, energy converting hydrogenase; *atp*, V/A-type H+-transporting ATPase; *nha*, Na+/K+/cation/H+ antiporter; *mtr*, tetrahydromethanopterin S-methyltransferase; *fpo*, $F_{420}H_2$ dehydrogenase/NADH dehydrogenase/NADH quinone oxidoreductase; *hya*, methanophenazine-reducing hydrogenase, H_4MPT, tetrahydromethanopterin; F_{420}, cofactor F_{420}; MF, methanofuran; CoBS-SCoM; CoB-CoM heterodisulfide; Fd, ferredoxin.



to tetrahydromethanopterin to form Formyl-H_4MPT. Next, N^5-formyl-H^4MPT is hydrolysed to N^5,N^{10}-methenyl-H_4MPT catalysed by N^5, N^{10}-methenyl-H_4MPT cyclohydrolase, (*mch*). Methenyl-H_4MPT is subsequently reduced to methylene-H_4MPT catalysed by methylene-H_4MPT dehydrogenase (*mtd*) and further to methyl-H_4MPT, both reactions using reduced F_{420} as the reductant. A second methylene-H_4MPT dehydrogenase (*hmd*) links methenyl-H_4MPT reduction directly with the oxidation of H_2 without using reduced F_{420}. The methylene-H_4MPT is reduced to methyl-H_4MPT catalysed by methylene-H_4MPT reductase (*mer*) using reduced F_{420}.

The methyl group is then transferred from N^5-methyl-H_4MPT to coenzyme M (HS-CoM) catalysed by a tetrahydromethanopterin S-methyltransferase (*mtrABCDEFGH*; *mtrA2*, *mtrH2*). The final reduction of methyl-CoM is catalysed by methyl-coenzyme M reductase (*mcrABCDG*; *mrtAGDB*) involving two coenzymes; B9 (HS-HTP) and factor F_{430}. The B9 acts as electron donor in the reduction of methyl-CoM to CH_4 and a mixed disulfide of HS-CoM and HS-HTP (CoM-S-S-HTP). The heterodisulfide (CoM-S-S-HTP) formed in the methylreductase reaction is reductively cleaved to regenerate HS-CoM and HS-HTP by the H_2-dependent heterodisulfide reductase system (*hdrABCD/E*).

A very unusual feature of the M1 genome was that it encoded two NADPH-dependent F_{420} dehydrogenase (*nplG1,2*) genes and three NADP-dependent alcohol dehydrogenase (*adh1, 2*, 3) genes. These genes encode enzymes which allow growth on ethanol or isopropanol in some non-rumen methanogens via $NADP^+$-dependent oxidation of alcohols coupled to F_{420} reduction of methenyl-H_4MPT to methyl-H_4MPT (Berk and Thauer, 1997). Although growth on ethanol or methanol had not previously been reported for M1 (Smith and Hungate, 1958), its growth was shown to be stimulated by ethanol and methanol in the presence of limiting amounts of H_2 + CO_2, although these alcohols did not support growth when H_2 was absent (Leahy et al., 2010). Because M1 lacked the methanol utilization genes (*mta*) it seems likely that the M1 Adh enzymes link the oxidation of alcohols to F_{420} reduction, and thereby increase the intracellular pool of reduced F_{420} and stimulate growth of M1 by sparing H_2 usually used to generate reduced F_{420}. The stimulation of M1 growth by short chain alcohols may represent an important ancillary capability for this abundant methanogen clade, and this point is elaborated later regarding similar activities observed in strains closely related to M1.

Like other methanogens, M1 has particular growth requirements, including the need for acetate, 2-methylbutyrate and co-enzyme M (CoM) in culture media (Bryant et al., 1971) and the M1 genome sequence has helped explain these growth requirements. Acetate is needed for cell carbon biosynthesis after activation to acetyl CoA via *acs* and *acsA*, followed by reductive carboxylation to pyruvate (*porABCDEF*). The branched chain volatile fatty acid, 2-methylbutyrate, is needed for isoleucine biosynthesis as M1 lacks a gene

encoding a homoserine kinase required for the usual pathway from threonine. Three genes needed in the CoM biosynthetic pathway (*comADE*) are also missing in M1 meaning that it needs exogenously supplied CoM for growth. These growth requirements probably reflect M1 adaptation to the rumen environment. Other rumen methanogens synthesize CoM, so it seems M1 has dispensed with its own CoM biosynthetic pathway and relies on uptake of this cofactor from its surroundings. Similarly, acetate and branched chain VFAs are produced in the rumen, so it also makes biological sense for M1 to obtain these compounds from its environment rather than synthesize them itself. M1 has a gene encoding a putative transporter SSS family protein (mru1786) which may encode a sodium-acetate symporter function for acetate uptake.

The cell envelope of M1 serves as a barrier to its external environment and mediates important interactions with rumen metabolites and other co-resident microorganisms. The cell surface proteins and polysaccharides of rumen methanogens are also of interest as they are potential targets for the development of anti-methanogen vaccines which may be able to reduce ruminant CH_4 (Leahy et al., 2010). Ultrastructural studies of M1 cells revealed that the cell wall is composed of three layers; a rough irregular outer layer (assumed to be composed of cell wall glycopolymers, wall-associated proteins and possibly other components), a thin electron-dense inner layer composed of compacted newly synthesised pseudomurein and a thicker less-electron-dense middle layer also composed of pseudomurein (Zeikus and Bowen, 1975; Miller, 2001; Graham and Beveridge, 1994). Outside of the three-layered M1 cell wall, there is an array of large adhesin-like proteins which are attached via different cell-anchoring domains, including a single LPxTG-domain containing protein, and multiple adhesins with pseudomurein-binding, C-terminal and C-terminal transmembrane anchors (Leahy et al., 2010). Some of these adhesins are very large; two are greater than 5000 amino acids (aa), and there are 11 over 3000 aa in length. The abundance of these very large proteins, and the cellular resources needed to synthesize them, suggests that they perform important functions in M1, probably mediating a range of interactions with their environment and with other rumen microorganisms. Indeed, M1 co-cultured with the xylan-degrading, H_2-producing bacterium, *Butyrivibrio proteoclasticus,* up-regulated the expression of six of these adhesin-like proteins and microscopic examination of the co-cultures showed co-aggregation of M1 and *B. proteoclasticus* cells (Leahy et al., 2010). One of these upregulated adhesins (Mru_1499) contains three bacterial immunoglobulin-like 1 (Big_1) domains that are commonly found in bacterial adhesins (Bodelón et al., 2013). Mru_1499 was subsequently shown to function as an adhesin in M1 and is capable of binding to a broad range of rumen protozoa (including *Epidinium* and *Entodinium*) and to *B. proteoclasticus* (Ng et al., 2016). Over 60 adhesin-like proteins are encoded in the M1 genome, several of them containing Big_1 domains, and although the

role(s) of these adhesin-like proteins is not known, it seems likely that they are important for methanogen ecology in the rumen. Like other hydrogenotrophic methanogens, M1 requires H_2 for growth and the observation that Mru_1499 mediates interactions with H_2-producing rumen microbes suggests that at least some of these proteins are involved in facilitating interaction with other H_2-producing bacteria and protozoa. It also seems probable that a multitude of other types of interactions are possible with various, potentially symbiotic, rumen organisms, or indeed the host animal itself.

During the sequencing of M1 an approximately 70 Kb region of the genome was highly over-represented in the sequencing reads. This region was subsequently identified as containing a prophage and was probably over-represented in the genome sequence due to the prophage excising and replicating during the large-scale cultivation used to isolate genomic DNA of M1. The prophage region was found to contain 70 ORFs (mru0256-0325) over a 62 Kb GC-rich (39% G+C content) region and was designated Ø-mru. Distinct modules encoding integration, DNA replication, DNA packaging, phage capsid, lysis and lysogenic functions were detected and within the lysis module, a gene encoding a putative lytic enzyme, endoisopeptidase PeiR (mru0320), was identified. PeiR represented a novel enzyme, as it did not show significant homology to any sequence in public databases. The ability of recombinant PeiR to lyse M1 cells in pure culture was subsequently demonstrated and the enzyme has been coupled to polyhydroxyalkanoate beads and used to inhibit CH_4 formation from a range of methanogen cultures *in vitro* (Altermann et al., 2018).

An unforeseen and novel feature of M1 is the presence of two large proteins (mru0068 and mru0351) showing the distinctive domain architecture of non-ribosomal peptide synthetases (NRPSs). NRPSs produce a wide variety of small molecule natural products that have biotechnological applications as peptide antibiotics, siderophores, immunosupressants or antitumor drugs (Amoutzias et al., 2008) and the M1 NRPSs were the first reported in an archaeal species. The NRPSs encoded by mru0068 are predicted to encode two modules, each containing condensation, adenylation and thiolation domains. The presence of a condensation domain in the first module is often associated with NRPSs that make *N*-acylated peptides (Fischbach and Walsh, 2006). The second module is followed by a terminal thioesterase domain which is thought to release the peptide from the final thiolation domain. Mru0068 is surrounded by genes that encode two serine phosphatases (mru0066, mru0071), an anti-sigma factor antagonist (mru0067) and a MatE efflux family protein (mru0069), which are likely to be involved in environment sensing, regulating NRPS expression and export of the NRP, respectively. Mru0068 displays full-length protein alignment with a putative NRPS from *Syntrophomonas wolfei* subsp. *wolfei* strain Göttingen, a gram-positive bacterium known to participate in syntrophic interactions with methanogens (McInerney et al., 1979). The second NRPS

gene (mru0351) contains 4 modules and a thioesterase domain. Downstream of mru0351 is another MatE efflux family protein (mru0352), presumably involved in NRP export. A third, smaller cluster of genes located elsewhere in the genome (mru0513-0516) appears to encode NRPS-associated functions. This cluster includes a phosphopantetheinyl transferase (mru0514) which primes NRPSs by adding a phosphopantetheinyl group to a conserved serine within the thiolation domain, an acyltransferase (mru0512) possibly involved in NRP acylation, a serine phosphatase (mru0515), an anti-sigma factor antagonist (mru0513), and an anti-sigma regulatory factor serine/threonine protein kinase (mru0516) that may function in sensing the environment and NRPS regulation. Although the products of each NRPS are unknown, an analysis of adenylation domain amino acid sequences predicts 10 residues which are important for substrate binding and catalysis. Horizontal gene transfer analyses (Darkhorse; Podell and Gaasterland, 2007) indicate that these genes may be bacterial in origin.

6 Other hydrogenotrophic methanogens

6.1 Methanobrevibacter olleyae YLM1

Mbb. olleyae YLM1 was isolated from a lamb rumen (Skillman et al., 2004). The YLM1 genome is very similar in size and its overall gene content is largely comparable to that of *Mbb. ruminantium* M1, suggesting that the basic metabolism of these two hydrogenotrophic methanogens is similar. YLM1 contains a prophage but is slightly smaller in size compared to Ø-mru at 40 Kb and encodes 2 clustered regularly interspaced short palindromic repeats (CRISPR) domains. It has 64 large adhesion-like proteins which may mediate interactions with other rumen microbes. YLM1 has two genomic regions that are predicted to be important in cell functionality: a 10-gene insertion encoding the CoB–CoM heterodisulfide reductase genes (*hdrABC*), enzymes involved in coenzyme M production (*comADE*), a methanogenesis marker protein 16, an adhesin-like protein, and two hypothetical proteins; and a second 9-gene region which encodes a set of formate dehydrogenase genes, a hydrogenase maturation protein, an ATPase and the *mrtBDGA* (methylcoenzyme M reductase II) operon which is thought to enable growth at high H_2 concentrations. The ability to synthesize its own CoM and to grow at high H_2 concentrations are likely to allow YLM1 to occupy a slightly different niche in the rumen compared to M1.

6.2 Methanobrevibacter sp. AbM4

AbM4 was originally isolated from the abomasal contents of a sheep and it is most closely related to the *Mbb. wolinii*-type strain SH (95% 16S rRNA gene

similarity). It was chosen as a representative of the *Mbb. wolinii* clade for genome sequencing, and its genome was found to be smaller than that of M1 (2.0 Mb versus 2.93 Mb); it encoded fewer open reading frames (ORFs; 1,671 versus 2,217) with a lower %G+C (29% versus 33%), but very similar to the genome of *Mbb. wolinii*-type strain SH^T (JGI Project ID: Gp0047017) of 2.0 Mb. The AbM4 and SH^T genomes are very syntenous and share many of the genes encoding the methanogenesis pathway (Fig. 2), suggesting that the metabolisms of these strains are highly similar. AbM4 and SH^T use only the McrI system for the final methyl-CoM reduction and appear adapted for growth at low levels of H_2. AbM4 is capable of growth on methanol but does not contain methanol:cobalamin methyltransferase genes (*mtaABC*), whereas SH^T encodes a methanol:corrinoid methyltransferase (T523DRAFT_00271) and a methyl-Co(III) methanol-specific corrinoid protein]:coenzyme M methyltransferase (T523DRAFT_00274). Like M1, AbM4 encodes two NADP-dependent F_{420} dehydrogenase genes (AbM4_0649 and AbM4_1626) and three alcohol dehydrogenase genes (AbM4_1002, AbM4_1297 and AbM4_1629; Leahy et al., 2013), which suggested that AbM4 can use alcohols as alternative sources of reducing potential for methanogenesis. When the medium was supplemented with both ethanol and methanol at 20 mM, AbM4 was able to grow without H_2, and this feature has allowed a high-throughput, microtitre plate-based bioassay to be developed for the screening of inhibitory compounds (Weimar et al., 2017). AbM4 has a complete CoM biosynthesis pathway but does not contain a prophage or any NRPS genes. However, it does have a large CRISPR region and several Type I and Type II restriction-modification system components. Unusually, AbM4 has DNA-directed RNA polymerase β'and β'' subunits that are joined, a feature only previously observed in some thermophilic *Archaea*. AbM4 has fewer genes encoding adhesin-like proteins which suggests it occupies a ruminal niche different from that of M1.

6.3 Methanobrevibacter millerae SM9

SM9 was isolated from a sheep on a fresh forage diet, and its 16S rRNA gene sequence is 99% similar to that of *Mbb. millerae* ZA-10^T (DSM 16643) which also has a genome sequence available (JGI GOLD genome ID 2593339167). These two organisms are representatives of the *Mbb. gottschalkii* clade (*Mbb. gottschalkii, Mbb. millerae* and *Mbb. thaueri*; Janssen and Kirs, 2008) which dominate the rumen with a mean abundance of 42.4% of archaeal rRNA gene sequences in the global rumen census (Henderson et al., 2015). The SM9 and ZA-10^T genomes are highly syntenous with each other, and both have methanogenesis genes very similar to *Mbb. ruminantium* M1. However, they differ by having the *mrtAGDB* methyl CoM reductase genes, 2 copies of F_{420}-dependent methylene tetrahydromethanopterin dehydrogenase (*mtd*), and

a second set of formate dehydrogenase genes (*fdhAB*). SM9 and ZA-10 have *comABCDE* genes for CoM biosynthesis and genes for cobalamin synthesis, and although they do not have the biotin biosynthesis genes, they do contain a *BioY* transporter for biotin uptake. Both genomes encode genes with high homology to an extracellular tannase (TanALp) from *Lactobacillus plantarum*, and therefore may have an advantage on diets with higher levels of plant tannins. Both SM9 and ZA-10^T encode NRPSs; SM9 has 2, while ZA-10^T has 3, but they do not share homology, although their NRPS do show weak homology to different NRPSs from M1. The combination of these features differentiates between the *Mbb. ruminantium* and *Mbb. millerae* clades and may allow them to occupy separate ruminal niches and explain why both groups are always found in 16S rRNA gene surveys of the rumen.

6.4 Methanobacterium formicicum BRM9

Methanobacterium sp. BRM9 was isolated from the rumen of a Friesian cow grazing a ryegrass/clover pasture in NZ and its 16S rRNA gene sequence is 99.8% similar to the *Mb. formicicum* type strain DSM 1535 (Jarvis et al., 2000). BRM9 is hydrogenotrophic and produces CH_4 from formate and H_2 + CO_2 only, and it is a non-motile, short rod which tends to become longer and irregular at later stages of growth. Its genes encoding the methanogenesis pathway are similar to other members of the family *Methanobacteriaceae* except it does not encode an [Fe]-hydrogenase/dehydrogenase (*hmd*) and it uses the *mrtAGDB* methyl CoM reductase system. A particularly notable feature of BRM9 is that it has many enzymes predicted to be involved in oxidative stress response, including a superoxide dismutase, a catalase/peroxidase and a peroxiredoxin (alkyl hydroperoxide reductase). It also has 3 ectoine biosynthetic genes (*ectABC*) producing the compound ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidin ecarboxylic acid) that has a function as an osmolyte helping organisms survive osmotic stress. BRM9 appears to monitor redox potential, oxygen and energy levels very closely as it has a large number of genes encoding components of histidine kinase/response regulator signal transduction systems sensing these conditions. BRM9 also seems to encode several nitrogen assimilation mechanisms: it has two ammonium transporters, a glutamine synthase (GS)/glutamate synthase (glutamine:2-oxoglutarate aminotransferase, GOGAT) pathway and surprisingly, it also has a *nif* operon encoding a nitrogenase and nitrogenase cofactor biosynthesis genes.

7 Methylotrophic methanogens

Methylotrophic methanogenesis involves CH_4 formation from methylated compounds including methanol, methylamines and methylated thiols,

and members of the orders *Methanobacteriales*, *Methanosarcinales* and *Methanomassiliicoccales* have this metabolism. Analysis of genome sequences of the new CH_4-forming phyla mentioned previously, the *Bathyarchaeota* and the *Verstraetearchaeota,* indicate they have methylotrophic CH_4 metabolisms (Evans et al., 2015; Vanwonterghem et al., 2016). In the rumen the main methyl-compounds found are methanol and methylamines. Methanol is present at around 0.8 mM in the rumen of cattle fed hay and grain (Vantcheva et al., 1970) and is thought to be derived from demethyoxylation of dietary pectins via the action of pectin methyl esterases (PMEs; EC3.1.1.11) encoded by rumen bacteria including *Lachnospira multipara* (Silley, 1985, 1986) and species of the genera *Butyrivibrio* and *Prevotella*. Mono-, di- and tri-methylamines are produced mainly as the end-product of plant phosphatidylcholine degradation (Dawson and Hemington, 1974) via choline. Methylamine has been measured at around 85 μM in the rumen fluid of dairy cows fed a cereal grain diet (Ametaj et al., 2010) and ranges from 28.5 to 703 μM in the rumen of cows fed varying amounts of barley grain (Bovine Rumen Metabolome Database, 2018).

Very little is known about how methylamines are produced in the rumen. It has been shown that labelled choline dosed into the rumen was rapidly metabolized to trimethylamine (TMA) by rumen microorganisms and the labelled methyl groups ended up as CH_4 (Broad and Dawson, 1976; Neill et al., 1978). A more recent study found a negative relationship between rumen *Methanomassiliicoccales* populations and urinary trimethylamine-*N*-oxide (TMAO) concentration (Morgavi et al., 2015), thought to be due to *Methanomassiliicoccales* using TMA for CH_4 formation in the rumen, and diverting it from being oxidised to TMAO in the liver.

7.1 Methanogens belonging to the order Methanomassiliicoccales

Tajima et al. (2001) were the first to notice a novel cluster of uncultured archaeal sequences distantly associated with *Thermoplasma* during their study on the diversity of *Archaea* in the bovine rumen. Wright et al. (2004) also observed these novel *Thermoplasma*-affiliated sequences from the rumen of sheep in Western Australia, Queensland, Australia (Wright et al., 2006) and in potato-fed feedlot cattle in Ontario and Prince Edward Island, Canada (Wright et al., 2007). The meta-analysis of all the available methanogen 16S rRNA gene sequences by Janssen and Kirs (2008) conclusively showed a large group of uncultured rumen *Archaea* affiliated with *Thermoplasmatales* making up almost 16% of the global dataset and proposed the name Rumen Cluster C (RCC). In later studies, *Thermoplasmatales*-affiliated sequences were found to dominate in

methanogen clone libraries from yak and cattle in the Qinghai-Tibetan plateau, China (Huang et al., 2012) while the *Methanomassiliicoccaceae* family members from *Mmc*. Group 10 and *Mmc*. Group 4 were dominant in amplicon sequences from yak and Tibetan sheep and *Mmc*. Group 12 were dominant in introduced cattle and crossbred sheep (Huang et al., 2016). In Jinnan cattle from China, *Thermoplasmatales*-affiliated sequences were widely distributed in the rumen epithelium, rumen solid and fluid fractions (Pei et al., 2010). *Thermoplasmatales*-affiliated sequences have been also found in rumen samples from a variety of ruminant species, and in some cases constituted the dominant rumen methanogen present (King et al., 2011; Gu et al., 2011; Franzolin et al., 2012; Jeyanathan et al., 2011; Cheng et al., 2009).

All of the information on the RCC-*Thermoplasmatales*-affiliated sequence up to this stage was inferred from 16S rRNA gene libraries, but as indicated above a strain of the *Thermoplasmatales*-associated lineage C (TALC) group, BRNA1, was enriched from the bovine rumen (Denman et al., 2011; unpublished data) and a pure culture, *Methanoplasma gallocaecorum* DOK-1, was obtained from the faeces of a chicken (Padmanabha et al., 2013) and used H_2 to reduce both methylamines and methanol to CH_4. The BRNA1 strain was part of a multispecies enrichment, but a draft sequence of the TALC organism was reassembled from metagenomic data of the mixed culture and its gene complement confirmed it was capable of CH_4 production. Poulsen et al. (2013) showed that *Thermoplasmata Archaea* in the bovine rumen were methylotrophic methanogens and that dietary supplementation with rapeseed oil in lactating cows reduced their numbers. Using a metatranscriptomic approach, *Thermoplasmata* 16S rRNA and *mcr* transcripts decreased at the same time as mRNAs of enzymes involved in methanogenesis from methylamines, indicating methylamine use. Methylotrophic methanogenesis was confirmed using *in vitro* incubations, where methylamine supplementation was accompanied by enhanced growth of the *Thermoplasmata* organisms and elevated CH_4 production. Subsequently, metagenome sequences from trimethylamine enrichments of rumen fluid from Brown Swiss cattle yielded reassembled genomes of two methanogenic *Methanomassiliicoccales* organisms (Söllinger et al., 2016). The RumEn M1 MAG consisted of 182 contigs with an estimated genome size of 2.21 Mbp and its 16S rRNA gene sequence confirmed it is a novel member of the family *Methanomassiliicoccaceae*. The second MAG, RumEn M2, contained 18 contigs with an estimated genome size of 1.28 Mbp and represented a novel member of a gastrointestinal (GIT) clade of the *Methanomassiliicoccales*. Another enrichment culture from bovine rumen contents which produced CH_4 from trimethylamine and methanol contained a '*Candidatus Methanomethylophilus*' sp. 1R26 and represented an additional member of the *Methanomassiliicoccales* order (Noel et al., 2016). More recently,

work with the methanogen inhibitor, 3-nitrooxy propanol (3-NOP) in cattle has shown that *Methanomassiliicoccales* decreased when this compound was fed to cattle (Martinez-Fernandez et al., 2018). At the same time, trimethylamine accumulated to around 1.2 mM in the rumen of 3-NOP-fed animals compared to 0.33 mM in control animals, supporting a role for *Methanomassiliicoccales* in trimethylamine metabolism.

Around the same time, Dridi et al. (2012) isolated a strain affiliated with the *Thermoplasmatales* from human faeces and called it *Methanomassiliicoccus luminyensis,* while Borrel et al. (2012) sequenced the genome of another RCC-related isolate, *Candidatus Methanomethylophilus alvus* Mx1201. Paul et al. (2012) pulled all the *Thermoplasmatales* 16S rRNA genes and *mcrA* gene sequences together, along with the enriched cultures from the higher termites, millipedes and the *Mms. luminyensis* isolate and proposed that they formed a seventh order of methanogenic *Archaea*, the *Methanomassiliicoccales*.

The previously mentioned methanogen culture work by Jeyanathan et al. (2011) generated 3 mixed enrichment cultures containing RCC-like organisms as the sole methanogens. The ISO4-H5 mixed culture contained a single *Methanomassiliicoccales* strain and a bacterial partner identified as a *Succinivibrio dextrinisolvens*, and genomic DNAs from this mixed culture were sequenced. *Methanomassiliicoccales*-related sequences were reassembled into 47 contigs in a single scaffold and its genome was closed (Li et al., 2016b). The *Methanomassiliicoccales* sp. ISO4-H5 genome was found to be 1.9 Mb and had 54% G + C content, similar to the genomes of Mx1201, B10 and BRNA1. ISO4-H5 encodes the genes enabling H_2-dependent methylotrophic methanogenesis by reduction of methyl substrates but does not have the ability to oxidize methyl substrates to CO_2. It is predicted to use H_2 and methanol, mono-, di-, tri-methylamine and methyl-3-methylthiopropionate giving ISO4-H5 more metabolic versatility than other methylotrophic methanogens in the rumen.

As described in other *Methanomassiliicoccales*, ISO4-H5 uses a heterodisulfide reductase (*hdrABC*) and a methyl-viologen hydrogenase (*mvhADG*) to recycle CoM, using reducing equivalents generated from the hydrogenase, and is coupled to a F_{420}-dehydrogenase Fpo-like complex to generate the membrane potential and ATP. ISO4-H5 does not encode CoM biosynthesis genes, which suggests it has adapted to the rumen environment, where CoM produced by other methanogens is available. It also lacks the genes encoding cofactor F_{420} synthesis, which agrees with microscopic observations that it does not fluoresce under illumination at 420 nm. ISO4-H5 can produce pyrrolysine and has the specific aminoacyl-tRNA synthetase that enables readthrough of the amber stop codon UAG. In-frame amber codons signalling pyrrolysine insertion are found in 46 ISO4-H5 genes, including many of the genes encoding methylamine and methanol use.

The ISO4-G1 enrichment culture from Jeyanathan et al. (2011) has also been genome sequenced, revealing a single 1.6 Mb circular chromosome, with a GC content of 55.5%, and 1501 predicted protein-coding genes, but does not contain any identifiable plasmid, prophage, or CRISPR sequences (Kelly et al., 2016c). Like ISO4-H5, ISO4-G1 relies on H_2-dependent methylotrophic methanogenesis to produce energy, with methanol and methylamines as substrates. It also lacks the genes for CoM production and tryptophan biosynthesis, but does encode many transporters, including 15 ABC transporters predicted to be involved in Fe^{3+} or siderophore uptake. ISO4-G1 has a complete operon (AUP07_0651- AUP07_0654) for pyrrolysine biosynthesis and the specific aminoacyl-tRNA synthetase and 25 genes encoding pyrrolysine-containing proteins, 9 of which are mono-/di-/tri-methylamine:corrinoid methyltransferases. ISO4-G1 also encodes an NRPS which is predicted to incorporate a pyrrolysine residue, but the function of its predicted NRP is not known.

The CH_4 formation pathway from methyl compounds in both ISO4-H5 and ISO4-G1 likely pumps only one ion across the cell membrane to generate a membrane gradient for every two CH_4 molecules that are formed, compared to two ions pumped by *Msp. stadtmanae*, which uses essentially the same metabolism. Therefore, it is predicted that ISO4-H5 and ISO4-G1 have much lower ATP yields and therefore slower growth than *Methanosphaera* spp. which agrees with the very low culture densities achieved by ISO4-H5 and ISO4-G1. However, the differing stoichiometries for CH_4 formation (Table 1) means that ISO4-H5 and ISO4-G1 can be expected to have lower thresholds for H_2, allowing them to grow at lower H_2 concentrations compared to *Methanosphaera* spp., their main methylotrophic competitor in the rumen. This differentiates the *Methanomassiliicoccales* ecologically from *Methanosphaera*. The wide range of fermentation conditions in the rumen when animals are feeding or ruminating likely presents opportunities for both these groups of methylotrophic methanogens to grow and co-exist in the rumen.

7.2 Methanosphaera sp. BMS

Methanosphaera sp. BMS was isolated from a methanol enrichment of a rumen sample collected from a Brahman steer in Australia (Hoedt, 2017). Growth tests showed that BMS is a H_2-dependent methylotroph using only methanol, and not methylated amines, acetate, formate, propanol or a mixture of H_2 + CO_2. BMS was unable to use ethanol as an alternative to H_2 to support methanogenesis, as has been observed in the isolate obtained from a kangaroo (*Methanosphaera* sp. WGK6; Hoedt et al., 2016). The BMS genome was larger (2.9 Mb, 2204 protein coding sequences) than the genomes from *Methanosphaera* isolates

of human and kangaroo origin (both ~1.7 Mb). KEGG pathway analyses for CH_4 metabolism indicated that BMS encodes all the genes required for H_2-driven methanol reduction and encodes the Type II *mrt* system along with the coenzyme M methyltransferase subunits *mtrABC*. *Methanosphaera* sp. MAGs were also reassembled from various metagenome datasets and H_2-dependent reduction of methanol to CH_4 appeared to be a common feature of all the *Methanosphaera* genomes. However, as indicated earlier, culture-based studies of the kangaroo *Methanosphaera* isolate WGK6 revealed it could use ethanol as a sole source of reducing power for methanol reduction and methanogenesis, using alcohol and aldehyde dehydrogenases (Hoedt et al., 2016). Homologues of these alcohol and aldehyde dehydrogenase genes were also detected in a *Methanosphaera* sp. MAG (SHI1033) assembled from the low CH_4 yield sheep metagenomes (Shi et al., 2014), but not in any of the other MAGs or the *Methanosphaera* sp. BMS and *Methanosphaera stadtmanae* DSMZ 3091^T genomes. This raises the interesting possibility that ethanol-driven methanogenesis by *Methanosphaera* sp. is a common feature of low CH_4-producing gut environments.

8 Acetoclastic methanogens: *Methanosarcina* sp. CM1

Acetate is not metabolized to CH_4 to any significant extent in the rumen (Hungate et al., 1970), and methanogens able to form CH_4 from acetate (via the acetoclastic methanogenesis pathway) are extremely rare in this environment (<0.015%; Henderson et al., 2015), supporting this notion. Despite these observations, *Methanosarcina* have been isolated from the rumen on several occasions (Patterson and Hespell, 1979; McInerney et al., 1981b; Jarvis et al., 2000) so their rarity in the rumen microbial community appears not to preclude their cultivation. *Methanosarcina* sp. CM1 (99% 16S rRNA gene similarity to that of the *Ms. barkeri* MS^T DSM 800) was isolated from the rumen of a NZ Friesian cow grazing a ryegrass/white clover pasture and grew as large cell aggregates in broth culture, the characteristic morphology associated with *Ms. barkeri*. It grew and produced CH_4 from H_2 + CO_2, acetate, methanol and methylamines, but not formate, even though it encodes a formate dehydrogenase gene. The CM1 genome is much larger than other rumen methanogens (4.5 Mb; 3656 predicted genes) and encodes acetoclastic, hydrogenotrophic and methylotrophic methanogenesis pathways. CM1 does not encode a [Fe]-hydrogenase/dehydrogenase (*hmd*), or *mrt*, but does encode a CoM biosynthesis pathway, although the pathway differs from that in other sequenced rumen *Methanobacteriales*. Several features make CM1 a rumen curiosity. It encodes pyrrolysine biosynthesis (*pylSBCD*) together with a specific pyrrolysine aminoacyl-tRNA synthetase and its methyl transferase genes are predicted to incorporate pyrrolysine into their amino acid sequences. Its cell surface is predicted to be composed of a protein S-layer rather than the

pseudomurein found in other rumen *Methanobacteriales*, and it has a glycoside hydrolase family 18 (GH18; chitinase) protein which is predicted to be secreted from the cell and may mediate interaction with the chitin-containing rumen anaerobic fungi. Although CM1 has an operon encoding a flagellum (*flaB-flaJ*) and associated chemotaxis genes, CM1 has not been observed as a motile organism, suggesting these flagellar proteins may serve a different purpose in CM1. Like *Mb. formicicum* BRM9, CM1 contains two different *nif* operons with nitrogenase and nitrogenase cofactor biosynthesis genes which function in nitrogen fixation. Having the ability to use a wide range of substrates marks CM1 as a generalist able to occupy a range of different environments, although it does not attain high populations and is practically undetectable in the rumen under most circumstances. However, when pectin is used to induce methanol production in the rumen, *Methanosarcina* can be enriched and isolated (Pol and Demeyer, 1988).

9 Conclusions

The realisation that CH_4 emissions from ruminant animals contribute to anthropogenic GHG emissions and global warming has seen a renewed interest in characterising the methanogens in the rumen and finding ways to reduce their activities. The summary of rumen ecology studies outlined here indicates that there have been numerous cultivations of rumen methanogens, but only a handful of these have been successfully isolated as pure cultures and even fewer have been fully described and deposited in internationally recognised and accessible culture collections. There is a strong need for new initiatives, such as the Hungate 1000 project (Seshadri et al., 2018), to get more rumen methanogens into culture and to carry out detailed phenotypic and genomic characterizations. A more comprehensive catalogue of methanogen strains is needed to better characterize their physiology and to further understand the detail of the different modes of CH_4 formation. While methanogen genome sequences go some way explaining why there are multiple methanogen types found in the rumen, there is a great deal more work required at the organism level to elucidate their growth characteristics and how they respond to varying levels of their main energy source, H_2, and to a lesser extent, short chain alcohols. Furthermore, understanding the interactions of H_2– and methyl-compound-producing rumen microbes with methanogens is crucial to defining the role of interspecies H_2 transfer and the extent to which this process governs CH_4 formation.

Most of the biochemistry of methanogenesis pathway enzymes has been carried out in non-rumen methanogens, principally *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri* (Thauer et al., 2008). It is important to at least confirm that the biochemistry of methanogenesis in

rumen methanogens proceeds via the same mechanisms and pathways as characterised elsewhere, and preferably to fully elucidate the enzymology of the main rumen methanogen groups. It would also be desirable to have genetically tractable strains of the main rumen methanogen groups to allow targeted inactivation of specific methanogen genes to support gene function experiments. In non-rumen *Archaea*, genetic modification systems have been developed which have subsequently become the workhorses of archaeal microbial research (Metcalf et al., 1997; Rother and Metcalf, 2005). Systems for genetic manipulation of rumen methanogens do not currently exist but these will be required if real progress is to be made in determining the role of the myriad methanogen genes without known function. Some of the raw materials for genetic systems exist in rumen methanogens in the form of plasmids, phage and CRISPR-Cas systems, so there needs to be a concerted effort to put these genetic elements to work to reveal the details of rumen methanogen biology.

The high throughput, NGS technologies undoubtedly have an important role to play in the future characterisation of rumen methanogen communities. NGS technologies are already making genome sequencing of cultivated methanogen species routine and are amplifying our ability to catalogue and analyse methanogen marker genes. They are also offering powerful new tools to retrieve methanogen metagenomic sequences to ever increasing depths, thereby allowing access to genomic information directly from rumen samples via metagenome re-assemblies. It is anticipated that these new approaches will produce large amounts of marker gene, genomic and metagenomic sequence data that will require interpretation in the context of methanogen biology before the derived understanding can be applied to practical treatments for CH_4 mitigation in the rumen. Well-annotated and curated reference genes and whole genome sequences organised by taxonomic and functional categories in dedicated databases, will be crucial to accurate interpretation of these new datasets. The ever-increasing amounts of data will also require significantly expanded computing capabilities coupled with better software to identify, extract and analyse these new datasets. While these challenges are significant, the opportunities these new datasets afford are considerable, and augur well for a much-enhanced understanding of the biology of rumen methanogens in the future.

10 References

Akhmanova, A., Voncken, F., Van Alen, T., Van Hoek, A., Boxma, B., Vogels, G., Veenhuis, M. and Hackstein, J. H. 1998. A hydrogenosome with a genome. *Nature* 396(6711), 527–8. doi:10.1038/25023.

Altermann, E., Schofield, L. R., Ronimus, R. S., Beattie, A. K. and Reilly, K. 2018. Inhibition of rumen methanogens by a novel archaeal lytic enzyme displayed

on tailored bionanoparticles. *Frontiers in Microbiology* 9, 2378. doi:10.3389/fmicb.2018.02378.

Ametaj, B. N., Zebeli, Q., Saleem, F., Psychogios, N., Lewis, M. J., Dunn, S. M., Xia, J. and Wishart, D. S. 2010. Metabolomics reveals unhealthy alterations in rumen metabolism with increased proportion of cereal grain in the diet of dairy cows. *Metabolomics* 6(4), 583–94. doi:10.1007/s11306-010-0227-6.

Amoutzias, G. D., Van de Peer, Y. and Mossialos, D. 2008. Evolution and taxonomic distribution of non-ribosomal peptide and polyketide synthases. *Future Microbiology* 3(3), 361–70. doi:10.2217/17460913.3.3.361.

Anderson, R. C., Rasmussen, M. A. and Allison, M. J. 1993. Metabolism of the plant toxins nitropropionic acid and nitropropanol by ruminal microorganisms. *Applied and Environmental Microbiology* 59(9), 3056–61. doi:10.1128/AEM.59.9.3056-3061.1993.

Anderson, R. C., Rasmussen, M. A. and Allison, M. J. 1996. Enrichment and isolation of a nitropropanol-metabolizing bacterium from the rumen. *Applied and Environmental Microbiology* 62(10), 3885–6. doi:10.1128/AEM.62.10.3885-3886.1996.

Anderson, R. C., Rasmussen, M. A., Jensen, N. S. and Allison, M. J. 2000. *Denitrobacterium detoxificans* gen. nov., sp. nov., a ruminal bacterium that respires on nitrocompounds. *International Journal of Systematic and Evolutionary Microbiology* 50(2), 633–8. doi:10.1099/00207713-50-2-633.

Balch, W. E. and Wolfe, R. S. 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Applied and Environmental Microbiology* 32(6), 781–91. doi:10.1128/AEM.32.6.781-791.1976.

Balch, W. E., Fo, G. E., Magrum, L. J., Woese, C. R. and Wolfe, R. S. 1979. Methanogens: reevaluation of a unique biological group. *Microbiological Reviews* 43(2), 260–96. doi:10.1128/MMBR.43.2.260-296.1979.

Barker, H. A. 1936a. On the biochemistry of the methane fermentation. *Archives of Microbiology* 7(1–5), 404–19. doi:10.1007/BF00407413.

Barker, H. A. 1936b. Studies upon the methane-producing bacteria. *Archives of Microbiology* 7(1–5), 420–38. doi:10.1007/BF00407414.

Barker, H. A. 1940. Studies upon the methane fermentation. IV. The isolation and culture of *Methanobacterium omelianski*. *Antonie van Leeuwenhoek* 6, 201–20.

Barker, H. A. 1941. Studies of the methane fermentation. V. Biochemical activities of *Methanobacterium omelianskii*. *Journal of Biological Chemistry* 137, 153–67.

Barker, H. A., Ruben, S. and Kamen, M. D. 1940. The reduction of radioactive carbon dioxide by methane-producing bacteria. *Proceedings of the National Academy of Sciences of the United States of America* 26(6), 426–30.

Battumur, U., Lee, M., Bae, G. S. and Ki, C. H. 2019. Isolation and characterization of a new *Methanoculleus bourgensis* strain KOR-2 from the rumen of Holstein steers. *Asian-Australasian Journal of Animal Sciences* 32(2), 241–8.

Bauchop, T. and Mountfort, D. O. 1981. Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. *Applied and Environmental Microbiology* 42(6), 1103–10. doi:0099-2240/81/121103-08.

Beijer, W. H. 1952. Methane fermentation in the rumen of cattle. *Nature* 170(4327), 576–7. doi:10.1038/170576a0.

Belanche, A., de la Fuente, G. and Newbold, C. J. 2014. Study of methanogen communities associated with different rumen protozoal populations. *FEMS Microbiology Ecology* 90(3), 663–77. doi:10.1111/1574-6941.12423.

Berk, H. and Thauer, R. K. 1997. Function of coenzyme F_{420}-dependent NADP reductase in methanogenic archaea containing an NADP-dependent alcohol dehydrogenase. *Archives of Microbiology* 168(5), 396–402. doi:10.1007/s002030050514.

Bodelón, G., Palomino, C. and Fernández, L. Á 2013. Immunoglobulin domains in *Escherichia coli* and other enterobacteria: from pathogenesis to applications in antibody technologies. *FEMS Microbiology Reviews* 37(2), 204–50. doi:10.1111/j.1574-6976.2012.00347.x.

Bokranz, M., Katz, J., Schröder, I., Roberton, A. M. and Kröger, A. 1983. Energy metabolism and biosynthesis of *Vibrio succinogenes* growing with nitrate or nitrite as terminal electron acceptor. *Archives of Microbiology* 135(1), 36–41. doi:10.1007/BF00419479.

Borrel, G., Harris, H. M. B., Tottey, W., Mihajlovski, A., Parisot, N., Peyretaillade, E., Peyret, P., Gribaldo, S., O'Toole, P. W. and Brugère, J. F. 2012. Genome sequence of "*Candidatus Methanomethylophilus alvus*" Mx1201, a methanogenic archaeon from the human gut belonging to a seventh order of methanogens. *Journal of Bacteriology* 194(24), 6944–5. doi:10.1128/JB.01867-12.

Böttger, E. C. 1989. Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. *FEMS Microbiology Letters* 53(1–2), 171–6. doi:10.1016/0378-1097(89)90386-8.

Bovine Rumen Metabolome Database. 2018. Available at: http://www.rumendb.ca (accessed on 29 June 2018).

Breznak, J. A. and Kane, M. D. 1990. Microbial H_2/CO_2 acetogenesis in animal guts: nature and nutritional significance. *FEMS Microbiology Reviews* 7(3–4), 309–13. doi:10.1111/j.1574-6968.1990.tb04929.x.

Broad, T. E. and Dawson, R. M. 1976. Role of choline in the nutrition of the rumen protozoon *Entodinium caudatum*. *Journal of General Microbiology* 92(2), 391–97. doi:10.1099/00221287-92-2-391.

Bryant, M. P. 1965. Rumen methanogenic bacteria. In: Dougherty, R. W., Allen, R. S., Burroughs, R. W., Jacobson, N. L. and McGilliard, A. D. (Eds), *Physiology of Digestion of the Ruminant*. Butterworths Publishing, Inc., Washington DC, pp. 411–8.

Bryant, M. P. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. *American Journal of Clinical Nutrition* 25(12), 1324–8. doi:10.1093/ajcn/25.12.1324.

Bryant, M. P., Wolin, E. A., Wolin, M. J. and Wolfe, R. S. 1967. *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. *Archives of Microbiology* 59(1), 20–31. doi:10.1007/bf00406313.

Bryant, M. P., Tzeng, S. F., Robinson, I. M. and Joyner, A. E. 1971. Nutritional requirements of methanogenic bacteria. In: Pohland, F. G. (Ed.), *Anaerobic Biological Treatment Processes*. Advances in Chemistry Series 105. American Chemical Society, Washington DC, pp. 23–40.

Chagan, I., Tokura, M., Jouany, J. P. and Ushida, K. 1999. Detection of methanogenic archaea associated with rumen ciliate protozoa. *Journal of General and Applied Microbiology* 45(6), 305–8. doi:10.2323/jgam.45.305.

Chaucheyras, F., Fonty, G., Bertin, G. and Gouet, P. 1995. *In vitro* H_2 utilization by a ruminal acetogenic bacterium cultivated alone or in association with an Archaea methanogen is stimulated by a probiotic strain of *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* 61(9), 3466–7. doi:10.1128/AEM.61.9.3466-3467.1995.

Cheng, Y. F., Mao, S. Y., Liu, J. X. and Zhu, W. Y. 2009. Molecular diversity analysis of rumen methanogenic archaea from goat in eastern China by DGGE methods using different primer pairs. *Letters in Applied Microbiology* 48(5), 585–92. doi:10.1111/j.1472-765X.2009.02583.x.

Cord-Ruwisch, R., Seitz, H.-J. and Conrad, R. 1988. The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. *Archives of Microbiology* 149(4), 350–7. doi:10.1007/BF00411655.

Dawson, R. M. C. and Hemington, N. L. 1974. Digestion of grass lipids and pigments in the sheep rumen. *British Journal of Nutrition* 32(2), 327–40. doi:10.1079/bjn19740086.

de la Fuente, G., Belanche, A., Girwood, S. E., Pinloche, E., Wilkinson, T. and Newbold, C. J. 2014. Pros and cons of Ion-Torrent next generation sequencing versus Terminal Restriction Fragment Length Polymorphism T-RFLP for studying the rumen bacterial community. *PLoS ONE* 9(7), e101435. doi:10.1371/journal.pone.0101435.

Denman, S. E., Tomkins, N. W. and McSweeney, C. S. 2007. Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiology Ecology* 62(3), 313–22. doi:10.1111/j.1574-6941.2007.00394.x.

Denman, S. E., Evans, P., Bragg, L., Padmanahba, J., McKenzie, M., Edwards, D., Krzycki, J., McSweeney, C. S. and Morrison, M. 2011. *Thermoplasmatales*-like gut symbionts are pyrrolysine-dependent-methanogens. Genbank CP002916.1, unpublished.

Dridi, B., Fardeau, M. L., Ollivier, B., Raoult, D. and Drancourt, M. 2012. *Methanomassiliicoccus luminyensis* gen. nov., sp. nov., a methanogenic archaeon isolated from human faeces. *International Journal of Systematic and Evolutionary Microbiology* 62(8), 1902–7. doi:10.1099/ijs.0.033712-0.

Evans, P. N., Parks, D. H., Chadwick, G. L., Robbins, S. J., Orphan, V. J., Golding, S. D. and Tyson, G. W. 2015. Methane metabolism in the archaeal phylum Bathyarchaeota revealed by genome-centric metagenomics. *Science* 350(6259), 434–8. doi:10.1126/science.aac7745.

Evans, P. N., Boyd, J. A., Leu, A. O., Woodcroft, B. J., Parks, D. H., Hugenholtz, P. and Tyson, G. W. 2019. An evolving view of methane metabolism in the Archaea. *Nature Reviews. Microbiology* 17(4), 219–32 doi:10.1038/s41579-018-0136-7.

Finlay, B. J., Esteban, G., Clarke, K. J., Williams, A. G., Embley, T. M. and Hirt, R. P. 1994. Some rumen ciliates have endosymbiotic methanogens. *FEMS Microbiology Letters* 117(2), 157–61. doi:10.1111/j.1574-6968.1994.tb06758.x.

Fischbach, M. A. and Walsh, C. T. 2006. Assembly-line enzymology for polyketide and non-ribosomal peptide antibiotics: logic, machinery and mechanisms. *Chemical Reviews* 106(8), 3468–96. doi:10.1021/cr0503097.

Fouts, D. E., Szpakowski, S., Purushe, J., Torralba, M., Waterman, R. C., MacNeil, M. D., Alexander, L. J. and Nelson, K. E. 2012. Next generation sequencing to define prokaryotic and fungal diversity in the bovine rumen. *PLoS ONE* 7(11), e48289. doi:10.1371/journal.pone.0048289.

Franzolin, R., St-Pierre, B., Northwood, K. and Wright, A. D. 2012. Analysis of rumen methanogens diversity in water buffaloes (*Bubalus bubalis*) under three different diets. *Microbial Ecology* 64(1), 131–9. doi:10.1007/s00248-012-0007-0.

Frey, J. C., Pell, A. N., Berthiaume, R., Lapierre, H., Lee, S., Ha, J. K., Mendell, J. E. and Angert, E. R. 2010. Comparative studies of microbial populations in the rumen, duodenum,

ileum and faeces of lactating dairy cows. *Journal of Applied Microbiology* 108(6), 1982–93. doi:10.1111/j.1365-2672.2009.04602.x.

Fricke, W. F., Seedorf, H., Henne, A., Krer, M., Liesegang, H., Hedderich, R., Gottschalk, G. and Thauer, R. K. 2006. The genome sequence of *Methanosphaera stadtmanae* reveals why this human intestinal archaeon is restricted to methanol and H_2 for methane formation and ATP synthesis. *Journal of Bacteriology* 188(2), 642–58. doi:10.1128/JB.188.2.642-658.2006.

Genthner, B. R., Davis, C. L. and Bryant, M. P. 1981. Features of rumen and sewage sludge strains of *Eubacterium limosum*, a methanol-and H_2-CO_2-utilizing species. *Applied and Environmental Microbiology* 42(1), 12–9. doi:10.1128/AEM.42.1.12-19.1981.

Gilbert, R. A., Ouwerkerk, D., Zhang, L. H., Klieve, A. V. and Cooperative Research Centre for Beef Genetic Technologies. 2010. *In vitro* detection and primary cultivation of bacteria producing materials inhibitory to ruminal methanogens. *Journal of Microbiological Methods* 80(2), 217–8. doi:10.1016/j.mimet.2009.12.004.

Graham, L. L. and Beveridge, T. J. 1994. Structural differentiation of the *Bacillus subtilis* 168 cell wall. *Journal of Bacteriology* 176(5), 1413–21. doi:10.1128/jb.176.5.1413-1421.1994.

Greening, R. C. and Leedle, J. A. Z. 1989. Enrichment and isolation of *Acetitomaculum ruminis*, gen. nov., sp. nov.: acetogenic bacteria from the bovine rumen. *Archives of Microbiology* 151(5), 399–406. doi:10.1007/bf00416597.

Gu, M. J., Alam, M. J., Kim, S. H., Jeon, C. O., Chang, M. B., Oh, Y. K., Lee, S. C. and Lee, S. S. 2011. Analysis of methanogenic archaeal communities of rumen fluid and rumen particles from Korean black goats. *Animal Science Journal = Nihon Chikusan Gakkaiho* 82(5), 663–72. doi:10.1111/j.1740-0929.2011.00890.x.

Gupta, A. and Chaudhary, U. B. 2010. Isolation and characterization of *Methanobrevibacter smithii* GMS-01 from rumen of goats. *Indian Veterinary Journal* 87, 1009–12.

Henderson, C. 1980. The influence of extracellular hydrogen on the metabolism of *Bacteroides ruminicola*, *Anaerovibrio lipolytica* and *Selenomonas ruminantium*. *Microbiology* 119(2), 485–91. doi:10.1099/00221287-119-2-485.

Henderson, G., Naylor, G. E., Leahy, S. C. and Janssen, P. H. 2010. Presence of novel, potentially homoacetogenic bacteria in the rumen as determined by analysis of formyltetrahydrofolate synthetase sequences from ruminants. *Applied and Environmental Microbiology* 76(7), 2058–66. doi:10.1128/AEM.02580-09.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Global Rumen Census Collaborators and Janssen, P. H. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific Reports* 5, 14567. doi:10.1038/srep14567.

Hess, M., Sczyrba, A., Egan, R., Kim, T. W., Chokhawala, H., Schroth, G., Luo, S., Clark, D. S., Chen, F., Zhang, T., Mackie, R. I., Pennacchio, L. A., Tringe, S. G., Visel, A., Woyke, T., Wang, Z. and Rubin, E. M. 2011. Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. *Science* 331(6016), 463–7. doi:10.1126/science.1200387.

Hoedt, E. C. 2017. Functional and comparative studies of members of the genus *Methanosphaera*, and their adaptations to the gut environment. PhD Thesis. The University of Queensland, Brisbane, Australia. doi:10.14264/uql.2017.366.

Hoedt, E. C., Ó Cuív, P. Ó, Evans, P. N., Smith, W. J. M., McSweeney, C. S., Denman, S. E. and Morrison, M. 2016. Differences down-under: alcohol-fuelled methanogenesis by archaea present in Australian macropodids. *ISME Journal* 10(10), 2376–88. doi:10.1038/ismej.2016.41.

Hoedt, E. C., Parks, D. H., Volmer, J. G., Rosewarne, C. P., Denman, S. E., McSweeney, C. S., Mui, J. G., Gibson, P. R., Cuív, P. Ó., Hugenholtz, P., Tyson, G. W. and Morrison, M. 2018. Culture- and metagenomics-enabled analyses of the *Methanosphaera* genus reveals their monophyletic origin and differentiation according to genome size. *ISME Journal* 12(12), 2942–53. doi:10.1038/s41396-018-0225-7.

Hook, S. E., Northwood, K. S., Wright, A. D. G. and McBride, B. W. 2009. Long-term monensin supplementation does not significantly affect the quantity or diversity of methanogens in the rumen of the lactating dairy cow. *Applied and Environmental Microbiology* 75(2), 374–80. doi:10.1128/AEM.01672-08.

Howard, B. H. and Hungate, R. E. 1976. *Desulfovibrio* of the sheep rumen. *Applied and Environmental Microbiology* 32(4), 598–602. doi:10.1128/AEM.32.4.598-602.1976.

Huang, X. D., Tan, H. Y., Long, R. J., Liang, J. B. and Wright, A. D. G. 2012. Comparison of methanogen diversity of yak (*Bos grunniens*) and cattle (*Bos taurus*) from the Qinghai-Tibetan Plateau, China. *BMC Microbiology* 12, 237. doi:10.1186/1471-2180-12-237.

Huang, X. D., Martinez-Fernandez, G., Padmanabha, J., Long, R. J., Denman, S. E. and McSweeney, C. S. 2016. Methanogen diversity in indigenous and introduced ruminant species on the Tibetan Plateau. *Archaea* 2016, 5916067 doi:10.1155/2016/5916067.

Huisingh, J., McNeill, J. J. and Matrone, G. 1974. Sulfate reduction by a *Desulfovibrio* species isolated from sheep rumen. *Applied Microbiology* 28(3), 489–97. doi:10.1128/AEM.28.3.489-497.1974.

Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. *Bacteriological Reviews* 14(1), 1–49. doi:10.1128/MMBR.14.1.1-49.1950.

Hungate, R. E. 1966. *The Rumen and Its Microbes*. Academic Press, New York, NY and London, UK.

Hungate, R. E. 1969. A roll-tube method for cultivation of strict anaerobes. *Methods in Microbiology* 3B, 117–32.

Hungate, R. E. 1975. The rumen microbial ecosystem. *Annual Review of Ecology and Systematics* 6(1), 39–66. doi:10.1146/annurev.es.06.110175.000351.

Hungate, R. E., Smith, W., Bauchop, T., Yu, I. and Rabinowitz, J. C. 1970. Formate as an intermediate in the rumen fermentation. *Journal of Bacteriology* 102, 384–97.

Huser, B. A., Wurmann, K. and Zehnder, A. J. B. 1982. *Methanothrix soehngenii* gen. nov. sp. nov., a new acetotrophic non-hydrogen-oxidizing methane bacterium. *Archives of Microbiology* 132(1), 1–9. doi:10.1007/BF00690808.

Hutten, T. J., Bongaerts, H. C. M., Van der Drift, C. and Vogels, G. D. 1980. Acetate, methanol and carbon dioxide as substrates for growth of *Methanosarcina barkeri*. *Antonie van Leeuwenhoek* 46(6), 601–10. doi:10.1007/bf00394016.

Iino, T., Tamaki, H., Tamazawa, S., Ueno, Y., Ohkuma, M., Suzuki, K., Igarashi, Y. and Haruta, S. 2013. *Candidatus* Methanogranum caenicola: a novel methanogen from the anaerobic digested sludge, and proposal of *Methanomassiliicoccaceae* fam. nov. and *Methanomassiliicoccales* ord. nov., for a methanogenic lineage of the class *Thermoplasmata*. *Microbes and Environments* 28(2), 244–50. doi:10.1264/jsme2.me12189.

Indugu, N., Bittinger, K., Kumar, S., Vecchiarelli, B. and Pitta, D. 2016. A comparison of rumen microbial profiles in dairy cows as retrieved by 454 Roche and Ion Torrent (PGM) sequencing platforms. *PeerJ* 4, e1599 doi:10.7717/peerj.1599.

Irbis, C. and Ushida, K. 2004. Detection of methanogens and proteobacteria from a single cell of rumen ciliate protozoa. *Journal of General and Applied Microbiology* 50(4), 203–12. doi:10.2323/jgam.50.203.

Iwamoto, M., Asanuma, N. and Hino, T. 2002. Ability of *Selenomonas ruminantium*, *Veillonella parvula*, and *Wolinella succinogenes* to reduce nitrate and nitrite with special reference to the suppression of ruminal methanogenesis. *Anaerobe* 8(4), 209–15. doi:10.1006/anae.2002.0428.

Jamieson, N. D. 1959. Rumen nitrate metabolism and the changes occurring in the composition of the rumen volatile fatty acids of grazing sheep. *New Zealand Journal of Agricultural Research* 2(2), 314–28, doi:10.1080/00288233.1959.10420320.

Janssen, P. H. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Animal Feed Science and Technology* 160(1–2), 1–22. doi:10.1016/j.anifeedsci.2010.07.002.

Janssen, P. H. and Kirs, M. 2008. Structure of the archaeal community of the rumen. *Applied and Environmental Microbiology* 74(12), 3619–25. doi:10.1128/AEM.02812-07.

Jarvis, G. N., Strompl, C., Burgess, D. M., Skillman, L. C., Moore, E. R. B. and Joblin, K. N. 2000. Isolation and identification of ruminal methanogens from grazing cattle. *Current Microbiology* 40(5), 327–32. doi:10.1007/s002849910065.

Jeyanathan, J. 2010. Investigation of rumen methanogens in New Zealand livestock. PhD Thesis. Massey University, Palmerston North, New Zealand.

Jeyanathan, J., Kirs, M., Ronimus, R. S., Hoskin, S. O. and Janssen, P. H. 2011. Methanogen community structure in the rumens of farmed sheep, cattle and red deer fed different diets. *FEMS Microbiology Ecology* 76(2), 311–26. doi:10.1111/j.1574-6941.2011.01056.x.

Jin, W., Cheng, Y. F., Mao, S. Y. and Zhu, W. Y. 2011. Isolation of natural cultures of anaerobic fungi and indigenously associated methanogens from herbivores and their bioconversion of lignocellulosic materials to methane. *Bioresource Technology* 102(17), 7925–31. doi:10.1016/j.biortech.2011.06.026.

Jin, W., Cheng, Y. F., Mao, S. Y. and Zhu, W. Y. 2014. Discovery of a novel rumen methanogen in the anaerobic fungal culture and its distribution in the rumen as revealed by real-time PCR. *BMC Microbiology* 14, 104. doi:10.1186/1471-2180-14-104.

Joblin, K. N. 1999. Ruminal acetogens and their potential to lower ruminant methane emissions. *Australian Journal of Agricultural Research* 50(8), 1307–13. doi:10.1071/AR99004.

Joblin, K. N. 2005. Methanogenic archaea. In: Makkar, H. P. S. and McSweeney, C. (Eds), *Methods in Gut Microbial Ecology for Ruminants*. Kluwer Academic Publishers, the Netherlands, pp. 47–53.

Johns, A. T. and Barker, H. A. 1960. Methane formation: fermentation of ethanol in the absence of carbon dioxide by *Methanobacillus omelianskii*. *Journal of Bacteriology* 80, 837–41. doi:10.1128/JB.80.6.837-841.1960.

Jones, G. A. 1972. Dissimilatory metabolism of nitrate by the rumen microbiota. *Canadian Journal of Microbiology* 18(12), 1783–7. doi:10.1139/m72-279.

Joshi, A., Lanjekar, V., Dhakephalkar, P. K. and Dagar, S. S. 2018. Cultivation of multiple genera of hydrogenotrophic methanogens from different environmental niches. *Anaerobe* 50, 64–8. doi:10.1016/j.anaerobe.2018.02.001.

Kamke, J., Kittelmann, S., Soni, P., Li, Y., Tavendale, M., Ganesh, S., Janssen, P. H., Shi, W., Froula, J., Rubin, E. M. and Attwood, G. T. 2016. Rumen metagenome and

metatranscriptome analyses of low methane yield sheep reveals a *Sharpea*-enriched microbiome characterised by lactic acid formation and utilisation. *Microbiome* 4(1), 56. doi:10.1186/s40168-016-0201-2.

Kelly, W. J., Leahy, S. C., Li, D., Perry, R., Lambie, S. C., Attwood, G. T. and Altermann, E. 2014. The complete genome sequence of the rumen methanogen *Methanobacterium formicicum* BRM9. *Standards in Genomic Sciences* 9(1), 15. doi:10.1186/1944-3277-9-15.

Kelly, W. J., Li, D., Lambie, S. C., Cox, F., Attwood, G. T., Altermann, E. and Leahy, S. C. 2016a. Draft genome sequence of the rumen methanogen *Methanobrevibacter olleyae* YLM1. *Genome Announcements* 4(2). doi:10.1128/genomeA.00232-16.

Kelly, W. J., Pacheco, D. M., Li, D., Attwood, G. T., Altermann, E. and Leahy, S. C. 2016b. The complete genome sequence of the rumen methanogen *Methanobrevibacter millerae* SM9. *Standards in Genomic Sciences* 11, 49. doi:10.1186/s40793-016-0171-9.

Kelly, W. J., Li, D., Lambie, S. C., Jeyanathan, J., Cox, F., Li, Y., Attwood, G. T., Altermann, E. and Leahy, S. C. 2016c. Complete genome sequence of methanogenic archaeon ISO4-G1, a member of the *Methanomassiliicoccales*, isolated from a sheep rumen. *Genome Announcements* 4(2). doi:10.1128/genomeA.00221-16.

Kennedy, P. M. and Milligan, L. P. 1978. Quantitative aspects of the transformations of sulphur in sheep. *British Journal of Nutrition* 39(1), 65–84. doi:10.1079/bjn19780013.

Kim, C. C.-H. 2012. Identification of rumen methanogens, characterization of substrate requirements and measurement of hydrogen thresholds. MSc Thesis. Massey University, Palmerston North, New Zealand.

King, E. E., Smith, R. P., St-Pierre, B. and Wright, A. D. 2011. Differences in the rumen methanogens populations of lactating Jersey and Holstein dairy cows under the same diet regimen. *Applied and Environmental Microbiology* 77(16), 5682–7. doi:10.1128/AEM.05130-11.

Kittelmann, S., Seedorf, H., Walters, W. A., Clemente, J. C., Knight, R., Gordon, J. I. and Janssen, P. H. 2013. Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLoS ONE* 8(2), e47879. doi:10.1371/journal.pone.0047879.

Kittelmann, S., Pinares-Patinő, C. S., Seedorf, H., Kirk, M. R., Ganesh, S., McEwan, J. C. and Janssen, P. H. 2014. Two different bacterial community types are linked with the low-methane emission trait in sheep. *PLoS ONE* 9(7), e103171. doi:10.1371/journal.pone.0103171.

Knight, M., Wolfe, R. S. and Elsden, S. R. 1966. The synthesis of amino acids by *Methanobacterium omelianskii*. *Biochemical Journal* 99(1), 76–86. doi:10.1042/bj0990076.

Lambie, S. C., Kelly, W. J., Leahy, S. C., Li, D., Reilly, K., McAllister, T. A., Valle, E. R., Attwood, G. T. and Altermann, E. 2015. The complete genome sequence of the rumen methanogen *Methanosarcina barkeri* CM1. *Standards in Genomic Sciences* 10, 57. doi:10.1186/s40793-015-0038-5.

Leahy, S. C., Kelly, W. J., Altermann, E. H., Ronimus, R. S., Yeoman, C. J., Pacheco, D. M., Li, D., Kong, Z., McTavish, S., Sang, C., Lambie, S. C., Janssen, P. H., Dey, D. and Attwood, G. T. 2010. The genome sequence of the rumen methanogen *Methanobrevibacter ruminantium* reveals new possibilities for controlling ruminant methane emissions. *PLoS ONE* 5(1), e8926. doi:10.1371/journal.pone.0008926.

Leahy, S. C., Kelly, W. J., Li, D., Li, Y., Altermann, E., Lambie, S. C., Cox, F. and Attwood, G. T. 2013. The complete genome sequence of *Methanobrevibacter* sp. AbM4. *Standards in Genomic Sciences* 8(2), 215–27. doi:10.4056/sigs.3977691.

Lee, H. J., Jung, J. Y., Oh, Y. K., Lee, S. S., Madsen, E. L. and Jeon, C. O. 2012. Comparative survey of rumen microbial communities and metabolites across one caprine and three bovine groups, using bar-coded pyrosequencing and ^{1}H nuclear magnetic resonance spectroscopy. *Applied and Environmental Microbiology* 78(17), 5983–93; doi:10.1128/AEM.00104-12.

Lee, J. H., Kumar, S., Lee, G. H., Chang, D. H., Rhee, M. S., Yoon, M. H. and Kim, B. C. 2013a. *Methanobrevibacter boviskoreani* sp. nov., isolated from the rumen of Korean native cattle. *International Journal of Systematic and Evolutionary Microbiology* 63(11), 4196–201. doi:10.1099/ijs.0.054056-0.

Lee, J. H., Rhee, M. S., Kumar, S., Lee, G. H., Chang, D. H., Kim, D. S., Choi, S. H., Lee, D. W., Yoon, M. H. and Kim, B. C. 2013b. Genome sequence of *Methanobrevibacter* sp. strain JH1, isolated from rumen of Korean native cattle. *Genome Announcements* 1(1). pii: e00002-13. doi:10.1128/genomeA.00002-13.

Li, Y. 2016. Comparative genomics of rumen methanogens. PhD Thesis. Massey University, Palmerston North, New Zealand. Available at: http://hdl.handle.net/10179/10953.

Li, F., Henderson, G., Sun, X., Cox, F., Janssen, P. H. and Guan, L. L. 2016a. Taxonomic assessment of rumen microbiota using total RNA and targeted amplicon sequencing approaches. *Frontiers in Microbiology* 7, 987. doi:10.3389/fmicb.2016.00987.

Li, Y., Leahy, S. C., Jeyanathan, J., Henderson, G., Cox, F., Altermann, E. H., Kelly, W. J., Lambie, S. C., Janssen, P. H., Rakonjac, J. and Attwood, G. T. 2016b. The complete genome sequence of the methanogenic archaeon ISO4-H5 provides insights into the methylotrophic lifestyle of a ruminal representative of the *Methanomassiliicoccales*. *Standards in Genomic Sciences* 11(1), 59. doi:10.1186/s40793-016-0183-5.

Lin, C., Raskin, L. and Stahl, D. A. 1997. Microbial community structure in gastrointestinal tracts of domestic animals: comparative analyses using rRNA-targeted oligonucleotide probes. *FEMS Microbiology Ecology* 22(4), 281–94. doi:10.1111/j.1574-6941.1997.tb00380.x.

Liu, Y. and Whitman, W. B. 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic Archaea. *Annals of the New York Academy of Sciences* 1125, 171–89. doi:10.1196/annals.1419.019.

Lloyd, D., Williams, A. G., Amann, R., Hayes, A. J., Durrant, L. and Ralphs, J. R. 1996. Intracellular prokaryotes in rumen ciliate protozoa: detection by confocal laser scanning after *in situ* hybridization with fluorescent 16S rRNA probes. *European Journal of Protistology* 32(4), 523–31. doi:10.1016/S0932-4739(96)80011-3.

Lovely, D. R., Greening, R. C. and Ferry, J. G. 1984. Rapidly growing rumen methanogenic organism that synthesizes coenzyme M and has a high affinity for formate. *Applied and Environmental Microbiology* 48(1), 81–7. doi:10.1128/AEM.48.1.81-87.1984.

Mackie, R. I. and Bryant, M. P. 1994. Acetogenesis and the rumen: syntrophic relationships. In: Drake, H. L. (Ed.), *Acetogenesis*. Chapman and Hall, New York, NY, pp. 331–64.

Martin, S. A. and Park, C. M. 1996. Effect of extracellular hydrogen on organic acid utilization by the ruminal bacterium *Selenomonas ruminantium*. *Current Microbiology* 32(6), 327–31. doi:10.1007/s002849900058.

Martinez-Fernandez, G., Duval, S., Kindermann, M., Schirra, H. J., Denman, S. E. and McSweeney, C. S. 2018. 3-NOP vs. halogenated compound: methane production, ruminal fermentation and microbial community response in forage fed cattle. *Frontiers in Microbiology* 9, 1582. doi:10.3389/fmicb.2018.01582.

McAllister, T. A., Cheng, K.-J., Okine, E. K. and Mathison, G. W. 1996. Dietary, environmental and microbiological aspects of methane production in ruminants. *Canadian Journal of Animal Science* 76(2), 231–43. doi:10.4141/cjas96-035.

McCabe, M. S., Cormican, P., Keogh, K., O'Connor, A., O'Hara, E., Palladino, R. A., Kenny, D. A. and Waters, S. M. 2015. Illumina MiSeq phylogenetic amplicon sequencing shows a large reduction of an uncharacterised *Succinivibrionaceae* and an increase of the *Methanobrevibacter gottschalkii* clade in feed restricted cattle. *PLoS ONE* 10(7), e0133234. doi:10.1371/journal.pone.0133234.

McInerney, M. J., Bryant, M. P. and Pfennig, N. 1979. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. *Archives of Microbiology* 122(2), 129–35. doi:10.1007/BF00411351.

McInerney, M. J., Bryan, M. P., Hespell, R. B. and Costerton, J. W. 1981a. *Syntrophomonas wolfei* gen nov., sp. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium. *Applied and Environmental Microbiology* 41(4), 1029–39. doi:10.1128/AEM.41.4.1029-1039.1981.

McInerney, M. J., Mackie, R. I. and Bryant, M. P. 1981b. Synthrophic association of a butyrate-degrading bacterium and *Methansarcina* enriched from bovine rumen fluid. *Applied and Environmental Microbiology* 41(3), 826–8. doi:10.1128/AEM.41.3.826-828.1981.

Metcalf, W. W., Zhang, J. K., Apolinario, E., Sowers, K. R. and Wolfe, R. S. 1997. A genetic system for Archaea of the genus *Methanosarcina*: liposome-mediated transformation and construction of shuttle vectors. *Proceedings of the National Academy of Sciences of the United States of America* 94(6), 2626–31. doi:10.1073/pnas.94.6.2626.

Miller, T. L. 2001. Genus II. *Methanobrevibacter*. In: Boone, D. R. and Castenholz, R. W. (Eds), *Bergey's Manual of Systematic Bacteriology Vol. 1.: The Archaea and the Deeply Branching and Phototrophic Bacteria*. Springer, New York, NY, pp. 218–26.

Miller, T. L. and Lin, C. 2002. Description of *Methanobrevibacter gottschalkii* sp. nov., *Methanobrevibacter thaueri* sp. nov., *Methanobrevibacter woesei* sp. nov. and *Methanobrevibacter wolinii* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 52(3), 819–22. doi:10.1099/00207713-52-3-819.

Miller, T. L., Wolin, M. J., Zhao, H. X. and Bryant, M. P. 1986. Characteristics of methanogens isolated from bovine rumen. *Applied and Environmental Microbiology* 51(1), 201–2. doi:10.1128/AEM.51.1.201-202.1986.

Morgavi, D. P., Rathahao-Paris, E., Popova, M., Boccard, J., Nielsen, K. F. and Boudra, H. 2015. Rumen microbial communities influence metabolic phenotypes in lambs. *Frontiers in Microbiology* 6, 1060.doi:10.3389/fmicb.2015.01060.

Morvan, B., Dore, J., Rieu-Lesme, F., Foucat, L., Fonty, G. and Gouet, P. 1994. Establishment of hydrogen-utilizing bacteria in the rumen of the newborn lamb. *FEMS Microbiology Letters* 117(3), 249–56. doi:10.1016/0378-1097(94)90567-3.

Morvan, B., Bonnemoy, F., Fonty, G. and Gouet, P. 1996. Quantitative determination of H_2-utilizing acetogenic and sulfate-reducing bacteria and methanogenic archaea from digestive tract of different mammals. *Current Microbiology* 32(3), 129–33. doi:10.1007/s002849900023.

Müller, M. 1993. The hydrogenosome. *Journal of General Microbiology* 139(12), 2879–89. doi:10.1099/00221287-139-12-2879.

Murray, R. M., Bryant, A. M. and Leng, R. A. 1978. Methane production in the rumen and lower gut of sheep given Lucerne chaff: effect of level of intake. *British Journal of Nutrition* 39(2), 337–45. doi:10.1079/bjn19780043.

Myer, P. R., Kim, M.-S., Freetly, H. C. and Smith, T. P. L. 2016. Evaluation of 16S rRNA amplicon sequencing using two next-generation sequencing technologies for phylogenetic analysis of the rumen bacterial community in steers. *Journal of Microbiological Methods* 127, 132–40. doi:10.1016/j.mimet.2016.06.004.

Neill, A. R., Grime, D. W. and Dawson, R. M. 1978. Conversion of choline methyl groups through trimethylamine into methane in the rumen. *Biochemical Journal* 170(3), 529–35. doi:10.1042/bj1700529.

Nelson, W. O., Oppermann, R. A. and Brown, R. E. 1958. *In vitro* studies on methanogenic rumen bacteria. II. Fermentation of butyric and valeric acid. *Journal of Dairy Science* 41(4), 545–51. doi:10.3168/jds.S0022-0302(58)90960-3.

Ng, F., Kittelmann, S., Patchett, M. L., Attwood, G. T., Janssen, P. H., Rakonjac, J. and Gagic, D. 2016. An adhesin from hydrogen-utilizing rumen methanogen *Methanobrevibacter ruminantium* M1 binds a broad range of hydrogen-producing microorganisms. *Environmental Microbiology* 18(9), 3010–21 doi:10.1111/1462-2920.13155.

Nicholson, M. J., Evans, P. N. and Joblin, K. N. 2007. Analysis of methanogen diversity in the rumen using temporal temperature gradient gel electrophoresis: identification of uncultured methanogens. *Microbial Ecology* 54(1), 141–50. doi:10.1007/s00248-006-9182-1.

Nobu, M. K., Narihiro, T., Kuroda, K., Mei, R. and Liu, W. T. 2016. Chasing the elusive Euryarchaeota class WSA2: genomes reveal a uniquely fastidious methyl-reducing methanogen. *ISME Journal* 10(10), 2478–87. doi:10.1038/ismej.2016.33.

Noel, S. J., Højberg, O., Urich, T. and Poulsen, M. 2016. Draft genome sequence of '*Candidatus* Methanomethylophilus' sp. 1R26, enriched from bovine rumen, a methanogenic archaeon belonging to the *Methanomassiliicoccales* order. *Genome Announcements* 4(1), e01734-15. doi:10.1128/genomeA.01734-15.

Ohene-Adjei, S., Teather, R. M., Ivan, M. and Forster, R. J. 2007. Post-inoculation protozoan establishment and association patterns of methanogenic archaea in the ovine rumen. *Applied and Environmental Microbiology* 73(14), 4609–18. doi:10.1128/AEM.02687-06.

Ohene-Adjei, S., Chaves, A. V., McAllister, T. A., Benchaar, C., Teather, R. M. and Forster, R. J. 2008. Evidence of increased diversity of methanogenic archaea with plant extract supplementation. *Microbial Ecology* 56(2), 234–42. doi:10.1007/s00248-007-9340-0.

Oppermann, R. A., Nelson, W. O. and Brown, R. E. 1957. *In vitro* studies on methanogenic rumen bacteria. *Journal of Dairy Science* 40(7), 779–88. doi:10.3168/jds.S0022-0302(57)94554-X.

Orpin, C. G. 1975. Studies on the rumen flagellate, *Neocallimastix frontalis*. *Journal of General Microbiology* 91(2), 249–62. doi:10.1099/00221287-91-2-249.

Ozutsumi, Y., Tajima, K., Takenaka, A. and Itabashi, H. 2012. The *mcrA* gene and 16S rRNA gene in the phylogenetic analysis of methanogens in the rumen of faunated and unfaunated cattle. *Animal Science Journal = Nihon Chikusan Gakkaiho* 83(11), 727–34. doi:10.1111/j.1740-0929.2012.01023.x.

Padmanabha, J., Liu, J., Kurekci, C., Denman, S. and McSweeney, C. 2013. A methylotrophic methanogen isolate from the *Thermoplasmatales* affiliated RCC clade may provide

insight into the role of this group in the rumen. *Proceedings of the 5th Greenhouse Gases and Animal Agriculture Conference*, Dublin, Ireland, 23–26 June 2013. Cambridge University Press, Cambridge, p. 259.

Patterson, J. A. and Hespell, R. B. 1979. Trimethylamine and methylamine as growth substrates for rumen bacteria and *Methanosarcina barkeri*. *Current Microbiology* 3(2), 79–83. doi:10.1007/BF02602436.

Paul, K., Nonoh, J. O., Mikulski, L. and Brune, A. 2012. *Methanoplasmatales*, *Thermoplasmatales*-related archaea in termite guts and other environments, are the seventh order of methanogens. *Applied and Environmental Microbiology* 78(23), 8245–53. doi:10.1128/AEM.02193-12.

Paynter, M. J. B. and Hungate, R. E. 1968. Characterization of *Methanobacterium mobilis*, sp. n., isolated from the bovine rumen. *Journal of Bacteriology* 95(5), 1943–51. doi:10.1128/JB.95.5.1943-1951.1968.

Pei, C. X., Mao, S. Y., Cheng, Y. F. and Zhu, W. Y. 2010. Diversity, abundance and novel 16S rRNA gene sequences of methanogens in rumen liquid, solid and epithelium fractions of Jinnan cattle. *Animal* 4(1), 20–9. doi:10.1017/S1751731109990681.

Podell, S. and Gaasterland, T. 2007. Darkhorse: a method for genome-wide prediction of horizontal gene transfer. *Genome Biology* 8(2), R16. doi:10.1186/gb-2007-8-2-r16.

Pol, A. and Demeyer, D. I. 1988. Fermentation of methanol in the sheep rumen. *Applied and Environmental Microbiology* 54(3), 832–4. doi:10.1128/AEM.54.3.832-834.1988.

Popova, M., Marti, C., Eugène, M., Mialon, M. M., Doreau, M. and Morgavi, D. P. 2011. Effect of fibre- and starch-rich finishing diets on methanogenic Archaea diversity and activity in the rumen of feedlot bulls. *Animal Feed Science and Technology* 166–167, 113–21. doi:10.1016/j.anifeedsci.2011.04.060.

Poulsen, M., Schwab, C., Borg Jensen, B. B., Engberg, R. M., Spang, A., Canibe, N., Højberg, O., Milinovich, G., Fragner, L., Schleper, C., Weckwerth, W., Lund, P., Schramm, A. and Urich, T. 2013. Methylotrophic methanogenic *Thermoplasmata* implicated in reduced methane emissions from bovine rumen. *Nature Communications* 4, 1428. doi:10.1038/ncomms2432.

Rea, S., Bowman, J. P., Popovski, S., Pimm, C. and Wright, A. D. 2007. *Methanobrevibacter millerae* sp. nov. and *Methanobrevibacter olleyae* sp. nov., methanogens from the ovine and bovine rumen that can utilize formate for growth. *International Journal of Systematic and Evolutionary Microbiology* 57(3), 450–6. doi:10.1099/ijs.0.63984-0.

Reeve, J. N., Nolling, J., Morgan, R. M. and Smith, D. R. 1997. Methanogenesis: genes, genomes, and who's on first? *Journal of Bacteriology* 179(19), 5975–86. doi:10.1128/jb.179.19.5975-5986.1997.

Regensbogenova, M., McEwan, N. R., Javorsky, P., Kisidayova, S., Michalowski, T., Newbold, C. J., Hackstein, J. H. P. and Pristas, P. 2004. A re-appraisal of the diversity of the methanogens associated with the rumen ciliates. *FEMS Microbiology Letters* 238(2), 307–13. doi:10.1016/j.femsle.2004.07.049.

Rieu-Lesme, F., Morvan, B., Collins, M. D., Fonty, G. and Willems, A. 1996. A new H_2/CO_2-using acetogenic bacterium from the rumen: description of *Ruminococcus schinkii* sp. nov. *FEMS Microbiology Letters* 140(2–3), 281–6. doi:10.1016/0378-1097(96)00195-4.

Rother, M. and Metcalf, W. W. 2005. Genetic technologies for Archaea. *Current Opinion in Microbiology* 8(6), 745–51. doi:10.1016/j.mib.2005.10.010.

Rowe, J. B., Loughnan, M. L., Nolan, J. V. and Leng, R. A. 1979. Secondary fermentation in the rumen of a sheep given a diet based on molasses. *British Journal of Nutrition* 41(2), 393–7. doi:10.1079/bjn19790048.

Seedorf, H., Kittelmann, S., Henderson, G. and Janssen, P. H. 2014. RIM-DB: a taxonomic framework for community structure analysis of methanogenic archaea from the rumen and other intestinal environments. *PeerJ* 2, e494. doi:10.7717/peerj.494.

Seedorf, H., Kittelmann, S. and Janssen, P. H. 2015. Few highly abundant operational taxonomic units dominate within rumen methanogenic archaeal species in New Zealand sheep and cattle. *Applied and Environmental Microbiology* 81(3), 986–95. doi:10.1128/AEM.03018-14.

Seshadri, R., Leahy, S. C., Attwood, G. T., Teh, K. H., Lambie, S. C., Cookson, A. L., Eloe-Fadrosh, E. A., Pavlopoulos, G. A., Hadjithomas, M., Varghese, N. J., Paez-Espino, D., Hungate1000 project collaborators, Perry, R., Henderson, G., Creevey, C. J., Terrapon, N., Lapebie, P., Drula, E., Lombard, V., Rubin, E., Kyrpides, N. C., Henrissat, B., Woyke, T., Ivanova, N. N. and Kelly, W. J. 2018. Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nature Biotechnology* 36(4), 359–67. doi:10.1038/nbt.4110.

Sharp, R., Ziemer, C. J., Stern, M. D. and Stahl, D. A. 1998 Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. *FEMS Microbiology Ecology* 26(1), 71–8. doi:10.1111/j.1574-6941.1998.tb01563.x.

Shi, W., Moon, C. D., Leahy, S. C., Kang, D., Froula, J., Kittelmann, S., Fan, C., Deutsch, S., Gagic, D., Seedorf, H., Kelly, W. J., Atua, R., Sang, C., Soni, P., Li, D., Pinares-Patiño, C. S., McEwan, J. C., Janssen, P. H., Chen, F., Visel, A., Wang, Z., Attwood, G. T. and Rubin, E. M. 2014. Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. *Genome Research* 24(9), 1517–25. doi:10.1101/gr.168245.113.

Shin, E. C., Choi, B. R., Lim, W. J., Hong, S. Y., An, C. L., Cho, K. M., Kim, Y. K., An, J. M., Kang, J. M., Lee, S. S., Kim, H. and Yun, H. D. 2004. Phylogenetic analysis of archaea in three fractions of cow rumen based on the 16S rDNA sequence. *Anaerobe* 10(6), 313–9. doi:10.1016/j.anaerobe.2004.08.002.

Silley, P. 1985. A note on the pectinolytic enzymes of *Lachnospira multiparus*. *Journal of Applied Bacteriology* 58(2), 145–9. doi:10.1111/j.1365-2672.1985.tb01441.x.

Silley, P. 1986. The production and properties of a crude pectin lyase from *Lachnospira multiparus*. *Letters in Applied Microbiology* 2(2), 29–31. doi:10.1111/j.1472-765X.1986.tb01509.x.

Simcock, D. C., Joblin, K. N., Scott, I., Burgess, D. M., Rogers, C. W., Pomroy, W. E. and Simpson, H. V. 1999. Hypergastrinaemia, abomasal bacterial population densities and pH in sheep infected with *Ostertagia circumcincta*. *International Journal for Parasitology* 29(7), 1053–63. doi:10.1016/s0020-7519(99)00065-x.

Skillman, L. C., Evans, P. N., Naylor, G. E., Morvan, B., Jarvis, G. N. and Joblin, K. N. 2004. 16S ribosomal DNA-directed PCR primers for ruminal methanogens and identification of methanogens colonising young lambs. *Anaerobe* 10(5), 277–85. doi:10.1016/j.anaerobe.2004.05.003.

Skillman, L. C., Evans, P. N., Strömpl, C. and Joblin, K. N. 2006. 16S rDNA directed PCR primers and detection of methanogens in the bovine rumen. *Letters in Applied Microbiology* 42(3), 222–8. doi:10.1111/j.1472-765X.2005.01833.x.

Smith, P. H. and Hungate, R. E. 1958. Isolation and characterization of *Methanobacterium ruminantium* n. sp. *Journal of Bacteriology* 75(6), 713–8. doi:10.1128/JB.75.6.713-718.1958.

Snelling, T. J., Genç, B., McKain, N., Watson, M., Waters, S. M., Creevey, C. J. and Wallace, R. J. 2014. Diversity and community composition of methanogenic Archaea in the rumen of Scottish Upland sheep assessed by different methods. *PLoS ONE* 9(9), e106491. doi:10.1371/journal.pone.0106491.

Soliva, C. R., Hindrichsen, I. K., Meile, L., Kreuzer, M. and Machmüller, A. 2003. Effects of mixtures of lauric and myristic acid on rumen methanogens and methanogenesis *in vitro*. Letters in Applied Microbiology 37, 35–9.

Söllinger, A., Schwab, C., Weinmaier, T., Loy, A., Tveit, A. T., Schleper, C. and Urich, T. 2016. Phylogenetic and genomic analysis of *Methanomassiliicoccales* in wetlands and animal intestinal tracts reveals clade-specific habitat preferences. *FEMS Microbiology Ecology* 92(1), fiv149. doi:10.1093/femsec/fiv149.

Sowers, K. R., Baron, S. F. and Ferry, J. G. 1984. *Methanosarcina acetivorans* sp. nov., an acetotrophic methane producing bacterium isolated from marine sediments. *Applied and Environmental Microbiology* 47(5), 971–8. doi:10.1128/AEM.47.5.971-978.1984.

Stewart, R. D., Auffret, M. D., Warr, A., Wiser, A. H., Press, M. O., Langford, K. W., Liachko, I., Snelling, T. J., Dewhurst, R. J., Walker, A. W., Roehe, R. and Watson, M. 2018. Assembly of 913 microbial genomes from metagenomic sequencing of the cow rumen. *Nature Communications* 9(1), 870. doi:10.1038/s41467-018-03317-6.

Tajima, K., Nagamine, T., Matsui, H., Nakamura, M. and Aminov, R. I. 2001. Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known methanogens. *FEMS Microbiology Letters* 200(1), 67–72. doi:10.1111/j.1574-6968.2001.tb10694.x.

Tanner, A. C. R., Badger, S., Lai, C.-H., Listgarten, M. A. X. A., Visconti, R. A. and Socransky, S. S. 1981. *Wolinella* gen. nov., *Wolinella succinogenes* (*Vibrio succinogenes* Wolin et al.) comb. nov., and description of *Bacteroides gracilis* sp. nov., *Wolinella recta* sp. nov., *Campylobacter concisus* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 31, 432–45.

Tatsuoka, N., Mohammed, N., Mitsumori, M., Hara, K., Kurihara, M. and Itabashi, H. 2004. Phylogenetic analysis of methyl coenzyme-M reductase detected from the bovine rumen. *Letters in Applied Microbiology* 39(3), 257–60. doi:10.1111/j.1472-765X.2004.01566.x.

Thauer, R. K. 1998. Biochemistry of methanogenesis: a tribute to Marjory Stephenson. 1998 Marjory Stephenson Prize Lecture. Microbiology 144(9), 2377–406. doi:10.1099/00221287-144-9-2377.

Thauer, R. K., Kaster, A. K., Seedorf, H., Buckel, W. and Hedderich, R. 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. *Nature Reviews Microbiology* 6(8), 579–91 doi:10.1038/nrmicro1931.

Tindall, B. J. 2007. Vacuum-drying and cryopreservation of prokaryotes. In: Day, J. G. and Stacey, G. N. (Eds), *Cryopreservation and Freeze-Drying Protocols. Methods in Molecular Biology* (vol. 368). Humana Press, Inc., Totowa, NJ. doi:10.1007/978-1-59745-362-2_5.

Tokura, M., Tajima, K. and Ushida, K. 1999. Isolation of *Methanobrevibacter* sp. as a ciliate-associated ruminal methanogen. *Journal of General and Applied Microbiology* 45(1), 43–7. doi:10.2323/jgam.45.43.

Tymensen, L. D., Beauchemin, K. A. and McAllister, T. A. 2012. Structures of free-living and protozoa-associated methanogen communities in the bovine rumen differ according to comparative analysis of 16S rRNA and *mcrA* genes. *Microbiology* 158(7), 1808–17. doi:10.1099/mic.0.057984-0.

Unden, G., Hackenberg, H. and Kröger, A. 1980. Isolation and functional aspects of the fumarate reductase involved in the phosphorylative electron transport of *Vibrio succinogenes*. *Biochimica et Biophysica Acta* 591(2), 275–88. doi:10.1016/0005-2728(80)90159-0.

Ungerfeld, E. M. and Kohn, R. A. 2006. The role of thermodynamics in the control of ruminal fermentation. In: Sejrsen, K., Hvelplund, T. and Nielsen, M. O. (Eds), *Ruminant Physiology*. Wageningen Academic Publishers, Wageningen, pp. 55–85.

Valle, E. R., Henderson, G., Janssen, P. H., Cox, F., Alexander, T. W. and McAllister, T. A. 2015. Considerations in the use of fluorescence in situ hybridization (FISH) and confocal laser scanning microscopy to characterize rumen methanogens and define their spatial distributions. *Canadian Journal of Microbiology* 61(6), 417–28. doi:10.1139/cjm-2014-0873.

Vantcheva, Z. M., Prodhan, K. and Hemken, R. W. 1970. Rumen methanol *in vivo* and *in vitro*. *Journal of Dairy Science* 53(10), 1511–4. doi:10.3168/jds.s0022-0302(70)86424-4.

Vanwonterghem, I., Evans, P. N., Parks, D. H., Jensen, P. D., Woodcroft, B. J., Hugenholtz, P. and Tyson, G. W. 2016. Methylotrophic methanogenesis discovered in the archaeal phylum Verstraetearchaeota. *Nature Microbiology* 1, 16170 | doi:10.1038/NMICROBIOL.2016.170.

Vogels, G. D., Hoppe, W. F. and Stumm, C. K. 1980. Association of methanogenic bacteria with rumen ciliates. *Applied and Environmental Microbiology* 40(3), 608–12. doi:10.1128/AEM.40.3.608-612.1980.

Wei, Y.-Q., Long, R.-J., Yang, H., Yang, H.-J., Hui Shen, X.-H., Shi, R.-F., Wang, Z.-Y., Du, J.-G., Qi, X.-J. and Ye, Q.-H. 2016. Fiber degradation potential of natural co-cultures of *Neocallimastix* frontalis and *Methanobrevibacter ruminantium* isolated from yaks (*Bos grunniens*) grazing on the Qinghai Tibetan Plateau. *Anaerobe* 39, 158–64.

Wei, Y. Q., Yang, H. J., Long, R. J., Wang, Z. Y., Cao, B. B., Re, Q. C. and Wu, T. T. 2017. Characterization of natural co-cultures of *Piromyces* with *Methanobrevibacter ruminantium* from yaks grazing on the Qinghai-Tibetan Plateau: a microbial consortium with high potential in plant biomass degradation. *AMB Express* 7(1), 160. doi:10.1186/s13568-017-0459-1.

Weimar, M. R., Cheung, J., Dey, D., McSweeney, C., Morrison, M., Kobayashi, Y., Whitman, W. B., Carbone, V., Schofield, L. R., Ronimus, R. S. and Cook, G. M. 2017. Development of multiwell-plate methods using pure cultures of methanogens to identify new inhibitors for suppressing ruminant methane emissions. *Applied and Environmental Microbiology* 83(15), e00396-17. doi:10.1128/AEM.00396-17.

Whitford, M. F., Teather, R. M. and Forster, R. J. 2001. Phylogenetic analysis of methanogens from the bovine rumen. *BMC Microbiology* 1, 5. doi:10.1186/1471-2180-1-5.

Wirth, R., Kádár, G., Kakuk, B., Maróti, G., Bagi, Z., Szilágyi, Á., Rákhely, G., Horváth, J. and Kovács, K. L. 2018. The planktonic core microbiome and core functions in the cattle rumen by next generation sequencing. *Frontiers in Microbiology* 9, 2285. doi:10.3389/fmicb.2018.02285.

Wolin, M. J., Wolin, E. A. and Jacobs, N. J. 1961. Cytochrome-producing anaerobic vibrio, *Vibrio succinogenes*, sp. n. *Journal of Bacteriology* 81, 911–7. doi:10.1128/JB.81.6.911-917.1961.

Wolin, M. J., Wolin, E. A. and Wolfe, R. S. 1963. ATP-dependent formation of methane from methylcobalamin by extracts of *Methanobacillus omelianskii*. *Biochemical and Biophysical Research Communications* 12(6), 464–8. doi:10.1016/0006-291X(63)90316-4.

Wolin, E. A., Wolfe, R. S. and Wolin, M. J. 1964a. Viologen dye inhibition of methane formation by *Methanobacillus omelianskii*. *Journal of Bacteriology* 87(5), 993–8. doi:10.1128/JB.87.5.993-998.1964.

Wolin, M. J., Wolin, E. A. and Wolfe, R. S. 1964b. The cobalamin product of the conversion of methylcobalamin to CH4 by extracts of *Methanobacillus omelianskii*. *Biochemical and Biophysical Research Communications* 15(5), 420–3. doi:10.1016/0006-291x(64)90478-4.

Wood, J. M., Allam, A. M., Brill, W. J. and Wolfe, R. S. 1965. Formation of methane from serine by cell-free extracts of *Methanobacillus omelianskii*. *Journal of Biological Chemistry* 240(12), 4564–9.

Wright, A. D. G., Williams, A. J., Winder, B., Christophersen, C. T., Rodgers, S. L. and Smith, K. D. 2004. Molecular diversity of rumen methanogens from sheep in Western Australia. *Applied and Environmental Microbiology* 70(3), 1263–70. doi:10.1128/aem.70.3.1263-1270.2004.

Wright, A. D., Toovey, A. F. and Pimm, C. L. 2006. Molecular identification of methanogenic archaea from sheep in Queensland, Australia reveal more uncultured novel archaea. *Anaerobe* 12(3), 134–9. doi:10.1016/j.anaerobe.2006.02.002.

Wright, A. D. G., Auckland, C. H. and Lynn, D. H. 2007. Molecular diversity of methanogens in feedlot cattle from Ontario and Prince Edward Island, Canada. *Applied and Environmental Microbiology* 73(13), 4206–10. doi:10.1128/AEM.00103-07.

Wright, A. D. G., Ma, X. and Obispo, N. E. 2008. *Methanobrevibacter* phylotypes are the dominant methanogens in sheep from Venezuela. *Microbial Ecology* 56(2), 390–4. doi:10.1007/s00248-007-9351-x.

Xia, Y., Kon, Y. H., Seviour, R., Forster, R. J., Kisidayova, S. and McAllister, T. A. 2014. Fluorescence in situ hybridization probing of protozoal *Entodinium* spp. and their methanogenic colonizers in the rumen of cattle fed alfalfa hay or triticale straw. *Journal of Applied Microbiology* 116(1), 14–22. doi:10.1111/jam.12356.

Yu, Z., García-González, R., Schanbacher, F. L. and Morrison, M. 2008. Evaluations of different hypervariable regions of archaeal 16S rRNA genes in profiling of methanogens by Archaea-specific PCR and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology* 74(3), 889–93. doi:10.1128/AEM.00684-07.

Zeikus, J. G. and Bowen, V. G. 1975. Comparative ultrastructure of methanogenic bacteria. *Canadian Journal of Microbiology* 21(2), 121–9. doi:10.1139/m75-019.

Zhou, M., Hernandez-Sanabria, E. and Guan, L. L. 2009. Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. *Applied and Environmental Microbiology* 75(20), 6524–33. doi:10.1128/AEM.02815-08.

Zhou, M., Hernandez-Sanabria, E. and Luo Guan, L. L. 2010. Characterization of variation in rumen methanogenic communities under different dietary and host feed efficiency conditions, as determined by PCR-denaturing gradient gel electrophoresis analysis. *Applied and Environmental Microbiology* 76(12), 3776–86. doi:10.1128/AEM.00010-10.

Zinder, S. H. 1993. Physiological ecology of methanogens. In: Ferry, J. G. (Ed.), *Methanogenesis:Ecology, Physiology, Biochemistry and Genetics*. Chapman and Hall, New York, NY, pp. 128–206.

Chapter 7

Ruminal-ciliated protozoa

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1 Introduction

Rumen protozoa were first reported by Gruby in 1843. Since then efforts in understanding the function of these organisms have increased, albeit at a slower rate than efforts applied to understand the rumen bacteria, due to the increased complexity of working with rumen protozoa. Rumen protozoa belong mainly within the phylum Ciliophora and belong to one of the two orders, Vestibuliferida and Entodiniomorphida (often referred to as the holotrich and entodiniomorphids, respectively). Although they are less enumerate than rumen bacteria they are larger in size and account for anywhere between 5 and 50% of microbial biomass in the rumen. It is also known that ruminants possess defined populations of rumen protozoa (types A, B, O and K), the exact reason being unknown, although host genomics and/or predatory behaviour (i.e. protozoa feeding on other protozoa) have been implicated. The rumen protozoa possess two types of nuclei, the micronuclei and the macronuclei, with the micronuclei behaving as a germline nucleus which is not involved in

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RNA transcription, whereas the macronuclei undergo RNA transcription for vegetative growth.

The presence of these protozoa in the rumen also has profound effects on the bacteria, fungi and archaea present. For example, many of the rumen protozoa display selective predation, resulting in changes in the rumen microbiome. Rumen methanogenic archaea also form close associations with the rumen protozoa, particularly the order Vestibuliferida, due to the fact that the ciliate protozoa possess hydrogenosomes, which compartmentalise the terminal reactions of energy metabolism resulting in hydrogen release, thus allowing the methanogens to utilise the hydrogen to form methane more effectively.

The rumen protozoa also interact closely with dietary plant material, for example, *Epidinium* spp. actively seek, engulf and store plant chloroplasts within their cytoplasm. The reason for this is unclear, but as the plant chloroplast is rich in lipids and protein it is likely due to their energy requirements. Many of the protozoa within the order Entodiniomorpha also possess the ability to degrade fibre, through possession of glycosyl hydrolases, which have likely been acquired through horizontal gene transfer from rumen bacteria. In vivo studies based mainly on defaunation studies (absence of rumen protozoa) and meta-analyses of a number of these in vivo studies, confirm that the rumen protozoa increase fibre degradation and methane emission, but reduce microbial protein supply and average daily weight gain suggesting a negative effect on host growth and environmental impact. Nonetheless, it should be noted that removal of all rumen protozoa, as a future methane mitigation strategy, may not be the best approach, and innovative technologies to eliminate the order Vestibuliferida may be favourable due to their larger role in methane emissions and minimal role in dietary fibre digestion. Given that removal of all protozoa is challenging, developing innovative technologies to target removal of specific protozoa will be even more problematic.

2 Discovery of rumen protozoa

The pioneering work of Antonie van Leeuwenhoek led to the first discovery of free-living protozoa in 1676. Nonetheless rumen protozoa were not reported until 1843 (Gruby and Delafond, 1843). This is likely due to the challenges of obtaining rumen samples and the surprising fact that protozoa survive in such a harsh environment, making it an environment in which scientists would be less likely to prospect for protozoa in the first instance. The rumen is warm (39°C), anoxic, contains an abundance of food particles and contracts in a regular and frequent manner to increase the flow of particles to the lower intestines. As such the rumen is analogous to a chemostat and the slow generation time of the protozoa, approximated as ranging from 5h to 15.7 h (Warner, 1962; Karnati

et al., 2007), coupled with a flow rate of approximately 2×10^{11} protozoal cells/day (Karnati et al., 2007), means that they have had to develop strategies to persist and survive in this environment. For example, they will often attach to fresh rumen digesta (Orpin, 1985) or sequester to the reticulo-rumen wall (Abe and Iriki, 1989) to avoid outflow from the rumen. The rumen protozoa can also adjust their generation time in response to changes in passage rate or substrate, which again aids their survival (Dehority, 1998, 2004; Karnati et al., 2007).

From earlier works, it is now clear that a small number of the cells described as protozoa were in fact zoospores produced by the other group of rumen eukaryotes, the rumen fungi, which are present at very low densities (Orpin, 1975). However, it is now well recognised that those confirmed as rumen protozoa are less dense than rumen bacteria (typically 10^4–10^6 cells/mL as compared with 10^9–10^{11} cells/mL for rumen bacteria). Yet they may account for anywhere between 5% and 50% of microbial biomass in the rumen, depending on the host animal's diet, due to the larger size of the protozoa (Williams and Coleman, 1992; Denton et al., 2015).

3 Rumen protozoal taxonomy and population 'types'

The rumen protozoa are mainly ciliates belonging to the phylum Ciliophora, with very low numbers of flagellates, such as *Trichomonas* spp., *Monocecromonas* spp. and *Chilomastix* spp. occasionally seen (Williams and Coleman, 1992; Fig. 1). Rumen ciliates form the part of a monophyletic group, together with ciliates seen in the foreguts of camelids (Kubesy and Dehority, 2002) and the hindgut of other herbivores, such as horses and rhinoceroses (Moon-van der Staay et al., 2014). They are classified into two main orders depending on their morphology, namely order Vestibuliferida (often called holotrichs) and order Entodiniomorphida (often called entodiniomorphs; Kamra, 2005; Fig. 1).

The order Vestibuliferida consists of four families: Isotrichidae, Blepharocorythidae, Beutschliidae and Paraisotrichidae. Of these, the Isotrichidae is far more widespread and has been reported to account for up to 40% of the ciliate population (Williams and Coleman, 1992). Within the order Vestibuliferida, family Isotrichidae tend to utilise soluble carbohydrates, are somewhat aero-tolerant, and can be physically characterised by cilia of uniform length, covering the entire cell surface (Williams and Coleman, 1992; Dehority, 2003; Fig. 2). These cilia are fused around the vestibulum and the flexible pellicles (Williams and Coleman, 1992; Dehority, 2003; Fig. 2). Three principal Isotrichidae species inhabit the rumen, namely *Isotricha intestinalis*, *I. prostoma* and *Dasytricha ruminantium*, with some other species making up a very minor portion (Hobson and Stewart, 1997; Figs. 1 and 2).

Phylum Ciliophora
Subphylum Intramacronucleata
Class Litostomatea
Subclass Trichostomatia
Order Vestibuliferida
Family *Isotrichidae*
Genus *Aviisotricha*
Genus *Dasytricha*
Genus *Isotricha*
Order Entodiniomorphida
Suborder *Entodiniomorphina*
Family *Ophryoscolecidae*
Sub-family *Entodiniinae*
Genus *Entodinium*
Sub-family *Diplodiniinae*
Genus *Diplodinium*
Eodinium
Eudiplodinium
Ostracodinium
Enoploplastron
Metadinium
Elytroplastron
Polyplastron
Subfamily *Ophryoscolecinae*
Genus *Epidinium*
Epiplastron
Opisthotrichum
Ophryoscolex
Caloscolex

Figure 1 Taxonomy of the dominant rumen-ciliate protozoa (adapted from Imai, 1998).

The order Entodiniomorphida is composed of family Ophryscolecidae, which contains most of the genera of rumen protozoal entodiniomorphs (Fig. 1). Nonetheless, two other genera, namely *Parentodinium* spp., which belong to the family Cycloposthiidae, and *Rhinozeta* spp., which belong to the family Rhinozetidae, are present in the rumen but are less enumerate than protozoa belonging to the family Ophryscolecidae (Imai, 1998).

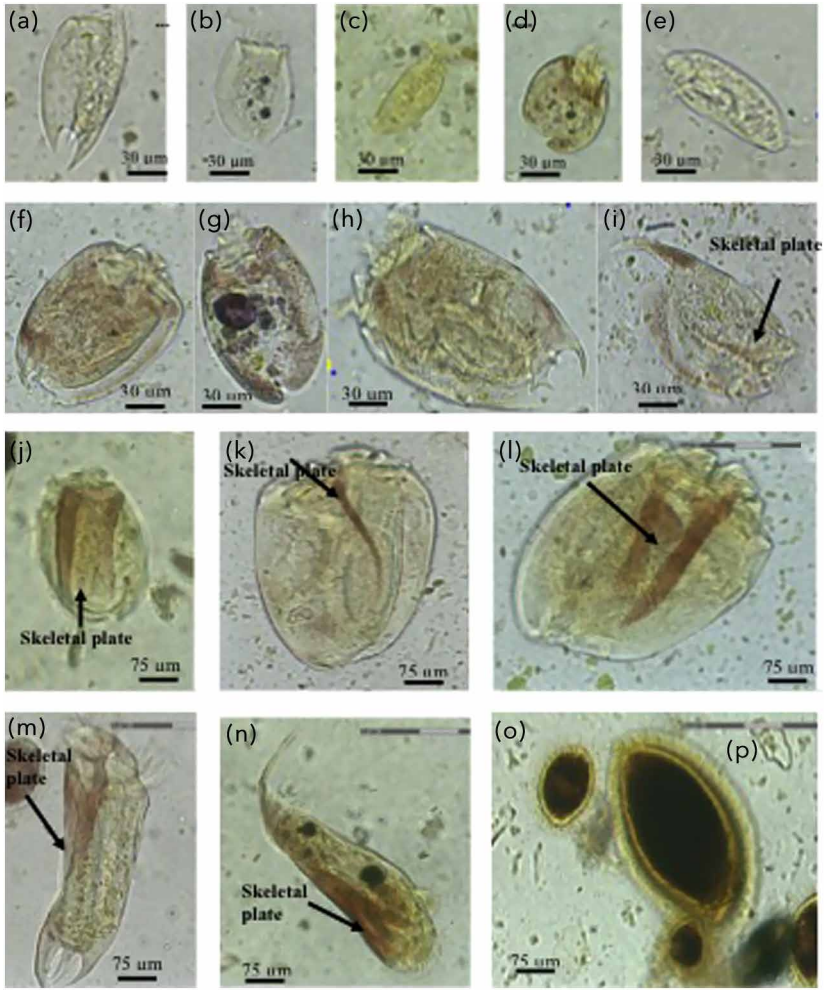

Figure 2 Light photomicrographs of various Lugol's stained rumen protozoa ciliates. From (a**)** to (e**)** *Entodinium* spp; from (f) to (h) *Diplodinium* spp; (i) *Eremoplastron* spp.; (j) *Ostracodinium* spp.; (k) *Eudiplodinium* spp.; (l) *Metadinium* spp; (m) and (n) *Epidinium* spp.; (o) *Dasytricha* spp.; and (p) *Isotricha* spp. Images are the author's own (Williams, 2018) and all belong to type B rumen protozoal populations.

These entodiniomorphid protozoa also differ morphologically from the Vestibuliferida, in that they display congregations of cilia at their anterior and/or posterior end and are classified using characteristics such as ciliary zones, the number of skeletal plates, caudal projections and the arrangement of cilia on the cell surface (Dehority, 2003; Fig. 2). The order Entodiniomorphida is also less flexible than the order Vestibuliferida, due to the thick outer pellicle that they possess, which is thought to be an adaptation for survival in the

harsh rumen environment (Williams and Coleman, 1992). There are several subfamilies within the Entodiniomorphida, such as Entodiniiae, Diplodiniiae, Epidiniinae, Opisthotrichinae, Ophryoscolecinae and Caloscolecinae, which are found in ruminants worldwide (Williams and Coleman, 1992; Henderson et al., 2015; Figs. 1 and 2). Identification of entodinomorphids at the species level is complex due to the intraspecific variation, for example *Entodinium caudatum* only produces a caudal spine in the presence of *Entodinium bursa* (Hobson and Stewart, 1997).

The 'rumen census' publication (Henderson et al., 2015) has provided, to date, the most comprehensive study of ruminant and camelid microbiomes across the geographical location, host and diet. This study examined the ruminant microbial communities in a total of 768 samples, using molecular based-sequencing analysis targeting all known bacteria, archaea and ciliates, with additional reference to the rumen fungi and viruses (Henderson et al., 2015). Over 99% of protozoal sequence data were assigned to 12 genus-equivalent groups: *Entodinium, Epidinium, Enoploplastron, Ophryscolex, Anoplodinium/Diplodinium, Eremoplastron/Diploplastron, Eudiplodinium, Metadinium, Ostracodinium, Polyplastron, Dasytricha* and *Isotricha*. The *Entodinium* spp. and *Epidinium* spp. dominated and were represented in 32.2% and 16.5% of the 768 samples respectively (Henderson et al., 2015). It was also apparent that the variation in protozoa was much more pronounced than that of bacteria in relation to the geographical location (Henderson et al., 2015).

Before the advent of advanced omic technologies, it has been shown that ruminants typically possess a distinct 'type' of rumen protozoa population, defined by the presence of certain genera only (Eadie, 1962; Williams and Coleman, 1992; Table 1). These rumen protozoal populations have been defined as types A, B, O and K, with types A and B being the most diverse in composition and also the predominant population types encountered in ruminants. Within these populations, individual genera, such as *Diplodinium* and *Ophryscolex*, may disappear for no apparent reason and the larger entodiniomorphid protozoa tend to vary most between animals, while the holotrichs remain relatively constant (Eadie, 1962; Williams and Coleman, 1992).

The reason that ruminants possess specific rumen protozoal 'types' coupled with the variability in the rumen protozoa across geographical locations is unclear. Nonetheless, it has been postulated that the genetics and physiology of the host animal, its diet, transfaunation (the introduction and spread of a spectrum of protozoal species) and antagonism among ciliate species are important factors in establishing both the composition and concentration of the rumen-ciliate community. Indeed, larger protozoa are capable of predatory behaviour through ingestion of smaller species of protozoa, which again may partially explain why large and small protozoa rarely exist together

Table 1 A classification scheme for protozoal population types found in cattle, sheep and goats

Type A	Type B	Type O	Type K[e] (cattle only)
Entodinium	*Entodinium*	*Entodinium*	*Entodinium*
Polyplastron multivesiculatum	*Epidinium* (except *E. tricaudaum*)		*Elytroplastron bubali*
Diploplastron affine	*Eudiplodinium maggii*		*Eremoplastron rostratum*
Orphryoscolex (cattle and sheep)	*Eremoplastron*		*Diplodinium*
Ostracodinium (cattle and sheep)	*Ostracodinium* (sheep)		
Eremoplastron (rarely cattle)	*Orphryoscolex* (goat)		
Diplodinium	*Diplodinium*		
Enoploplastron	*Enoploplastron* (rarely cattle)		
Eodinium	*Diploplastron*		
	Eodinium		

Source: adapted from Williams and Coleman (1992).

in the rumen (Williams and Coleman, 1992). Eadie (1967) demonstrated that *Polyplastron multivesiculatum* predated upon *Epidinium* spp., *Eudiplodinium magii*, *Eremoplastron bovis* and *Ostracodinium* spp. Therefore, the presence or absence of *P. multivesiculatum* appears to define whether a ruminant possess type A or Type B protozoal populations.

4 Rumen protozoal genomics

The rumen protozoa possess two types of nuclei: the macronuclei and the micronuclei. In non-rumen ciliates, the micronucleus has been shown to act as the germline nucleus and does not express its genes, whereas the macronucleus transcribes RNA for vegetative growth (Prescott, 1994), providing the genes encoding all enzymes that the protozoa express. It is assumed that this is also the case in the macro and micronucleus of the rumen ciliates. The macronucleus is the larger of the two nuclei, despite only containing a sub-component of the genetic material in the micronucleus. This is a consequence of it possessing multiple copies of many of the DNA sequences present in the micronucleus. Some non-ruminant ciliates have single gene chromosomes, chromosomes containing a single gene flanked by telomeres. Rumen ciliates, on the other hand, have relatively short chromosomes, but these are considerably longer than single-gene chromosomes (Thomas et al., 2004) and have been referred to as midi-chromosomes. Both nuclei are known to be extremely AT-rich, with

the coding regions showing a strong preference for codons using A or T where possible and only using a limited subset of the available codons (McEwan et al., 2000a,b). In the past, the A-T richness of the rumen protozoa has proved a limiting factor when applying molecular techniques. DNA with a low GC content (or AT richness) is less stable than that with a higher percentage mainly due to interactions during base stacking. This has caused problems with sequencing AT-rich genomes as read coverage is biased in favour of GC-balanced regions, often leading to few or no reads from AT-rich or GC-rich regions (Chen et al., 2013).

Due to the similarity in size between micronuclei and larger bacteria, micronuclei have proved difficult to purify, although macronuclei have been successfully isolated (Young et al., 2015). Consequently, it is only recently that the first, and only, draft macronuclear genome sequence of the rumen protozoa was published (Park et al., 2018). This draft macronuclear genome of the ruminal ciliate *Entodinium caudatum* is the first of its kind, providing sequencing data from the transcriptionally active nucleus. Gene prediction was trained using *Tetrahymena thermophila* and showed similarity to *Oxytricha trifallax*. While these data provide an excellent foundation, this annotation is less than ideal as the differences between symbiotic ciliates of the ruminant gut are likely to be significant relative to that of a free-living protozoa. The lack of reference sequences for the rumen protozoa often results in their mis- or under-representation in metagenomic datasets as well as poor coverage during annotation. Similar problems have been encountered with the genome of the rumen anaerobic fungus *Orpinomyces* which contains 80–85% AT bases (Nicholson et al., 2005; Quail et al., 2012). Nonetheless, this is a major step forward in our gene-based functional understanding of the rumen protozoa and provides a foundation for macronuclear sequencing of other rumen protozoa in the future.

5 Ecological fluctuations in protozoa populations

In response to feeding, the entodiniomorphid population decreases by more than 50% in the 6 hours post-feeding, after which the population increases again over 24 hours to reach pre-feeding levels (Williams and Coleman, 1992). It is thought that this is due to an increased amount of saliva and water entering the rumen which dilutes the microorganisms; however, the protozoa recover quickly (usually in approx. 8 hours) due to the abundance of nutrients (Williams and Coleman, 1992). This pattern appears to be consistent regardless of how many times a day an animal is fed, taking longer when fed once a day but occurring more rapidly when fed several times (Williams and Coleman, 1992).

The two orders of rumen protozoa also display their own, independent diurnal cycles. Purser and Moir (1959) noted that entodiniomorph densities

decreased at feeding and then rose again post-feeding, while Purser (1961) later observed that holotrich densities of the Vestibuliferida peak at feeding time and then gradually diminish. It is thought that the contractions of the reticulum wall before and during feeding, coupled with the presence of soluble sugars, stimulate the migration of protozoa belonging to the order Vestibuliferida in the rumen (Hobson and Stewart, 1997). Predominantly this happens with those protozoa which graze on bacteria and can digest components of plant material (cellulose, hemicelluloses, fructosans, pectin, starch, insoluble sugars, proteins and lipids).

Although rumen protozoa are a natural, and indeed normal, component of the rumen ecosystem, it is possible for animals to survive without a protozoal population. This can be achieved either by rearing animals in isolation from other ruminants (Belanche et al., 2015) and preventing the population developing (non-faunated animals), or removing the protozoal population later in life (defaunated animals) by chemical treatments such as the use of 2% (w/v) copper sulphate and dioctyl sodium sulphosuccinate, delivered to the rumen via a nasogastric tube or fistula (Aban and Bestil, 2016). While bacteria and fungi alone are able to perform the digestion of a plant material, experiments with defaunated or non-faunated animals show a significant decrease in fibre digestibility overall, with compensatory digestion in the large intestine and caecum (Demeyer, 1981).

6 Protozoa interactions in the rumen

6.1 Rumen bacteria–protozoa interactions

Observations by Bryant and Small (1960) showed a doubling of rumen bacteria from 6.0×10^9 bacteria/mL in calves inoculated with whole rumen contents (i.e. including protozoa) to 13.6×10^9 bacteria/mL in defaunated calves. This highlights the considerable effect that protozoa have on the bacterial population, with some protozoa fully digesting bacteria and releasing H_2, which contribute towards CH_4 production and release (Williams, 1986; van Zijderveld et al., 2011). Bacteria are possibly the most important source of nitrogenous compounds for protozoa, and genera within the orders Entodiniomorphida and Vestibuliferida show a distinct preference in bacterial species, for example, Entodiniomorphids will ingest *Selenomonas ruminantium* and *Butyrivibrio fibrisolvens* much quicker than other bacteria (Coleman, 1964). The protozoa utilise a range of specific and active enzymes in the digestion of rumen bacteria and fungi. Both exo- and endo-chitinolytic enzymes are produced by *Eudiplodinium maggii*, for example, which serve the sole purpose of breaking down fungal cell walls (Miltko et al., 2012). Metatranscriptomes constructed from the rumen protozoa have also revealed cathepsins (B and F) which show predominantly the lysosomal activity and are likely to be used in the breakdown

of bacterial cell walls (Williams, 2018). The fate of the engulfed bacteria also depends upon the bacterial species, for example, *E. caudatum* has been observed to kill *Escherichia coli* and *Klebsiella aerogenes* rapidly, while *Proteus mirabilis* appears relatively resistant to digestion (White, 1969). Irrespective of the effects on bacterial densities and diversity, it has also been shown that the rumen protozoa harbour unique intracellular endosymbiotic bacteria, different to those found in the planktonic phase with class Entomicrobia, symbiotic bacteria of termites being found intracellularly in low abundance (Levy and Jami, 2018).

6.2 Rumen fungi–protozoa interactions

The meta-analysis performed by Newbold et al. (2015) suggested that rumen defaunation causes a reduction in the cellulolytic microorganisms, including fungi. Newbold et al. (2015) suggest that this observation may be due to the fact that fibre digestion in the rumen is complex and requires the symbiotic collaboration of several fibrolytic microbes, including rumen protozoa. Therefore, the absence of rumen protozoa may have a detrimental effect on the fibrolytic consortium and ultimately in fibre digestion. However, Hsu et al. (1991) reported an increase in ruminal fungal zoospores in defaunated animals, which they hypothesised, was due to the removal of protozoal predation and competition for nutrients. In support of protozoal predation of rumen fungi, Morgavi et al. (1994) showed that rumen protozoa possessed chitinases capable of degrading the chitin found in the cell wall of rumen fungi.

6.3 Rumen methanogen–protozoa interactions

Rumen protozoa possess hydrogenosomes, which compartmentalise the terminal reactions of energy metabolism resulting in hydrogen release, allowing the methanogens to utilise the hydrogen to form methane more effectively. This means that the protozoa have strong intra and extracellular interaction with the rumen protozoa, giving them direct access to the hydrogen and thus increasing the methane output (Fenchel and Finlay, 2006; Belanche et al., 2014). In a recent publication it has also been demonstrated that the dominant methanogen found in association with rumen protozoa is hydrogenotrophic *Methanobrevibacter* (Tymensen et al., 2012; Belanche et al., 2014; Wang et al., 2017). Holotrich protozoa have also been shown to harbour slightly different endosymbiotic methanogens than entodiniomorphids (Belanche et al., 2014), possibly due to the fact that holotrichs have more active hydrogenosomes than entodiniomorphids (Paul et al., 1990) and/or rapid synthesis of glycogen by holotrichs in the presence of excess carbohydrates, resulting in increased hydrogen generation (Hall, 2011; Denton et al., 2015). In a subsequent study by Belanche et al. (2014), holotrichs were introduced to defaunated sheep,

and it was observed that methane emissions increased to a greater extent than animals which were inoculated with whole protozoal populations (holotrich and entodiniomorphs).

6.4 Rumen plant–protozoal interactions

It is well documented that the rumen microbiome shows a distinct and rapid response to feeding, to which the protozoa are no exception (Williams and Coleman, 1992; Newbold et al., 2015; Nikkhah, 2016). The larger members of the order Entodiniomorphida generally prefer particulate matter and often associate closely with plant fibres during digestion, for example, *Epidinium* and *Polyplastron* spp. (Huws et al., 2009, 2012, 2018). *Epidinium* spp. have been shown to swim through fresh perennial ryegrass ad orchard grass leaves, engulfing plant chloroplasts as they move (Akin and Amos, 1979; Huws et al., 2018; Fig. 3). Bauchop (1980) also noted that the association of *Epidinium* spp. with plant material in vitro was rapid and after prolonged incubation, digestion of the mesophyll tissue occurred in the presence of antibiotics, which suppressed the activity of rumen bacteria, suggesting that these ciliates were major digesters of the mesophyll tissue.

It is postulated that the rumen protozoa play a role in starch, cellulose, cellobiose, pectin and hemicellulose breakdown (Wright, 1960; Bailey et al., 1962; Coleman, 1978, 1985; Forsberg et al., 1984; Orpin, 1984; Veira, 1986; Varel and Dehority, 1989; Jouany and Ushida, 1999; Wang and McAllister, 2002; Newbold et al., 2015). In fact, it has been proposed that the rumen bacteria and fungi are responsible for 80% of plant cell wall degradation while the protozoa contribute to around 20% of degradative activity (Wang and McAllister, 2002; Newbold et al., 2015). However, these results may underestimate the protozoal role, as attempts to study protozoal enzymes have been hampered in the past by difficulties in successfully culturing and maintaining the organisms in vitro (Wang and McAllister, 2002). Regardless, many rumen protozoa have been shown to exhibit potential cellulolytic, hemicellulolytic and pectinolytic activities simultaneously: *Eudiplodinium maggii*, *Ostracodinium dilobum*, *Metadinium affine*, *Eudiplodinium bovis*, *Orphryoscolex caudatus*, *Polyplastron multivesiculatum*, *Isotricha intestinalis* and *Isotricha prostoma* (Wang and McAllister, 2002). In addition to these species are *Entodinium caudatum, Diplodinium pentacanthum, Endoploplastron triloricatum, Orphyroscolex tricoronatus, Ostracodinium gracile* and *Entodinium caudatum* (Dehority, 1993).

The majority of these studies concerning carbohydrate-active enzymes of the rumen protozoa span the period between 1960 and 2000, pre-dating the genomic era (Guttmacher and Collins, 2003; Konstantinidis et al., 2006). As such, very little sequence data are available and most research uses faunated versus defaunted animals or simple agar-based assays with pure cultures to infer

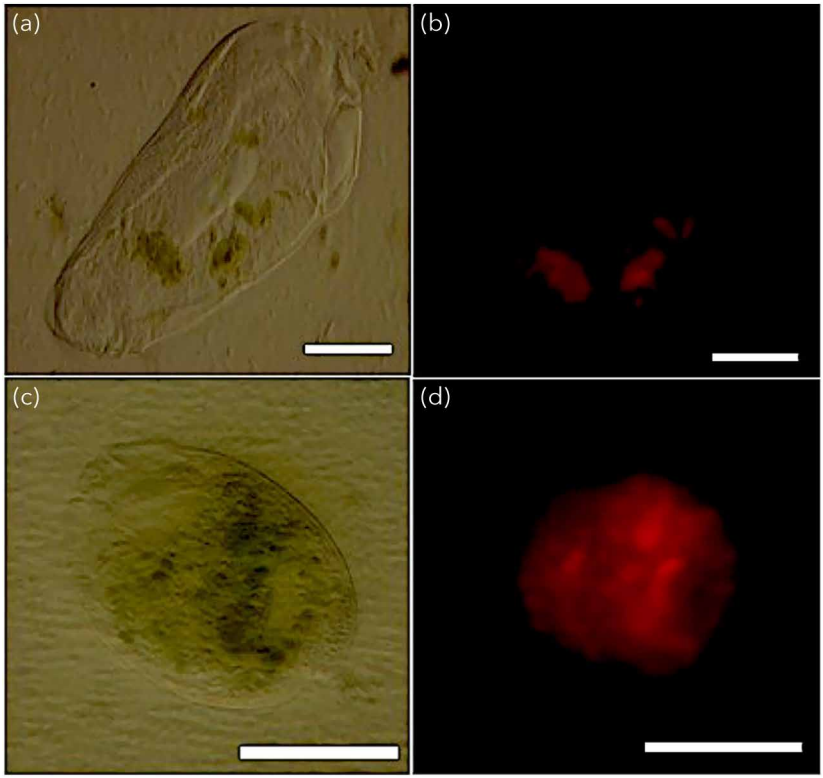

Figure 3 Light microscopy images of an (a) *Epidinium* spp. fractionated from a steer 2 h post grass feeding saturated with intracellular chloroplasts (scale bar = 10μM) and (b) the contrasting epifluorescent image. (c) Light microscopy images of an *Entodinium* sp. fractionated from a steer 2 h post grass feeding saturated with intracellular chloroplasts (scale bar = 10μM) and (d) the contrasting epifluorescent image.

activity. Nonetheless, such studies have reasonably provided a solid evidence of protozoal involvement in fibrolytic breakdown in the rumen (Jouany and Senaud, 1979). Indeed, wide ranges of plant cell wall polysaccharases have been found in the *Epidinium* genus based on zymogram studies. A study by Bailey and Gaillard (1965) showed that three hemicelluloses in the grass were hydrolysed by cell-free extracts of *E. caudatum,* and determined that arabinofuranosidases, endo-1, 4-β-xylanases and endo-1, 4-β-xylodextrinases were being produced. In the same suspension, cellobiose was released from B hemicellulose suggesting cellodextrinase activity, in addition to β-1,3-glucosidase activity. Another study by Bailey et al. (1962) confirmed the hydrolysis of wheat xylan with the release of arabinose, xylobiose and xylose in similar, cell-free extracts of *E. ecaudatum*. Research concerning other protozoal species has also been carried out: cell-free extracts of *Eremoplastron bovis* demonstrated xylanase,

xylodextrinase, cellodextrinase and exo-1,4–β-glucanase activity, and similar suspensions of *E. maggii* were shown to digest cellulose and pectate (Hungate, 1942, 1943; Bailey and Clarke, 1963; Coleman, 1978, 1986).

In the post-genomic era, a number of different fibrolytic genes have been identified in rumen protozoa, especially the larger entodiniomorphs (Devillard et al., 1999; Ricard et al., 2006; Wereszka et al., 2004, 2006). For example, Devillard et al. (1999) constructed a metagenomic library from the rumen protozoan *P. multivesiculatum,* the study successfully identified and characterised GH family 11 xylanases from the *P. multivesiculatum* cDNA library. But upon construction of a phylogenetic tree from family 11 xylanase catalytic domains, it was clear that the *Polyplastron* xylanase was more closely related to sequences from the rumen bacteria *Ruminococcus flavefaciens* and *Bacillus pumilus* than to other xylanases of eukaryotic origin. This raises the possibility that the protozoal glycosyl hydrolases may have been acquired via horizontal gene transfer (HGT), perhaps from ingested bacteria (Devillard et al., 1999; Friedman and Ely, 2012). More recently, a glutamate dehydrogenase cDNA clone was isolated from *Entodinium caudatum* and was assumed to have been acquired by the ciliate via HGT (Newbold et al., 2005).

On a larger scale, a study by Ricard et al. (2006) investigated the concept of HGT from rumen bacteria into ciliates using approximately 4 000 Expressed Sequence Tags (ESTs) from representatives from the main groups of rumen ciliates. The study selected some of the more abundant and common ciliates: *E. simplex, E. caudatum, E. maggii, Metadinium medium, D. affine, P. multivesiculatum E. ecaudatum, I. prostoma, I. intestinalis* and *D. ruminantium* to ensure maximum coverage when constructing cDNA libraries. Phylogenetic trees were constructed for each cluster, which revealed those that clustered within the bacterial portion of the tree. Among the HGT candidates was an over-representation of enzymes involved in carbohydrate transfer and metabolism (around three-quarters), and that of all enzymes encoded, 35% of these were glycosyl hydrolases. Interestingly, a significant difference was observed between the enzyme profiles of the orders Entodiniomorphida and Vestibuliferida with the Vestibuliferida expressing enolases, fructokinases and glucokinases that were absent in the Entodiniomorphida. The Entodiniomorphida on the other hand, expressed cellobiose phosphorylases, cellulases, xylanases, pectate lyase, aspartate-ammonia lyase and nitroreductase that were absent in the order Vestibuliferida. In fact, the only GHs found in the order Vestibuliferida were not fibrolytic, supporting the conclusion that this type of protozoa does not digest fibres to a major extent. This study by Ricard et al. (2006) suggests that HGT is an important process in the adaptation of the rumen protozoa to a carbohydrate-rich environment, with 4.1% of ESTs being the result of HGT.

It is also widely acknowledged that in fast-growing microorganisms, such as the rumen bacteria, codon biases occur either by neutral mutation or by natural

selection for optimal translation efficiency (Rocha, 2004; Behura and Severson, 2013; Brandis and Hughes, 2016). A more comprehensive investigation of this unusual codon usage pattern was conducted by McEwan et al. (2000b) where it was shown that the rumen ciliates follow the 'universal' codon code, unlike certain other ciliates which use TAA, TAG or TGA to encode amino acids. As would be expected in organisms with a high AT content, the preferred stop codon is TAA. Although all 61 codons in the 'universal' codon code were used, there was a distinct preference for a sub-group of codons, most interestingly in terms of their preferential usage of AGA to encode arginine, a codon whose usage is often minimised in many other organisms. In addition, the rumen ciliates make use of unusual nucleotide patterns within their 3′ untranslated regions of mRNA, for example, not using the 'universal' polyadenylation signal (Destables et al., 2005).

7 Challenges of working with rumen protozoa

The rumen protozoa present a number of challenges in terms of genetic studies and cultivation experiments. Many of the issues related to genetic studies have been discussed previously: dual-nuclear structures, unusual codon usage patterns, non-standard polyadenylation and so on. In terms of culturing rumen protozoa in vitro it is currently not possible to grow them as axenic cultures (i.e. in the absence of bacteria) or as monoaxenic cultures (i.e. one bacterial species present). Many studies involving individual species of rumen protozoa have involved introducing single species of protozoa into the rumen of defaunated or non-faunated animals to achieve animals with a mono-faunated status allowing their study. This can be achieved by using a micromanipulator to pick out individual protozoal cells which share sufficient morphological identity to suggest they are a single species. These can then be used to inoculate host ruminants. The animals being used as hosts must be free of protozoa in the rumen before inoculation; however, it is also important that if they have ever had a protozoal population that the omasum (third chamber of the ruminant foregut) is also free of protozoa before introducing the inoculum (Michałowski et al., 1991). This can be problematic where more than one species shares very similar morphological structures, with the potential for inclusion of more than one species in the initial inoculum being a serious issue. Even when there is a single species in the initial inoculum introduced into the rumen, the host animals must be maintained in isolation from other ruminants to avoid contamination with other protozoal species.

Coleman et al. (1976) detailed methods for the culture of six species of rumen protozoa (*Enoploplastron triloricatum, Eudiplodinium maggii, Diploplastron affine, E. caudatum, Diplodinium monacanthum* and *Diplodinium pentacanthum*) along with a screening method for cellulolytic activity using

α-[^{14}C] cellulose in the form of ^{14}C-labelled perennial ryegrass. Five species showed activity against the cellulose, although the assay was not successful in the case of *D. monacanthum*. As always, it was challenging to truly discriminate between protozoal and bacterial activity but after centrifugation to remove bacteria, cell-free extracts of these organisms continued to show cellulolytic activity as measured by sugar production. However, there still remained the potential for a few bacterial cells to persist in the protozoal fraction following centrifugation.

Alternatively, the micromanipulator method used to pick individual cells and establish monofaunated populations in the rumen of otherwise protozoa-free animals may also be used to establish in vitro cultures. A number of different approaches involving salt solutions have been used with some success for in vitro growth of rumen protozoa: Hungate salt solutions (Hungate, 1942), 'caudatum' salt solution (Coleman et al., 1972), 'simplex' type solution (Coleman et al., 1972) and 'artificial rumen fluid' (Michałowski et al., 1999). However, these approaches can be difficult to establish and again normally rely on inoculation with several morphologically similar cells – which again may or may not actually be a single species. Even after successfully establishing a culture, many protozoa will die after a number of rounds of cell division. In non-rumen ciliates this has been attributed to a lack of conjugation taking place between cells to produce new sexual nuclei (micronuclei), with cultures relying on asexual reproduction alone having a limited level of stability and longevity. Although, it is assumed that rumen ciliates utilise conjugation as a means of sexual reproduction, this has not been reported.

8 The effects of protozoal function on ruminant nutrition, health and emissions

Irrespective of the challenges of understanding the function of rumen protozoa, Gruby and Delafond (1843) hypothesised that due to the dramatic appearance of these protozoa, they must play a role in host nutrition. Subsequently, it has been demonstrated that the rumen protozoa play an important role in ruminant function, despite not being essential for survival (Newbold et al., 2015; Li et al., 2018). Previously discussed protozoal functionality within this chapter is largely based on fundamental lab-based studies; in this section data from largely in vivo studies are discussed. Defaunation studies provide the bulk of available information with regard to assessing the overall impact of the rumen protozoa on animal production. Recent meta-analyses based on defaunation experiments have also been published, building strength in our understanding of the role of the rumen protozoa in host phenotype, due to the amalgamation of available datasets (Eugène et al., 2004; Newbold et al., 2015; Li et al., 2018).

8.1 Rumen protozoa and Nitrogen-use efficiency

Research into protozoal protein metabolism is limited and often contradictory, for example, Forsberg et al. (1984) and Coleman (1983) found little activity of protozoal enzymes on casein at a neutral pH, whereas others had no difficulty producing enzymatic activity under the same conditions (Brock et al., 1982). Although results are ambiguous, evidence suggests that entodiniomorphid protozoa do indeed produce proteolytic enzymes of the 'thiol' and 'carboxyl' types which show weak activity overall (Coleman, 1983; Forsberg et al., 1984). It must be noted that such early studies were unable to differentiate between enzymatic activity of ingested bacteria and enzymes produced by the protozoa themselves, as such; older results may not be as reliable as results from more recent studies (Hess et al., 2011).

Defaunation has been shown in many studies to increase rumen nitrogen retention (Eugène et al., 2004; Newbold et al., 2015; Li et al., 2018), but in others no difference has been seen (Nguyen et al., 2016). These discrepancies in the data could be a result of many reasons, including using different host breeds, diets, length of defaunation and so on, which makes meta-analytical approaches all the more powerful in obtaining a more holistic outlook. Based on a varying number of studies and inclusion/exclusion factors, three meta-analyses have been published, all of which demonstrate that the absence of rumen protozoa increases ammonia N concentrations in the rumen (Eugène et al., 2004; Newbold et al., 2015; Li et al., 2018). This is likely due to their capacity to digest rumen bacteria and fungi, subsequently releasing high-quality microbial protein for use by the host. As a result of bacterial digestion, soluble protein is also supplied back to the rumen microbiome for growth as rumen protozoa cannot utilise ammonia nitrogen (Ivan et al., 1991; Demeyer and Fievez, 2004). The rumen protozoa have also been shown to contribute towards the flow of microbial nitrogen from the foregut into the duodenum, due to their ability to engulf large molecules, such as proteins and carbohydrates (Bach et al., 2005). Nonetheless, because protozoa are selectively retained in the rumen their contribution to the total supply of crude protein is approximately 11% (Shabi et al., 2000).

8.2 Rumen protozoa and fibre digestion

Evidence has been found that the removal of protozoa from the rumen causes a significant decrease in organic matter degradation and that fibre digestion is a complex task requiring the cooperation of several fibrolytic microbes, the protozoa being responsible for the initial stages of fibre colonization and digestion (Newbold et al., 2015). While the activity of the holotrichs is lower than that seen in entodiniomorphids (possibly due to relative abundances), they do actively participate in metabolic processes. Holotrich densities have been observed to greatly increase when the animals' diet contains high levels of

soluble carbohydrates. In general, the holotrichs are heavily influenced by the host diet. Although, it is worth noting that most studies of metabolic activity in this group have been performed using only the three principal holotrich species mentioned above. Therefore, it is well documented that these species are mostly involved in the utilisation of soluble sugars and non-structural polysaccharides and will migrate towards this material using chemotaxis. They also associate with ingested plant material but probably have a limited ability to degrade plant cell wall polysaccharides (Hobson and Stewart, 1997). Defaunation also has an impact on the whole microbial community with fungal concentrations greatly decreasing while populations of cellulolytic bacteria increase (Newbold et al., 2015). The effect of defaunation on feed conversion rate is disputed. Williams and Coleman (1992) reported an improved conversion and greater average daily gain; however, this may be due to fewer nutrients being absorbed (because of poor digestibility) so those which are obtained must be used more efficiently. Another significant result of defaunation is the increased flow of microbial protein to the duodenum. This is because no bacteria are being ingested by protozoa, which also results in a larger and more diverse rumen bacterial population (Williams and Coleman, 1992; Newbold et al., 2015).

8.3 Rumen protozoa and healthiness of ruminant products

The role of the rumen protozoa in lipid metabolism is debated, as the protozoa have been found to be rich in human health beneficial polyunsaturated fatty acids (PUFA) (Huws et al., 2009). It is thought that this is not due to direct involvement in lipid metabolism but rather their tendency to saturate themselves with PUFA-rich chloroplasts, which leads to their high C18:3 *n*-3 content (Huws et al., 2009; Fig. 3), as discussed previously. After engulfment some chloroplasts are partially metabolised by the engulfed bacteria, which can also result in the accumulation of conjugated linoleic acid (CLA) and *cis*-9, *trans*-11 vaccenic acid, which have also been shown to have some human health benefits, within the protozoal cell (Lourenco et al., 2010). The rumen protozoa effectively protect PUFA in the rumen from lipolysis and biohydrogenation, thus increasing the chloroplast content of protozoa via diet consequently providing the opportunity to enhance the flow of PUFA to the duodenum and into meat and milk products as suggested in studies by Huws et al. (2009, 2012). Indeed, a recent study by Francisco et al. (2019) showed that rumen protozoal densities were positively correlated with deposition of human health beneficial fatty acids in meat. Nonetheless, many studies show that rumen protozoa are retained in the rumen, therefore the intra-protozoal chloroplastic PUFA flow to the duodenum is minimal (Huws et al., 2012), and consequently absorption of human health beneficial fatty acids is not increased in the duodenum in the presence of protozoa (Huws et al., 2012).

8.4 Rumen protozoa and methane emissions

Ruminants are responsible for a significant proportion of agricultural methane (approx. 14% of methane released annually into the atmosphere) (Huws et al., 2018). Within these animals the principal site of methane production is in the foregut, most notably the rumen. This methane is produced by a population of archaea (methanogens) which inhabit the rumen ecosystem. As noted previously many of these archaea are associated with the rumen protozoa, either on the surface of the cells or endosymbiotically. This interaction has been shown to be highly dependent on the gaseous component of rumen, and when the rumen is high in $N_{2,}$ methanogens have a high association with ciliate protozoa, conversely when the rumen is high in H_2 the association is reduced and the methanogens are found in higher concentrations in the planktonic (Stumm et al., 1982). This study suggests that association of rumen methanogens with protozoa is an evolutionary adaptation to ensure access to hydrogen when concentrations are low in the rumen itself. Irrespective, targeting the rumen protozoa has been proposed as a mechanism to reduce methane levels, albeit in an indirect manner. While defaunation does not have a significant effect on levels of methanogens, methane production is reduced; a possible explanation for this lies in the elimination of endosymbiotic, protozoa-associated methanogens (Finlay et al., 1994). Indeed, there is a linear relationship between protozoal concentration and methane emissions, which has prompted research into methane mitigation via manipulation of the rumen protozoa (Newbold et al., 2015).

9 Case study: manipulating the rumen ciliates

The current data on function of the rumen protozoa suggest that lowering their densities or removing them entirely from the rumen microbiome may be an effective strategy to ensure high production and lower environmental impact of ruminants. The rumen protozoa are passed between animals by direct transfer through saliva (Becker and Hsiung, 1929; Yáñez-Ruiz et al., 2015). Ciliate protozoa can normally be seen in the rumen of young ruminants within 2 weeks of birth with small Entodinia established before the large protozoa (Eadie, 1962). As such, protozoal-free rumen populations may be established in calves or lambs by rearing them in an environment devoid of other ruminants with a normal protozoal population from an early age (typically around 24–48 hours after birth, following initial suckling to expose them to maternal colostrum). Likewise, chemical treatment of the foregut can be used to remove the protozoal population later in life. While both of these approaches can be achieved in a research environment, neither is particularly practical in an agricultural setting. Instead manipulation, including either reduction or eradication, of the rumen ciliate population must be achieved by alternative means to make it practical.

One of the most successfully implemented examples of the use of supplements to reduce protozoal numbers and improve ruminant production efficiency, while minimising environmental impact, are the ionophores (including monensin) (Nagaraja et al., 1997). Ionophores have growth-promoting effect on ruminants. Their mode of action is, to date, not fully understood although monensin has been shown to inhibit proteolysis within storage vesicles in the non-rumen ciliate *Paramecium* (Fok and Ueno, 1987) and inhibits lysosomal fusion of the food vacuole (Gautier et al., 1994). As a consequence, it is assumed that monensin works in a similar way against the related rumen protozoa, albeit this has not been confirmed. Nonetheless, the rumen protozoa have also been shown to adapt to the addition of ionophores, thus reducing their ability to reduce protozoal density (Nagaraja et al., 1997). This protozoal adaptation coupled with a ban in 2006 for ionophore use in livestock production in the European Union due to concerns on the development of antimicrobial resistance means that alternatives need to be found.

Following the ban on ionophores, a substantial focus was placed onto evaluating phytochemicals as replacements. In particular, much emphasis has been given to the plant saponins, which consist of an aglycone or sapogenin linked to one or more sugar moieties through a glycosidic bond (Francis et al., 2002). Different sources of saponins have been shown to have different levels of effect (Wallace et al., 2002), for example, saponins from alfalfa have been shown to alter fermentation in a continuous culture (Lu et al., 1987) and reduce both protozoal numbers and methane production (Patra and Saxena, 2009). However, the results of application of saponins in the ruminant diet are mixed, with many studies reporting no effects (Śliwiński et al., 2002; Holtshausen et al., 2009) but some reporting reductions in methane of 6.7% (Santoso et al., 2004). It is suggested that reductions in methane production are indirect and are a result of an increase in propionate production and reduction in protozoal numbers (Hristov et al., 1999). It is hypothesised that this variation in results is due to the differing sources and concentrations used in studies, for example, extracts from the fruit *Sapindus saponaria* were used in a study by Hess et al., (2004) which resulted in an inclusion rate of 0.75% which are much higher than the levels occurring in Yucca extracts (Śliwiński et al., 2002; Goel and Makkar, 2012). There is also the added complication of the accompanying diets in these studies, which vary from grazed pasture to forage/concentrate diets (Goel and Makkar, 2012).

Tannins work in a similar way to saponins as naturally occurring plant secondary metabolites with antimicrobial action and effects on rumen fermentation pathways. Two types of tannins exist – condensed and hydrolysable, both of which may have adverse and beneficial effects on the animal simultaneously. To that end, condensed tannin-rich plants or their extracts are mostly used to reduce the risk of toxicity (Beauchemin et al., 2008). It is suggested that the methane-reducing effects of tannins may be a result of one

of two actions: by direct effects on the rumen methanogens or by reducing feed degradation which lowers hydrogen production (Tavendale et al., 2005). The negative effects of tannin application on ruminal fibre degradation are likely due to a reduction in cellulolytic bacteria and the formation of recalcitrant tannin-cellulose complexes (McSweeney et al., 2001; Makkar et al., 1995). However, as with studies investigating the effects of saponins, the research surrounding tannins can be inconsistent and contradictory, with conflicting evidence for effects on the rumen protozoa and the magnitude of effects observed (Benchaar et al., 2008; Goel and Makkar, 2012).

Other dietary supplements have also been used with some degree of success in reducing protozoal numbers (Benchaar et al., 2008). This has included essential oils which have previously been shown to be effective against certain populations of rumen bacteria, and also tannins. The main issue in applying essential oils to the diet of animals is a lack of clarity about their exact mechanism and the contradictory research that has been published. While some studies are able to demonstrate significant effects, others are unable to replicate these results (Patra, 2011). There are also regulatory and safety concerns associated with the use of essential oils, as often high concentrations are required to produce a significant result which may lead to toxic effects as well as feeding into poor profitability.

Despite the research conducted on phytochemical supplementation of ruminant diets, these supplements are currently not used extensively in practice, mainly due to regulatory challenges, practical and financial concerns and limited feed industry uptake, although a company has been established (Delacon), which sell phytogenic supplements for enhancing ruminant production and health. It should also be noted that future strategies should aim to target removal of the order Vestibuliferida, due to their association with methanogens. Conversely, the current data suggest that due to their fibrolytic capacity, the order Entodiniomorphida should be retained.

10 Future trends and conclusions

With the global human population predicted to reach around 9.8 billion in 2050 one of the primary concerns facing society is the provision of adequate and nutritional food (UN, 2017). As a result, the agricultural industry will likely see a major surge in demand for food of animal origin, while concurrently experiencing pressure to increase sustainability and minimise environmental impact. One method of addressing this issue is to maximise rumen function and efficiency, however, to achieve improvements it is essential that we first have a complete understanding of the rumen microbiome. As previously highlighted, our knowledge of the rumen protozoa is far from complete, as such, further research using the latest sequencing and molecular techniques is vital. These methods offer the greatest potential to help truly elucidate the role of the protozoa in the

rumen and therefore how best to manipulate the microbial population in our quest to improve sustainability. Meta-omic technologies such as metagenomics, metabolomics, metaproteomics and metatranscriptomics offer the opportunity to examine whole populations as opposed to the more limited methods that use pure cultures. Used in conjunction, these methods could provide an in-depth meta-analysis detailing interactions within the microbiome as well as between the microbiome and the host or ingested plant material. The biggest advantage of these technologies, in terms of the rumen protozoa, is their ability to bypass the roadblock of developing and maintaining a protozoal culture (Newbold et al., 2015). Despite the development of advanced, culture-free techniques, the current representation of the rumen protozoa in sequence libraries is poor and limited to one macronuclear genome sequence of *Entodinium caudatum* alongside a handful of fibrolytic proteins (Park et al., 2018). As such, during bioinformatic analyses it is feasible that the protozoal fraction of a sample is significantly mis- or under-represented leading to unrepresentative results and a smaller perceived role in many metabolic processes (Comtet-Marre et al., 2017). It is therefore important that research utilises NGS and omic technologies to their fullest to deliver further functional annotation and genome sequencing of the rumen inhabitants – specifically the protozoa. The collection of more, in-depth and robust data seems to be the key to many potential interventions, including the development of microbiome biomarkers, protozoal-methanogen relationships and host-microbiome interaction, all of which affect the efficiency and environmental impact of the animal.

There is also a gap in the literature for more in-depth characterisation of the interactions between protozoa and the other microbial groups in the rumen, in particular the relationship of the methanogen populations with protozoa. It is currently established that abundance and structure of methanogen colonisation of the rumen protozoa differ depending on the genera, for example, smaller *Entodinium* spp. have been associated with higher levels of methane production than larger species, such as *P. multivesiculatum* (Ranilla et al., 2007).

The relationship between the host genotype and microbiome also remains to be fully elucidated, particularly in relation to methane production. When examining the effect of host genetics and the microbiome on methane production, a study by Difford et al. (2018) attributed 13% of variation to the rumen microbiome and 21% to host genetics, further suggesting that the two may not be linked. If this were the case then these two factors could be targeted independently. In summary, working with rumen protozoa is challenging, leading to difficulties for enhancing our understanding of their exact role in the rumen. Therefore, research on these unique protozloa still needs to be enhanced in order to fully understand their function and allow development of innovative strategies to target specific rumen protozoa to improve host phenotype and environmental impact.

11 Where to look for further information

Key studies:

- Hobson, P. N. and Stewart, C. S. 1997. *The Rumen Microbial Ecosystem* (2nd edn.). Blackie Academic and Professional, UK.
- Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., Muñoz-Tamayo, R., Forano, E., Waters., S. M., Hess, M., Tapio, I., Smidt, H., Krizsan, S. J., Yáñez-Ruiz, D. R., Belanche, A., Guan, L., Gruninger, R. J., McAllister, T. A., Newbold, C. J., Roehe, R., Dewhurst, R. J., Snelling, T. J., Watson, M., Suen, G., Hart, E. H., Kingston-Smith, A. H., Scollan, N. D., do Prado, R. M., Pilau, E. J., Mantovani, H. C., Attwood, G. T., Edwards, J. E., McEwan, N. R., Morrisson, S., Mayorga, O. L., Elliott, C. and Morgavi, D. P. 2018. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present and future. *Front. Microbiol.* 9, 1–33.
- Kamra, D. N. and Agarwal, N. 2003. *Techniques in Rumen Microbiology*. Indian Veterinary Research Institute, India.
- Newbold, C. J., de la Fuente, G., Belanche, A., Ramos-Morales, E. and McEwan, N. R. 2015. The role of ciliate protozoa in the rumen. *Front. Microbiol* 6.
- Russell, J. B. 2002. *Rumen Microbiology*. James B. Russell, Ithaca, NY.
- Williams, A. G. and Coleman, G. S. 1992. *The Rumen Protozoa* (1st edn.). Springer-Verlag, New York, NY.

Key conferences/journals:

- Conference: INRA-Rowett conference on gastrointestinal tract function held biannually between the Rowett Institute, Aberdeen University and INRA-Theix, France.
- Conference: Congress on Gastrointestinal Function held biannually in the University of Chicago, Illinois.
- Journals: *Microbiome*; *Animal Microbiome*.

12 References

Aban, M. L. and Bestil, L. C. 2016. Rumen defaunation: determining the level and frequency of *Leucaena leucocephala* Linn. forage. *Int. J. Food. Eng.* 2(1), 55–60. doi:10.18178/ijfe.2.1.55-60.

Abe, M. and Iriki, T. 1989. Mechanism whereby holotrich ciliates are retained in the reticulo-rumen of cattle. *Br. J. Nutr.* 62(3), 579–87. doi:10.1079/bjn19890059.

Akin, D. E. and Amos, H. E. 1979. Mode of attack on Orchard grass leaf blades by rumen protozoa. *Appl. Environ. Microbiol.* 37(2), 332–8. doi:10.1128/AEM.37.2.332-338.1979.

Bach, A., Calsamiglia, S. and Stern, M. D. 2005. Nitrogen metabolism in the rumen. *J. Dairy Sci.* 88 (Suppl. 1), E9–E21. doi:10.3168/jds.S0022-0302(05)73133-7.

Bailey, R. W. and Clarke, R. T. J. 1963. Carbohydrase activity of tureen *Entodinium* spp. from sheep on a starch-free diet. *Nature* 198(4882), 787. doi:10.1038/198787a0.

Bailey, R. W. and Gaillard, B. D. 1965. Carbohydrases of the rumen ciliate *Epidinium ecaudatum* (Crawley). Hydrolysis of plant hemicellulose fractions and beta-linked glucose polymers. *Biochem. J.* 95(3), 758–66. doi:10.1042/bj0950758.

Bailey, R. W., Clarke, R. T. J. and Wright, D. E. 1962. Carbohydrases of the rumen ciliate *Epidinium ecaudatum* (Crawley). *Biochem. J.* 83(3), 517–23.

Bauchop, T. 1980. *Scanning Electron Microscopy in the Study of Microbial Digestion of Plant Fragments in the Gut*. New York Academic Press, London, UK.

Beauchemin, K. A., Kreuzer, M., O'Mara, F. and McAllister, T. A. 2008. Nutritional management for enteric methane abatement: a review. *Aust. J. Exp. Agric.* 48(2), 21–7. doi:10.1071/EA07199.

Becker, E. R. and Hsiung, T. S. 1929. The method by which ruminants acquire their fauna of infusoria, and remarks concerning experiments on the host-specificity of these protozoa. *Proc. Natl. Acad. Sci. U. S. A.* 15(8), 684–90. doi:10.1073/pnas.15.8.684.

Behura, S. K. and Severson, D. W. 2013. Codon usage bias: causative factors, quantification methods and genome-wide patterns: with emphasis on insect genomes. *Biol. Rev. Camb. Philos. Soc.* 88(1), 49–61. doi:10.1111/j.1469-185X.2012.00242.x.

Belanche, A., De La Fuente, G. and Newbold, C. J. 2014. Study of methanogen communities associated with different rumen protozoal populations. *FEMS. Microbiol. Ecol.* 90(3), 663–77. doi:10.1111/1574-6941.12423.

Belanche, A., De La Fuente, G. and Newbold, C. J. 2015. Effect of progressive inoculation of fauna-free sheep with *Vestibuliferida protozoa* and total-fauna on rumen fermentation, microbial diversity and methane emissions. *FEMS. Microbiol. Ecol.* 91(3). doi:10.1093/femsec/fiu026.

Benchaar, C., McAllister, T. A. and Chouinard, P. Y. 2008. Digestion, ruminal fermentation, ciliate protozoal populations, and milk production from dairy cows fed cinnamaldehyde, quebracho condensed tannin, or *Yucca schidigera* Saponin extracts. *J. Dairy Sci.* 91(12), 4765–77. doi:10.3168/jds.2008-1338.

Brandis, G. and Hughes, D. 2016. The selective advantage of synonymous codon usage bias in *Salmonella*. *PLoS Genet.* 12(3), e1005926. doi:10.1371/journal.pgen.1005926.

Brock, F. M., Forsberg, C. W. and Buchanan-Smith, J. G. 1982. Proteolytic activity of the rumen microorganisms and effects of proteinase inhibitors. *Appl. Environ. Microbiol.* 44, 561–9.

Bryant, M. P. and Small, N. 1960. Observations on the ruminal microorganisms of isolated and inoculated calves. *J. Dairy Sci.* 43(5), 654–67. doi:10.3168/jds.S0022-0302(60)90216-2.

Chen, Y.-C., Liu, T., Yu, C.-H., Chiang, T.-Y. and Hwang, C.-C. 2013. Effects of GC bias in next-generations-sequencing data on *de novo* genome assembly. *PLoS ONE* 8(4). doi:10.1371/journal.pone.0062856.

Coleman, G. S. 1964. The metabolism of *Escherichia coli* and other bacteria by *Entodinium caudatum*. *J. Gen. Microbiol.* 37(2), 209–23. doi:10.1099/00221287-37-2-209.

Coleman, G. S. 1978. The metabolism of cellulose, glucose and starch by the tureen ciliate protozoon *Eudiplodinium maggii*. *J. Gen. Microbiol.* 107(2), 359–66. doi:10.1099/00221287-107-2-359.

Coleman, G. S. 1983. Hydrolysis of fraction 1 leaf protein and casein by rumen entodiniomorphid protozoa. *J. Appl. Bacteriol.* 55(1), 111–8. doi:10.1111/j.1365-2672.1983.tb02654.x.

Coleman, G. S. 1985. The cellulose content of 15 species of entodiniomorphid protozoa, mixed bacteria and plant debris isolated from the ovine rumen. *J. Agric. Sci.* 164, 349–60.

Coleman, G. S. 1986. The distribution of carboxymethyl cellulase between fractions taken from the rumens of sheep containing no protozoa or one of five different protozoal populations. *J. Agric. Sci.* 106(1), 121–7. doi:10.1017/S0021859600061827.

Coleman, G. S., Davies, J. I. and Cash, M. A. 1972. The cultivation of rumen ciliates *Epidinium ecaudatum caudatum* and *Polyplastron multivesiculatum in vitro*. *J. Gen. Microbiol.* 73(3), 509–21. doi:10.1099/00221287-73-3-509.

Coleman, G. S., Laurie, J. I., Bailey, J. E. and Holdgate, S. A. 1976. The cultivation of cellulolytic protozoa isolated from the rumen. *J. Gen. Microbiol.* 95(1), 144–50. doi:10.1099/00221287-95-1-144.

Comtet-Marre, S., Parisot, N., Lepercq, P., Chaucheyras-Durand, F., Mosoni, P., Peyretaillade, E., Bayat, A. R., Shingfield, K. J., Peyret, P. and Forano, E. 2017. Metatranscriptomics reveals the active bacterial and eukaryotic fibrolytic communities in the rumen of dairy cow fed a mixed diet. *Front. Microbiol.* 8(67), 67. doi:10.3389/fmicb.2017.00067.

Dehority, B. A. 1993. Microbial ecology of cell wall fermentation. In: Jung, H. G., Buxton, D. R., Hatfield, R. D. and Ralph, J. (Eds), *Forage Cell Wall Structure and Digestibility*. Wiley, pp. 425–53.

Dehority, B. A. 1998. Generation times of *Epidinium caudatum* and *Entodinium caudatum*, determined *in vitro* by transferring at various time intervals. *J. Anim. Sci.* 76(4), 1189–96. doi:10.2527/1998.7641189x.

Dehority, B. A. 2003. *Rumen Microbiology*. Nottingham University Press, Nottingham, UK.

Dehority, B. A. 2004. *In vitro* determination of generation times for *Entodinium exiguum*, *Ophryoscolex purkynjei* and *Eudiplodinium maggii*. *J. Eukaryot. Microbiol.* 51(3), 333–8. doi:10.1111/j.1550-7408.2004.tb00575.x.

Demeyer, D. I. 1981. Rumen microbes and digestion of plant cell walls. *Agric. Environ.* 6(2–3), 295–337. doi:10.1016/0304-1131(81)90020-5.

Demeyer, D. and Fievez, V. 2004. Is the synthesis of rumen bacterial protein limited by the availability of pre-formed amino acids and/or peptides? *Br. J. Nutr.* 91(2), 175–6. doi:10.1079/BJN20031073.

Denton, B. L., Diese, L. E., Firkins, J. L. and Hackmann, J. 2015. Accumulation of reserve carbohydrate by rumen protozoa and bacteria in competition for glucose. *Am. Soc. Microbiol.* 81(5), 1832–8.

Destables, E., Thomas, N. A., Boxma, B., van Alen, T. A., van der Staay, G. W. M., Hackstein, J. H. P. and McEwan, N. R. 2005. The 3′ untranslated region of mRNAs from the ciliate *Nyctotherus ovalis*. *Acta Protozolog.* 44, 231–6.

Devillard, E., Newbold, C. J., Scott, K. P., Forano, E., Wallace, R. J., Jouany, J. P. and Flint, H. J. 1999. A xylanase produced by the rumen anaerobic protozoan *Polyplastron multivesiculatum* shows close sequence similarity to family 11 xylanases from gram-positive bacteria. *FEMS Microbiol. Lett.* 181(1), 145–52. doi:10.1111/j.1574-6968.1999.tb08837.x.

Difford, G. F., Plichta, D. R., Lovendahl, P., Lassen, J., Noel, S. J., Hojberg, O., Wright, A.-D. G., Zhu, Z., Kristensen, L., Nielsen, H. B., Guldbrandtsen, B. and Sahana, G. 2018. Host genetics and the rumen microbiome jointly associate with methane emissions in dairy cows. *PLoS Genet.* 14(10), e1007580.

Eadie, J. M. 1962. Inter-relationships between certain rumen ciliate protozoa. *Microbiology* 29(4), 579–88. doi:10.1099/00221287-29-4-579.

Eadie, J. M. 1967. Studies on the ecology of certain rumen ciliate protozoa. *J. Gen. Microbiol.* 49(2), 175–94. doi:10.1099/00221287-49-2-175.

Eugène, M., Archimede, H. and Sauvant, D. 2004. Quantitative meta-analysis on the effects of defaunation of the rumen on growth, intake and digestion in ruminants. *Livest. Prod. Sci.* 85(1), 81–97. doi:10.1016/S0301-6226(03)00117-9.

Fenchel, T. and Finlay, B. J. 2006. The diversity of microbes: resurgence of the phenotype. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 361(1475), 1965–73. doi:10.1098/rstb.2006.1924.

Finlay, B. J., Esteban, G., Clarke, K. J., Williams, A. G., Embley, T. M. and Hirt, R. P. 1994. Some rumen ciliates have endosymbiotic methanogens. *FEMS Microbiol. Lett.* 117(2), 157–61. doi:10.1111/j.1574-6968.1994.tb06758.x.

Fok, A. K. and Ueno, M. S. 1987. Ionophores and weak bases inhibit phagolysosomal proteolysis in *Paramecium*. *Eur. J. Cell Biol.* 45(1), 145–50.

Forsberg, C. W., Lovelock, L. K., Krumholz, L. and Buchanan-Smith, J. G. 1984. Protease activities of rumen protozoa. *Appl. Environ. Microbiol.* 47(1), 101–10. doi:10.1128/AEM.47.1.101-110.1984.

Francis, G., Kerem, Z., Makkar, H. P. and Becker, K. 2002. The biological action of saponins in animal systems: a review. *Br. J. Nutr.* 88(6), 587–605. doi:10.1079/BJN2002725.

Francisco, A. E., Santos-Silva, J. M., V Portugal, A. P., Alves, S. P. and B Bessa, R. J. 2019. Relationship between rumen ciliate protozoa and biohydrogenation fatty acid profile in rumen and meat of lambs. *PLoS ONE* 14(9), e0221996. doi:10.1371/journal.pone.0221996.

Friedman, R. and Ely, B. 2012. Codon usage methods for horizontal gene transfer detection generate an abundance of false positive and false negative results. *Curr. Microbiol.* 65(5), 639–42. doi:10.1007/s00284-012-0205-5.

Gautier, M. C., Garreau de Loubresse, N., Madeddu, L. and Sperling, L. 1994. Evidence for defects in membrane traffic in Paramecium secretory mutants unable to produce functional storage granules. *J. Cell Biol.* 124(6), 893–902. doi:10.1083/jcb.124.6.893.

Goel, G. and Makkar, H. P. 2012. Methane mitigation from ruminants using tannins and saponins. *Trop. Anim. Health. Pro.* 44(4), 729–39.

Gruby, D. and Delafond, H. M. O. 1843. Recherches ser des animalcules se développant en grand nombre dans 1'estomac et dans les intestins, pedant la digestion des animaux herbivores et carnivores. *Compt. Rend. Acad. Sci.* 17, 1304–8.

Guttmacher, A. E. and Collins, F. S. 2003. Welcome to the genomic era. *New Engl. J. Med.* 349(10), 996–8. doi:10.1056/NEJMe038132.

Hall, M. B. 2011. Isotrichid protozoa influence conversion of glucose to glycogen and other microbial products. *J. Dairy Sci.* 94(9), 4589–602. doi:10.3168/jds.2010-3878.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Collaborators, G.R.C., Abecia, L., Angarita, E., Aravena, P., Arenas, G. N. and Ariza, C. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep. UK* 5, 14567.

Hess, H. D., Beuret, R. A., Lötscher, M., Hindrichsen, I. K., Machmüller, A., Carulla, J. E., Lascano, C. E. and Kreuzer, M. 2004. Ruminal fermentation, methanogenesis and nitrogen utilization of sheep receiving tropical grass hay-concentrate diets offered with *Sapindus saponaria* fruits and *Cratylia argentea* foliage. *Anim. Sci.* 79(1), 177–89. doi:10.1017/S1357729800054643.

Hess, M., Sczyrba, A., Egan, R., Kim, T. W., Chokhawala, H., Schroth, G., Luo, S., Clark, D. S., Chen, F., Zhang, T., Mackie, R. I., Pennacchio, L. A., Tringe, S. G., Visel, A., Woyke, T., Wang, Z. and Rubin, E. M. 2011. Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. *Science* 331(6016), 463–7. doi:10.1126/science.1200387.

Hobson, P. N. and Stewart, C. S. 1997. *The Rumen Microbial Ecosystem* (2nd edn.). Blackie Academic and Professional, UK.

Holtshausen, L., Chaves, A. V., Beauchemin, K. A., McGinn, S. M., McAllister, T. A., Odongo, N. E., Cheeke, P. R. and Benchaar, C. 2009. Feeding saponin-containing *Yucca schidigera* and *Quillaja saponaria* to decrease enteric methane production in dairy cows. *J. Dairy Sci.* 92(6), 2809–21. doi:10.3168/jds.2008-1843.

Hristov, A. N., McAllister, T. A., Van Herk, F. H., Cheng, K. J., Newbold, C. J. and Cheeke, P. R. 1999. Effect of *Yucca schidigera* on ruminal fermentation and nutrient digestion in heifers. *J. Anim. Sci.* 77(9), 2554–63. doi:10.2527/1999.7792554x.

Hungate, R. E. 1942. The culture of *Eudiplodinium neglectum* with experiments on the digestion of cellulose. *Biol. Bull.* 83(3), 303–19. doi:10.2307/1538229.

Hungate, R. E. 1943. Further experiments on cellulose digestion by the protozoa in the rumen of cattle. *Biol. Bull.* 84(2), 157–63. doi:10.2307/1538178.

Hsu, J. T., Fahey, G. C., Merchen, N. R. and Mackie, R. I. 1991. Effects of defaunation and various nitrogen supplementation regimens on microbial numbers and activity in the rumen of sheep. *J. Anim. Sci.* 69(3), 1279–89. doi:10.2527/1991.6931279x.

Huws, S. A., Kim, E. J., Kingston-Smith, A. H., Lee, M. R. F., Muetzel, S. M., Cookson, A. R., Newbold, C. J., Wallace, R. J. and Scollan, N. D. 2009. Rumen protozoa are rich in polyunsaturated fatty acids due to the ingestion of chloroplasts. *FEMS Microbiol. Ecol.* 69(3), 461–71. doi:10.1111/j.1574-6941.2009.00717.x.

Huws, S. A., Lee, M. R. F., Kingston-Smith, A. H., Kim, E. J., Scott, M. B., Tweed, J. K. and Scollan, N. D. 2012. Ruminal protozoal contribution to the duodenal flow of fatty acids following feeding of steers on forages differing in chloroplast content. *Br. J. Nutr.* 108(12), 2207–14. doi:10.1017/S0007114512000335.

Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., Muñoz-Tamayo, R., Forano, E., Waters., S. M., Hess, M., Tapio, I., Smidt, H., Krizsan, S. J., Yáñez-Ruiz, D. R., Belanche, A., Guan, L., Gruninger, R. J., McAllister, T. A., Newbold, C. J., Roehe, R., Dewhurst, R. J., Snelling, T. J., Watson, M., Suen, G., Hart, E. H., Kingston-Smith, A. H., Scollan, N. D., do Prado, R. M., Pilau, E. J., Mantovani, H. C., Attwood, G. T., Edwards, J. E., McEwan, N. R., Morrisson, S., Mayorga, O. L., Elliott, C. and Morgavi, D. P. 2018. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present and future. *Front. Microbiol.* 9, 1–33.

Imai, S. 1998. Phylogenetic taxonomy of rumen ciliate protozoa based on their morphology and distribution. *J. Appl. Anim. Res.* 13(1–2), 17–36. doi:10.1080/09712119.1998.9706670.

Ivan, M., Charmley, L. L., Neill, L. and Hidiroglou, M. 1991. Metabolic changes in the rumen following protozoal inoculation of fauna-free sheep fed a corn silage diet supplemented with casein or soybean meal. *Ann. Rech. Vet.* 22(2), 227–38.

Jouany, J. P. and Senaud, J. 1979. Role of rumen protozoa in the digestion of food cellulosic materials. *Ann. Rech. Vet.* 10(2–3), 261 –3.

Jouany, J. P. and Ushida, K. 1999. The role of protozoa in feed digestion – review. *Asian Australas. J. Anim. Sci* 12(1), 113–28. doi:10.5713/ajas.1999.113.

Kamra, D. N. 2005. Rumen microbial ecosystem. *Curr. Sci. India* 89(1), 124–35.

Karnati, S. K. R., Sylvester, J. T., Noftsger, S. M., Yu, Z., St-Pierre, N. R. and Firkins, J. L. 2007. Assessment of ruminal bacterial populations and protozoal generation time in cows fed different methionine sources. *J. Dairy Sci.* 90(2), 798–809. doi:10.3168/jds.S0022-0302(07)71564-3.

Konstantinidis, K. T., Ramette, A. and Tiedje, J. M. 2006. The bacterial species definition in the genomic era. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 361(1475), 1929–40. doi:10.1098/rstb.2006.1920.

Kubesy, A. A. and Dehority, B. A. 2002. Forestomach ciliate Protozoa in Egyptian dromedary camels (*Camelus dromedarius*). *Zootaxa* 51(1), 1–12. doi:10.11646/zootaxa.51.1.1.

Levy, B. and Jami, E. 2018. Exploring the prokaryotic community associated with the rumen ciliate protozoa population. *Front. Microbiol.* 9(9), 2526. doi:10.3389/fmicb.2018.02526.

Li, Z., Deng, Q., Liu, Y., Yan, T., Li, F., Cao, Y. and Yao, J. 2018. Dynamics of methanogenesis, ruminal fermentation and fiber digestibility in ruminants following elimination of protozoa: a meta-analysis. *J. Anim. Sci. Biotechnol.* 9(9), 89. doi:10.1186/s40104-018-0305-6.

Lourenco, M., Ramos-Morales, E. and Wallace, R. J. 2010. The role of microbes in rumen lipolysis and biohydrogenation and their manipulation. *Animal* 4(7), 1008–23. doi:10.1017/S175173111000042X.

Lu, C. D., Tsai, L. S., Schaefer, D. M. and Jorgensen, N. A. 1987. Alteration of fermentation in continuous culture of mixed rumen bacteria by isolated alfalfa saponins. *J. Dairy Sci.* 70(4), 799–805. doi:10.3168/jds.S0022-0302(87)80076-0.

Makkar, H. P. S., Blümmel, M. and Becker, K. 1995. *In vitro* effects of and interactions between tannins and saponins and fate of tannins in the rumen. *J. Sci. Food Agric.* 69(4), 481–93. doi:10.1002/jsfa.2740690413.

McEwan, N. R., Eschenlauer, S. C. P., Calza, R. E., Wallace, R. J. and Newbold, C. J. 2000a. The 3′ untranslated region of messages in the rumen protozoan *Entodinium caudatum*. *Protist* 151(2), 139–46. doi:10.1078/1434-4610-00014.

McEwan, N. R., Gatherer, D., Eschenlauer, S. C. P., McIntosh, F. M., Calza, R. E., John Wallace, R. and Newbold, C. J. 2000b. An unusual codon usage pattern in the ciliate family *Ophryoscolecidae* and its implications for determining the source of cloned DNA. *Anaerobe* 6(1), 21–8. doi:10.1006/anae.1999.0310.

McSweeney, C. S., Palmer, B., McNeill, D. M. and Krause, D. O. 2001. Microbial interactions with tannins: nutritional consequences for ruminants. *Anim. Feed Sci. Tech.* 91(1–2), 83–93. doi:10.1016/S0377-8401(01)00232-2.

Michałowski, T., Muszyński, P. and Landa, I. 1991. Factors influencing the growth of rumen ciliates *Eudiplodinium maggii in vitro*. *Acta. Protozool.* 25, 419–26.

Michałowski, T., Harmeyer, J. and Bełżecki, G. 1999. The importance of washing the omasum for successful defaunation of sheep. *J. Anim. Feed. Sci.* 8(4), 611–9. doi:10.22358/jafs/69185/1999.

Miltko, R., Belezecki, G. and Michalowski, T. 2012. Chitinolytic enzymes of the rumen ciliate *Eudiplodinium maggii*. *Folia Microbiol. (Praha)* 57(4), 317–9. doi:10.1007/s12223-012-0133-6.

Moon-van der Staay, S. Y., van der Staay, G. W. M., Michałowski, T., Jouany, J. P., Pristas, P., Javorsky, P., Kišidayová, S., Váradyová, Z., McEwan, N. R., Newbold, C. J., van Alen, T., de Graaf, R., Schmid, M., Huynen, M. A. and Hackstein, J. H. P. 2014. The symbiotic intestinal ciliates and the evolution of their hosts. *Eur. J. Protistol.* 50(2), 166–73. doi:10.1016/j.ejop.2014.01.004.

Morgavi, D. P., Sakurada, M., Tomita, Y. and Onodera, R. 1994. Presence in rumen bacterial and protozoal populations of enzymes capable of degrading fungal cell walls. *Microbiology (Reading, Engl.)* 140(3), 631–6. doi:10.1099/00221287-140-3-631.

Nagaraja, T. G., Newbold, C. J., Van Nevel, C. J. and Demeyer, D. I. 1997. Manipulation of ruminal fermentation. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem*. Blackie Academic and Professional, London, UK, pp. 523–632.

Newbold, C. J., McEwan, N. R., Calza, R. E., Chareyron, E. N., Duval, S. M., Eschenlauer, S. C. P., McIntosh, F. M., Nelson, N., Travis, A. J. and Wallace, R. J. 2005. An NAD^+-dependent glutamate dehydrogenase cloned from the ruminal ciliate protozoan, *Entodinium caudatum*. *FEMS Microbiol. Lett.* 247(2), 113–21. doi:10.1016/j.femsle.2005.04.034.

Newbold, C. J., de la Fuente, G., Belanche, A., Ramos-Morales, E. and McEwan, N. R. 2015. The role of ciliate protozoa in the rumen. *Front. Microbiol.* 6, 1313. doi:10.3389/fmicb.2015.01313.

Nguyen, S. H., Li, L. and Hegarty, R. S. 2016. Effects of rumen protozoa of Braham heifers and nitrate on fermentation and in vitro methane production. *Asian-Australas. J. Anim. Sci.* 29(6), 807–13. doi:10.5713/ajas.15.0641.

Nicholson, M. J., Theodorou, M. K. and Brookman, J. L. 2005. Molecular analysis of the anaerobic rumen fungus *Orpinomyces* – insights in an AT-rich genome. *Microbiology (Reading, Engl.)* 151(1), 121–33. doi:10.1099/mic.0.27353-0.

Nikkhah, A. 2016. Rumen microbial protein synthesis in multigrain and barley fed lactating dairy cows. *J. Anim. Sci.* 94(suppl_2), 160. doi:10.2527/msasas2016-340.

Orpin, C. G. 1975. Studies on the rumen flagellate *Neocallimastix frontalis*. *J. Gen. Microbiol.* 91(2), 249–62. doi:10.1099/00221287-91-2-249.

Orpin, C. G. 1984. The role of ciliate protozoa and fungi in the rumen digestion of plant cell walls. *Anim. Feed. Sci. Tech.* 10(2–3), 121–43. doi:10.1016/0377-8401(84)90003-8.

Orpin, C. G. 1985. Association of rumen ciliate populations with plant particles *in vitro*. *Microb. Ecol.* 11(1), 59–69. doi:10.1007/BF02015109.

Park, T., Wijeratne, S., Meulia, T., Firkins, J. and Yu, Z. 2018. Draft macronuclear genome sequence of the ruminal ciliate *Entodinium caudatum*. *Microbiol. Res. Ann.* 7(1), e00826-18. doi:10.1128/MRA.00826-18.

Patra, A. K. 2011. Effects of essential oils on rumen fermentation, microbial ecology and ruminant production. *Asian J. Anim. Vet. Adv.* 6(5), 416–28. doi:10.3923/ajava.2011.416.428.

Patra, A. K. and Saxena, J. 2009. The effect and mode of action of saponins on the microbial populations and fermentation in the rumen and ruminant production. *Nutr. Res. Rev.* 22(2), 204–19. doi:10.1017/S0954422409990163.

Paul, R. G., Williams, A. G. and Butler, R. D. 1990. Hydrogenosomes in the rumen Entodiniomorphid ciliate *Polyplastron multivesiculatum*. *J. Gen. Microbiol.* 136(10), 1981–9. doi:10.1099/00221287-136-10-1981.

Prescott, D. M. 1994. The DNA of ciliated protozoa. *Microbiol. Rev.* 58(2), 233–67. doi:10.1128/MMBR.58.2.233-267.1994.

Purser, D. B. 1961. A diurnal cycle for Vestibuliferida protozoa of the rumen. *Nature* 190, 831–2. doi:10.1038/190831a0.

Purser, D. and Moir, R. 1959. Ruminal flora studies in the sheep. IX. The effect of pH on the ciliate population of the rumen *in vivo*. *Aust. J. Agric. Res.* 10(4). doi:10.1071/AR9590555.

Quail, M. A., Smith, M., Coupland, P., Otto, T. D., Harris, S. R., Connor, T. R., Bertoni, A., Swerdlow, H. P. and Gu, Y. 2012. A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics* 13, 341. doi:10.1186/1471-2164-13-341.

Ranilla, M. J., Jouany, J. P.. and Morgavi, D. P. 2007. Methane production and substrate degradation by rumen microbial communities containing single protozoal species *in vitro*. *Lett. Appl. Microbiol.* 45(6), 675–80. doi:10.1111/j.1472-765X.2007.02251.x.

Ricard, G., McEwan, N. R., Dutilh, B. E., Jouany, J. P., Macheboeuf, D., Mitsumori, M., McIntosh, F. M., Michałowski, T., Nagamine, T., Nelson, N., Newbold, C. J., Nsabimana, E., Takenaka, A., Thomas, N. A., Ushid, K., Hackstein, J. H. P. and Huynen, M. A. 2006. Horizontal gene transfer from bacteria to rumen ciliates indicates adaptation to their anaerobic, carbohydrates-rich environment. *BMC Genomics* 7, 22. doi:10.1186/1471-2164-7-22.

Rocha, E. P. C. 2004. Codon usage bias from tRNA's point of view: redundancy, specialization and efficient decoding for translational optimization. *Genome Res.* 14(11), 2279 –86. doi:10.1101/gr.2896904.

Santoso, B., Mwenya, B., Sar, C., Gamo, Y., Kobayashi, T., Morikawa, R., Kimura, K., Mizukoshi, H. and Takahashi, J. 2004. Effects of supplementing galacto-oligosaccharides, Yucca schidigera or nisin on rumen methanogenesis, nitrogen and energy metabolism in sheep. *Livest. Prod. Sci.* 91(3), 209–17. doi:10.1016/j.livprodsci.2004.08.004.

Shabi, Z., Tagari, H., Murphy, M. R., Bruckental, I., Mabjeesh, S. J., Zamwel, S., Celik, K. and Arieli, A. 2000. Partitioning of amino acids flowing to the abomasum into feed, bacterial, protozoal, and endogenous fractions. *J. Dairy Sci.* 83(10), 2326–34. doi:10.3168/jds.S0022-0302(00)75120-4.

Śliwiński, B. J., Soliva, C. R., Machmüller, A. and Kreuzer, M. 2002. Efficacy of plant extracts rich in secondary constituents to modify rumen fermentation. *Anim. Feed Sci. Tech.* 101(1–4), 101–14. doi:10.1016/S0377-8401(02)00139-6.

Stumm, C. K., Gijzen, H. J. and Vogels, G. D. 1982. Association of methanogenic bacteria with ovine rumen ciliates. *Br. J. Nutr.* 47(1), 95–9. doi:10.1079/bjn19820013.

Tavendale, M. H., Meagher, L. P., Pacheco, D., Walker, N., Attwood, G. T. and Sivakumaran, S. 2005. Methane production from in vitro rumen incubations with *Lotus pedunculatus* and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. *Anim. Feed Sci. Tech.* 123–124, 403–19. doi:10.1016/j.anifeedsci.2005.04.037.

Thomas, N. A., Regensbogenova, M., de Graaf, R. M., Devillard, E., Pristas, P., van der Staay, G. W. M., Javorsky, P., Hackstein, J. H. P., Newbold, C. J. and McEwan, N. R. 2004. Diversity in the length of macronuclear chromosomes in the phylum Ciliophora; rumen ciliates and *Nyctotherus* – a case study. *Reprod. Nutr. Dev.* 44 (Suppl. 31).

Tymensen, L. D., Beauchemin, K. A. and McAllister, T. A. 2012. Structures of free-living and protozoa-associated methanogen communities in the bovine rumen differ according to comparative analysis of 16S rRNA and mcrA genes. *Microbiology (Reading, Engl.)* 158(7), 1808–17. doi:10.1099/mic.0.057984-0.

UN. 2017. *2017 Revision of World Population Prospects: Key Findings and Advance Tables*.

van Zijderveld, S. M., Fonken, B., Dijkstra, J., Gerrits, W. J., Perdok, H. B., Fokkink, W. and Newbold, J. R. 2011. Effects of a combination of feed additives on methane production, diet digestibility and animal performance in lactating dairy cows. *J. Dairy Sci.* 94(3), 1445–54. doi:10.3168/jds.2010-3635.

Varel, V. H. and Dehority, B. A. 1989. Ruminal cellulolytic bacteria and protozoa from bison, cattle-bison hybrids and cattle fed three alfalfa-corn diets. *Appl. Environ. Microbiol.* 55(1), 148–53. doi:10.1128/AEM.55.1.148-153.1989.

Veira, D. M. 1986. The role of ciliate protozoa in nutrition of the ruminant. *J. Anim. Sci.* 63(5), 1547–60. doi:10.2527/jas1986.6351547x.

Wallace, R. J., McEwan, N. R., McIntosh, F. M., Teferedegne, B. and Newbold, C. J. 2002. Natural products as manipulators of rumen fermentation. *Asian Australas. J. Anim. Sci* 15(10), 1458–68. doi:10.5713/ajas.2002.1458.

Wang, Y. and McAllister, T. A. 2002. Rumen microbes, enzymes and feed digestion – a review. *Asian Australas. J. Anim. Sci* 15(11), 1659–76. doi:10.5713/ajas.2002.1659.

Wang, Z., Elekwachi, C. O., Jiao, J., Wang, M., Tang, S., Zhou, C., Tan, Z. and Forster, R. J. 2017. Investigation and manipulation of metabolically active methanogen community composition during rumen development in black goats. *Sci. Rep.* 7(1), 422. doi:10.1038/s41598-017-00500-5.

Warner, A. C. I. 1962. Some factors influencing the rumen microbial population. *J. Gen. Microbiol.* 28(1), 129–46. doi:10.1099/00221287-28-1-129.

Wereszka, K., McIntosh, F. M., Michałowski, T., Jouany, J.-P., Nsabimana, E., Macheboeuf, D., McEwan, N. R. and Newbold, C. J. 2004. A cellulase produced by the rumen anaerobic protozoan *Epidinium ecaudatum* has an unusual pH optimum. *Endocyt. Cell Res.* 15, 561–9.

Wereszka, K., Michałowski, T., Newbold, C. J. and McEwan, N. R. 2006. Xylanolytic activity of the rumen protozoan *Diploplastron affine*. *J. Anim. Feed Sci.* 15, S43–6.

White, R. W. 1969. Viable bacteria inside the rumen ciliate *Entodinium caudatum*. *J. Gen. Microbiol.* 56(3), 403–8. doi:10.1099/00221287-56-3-403.

Williams, A. G. 1986. Rumen vestibuliferida ciliate protozoa. *Am. Soc. Microbiol.* 50(1), 25–49.

Williams, C. L. 2018. Prospecting the rumen protozoa for hydrolytic enzymes and their evolutionary origins. Ph.D. Thesis. Aberystwyth University.

Williams, A. G. and Coleman, G. S. 1992. *The Rumen Protozoa* (1st edn.). Springer-Verlag, New York, NY.

Wright, D. E. 1960. Pectic enzymes in rumen protozoa. *Arch. Biochem. Biophys.* 86, 251–4. doi:10.1016/0003-9861(60)90413-6.

Yáñez-Ruiz, D. R., Abecia, L. and Newbold, C. J. 2015. Manipulating rumen microbiome and fermentation through interventions during early life: a review. *Front. Microbiol.* 6(6), 1133. doi:10.3389/fmicb.2015.01133.

Young, K. W., Thomas, N. A., Duval, S. M., Newbold, C. J., Fried, J., Brown, D. S., Hackstein, J. H. P., Michalowski, T. and McEwan, N. R. 2015. Isolation of macro-nuclear DNA from the rumen ciliate *Entodinium caudatum*. *Endocyt. Cell Res.* 26, 50–5.

Chapter 8

The anaerobic rumen fungi

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1 Introduction

Members of the phylum Neocallimastigomycota have been shown to be widely distributed in rumen and hindgut of herbivorous mammals and play key roles in digestion of fibrous feeds (Gruninger et al., 2014). These strictly anaerobic fungi are the first colonizers of ingested plant materials and act as a biological crowbar to break down fibrous materials through their hyphal tips bearing high concentrations of fibrolytic enzymes, thus increasing access to the plant carbohydrates for other cellulolytic microbes. Despite accounting for very small percentages of the gut microbiota in their hosts (7–9%), anaerobic fungi release more than 50% of the fermentable sugars from ingested plant matter and thus

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play a critical role in the digestion of fibrous feed (Theodorou et al., 1996). It was shown that removal of anaerobic fungi from the digestive tract of sheep decreased feed intake severely (Gordon and Phillips, 1993).

2 The life cycle of anaerobic fungi

Rumen fungi reproduce asexually and possess a life cycle consisting of a motile flagellate (zoospore) stage and a non-motile vegetative and reproductive (thallus) stage (Mountfort, 1987). It is during the non-motile stage that rumen fungi colonize and degrade fibrous feeds, thus enabling them to play a role in the digestion of fiber in the rumen. The life cycle begins with the differentiation of the reproductive bodies (sporangia) into zoospores as response to increased concentrations of soluble carbohydrates, haem, and other related porphyrins released into the rumen liquid (Orpin and Greenwood, 1986). Abundance of zoospores, which can be monoflagellate or polyflagellate, peaks after 30–60 min of feeding (Orpin and Joblin, 1997). Although zoospores remain motile for several hours (Lowe et al., 1987), they usually attach and encyst on plant fragments within 30 min after they have been released from a sporangium (Heath et al., 1986). Motile zoospores accumulate at the potential attachment site on the ingested fiber by chemotaxis following a gradient of soluble sugars and phenolics (Wubah and Kim, 1996). Once attached, they increase in size, shed their flagella, and form cysts by thickening their cell wall, but remain capable of ameboid movement. Cyst germination, which involves the production of a germ tube at the polar end opposite from where the flagella originated, differs between rumen fungi with one sporangium (monocentric) and multiple sporangia (polycentric). Monocentric fungi undergo an endogenous cyst germination, during which the nucleus remains within the cyst producing a zoosporangium and an anucleate rhizoid system that acts as an anchor and as trophic interface (Ho and Barr, 1995). After zoosporogenesis, the remaining thallus is autolyzed (Lowe et al., 1987). Polycentric fungi display an exogeneous cyst germination during which nuclei migrate into the rhizoids system enabling the formation of multiple sporangia (Barr et al., 1989). The life cycle of monocentric anaerobic fungi (zoospore attachment-cyst germination-thallus development-reproductive stage-zoospore release) is about 14–32 h (Lowe et al., 1987; Ho et al., 1996). The polycentric anaerobic fungi besides producing zoospores for reproduction are capable of reproducing through vegetative growth as mycelia contain nucleus and hence capable of indeterminate growth. In case of two bulbous genera (*Caecomyces* and *Cyllamyces*), nuclei are present in holdfast and sporangiophores consistent with exogenous development and development of thalli in these two genera are not strictly determinated as in the case of monocentric genera but is more limited than polycentric genera.

Besides their biological relevance, zoospores have also been used as a technique to determine the abundance of anaerobic fungi in strained rumen fluid using agar roll tubes (Bauchop, 1979; Joblin, 1981). Like other methods, this approach only provides an estimate, as there are considerable fluctuations of zoospores abundance over a 24 h period and the relation between zoospores per sporangium is still not fully understood. Despite its limitations, this method has the advantage that morphological phenotypes can be observed, facilitating a preliminary identification and classification, and it has been used successfully to isolate various different genera of anaerobic fungi (Phillips and Gordon, 1988, 1995; Borneman et al., 1989). An alternative approach to the roll tube technique is to inoculate agar plate in an anaerobic chamber (Borneman et al., 1989). Taking into consideration that different anaerobic fungi have been isolated from the distinct compartments of the gastrointestinal (GI) tract of herbivores, the GI tract versus feces, and from domesticated versus wild herbivores, it is possible that these differences are caused by employing different isolation techniques. A combination of these techniques should be considered to maximize the success of any isolation effort and the most accurate representation of fungal isolates from these niches.

A number of means, such as saliva, feces, and aerosols, have been suggested to facilitate the transfer between animals (Lowe et al., 1987; Orpin, 1989). Milne et al. for example reported that it was possible to isolate anaerobic fungi from sheep saliva stored at 39°C for up to 8 h and from dried sheep feces stored at 39°C for up to 128 days (Milne et al., 1989). Feces were reported to contain substantial counts of fungal sporangia, with counts declined very slowly and maintaining viability for up to 10 months once feces were dried (Theodorou et al., 1990). Based on the observation that survival rate in dried feces were higher compared to those determined for feces stored under moist conditions, Trinci et al. (1988) hypothesized that the drying process stimulates the formation of resistant structures (Trinci et al., 1988). This in combination with the loss of fungal viability due to bacterial activity under moist conditions (McGranaghan et al., 1999) might explain increases in fungal survival under low moisture conditions. The ability of anaerobic fungi to form aero-tolerant survival structures, such as the multi-chambered spores of *Anaeromyces* (Brookman et al., 2000; Ozkose et al., 2001), would explain the presence of anaerobic fungi in landfill sites (McDonald et al., 2012), deep-sea sediments (Nagahama and Nagano, 2012), and as part of the microbial community in biogas reactors (Haitjema et al., 2014). It would also explain the difficulty of maintaining the GI tract of ruminant animals free of anaerobic fungi, although the processes responsible for the formation and later germination of these spores remain currently unknown (Gruninger et al., 2014).

3 Taxonomy and morphological features of anaerobic fungi

The historical pilgrimage of rumen fungi and their taxonomic classification system lasted for almost a century. When flagellate microbes from rumen fluid were described for the first time early in the twentieth century, they were classified as protozoa (Liebetanz, 1910; Braune, 1913); although the observed cells were much smaller than true flagellate protozoa cultured from rumen liquor (Jensen and Hammond, 1964). It was not until 1975 that Colin Orpin described the stages of their life cycle (Orpin, 1975), discovered chitin as their main cell-walls structural polysaccharide (Orpin, 1977a), and reclassified them correctly as the first species of anaerobic fungi (Orpin, 1976, 1977b). The reclassification of these microbes as true fungi contradicted the mycological dogma of that time (Foster, 1949; Vavra and Joyon, 1966), which claimed that fungi are highly oxidative in nature and are unable to metabolize carbohydrates in the absence of oxygen.

In 1980, the rumen fungi were assigned to the class Chytridiomycetes, order Spizellomycetales (Barr, 1980), which was supported later based on the sequence of their 18S ribosomal RNA (rRNA; Dore and Stahl, 1991; Bowman et al., 1992; Li and Heath, 1992). Despite the molecular evidence, unique phenotypic features of rumen fungi, like their strict anaerobiosis, the absence of mitochondria, the presence of hydrogenosomes and polyflagellate zoospores (Li et al., 1993), not found in other Spizellomycetales ultimately raised doubts about Barr's classification system (Barr, 1980, 1988). This disagreement led to a new order inside the class Chytridiomycetes, namely the Neocallimastigales with a single family the Neocallimastigaceae, accommodating only anaerobic fungi (Li et al., 1993).

Significant advances in fungal systematics were achieved by the 'Assembling the Fungal Tree of Life' project that employed a multigene approach to decipher the low-level evolutionary phylogenetic relationships between members of the fungal kingdom (James et al., 2006). A combination of a 'six-gene phylogeny', four genes from the rRNA operon (i.e. 18S rRNA, 28S rRNA, ITS) and two protein-coding genes (i.e. EF1α, RNA polymerase II largest subunit RPB1, and its second largest subunit RPB2), and the distinct morphological features of the rumen fungi ultimately led to the separation of anaerobic fungi from the Chytridiomycota and the formation of a new phylum, the Neocallimastigomycota. This phylum is comprised of the Neocallimastigomycetes, Neocallimastigales, and Neocallimastigaceae on the class, order, and family level, respectively (Hibbett et al., 2007). In contrast to this six-gene phylogeny, a classification based on the three nuclear ribosomal regions (i.e. ITS, LSU, and SSU) and a region of one protein-coding gene (i.e. RPB1) (Schoch et al., 2012) as well as a phylogenomic classification based on 46 slowly evolving and 107 moderately evolving, orthologous, protein-coding

Table 1 Commonly used taxonomic classifications of anaerobic fungi

	Tedersoo et al. (2018)	Hibbett et al. (2007)	NCBI taxonomy
Kingdom	Fungi	Fungi	Fungi
Subkingdom	Chytridiomycota[a]	–	–
Phylum	Neocallimastigomycota[b]	Neocallimastigomycota[b]	Chytridiomycota[c]
Subphylum	Neocallimastigomycotina[d]		
Class	Neocallimastigomycetes[e]	Neocallimastigomycetes[e]	Neocallimastigomycetes[e]
Order	Neocallimastigales[f]	Neocallimastigales[f]	Neocallimastigales[f]
Family	*Neocallimastigaceae*[g]	*Neocallimastigaceae*[g]	*Neocallimastigaceae*[g]

[a] Subkingdom: Chytridiomycota Tedersoo et al. subkgd. nov. (Tedersoo et al., 2018).
[b] Phylum: Neocallimastigomycota M. J. Powell, phylum nov. (Hibbett et al., 2007).
[c] Phylum: Chytridiomycota (Barr, 2001).
[d] Subphylum: Neocallimastigomycotina Tedersoo et al. subphyl. nov. (Tedersoo et al., 2018).
[e] Class: Neocallimastigomycetes M. J. Powell, class. nov. (Hibbett et al., 2007).
[f] Order: Neocallimastigales (Li et al., 1993).
[g] Family: *Neocallimastigaceae* (Li et al., 1993).

genes (Ebersberger et al., 2012) suggest a monophyletic origin of the zoosporic chitinous fungi. Tedersoo et al. (2018) found sufficient support for a separate phylum of the anaerobic fungi and proposed nomenclatural changes at the higher taxonomic level that led them to the introduction of fungal subkingdoms (Table 1). Based on molecular phylogenies, divergence time, and monophyly criterion, they proposed the new subkingdom Chytridiomyceta comprised of three phyla, namely the Chytridiomycota, Monoblepharomycota, and Neocallimastigomycota (Tedersoo et al., 2018).

To determine the most recent common ancestor of the anaerobic fungi, Wang et al. (2018) used genome and transcriptome data from 27 Neocallimastigomycota to calculate the divergence time of anaerobic fungi. Their analysis suggest that anaerobic fungi diverged approximately 73.5 ± 5 million years ago, which corresponds to the estimated time when mammalian herbivory evolved.

4 Genera and species of anaerobic fungi

Eighteen genera of anaerobic fungi have been described until today, although culture-independent studies have suggested at least 25 anaerobic fungal genera. Due to the challenges associated with the classification of anaerobic fungi, the number of described species ranges between 31 and 41. Considering the challenges associated with the accurate classification of anaerobic fungi and the possibility that some of the described species might be redundant, it appears advisable to consider the lower number to be correct. A more precise classification will evolve with the development of more advanced isolation

and cultivation approaches and with new molecular techniques targeting specifically anaerobic fungi.

Morphological features have been the key for the classification of anaerobic fungi, and genera were defined based on the formation of a mono- or polycentric thallus, filamentous or bulbous rhizoids, the shape of sporangia, and if an organism forms mono- or polyflagellate zoospores. However, the morphological approach to the systematic differentiation of anaerobic fungi is encumbered with difficulties, mainly from the extensive morphological variations, pleomorphism in sporangial and rhizoidal structures, similarities in morphological features of monocentric and uniflagellate genera, failure to produce sporangia, and the absence of zoosporogenesis in some polycentric species. Key morphological features of currently recognized anaerobic fungal genera are summarized in Table 2.

Most of the culture-dependent and culture-independent studies performed to date have used ITS-based sequences. Recent studies have confirmed that internal transcribed spacer (ITS) sequences analysis is inferior for species-level differentiation of several anaerobic fungal genera to the sequence analysis based on the ribosomal large subunit (LSU), although the number of curated LSU sequences for comparative is currently still limited. Morphologically independent molecular approach based on DNA analyses, thus, represents an extremely powerful tool to shed light into the otherwise cumbersome classification system, and the differentiation and relationship of the individual anaerobic fungi. A LSU sequence-based phylogenetic analysis has been performed recently by Hanafy et al. (2019) to visualize the evolutionary relationship of all currently known 18 genera of anaerobic rumen fungi (Fig. 1).

5 Monocentric genera

5.1 Neocallimastix

Neocallimastix is the first described genus of anaerobic fungi, named after *Callimastix frontalis* originally classified as flagellate protozoan by Braune (1913). This isolate was renamed in 1966 into *Neocallimastix frontalis* but remained classified as protozoa (Vavra and Joyon, 1966) until reclassified as fungus by Orpin (1975). A more detailed description of *N. frontalis* was performed later by Heath et al. (1983). This strain, however, differed from those studied originally by Orpin, which led Orpin to rename his culture to *N. patriciarum* (Orpin and Munn, 1986). Neocallimastix is widely distributed among ruminant as well as non-ruminant herbivores and represents the best-studied anaerobic fungus to date.

Species: Over the years, seven distinct species have been reported inside the genus *Neocallimastix* (MB#25486), including *Neocallimastix frontalis* (Heath

Table 2 Key morphological features of currently recognized anaerobic fungal genera

Genus (reference)	Zoospore/thallus morphology	Other features
Neocallimastix (Heath et al., 1983)	Polyflagellate/ monocentric, filamentous	Rhizoid tubular or inflated below the neck of sporangia, sporangia located on unbranched or branched sporangiophores
Piromyces (Barr et al., 1989)	Uniflagellate/ monocentric, filamentous	Bi or quadriflagellate zoospores, both endogenous and exogenous zoosporangial development, rhizoids with or without subsporangial swelling, septum often in mature zoosporangia
Oontomyces (Dagar et al., 2015)	Uniflagellate/ monocentric, filamentous	Intercalary rhizoidal swellings, sporangia never mucronated, formed terminally, long sporangiophores can be separated from the rhizomycelium by distinct constriction
Buwchfawromyces (Callaghan et al., 2015)	Uniflagellate/ monocentric, filamentous	Extensive rhizoidal system with twisted rhizoids, sporangia with no apical projections, septum can be visible, nuclei located in sporangia, but not observed in sporangiophores or rhizoids
Pecoramyces (Hanafy et al., 2017)	Uniflagellate/ monocentric, filamentous	Biflagellate zoospores, both endogenous and exogenous zoosporangial development, single terminal sporangium formed per thallus, sporangiophores unbranched, often forming apophysis-like or eggcup-like swelling below sporangium. Extensive anucleate rhizoidal system lacks rhizoidal swellings or constrictions
Liebetanzomyces (Joshi et al., 2018)	Uniflagellate/ monocentric, filamentous	Both endogenous and exogenous zoosporangial development, extensive anucleate rhizoidal system without constrictions, single terminal sporangium per thallus, sporangium with septum on sporangiophore of variable length, sometimes forming eggcup-like structure below the sporangium or showing cyst-like structure. Pleomorphism in sporangial and rhizoidal structures on different substrates is typical
Feramyces (Hanafy et al., 2018a, b)	Polyflagellate/ monocentric, filamentous	Extensive highly branched rhizoidal system with wide and narrow hyphae, wide hyphae with constrictions at irregular intervals, single terminal sporangium per thallus with the occasional formation of pseudo-intercalary sporangia, sporangiophores frequently coiled or wide and flattened, often forming an apophysis-like or eggcup-like swelling below the sporangium, both endogenous and exogenous zoosporangial development, zoospores are released through apical pore with the sporangial wall staying intact, or through detachment of the whole sporangium
Agriosomyces (Hanafy et al., 2019)	Uniflagellate/ monocentric, filamentous	Both endogenous and exogenous zoosporangial development, rhizoids are swollen below the sporangial tightly constricted neck, swollen sporangiophores

(*Continued*)

Table 2 (*Continued*)

Genus (reference)	Zoospore/thallus morphology	Other features
Aklioshbomyces (Hanafy et al., 2019)	Uniflagellate/ monocentric, filamentous	Bi or triflagellate zoospores, both endogenous and exogenous zoosporangial development, papillated sporangia, pseudo-intercalary endogenous sporangia occasionally, unbranched sporangiophores
Capellomyces (Hanafy et al., 2019)	Uniflagellate/ monocentric, filamentous	Both endogenous and exogenous zoosporangial development, unbranched sporangiophores can exhibit subsporangial swelling, zoospores released through apical pore
Ghazallomyces (Hanafy et al., 2019)	Polyflagellate/ monocentric, filamentous	Both endogenous and exogenous zoosporangial development, highly branched rhizoids, unbranched sporangiophores, pleomorphic sporangia with septum, sporangial necks constricted with narrow port, zoospores released through apical pore
Joblinomyces (Hanafy et al., 2019)	Uniflagellate/ monocentric, filamentous	Biflagellate zoospores, both endogenous and exogenous zoosporangial development, sporangiophores vary in length, zoospores released through wide apical pore resulting in empty cup-shaped sporangium
Khoyollomyces (Hanafy et al., 2019)	Uniflagellate/ monocentric, filamentous	Both endogenous and exogenous zoosporangial development, highly branched rhizoids, intercalary swellings in broad hyphae, multisporangiate thallus, branched sporangiophores with two to four sporangia, zoospores released through wide apical pore
Tahromyces (Hanafy et al., 2019)	Uniflagellate/ monocentric, filamentous	Bi or triflagellate zoospores, both endogenous and exogenous zoosporangial development, branched rhizoids, short swollen sporangiophores, sporangia with septum, sporangial necks constricted
Anaeromyces (Breton et al., 1990)	Uniflagellate/polycentric, filamentous	Sporangia with acuminate (mucronate) apex, can be located on erect, solitary, unbranched sporangiophore, hyphae are highly branched, often with numerous constrictions (sausage-like appearance), sometimes with root-like appearance
Orpinomyces (Barr et al., 1989)	Polyflagellate/polycentric, filamentous	Polynucleate rhizomycelium of extensively branched hyphae, wider hyphae can have tightly constricted points at close intervals (bead-like or sausage-like appearance)
Caecomyces (Gold et al., 1988)	Uniflagellate/ monocentric, bulbous	Bi or quadriflagellate zoospores, vegetative stage is absent of developed branching rhizoidal system, consists of spherical or ovoid bodies (holdfast or haustoria), tubular sporangiophores and bulbous rhizoids, nuclei usually present both in sporangia and vegetative cells
Cyllamyces (Ozkose et al., 2001)	Uniflagellate/polycentric, bulbous	Bi or triflagellate zoospores, bulbous holdfast without rhizoids with multiple sporangia, which can be born on a single elongate or branched sporangiophore, nuclei present in bulbous holdfast and sporangiophores

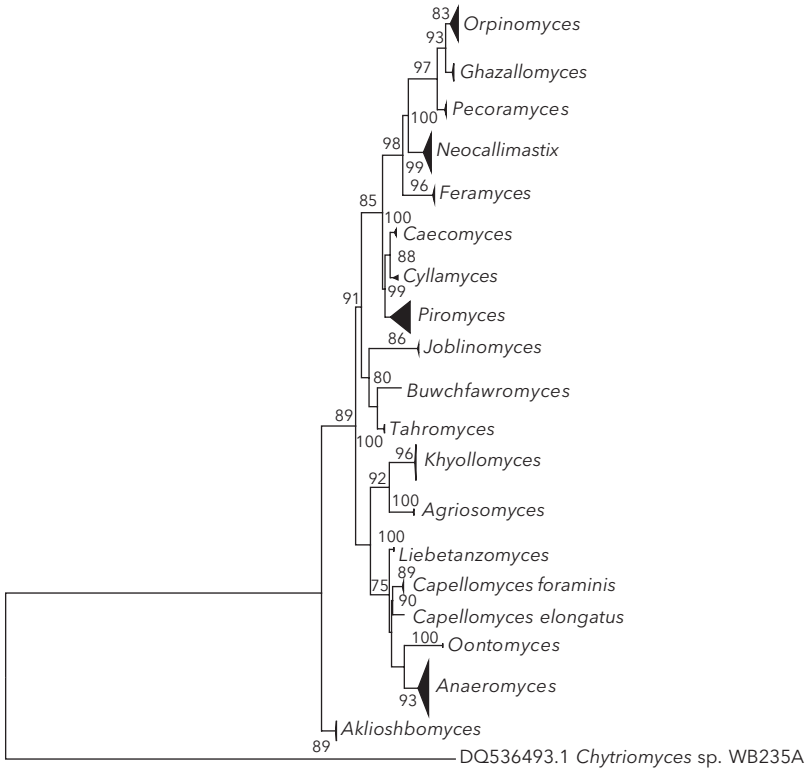

Figure 1 Evolutionary relationship of all currently known genera of anaerobic rumen fungi based on their LSU sequence. Source: adapted with permission from Hanafy et al. (2019). Sequences were aligned in MAFFT (Nakamura et al., 2018) and phylogenetic trees were constructed in MEGA7 (Kumar et al., 2016) using a maximum likelihood approach. Bootstrap values from 100 replicates are shown for nodes with more than 70% bootstrap support.

et al., 1983), *Neocallimastix patriciarum* (Orpin and Munn, 1986), *Neocallimastix joyonii* (Breton et al., 1989), *Neocallimastix hurleyensis* (Webb and Theodorou, 1991), *Neocallimastix variabilis* (Ho et al., 1993b), *Neocallimastix cameroonii* (Ariyawansa et al., 2015), and *Neocallimastix californiae* (Li et al., 2016). *Neocallimastix joyonii* was found to be identical with *Orpinomyces,* which caused this strain to be renamed to *Orpinomyces joyonii* (Li et al., 1991). In 1991, a direct comparison of the type cultures of *N. patriciarum* and *N. frontalis* revealed those to be morphologically and culturally indistinguishable (Wubah et al., 1991). Ho and Barr later proposed to place both *Neocallimastix patriciarum* and *N. variabilis* in synonymy with *N. frontalis* and they questioned the status of *N. hurleyensis* as a distinct species (Ho and Barr, 1995). A phylogenetic analysis of three loci published in 2017 provides molecular evidence that *N. hurleyensis*

should indeed be synonymous with *N. frontalis* (Wang et al., 2017), which implies that *N. frontalis*, *N. cameroonii,* and *N. californiae* are the only three species successfully isolated and cultured from the genus *Neocallimastix* to date.

5.2 Piromyces

Monoflagellate protozoans from the rumen were reported for the first time more than 100 years ago (Liebetanz, 1910), but it was not until the late 1970s that these were recognized as fungi (Orpin, 1977a). Orpin named his isolate *Piromonas communis* and assigned it its own genus, the *Piromonas*. To underline the fungal kinship, *Piromonas* was later renamed *Piromyces* (Gold et al., 1988) and a detailed description of *P. communis* was published the following year (Barr et al., 1989). *Piromyces* appears to be the most heterogeneous genus among the anaerobic fungi, but since phylogenetic analysis and alignments indicate that isolates of *Piromyces* are polyphyletic (Wang et al., 2017), a detailed revision of this genus might be required.

Species: Within the genus *Piromyces* (MB#25332), nine species have been described as of now, namely *P. communis* (Barr et al., 1989), *P. mae* (Li et al., 1990), *P. dumbonicus* (Li et al., 1990), *P. rhizinflatus* (Breton et al., 1991), *P. spiralis* (Ho et al., 1993c), *P. minutus* (Ho et al., 1993d), *P. polycephalus* (Chen et al., 2002), *P. irregularis* (Ariyawansa et al., 2015), and *P. finnis* (Li et al., 2016). An additional species (i.e. *P. cryprodigmaticus*) has been detected by 16S rRNA amplicon sequencing.

5.3 Oontomyces

Dagar et al. (2015) isolated the first cultured representative of this genus from the forestomach of an Indian camel. Cultures were morphologically similar to members of the genus *Piromyces*. Genetic analyses, however, demonstrated this genus to be closer related to members of the polycentric *Anaeromyces* clade. To date, sequences identical or similar to those associated with this genus have been reported exclusively for samples derived from camels, suggesting fungus may be specific to camelids.

Species: Within the genus *Oontomyces* (MB#550795), a sole species, *Oontomyces anksri* (Dagar et al., 2015), has been described to date.

5.4 Buwchfawromyces

A clade previously denoted as SK2 and that contained only uncultured anaerobic fungi (Kittelmann et al., 2012; Koetschan et al., 2014). It was renamed *Buwchfawromyces* after a cultivar that was obtained in 2015 (Callaghan et al.,

2015). This genus contains representatives, which are morphologically similar to *Piromyces* and that have been isolated from feces of cows, sheep, and horses. However, they are genetically different to *Piromyces* and form a sister clade of the *Anaeromyces* instead.

Species: Buwchfawromyces eastonii (Callaghan et al., 2015) is currently the only characterized representative of the genus *Buwchfawromyces* (MB#550797).

5.5 Pecoramyces

Previously denoted as *Orpinomyces* 5 (Kittelmann et al., 2012), then renamed *Orpinomyces* sp. C1A (Youssef et al., 2013) and reclassified as *Pecoramyces ruminantium* in 2017 (Hanafy et al., 2017). Morphologically its sole representative is similar to members of other anaerobic fungi, but genetically it is unique, forming a monophyletic cluster with strong bootstrap support as a sister clade to the genus *Orpinomyces*.

Species: Pecoramyces ruminantium (Hanafy et al., 2017) is currently the only characterized representative for the genus *Pecoramyces* (MB#552530).

5.6 Liebetanzomyces

Clade previously denoted as SP4 (Paul et al., 2018) but renamed within the same year to *Liebetanzomyces* after its first cultured representative (Joshi et al., 2018). Its cultured isolate, derived from rumen digesta of a goat, was morphologically similar to other monocentric genera, but genetically quite distinct, forming sister clade of *Anaeromyces*.

Species: Inside the genus *Liebetanzomyces* (MB#554794), only species *Liebetanzomyces polymorphus* (Joshi et al., 2018) has been described to date.

5.7 Feramyces

Clade of uncultured anaerobic fungi detected from environmental sequences (Liggenstoffer et al., 2010) and classified later as AL6 (Kittelmann et al., 2012). In 2018, its first and currently only cultured isolate was described and the genus was renamed accordingly to *Feramyces* (Hanafy et al., 2018a). Cultivars were isolated from fecal samples and rumen digesta of wild, undomesticated Barbary sheep and fallow deer. Morphological representatives of *Feramyces* are similar to other genera of anaerobic fungi, but genetically it is unique, forming a distinct lineage basal to the *Neocallimastix-Pecoramyces-Orpinomyces* clade.

Species: Inside the genus *Feramyces* (MB#823650), only species *Feramyces austinii* (Hanafy et al., 2018a) has been described to date.

5.8 Agriosomyces

Isolated from the feces of the mouflon and wild boer goat (Hanafy et al., 2019). Based on the LSU sequences, *Agriosomyces* forms a sister clade of *Khoyollomyces*, clustering with the *Oontomyces-Anaeromyces-Liebetanzomyces* clade. Based on the phylogenetic analysis of its ITS 1 region, this genus forms a sister clade to the *Ghazallomyces-Orpinomyces-Pecoramyces* cluster.

Species: Inside the genus *Agriosomyces* (MB#830737), only species *Agriosomyces longus* has been described up to now (Hanafy et al., 2019).

5.9 Aklioshbomyces

This genus exhibits some morphological similarities to *Oontomyces* and *Feramyces* (i.e. pseudo-intercalary endogenous sporangia) and *Piromyces mae* (i.e. papillated sporangia), but phylogenetically it appears to be rather distant, forming a sister clade to all hitherto-cultured anaerobic fungi (Hanafy et al., 2019).

Species: As of now, only one species (*Aklioshbomyces papillarum*) had been described within the genus *Aklioshbomyces* (MB#830735).

5.10 Capellomyces

A glade represented by two species isolated from the feces of the wild boar and domesticated goat. Morphological representatives share some features with *Piromyces rhizinflatus* (Ho and Barr, 1995) and *Neocallimastix frontalis* (Barr et al., 1995), but they are genetically unique. LSU sequences place this fungal genus into the *Oontomyces-Anaeromyces-Liebetanzomyces* supragenus clade.

Species: Inside the genus *Capellomyces* (MB#830739), two species, namely *Capellomyces foraminis* and *Capellomyces elongatus*, have been described up to now (Hanafy et al., 2019).

5.11 Ghazallomyces

A genus described only recently with one representative isolated from the feces of the wild axis deer by Hanafy et al. (2019). This genus forms a monophyletic cluster with strong bootstrap support as a sister clade to *Orpinomyces* and *Pecoramyces*.

Species: Inside the genus *Ghazallomyces* (MB#830733), only species *Ghazallomyces constrictus* has been described up to now (Hanafy et al., 2019).

5.12 Joblinomyces

A genus represented currently by one species (*Joblinomyces apicalis*) isolated from the feces of the domesticated goat and sheep by Hanafy

et al. (2019). Exhibits zoospore release pattern and sporangia morphology similar to those described for *Piromyces minutus* (Ho and Barr, 1995), but genetically is unique. Based on its ITS 1 sequence, this genus forms a separate clade distantly related to the cluster of the *Neocallimastix*, *Feramyces*, *Agriosomyces*, *Ghazallomyces*, *Orpinomyces,* and *Pecoramyces*. Based on the LSU sequences *Joblinomyces* forms a sister clade of the *Buwchfawromyces* and *Tahromyces*.

Species: Inside the genus *Joblinomyces* (MB#830867), only species *Joblinomyces apicalis* has been described up to now (Hanafy et al., 2019).

5.13 Khoyollomyces

A clade first described by Liggenstoffer et al. (2010) from environmental sequences, later denoted as AL1 (Kittelmann et al., 2012), and renamed *Khoyollomyces* based on the name of its first cultured representatives that was isolated from the feces of the domesticated horse and zoo-housed zebra (Hanafy et al., 2019). Based on ITS 1 sequence analysis, this genus forms a distinct clade. Based on the LSU sequences, *Khoyollomyces* forms a monophyletic cluster with strong bootstrap support as a sister clade to the genus *Agriosomyces*.

Species: Inside the genus *Khoyollomyces* (MB#830741), only species *Khoyollomyces ramosus* has been described up to now (Hanafy et al., 2019).

5.14 Tahromyces

A recently described genus with one representative species was isolated from the feces of the wild nilgiri tahr by Hanafy et al. (2019). Morphologically is similar to genera *Piromyces*, *Buwchfawromyces* and *Neocallimastix*, but genetically unique and forming a monophyletic cluster with bootstrap support as a sister clade to the genus *Buwchfawromyces*.

Species: Inside the genus *Tahromyces* (MB#830865), only species *Tahromyces munnarensis* has been described up to now (Hanafy et al., 2019).

5.15 Morphology of monocentric genera

Fungi displaying a monocentric thallus have either monoflagellate (*Piromyces*, *Oontomyces*, *Buwchfawromyces*, *Pecoramyces*, *Liebetanzomyces*, *Aklioshbomyces*, *Agriosomyces*, *Capellomyces*, *Joblinomyces*, *Khoyollomyces*, *Tahromyces*) or polyflagellate (*Neocallimastix*, *Feramyces*, *Ghazallomyces*) zoospores of spherical to broadly ellipsoidal shapes (4–13 μm dia), which may germinate either endogenously or exogenously. Occasional (6–9%) biflagellate zoospores are formed (Hanafy et al., 2017, 2019) and for *Piromyces* even

four flagella have been observed (Barr et al., 1989). Polyflagellate zoospores can carry 7–17 flagella. Length of flagella, which become detached from the cells prior encystment, range between 15 and 37 μm. The vegetative stage of monocentric genera is anucleate and highly branched with sporangia of globose, ovoid, ellipsoidal, clavate, triangular, pyriform, heart-shaped, egg-shaped, subcylindrical or irregular shapes (40–185 μm long, 20–100 μm wide). The main rhizoid can be tubular or inflated below the neck of the sporangia. Ovoid-to-subovoid intercalary rhizoidal swellings were observed for *Oontomyces* (Dagar et al., 2015) and *Khoyollomyces* (Hanafy et al., 2019). The wide hyphae can display multiple constrictions at irregular intervals. Sporangia can be located on unbranched or branched sporangiophores. Sporangiophores vary in length (15–600 μm), frequently are coiled or wide and flattened, often forming an apophysis-like or eggcup-like swelling below the sporangium (Hanafy et al., 2017, 2018a,b; Joshi et al., 2018). A septum is often visible in mature zoosporangia, separating the zoosporangium from the sporangiophore (Heath et al., 1983; Callaghan et al., 2015; Joshi et al., 2018). Pleomorphism in sporangial and rhizoidal structures on different substrates is typical (Joshi et al., 2018).

6 Polycentric genera

6.1 Orpinomyces

Barr et al. (1989) described *Orpinomyces bovis* as type species of this genus and in the same year Breton et al. (1989) described *Neocallimastix joyonii* with highly similar specification. This strain was recognized to be synonymous with Barr's strain and 2 years later *N. joyonii* was placed in the new genus *Orpinomyces* and renamed to *Orpinomyces joyonii* (Li et al., 1991).

Species: Inside the genus *Orpinomyces* (MB#25326), three species have been described up to now, namely *O. bovis* (Barr et al., 1989), *O. joyonii* (Li et al., 1991), and *O. intercalaris* (Ho et al., 1994). *Orpinomyces bovis,* however, was later identified to be a synonym of *O. joyonii*.

6.2 Anaeromyces

In 1990, two papers described isolates from a new polycentric anaerobic fungal genus: *Anaeromyces mucronatus* isolated from the rumen content of a cow (Breton et al., 1990) and *Ruminomyces elegans* isolated from the rumen of a steer (Ho et al., 1990). Based on some morphological differences, *R. elegans* was described initially as a new species inside the *Anaeromyces* genera and renamed *Anaeromyces elegans* (Ho et al., 1993a). Later, Ho and Barr (1995) expressed doubts about the status of *A. elegans*. Since no culture or DNA of

A. elegans was available to obtain further clarification, it remains uncertain whether this species is synonymous to *A. mucronatus* or not. It was suggested that these two genera are synonymous and *Anaeromyces mucronatus* was determined as the valid species name.

Species: Inside the genus *Anaeromyces* (MB#27188), four species have been described up to now including *A. mucronatus* (Breton et al., 1990), *A. polycephalus*, *A. robustus* (Li et al., 2016), and *A. contortus* (Hanafy et al., 2018b). In 2012, it was suggested that the previously described *Piromyces polycephalus* (Chen et al., 2002) should be renamed into *Anaeromyces polycephalus* (Kirk, 2012) based in its ITS sequence. However, since this species is morphologically and genetically distinct from members of the *Piromyces* and *Anaeromyces*, it is more likely that it belongs to neither of these genera and should be reclassified.

Another candidate for reclassification within the *Anaeromyces is A. robustus*, which together with *Oontomyces* forms the sister clade of *Anaeromyces*. Analysis of its partial 28S rRNA sequence, however, places *A. robustus* as a unique out-group unrelated to *Anaeromyces* genus (Li et al., 2016).

6.3 Morphology of polycentric genera

Fungi with polycentric thallus have either monoflagellate (*Anaeromyces*) or polyflagellate (*Orpinomyces*) zoospores of spherical or oval shape (8–16 µm dia). Polyflagellate zoospores can carry 10–25 flagella (30–50 µm long). The vegetative stage consists of dense polynucleate rhizomycelium of extensively branched hyphae that are considerably larger than those of monocentric species. These hyphae are highly branched, large, often with numerous constrictions imparting a bead-like or sausage-like appearance (Barr et al., 1989; Breton et al., 1990; Fig. 2). Hyphae with delicate and root-like appearance were observed for *Anaeromyces* (Breton et al., 1990). Sporangia have globose, subglobose, ellipsoidal, or irregular shape (30–120 µm long, 8–80 µm wide), in *Anaeromyces* with typical acuminate (mucronate) apex (Breton et al., 1990). Sporangia of *Orpinomyces* are terminal, formed at the apices of the simple or branched sporangiophore complexes (Li et al., 1991) or intercalary developed as a small swelling in the hyphae or as a lateral outgrowth of a hypha (Ho et al., 1994). Sporangia of *Anaeromyces* can be located on erect, solitary, unbranched sporangiophore (5–100 µm long) arising laterally or terminally from the hyphae (Breton et al., 1990). Some cultures, however, fail to produce mature sporangia, and zoospores are rarely seen making classification by morphologic means difficult (Ho and Bauchop, 1991).

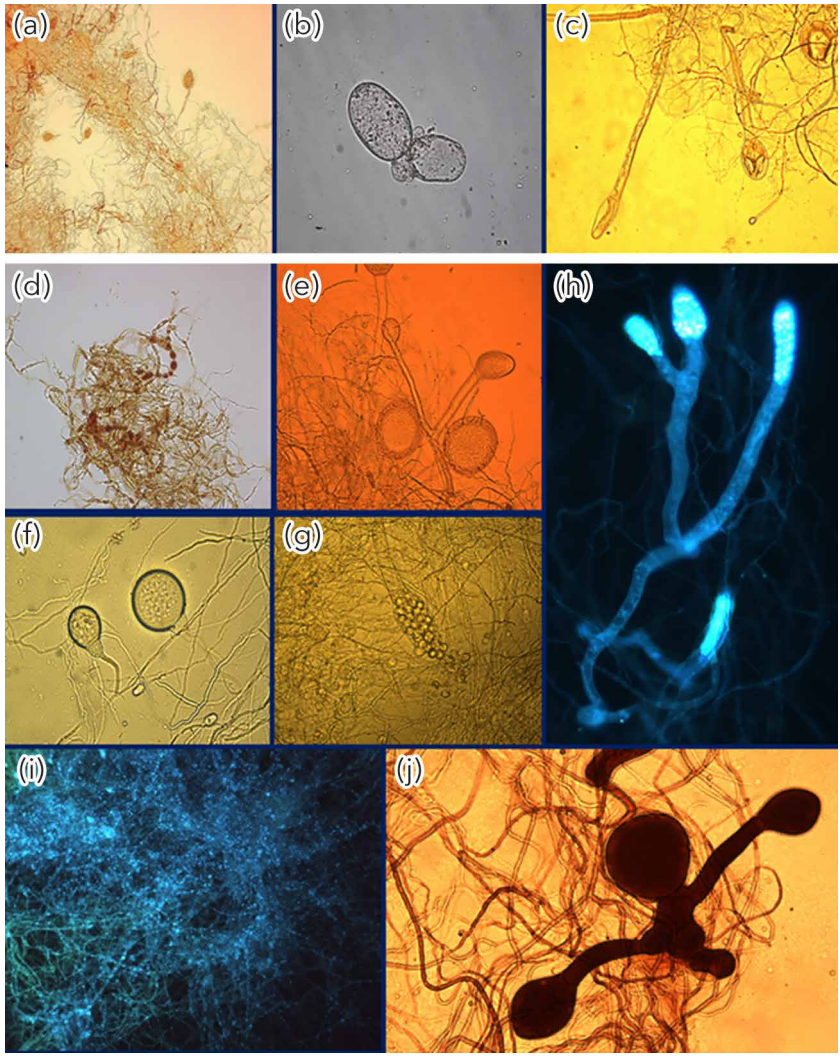

Figure 2 Features of anaerobic fungi examined by light microscopy. (a) *Anaeromyces*: sporangia with acuminate (mucronate) apex located on unbranched sporangiophore. (b) *Caecomyces*: bulbous rhizoid. (c) *Piromyces*: long unbranched sporangiophore. (d) *Anaeromyces*: hyphae with numerous constrictions imparting a sausage-like or bead-like appearances. (e) *Piromyces*: bifurcated sporangiophore. (f) *Piromyces*: sporangiophores forming an eggcup-like swelling below the sporangium. (g) *Piromyces*: release of zoospores from sporangium. (h) *Piromyces*: branched sporangiophore with nuclei concentrated in the tips (DAPI staining). (i) *Orpinomyces*: dense polynucleate rhizomycelium of extensively branched hyphae (DAPI staining). (j) *Piromyces*: irregular shape of sporangia (lugol staining).

7 Bulbous genera

7.1 Caecomyces

Strains exhibiting the *Caecomyces* morphology were originally described as *Sphaeromonas*. The generic name *Sphaeromonas* was first used by Liebetanz (1910) for flagellate protozoa and later by Orpin (1976) for fungal isolates with zoospores similar to the cultures described by Liebetanz. To emphasize that these isolates belong to the anaerobic fungi, a new genus *Caecomyces* was established by Gold et al. (1988) and *Sphaeromonas communis* was renamed to *Caecomyces communis*.

Species: Inside the genus *Caecomyces* (MB#25287), four species have been described up to now including *C. communis* (Gold et al., 1988), *C. equi* (Gold et al., 1988), *C. sympodialis* (Chen et al., 2007), and *C. churrovis* (Henske et al., 2017). The status of *C. equi* as a distinct species was questioned by Ho and Barr (1995); however, since no culture of *C. equi* is available for further study, it is difficult to determine whether this species is synonymous to *C. communis* or not.

7.2 Cyllamyces

The first representative of the genus *Cyllamyces,* capable to form a bulbous mycelium, was isolated from fresh cattle feces of milking cows in Wales (Ozkose et al., 2001). *Cyllamyces* sp. have been also been reported from rumen fluid and fresh feces of cattle and buffaloes (Sridhar et al., 2007) and from feces of bison and antelope (Liggenstoffer et al., 2010). Increased relative abundance of *Cyllamyces* was reported in dairy cows fed high-energy diet (Kumar et al., 2015). However, according to current knowledge, *Cyllamyces* is a genus that is not ubiquitous among herbivores.

Species: Inside the genus *Cyllamyces* (MB#28540), only two species, namely *C. aberensis* (Ozkose et al., 2001) and *C. icaris* (Sridhar et al., 2014), have been described up to now.

7.3 Morphology of bulbous genera

Bulbous morphology is typical for monocentric *Caecomyces* and polycentric *Cyllamyces*, both having monoflagellate zoospores of spherical, oval, ellipsoid, or amoeboid (*Caecomyces*) shape (7–9 µm dia; Fig. 2). Occasionally two or three flagella (20–30 µm long) can occur, zoospores of *Caecomyces sympodialis* can be even quadriflagellate (Chen et al., 2007). The vegetative stage of *Caecomyces* is absent in a developed branching rhizoidal system and consists of spherical or ovoid bodies (holdfast or haustoria), tubular sporangiophores, and bulbous

rhizoids. Nuclei are usually present both in sporangia (22–33 μm dia) and vegetative cells (Gold et al., 1988). Thallus development may terminate at the unisporangiate or multisporangiate stage (Gold et al., 1988). The vegetative stage of *Cyllamyces* consists of bulbous holdfast (30–50 μm dia) without rhizoids with multiple spherical or ovoid sporangia (12–15 μm dia; Ozkose et al., 2001). Sporangia can be born on a single elongate or branched sporangiophore (85-μm long). Even if authors described the *Cyllamyces* thallus as polycentric, thallus development is considered as monocentric-polysporangiate, because nuclei are present in the vegetative part of the thallus (bulbous holdfast and sporangiophores) and numerous sporangia are consistently produced (Ozkose et al., 2001).

8 Genomics of anaerobic fungi

Anaerobic fungi are recognized as key players in the decomposition of lignocelluloses, which they accomplish by a combination of physical penetration and the enzymatic activity of a diverse repertoire of cell wall-degrading enzymes. Advances in molecular and computational biology techniques resulted in the experimental and technological framework, respectively, that now enables us to decipher complex fungal genomes and the biological role of individual fungi without the need of an extensive bioinformatics network and highly trained and specialized staff. The unusually high A+T content, high number of non-coding intergenic regions, and gene duplications within fungal genomes, as well as the unknown ploidy and complex life cycles of anaerobic fungi, have made nucleotide sequencing and analysis of the obtained data rather challenging (Brownlee, 1989; Chen et al., 2006; Youssef et al., 2013; Gruninger et al., 2014).

In the past, these challenges were solved by brute force sequencing efforts, affordable to only a few institutes. An excellent example of the encountered obstacles and how they were addressed is the draft genome of *Piromyces* sp. E2, a highly prolific biomass degrader. An initial assembly using Sanger data was generated by the US Department of Energy Joint Genome Institute in 2011 (https://genome.jgi.doe.gov/PirE2_1/ PirE2_1.info.html). It took another 6 years before data generated using Illumina's short-read sequencing technology allowed to complete the initial assembly to provide useful insights into the biology of and the molecular machinery employed by *Piromyces* sp. E2 (Haitjema et al., 2017).

More recently, 'single molecule real time' (SMRT) sequencing, a technology developed by PacBio, has opened new possibilities for genome sequencing. The high error rate associated with the long reads generated with the SMRT technology can be addressed by polishing SMRT data with Illumina. The combination of the various types of 'next generation sequencing' (NGS) technologies has been used successfully to yield genomes of a variety of anaerobic rumen fungi, such

as *Pecoramyces ruminantium* (formerly classified as *Orpinomyces* sp. strain C1A; Youssef et al., 2013; Hanafy et al., 2017) as well as *Anaeromyces robustus*, *Piromyces finnis*, and *Neocallimastix californiae* (Haitjema et al., 2017).

A summary of the genomes from anaerobic rumen fungi that have been sequenced to date is provided in Table 3. Findings for these genome studies, which so far have been limited to members belonging to the *Orpinomyces*, with the exception of *Pecoramyces ruminantium* as a representative of the *Pecoramyces*, confirmed the hypothesis that cellulosomes, which are large multiprotein complexes that consolidate a wide array of plant biomass-degrading enzymes for improved biomass hydrolysis (Artzi et al., 2017), are employed by members of the Neocallimastigaceae.

8.1 Single-cell genomics

Sequencing of individual cells has become a powerful tool for deciphering genomic variation in human cells since the 1990s and a decade later when basic PCR-based methods and advanced isothermal DNA amplification approaches were developed, respectively (Zhang et al., 1992; Dean et al., 2001). It did not take much longer until single-cell genomics (SCG) was adapted successfully to sequence genomes from archaea and bacteria (Raghunathan et al., 2005; Zhang et al., 2006; Podar et al., 2013). Even draft genomes of an uncultured rumen microbe (Hess et al., 2011) and unicellular marine eukaryotes (Yoon et al., 2011; Strassert et al., 2018) were assembled. Challenges associated with SCG of fungi remain the same as those encountered with traditional sequencing approaches (e.g., higher ploidy, mitochondrial genomes, multiple chromosomes, transposable elements, and wide GC variation). However, with SCG the highly robust fungal cell walls are a more significant problem, since it hinders access to the DNA for subsequent amplification rendering only a limited number of cells of the target organism available for the downstream process

Table 3 Statistics of currently available genomes from anaerobic fungi

Name of organism	Genome size[a] (base pairs)	Numbers of genes[a]	Reference
Anaeromyces robustus	71 685 009	12 832	Haitjema et al. (2017)
Caecomyces churrovis	165 495 782	15 009	Henske et al. (2017)
Neocallimastix californiae	193 495 782	20 219	Haitjema et al. (2017)
Pecoramyces ruminantium (formerly *Orpinomyces* sp. C1A)	100 954 185	18 936	Youssef et al. (2013)
Piromyces finnis	56 455 805	10 992	Haitjema et al. (2017)
Piromyces sp. E2	71 019 055	14 648	Haitjema et al. (2017)

[a] https://genome.jgi.doe.gov/neocallimastigomycota/.

(Ahrendt et al., 2018). To overcome this obstacle, Ahrendt et al. developed a robust capture and *de-novo* assembly protocol that enabled the assembly of genomes from individual single cells of aerobic fungi up to a completeness of 88%; with an additional improvement to ~97% when combining multiple cells of the target organisms prior to sequencing (Ahrendt et al., 2018). It appears to be only a question of time before this highly powerful technology will be applied to capture genomes from anaerobic fungi.

8.2 Transcriptomics

Transcriptome as well as proteome studies, in which gene expression and protein profiles of individual organisms under a set of defined metabolic conditions are determined and compared to each other, provide a more functionalistic view than the traditional genome studies in which the overall metabolic potential of organisms is determined. The additional benefit of those two approaches when applied to eukaryotes, including anaerobic fungi, is that they render the assembly of introns and longer repetitive reads, both a major challenge in the assembly of fungal genomes, obsolete (Gruninger et al., 2014).

Hybridization-based approaches to determine the gene expression profile of bacteria were used widely in late 1990s, but gained little traction for mycology (Nilsson et al., 2019). Function-based hybridization arrays, such as the CAZyChip, FibroChip, and GeoChip, were developed to quickly quantify the expression of functional key enzymes even by complex microbial communities (Tu et al., 2014; Abot et al., 2016; Comtet-Marre et al., 2018). These arrays contain probes that allow to quantify the expression of genes that are similar enough in their sequence to bind to the probe attached to the array matrix. The need for a relatively high similarity of bait and target is a significant drawback since key genes might not possess sufficient affinity. As the cost for nucleotide sequencing and sequence analysis continuous to decrease, it is expected that these hybridization arrays will become less common.

Transcriptomic analysis of *Anaeromyces robustus*, *Neocallimastix californiae,* and *Piromyces finnis* suggested that *N. californiae* and *P. finnis* had a threefold increase in the xylanolytic activity compared to commercially available *Aspergillus* enzyme mixtures, with approximately 2% of the transcripts coding for lignocellulolytic glycoside hydrolase (GH) and other carbohydrate-active enzymes (CAZymes; Solomon et al., 2016). All three of these anaerobic fungi expressed an increased number of hemicellulases (i.e. GH10) as well as pectin-degrading enzymes compared to *Trichoderma* and *Aspergillus*, rendering them as superior in removing hemicelluloses and pectin to access the energy-rich carbohydrates of plant cells. Interestingly, *A. robustus* preferred glucose

over the more complex carbohydrates and this for simpler sugars suggests that this fungus might be more important for the degradation plant material once the more complex components of the plant fiber have been digested by other microbes (Solomon et al., 2016).

In addition to the wide array of transcripts-encoding GH families categorized as cellulases and hemicellulases, transcriptomics also revealed an increased number of genes for auxiliary enzymes (AAs) and carboxylesterase (CE) being expressed by these fungi. This explains why members of the Neocallimastigomycota, including *Pecoramyces ruminantium*, can degrade an array of lignin-rich grasses without prior pretreatment (Couger et al., 2015; Solomon et al., 2016). Wang et al. used a combination of Roche's and Illumina's NGS technologies to identify 288 unique GH-like contigs actively expressed by the anaerobic rumen fungus *Neocallimastix patriciarum* W5 in the presence of rice straw (Wang et al., 2011). These GH-like contigs were predicted to encode a variety of cellulases, hemicellulases, including members belonging to the GH10 family, chitinases, and open reading frames containing non-catalytic dockerin domains (NCDDs), the latter being characteristic for cellulosomes (Gilmore et al., 2015).

Gruninger et al. (2018) identified a large repertoire of CAZymes, accounting for 8.1%, 9%, 11.2%, and 8.9% of the total transcripts from *Anaeromyces mucronatus, Neocallimastix frontalis, Orpinomyces joyonii,* and *Piromyces rhizinflata*. Furthermore, Gruninger et al. were able to identify 12 CAZy families and ten carbohydrate-binding modules (CBMs) that were more than twofold more abundant in the transcriptome of these three anaerobic rumen fungi when compared to transcriptomes of aerobic fungi, non-rumen bacteria, and rumen bacteria (Table 4). Of these enriched CAZyme families, GH6, GH11, GH48, and CE1 as well as CBM1, CBM26, and CBM29 were even fivefold more within transcriptomes of these anaerobic fungi. More strikingly, transcripts for the cellulose-specific CBM10 and the chitin-binding CBM18 were more than 20-fold more abundant in these expression profiles (Table 4).

Due to their lack of overall similarity of these NCDDs that are part of cellulosomes to their bacterial counterparts, it was suggested previously that fungal cellulosomes evolved independently, but acquired some of the beneficial properties (i.e. catalytic domains) via horizontal gene transfer from their bacterial neighbors that coexist with the anaerobic fungi in the rumen ecosystem (Haitjema et al., 2017). First insights into the gene expression of *Caecomyces churrovis,* an anaerobic fungus that lacks the extensive rhizoidal system that is characteristic for many of the other known gut fungi, were obtained via Illumina's short-read data (Henske et al., 2017). Henske et al. were able to show that *C. churrovis* contained the significant lower fraction (15%) of CAZyme transcripts that contained NCDDs, compared to the ~30%

Table 4 CAZyme profile of anaerobic fungi

		Gruninger et al. (2018)				Solomon et al. (2016)[h]/Haitjema et al. (2017)[i]					Wang et al. (2011)[j]	Henske et al. (2017)[k]	Couger et al. (2015)[h]
	Main known activity[c]	*Anaeromyces mucranatus*[d]	*Neocallimastix frontalis*[d]	*Orpinomyces joyonii*[d]	*Piromyces rhizinflata*[d]	*Anaeromyces robustus*	*Neocallimastix californiae*	*Pecoramyces ruminantium*	*Piromyces finnis*	*Piromyces* sp. E2	*Neocallimastix patriciarium* W5	*Caecomyces churrovis*	*Pecoramyces ruminantium*
Glycoside hydrolase (GH) families[a]													
Cellulases													
GH5	Cellulase	24[e]	40[e]	31[e]	49[e]	22/10	44/33	45/24	25/14	NR/9	20	19(1)	36
GH6	Endoglucanase	12[f]	35[f]	31[f]	27[f]	3/12	18/9	46/32	1/17	NR/22	35	27(8)	18
GH7	Endoglucanase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	0	0	NR
GH9	Endoglucanase	14[e]	13[e]	11[e]	14[e]	14/8	24/13	14	8	NR/11	12	29(11)	21
GH44	Endoglucanase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
GH45	Endoglucanase	14[e]	22[e]	16[e]	20[e]	13/7	23/15	13/11	7/9	NR/5	14	26(12)	16
GH48	Endo-processive cellulase	6[f]	22[f]	19[f]	16[f]	7/6	15/16	14/6	313	NR/11	12	25(5)	17
	Total	**70**	**132**	**108**	**126**	**59/43**	**124/86**	**132/81**	**44/64**	**-/58**	**93**	**126(37)**	**108**
Hemicellulases													
GH8	Endoxylanase	1	2	4	2	1/2	4/1	1/0	2/1	NR/1	NR	2(1)	2
GH10	Endo-1,4-β-xylanase	10[e]	35[e]	23[e]	25[e]	14/6	60/24	30/11	24/12	NR/4	21	15(2)	28
GH11	Xylanase	24[f]	41[f]	23[f]	49[f]	24/11	47/9	43/15	11/10	NR/27	11	123(12)	24
GH12	Endoglucanase and xyloglucan hydrolase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	0	NR	NR
GH26	β-Mannanase and xylanase	3	10	1	7	NR/2	NR/12	NR/0	NR/2	NR/4	4	NR	7
GH28	Galacturonase	0	0	1	0	0/NR	0/NR	0/NR	0/NR	NR/NR	NR	NR	5
GH53	Endo-1,4-b-galactanase	1	2	1	2	NR/1	NR/2	NR/0	NR/1	NR/0	1	NR	2
	Total	**39**	**90**	**53**	**85**	**39/22**	**111/48**	**74/26**	**37/26**	**-/36**	**37**	**126(15)**	**68**

Debranching enzymes													
GH51	α-*L*-arabinofuranosidase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	0	NR	NR
GH54	α-*L*-arabinofuranosidase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	0	NR	NR
GH62	α-*L*-arabinofuranosidase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	0	NR	NR
GH67	α-glucuronidase	1	0	0	1	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	0	NR	1
GH78	α-L-rhamnosidase	1	1	1	1	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	1
	Total	**2**	**1**	**1**	**2**	**-**	**-**	**-**	**-**	**-**	**0**	**0**	**-**
Oligosaccharide-degrading enzymes													
GH1	β-glucosidase and other β-linked dimers	5	11	11	6	10/NR	14/NR	16/NR	4/NR	NR/NR	7	20(0)	9
GH2	β-glucosidase and other β-linked dimers	2	2	1	1	NR/0	NR/6	NR/0	NR/0	NR/1	NR	NR	1
GH3	mainly β-glucosidases	13	18	15	10	16/3	30/3	17/2	13/3	NR/2	10	16(3)	17
GH29	α-*L*-fucosidase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	0	0	NR
GH35	β-galactosidase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
GH38	α-mannosidase	1	1	1	1	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	1
GH39	β-xylosidase	2	4	6	3	4/5	7/6	0/1	1/2	NR/3	NR	3(2)	9
GH42	β-galactosidase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
GH43	Arabinases and xylosidases	24[e]	37[e]	30[e]	18[e]	15/8	32/14	24/6	7/10	NR/7	20	59(10)	32
GH52	β-xylosidase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
	Total	**47**	**73**	**64**	**39**	**45/16**	**83/29**	**57/9**	**25/15**	**-/13**	**37**	**98(15)**	**-**

(*Continued*)

Table 4 (*Continued*)

		Gruninger et al. (2018)				Solomon et al. (2016)[h]/Haitjema et al. (2017)[i]					Wang et al. (2011)[j]	Henske et al. (2017)[k]	Couger et al. (2015)[h]
	Main known activity[c]	*Anaeromyces mucranatus*[d]	*Neocallimastix frontalis*[d]	*Orpinomyces joyonii*[d]	*Piromyces rhizinflata*[d]	*Anaeromyces robustus*	*Neocallimastix californiae*	*Pecoramyces ruminantium*	*Piromyces finnis*	*Piromyces* sp. E2	*Neocallimastix patriciarium* W5	*Caecomyces churrovis*	*Pecoramyces ruminantium*
Auxiliary activities (AA) families[b]													
AA1	Laccase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
AA2	Manganese peroxidase and lignin peroxidase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
AA3	Cellobiose dehydrogenase and aryl alcohol oxidase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
AA4	Vanillyl-alcohol oxidase	1	2	1	1	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
AA5	Galactose oxidase and alcohol oxidase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
AA6	1,4-benzoquinone reductase	1	1	1	2	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
AA7	Glucooligos-accharide oxidase and chitooligos-accharide oxidase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
AA8	Iron reductase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
AA9 (formerly GH61)	Copper-dependent lytic polysaccharide monooxygenases (LPMOs)	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR

AA10 (formerly CBM33)	Copper-dependent lytic polysaccharide monooxygenases (LPMOs)	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
AA11	Copper-dependent lytic polysaccharide monooxygenases (LPMOs)	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
AA12	Pyrroloquinoline quinone-dependent oxidoreductase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
AA13	Copper-dependent lytic polysaccharide monooxygenases (LPMOs)	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
	Total	**2**	**3**	**2**	**3**	-	-	-	-	-	-	-	-
Carboxyl esterase (CE) families[b]													
CE1	Acetyl xylan esterase and cinnamoyl esterase	22[f]	29[f]	21[f]	17[f]	NR/13	NR/25	NR/20	NR/10	NR/16	NR	NR	67
CE2	Acetyl xylan esterase	2[e]	8[e]	3[e]	5[e]	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	4
CE3	Acetyl xylan esterase	6	8	2	5	NR/0	NR/1	NR/1	NR/0	NR/1	NR	NR	9
CE4	Acetyl xylan esterase and chitin deacetylase	34[e]	53[e]	32[e]	49[e]	53/1	85/0	48/1	41/3	NR/0	NR	NR	66

(*Continued*)

Table 4 (*Continued*)

		Gruninger et al. (2018)				Solomon et al. (2016)[h]/Haitjema et al. (2017)[i]					Wang et al. (2011)[j]	Henske et al. (2017)[k]	Couger et al. (2015)[h]
	Main known activity[c]	*Anaeromyces mucranatus*[d]	*Neocallimastix frontalis*[d]	*Orpinomyces joyonii*[d]	*Piromyces rhizinflata*[d]	*Anaeromyces robustus*	*Neocallimastix californiae*	*Pecoramyces ruminantium*	*Piromyces finnis*	*Piromyces* sp. E2	*Neocallimastix patriciarium* W5	*Caecomyces churrovis*	*Pecoramyces ruminantium*
CE5	Acetyl xylan esterase and cutinase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
CE6	Acetyl xylan esterase	8[e]	12[e]	12[e]	19[e]	NR/8	NR/8	NR/4	NR/4	NR/7	NR	NR	10
CE7	Acetyl xylan esterase and cephalosporin-C deacetylase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	4
CE8	Pectin methylesterase	4	3	2	9	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	5
CE9	*N*-acetylglu-cosamine 6-phosphate deacetylase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	3
CE11	UDP-3-*O*-acyl *N*-acetylglu-cosamine deacetylase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
CE12	Pectin acetylesterase and acetyl xylan esterase	8	4	6	8	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	4
CE13	Pectin acetylesterase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR

CE14	*N*-acetyl-1-*D*-myo-inosityl-2-amino-2-deoxy-α-*D*-glucopyranoside deacetylase	1	1	1	1	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	1
CE15	4-*O*-methyl-glucuronoyl methylesterase	3	4	3	3	NR/2	NR/4	NR/0	NR/1	NR/0	NR	NR	2
CE16	Acetylesterase	6	9	3	14	NR/2	NR/3	NR/1	NR/1	NR/0	NR	NR	12
	Total	**94**	**131**	**85**	**130**	**53/26**	**85/41**	**48/27**	**41/19**	**0/24**	**-**	**-**	**187**
Polysaccharide lyase (PL) families[b]													
PL1	Pectate lyase and pectin lyase	6	9	8	13	NR/1	NR/0	NR/0	NR/1	NR/0	NR	NR	21
PL3	Pectate lyase	1	5	5	10	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	9
PL4	Rhamnogalac-turonan endolyase	3	4	3	7	NR/1	NR/1	NR/1	NR/1	NR/1	NR	NR	4
PL9	Pectate lyase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	1
PL10	Pectate lyase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	1
PL11	Rhamnogala-cturonan lyase	0	1	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	1
PL22	Oligogalacturonate lyase	0	0	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	5
	Total	**10**	**19**	**16**	**30**	**-/2**	**-/1**	**-/1**	**-/2**	**-/1**	**-**	**-**	**42**
Carbohydrate-binding module (CBM) families[b]													
CBM1	Cellulose and chitin	81[f]	106[f]	85[f]	119[f]	NR/47	NR/26	NR/24	NR/46	NR/16	1	NR	NR
CBM2	Cellulose, chitin, and xylan	2	2	1	3	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR

(*Continued*)

Table 4 (*Continued*)

		Gruninger et al. (2018)				Solomon et al. (2016)[h]/Haitjema et al. (2017)[i]					Wang et al. (2011)[j]	Henske et al. (2017)[k]	Couger et al. (2015)[h]
	Main known activity[c]	*Anaeromyces mucranatus*[d]	*Neocallimastix frontalis*[d]	*Orpinomyces joyonii*[d]	*Piromyces rhizinflata*[d]	*Anaeromyces robustus*	*Neocallimastix californiae*	*Pecoramyces ruminantium*	*Piromyces finnis*	*Piromyces* sp. E2	*Neocallimastix patriciarium* W5	*Caecomyces churrovis*	*Pecoramyces ruminantium*
CBM6	Cellulose and β-1,4-xylan	10[e]	11[e]	15[e]	5[e]	NR/4	NR/5	NR/4	NR/6	NR/5	1	NR	NR
CBM10	Cellulose	275[g]	424[g]	283[g]	390[g]	NR/3	NR/8	NR/10	NR/10	NR/0	16	NR	NR
CBM12	Chitin	3	1	1	2	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
CBM13	Galactose, mannose, and xylan	27[e]	41[e]	35[e]	24[e]	NR/17	NR/38	NR/15	NR/15	NR/6	NR	NR	NR
CBM18	Chitin	95[g]	193[g]	105[g]	159[g]	NR/1	NR/3	NR/1	NR/0	NR/4	NR	NR	NR
CBM20	Starch	3	4	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
CBM21	Starch	6[e]	11[e]	5[e]	6[e]	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
CBM22	Xylan and mixed β-1,3/β-1,4-glucans	2	1	1	0	NR/0	NR/1	NR/0	NR/1	NR/0	NR	NR	NR
CBM25	Starch	2[e]	3[e]	8[e]	1[e]	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
CBM26	Starch	6[f]	11[f]	22[f]	2[f]	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
CBM29	Mannan/ glucomannan	7[f]	9[f]	9[f]	16[f]	NR/1	NR/8	NR/9	NR/5	NR/2	NR	NR	NR
CBM32	Galactose, lactose and polygalacturonic acid	0	3	0	2	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
CBM35	Xylan and mannoolig-osaccharides, β-galactan	3	3	2	8	NR/2	NR/6	NR/0	NR/2	NR/2	NR	NR	NR
CBM36	Xylans and xylooligosacc-harides	0	1	0	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR

CBM48	Glycogen	6	8	5	6	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
CBM50	Chitopentaose	2	4	4	5	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
CBM52	β-1,3-glucan	3	6	4	5	NR/1	NR/1	NR/0	NR/1	NR/0	NR	NR	NR
CBM61	β-1,4-galactan	3	1	1	2	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
CBM63	Cellulose	8[e]	5[e]	5[e]	8[e]	NR/1	NR/3	NR/2	NR/2	NR/0	NR	NR	NR
CBM66	Fructans	1	0	1	0	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
CBM67	L-rhamnose	1	1	1	1	NR/NR	NR/NR	NR/NR	NR/NR	NR/NR	NR	NR	NR
	Total	**546**	**849**	**593**	**764**	**-/77**	**99**	**-/65**	**-/88**	**-/35**	**-**	**-**	**-**

[a] Allgaier et al. (2010).
[b] Gruninger et al. (2018).
[c] Main substrate for carbohydrate-binding modules.
[d] Number of detected transcripts.
[e] Carbohydrate active enzyme families and carbohydrate binding module families for which transcripts were more than two-times more abundant in transcriptomes from anaerobic fungi than in those from aerobic fungi or anaerobic or aerobic bacteria.
[f] Carbohydrate active enzyme families and carbohydrate binding module families for which transcripts were more than five-times more abundant in transcriptomes from anaerobic fungi than in those from aerobic fungi or anaerobic or aerobic bacteria.
[g] Carbohydrate active enzyme families and carbohydrate binding module families for which transcripts were >20-times more abundant in transcriptomes from anaerobic fungi than in those from aerobic fungi or anaerobic or aerobic bacteria.
[h] Number of detected transcripts.
[i] Number of detected genes with non-catalytic dockerin domain.
[j] Number of expressed genes encoding for carbohydrate-active enzyme family.
[k] Number of detected transcripts (number of transcripts containing at least one dockerin domain).
NR = Not reported.

Figure 3 The carbohydrate-active enzyme (CAZyme) repertoire of anaerobic rumen fungi. (a) Transcripts of CAZymes detected in the expression profile of *Anaeromyces mucranatus*, *Neocallimastix frontalis, Orpinomyces joyonii,* and *Piromyces rhizinflata* (Gruninger et al., 2018). Only the five most abundant CAZyme families detected in the transcriptomes of all four organisms are reported. (b) CAZymes detected in the expression profile of *A. robustus, N. californiae, Pecoramyces ruminantium,* and *P. finnis* (Solomon et al., 2016) and in the genomes of *A. robustus, N. californiae, Pecoramyces ruminantium*, *P. finnis*, and *Piromyces* sp. E2 (Haitjema et al., 2017). Only the five most abundant CAZyme families detected in both the transcriptomes of *A. robustus, N. californiae, Pecoramyces ruminantium,* and *P. finnis* and in the genomes of all five organisms (i.e. *A. robustus, N. californiae, Pecoramyces ruminantium*, *P. finnis,* and *Piromyces* sp. E2) are reported. (c) CAZymes detected in the expression profile of *Neocallimastix patriciarium* W5 (Wang et al., 2011). Only the five most abundant CAZyme families detected are reported. (d) CAZymes detected in the expression profile of *Caecomyces churrovis* (Henske et al., 2017). Only the five most abundant CAZyme families detected are reported. (e) CAZymes detected in the expression profile of *Pecoramyces ruminantium* (Couger et al., 2015). Only the five most abundant CAZyme families detected are reported.

of CAZyme transcripts associated with at least one NCDD that has been reported for rhizoid forming anaerobic fungi. This observation let Henske et al. (2017) hypothesize that instead of a cellulosome-based biomass-degradation strategy, *C. churrovis* appears to rely more heavily on free CAZymes to degrade plant carbohydrates.

While recent genome and transcriptome studies have provided significant insights into the molecular machinery employed by a number of anaerobic fungi, the differences in data acquisition, analysis, and reporting make a direct comparison of the data rather difficult. Despite these challenges, results from these studies have confirmed that fungal CBMs as well as fungal CAZymes-containing catalytic domains play a key role in the degradation of complex plant material in the anaerobic rumen ecosystem. GH families belonging to the cellulases reported for all anaerobic fungi for which genomes are available are GH5, GH6, GH9, GH45, and GH48. Interestingly, representatives of these GHs were also always found to be associated with cellulosome-specific CBMs in studies that reported sufficient details (Fig. 3 and Table 4). The same is the case for GH10 and GH11, which are predicted to target xylose and a set of GH classified as oligosaccharide-degrading (Table 4). Although the repertoire of hemicellulases employed by the anaerobic fungi always contained members of the GH10 and GH11, a slight variation in the set of additional GH families was observed for the degradation of hemicelluloses components by the individual fungi (Fig. 3). These additional hemicellulases belonged to either the GH8, GH26, and GH53, which are classified as families comprised of enzymes possessing endoxylanase, xylanase, and endo-1,4-β-galactanase activity, respectively.

Despite these ubiquitous CAZymes, more detailed transcriptome studies have revealed the importance of the previously often neglected AAs, CEs, and polysaccharide lyases (PLs). These enzymes enhance the catalytic activity of the historically more studied GH by breaking down or modifying the recalcitrant structural components surrounding the crystalline cellulose core, thus mechanically weakening the cellulose structure (Ochiai et al., 2007; Gilbert, 2010; Makela et al., 2018). Transcripts classified as AA4 and AA6 were identified for *A. mucranatus, N. frontalis, O. joyonii,* and *P. rhizinflata* (Gruninger et al., 2018), whereas CEs (i.e. CE1, CE4, and CE6) were reported from the transcriptome of *Pecoramyces ruminantium* (Couger et al., 2015), *A. robustus, N. californiae, P. finnis,* and the genome of *Piromyces* sp. E2 (Solomon et al., 2016; Haitjema et al., 2017). PLs of the PL1, PL3, and PL4 family were reported for *A. mucranatus, N. frontalis, O. joyonii,* and *P. rhizinflata* (Gruninger et al., 2018) and *Pecoramyces ruminantium*, with PL5 and PL9 transcripts also detected from *Pecoramyces ruminantium* (Couger et al., 2015). Members of the PL4-containing NCDD were also detected in the genomes of *A. robustus*, *N.*

californiae, *Pecoramyces ruminantium*, *P. finnis*, and *Piromyces* sp. E2 (Haitjema et al., 2017). Figure 3 shows the most abundant CAZyme families detected across the different transcriptomes and genomes of anaerobic fungi. A more detailed breakdown of the different CAZyme families identified from each individual anaerobic rumen fungus can be found in Table 4.

Gene expression is a complex and tightly regulated process that remains poorly understood. Transcription of non-coding RNA strand that then interacts with the coding strand has been reported as a major mechanism for regulating gene expression control across all domains of life (Wagner and Simons, 1994; Katayama et al., 2005; Donaldson and Saville, 2012; Britto-Kido Sde et al., 2013). Antisense-based expression regulation has been shown to adapt rapidly to evolutionary pressure (Yan and Wang, 2012), but more importantly can achieve a switch-like response (Pelechano and Steinmetz, 2013), which explains why natural antisense transcripts (NATs) have been found throughout fungal genomes and to play vital roles in their metabolism. A recent study (Solomon et al., 2018) revealed that NAT-mediated gene regulation is conserved within the early branching anaerobic rumen fungi (i.e., *A. robustus*, *N. californiae*, and *P. finnis*). Although ubiquitous in the three studied specimen, overall NAT abundance was low compared to other fungi, which led the authors to the conclusion that this might reflect the early divergence of anaerobic fungi and the significant rate of horizontal gene transfer with bacteria. Of the NAT-regulated processes identified during the comparative analysis, 10% were associated with lignocellulose degradation, which suggests that in order to fully understand the biomass-degrading phenotype, and most likely other phenotypes as well, of anaerobic fungi might require a thorough analysis of the corresponding NAT profiles.

To our knowledge, there is no stand-alone proteomic survey that aims to generate a global protein profile or identify previously unknown enzymes of anaerobic fungi as of today. Protein profiles generated to date have only been used to determine which of the previously identified CAZyme families or solute transporters are involved in the carbohydrate metabolism of *Neocallimastix patriciarium* W5, *A. robustus, N. californiae, Pecoramyces ruminantium,* and *P. finnis* (Wang et al., 2011; Seppala et al., 2016; Solomon et al., 2016). Secretion of proteins belonging to the cellulases (i.e. GH6, GH9, GH45, and GH48), hemicellulases, (i.e. GH10 and GH11), as well as other oligosaccharide-degrading enzyme families (i.e. GH1, GH3, and GH43) were verified via mass spectrometry for *N. patriciarum* W5 (Wang et al., 2011). *Anaeromyces robustus, N. californiae,* and *P. finnis* secreted the same repertoire of cellulases and hemicellulases (Solomon et al., 2016), providing additional support to the hypothesis that similar protein repertoires are employed by these organisms to overcome the recalcitrance of complex plant polymers.

9 Meta-omics of anaerobic fungi

9.1 Metagenomics

Utilizing nucleotide sequencing to obtain a better understanding of the assemblage and the potential function of a microbial community in a given environment has become a standard approach in microbial ecology. In the simplistic form of metagenomics that has been well established for several years now, amplicons are generated from hypervariable regions of the 16S rRNA gene, which then facilitate to generate abundance profiles of the prokaryotic population at relatively low costs. Although shotgun metagenomics, in which whole genomes of archaea, bacteria, and even viruses can be assembled from the generated short-read data, amplicon sequencing often remains the first step to obtain initial insights into the microbial community and its potential response to external factors.

Despite the limitation of amplicon sequencing, such as PCR bias and limited resolution, the obtained data remains of great value, when having to plan subsequent experimental and analyses approaches for more complex studies that involve shotgun metagenomics or transcriptomics, since those are more resource intensive (Turaev and Rattei, 2016; Staley and Sadowsky, 2018). For fungal community profiles, the ITS region of the nuclear rRNA operon is commonly used as the taxonomic barcode of choice (Schoch et al., 2012). Molecular surveys of anaerobic fungi have mainly focused on the ITS1 region and have provided insights into the diversity and structure of the fungal community associated with the digestive tract of a variety of herbivorous animals (Dill-McFarland et al., 2019; Mura et al., 2019).

When only a limited number of known fungi are of interest, probe-specific approaches such as the automated ribosomal intergenic spacer region analysis developed by Denman et al. (2008) can be sufficient and allow to track abundances of the targets over an extended period. Recently, Dollhofer et al. (2016) published a PCR-based approach that allows a quick and rather inexpensive option for an initial assessment of the assemblage of the fungal population and its cellulolytic activity by targeting regions of the 18S rRNA and 28S rRNA genes as well as a gene that codes for a endoglucanase of the GH5. Despite these advances in the area of fungal amplicon sequencing, the major challenge remains the rather poor resolution that can be achieved with any of the fungal taxonomic marker genes, a problem that might eventually be overcome with an increasing number of cultivars and, therefore, with increasing reference databases of these marker genes.

Shotgun metagenomics in which the environmental DNA is directly sequenced and then assembled into long contagious DNA fragments (contigs) and eventually into draft genomes provides the opportunity to obtain a more

holistic understanding of the functional potential of the microbiome associated with any given environment. Morphological and genomic characteristics that have hindered the acquisition and analysis of genomic information from individual anaerobic fungi as well as the low natural abundance of anaerobic fungi and the use of sample preparation techniques, such *in-sacco* techniques that use nylon bags with pores sizes that hinder the larger and slower replication rumen fungi from colonizing rumen incubated biomass, might be significant factors that have prevented the co-assembly of fungal contigs from metagenome data. There have been the few occasional reports of successful co-assembly of longer eukaryotic fragments from metagenome data, but these occasions have been rare (Sharon et al., 2013; Kantor et al., 2015, 2017; Quandt et al., 2015; Mosier et al., 2016; Raveh-Sadka et al., 2016). In order to assemble eukaryotic fragments from metagenome data of diverse microbial communities, the Banfield Lab at UC Berkeley developed a *k-mer*-based approach called *EukRep* to identify assembled eukaryotic sequences in data sets from diverse environmental samples, which then improve the quality of gene predictions and further binning decisions (West et al., 2018). When they tested this approach on 268 Gbp of rumen metagenome data they published previously (Hess et al., 2011), *EukRep* did not identify any eukaryotic assemblies, emphasizing the importance of sampling strategy (unpublished data). In this particular case, plant material was incubated for 72 h in rumen of a fistulated cow after the biomass was ground and placed in an *in-situ* nylon bag with a pore size of 50 µm, which allowed rumen prokaryotes to enter and colonize the ground biomass, but might have resulted in insufficient colonization of the fiber by anaerobic rumen fungi in order to detect their genetic material in the generated metagenome.

9.2 Metatranscriptomics

Similar to expression profile analyses of individual fungal isolates, many of the bioinformatic challenges associated with fungal metagenomics can be addressed via metatranscriptomics approaches. However, as of today, insights into the expression profile of the fungal population associated with the rumen are rather a side product of whole community expression profiles in which prokaryotic transcripts make up the majority of the generated reads. In a benchmark paper, Qi et al. (2011) reported 59 129 contigs, with an average length of 310 base pairs (bp) that were assembled from 2.8 Gbp of metatranscriptome sequence data (Qi et al., 2011). These data were generated after total mRNA of solid rumen content from two muskoxen was enriched for polyadenylated mRNA, a feature of RNA processing in eukaryotes (Stewart, 2019). Of the reads predicted to encode proteins, ~14.4% were classified as fungal, with approximately half of them being derived from members belonging

to the Neocallimastigomycota phylum. Further analysis revealed the presence of a variety of CAZymes, including GH classified as cellulases (i.e. GH5, GH6, GH7, GH9, GH45, and GH48), endocellulases (i.e. GH8, GH10, GH11, GH26, and GH28), debranching enzymes (i.e. GH67 and GH78), and oligosaccharide-degrading enzymes (i.e. GH1, GH2, GH3, and GH38), as well as a variety of CEs, PLs and CBMs, such as CBM1, CBM6, CBM10, CBM13, CBM18, and CBM29. Qi et al. also identified several highly expressed genes that contained multiple CAZyme domains, in many cases a combination of a CBM10 and a GH6 or GH48, suggesting that anaerobic fungi utilize cellulosome that contain GH6 or GH48 catalytic domains. Overall, this work provided firsthand *omics* evidence that the fungal population is a major player in the degradation of complex carbohydrates and utilizes a multi-modular machinery of enzymes that facilitate the breakdown of recalcitrant plant material within the rumen ecosystem.

Metatranscriptome data of a mixed microbial community generated from the rumen of two dairy cows 1 h after feeding, when active degradation in the rumen is known to occur, revealed that only ~0.12% of the total reads were derived from rumen fungi (Dai et al., 2015). Of these fungal reads, the majority were assigned to be from members of the *Piromyces* or the *Neocallimastix*, with 54% and 41% of the fungal reads, respectively. Despite the low abundance of the fungal transcripts, they represented ~14% of the total GH48 that were identified, suggesting that they are a major producer of these proteins. Furthermore, fungal transcripts-encoding cellulases (i.e. GH6, GH9, GH48), hemicellulases (i.e. GH10 and GH11), β-glucosidases (i.e. GH1 and GH3), as well as CBM6 and the cellulosome-specific CBM10 were detected. A similar study that utilized Illumina's paired-end sequencing technology to generate an expression profile from mixed-community mRNA revealed that anaerobic fungi contribute ~7.5% of the active free-floating community in lactating dairy cows with members of the *Neocallimastigacea* contributing to the gene expression profile with significant amounts of cellulases transcripts (Sollinger et al., 2018). These findings suggest that the role of anaerobic fungi in plant biomass degradation within the rumen ecosystem has been underestimated. The increased abundance of fungal cellulase transcripts in both these metatranscriptomes from mixed-rumen mRNA correlates with their previously suggested importance in rendering recalcitrant biomass more accessible, via mechanical and enzymatic means, for subsequent colonization by microbes such as *Prevotellacea* that possess genomes sporting a rich repertoire of genes related to hemicelluloses, starch, and protein degradation but not for cellulose degradation (Flint et al., 2012; Accetto and Avgustin, 2015). However, it will be essential to obtain a more detailed insight into the fungal repertoire of genes that are actively expressed during biomass degradation and more fungi-targeted meta-omics approaches such as the one by Foster et al. (Qi et al.,

2011) will be essential. Otherwise, we will keep evaluating the tip of the fungal iceberg.

10 Interactions between rumen fungi and other components of the rumen ecosystem

10.1 Interaction between anaerobic fungi and methanogens

In the rumen, anaerobic fungi coexist with a diverse population of bacteria, protozoa, methanogenic archaea, and bacteriophages. Except for methanogens and bacteriophages, these microbes produce enzymes required for the breakdown and fermentation of feed material, resulting in the production of end products such as volatile fatty acids (mainly acetate, propionate, and butyrate), hydrogen, and carbon dioxide. When grown with methanogens, the fermentation profile of anaerobic fungi was shifted from electron sink products (i.e. ethanol and lactate) to more reduced products (i.e. acetate and formate; Theodorou et al., 1996). In methanogenic coculture of anaerobic rumen fungi, acetate was a major product and carbon dioxide increased, whereas lactate and ethanol decreased (Bauchop and Mountfort, 1981). In addition to the changes in fermentation, methanogenic coculture showed significant increase in fungal biomass because of the removal of fermentation inhibitory intermediates (i.e. ethanol, formate, and lactate).

Major metabolic products of anaerobic fungi include acetate, formate, hydrogen, and carbon dioxide. Hence, it is not surprising that methanogens have been reported to be physically associated with their surface where they can mediate interspecies hydrogen transfer and contribute to an energetically favorable disposal of electrons through methanogenesis (Edwards et al., 2017; Li et al., 2019). Many studies have now found syntrophic associations of methanogens with anaerobic fungi (Cheng et al., 2009; Jin et al., 2011, 2014; Leis et al., 2014). A novel rumen methanogen species belonging to uncultured archaea group 'Rumen Cluster C', subsequently classified as *Methanomassiliicoccus* (Seedorf et al., 2014; Paul et al., 2015), was found associated with anaerobic fungal cultures (Jin et al., 2014). Jin et al. (2011) reported *Methanobrevibacter* spp. as a dominant methanogenic group associated with the *Piromyces*, *Anaeromyces,* and *Neocallimastix*. Leis et al. (2014) established a syntrophic coculture of *Caecomyces communis* with methanogens belonging to the order of *Methanobacteriales*. Sun et al. (2014) also obtained three cocultures of *Methanobrevibacter olleyae* such as strains with *Piromyces*, *Neocallimastix,* and *Caecomyces*, and one *Neocallimastix/ Methanobrevibacter thaueri* coculture. The positive effect of this syntrophic association has also been observed on fungal growth (Cheng et al., 2009; Li et al., 2019), substrate utilization (Bootten et al., 2011), enzyme production

(Teunissen et al., 1992), fermentation products (Cheng et al., 2013), and resistance to toxic carboxylic ionophores (Stewart and Richardson, 1989). However, the interactions of these fungi with protozoa and some rumen bacteria, especially the ones competing for the same substrate, have mainly been classified as competitive. Conversely, certain groups of rumen bacteria that lack efficient lignocellulolytic machinery benefit positively from anaerobic fungi because of increased access to internal parts of lignocelluloses following physical degradation by fungal rhizoids.

10.2 Effect of feed composition on fungal rumen population

The addition of grain to herbage being consumed by ruminants is a common means of increasing both the energy density of diet and the intake of available carbohydrates, but the inclusion of easily fermentable starchy concentrates in diet had different effects on anaerobic fungi. Grain additions supported slightly lower populations of anaerobic fungi in the rumen (Orpin, 1977a; Gordon, 1985; Grenet et al., 1989), while addition of maize to a sorghum-silage diet increased the degradation ability of anaerobic fungi, when tested *in vitro* (Akin and Windham, 1989). Also, the addition of a mainly grain-containing concentrate to a hay diet substantially increased the count of fungal zoospores in the rumen of sheep (>20-fold) but the increase in fungal biomass was only one- to twofold (Faichney et al., 1997). A possible explanation for these apparent differences may be that only some anaerobic fungi, generally species of the genera *Neocallimastix*, *Piromyces,* and *Orpinomyces*, produced amylases and, therefore, possessed the ability to ferment starch (Phillips and Gordon, 1988, 1995; Yanke et al., 1993; Mountfort, 1994). The attack on cereal grains by amylolytic anaerobic fungi was documented by McAllister et al. (1993), and it seems to be apparent that complex interactions occur within the rumen microbiome. Currently, there is no common conclusion relating to effects on rumen fungal populations. The feeding of free lipid to ruminants can have a detrimental effect on rumen fermentation, retarding fiber degradation (Jenkins, 1993), an important consideration given the increasing use of lipid-containing oilseed meals. Anaerobic fungi, as one component of the rumen microbial population, were adversely affected by the addition of lipid to the diet. The addition of rapeseed oil led to a considerable decrease in the fungal population but the mechanism was not elucidated (Fonty and Grenet, 1994). Elliott et al. (1987) and Calderon-Cortes et al. (1989) found that feeding a supplement of sunflower meal to sheep consuming a barley straw diet resulted in depression of the fungal population in the rumen to below detectable levels. Fungal zoospore counts in the rumen were reduced below detectable levels and fungal DNA could not be detected when a supplement of cottonseed meal was provided to sheep. The feeding of calcium salts of medium-chain

fatty acids (C6-l2) to sheep resulted in reduced numbers of fungal zoospores in the rumen, whereas the salts of long-chain fatty acids (C12-14) had no effect on anaerobic fungi (Ushida et al., 1991), indicating that the inhibitory effects of the long-chain fatty acids common in oilseed meals can be alleviated, at least partly, by chemical pretreatment.

10.3 Effect of anaerobic rumen fungi on metabolic intermediates for host utilization

Studies have shown an increase in the anaerobic fungal population in animals fed recalcitrant lignocelluloses diets, which can be attributed to the unique ability of these fungi to penetrate, colonize lignified tissues, and solubilize plant cell walls (Akin et al., 1983; Grenet et al., 1989), thus facilitating subsequent plant fermentation by bacteria. Fungi are abundant in animals fed on stalky, hard stem diets and low in numbers when fed on soft leafy diets (Grenet et al., 1989). The removal of fungi from sheep rumen decreased straw digestion (Calderon-Cortes et al., 1989) and was reversed when fungi were reintroduced in the system. The *in-vivo* digestibility increased from 3% to 8% in the presence of anaerobic fungi (Elliott et al., 1987; Gordon and Phillips, 1993; Kumar et al., 2004; Dayananda et al., 2007; Tripathi et al., 2007; Sehgal et al., 2008; Saxena et al., 2010). *Neocallimastix* sp. and *Piromonas* sp. were better than *Caecomyces* sp. in degrading fragments of plant tissues, possibly because filamentous rhizoids are more effective than bulbous rhizoids at penetrating harder tissues (Orpin, 1989), and addition of *Neocallimastix* to mixed-rumen bacteria during culturing increased the degradation rate of wheat straw by up to 15% (Hillaire and Jouany, 1989). Taking these observations into consideration, it appears to be extremely likely that anaerobic rumen fungi play a significant role in the degradation of fibrolytic feedstuff and rendering otherwise not digested carbohydrates available for further metabolic use in the host animal.

Anaerobic fungi also contribute to the protein supply of the host animal, both through the production of proteolytic enzymes and as a microbial protein synthesized in rumen that passes to the abomasum and intestines for digestion and absorption. Unlike bacteria, rumen fungi are protease-positive and, hence, penetrate the proteinaceous part of feed via rhizoids, and they play an important role in degrading fiber-associated protein or tannin-protein complex (Wallace and Munro, 1986; Gordon et al., 1995). However, the extent of fungal contribution to proteolysis in rumen is not yet determined (Bonnemoy et al., 1993). Rumen fungi have also been shown to have aminopeptidase activity (Michel et al., 1993). A high proportion of the protein component of the monocentric anaerobic fungi, *Neocallimastix*, *Piromyces,* and *Caecomyces*, was digested and absorbed by the intestine of sheep (Gulati et al., 1988, 1989). Although the overall contribution of anaerobic fungi to microbial protein to host

was minor, the fungi-derived protein was of high quality and readily available to the host (Faichney et al., 1997). Since anaerobic fungi can use ammonia as a sole source of nitrogen (Lowe et al., 1985; Guliye and Wallace, 2007), it is possible to manipulate the rumen fungi population, either by direct inoculation of the strains or by stimulating their activity through dietary supplementation, in both cases with the result to enhance the supply of high-quality microbial protein to the host ruminant.

Fermentation by anaerobic fungi was governed by the substrate and presence of other microbes (Theodorou et al., 1996; Sirohi et al., 2012). The major fermentation end products were acetate, ethanol, formate, lactate, succinate, carbon dioxide, and hydrogen when glucose was used as a substrate (Lowe et al., 1987). Polycentric anaerobic fungi produced less lactate than monocentric fungi (Borneman et al., 1989; Phillips and Gordon, 1995) and *Piromyces* did not produce any lactate (Ho et al., 1996). On glucose and xylose, *Neocallimastix* sp. produced formate, acetate, lactate, and ethanol (Lowe et al., 1987). Borneman et al. (1989) concluded that accumulation of fermentation products was concomitant with substrate utilization. The major fermentation products were formate, acetate, D(-)lactate, ethanol, carbon dioxide, and hydrogen. All fungal strains, irrespective of their origin, produce lactate except *Piromyces* strain isolated from the cecum of donkey that did not produce lactate (Julliand et al., 1998).

Within half an hour of feeding, zoospores of anaerobic fungi are released and attach to the plant cell and damaged surfaces (Edwards et al., 2008). After attachment, the zoospores first penetrate the plant tissue and then are branched to form a mesh-like pseudo-root structure (Dollhofer et al., 2015; Cheng et al., 2018). Fungal zoospores show a chemotactic response to phenolic acids (*p*-coumaric acid, ferulic and syringic acids) that are found in the lignified tissues (Wubah and Kim, 1996) and attack lignin to release phenolic acids from the cell wall. The locomotion of anaerobic fungal zoospores has a chemical tendency to attach soluble sugars and phenolic acid (Orpin and Bountiff, 1978) that are released from plant material. After the zoospores attach to the plant tissue, the flagellum falls off and a cyst is formed. The germ tubes are generated at the position of the original flagella, and subsequently develop a branched rhizoidal system that can penetrate the plant tissues by the combination of enzymes and rhizoides (Ho et al., 1988). This characteristic of anaerobic fungi allows them to quickly erode plant tissues that enter the digestive tract before other microorganisms (Edwards et al., 2008; Cheng et al., 2018). Phenolic monomers are toxic to rumen fungi at a high concentration, although resistance to these acids is much higher than that of other rumen microbes (Akin and Rigsby, 1987; Paul et al., 2003). Rumen fungi produce extracellular feruloyl and *p*-coumaroyl esterase enzymes that release hydroxycinnamic acids from arabinoxylans (Borneman et al., 1990). The ability of *Neocallimastix*

patriciarium to digest model lignin compounds and lignified plant material was studied by McSweeney et al. (1994). The fungus did not degrade model lignin compounds nor did it solubilize acid detergent lignin isolated from spear grass. The fungus cleaved the ester bonds that cross-link lignin and polysaccharides but failed to cleave ether bonds between lignin and polysaccharides. Akin and Benner (1988) and Chesson (1993) also concluded that rumen fungi are able to solubilize but not to degrade it to end products of metabolism.

10.4 Manipulation of rumen fungal population for increased lignocelluloses degradation

The application of anaerobic fungi as direct-fed microbes has been studied, in ruminant and non-ruminant livestock production, to improve utilization of low-quality forages and dosing fungus-free sheep with fungi increased to 40% intake of straw-based diets (Gordon and Phillips, 1998). The results of *in-vitro* fiber digestibility and rumen fermentation analyses have also revealed the positive effect of fungal supplementation in increasing acetate, *in-vitro* dry-matter digestibility, partition factor values, and microbial biomass synthesis levels (Sirohi et al., 2013). Administration of cultures of anaerobic fungi in the diets of ruminants has been shown to improve feed intake, animal growth rate, feed efficiency, and milk production (Lee et al., 2000; Dey et al., 2004; Paul et al., 2004; Tripathi et al., 2007; Sehgal et al., 2008; Saxena et al., 2010; Paul et al., 2011; Gao et al., 2013; Kumar et al., 2015). The benefits of dosing with anaerobic fungi were higher in young ruminant (Sehgal et al., 2008) and sheep devoid of anaerobic fungi (Elliott et al., 1987; Gordon and Phillips, 1993). Studies illustrate that the application of anaerobic fungi as a direct-fed microbes can be used to improve digestibility by enhancing rumen fermentation characteristics (pH, volatile fatty acids, ammonia-N), microbial populations, and cellulolytic enzyme activities. On the contrary, the inclusion of enzymes secreted by anaerobic fungi alone does not alter rumen fermentation, highlighting the importance of using viable cultures as a ruminant feed additive (Lee et al., 2000). However, feasibility of improving fiber digestion in rumen is still questionable, considering the difficulty in culturing and administration of these strict anaerobic fungi.

The sulfur content of hay diets or pasture was a significant factor in governing the fungal population in rumen (Akin et al., 1983; Gordon, 1985). When the sulfur level in the diet was at 1.0 g S/kg organic matter or less, anaerobic fungi were absent from the rumen of sheep fed on hay made from the tropical pasture grass *Digitaria pentzii* (Akin et al., 1983). The anaerobic fungal population in rumen increased considerably after either an application of a sulfur fertilizer to the pasture used to make the hay (Akin et al., 1983) or a sulfur-containing dietary supplement to the low-sulfur hay (Gordon et al., 1983). Fertilization of

the pasture also resulted in an average increase of 38% in ad lib. feed intake (Akin et al., 1983; Gordon, 1985). A diet of another tropical grass hay that had a low sulfur content also resulted in an undetectable population in rumen fungi (Morrison et al., 1990). Alternatively, diets of cereal straw that were low in sulfur supported a low, but detectable, population of anaerobic fungi (Gordon et al., 1983; Gulati et al., 1985; Weston et al., 1988). The relation between a reduced sulfur content of pasture and a declining rumen fungal population did not apply to a ryegrass pasture (Millard et al., 1987), where anaerobic fungal populations were higher in sheep consuming an unfertilized hay (containing 0.9 g S/kg DM) than in those fed a hay prepared from fertilized pasture (2–4 g S/kg DM). The additional sulfur of fertilized pasture was predominantly contained in the sulfate and non-protein fractions which was similar to the sulfur distribution in the fertilized *D. pentzii* used by Akin et al. (1983), where the additional sulfur was in the soluble, nonprotein fraction. It is likely that the form and distribution of sulfur in herbage of low total sulfur content was as important as the total sulfur content in determining the size of the fungal population in rumen. Diets of low-sulfur *Digitaria* when supplemented with methionine and elemental sulfur (Gordon, 1985), and cereal straw supplemented with either methionine (Gordon et al., 1983; Gulati et al., 1985) or sulfate (Weston et al., 1988), also supported anaerobic fungi in rumen. Supplementation of spear grass with sulfate supported a higher fungal population compared to hay when un-supplemented (Morrison et al., 1990). Importantly, anaerobic fungi grown *in vitro* required reduced sulfur (Orpin and Greenwood, 1986; Gordon and Phillips, 1995), indicating the need for reduction of supplementary sulfate in rumen before it is available for anaerobic fungi. Sulfur content of dietary supplements (elemental sulfur, sulfate, or methionine) used so far to stimulate anaerobic fungi in rumen were potentially available to all rumen microbiota. Two organic sulfur nutrients mercapto-1-propionic acid (MPA) and 3-mercapto-1-propanesulfonic acid (MPS) were tested in cattle trials and compared to an inorganic sulfur supplement. It was reported that these organic sulfur sources improved N utilization and microbial protein production, but surprisingly this was due to a general improvement in the efficiency of microbial fermentation of lignocellulose and not from specific stimulation of fungi (McSweeney and Denman, 2007). A sulfur supplement specific for anaerobic rumen fungi remains to be identified.

The feasibility of transfer of anaerobic fungi between different ruminant species has been demonstrated (Orpin, 1989). Lee et al. (2000) reported that inoculation of goat originated *Orpinomyces* strain to sheep rumen increased nutrient digestibility. Similarly, Paul et al. (2004) reported that feeding of cultures of an elite anaerobic fungi (*Piromyces* sp. FNG5; isolated from feces of wild blue bull), having higher lignocellulolytic activities than those normally isolated from buffalo, to buffaloes, resulted in increased digestibility. On intra-rumen

dosing of the fungal isolate, concentrations of volatile fatty acids increased substantially. There was also an increase in cellulolytic, hemicellulolytic activities and total bacterial and fungal counts on administration of fungus. Activities of carboxymethyl cellulase, xylanase, microcrystalline cellulase, acetyl esterase, feruloyl esterase, and protease of rumen content also increased. Gordon et al. (2000) noticed that dosing of sheep with a nonindigenous anaerobic fungus (*Piromyces* sp. CS15 isolated from cattle), having higher cellulolytic activities than isolated from sheep, resulted in a 12% increase in voluntary dry-matter intake.

11 Conclusion

Although the ecological importance of anaerobic rumen fungi has been widely accepted, very little research has been aimed at this group of microorganisms. Recently developed advanced molecular techniques have improved the ability to detect and classify fungi in general and they have provided the first insights into the true phylogenetic and functional diversity of anaerobic rumen fungi. The pace at which new isolates of hitherto unknown fungal genera has been proceeding recently as well as the identification and enzymes and enzyme repertoires that were previously thought to be absent in anaerobic fungi and have opened up new possibilities. These will hopefully encourage the scientific community to pay more attention to this currently, still-poorly understood group of microorganisms and their role in the rumen ecosystem.

12 Where to look for further information

Our knowledge of anaerobic fungi is still relatively limited but there are several articles available that provide a good introduction in the biology of these intriguing organisms. Furthermore, latest advances in omics technology have resulted in some significant insights into the molecular processes of these recalcitrant biomass degraders. A very good place to stay up-to-date with the latest developments in the area of anaerobic fungi is the website of the Anaerobic Fungi Network (https://anaerobicfungi.org/). This site contains a list of most of the researchers working on anaerobic fungi. A general introduction (https://youtu.be/VEisRWzNyfI) and summaries of the life cycle (https://youtu.be/x8jJbkT7t3o) and taxonomy (https://youtu.be/vMhb4QL5zRQ) of anaerobic fungi by Dr. Joan Edwards can be found on You tube for those of us who want to get a quick introduction into the field. Another useful resource on anaerobic eukaryotes is a research topic site containing open access peer-reviewed articles on anaerobic fungi and other eukaryotes that inhabit the gut of herbivores. The URL of this research topic site is https://www.frontiersin.org/research-topics/9250. An excellent collection of protocols

for fungal genomics, including genomics of anaerobic fungi, can be found in *Fungal Genomics* by de Vries et al. (2018). A recent paper by Wang et al. (2019) utilizes a comparative omics approach to shed light onto the timepoint of when anaerobic fungi emerged and the potential role of horizontal gene transfer in the evolution and exceptional metabolic abilities of anaerobic fungi. Work by the group of Prof. Elshahed has resulted in a significant expansion of the phylogeny of anaerobic fungi (Hanafy et al., 2019). Last but not least, two hallmark publications that are highly recommended are Orpin (1975) and Gordon and Philipps (1998).

13 References

Abot, A., Arnal, G., Auer, L., Lazuka, A., Labourdette, D., Lamarre, S., Trouilh, L., Laville, E., Lombard, V., Potocki-Veronese, G., Henrissat, B., O'Donohue, M., Hernandez-Raquet, G., Dumon, C. and Leberre, V. A. 2016. CAZyChip: dynamic assessment of exploration of glycoside hydrolases in microbial ecosystems. *BMC Genomics* 17, 671. doi:10.1186/s12864-016-2988-4.

Accetto, T. and Avgustin, G. 2015. Polysaccharide utilization locus and CAZYme genome repertoires reveal diverse ecological adaptation of *Prevotella* species. *Syst. Appl. Microbiol.* 38(7), 453–61. doi:10.1016/j.syapm.2015.07.007.

Ahrendt, S. R., Quandt, C. A., Ciobanu, D., Clum, A., Salamov, A., Andreopoulos, B., Cheng, J. F., Woyke, T., Pelin, A., Henrissat, B., Reynolds, N. K., Benny, G. L., Smith, M. E., James, T. Y. and Grigoriev, I. V. 2018. Leveraging single-cell genomics to expand the fungal tree of life. *Nat. Microbiol.* 3(12), 1417–28. doi:10.1038/s41564-018-0261-0.

Akin, D. E. and Benner, R. 1988. Degradation of polysaccharides and lignin by ruminal bacteria and fungi. *Appl. Environ. Microbiol.* 54(5), 1117–25.

Akin, D. E. and Rigsby, L. L. 1987. Mixed fungal populations and lignocellulosic tissue degradation in the bovine rumen. *Appl. Environ. Microbiol.* 53(9), 1987–95.

Akin, D. E. and Windham, W. R. 1989. Influence of diet on rumen fungi. In: Nolan, J. V., Leng, R. A. and Demeyer, D. I. (Eds), *The Role of Protozoa and Fungi in Ruminant Digestion*. Penumbul Books, Armidale, New South Wales, Australia, pp. 75–82.

Akin, D. E., Gordon, G. L. and Hogan, J. P. 1983. Rumen bacterial and fungal degradation of *Digitaria pentzii* grown with or without sulfur. *Appl. Environ. Microbiol.* 46(3), 738–48.

Ariyawansa, H. A., Hyde, K. D., Jayasiri, S. C., Buyck, B., Chethana, K. W. T., Dai, D. Q., Dai, Y. C., Daranagama, D. A., Jayawardena, R. S., Lücking, R., Ghobad-Nejhad, M., Niskanen, T., Thambugala, K. M., Voigt, K., Zhao, R. L., Li, G.-J., Doilom, M., Boonmee, S., Yang, Z. L., Cai, Q., Cui, Y.-Y., Bahkali, A. H., Chen, J., Cui, B. K., Chen, J. J., Dayarathne, M. C., Dissanayake, A. J., Ekanayaka, A. H., Hashimoto, A., Hongsanan, S., Jones, E. B. G., Larsson, E., Li, W. J., Li, Q.-R., Liu, J. K., Luo, Z. L., Maharachchikumbura, S. S. N., Mapook, A., McKenzie, E. H. C., Norphanphoun, C., Konta, S., Pang, K. L., Perera, R. H., Phookamsak, R., Phukhamsakda, C., Pinruan, U., Randrianjohany, E., Singtripop, C., Tanaka, K., Tian, C. M., Tibpromma, S., Abdel-Wahab, M. A., Wanasinghe, D. N., Wijayawardene, N. N., Zhang, J.-F., Zhang, H., Abdel-Aziz, F. A., Wedin, M., Westberg, M., Ammirati, J. F., Bulgakov, T. S., Lima, D. X., Callaghan, T. M., Callac, P., Chang,

C.-H., Coca, L. F., Dal-Forno, M., Dollhofer, V., Fliegerová, K., Greiner, K., Griffith, G. W., Ho, H.-M., Hofstetter, V., Jeewon, R., Kang, J. C., Wen, T.-C., Kirk, P. M., Kytövuori, I., Lawrey, J. D., Xing, J., Li, H., Liu, Z. Y., Liu, X. Z., Liimatainen, K., Lumbsch, H. T., Matsumura, M., Moncada, B., Nuankaew, S., Parnmen, S., de Azevedo Santiago, A. L. C. M., Sommai, S., Song, Y., de Souza, C. A. F., de Souza-Motta, C. M., Su, H. Y., Suetrong, S., Wang, Y., Wei, S.-F., Wen, T. C., Yuan, H. S., Zhou, L. W., Réblová, M., Fournier, J., Camporesi, E., Luangsa-ard, J. J., Tasanathai, K., Khonsanit, A., Thanakitpipattana, D., Somrithipol, S., Diederich, P., Millanes, A. M., Common, R. S., Stadler, M., Yan, J. Y., Li, X., Lee, H. W., Nguyen, T. T. T., Lee, H. B., Battistin, E., Marsico, O., Vizzini, A., Vila, J., Ercole, E., Eberhardt, U., Simonini, G., Wen, H.-A., Chen, X.-H., Miettinen, O., Spirin, V. and Hernawati 2015. Fungal diversity notes 111–252—taxonomic and phylogenetic contributions to fungal taxa. *Fungal Divers.* 75(1), 27–274. doi:10.1007/s13225-015-0346-5.

Artzi, L., Bayer, E. A. and Morais, S. 2017. Cellulosomes: bacterial nanomachines for dismantling plant polysaccharides. *Nat. Rev. Microbiol.* 15(2), 83–95. doi:10.1038/nrmicro.2016.164.

Barr, D. J. S. 1980. An outline for the reclassification of the Chytridiales, and for a new order, the Spizellomycetales. *Can. J. Bot.* 58(22), 2380–94. doi:10.1139/b80-276.

Barr, D. J. S. 1988. How modern systematics relates to the rumen fungi. *Biosystems* 21(3–4), 351–6. doi:10.1016/0303-2647(88)90032-9.

Barr, A. J. 2001. Chytridiomycota. In: McLaughlin, D. and Spatafora, J. W. (Eds), *The Mycota VII – Systematics and Evolution – Part A*. Springer-Verlag, Berlin, pp. 93–112.

Barr, D. J. S., Kudo, H., Jakober, K. D. and Cheng, K.-J. 1989. Morphology and development of rumen fungi: *Neocallimastix* sp., *Piromyces communis*, and *Orpinomyces bovis* gen.nov., sp.nov. *Can. J. Bot.* 67(9), 2815–24. doi:10.1139/b89-361.

Barr, D. J. S., Yanke, L. J., Bae, H. D., McAllister, T. A. and Cheng, K.-J. 1995. Contributions on the morphology and taxonomy of some rumen fungi from Canada. *Mycotaxon* 54, 203–14.

Bauchop, T. 1979. Rumen anaerobic fungi of cattle and sheep. *Appl. Environ. Microbiol.* 38(1), 148–58.

Bauchop, T. and Mountfort, D. O. 1981. Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. *Appl. Environ. Microbiol.* 42(6), 1103–10.

Bonnemoy, F., Fonty, G., Michel, V. and Gouet, P. 1993. Effect of anaerobic fungi on the ruminal proteolysis in gnotobiotic lambs. *Reprod. Nutr. Dev.* 33(6), 551–5. doi:10.1051/rnd:19930606.

Bootten, T. J., Joblin, K. N., McArdle, B. H. and Harris, P. J. 2011. Degradation of lignified secondary cell walls of Lucerne (*Medicago sativa* L.) by rumen fungi growing in methanogenic co-culture. *J. Appl. Microbiol.* 111(5), 1086–96. doi:10.1111/j.1365-2672.2011.05127.x.

Borneman, W. S., Akin, D. E. and Ljungdahl, L. G. 1989. Fermentation products and plant cell wall-degrading enzymes produced by monocentric and polycentric anaerobic ruminal fungi. *Appl. Environ. Microbiol.* 55(5), 1066–73.

Borneman, W. S., Hartley, R. D., Morrison, W. H., Akin, D. E. and Ljungdahl, L. G. 1990. Feruloyl and p-coumaroyl esterase from anaerobic fungi in relation to plant cell wall degradation. *Appl. Microbiol. Biotechnol.* 33(3), 345–51. doi:10.1007/BF00164534.

Bowman, B. H., Taylor, J. W., Brownlee, A. G., Lee, J., Lu, S. D. and White, T. J. 1992. Molecular evolution of the fungi: relationship of the Basidiomycetes, Ascomycetes,

and Chytridiomycetes. *Mol. Biol. Evol.* 9(2), 285–96. doi:10.1093/oxfordjournals.molbev.a040720.

Braune, R. A. 1913. Untersuchungen über die im Wiederkäuermagen vorkommenden Protozoen. *Arch. Protistenkunde* 32, 111–70.

Breton, A., Bernalier, A., Bonnemoy, F., Fonty, G., Gaillard, B. and Gouet, P. 1989. Morphological and metabolic characterization of a new species of strictly anaerobic rumen fungus: *Neocallimastix joyonii*. *FEMS Microbiol. Lett.* 58(2-3), 309–14. doi:10.1111/j.1574-6968.1989.tb03065.x.

Breton, A., Bernalier, A., Dusser, M., Fonty, G., Gaillard-Martinie, B. and Guillot, J. 1990. *Anaeromyces mucronatus* nov. gen., nov. sp. A new strictly anaerobic rumen fungus with polycentric thallus. *FEMS Microbiol. Lett.* 58(2), 177–82. doi:10.1111/j.1574-6968.1990.tb13974.x.

Breton, A., Dusser, M., Gaillard-Martinie, B., Guillot, J., Millet, L. and Prensier, G. 1991. *Piromyces rhizinflata* nov. sp., a strictly anaerobic fungus from faeces of the Saharian ass: a morphological, metabolic and ultrastructural study. *FEMS Microbiol. Lett.* 66(1), 1–8. doi:10.1111/j.1574-6968.1991.tb04830.x.

Britto-Kido Sde, A., Neto, J. R. F., Pandolfi, V., Marcelino-Guimaraes, F. C., Nepomuceno, A. L., Vilela Abdelnoor, R., Benko-Iseppon, A. M. and Kido, E. A. 2013. Natural antisense transcripts in plants: a review and identification in soybean infected with Phakopsora pachyrhizi SuperSAGE library. *Sci. World J.* 2013:219798. doi:10.1155/2013/219798.

Brookman, J. L., Ozkose, E., Rogers, S., Trinci, A. P. J. and Theodorou, M. K. 2000. Identification of spores in the polycentric anaerobic gut fungi which enhance their ability to survive. *FEMS Microbiol. Ecol.* 31(3), 261–7. doi:10.1111/j.1574-6941.2000.tb00692.x.

Brownlee, A. G. 1989. Remarkably AT-rich genomic DNA from the anaerobic Fungus *Neocallimastix*. *Nucleic Acids Res.* 17(4), 1327–35. doi:10.1093/nar/17.4.1327.

Calderon-Cortes, J. F., Elliott, R. and Ford, C. W. 1989. Influence of rumen fungi on the nutrition of sheep fed forage diets. In: Nolan, J. V., Leng, R. A. and Demeyer, D. I. (Eds), *The Roles of Protozoa and Fungi in Ruminant Digestion*. Penumbul Books, Armidale, Australia, pp. 181–7.

Callaghan, T. M., Podmirseg, S. M., Hohlweck, D., Edwards, J. E., Puniya, A. K., Dagar, S. S. and Griffith, G. W. 2015. *Buwchfawromyces eastonii* gen. nov., sp. nov.: a new anaerobic fungus (Neocallimastigomycota) isolated from buffalo faeces. *MycoKeys* 9, 11–28. (doi:10.3897/mycokeys.9.9032).

Chen, Y.-C., Hseu, R.-S. and Chien, C.-Y. 2002. *Piromyces polycephalus* (Neocallimastigaceae), a new rumen fungus. *Nova Hedwigia* 75(3), 409–14. doi:10.1127/0029-5035/2002/0075-0409.

Chen, H., Hopper, S. L., Li, X. L., Ljungdahl, L. G. and Cerniglia, C. E. 2006. Isolation of extremely AT-rich genomic DNA and analysis of genes encoding carbohydrate-degrading enzymes from *Orpinomyces* sp. strain PC-2. *Curr. Microbiol.* 53(5), 396–400. doi:10.1007/s00284-006-0098-2.

Chen, Y. C., Tsai, S. D., Cheng, H. L., Chien, C. Y., Hu, C. Y. and Cheng, T. Y. 2007. *Caecomyces sympodialis* sp. nov., a new rumen fungus isolated from Bos indicus. *Mycologia* 99(1), 125–30. doi:10.3852/mycologia.99.1.125.

Cheng, Y. F., Edwards, J. E., Allison, G. G., Zhu, W. Y. and Theodorou, M. K. 2009. Diversity and activity of enriched ruminal cultures of anaerobic fungi and methanogens grown together on lignocellulose in consecutive batch culture. *Bioresour. Technol.* 100(20), 4821–8. doi:10.1016/j.biortech.2009.04.031.

Cheng, Y. F., Jin, W., Mao, S. Y. and Zhu, W. Y. 2013. Production of citrate by anaerobic fungi in the presence of co-culture methanogens as revealed by (1)H NMR spectrometry. *Asian-Australas. J. Anim. Sci.* 26(10), 1416–23. doi:10.5713/ajas.2013.13134.

Cheng, Y., Shi, Q., Sun, R., Liang, D., Li, Y., Li, Y., Jin, W. and Zhu, W. 2018. The biotechnological potential of anaerobic fungi on fiber degradation and methane production. *World J. Microbiol. Biotechnol.* 34(10), 155. doi:10.1007/s11274-018-2539-z.

Chesson, A. 1993. Mechanistic models of forage cell wall degradation. In: Jung, H. G., Buxton, D. R., Hatfield, R. D. and Ralph, J. (Eds), *Forage Cell Wall Structure and Digestibility*. American Society of Agronomy, Madison, WI, pp. 347–76.

Comtet-Marre, S., Chaucheyras-Durand, F., Bouzid, O., Mosoni, P., Bayat, A. R., Peyret, P. and Forano, E. 2018. FibroChip, a functional DNA microarray to monitor cellulolytic and hemicellulolytic activities of rumen microbiota. *Front. Microbiol.* 9, 215. doi:10.3389/fmicb.2018.00215.

Couger, M. B., Youssef, N. H., Struchtemeyer, C. G., Liggenstoffer, A. S. and Elshahed, M. S. 2015. Transcriptomic analysis of lignocellulosic biomass degradation by the anaerobic fungal isolate *Orpinomyces* sp. strain C1A. *Biotechnol. Biofuels* 8, 208. doi:10.1186/s13068-015-0390-0.

Dagar, S. S., Kumar, S., Griffith, G. W., Edwards, J. E., Callaghan, T. M., Singh, R., Nagpal, A. K. and Puniya, A. K. 2015. A new anaerobic fungus (*Oontomyces anksri* gen. nov., sp. nov.) from the digestive tract of the Indian camel (*Camelus dromedarius*). *Fungal Biol.* 119(8), 731–7. doi:10.1016/j.funbio.2015.04.005.

Dai, X., Tian, Y., Li, J., Luo, Y., Liu, D., Zheng, H., Wang, J., Dong, Z., Hu, S. and Huang, L. 2015. Metatranscriptomic analyses of plant cell wall polysaccharide degradation by microorganisms in the cow rumen. *Appl. Environ. Microbiol.* 81(4), 1375–86. doi:10.1128/AEM.03682-14.

Dayananda, T. L., Nagpal, R., Puniya, A. K., Sehgal, J. P. and Singh, K. 2007. Biodegradation of urea-NH3 treated wheat straw using anaerobic rumen fungi. *J. Anim. Feed Sci.* 16(3), 484–9. doi:10.22358/jafs/66804/2007.

Dean, F. B., Nelson, J. R., Giesler, T. L. and Lasken, R. S. 2001. Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. *Genome Res.* 11(6), 1095–9. doi:10.1101/gr.180501.

Denman, S. E., Nicholson, M. J., Brookman, J. L., Theodorou, M. K. and McSweeney, C. S. 2008. Detection and monitoring of anaerobic rumen fungi using an ARISA method. *Lett. Appl. Microbiol.* 47(6), 492–9. doi:10.1111/j.1472-765X.2008.02449.x.

de Vries, R. P., Tsang, A. and Grigoriev, I. V. (Eds). 2018. *Fungal Genomics: Methods and Protocols*. Humana Press. Available at: https://www.springer.com/us/book/9781493978038.

Dey, A., Sehgal, J. P., Puniya, A. K. and Singh, K. 2004. Influence of an anaerobic fungal culture (*Orpinomyces* sp.) administration on growth rate, ruminal fermentation and nutrient digestion in calves. *Asian Australas. J. Anim. Sci* 17(6), 820–4. doi:10.5713/ajas.2004.820.

Dill-McFarland, K. A., Weimer, P. J., Breaker, J. D. and Suen, G. 2019. Diet influences early microbiota development in dairy calves without long-term impacts on milk production. *Appl. Environ. Microbiol.* 85(2): doi:10.1128/AEM.02141-18.

Dollhofer, V., Podmirseg, S. M., Callaghan, T. M., Griffith, G. W. and Fliegerova, K. 2015. Anaerobic fungi and their potential for biogas production. *Adv. Biochem. Eng. Biotechnol.* 151, 41–61. doi:10.1007/978-3-319-21993-6_2.

Dollhofer, V., Callaghan, T. M., Dorn-In, S., Bauer, J. and Lebuhn, M. 2016. Development of three specific PCR-based tools to determine quantity, cellulolytic transcriptional activity and phylogeny of anaerobic fungi. *J. Microbiol. Methods* 127, 28–40. doi:10.1016/j.mimet.2016.05.017.

Donaldson, M. E. and Saville, B. J. 2012. Natural antisense transcripts in fungi. *Mol. Microbiol.* 85(3), 405–17. doi:10.1111/j.1365-2958.2012.08125.x.

Dore, J. and Stahl, D. A. 1991. Phylogeny of anaerobic rumen Chytridiomycetes inferred from small subunit ribosomal RNA sequence comparisons. *Can. J. Bot.* 69(9), 1964–71. doi:10.1139/b91-246.

Ebersberger, I., de Matos Simoes, R., Kupczok, A., Gube, M., Kothe, E., Voigt, K. and von Haeseler, A. 2012. A consistent phylogenetic backbone for the fungi. *Mol. Biol. Evol.* 29(5), 1319–34. doi:10.1093/molbev/msr285.

Edwards, J. E., Kingston-Smith, A. H., Jimenez, H. R., Huws, S. A., Skot, K. P., Griffith, G. W., McEwan, N. R. and Theodorou, M. K. 2008. Dynamics of initial colonization of nonconserved perennial ryegrass by anaerobic fungi in the bovine rumen. *FEMS Microbiol. Ecol.* 66(3), 537–45. doi:10.1111/j.1574-6941.2008.00563.x.

Edwards, J. E., Forster, R. J., Callaghan, T. M., Dollhofer, V., Dagar, S. S., Cheng, Y., Chang, J., Kittelmann, S., Fliegerova, K., Puniya, A. K., Henske, J. K., Gilmore, S. P., O'Malley, M. A., Griffith, G. W. and Smidt, H. 2017. PCR and omics based techniques to study the diversity, ecology and biology of anaerobic fungi: insights, challenges and opportunities. *Front. Microbiol.* 8, 1657. doi:10.3389/fmicb.2017.01657.

Elliott, R., Ash, A. J., Calderon-Cortes, F., Norton, B. W. and Bauchop, T. 1987. The influence of anaerobic fungi on rumen volatile fatty acid concentrations *in vivo*. *J. Agric. Sci.* 109(1), 13–7. doi:10.1017/S0021859600080928.

Faichney, G. J., Poncet, C., Lassalas, B., Jouany, J. P., Millet, L., Doré, J. and Brownlee, A. G. 1997. Effect of concentrates in a hay diet on the contribution of anaerobic fungi, protozoa and bacteria to nitrogen in rumen and duodenal digesta in sheep. *Anim. Feed Sci. Technol.* 64(2–4), 193–213. doi:10.1016/S0377-8401(96)01059-0.

Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P. and Forano, E. 2012. Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* 3(4), 289–306. doi:10.4161/gmic.19897.

Fonty, G. and Grenet, E. 1994. Effects of diet on the fungal population of the digestive tract of ruminants. In: Mountfort, D. O. and Orpin, C. G. (Eds), *Anaerobic Fungi: Biology, Ecology and Function*. Marcel Dekker, New York, pp. 229–39.

Foster, J. 1949. *Chemical Activities of the Fungi*. Academic Press, New York.

Gao, A. W., Wang, H. R., Yang, J. L. and Shi, C. X. 2013. The effects of elimination of fungi on microbial population and fiber degradation in sheep rumen. *Appl. Mech. Mater.* 295–298, 224–31. doi:10.4028/www.scientific.net/AMM.295-298.224.

Gilbert, H. J. 2010. The biochemistry and structural biology of plant cell wall deconstruction. *Plant Physiol.* 153(2), 444–55. doi:10.1104/pp.110.156646.

Gilmore, S. P., Henske, J. K. and O'Malley, M. A. 2015. Driving biomass breakdown through engineered cellulosomes. *Bioengineered* 6(4), 204–8. doi:10.1080/21655979.2015.1060379.

Gold, J. J., Heath, I. B. and Bauchop, T. 1988. Ultrastructural description of a new chytrid genus of caecum anaerobe, *Caecomyces equi* gen. nov., sp. nov., assigned to the Neocallimasticaceae. *Biosystems* 21(3-4), 403–15. doi:10.1016/0303-2647(88)90039-1.

Gordon, G. L. R. 1985. The potential for manipulation of rumen fungi. In: Leng, R. A., Barker, J. S. F., Adams, D. B. and Hutchinson, K. J. (Eds), *Biotechnology and Recombinant*

DNA Technology in the Animal Production Industries. Reviews in Rural Science No. 6. University of New England, Armidale, Australia, pp. 124–8.

Gordon, G. L. R. and Phillips, M. W. 1993. Removal of anaerobic fungi from the rumen of sheep by chemical treatment and the effect on feed consumption and *in vivo* fibre digestion. *Lett. Appl. Microbiol.* 17(5), 220–3. doi:10.1111/j.1472-765X.1993.tb01451.x.

Gordon, G. L. R. and Phillips, M. W. 1995. New approach for the manipulation of anaerobic fungi in the rumen. In: Corbett, J. L., Choct, M., Nolan, J. V. and Rowe, J. B. (Eds), *Recent Advances in Animal Nutrition in Australia*. University of New England, Armidale, Australia, pp. 108–15.

Gordon, G. L. and Phillips, M. W. 1998. The role of anaerobic gut fungi in ruminants. *Nutr. Res. Rev.* 11(1), 133–68. doi:10.1079/NRR19980009.

Gordon, G. L. R., Gulati, S. K. and Ashes, J. R. 1983. Influence of low-sulphur straw on anaerobic fungal numbers in a sheep rumen. In: *Proceedings of the Nutritional Society of Australia*.

Gordon, G. L. R., Wong, H. and Phillips, M. V. 1995. *In vitro* degradation of 14C lignocellulose by polycentric and monocentric ruminal anaerobic fungi is inhibited differently by phenolic monomers. *Ann. Zootech.* 44(Suppl. 1), 152–. doi:10.1051/animres:199505120.

Gordon, G. L. R., Phillips, M. W., Rintoul, A. J. and White, S. W. 2000. Increased intake of fibrous feed by sheep orally dosed with a culture of an elite non-indigenous anaerobic gut fungus. *Asian Aust. J. Anim. Sci.* 13(143).

Grenet, E., Breton, A., Barry, P. and Fonty, G. 1989. Rumen anaerobic fungi and plant substrate colonization as affected by diet composition. *Anim. Feed Sci. Technol.* 26(1–2), 55–70. doi:10.1016/0377-8401(89)90006-0.

Gruninger, R. J., Puniya, A. K., Callaghan, T. M., Edwards, J. E., Youssef, N., Dagar, S. S., Fliegerova, K., Griffith, G. W., Forster, R., Tsang, A., McAllister, T. and Elshahed, M. S. 2014. Anaerobic fungi (phylum *Neocallimastigomycota*): advances in understanding their taxonomy, life cycle, ecology, role and biotechnological potential. *FEMS Microbiol. Ecol.* 90(1), 1–17. doi:10.1111/1574-6941.12383.

Gruninger, R. J., Nguyen, T. T. M., Reid, I. D., Yanke, J. L., Wang, P., Abbott, D. W., Tsang, A. and McAllister, T. 2018. Application of transcriptomics to compare the carbohydrate active enzymes that are expressed by diverse genera of anaerobic fungi to degrade plant cell wall carbohydrates. *Front. Microbiol.* 9, 1581. doi:10.3389/fmicb.2018.01581.

Gulati, S. K., Ashes, J. R., Gordon, G. L. R. and Phillips, M. W. 1985. Possible contribution of rumen fungi to fibre digestion in sheep. *Proc. Nutr. Soc. Aust.* 10, 96.

Gulati, S. K., Ashes, J. R. and Gordon, G. L. R. 1988. Digestibility of sulphur amino acids in rumen fungal species. *Proc. Nutr. Soc. Aust.* 13(133).

Gulati, S. K., Ashes, J. R., Gordon, G. L. R., Connell, P. J. and Rogers, P. L. 1989. Nutritional availability of amino acids from the rumen anaerobic fungus *Neocallimastix* sp. LM1 in sheep. *J. Agric. Sci.* 113(3), 383–7. doi:10.1017/S002185960007009X.

Guliye, A. Y. and Wallace, R. J. 2007. Effects of aromatic amino acids, phenylacetate and phenylpropionate on fermentation of xylan by the rumen anaerobic fungi, *Neocallimastix frontalis* and *Piromyces communis*. *J. Appl. Microbiol.* 103(4), 924–9. doi:10.1111/j.1365-2672.2007.03327.x.

Haitjema, C. H., Solomon, K. V., Henske, J. K., Theodorou, M. K. and O'Malley, M. A. 2014. Anaerobic gut fungi: advances in isolation, culture, and cellulolytic enzyme discovery for biofuel production. *Biotechnol. Bioeng.* 111(8), 1471–82. doi:10.1002/bit.25264.

Haitjema, C. H., Gilmore, S. P., Henske, J. K., Solomon, K. V., de Groot, R., Kuo, A., Mondo, S. J., Salamov, A. A., LaButti, K., Zhao, Z., Chiniquy, J., Barry, K., Brewer, H. M., Purvine, S. O., Wright, A. T., Hainaut, M., Boxma, B., van Alen, T., Hackstein, J. H. P., Henrissat, B., Baker, S. E., Grigoriev, I. V. and O'Malley, M. A. 2017. A parts list for fungal cellulosomes revealed by comparative genomics. *Nat. Microbiol.* 2, 17087. doi:10.1038/nmicrobiol.2017.87.

Hanafy, R. A., Elshahed, M. S., Liggenstoffer, A. S., Griffith, G. W. and Youssef, N. H. 2017. *Pecoramyces ruminantium*, gen. nov., sp. nov., an anaerobic gut fungus from the feces of cattle and sheep. *Mycologia* 109(2), 231–43. doi:10.1080/00275514.2017.1317190.

Hanafy, R. A., Elshahed, M. S. and Youssef, N. H. 2018a. *Feramyces austinii*, gen. nov., sp. nov., an anaerobic gut fungus from rumen and fecal samples of wild Barbary sheep and fallow deer. *Mycologia* 110(3), 513–25. doi:10.1080/00275514.2018.1466610.

Hanafy, R. A., Johnson, B., Elshahed, M. S. and Youssef, N. H. 2018b. *Anaeromyces contortus*, sp. nov., a new anaerobic gut fungal species (Neocallimastigomycota) isolated from the feces of cow and goat. *Mycologia* 110(3), 502–12. doi:10.1080/00275514.2018.1465773.

Hanafy, R., Lanjekar, V., Dhakephalkar, P., Callaghan, T., Dagar, S., Griffith, G., Elshahed, M. and Youssef, N. 2019. Seven new Neocallimastigomycota genera from fecal samples of wild, zoo-housed, and domesticated herbivores: description of *Ghazallomyces constrictus* gen. nov., sp. nov., *Aklioshbomyces papillarum* gen. nov., sp. nov., *Agriosomyces longus* gen. nov., sp. nov., *Capellomyces foraminis* gen. nov., sp. nov. and *Capellomyces elongatus* sp. nov., *Joblinomyces apicalis* gen. nov., sp. nov., *Khoyollomyces ramosus* gen. nov., sp. nov., and *Tahromyces munnarensis* gen. nov., sp. nov. *bioRxiv*. doi:10.1101/642694:642694.

Heath, I. B., Bauchop, T. and Skipp, R. A. 1983. Assignment of the rumen anaerobe *Neocallimastix frontalis* to the Spizellomycetales (Chytridiomycetes) on the basis of its polyflagellate zoospore ultrastructure. *Can. J. Bot.* 61(1), 295–307. doi:10.1139/b83-033.

Heath, I. B., Kaminskyj, S. G. and Bauchop, T. 1986. Basal body loss during fungal zoospore encystment: evidence against centriole autonomy. *J. Cell Sci.* 83(1), 135–40.

Henske, J. K., Gilmore, S. P., Knop, D., Cunningham, F. J., Sexton, J. A., Smallwood, C. R., Shutthanandan, V., Evans, J. E., Theodorou, M. K. and O'Malley, M. A. 2017. Transcriptomic characterization of *Caecomyces churrovis*: a novel, non-rhizoid-forming lignocellulolytic anaerobic fungus. *Biotechnol. Biofuels* 10, 305. doi:10.1186/s13068-017-0997-4.

Hess, M., Sczyrba, A., Egan, R., Kim, T. W., Chokhawala, H., Schroth, G., Luo, S., Clark, D. S., Chen, F., Zhang, T., Mackie, R. I., Pennacchio, L. A., Tringe, S. G., Visel, A., Woyke, T., Wang, Z. and Rubin, E. M. 2011. Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. *Science* 331(6016), 463–7. doi:10.1126/science.1200387.

Hibbett, D. S., Binder, M., Bischoff, J. F., Blackwell, M., Cannon, P. F., Eriksson, O. E., Huhndorf, S., James, T., Kirk, P. M., Lücking, R., Thorsten Lumbsch, H., Lutzoni, F., Matheny, P. B., McLaughlin, D. J., Powell, M. J., Redhead, S., Schoch, C. L., Spatafora, J. W., Stalpers, J. A., Vilgalys, R., Aime, M. C., Aptroot, A., Bauer, R., Begerow, D., Benny, G. L., Castlebury, L. A., Crous, P. W., Dai, Y. C., Gams, W., Geiser, D. M., Griffith, G. W., Gueidan, C., Hawksworth, D. L., Hestmark, G., Hosaka, K., Humber, R. A., Hyde, K. D.,

Ironside, J. E., Kõljalg, U., Kurtzman, C. P., Larsson, K. H., Lichtwardt, R., Longcore, J., Miadlikowska, J., Miller, A., Moncalvo, J. M., Mozley-Standridge, S., Oberwinkler, F., Parmasto, E., Reeb, V., Rogers, J. D., Roux, C., Ryvarden, L., Sampaio, J. P., Schüssler, A., Sugiyama, J., Thorn, R. G., Tibell, L., Untereiner, W. A., Walker, C., Wang, Z., Weir, A., Weiss, M., White, M. M., Winka, K., Yao, Y. J. and Zhang, N. 2007. A higher-level phylogenetic classification of the Fungi. *Mycol. Res.* 111(5), 509–47. doi:10.1016/j.mycres.2007.03.004.

Hillaire, M. C. and Jouany, J. P. 1989. Effects of rumen anaerobic fungi on the digestion of wheat straw and the end products of microbial metabolism, studies in a semi-continuous in in vitro system. In: Nolan, J. V., Leng, R. A. and Demeyer, D. I. (Eds), *The Role of Protozoa and Fungi in Ruminant Digestion*. Penumbul Books, Armidale, Australia, pp. 269–71.

Ho, Y. W. and Barr, D. J. S. 1995. Classification of anaerobic gut fungi from herbivores with emphasis on rumen fungi from Malaysia. *Mycologia* 87(5), 655–77. doi:10.1080/00275514.1995.12026582.

Ho, Y. W. and Bauchop, T. 1991. Morphology of three polycentric rumen fungi and description of a procedure for the induction of zoosporogenesis and release of zoospores in cultures. *J. Gen. Microbiol.* 137(1), 213–7. doi:10.1099/00221287-137-1-213.

Ho, Y. W., Abdullah, N. and Jalaludin, S. 1988. Colonization of guinea grass by anaerobic rumen fungi in swamp buffalo and cattle. *Anim. Feed Sci. Technol.* 22(1–2), 161–71. doi:10.1016/0377-8401(88)90083-1.

Ho, Y. W., Bauchop, T., Abdullah, N. and Jalaludin, S. 1990. *Ruminomyces elegans* gen. et sp. nov,, a polycentric anaerobic rumen fungus from cattle. *Mycotaxon* 38, 397–405.

Ho, Y. W., Barr, D. J. S., Abdullah, N. and Jalaludin, S. 1993a. Anaeromyces, an earlier name for *Ruminomyces*. *Mycotaxon* 47, 283–4.

Ho, Y. W., Barr, D. J. S., Abdullah, N., Jalaludin, S. and Kudo, H. 1993b. *Neocallimastix variabilis*, a new species of anaerobic fungus from the rumen of cattle. *Mycotaxon* 46, 241–58.

Ho, Y. W., Barr, D. J. S., Abdullah, N., Jalaludin, S. and Kudo, H. 1993c. *Piromyces spiralis*, a new species of anaerobic fungus from the rumen of goat. *Mycotaxon* 47, 285–93.

Ho, Y. W., Barr, D. J. S., Abdullah, N., Jalaludin, S. and Kudo, H. 1993d. A new species of *Piromyces* from the rumen of deer in Malaysia. *Mycotaxon* 47, 285–93.

Ho, Y. W., Barr, D. J. S., Abdullah, N., Jalaludin, S. and Kudo, H. 1994. *Orpinomyces intercalaris*, a new species of polycentric anaerobic rumen fungus from cattle. *Mycotaxon* 50, 139–50.

Ho, Y. W., Abdullah, N. and Jalaludin, S. 1996. Microbial colonisation and degradation of some fibrous crop residues in the rumen of goats. *Asian Australas. J. Anim. Sci* 9(5), 519–24. doi:10.5713/ajas.1996.519.

James, T. Y., Kauff, F., Schoch, C. L., Matheny, P. B., Hofstetter, V., Cox, C. J., Celio, G., Gueidan, C., Fraker, E., Miadlikowska, J., Lumbsch, H. T., Rauhut, A., Reeb, V., Arnold, A. E., Amtoft, A., Stajich, J. E., Hosaka, K., Sung, G. H., Johnson, D., O'Rourke, B., Crockett, M., Binder, M., Curtis, J. M., Slot, J. C., Wang, Z., Wilson, A. W., Schüßler, A., Longcore, J. E., O'Donnell, K., Mozley-Standridge, S., Porter, D., Letcher, P. M., Powell, M. J., Taylor, J. W., White, M. M., Griffith, G. W., Davies, D. R., Humber, R. A., Morton, J. B., Sugiyama, J., Rossman, A. Y., Rogers, J. D., Pfister, D. H., Hewitt, D., Hansen, K., Hambleton, S., Shoemaker, R. A., Kohlmeyer, J., Volkmann-Kohlmeyer, B.,

Spotts, R. A., Serdani, M., Crous, P. W., Hughes, K. W., Matsuura, K., Langer, E., Langer, G., Untereiner, W. A., Lücking, R., Büdel, B., Geiser, D. M., Aptroot, A., Diederich, P., Schmitt, I., Schultz, M., Yahr, R., Hibbett, D. S., Lutzoni, F., McLaughlin, D. J., Spatafora, J. W. and Vilgalys, R. 2006. Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature* 443(7113), 818–22. doi:10.1038/nature05110.

Jenkins, T. C. 1993. Lipid metabolism in the rumen. *J. Dairy Sci.* 76(12), 3851–63. doi:10.3168/jds.S0022-0302(93)77727-9.

Jensen, E. A. and Hammond, D. M. 1964. A morphological study of trichomonads and related flagellates from the bovine digestive tract. *J. Protozool.* 11(3), 386–94. doi:10.1111/j.1550-7408.1964.tb01768.x.

Jin, W., Cheng, Y. F., Mao, S. Y. and Zhu, W. Y. 2011. Isolation of natural cultures of anaerobic fungi and indigenously associated methanogens from herbivores and their bioconversion of lignocellulosic materials to methane. *Bioresour. Technol.* 102(17), 7925–31. doi:10.1016/j.biortech.2011.06.026.

Jin, W., Cheng, Y. F., Mao, S. Y. and Zhu, W. Y. 2014. Discovery of a novel rumen methanogen in the anaerobic fungal culture and its distribution in the rumen as revealed by real-time PCR. *BMC Microbiol.* 14(1), 104. doi:10.1186/1471-2180-14-104.

Joblin, K. N. 1981. Isolation, enumeration, and maintenance of rumen anaerobic fungi in roll tubes. *Appl. Environ. Microbiol.* 42(6), 1119–22.

Joshi, A., Lanjekar, V. B., Dhakephalkar, P. K., Callaghan, T. M., Griffith, G. W. and Dagar, S. S. 2018. *Liebetanzomyces polymorphus* gen. et sp. nov., a new anaerobic fungus (Neocallimastigomycota) isolated from the rumen of a goat. *MycoKeys* 40(40), 89–110. (doi:10.3897/mycokeys.40.28337).

Julliand, V., Riondet, C., de Vaux, A., Alcaraz, G. and Fonty, G. 1998. Comparison of metabolic activities between *Piromyces citronii*, an equine fungal species, and *Piromyces communis*, a ruminal species. *Anim. Feed Sci. Technol.* 70(1–2), 161–8. doi:10.1016/S0377-8401(97)00043-6.

Kantor, R. S., van Zyl, A. W., van Hille, R. P., Thomas, B. C., Harrison, S. T. and Banfield, J. F. 2015. Bioreactor microbial ecosystems for thiocyanate and cyanide degradation unravelled with genome-resolved metagenomics. *Environ. Microbiol.* 17(12), 4929–41. doi:10.1111/1462-2920.12936.

Kantor, R. S., Huddy, R. J., Iyer, R., Thomas, B. C., Brown, C. T., Anantharaman, K., Tringe, S., Hettich, R. L., Harrison, S. T. and Banfield, J. F. 2017. Genome-resolved meta-omics ties microbial dynamics to process performance in biotechnology for thiocyanate degradation. *Environ. Sci. Technol.* 51(5), 2944–53. doi:10.1021/acs.est.6b04477.

Katayama, S., Tomaru, Y., Kasukawa, T., Waki, K., Nakanishi, M., Nakamura, M., Nishida, H., Yap, C. C., Suzuki, M., Kawai, J., Suzuki, H., Carninci, P., Hayashizaki, Y., Wells, C., Frith, M., Ravasi, T., Pang, K. C., Hallinan, J., Mattick, J., Hume, D. A., Lipovich, L., Batalov, S., Engstrom, P. G., Mizuno, Y., Faghihi, M. A., Sandelin, A., Chalk, A. M., Mottagui-Tabar, S., Liang, Z., Lenhard, B., Wahlestedt, C., RIKEN Genome Exploration Research Group, Genome Science Group (Genome Network Project Core Group) and FANTOM Consortium. 2005. Antisense transcription in the mammalian transcriptome. *Science* 309(5740), 1564–6. doi:10.1126/science.1112009.

Kirk, P. M. 2012. *Anaeromyces polycephalus* (Chen YC, Chien CY, Hseu RS) Flieg., K. Voigt & P.M. Kirk. IF550012. *Index Fungorum* 1, 1.

Kittelmann, S., Naylor, G. E., Koolaard, J. P. and Janssen, P. H. 2012. A proposed taxonomy of anaerobic fungi (class Neocallimastigomycetes) suitable for large-scale

sequence-based community structure analysis. *PLoS ONE* 7(5), e36866. doi:10.1371/journal.pone.0036866.

Koetschan, C., Kittelmann, S., Lu, J., Al-Halbouni, D., Jarvis, G. N., Muller, T., Wolf, M. and Janssen, P. H. 2014. Internal transcribed spacer 1 secondary structure analysis reveals a common core throughout the anaerobic fungi (Neocallimastigomycota). *PLoS ONE* 9(3), e91928. doi:10.1371/journal.pone.0091928.

Kumar, B. M., Puniya, A. K., Singh, K. and Sehgal, J. P. 2004. *In vitro* degradation of cell-wall and digestibility of cereal straws treated with anaerobic ruminal fungi. *Indian J. Exp. Biol.* 42(6), 636–8.

Kumar, S., Sehgal, J. P., Puniya, A. and Kumari, R. 2015. Growth performance and fibre utilization of Murrah male buffalo calves fed wheat straw based complete feed blocks incorporated with superior anaerobic fungal zoospores (*Neocallimastix* sp. GR-1). *Indian J. Anim. Sci.* 85(3), 275–81.

Kumar, S., Stecher, G. and Tamura, K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33(7), 1870–4. doi:10.1093/molbev/msw054.

Lee, S. S., Ha, J. K. and Cheng, K.-J. 2000. Influence of an anaerobic fungal culture administration on in vivo ruminal fermentation and nutrient digestion. *Anim. Feed Sci. Technol.* 88(3-4), 201–17: doi:10.1016/S0377-8401(00)00216-9.

Leis, S., Dresch, P., Peintner, U., Fliegerová, K., Sandbichler, A. M., Insam, H. and Podmirseg, S. M. 2014. Finding a robust strain for biomethanation: Anaerobic fungi (Neocallimastigomycota) from the Alpine ibex (*Capra ibex*) and their associated methanogens. *Anaerobe* 29, 34–43. doi:10.1016/j.anaerobe.2013.12.002.

Li, J. and Heath, I. B. 1992. The phylogenetic relationships of the anaerobic chytridiomycetous gut fungi (Neocallimasticaceae) and the Chytridiomycota. I. Cladistic analysis of rRNA sequences. *Can. J. Bot.* 70(9), 1738–46. doi:10.1139/b92-215.

Li, J., Heath, I. B. and Bauchop, T. 1990. *Piromyces mae* and *Piromyces dumbonica*, two new species of uniflagellate anaerobic chytridiomycete fungi from the hindgut of the horse and elephant. *Can. J. Bot.* 68(5), 1021–33. doi:10.1139/b90-129.

Li, J., Heath, I. B. and Cheng, K.-J. 1991. The development and zoospore ultrastructure of a polycentric chytridiomycete gut fungus, *Orpinomyces joyonii* comb.nov. *Can. J. Bot.* 69(3), 580–9. doi:10.1139/b91-079.

Li, J., Heath, I. B. and Packer, L. 1993. The phylogenetic relationships of the anaerobic chytridiomycetous gut fungi (Neocallimasticaceae) and the Chytridiomycota. II. Cladistic analysis of structural data and description of *Neocallimasticales* ord. nov. *Can. J. Bot.* 71(3), 393–407. doi:10.1139/b93-044.

Li, G. J., Hyde, K. D., Zhao, R. L., Hongsanan, S., Abdel-Aziz, F. A., Abdel-Wahab, M. A., Alvarado, P., Alves-Silva, G., Ammirati, J. F., Ariyawansa, H. A., Baghela, A., Bahkali, A. H., Beug, M., Bhat, D. J., Bojantchev, D., Boonpratuang, T., Bulgakov, T. S., Camporesi, E., Boro, M. C., Ceska, O., Chakraborty, D., Chen, J. J., Chethana, K. W. T., Chomnunti, P., Consiglio, G., Cui, B. K., Dai, D. Q., Dai, Y. C., Daranagama, D. A., Das, K., Dayarathne, M. C., De Crop, E., De Oliveira, R. J. V., de Souza, C. A. F., de Souza, J. I., Dentinger, B. T. M., Dissanayake, A. J., Doilom, M., Drechsler-Santos, E. R., Ghobad-Nejhad, M., Gilmore, S. P., Góes-Neto, A., Gorczak, M., Haitjema, C. H., Hapuarachchi, K. K., Hashimoto, A., He, M. Q., Henske, J. K., Hirayama, K., Iribarren, M. J., Jayasiri, S. C., Jayawardena, R. S., Jeon, S. J., Jerônimo, G. H., Jesus, A. L., Jones, E. B. G., Kang, J. C., Karunarathna, S. C., Kirk, P. M., Konta, S., Kuhnert, E., Langer, E.,

Lee, H. S., Lee, H. B., Li, W. J., Li, X. H., Liimatainen, K., Lima, D. X., Lin, C. G., Liu, J. K., Liu, X. Z., Liu, Z. Y., Luangsa-ard, J. J., Lücking, R., Lumbsch, H. T., Lumyong, S., Leaño, E. M., Marano, A. V., Matsumura, M., McKenzie, E. H. C., Mongkolsamrit, S., Mortimer, P. E., Nguyen, T. T. T., Niskanen, T., Norphanphoun, C., O'Malley, M. A., Parnmen, S., Pawłowska, J., Perera, R. H., Phookamsak, R., Phukhamsakda, C., Pires-Zottarelli, C. L. A., Raspé, O., Reck, M. A., Rocha, S. C. O., de Santiago, A. L. C. M. A., Senanayake, I. C., Setti, L., Shang, Q. J., Singh, S. K., Sir, E. B., Solomon, K. V., Song, J., Srikitikulchai, P., Stadler, M., Suetrong, S., Takahashi, H., Takahashi, T., Tanaka, K., Tang, L. P., Thambugala, K. M., Thanakitpipattana, D., Theodorou, M. K., Thongbai, B., Thummarukcharoen, T., Tian, Q., Tibpromma, S., Verbeken, A., Vizzini, A., Vlasák, J., Voigt, K., Wanasinghe, D. N., Wang, Y., Weerakoon, G., Wen, H. A., Wen, T. C., Wijayawardene, N. N., Wongkanoun, S., Wrzosek, M., Xiao, Y. P., Xu, J. C., Yan, J. Y., Yang, J., Da Yang, S., Hu, Y., Zhang, J. F., Zhao, J., Zhou, L. W., Peršoh, D., Phillips, A. J. L. and Maharachchikumbura, S. S. N. 2016. Fungal diversity notes 253–366: taxonomic and phylogenetic contributions to fungal taxa. *Fungal Divers.* 78(1), 1–237. doi:10.1007/s13225-016-0366-9.

Li, Y., Li, Y., Jin, W., Sharpton, T. J., Mackie, R. I., Cann, I., Cheng, Y. and Zhu, W. 2019. Combined genomic, transcriptomic, proteomic, and physiological characterization of the growth of *Pecoramyces* sp. F1 in monoculture and co-culture with a syntrophic methanogen. *Front. Microbiol.* 10, 435. doi:10.3389/fmicb.2019.00435.

Liebetanz, E. 1910. Die parasitischen Protozoen des Wiederkauermagens. *Arch. Prot.* 19, 19–90.

Liggenstoffer, A. S., Youssef, N. H., Couger, M. B. and Elshahed, M. S. 2010. Phylogenetic diversity and community structure of anaerobic gut fungi (phylum *Neocallimastigomycota*) in ruminant and non-ruminant herbivores. *ISME J.* 4(10), 1225–35 doi:10.1038/ismej.2010.49.

Lowe, S. E., Theodorou, M. K., Trinci, A. P. J. and Hespell, R. B. 1985. Growth of anaerobic rumen fungi on defined and semi-defined media lacking rumen fluid. *Microbiology* 131(9), 2225–9. doi:10.1099/00221287-131-9-2225.

Lowe, S. E., Theodorou, M. K. and Trinci, A. P. J. 1987. Growth and fermentation of an anaerobic rumen fungus on various carbon sources and effect of temperature on development. *Appl. Environ. Microbiol.* 53(6), 1210–5.

Makela, M. R., Dilokpimol, A., Koskela, S. M., Kuuskeri, J., de Vries, R. P. and Hilden, K. 2018. Characterization of a feruloyl esterase from *Aspergillus terreus* facilitates the division of fungal enzymes from carbohydrate esterase family 1 of the carbohydrate-active enzymes (CAZy) database. *Microb. Biotechnol.* 11(5), 869–80. doi:10.1111/1751-7915.13273.

McAllister, T. A., Dong, Y., Yanke, L. J., Bae, H. D., Cheng, K. J. and Costerton, J. W. 1993. Cereal grain digestion by selected strains of ruminal fungi. *Can. J. Microbiol.* 39(4), 367–76. doi:10.1139/m93-054.

McDonald, J. E., Houghton, J. N. I., Rooks, D. J., Allison, H. E. and McCarthy, A. J. 2012. The microbial ecology of anaerobic cellulose degradation in municipal waste landfill sites: evidence of a role for fibrobacters. *Environ. Microbiol.* 14(4), 1077–87. doi:10.1111/j.1462-2920.2011.02688.x.

McGranaghan, P., Davies, J. C., Griffith, G. W., Davies, D. R. and Theodorou, M. K. 1999. The survival of anaerobic fungi in cattle faeces. *FEMS Microbiol. Ecol.* 29(3), 293–300. doi:10.1111/j.1574-6941.1999.tb00620.x.

McSweeney, C. S. and Denman, S. E. 2007. Effect of sulfur supplements on cellulolytic rumen micro-organisms and microbial protein synthesis in cattle fed a high fibre diet. *J. Appl. Microbiol.* 103(5), 1757–65. doi:10.1111/j.1365-2672.2007.03408.x.

McSweeney, C. S., Dulieu, A., Katayama, Y. and Lowry, J. B. 1994. Solubilization of lignin by the ruminal anaerobic fungus *Neocallimastix patriciarum*. *Appl. Environ. Microbiol.* 60(8), 2985–9.

Michel, V., Fonty, G., Millet, L., Bonnemoy, F. and Gouet, P. 1993. *In vitro* study of the proteolytic activity of rumen anaerobic fungi. *FEMS Microbiol. Lett.* 110(1), 5–9. doi:10.1111/j.1574-6968.1993.tb06287.x.

Millard, P., Gordon, A. H., Richardson, A. J. and Chesson, A. 1987. Reduced ruminal degradation of ryegrass caused by sulphur limitation. *J. Sci. Food Agric.* 40(4), 305–14. doi:10.1002/jsfa.2740400403.

Milne, A., Theodorou, M. K., Jordan, M. G. C., Kingspooner, C. and Trinci, A. P. J. 1989. Survival of anaerobic fungi in feces, in saliva, and in pure culture. *Exp. Mycol.* 13(1), 27–37: doi:10.1016/0147-5975(89)90005-4.

Morrison, M., Murray, R. M. and Boniface, A. N. 1990. Nutrient metabolism and rumen micro-organisms in sheep fed a poor-quality tropical grass hay supplemented with sulphate. *J. Agric. Sci.* 115(2), 269–75. doi:10.1017/S0021859600075237.

Mosier, A. C., Miller, C. S., Frischkorn, K. R., Ohm, R. A., Li, Z., LaButti, K., Lapidus, A., Lipzen, A., Chen, C., Johnson, J., Lindquist, E. A., Pan, C., Hettich, R. L., Grigoriev, I. V., Singer, S. W. and Banfield, J. F. 2016. Fungi contribute critical but spatially varying roles in nitrogen and carbon cycling in acid mine drainage. *Front. Microbiol.* 7, 238. doi:10.3389/fmicb.2016.00238.

Mountfort, D. O. 1987. The rumen anaerobic fungi. *FEMS Microbiol. Lett.* 46(4), 401–8. doi:10.1111/j.1574-6968.1987.tb02476.x.

Mountfort, D. O. 1994. Regulatory constraints in the degradation and fermentation of carbohydrates by anaerobic fungi. In: Mountfort, D. O. and Orpin, C. G. (Eds), *Anaerobic Fungi: Biology, Ecology and Function*. Marcel Dekker, New York, pp. 147–68.

Mura, E., Edwards, J., Kittelmann, S., Kaerger, K., Voigt, K., Mrazek, J., Moniello, G. and Fliegerova, K. 2019. Anaerobic fungal communities differ along the horse digestive tract. *Fungal Biol.* 123(3), 240–6. doi:10.1016/j.funbio.2018.12.004.

Nagahama, T. and Nagano, Y. 2012. Cultured and uncultured fungal diversity in deep-sea environments. *Prog. Mol. Subcell. Biol.* 53, 173–87. doi:10.1007/978-3-642-23342-5_9.

Nakamura, T., Yamada, K. D., Tomii, K. and Katoh, K. 2018. Parallelization of MAFFT for large-scale multiple sequence alignments. *Bioinformatics* 34(14), 2490–2. doi:10.1093/bioinformatics/bty121.

Nilsson, R. H., Anslan, S., Bahram, M., Wurzbacher, C., Baldrian, P. and Tedersoo, L. 2019. Mycobiome diversity: high-throughput sequencing and identification of fungi. *Nat. Rev. Microbiol.* 17(2), 95–109. doi:10.1038/s41579-018-0116-y.

Ochiai, A., Itoh, T., Kawamata, A., Hashimoto, W. and Murata, K. 2007. Plant cell wall degradation by saprophytic *Bacillus subtilis* strains: gene clusters responsible for rhamnogalacturonan depolymerization. *Appl. Environ. Microbiol.* 73(12), 3803–13. doi:10.1128/AEM.00147-07.

Orpin, C. G. 1975. Studies on the rumen flagellate *Neocallimastix frontalis*. *J. Gen. Microbiol.* 91(2), 249–62. doi:10.1099/00221287-91-2-249.

Orpin, C. G. 1976. Studies on the rumen flagellate *Sphaeromonas communis*. *J. Gen. Microbiol.* 94(2), 270–80. doi:10.1099/00221287-94-2-270.

Orpin, C. G. 1977a. The occurrence of chitin in the cell walls of the rumen organisms *Neocallimastix frontalis*, *Piromonas communis* and *Sphaeromonas communis*. *J. Gen. Microbiol.* 99(1), 215–8. doi:10.1099/00221287-99-1-215.

Orpin, C. G. 1977b. The rumen flagellate *Piromonas communis*: its life-history and invasion of plant material in the rumen. *J. Gen. Microbiol.* 99(1), 107–17. doi:10.1099/00221287-99-1-107.

Orpin, C. G. 1989. Ecology of rumen anaerobic fungi in relation to the nutrition of the host animal. In: Nolan, J. V., Leng, R. A. and Demeyer, D. I. (Eds), *The Role of Protozoa and Fungi in Ruminant Digestion*. Penumbul Books, Armidale, Australia, pp. 29–37.

Orpin, C. G. and Bountiff, L. 1978. Zoospore chemotaxis in the rumen phycomycete *Neocallimastix frontalis*. *J. Gen. Microbiol.* 104(1), 113–22. doi:10.1099/00221287-104-1-113.

Orpin, C. G. and Greenwood, Y. 1986. The role of haems and related compounds in the nutrition and zoosporogenesis of the rumen chytridiomycete *Neocallimastix frontalis* H8. *Microbiology* 132(8), 2179–85:doi:10.1099/00221287-132-8-2179.

Orpin, C. G. and Joblin, K. N. 1997. The rumen anaerobic fungi. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem*. Blackie Academic and Professional, pp. 140–95.

Orpin, C. G. and Munn, E. A. 1986. *Neocallimastix patriciarum* sp.nov., a new member of the Neocallimasticaceae inhabiting the rumen of sheep. *Trans. Br. Mycol. Soc.* 86(1), 178–81. doi:10.1016/S0007-1536(86)80138-3.

Ozkose, E., Thomas, B. J., Davies, D. R., Griffith, G. W. and Theodorou, M. K. 2001. *Cyllamyces aberensis* gen.nov sp.nov., a new anaerobic gut fungus with branched sporangiophores isolated from cattle. *Can. J. Bot.* 79(6), 666–73 doi:10.1139/b01-047.

Paul, S. S., Kamra, D. N., Sastry, V. R. B., Sahu, N. P. and Kumar, A. 2003. Effect of phenolic monomers on biomass and hydrolytic enzyme activities of an anaerobic fungus isolated from wild nil gai (*Baselophus tragocamelus*). *Lett. Appl. Microbiol.* 36(6), 377–81. doi:10.1046/j.1472-765X.2003.01331.x.

Paul, S. S., Kamra, D. N., Sastry, V. R. B., Sahu, N. P. and Agarwal, N. 2004. Effect of administration of an anaerobic gut fungus isolated from wild blue bull (*Boselaphus tragocamelus*) to buffaloes (*Bubalus bubalis*) on *in vivo* ruminal fermentation and digestion of nutrients. *Anim. Feed Sci. Technol.* 115(1–2), 143–57. doi:10.1016/j.anifeedsci.2004.01.010.

Paul, S. S., Deb, S. M., Punia, B. S., Das, K. S., Singh, G., Ashar, M. N. and Kumar, R. 2011. Effect of feeding isolates of anaerobic fungus *Neocallimastix* sp. CF 17 on growth rate and fibre digestion in buffalo calves. *Arch. Anim. Nutr.* 65(3), 215–28. doi:10.1080/1745039x.2011.559722.

Paul, S. S., Deb, S. M., Dey, A., Somvanshi, S. P. S., Singh, D., Rathore, R. and Stiverson, J. 2015. 16S rDNA analysis of archaea indicates dominance of *Methanobacterium* and high abundance of *Methanomassiliicoccaceae* in rumen of Nili-Ravi buffalo. *Anaerobe* 35(B), 3–10. doi:10.1016/j.anaerobe.2015.06.002.

Paul, S. S., Bu, D., Xu, J., Hyde, K. D. and Yu, Z. 2018. A phylogenetic census of global diversity of gut anaerobic fungi and a new taxonomic framework. *Fungal Divers.* 89(1), 253–66. doi:10.1007/s13225-018-0396-6.

Pelechano, V. and Steinmetz, L. M. 2013. Gene regulation by antisense transcription. *Nat. Rev. Genet.* 14(12), 880–93. doi:10.1038/nrg3594.

Phillips, M. W. and Gordon, G. L. R. 1988. Sugar and polysaccharide fermentation by rumen anaerobic fungi from Australia, Britain and New Zealand. *Biosystems* 21(3–4), 377–83. doi:10.1016/0303-2647(88)90036-6.

Phillips, M. W. and Gordon, G. L. R. 1995. Carbohydrate fermentation by three species of polycentric ruminal fungi from cattle and water buffalo in tropical Australia. *Anaerobe* 1(1), 41–7. doi:10.1016/S1075-9964(95)80418-8.

Podar, M., Makarova, K. S., Graham, D. E., Wolf, Y. I., Koonin, E. V. and Reysenbach, A. L. 2013. Insights into archaeal evolution and symbiosis from the genomes of a nanoarchaeon and its inferred crenarchaeal host from Obsidian Pool, Yellowstone National Park. *Biol. Direct* 8, 9. doi:10.1186/1745-6150-8-9.

Qi, M., Wang, P., O'Toole, N., Barboza, P. S., Ungerfeld, E., Leigh, M. B., Selinger, L. B., Butler, G., Tsang, A., McAllister, T. A. and Forster, R. J. 2011. Snapshot of the eukaryotic gene expression in muskoxen rumen -a metatranscriptomic approach. *PLoS ONE* 6(5), e20521. doi:10.1371/journal.pone.0020521.

Quandt, C. A., Kohler, A., Hesse, C. N., Sharpton, T. J., Martin, F. and Spatafora, J. W. 2015. Metagenome sequence of *Elaphomyces granulatus* from sporocarp tissue reveals *Ascomycota ectomycorrhizal* fingerprints of genome expansion and a Proteobacteria-rich microbiome. *Environ. Microbiol.* 17(8), 2952–68. doi:10.1111/1462-2920.12840.

Raghunathan, A., Ferguson Jr., H. R., Bornarth, C. J., Song, W., Driscoll, M. and Lasken, R. S. 2005. Genomic DNA amplification from a single bacterium. *Appl. Environ. Microbiol.* 71(6), 3342–7. doi:10.1128/AEM.71.6.3342-3347.2005.

Raveh-Sadka, T., Firek, B., Sharon, I., Baker, R., Brown, C. T., Thomas, B. C., Morowitz, M. J. and Banfield, J. F. 2016. Evidence for persistent and shared bacterial strains against a background of largely unique gut colonization in hospitalized premature infants. *ISME J.* 10(12), 2817–30. doi:10.1038/ismej.2016.83.

Saxena, S., Sehgal, J. P., Puniya, A. K. and Singh, K. 2010. Effect of administration of rumen fungi on production performance of lactating buffaloes. *Benefic. Microbes* 1(2), 183–8. doi:10.3920/BM2009.0018.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., Fungal Barcoding Consortium and Fungal Barcoding Consortium Author List. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc. Natl Acad. Sci. U. S. A.* 109(16), 6241–6. doi:10.1073/pnas.1117018109.

Seedorf, H., Kittelmann, S., Henderson, G. and Janssen, P. H. 2014. RIM-DB: a taxonomic framework for community structure analysis of methanogenic archaea from the rumen and other intestinal environments. *PeerJ* 2, e494. doi:10.7717/peerj.494.

Sehgal, J. P., Jit, D., Puniya, A. K. and Singh, K. 2008. Influence of anaerobic fungal administration on growth, rumen fermentation and nutrient digestion in female buffalo calves. *J. Anim. Feed Sci.* 17(4), 510–8. doi:10.22358/jafs/66678/2008.

Seppala, S., Solomon, K. V., Gilmore, S. P., Henske, J. K. and O'Malley, M. A. 2016. Mapping the membrane proteome of anaerobic gut fungi identifies a wealth of carbohydrate binding proteins and transporters. *Microb. Cell Fact.* 15(1), 212. doi:10.1186/s12934-016-0611-7.

Sharon, I., Morowitz, M. J., Thomas, B. C., Costello, E. K., Relman, D. A. and Banfield, J. F. 2013. Time series community genomics analysis reveals rapid shifts in bacterial

species, strains, and phage during infant gut colonization. *Genome Res.* 23(1), 111–20. doi:10.1101/gr.142315.112.

Sirohi, S. K., Singh, N., Dagar, S. S. and Puniya, A. K. 2012. Molecular tools for deciphering the microbial community structure and diversity in rumen ecosystem. *Appl. Microbiol. Biotechnol.* 95(5), 1135–54. doi:10.1007/s00253-012-4262-2.

Sirohi, S. K., Choudhury, P. K., Dagar, S. S., Puniya, A. K. and Singh, D. 2013. Isolation, characterization and fibre degradation potential of anaerobic rumen fungi from cattle. *Ann. Microbiol.* 63(3), 1187–94. doi:10.1007/s13213-012-0577-6.

Sollinger, A., Tveit, A. T., Poulsen, M., Noel, S. J., Bengtsson, M., Bernhardt, J., Frydendahl Hellwing, A. L., Lund, P., Riedel, K., Schleper, C., Hojberg, O. and Urich, T. 2018. Holistic assessment of rumen microbiome dynamics through quantitative metatranscriptomics reveals multifunctional redundancy during key steps of anaerobic feed degradation. *mSystems* 3(4): doi:10.1128/mSystems.00038-18.

Solomon, K. V., Haitjema, C. H., Henske, J. K., Gilmore, S. P., Borges-Rivera, D., Lipzen, A., Brewer, H. M., Purvine, S. O., Wright, A. T., Theodorou, M. K., Grigoriev, I. V., Regev, A., Thompson, D. A. and O'Malley, M. A. 2016. Early-branching gut fungi possess a large, comprehensive array of biomass-degrading enzymes. *Science* 351(6278), 1192–5. doi:10.1126/science.aad1431.

Solomon, K. V., Henske, J. K., Gilmore, S. P., Lipzen, A., Grigoriev, I. V., Thompson, D. and O'Malley, M. A. 2018. Catabolic repression in early-diverging anaerobic fungi is partially mediated by natural antisense transcripts. *Fungal Genet. Biol.* 121, 1–9. doi:10.1016/j.fgb.2018.09.004.

Sridhar, M., Kumar, D., Anandan, S., Prasad, C. S. and Sampath, K. T. 2007. Occurrence and prevalence of *Cyllamyces* genus – a putative anaerobic gut fungus in Indian cattle and buffaloes. *Curr. Sci.* 92(10), 1356–8.

Sridhar, M., Kumar, D. and Anandan, S. 2014. *Cyllamyces icaris* sp. nov., a new anaerobic gut fungus with nodular sporangiophores isolated from Indian water buffalo (*Bubalus bubalis*). *Int. J. Curr. Res. Aca. Rev.* 2, 7–24.

Staley, C. and Sadowsky, M. J. 2018. Practical considerations for sampling and data analysis in contemporary metagenomics-based environmental studies. *J. Microbiol. Methods* 154, 14–8. doi:10.1016/j.mimet.2018.09.020.

Stewart, M. 2019. Polyadenylation and nuclear export of mRNAs. *J. Biol. Chem.* 294(9), 2977–87. doi:10.1074/jbc.REV118.005594.

Stewart, C. S. and Richardson, A. J. 1989. Enhanced resistance of anaerobic rumen fungi to the ionophores monensin and lasalocid in the presence of methanogenic bacteria. *J. Appl. Bacteriol.* 66(1), 85–93. doi:10.1111/j.1365-2672.1989.tb02458.x.

Strassert, J. F. H., Karnkowska, A., Hehenberger, E., Del Campo, J., Kolisko, M., Okamoto, N., Burki, F., Janouskovec, J., Poirier, C., Leonard, G., Hallam, S. J., Richards, T. A., Worden, A. Z., Santoro, A. E. and Keeling, P. J. 2018. Single cell genomics of uncultured marine alveolates shows paraphyly of basal dinoflagellates. *ISME J.* 12(1), 304–8. doi:10.1038/ismej.2017.167.

Sun, M., Jin, W., Li, Y., Mao, S., Cheng, Y. and Zhu, W. 2014. Isolation and identification of cellulolytic anaerobic fungi and their associated methanogens from holstein cow. *Wei Sheng Wu Xue Bao* 54(5), 563–71.

Tedersoo, L., Sánchez-Ramírez, S., Kõljalg, U., Bahram, M., Döring, M., Schigel, D., May, T., Ryberg, M. and Abarenkov, K. 2018. High-level classification of the Fungi and a tool for evolutionary ecological analyses. *Fungal Divers.* 90(1), 135–59. doi:10.1007/s13225-018-0401-0.

Teunissen, M. J., Kets, E. P. W., Op den Camp, H. J. M., Huis in't Veld, J. H. J. and Vogels, G. D. 1992. Effect of coculture of anaerobic fungi isolated from ruminants and non-ruminants with methanogenic bacteria on cellulolytic and xylanolytic enzyme activities. *Arch. Microbiol.* 157(2), 176–82. doi:10.1007/BF00245287.

Theodorou, M. K., Gill, M., King-Spooner, C. and Beever, D. E. 1990. Enumeration of anaerobic chytridiomycetes as thallus-forming units: novel method for quantification of fibrolytic fungal populations from the digestive tract ecosystem. *Appl. Environ. Microbiol.* 56(4), 1073–8.

Theodorou, M. K., Zhu, W. Y., Rickers, A., Nielsen, B. B., Gull, K. and Trinci, A. P. J. 1996. Biochemistry and ecology of anaerobic fungi. In: Howard, D. H. and Miller, J. D. (Eds), *Human and Animal Relationships*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 265–95.

Trinci, A. P., Lowe, S. E., Milne, A. and Theodorou, M. K. 1988. Growth and survival of rumen fungi. *Biosystems* 21(3-4), 357–63. doi:10.1016/0303-2647(88)90033-0.

Tripathi, V. K., Sehgal, J. P., Puniya, A. K. and Singh, K. 2007. Effect of administration of anaerobic fungi isolated from cattle and wild blue bull (*Boselaphus tragocamelus*) on growth rate and fibre utilization in buffalo calves. *Arch. Anim. Nutr.* 61(5), 416–23. doi:10.1080/17450390701556759.

Tu, Q., Yu, H., He, Z., Deng, Y., Wu, L., Van Nostrand, J. D., Zhou, A., Voordeckers, J., Lee, Y. J., Qin, Y., Hemme, C. L., Shi, Z., Xue, K., Yuan, T., Wang, A. and Zhou, J. 2014. GeoChip 4: a functional gene-array-based high-throughput environmental technology for microbial community analysis. *Mol. Ecol. Resour.* 14(5), 914–28. doi:10.1111/1755-0998.12239.

Turaev, D. and Rattei, T. 2016. High definition for systems biology of microbial communities: metagenomics gets genome-centric and strain-resolved. *Curr. Opin. Biotechnol.* 39, 174–81. doi:10.1016/j.copbio.2016.04.011.

Ushida, K., Jouany, J. P. and Demeyer, D. I. 1991. Physiological aspects of digestion and metabolism in ruminants. In: *Proceedings of the Seventh International Symposium on Ruminant Physiology*, New York, pp. 625–54.

Vavra, J. and Joyon, L. 1966. Etude sur la changes in the chemical composition of a cambial morphologie, le cycle evolutif et la position syscell during its differentiation into xylem and phloem tematique de *Callimastix cyclops*, Weissenberg, tissue in trees. *Protistologica* 2, 5–16.

Wagner, E. G. and Simons, R. W. 1994. Antisense RNA control in bacteria, phages, and plasmids. *Annu. Rev. Microbiol.* 48, 713–42. doi:10.1146/annurev.mi.48.100194.003433.

Wallace, R. J. and Munro, C. A. 1986. Influence of the rumen anaerobic fungus *Neocallimastix frontalis* on the proteolytic activity of a defined mixture of rumen bacteria growing on a solid substrate. *Lett. Appl. Microbiol.* 3(2), 23–6. doi:10.1111/j.1472-765X.1986.tb01539.x.

Wang, T. Y., Chen, H. L., Lu, M. J., Chen, Y. C., Sung, H. M., Mao, C. T., Cho, H. Y., Ke, H. M., Hwa, T. Y., Ruan, S. K., Hung, K. Y., Chen, C. K., Li, J. Y., Wu, Y. C., Chen, Y. H., Chou, S. P., Tsai, Y. W., Chu, T. C., Shih, C. A., Li, W. H. and Shih, M. C. 2011. Functional characterization of cellulases identified from the cow rumen fungus *Neocallimastix patriciarum* W5 by transcriptomic and secretomic analyses. *Biotechnol. Biofuels* 4, 24. doi:10.1186/1754-6834-4-24.

Wang, X., Liu, X. and Groenewald, J. Z. 2017. Phylogeny of anaerobic fungi (phylum *Neocallimastigomycota*), with contributions from yak in China. *Antonie Leeuwenhoek* 110(1), 87–103. doi:10.1007/s10482-016-0779-1.

Wang, Y., Youssef, N., Couger, M. B., Hanafy, R., Elshahed, M. and Stajich, J. E. 2018. Comparative genomics and divergence time estimation of the anaerobic fungi in herbivorous mammals. *bioRxiv*, 401869. doi:10.1101/401869.

Wang, Y., Youssef, N. H., Couger, M. B., Hanafy, R. A., Elshahed, M. S. and Stajich, J. E. 2019. Molecular dating of the emergence of anaerobic rumen fungi and the impact of laterally acquired genes. *mSystems* 4, e00247-19. doi:10.1128/mSystems.00247-19.

Webb, J. and Theodorou, M. K. 1991. Neocallimastix hurleyensis sp.nov., an anaerobic fungus from the ovine rumen. *Can. J. Bot.* 69(6), 1220–4. doi:10.1139/b91-156.

West, P. T., Probst, A. J., Grigoriev, I. V., Thomas, B. C. and Banfield, J. F. 2018. Genome-reconstruction for eukaryotes from complex natural microbial communities. *Genome Res.* 28(4), 569–80. doi:10.1101/gr.228429.117.

Weston, R. H., Lindsay, J. R., Purser, D. B., Gordon, G. L. R. and Davis, P. 1988. Feed intake and digestion responses in sheep to the addition of inorganic sulfur to a herbage diet of low sulfur content. *Aust. J. Agric. Res.* 39(6), 1107–19. doi:10.1071/AR9881107.

Wubah, D. A. and Kim, D. S. H. 1996. Chemoattraction of anaerobic ruminai fungi zoospores to selected phenolic acids. *Microbiol. Res.* 151(3), 257–62. doi:10.1016/S0944-5013(96)80022-X.

Wubah, D. A., Fuller, M. S. and Akin, D. E. 1991. Studies on Caecomyces communis: morphology and development. *Mycologia* 83(3), 303–10. doi:10.1080/00275514.1991.12026014.

Yan, B. and Wang, Z. 2012. Long noncoding RNA: its physiological and pathological roles. *DNA Cell Biol.* 31 (Suppl. 1), S34–41. doi:10.1089/dna.2011.1544.

Yanke, L. J., Dong, Y., McAllister, T. A., Bae, H. D. and Cheng, K. J. 1993. Comparison of amylolytic and proteolytic activities of ruminal fungi grown on cereal grains. *Can. J. Microbiol.* 39(8), 817–20. doi:10.1139/m93-121.

Yoon, H. S., Price, D. C., Stepanauskas, R., Rajah, V. D., Sieracki, M. E., Wilson, W. H., Yang, E. C., Duffy, S. and Bhattacharya, D. 2011 Single-cell genomics reveals organismal interactions in uncultivated marine protists. *Science* 332(6030), 714–7. doi:10.1126/science.1203163.

Youssef, N. H., Couger, M. B., Struchtemeyer, C. G., Liggenstoffer, A. S., Prade, R. A., Najar, F. Z., Atiyeh, H. K., Wilkins, M. R. and Elshahed, M. S. 2013. The genome of the anaerobic fungus *Orpinomyces* sp. strain C1A reveals the unique evolutionary history of a remarkable plant biomass degrader. *Appl. Environ. Microbiol.* 79(15), 4620–34. doi:10.1128/AEM.00821-13.

Zhang, L., Cui, X., Schmitt, K., Hubert, R., Navidi, W. and Arnheim, N. 1992. Whole genome amplification from a single cell: implications for genetic analysis. *Proc. Natl Acad. Sci. U. S. A.* 89(13), 5847–51. doi:10.1073/pnas.89.13.5847.

Zhang, K., Martiny, A. C., Reppas, N. B., Barry, K. W., Malek, J., Chisholm, S. W. and Church, G. M. 2006. Sequencing genomes from single cells by polymerase cloning. *Nat. Biotechnol.* 24(6), 680–6. doi:10.1038/nbt1214.

Chapter 9

Ruminal viruses and extrachromosomal genetic elements

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1 Introduction

It was noted by Marvin Bryant in the 1950s that the rumen is a natural microbial habitat that 'appears to be more susceptible to analysis by microbial ecologists than many other microbial habitats' (Bryant 1959). To this day this interest remains, and for good reasons: both economic and environmental. The rumen of herbivores is now known to contain a diverse microbial community of prokaryotes (bacteria, archaea) and eukaryotes (protozoa, fungi), which contribute to the efficient fermentation of plant-based feeds which would be otherwise indigestible. Many of these microbes have been characterised on a genetic and functional (enzymatic) basis, with a focus on the development of strategies to improve rumen function and feed efficiency and reduce the environmental impact of ruminant livestock production.

Despite the sustained research focus on rumen microbial ecology, there is still a relative lack of knowledge surrounding the rumen mobilome including the major factors that contribute to the mobilome (e.g. viruses and plasmids) and the extent to which the mobilome impacts on rumen function. Viral populations have been shown to co-exist with, or predate on, the rumen microbiota; however, there is still a relative lack of basic biological and genetic

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information. Even more striking is the paucity of information regarding non-viral extrachromosomal elements, such as plasmids, which are often intrinsically linked with rumen microbial populations. This chapter aims to review the current understanding of rumen viral populations and extrachromosomal elements, and describes the carriers of mobile genetic elements (MGEs), such as extracellular membrane vesicles (MVs). Building on research conducted in rumen-based studies and advances in other microbial ecosystems, the possible impacts of the mobilome on rumen function are also explored.

2 Extrachromosomal elements

The term 'extrachromosomal elements', most simply, describes the genetic elements present within a cell that are not integrated into the chromosome. Extrachromosomal elements occur in eukaryote and prokaryote cells and may be able to self-replicate. Extrachromosomal elements are in constant exchange with the more stable chromosome and constitute an important feature of genomes, contributing to the genetic capacity of cells and gene transfer between organisms (horizontal gene transfer or HGT). In this regard, extrachromosomal elements can also be described as MGEs and collectively as the mobilome (Koonin and Wolf 2008), with all of these elements contributing to HGT and ultimately microbial evolution (Brussow et al. 2004; Koonin and Dolja 2014; Croucher et al. 2016; Krupovic et al. 2019).

There are four main strategies for HGT: (1) transformation: the natural ability of cells to take up exogenous DNA from the environment; (2) transduction: the transfer of DNA from one cell to another via, for example, viruses; (3) conjugation: the contact-dependent unidirectional transfer of DNA from a donor cell to a recipient cell via a conjugation (mating) apparatus extruding from the donor; and (4) fusion: joining of either two cells or cells with DNA-containing vesicles, such as MVs (reviewed by Bellanger et al. 2014; Johnson and Grossman 2015; Guedon et al. 2017; Rowan-Nash et al. 2019). All of these strategies are likely to occur in the diverse and densely populated microbial ecosystem of the rumen (Morrison 1996), with extrachromosomal elements playing an integral role. In the context of previous studies undertaken with rumen microbes, the term 'extrachromosomal elements' encompasses all of those contributing to the mobilome: (1) viral genomes (intact and remnants of viral genomes); (2) plasmids; and (3) other genetic material, such as DNA contained in extracellular MVs (Fig. 1).

2.1 Viral genomes

Viruses of prokaryotes are the most well-studied and understood extrachromosomal elements found in the rumen, so much so that they are

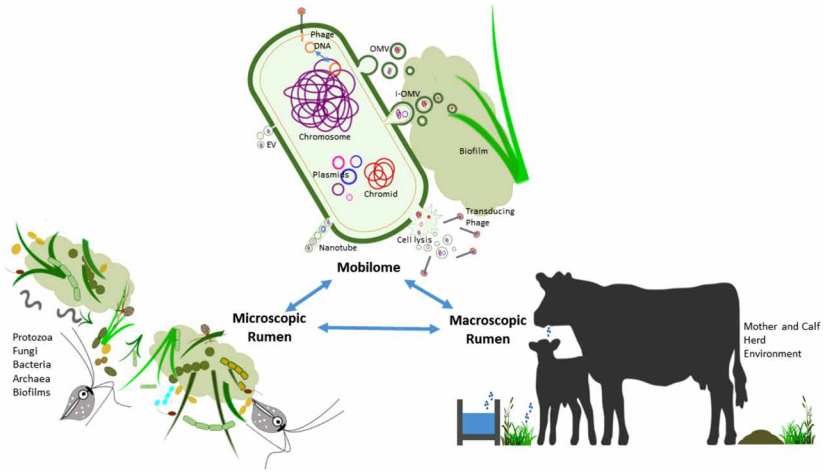

Figure 1 The rumen mobilome: macroscopic and microscopic interactions explained. The mobilome contributes to genetic transfer via mechanisms such as recombination, chromosomal integration and transduction, at the sub-microscopic genetic level. The mobilome includes microbial viruses (e.g. lytic, lysogenic and transducing phages), plasmids (e.g. extrachromosomal and episomal), large chromids, as well as EVs extruded directly from the cell or within a nanotube structure, outer membrane vesicles (OMVs), and outer-inner-membrane vesicles (O-IMVs). Elements of the mobilome may also be released following microbial cell lysis and be captured by biofilms. At the microscopic level, mobilome elements are produced by, and can potentially be transferred between, rumen populations of prokaryotes (bacteria, archaea) and eukaryotes (fungi and protozoa). At the macroscopic level, the mobilome is transferred via mother:calf interactions (e.g. saliva, aerosols); interactions between individual members of a herd (e.g. saliva, aerosols, faecal material); and by interactions with the environment (e.g. food, water).

usually described as independent entities (viruses or phages), rather than being referred to as extrachromosomal elements. Intracellular viral genomes or prophages can be found in cells as episomes (both integrated into the chromosomal and extrachromosomal elements in a population) or as plasmids (solely extrachromosomal) (Deutsch et al. 2018). In this regard, viral genomes may also be grouped with and described as, integrative and conjugative elements (ICEs) (Delavat et al. 2017), with phages capable of integrating new DNA into their genome also described as transducing phage (Toussaint and Rice 2017). The broader definition of ICEs, however, also includes modular genetic elements such as conjugative transposons, which typically contain genes for conjugation (type IV secretion system relaxase or proteins for replication initiation and translocation) and integration (integrases of the tyrosine, serine or DDE family) (Liu et al. 2019) and are often derived from defective prophages (Bobay et al. 2014). Although prophages, which confer a selective advantage, may avoid mutation and maintain viability, selective pressures tend to operate

on prophages, as extrachromosomal and integrated prophage sequences can represent a genetic, replicative burden to the host. As a result, prophages may be susceptible to accumulating mutations that render them defective (Winstanley et al. 2009; Koonin and Dolja 2014).

Defective prophages can be readily detected in rumen bacterial genomes (Gilbert and Klieve 2015). These appear as prophage-like sequences; however, either single genes or whole genetic modules, usually those encoding for structural proteins (coat and tail proteins), can be lost and only the integration and replication proteins remain. These defective prophages may be identified using computational tools for the detection of prophages in prokaryote genome sequences, for example PHASTer and PhiSpy (Akhter et al. 2012; Arndt et al. 2016). However if these tools are used, efforts to more comprehensively annotate prophage-associated genes and identify expected phage gene modules (Gilbert et al. 2017) are required to determine if the identified prophages possess a full complement of phage-related genes. Defective prophages, however, may be the basis for the formation of new ICEs and, therefore, contribute to HGT between rumen bacteria. The extent to which viruses (intact or defective) contribute to HGT in the rumen is difficult to fully ascertain; however, their impact is expected to be significant given the extent to which they occur (Seshadri et al. 2018). As rumen viruses are the most extensively studied genetic elements contributing to the rumen mobilome, developments in the field of rumen virus research are reviewed further in Section 3.

2.2 Plasmids

The first report of a plasmid being associated with a rumen bacterial isolate was made by Teather (1982), who found the type strain of *Butyrivibrio fibrisolvens* ATCC19171 and six new bovine isolates of *B. fibrisolvens* – all contained plasmids. This report also suggested that these plasmids could be important in conferring competitive fitness to this common rumen bacterial genus and could affect its ability to ferment sugars and polysaccharides and degrade proteins. Further studies followed, with several plasmids detected in other isolates of the genus *Ruminococcus* (Asmundson and Kelly 1987), and with the advent of DNA sequencing, plasmid sequences were reported for the isolates of *Ruminococcus*, *Selenomonas*, *Butyrivibrio* and *Prevotella* (later re-classified as *Bacteroides*) (Champion et al. 1988; Attwood and Brooker 1992; Hefford et al. 1993; May et al. 1996; Ogata et al. 1996; Ohara et al. 1998). These sequences showed the presence of modules of replication and mobilization proteins, as well as often unidentified accessory proteins (Ohara et al. 1998) as would be expected for a self-replicating extrachromosomal element (Thomas et al. 2017). There were also some investigations into the transfer of plasmids encoding

antibiotic (tetracycline) resistance between strains of *Bacteroides ruminicola* (Flint et al. 1988). Much of this research was driven by the trend towards biotechnology at that time, with the goal of discovering new enzymes involved in carbohydrate breakdown and developing tools for genetically transforming rumen bacteria in order to increase the efficiency of fibre breakdown (Morrison 1996; Flint 1997).

While interest in genetic transformation has waned, interest in the plasmids of rumen bacteria has continued. With improvements in genome sequencing technologies, plasmid sequences are being reported for many rumen bacterial isolates, including the original type strains of *Ruminococcus albus* (Suen et al. 2011; Dassa et al. 2014), as well as metabolically diverse, complex fibre-degrading strains of *Butyrivibrio* (Kelly et al. 2010; Palevich et al. 2017), and the resistant starch degrader *Bifidobacterium choerinum* (Jung et al. 2018). As genome annotation has also significantly improved since the original attempts for the isolation and sequencing of plasmids in the 1990s, the genes these newly identified plasmids carry are being assigned functional identity. For example, the plasmid associated with *B. choerinum* FMB-1 carries a phenicol efflux pump gene associated with antibiotic resistance (Jung et al. 2018). Genome sequencing of several *R. albus* strains have shown that plasmids found in this species, for example, *R. albus* strain 7, contain genes for dockerins (Dassa et al. 2014), which are required for the formation of cellulose-degrading, multi-enzyme cellulosome complexes (Nash et al. 2016). In contrast, two megaplasmids associated with *Butyrivibrio proteoclasticus* B316 appeared to encode genes important for survival in the rumen but made no detectable contribution to plant polysaccharide degradation (Kelly et al. 2010).

Interestingly, a relatively large percentage (7.7%) of the *Butyrivibrio hungatei* MB2003 genome is not directly chromosome associated, as it contains four replicons, a single chromosome (3 143 784 bp), a smaller chromid or secondary chromosome (91 776 bp), a megaplasmid (144 470 bp) and a small plasmid (6284 bp) (Palevich et al. 2017). Of these extrachromosomal elements, the chromid was found to encode genes for the intracellular breakdown of carbohydrates (β-glucosidase, β-galactosidase and a polysaccharide deacetylase). Chromids have been previously described in prokaryotes (Harrison et al. 2010) and differ from smaller plasmids, having a GC content similar to that of the main chromosome, yet containing plasmid-like genes for maintenance and replication. As more attention is focussed on annotating and characterising the plasmids associated with rumen microbial genomes, it can be anticipated that a greater understanding of the impact of these extrachromosomal elements on rumen function will be developed.

In an alternative approach to characterising and understanding the role of plasmids in the rumen, metagenomic sequencing has been undertaken of a plasmid fraction (plasmidome) purified from the rumen fluid from dairy cows

(Kav et al. 2012). This study developed new methodology for the purification of plasmids from the rumen fluid, examined the types of rumen microbes carrying plasmids and determined plasmid gene function. Results indicated that rumen bacteria were more likely to carry plasmids than archaea and eukaryotes, with the distribution of dominant bacterial phyla associated with plasmids being Firmicutes (47%), Bacteroidetes (22%), Proteobacteria (20%) and Actinobacteria (9%). Functional analysis of plasmid-associated genes using SEED assignment (Overbeek et al. 2005), as well as comparison to plasmid databases, showed a high representation of subsystems for amino acids; DNA metabolism; cofactors, vitamins etc.; cell wall and capsule; carbohydrates; respiration; and protein metabolism. This functional analysis indicated that plasmids of rumen bacteria may contain a mixture of plasmid-associated genes including replication and lateral gene transfer (LGT), as well as accessory functional genes, normally associated with survival and substrate utilisation in the rumen (including cell wall glycosyltransferases, capsular polysaccharides, enzymes for protein metabolism and carbohydrate-degrading enzymes).

Most recently, with advances in metagenomics and bioinformatics, metagenome assembled genomes (MAGSs) have been obtained from bovine rumen samples, together with plasmid sequences (Stewart et al. 2018). Although obtaining and analysing plasmids was not the major focus of this investigation, two plasmid contigs were identified which showed similarity to a *R. albus* 7 plasmid (pRUMAL02) (Dassa et al. 2014), which also included genes for dockerin proteins. In addition, these plasmid contigs appeared to be shared across four other assembled genomes including three which were classified as Clostridiales and one *R. flavefaciens* (Stewart et al. 2018). While the genus *Ruminococcus* is also classified in the bacterial order Clostridiales, these results suggest that plasmids provide a mechanism for cross-species transfer of key proteins responsible for carbohydrate breakdown in the rumen.

2.3 Other extrachromosomal elements and membrane vesicles

The extent to which rumen microbes can uptake genetic material via extracellular elements other than viruses and plasmids is still largely unknown and to date has not been an active area of investigation. This may, in part, be attributed to the nature of the rumen fluid itself, from the considerable phylogenetic and phenotypic diversity of the microbes present, to the physical complexity this diversity entails. Within the rumen there is stratification between the raft of plant material (digesta) and fluid fractions, formation of biofilms and production of extracellular polysaccharide slime (polymeric matrix), as well as interactions with the rumen epithelium (Bryant 1959; Russell and Rychlik 2001; Huws et al. 2018). It has been well established that microbial cells within the rumen can lyse, releasing their contents into the rumen fluid (Wells and Russell

1996), and the protein components are quickly absorbed by other microbes (Leng and Nolan 1984). The nucleic acids released from lysed cells are very vulnerable to degradation in the rumen (Russell and Wilson 1988); therefore, it was hypothesised that physical structures, such as MVs, also known as outer membrane vesicles (OMVs), extracellular vesicles (EVs) and the double-layered inner-outer membrane vesicles (I-OMVs) (Fig. 1), may play a major role in protecting nucleic acids from degradation in the rumen (Klieve et al. 2005). In this way, MVs may play a major role in the transport of extrachromosomal elements and facilitate gene transfer in the rumen.

MVs may be packed not only with DNA, but they can also contain proteins and lipids (reviewed by Willms et al. 2016; Clarke 2018; Volgers et al. 2018). MVs are spherical, bilayer structures, ranging from 20 to 400 nm in size (Toyofuku et al. 2019). MVs are usually formed by gram-negative bacteria, where they slough or bleb away from the cell surface, but the ability to release MVs from the cell surface is conserved across all prokaryotes (bacteria, archaea) and microbial eukaryotes (fungi and protozoa) (Deatherage and Cookson 2012). MVs may be used by microbes to acquire nutrients, defend against other microbes, enable a method for cell-to-cell communication, resist pressures from the host immune system and contribute to biofilm formation (Ellis and Kuehn 2010; Pope et al. 2011). They may also be used as a defence against virus or phage infection, with receptors on the MV providing a decoy for particle attachment; however, MVs can export and carry virus particles, as well as extracellular DNA (Toyofuku et al. 2019).

While MVs have been shown to be produced by rumen fungi, such as *Sphaeromonas communis*, during the colonization of straw (Gaillard et al. 1989), the majority of research into MVs in the rumen has focused on the ability of bacterial MVs to transport enzymes involved in feed breakdown. This has been experimentally shown for the rumen bacteria *R. albus* (Kim et al. 2001) and *Fibrobacter succinogenes* (Groleau and Forsberg 1981; Gong and Forsberg 1993; Arntzen et al. 2017). MVs are also produced by *Bacteroides ruminicola*; however, their role in plant degradation or DNA transfer is unknown (Huws et al. 2018). It is likely that many more species of rumen bacteria produce MVs, with these MVs assisting with feed breakdown but also actively contributing to the transport and LGT of extrachromosomal elements within the rumen.

3 Rumen viruses

Although some viruses may be ingested into the rumen from the environment via feed, water, aerosols, and between-animal transmission (e.g. saliva, contact with faecal material; Fig. 1), the majority of viruses found in the rumen are those which actively infect and replicate within the microbes. As the most dominant microbes present in the rumen are bacteria (Hungate 1966; Seshadri

et al. 2018), the vast majority of viruses present in the rumen fluid are viruses infecting bacteria or bacteriophages (phages) (Adams et al. 1966; Hoogenraad et al. 1967). Archaeal populations found in the rumen have also been shown to be infected by archaeal viruses (Baresi and Bertani 1984; Attwood et al. 2008) and are sometimes also referred to as phage or archaeaphage (Abedon and Murray 2013). Given findings from other microbial ecosystems (Ghabrial et al. 2015; Grybchuk et al. 2018), the eukaryote populations of the rumen (protozoa and fungi) are also likely to be infected by viruses. However, eukaryotic viruses are yet to be isolated from the rumen, with their existence shown only *in silico* through the detection of genes related to known protozoal viruses in the metagenomic analyses of the rumen virome (Berg Miller et al. 2012; Anderson et al. 2017).

Advances in rumen phage research have closely followed advances in the technologies available for their study. With the development of the transmission electron microscope (TEM), researchers began examining rumen samples from domesticated herbivores (cattle and sheep) and reindeer in order to establish whether viruses existed and undertook morphological surveys of the virus-like particles they observed (Adams et al. 1966; Hoogenraad and Hird 1970; Tarakanov 1972). Although the schematics for viral classification have changed with time, these studies were the first to determine that the rumen was dominated by tailed phages of the viral order Caudovirales, including long-tailed Siphoviridae, contractile-tailed Myoviridae and short-tailed Podoviridae.

A TEM morphological survey of a sample obtained from an anaerobic fermentation system started with an inoculum of goat rumen fluid (Fig. 2) showed the presence of Siphoviridae, Myoviridae and Podoviridae. While it can sometimes be difficult to distinguish the small tail structures of Podoviridae using TEM, this survey also found examples of non-tailed viral particles, possibly belonging to the viral families Tectiviridae, Corticoviridae and Microviridae, which are also known to infect bacteria (Ackermann and Prangishvili 2012). These particles have been previously observed by our laboratory during TEM of the bovine rumen fluid; however, these phages have not been isolated from the rumen. Filamentous phages (Inoviridae) have also been observed in the rumen fluid; however, it is difficult to distinguish these long, thin particles, lacking a head structure, in crude viral preparations from rumen fluid, due their similarity to broken phage tails and bacterial extracellular filamentous-like structures (thin pili and flagella).

During the era of TEM, researchers also began to isolate and purify infective phages from rumen fluid and introduced these phages into culture collections. As the isolation of individual phage requires the use of a bacterial host which is susceptible to infection and phages may have a limited host range (De Jonge et al. 2019), the choice of microbial host to use for phage isolation can dictate the chances of successful phage isolation. Rather than using a

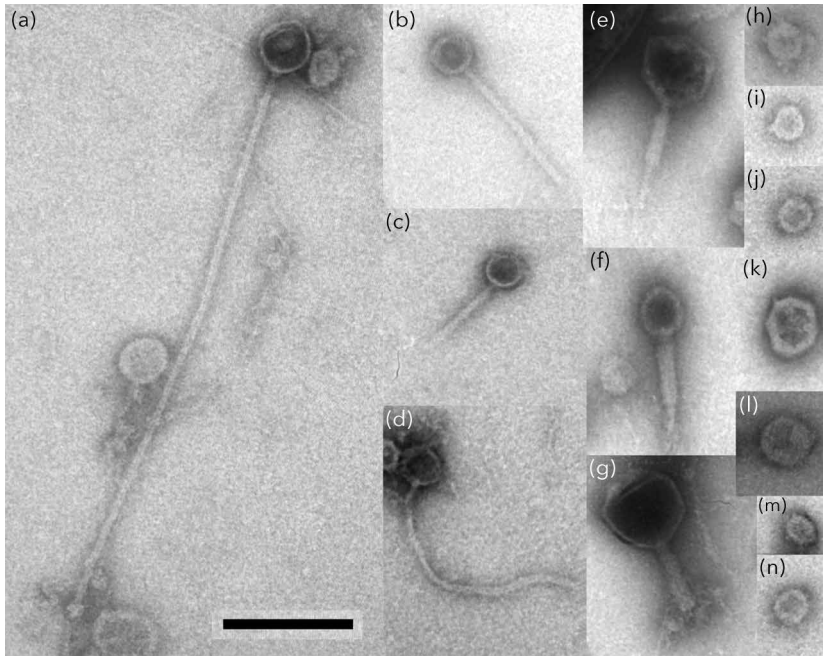

Figure 2 Example of common rumen viral morphotypes including Siphoviridae (a–d), Myoviridae (e–g), Podoviridae (h,i) and potentially non-tailed phages (j–n), such as Corticoviridae and Microviridae (scale bar 200 nm). All particles were purified from a single sample collected from an *in vitro* fermentation system (Infors), started with the rumen fluid obtained from a wild goat. Phage particles were fixed with 2.5% glutaraldehyde and stained with 1% ammonium molybdate pH 7.0, with all images taken at the same magnification using a Joel 1400 transmission electron microscope. Images taken with the assistance of Dr Kathy Crew, DAF.

broad-spectrum approach, employing a wide variety of rumen bacterial host strains, most isolation studies focused on using a limited number of bacterial genera of specific interest, reviewed by Gilbert and Klieve (2015). The majority of isolated phages have, therefore, been selected on bacterial hosts with the intent of developing phage therapies to control problematic rumen microbes (e.g. *Streptococcus bovis*), or for use in biotechnological approaches, for example, to introduce genes for plant detoxification (*Bacteroides ruminantium*, *Bacteroides ruminicola*) (Gregg et al. 1994; Tarakanov 1994; Wong et al. 2003).

The majority of phages isolated from the rumen to date have been Siphoviridae, infecting *Streptococcus* strains sourced from the rumen, formally classified as *S. bovis* and now classified as *S. equinus* (Schlegel et al. 2003). These studies were undertaken in several different countries and therefore encompass phages sourced from ruminants (cattle, including dairy cows, and sheep) being maintained on different diets (Adams et al. 1966; Brailsford

and Hartman 1968; Iverson and Millis 1976a; Tarakanov 1976; Styriak et al. 1989; Klieve and Bauchop 1991). Phage host ranges are often narrow; that is, each phage isolate can infect only a limited number of *Streptococcus* host strains (Klieve et al. 1999), although there are exceptions, with one report of a phage infecting five out of ten *S. equinus* strains examined (Styriak et al. 1994). From the bacterial perspective there are also exceptions, with the *S. equinus* strain 2B (Iverson and Millis 1976a) appearing to be more receptive to phage infection than other strains of *Streptococcus* (Klieve et al. 1999; Gilbert et al. 2017).

The number of reports describing the isolation and characterisation of phages from the rumen has decreased considerably with the advent of molecular and genetic methods to characterise rumen phage populations; however, much can be learnt about the biology of phages in the rumen using phage isolates. For example, early culture-based studies of rumen phage infecting *Streptococcus* determined not only host range but also attributes such as the growth rate (replication time from phage infection to host cell lysis) and burst size (the number of particles released following infection and replication) (Iverson and Millis 1976a). Other studies using phage isolates determined the rate of viral particle decay in rumen fluid (Orpin and Munn 1974; Tarakanov 1976; Swain 1999), the development of phage resistance (Klieve and Bauchop 1991) and the interactions of phages with rumen epithelial cells (Styriak et al. 1991).

With the advent of molecular biology, research shifted away from TEM surveys and culture-based studies, instead of using DNA-based methods to enumerate and obtain an overall picture, or snapshot, of the rumen phage population within any one sample or at a specific point in time (Klieve and Gilbert 2005). TEM studies had estimated rumen phage populations to be at least 5×10^7 phages per mL cattle rumen fluid (Paynter et al. 1969) and between 2×10^7 and 1×10^8 phages per mL cattle and sheep rumen fluid (Klieve and Bauchop 1988), with the highest estimates for cattle being $> 10^9$ phage particles per mL rumen fluid (Ritchie et al. 1970). The enumeration of rumen phage populations based on phage DNA concentrations estimated phage populations to be slightly higher than those from the previous TEM studies, with 3×10^9 and 1.6×10^{10} phage particles per mL rumen fluid reported (Klieve and Swain 1993). These methods also enabled a profile or fingerprint of phage populations to be obtained based on the genome length and showed two major components consistently occurring in the rumen fluid: first, a broad range of genome lengths (30–200 kb) representing DNA genomes from many different phages and encompassing the expected genome sizes of tailed phages; and, second, discrete bands of DNA arising from a single or several phages and presumably representing blooms of phage particle production (Swain et al. 1996).

These studies also demonstrated for the first time that diet and dietary components may affect phage numbers in the rumen, with sheep grazing green pasture having significantly higher phage populations than sheep fed a basal diet of chopped hay (oats:lucerne 70:30). They also showed differences occurring in overall phage numbers between individual animals and between ruminant species (sheep, cattle and goats) (Swain et al. 1996). The dynamics of phage populations in once-a-day fed sheep were also determined, showing that phage numbers in the rumen were not always constant and changed in response to feeding, with increases in total phage numbers occurring 4 h post-feeding, with maximum phage numbers occurring 8 to 10 h post-feeding. These patterns were similar to those previously observed for rumen bacterial populations in once-a-day fed cattle (Leedle et al. 1982), and it was hypothesised that phage numbers were fluctuating in response to changes in total bacterial numbers, albeit with phage numbers peaking approximately 2 h after the peak in bacterial numbers. This time delay is thought to be a consequence of the time taken for phage replication and host cell lysis.

At this time *in vitro* studies were also undertaken to develop models for manipulating rumen phage numbers, using dietary compounds or feed supplements to either stimulate phage lysis or remove phage particles from the rumen (Swain 1999). Compounds examined included secondary plant compounds (saponin, rutin, catechin, quercetin and tannic acid), the plant hormone phenyl acetic acid, the ionophore antibiotic monensin and the montmorillonite clay bentonite. Of these compounds, none appeared to be able to increase phage numbers; however, bentonite appeared to precipitate phage particles, binding and removing them from the rumen fluid. Similarly tannic acid also reduced phage numbers, presumably by binding the protein components of phage particles. A consequent animal trial with sheep (Swain 1999) showed that the intra-ruminal addition of tannic acid reduced phage population concentrations over a 24-h period (Fig. 3). These studies provided an insight into how dietary compounds may chemically affect or physically interact with phage particles in the rumen, and although there have been many more recent studies into the effect of plant compounds on rumen microbial populations (Hart et al. 2008; Huws et al. 2009; Belanche et al. 2012; Wang et al. 2019), the effect of most dietary compounds on rumen phage populations has remained unstudied.

Advances in bacterial genome sequencing technologies have also impacted our understanding of rumen phages. This is due to the fact that common phage replication strategies can result in the incorporation of phage genomic DNA into the genome of the host cell. Whole genome sequencing technologies can effectively include this phage genetic material in the total bacterial genome sequence data, and the interrogation of genomic sequence data can lead to the detection of phage-related genetic material (Koonin and Wolf 2008). While

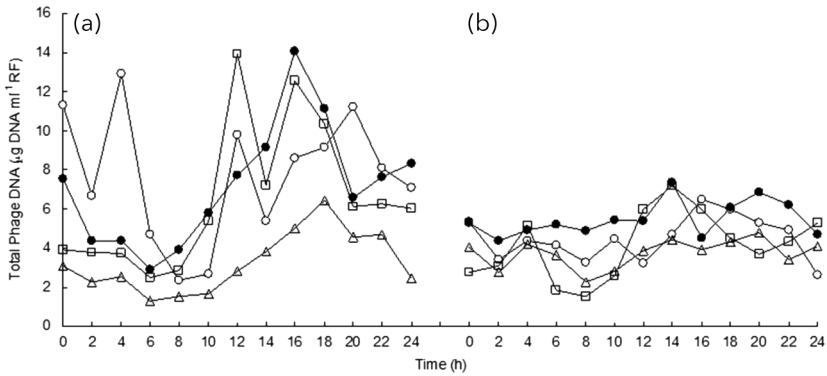

Figure 3 Effect of the model secondary plant compound, tannic acid, on rumen phage populations over a 24-h sampling period based on total phage DNA concentrations (µg mL^{-1} RF) for (a) four sheep without intra-ruminal tannic acid addition, and (b) the same four sheep with intra-ruminal tannic acid addition (50 mL of 8% wt/vol. solution) at 4 h (09:00). All sheep were fed once daily (Oaten:Lucerne chaff diet, 70:30 ratio) at 4 h (09:00) with (○) Sheep A, (Δ) Sheep B, (□) Sheep C and (●) Sheep D. Figure modified from Swain (1999).

the terminology used to describe phage replication strategies is sometimes inconsistent and under review (Hobbs and Abedon 2016), the ability of phages to infect and incorporate their genome into that of the host cell, resulting in a stable, heritable state, is most commonly referred to as lysogeny, and this replication cycle is called the lysogenic cycle (Fig. 3). Phages which undergo lysogeny are often referred to as a temperate, and the phage genome residing in the host cell is referred to as a prophage (Zabriskie 1964; Howard-Varona et al. 2017). Prophages either integrate their DNA into the host chromosome or become circularised in the cytosol as a plasmid, with these states being inter-changeable (episomal) (Deutsch et al. 2018). Lysogenic phages were first shown to exist in the rumen in the 1970s, with reports of intact phage-like particles being detected in *S. equinus* strains following the application of the chemical inducing agent, mitomycin C (Tarakanov 1974; Iverson and Millis 1976b). A study examining 38 strains of rumen bacteria, representing five different genera, shows that nine strains (23.7%) could produce intact phage-like particles following the application of mitomycin C (Klieve et al. 1989). Alternatives to lysogeny, such as carrier states and chronic infections, whereby phages can cause persistent infections and the phage can be maintained and co-exist with the microbial host (Weinbauer 2004; Clokie et al. 2011), have also been shown in rumen bacteria such as *Bacteroides ruminicola* (Klieve et al. 1991). While the existence of these states can be suggested using bacterial genomics (i.e. the presence of an intact prophage sequence), further

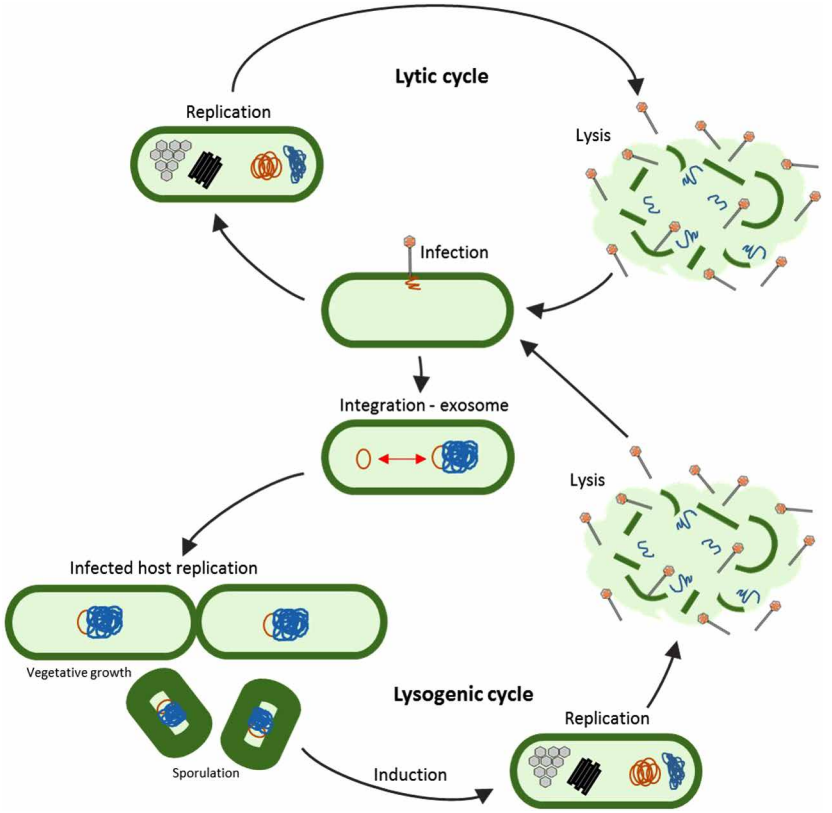

Figure 4 Diagrammatic representation of phage replication cycles previously shown to occur in the rumen. Following phage adsorption and infection, the lytic cycle may immediately commence, with the phage genome being transcribed, replicated and phage proteins produced, enabling the assembly of mature phage particles which are released following host cell lysis. The lysogenic cycle involves the establishment of stable, heritable state, with the phage genome forming an episome, either integrating into the host chromosome or circularising, that is referred to as a prophage and is retained during vegetative growth or sporulation. Most phage genes are transcriptionally repressed in prophages, but following induction, this repression is removed, resulting in phage replication and the lysis of the host cell to release mature phage particles, as in the lytic cycle.

culture-based experimental approaches are required to validate the use of these replicative lifestyles (Fig. 4).

To date, there are very few rumen phage genome sequences deposited in publicly available genetic databases. These sequences include lytic phages infecting the rumen microbial genera – *Streptococcus*, *Bacteroides* and *Ruminococcus* – and represent phages of the Siphoviridae and Podoviridae families (Gilbert et al. 2017). Of these phages, the *Streptococcus* phage φSb01,

originally sourced from the bovine rumen fluid, has been the most characterised biologically (Klieve and Bauchop 1991). This phage has a typical Siphovirus morphology and lacks any genetic modules for developing and maintaining lysogeny (Fig. 5). In anaerobic culture, φSb01 can lyse the host strain *S. equinus* 2B within 3 h post-infection, with phage-resistant cells being developed following the continued incubation of infected cultures. The phage-resistant cells change their growth habit to a clumping growth, with the formation of thick polysaccharide capsules (Klieve and Bauchop 1991). Phage particles of φSb01 have been shown to be degraded in rumen fluid (Fig. 5), with particle decay rates ranging from 36.6% to 53.0% per hour in repeated experiments (Swain 1999).

Technological developments in whole genome sequencing of rumen microbes (bacteria and archaea), however, have rapidly expanded the rate at which prophages can be detected. Surveys of the prevalence of prophage sequences in publicly available genome sequences of cultured rumen bacteria and archaea (Berg Miller et al. 2012; Gilbert and Klieve 2015; Seshadri et al. 2018) have revealed that the majority of rumen bacterial genera include strains which maintain prophage-related sequences within their genome. Whether these prophage sequences can encode for intact, viable phage particles, however, remains to be experimentally shown for the majority of these prophages. Although lytic phages infecting methanogenic archaea are known to exist (Meile et al. 1989; Nölling et al. 1993; Weidenbach et al. 2017; Wolf et al. 2019), to date there has only been one publication describing a lytic archaeal virus infecting the rumen archaea (Baresi and Bertani 1984), and no further work has been reported for this virus. Several prophages have been reported to be present in the genome sequences of methanogenic rumen archaea, including *M. ruminantium* M1, *Methanobrevibacter* sp. JH1 and *Methanobacterium formicicum* BRM9 (Attwood et al. 2008; Leahy et al. 2013; Kelly et al. 2014). All of these prophages genetically appear to represent tailed phages; however, they have proved difficult to be induced or cultivated *in vitro*. Despite the difficulty in obtaining intact phage particles, archaeal prophage–encoded proteins have been experimentally expressed and shown to specifically hydrolyse the pseudomurein cell wall component of the methanogenic archaea (Altermann et al. 2018).

Whole genome sequencing of bacteria and archaea has also revealed the presence of defence systems against phage infection, including restriction modification (RM) systems and clustered regularly interspaced short palindromic repeat sequences (CRISPR) and CRISPR-associated (*Cas*) proteins. RM systems enable specific and non-specific methylation and/or cleavage of incoming foreign DNA (Koonin et al. 2017), thus protecting against phage infection. RM systems have been identified in several rumen strains including *Streptococcus*, *Ruminococcus*, *Bacteroides, Megasphaera, Mitsuokella*,

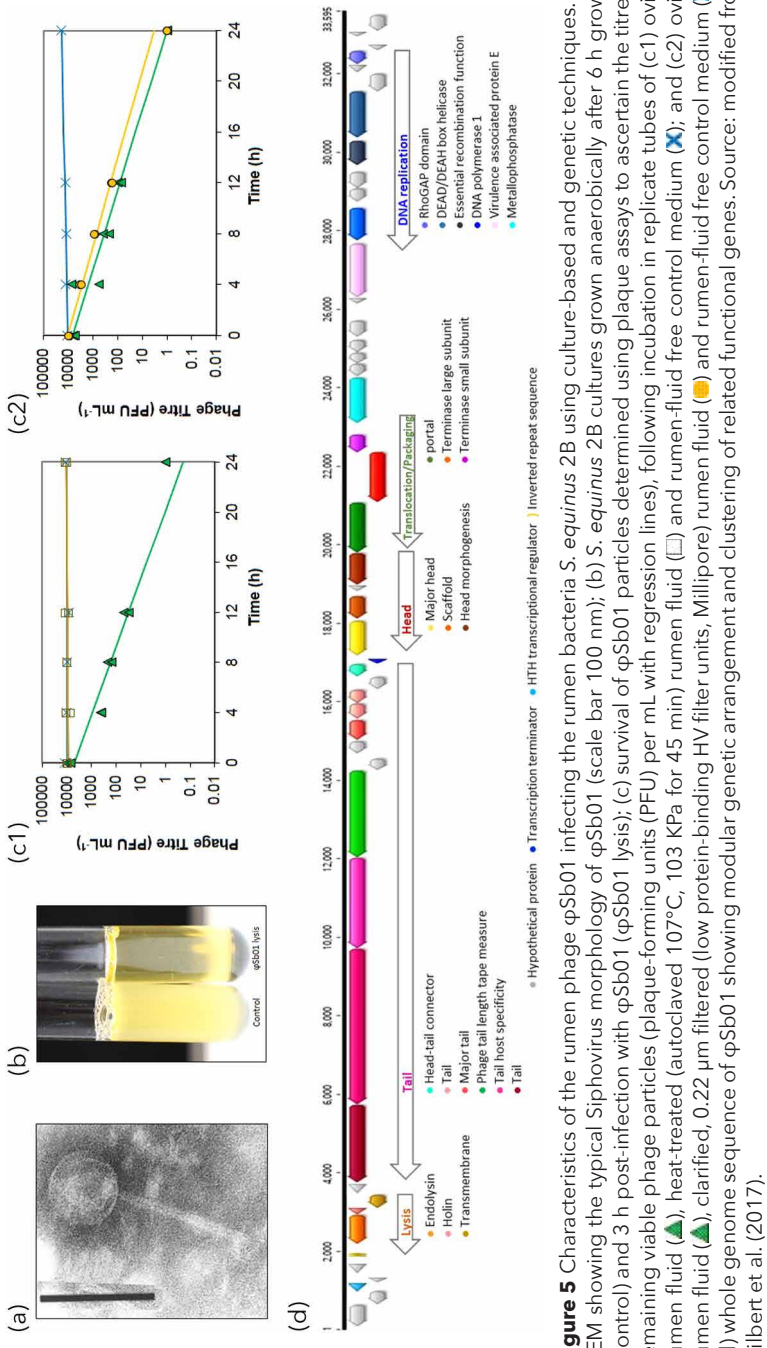

Figure 5 Characteristics of the rumen phage φSb01 infecting the rumen bacteria *S. equinus* 2B using culture-based and genetic techniques. (a) TEM showing the typical Siphovirus morphology of φSb01 (scale bar 100 nm); (b) *S. equinus* 2B cultures grown anaerobically after 6 h growth (control) and 3 h post-infection with φSb01 (φSb01 lysis); (c) survival of φSb01 particles determined using plaque assays to ascertain the titre of remaining viable phage particles (plaque-forming units (PFU) per mL with regression lines), following incubation in replicate tubes of (c1) ovine rumen fluid (▲), heat-treated (autoclaved 107°C, 103 KPa for 45 min) rumen fluid (□) and rumen-fluid free control medium (✕); and (c2) ovine rumen fluid (▲), clarified, 0.22 µm filtered (low protein-binding HV filter units, Millipore) rumen fluid (●) and rumen-fluid free control medium (✕); (d) whole genome sequence of φSb01 showing modular genetic arrangement and clustering of related functional genes. Source: modified from Gilbert et al. (2017).

Selenomonas and *Treponema* (Styriak et al. 1998; Piknova et al. 2004; Pristas and Piknova 2005; Gilbert et al. 2017), with species-specific differences in the types and abundance of RM systems observed, indicating that some species of rumen bacteria may be more susceptible to phage infection than others.

The presence of CRISPR-Cas systems in rumen microbes have also been noted in several studies of rumen bacterial genomes (Attwood et al. 2008; Berg Miller et al. 2012; Kelly et al. 2014; Gilbert et al. 2017; Seshadri et al. 2018). CRISPR-Cas systems are found in archaea and bacteria (Sorek et al. 2008) and provide microbes with immunity to incoming DNA, with immunity built up over time through the acquisition of short stretches (termed 'spacers') of invasive nucleic acids into CRISPR loci (Barrangou 2013). This provides a mechanism to recognise (via homology to spacer regions) and cleave (via *Cas* proteins) incoming DNA, including viral DNA. The most comprehensive study of CRISPR sequences in rumen bacterial genomes (Seshadri et al. 2018) showed that of the 410 genomes examined, 241 of these contained a total of 6344 CRISPR spacer sequences. Associations were noted between 83 viral operational taxonomic units (OTUs) and 31 Hungate hosts, following searches against the IMG/VR database (Paez-Espino et al. 2017), and only a single instance of identical spacer sequences, occurring between isolates of Enterobacteriales and Pseudomonadales. These findings further indicate the highly diverse and genetically heterogeneous nature of rumen phage populations, with very few cultured strains having developed immunity to, or experienced infection by, highly related phages.

Another advance in genetic technology which has contributed to our understanding of rumen phage populations has been the development of viral metagenomics, with the resulting dataset sometimes referred to as a virome. Metagenomics involves obtaining sequence data for all the genetic material in an environmental sample, including viruses. However with their considerably smaller genome length, viruses tend to be relatively under-represented in metagenomic datasets. Viral metagenomics usually involves pre-processing of the environmental sample to concentrate or 'enrich' the virus particles and reduce concentrations of contaminating, non-viral DNA (Edwards and Rohwer 2005; Thurber et al. 2009). Unless specific steps are introduced to clone and sequence ssDNA and transcribe and amplify any viral RNA contained in these enriched viral samples (Edwards and Rohwer 2005; Bolduc et al. 2012), these ssDNA and RNA viruses will not be detected by the DNA sequencing technologies. This may preclude the detection of families of viruses with RNA genomes which may infect rumen-associated eukaryotes (fungi, protozoa), such as the Partitiviridae and Totiviridae (Nibert et al. 2009; Goodman et al. 2011), and RNA viruses infecting bacteria and archaea such as the Cystoviridae and Leviviridae (Krupovic et al. 2011). Viral metagenomics has the advantage of overcoming the limitations of traditional culture-based approaches for the

detection of new viruses and has already revealed the enormous diversity and abundance of viruses in the rumen and microbial ecosystems in nature (Paez-Espino et al. 2016).

To date, viral metagenomes have been reported for rumen samples from dairy cows (Berg Miller et al. 2012; Ross et al. 2013), buffalo (Parmar et al. 2016), cattle (Anderson et al. 2017) and goats (Namonyo et al. 2018). Viromes have also been generated from the rumen metagenomes of dairy cows (Dinsdale et al. 2008), sheep (Yutin et al. 2015) and moose (Solden et al. 2018). An alternative approach to metagenomic analysis using viral signatures, generated using annotation-independent kmers (short nucleotide sequences), has also been undertaken using rumen metagenomes from dairy cows (Willner et al. 2009), showing that rumen viral populations are quite distinct to those occurring in other environments. Despite differences in diet and breed and technical differences in viral sample preparation and sequencing technologies (454-pyrosequencing, Illumina HiSeq and Ion torrent), all of these studies have indicated that rumen viral populations are dominated by populations of tailed phages belonging to the viral order Caudovirales. Siphoviridae are usually the most abundant tailed phage family, followed by Myoviridae and Podoviridae. These findings are, therefore, in accordance with the early TEM studies of rumen viral morphology where tailed phage morphologies were found to predominate rumen samples from cattle, sheep and more recently goats (Fig. 2). To date, most viral metagenomics studies have used a limited number of rumen samples to explore viral diversity (Berg Miller et al. 2012; Parmar et al. 2016; Namonyo et al. 2018) and kept experimental conditions such as diet, breed and stage of lactation constant (Ross et al. 2013). Only one recent study has examined larger numbers of samples and has incorporated more experimental variables, for example the effect of diet (Anderson et al. 2017). Despite these limitations, however, the depth of information obtained using high-throughput sequencing and bioinformatics techniques undoubtedly surpasses all previous technologies used to describe the composition of rumen viral populations. Viral metagenomics and metagenomic studies without viral enrichment enable the identification, functional classification and taxonomic assignment of viral genes as well as the assembly of partial and/or near-complete viral genomes.

All of the viral metagenome studies undertaken so far have shown that the rumen contains numerous and highly diverse viral populations. The use of gene-based methodologies for determining the numbers of different virus types (species richness), for example Phage Communities from Contig Spectrum (PHACCS) and CatchAll (Angly et al. 2005; Bunge et al. 2012), have resulted in considerable variation in the numbers of phage types present; however, all reports agree that there are thousands of different viral types present (Berg Miller et al. 2012; Ross et al. 2013; Anderson et al. 2017). As sequencing technologies improve to better capture viral populations, through

increased sequencing depth and the ability to assemble more complete viral contigs, for example (Solden et al. 2018), viral species estimation techniques may no longer be required.

Rumen viromes typically contain a high abundance of genes which are classified within the SEED subsystem grouping of Phages, Prophages, Transposable elements and Plasmids (Aziz et al. 2008; Berg Miller et al. 2012). Specific viral functional genes identified include functional genes relating to viral replication (DNA replication and control proteins) and structural genes contributing to viral particle formation (head and tail proteins), and cell lysis proteins (including lysins and endoisopeptidases) (Ross et al. 2013). Interestingly, a recent microbial and viral metagenome study of the moose rumen (Solden et al. 2018) also compiled a metabolic reconstruction and used metaproteomics to detect the expression of viral genes (viral protein production). While most (80%) of the viral proteins detected were of unknown function, some of the detected proteins could be classified as structural proteins (e.g. capsid proteins) (Solden et al. 2018).

As well as the three major Caudovirales families rumen viral metagenomes have also detected non-tailed virus families, such as Tectiviridae (Berg Miller et al. 2012; Ross et al. 2013; Solden et al. 2018), which have previously been observed in rumen fluid but never cultivated. Rumen viral metagenomics studies have also discovered genes highly related to very large or giant viruses (e.g. Mimiviruses) (Berg Miller et al. 2012; Anderson et al. 2017), which are thought to infect and replicate in rumen protozoal populations. The methods usually used for the purification of viral particles from rumen fluid samples, including filtration through 0.22 μm filters, would be expected to exclude these very large viruses (e.g. *Acanthamoeba polyphaga* mimivirusis has a diameter of 400 nm). Whether these giant virus genes are actually homologous genes arising from smaller, novel rumen viruses which are otherwise absent from genetic databases is not known. A study which mined a microbial metagenome of sheep rumen fluid detected the presence of novel virophages, which parasitise the giant viruses of the Mimiviridae family (Yutin et al. 2015). In this way, metagenomics is rapidly expanding our understanding of the types of viruses associated with rumen microbial populations.

The other way rumen virus:host interactions can be studied in the absence of any virus:microbe cultivation uses CRISPR spacers (described earlier). Viral metagenome sequences or assembled viral contigs are compared for sequence homology to CRISPR spacer sequence databases or CRISPR spacer sequences derived from bacterial genome sequences (Berg Miller et al. 2012; Anderson et al. 2017). This approach has sometimes shown little similarity between rumen viral populations and known CRISPR spacer regions; however, the most recent viral metagenome study, which also assembled microbial genomes from the same rumen fluid sample, used a CRISPR-based approach

to associate 113 viral contigs (from the 1907 viral contigs >10 kb obtained in the study) with microbial hosts from four phyla (Seshadri et al. 2018). In this way, this study showed, using a metagenomic approach, that viral predation in the rumen could affect major microbial populations involved in feed breakdown, including those involved in the breakdown of complex polymers (xyloglycan, hemicellulose polymers and sugars, such as xylose).

4 Role and impact of the mobilome on rumen function

Much can be learned about the potential roles and impact of the mobilome in the rumen from studies undertaken in other microbial ecosystems, such as soil, water and other gut ecosystems (Wommack et al. 1996; Srinivasiah et al. 2008; Letarov 2012; Roux and Brum 2019; Shkoporov and Hill 2019). However, there are some physical attributes of the rumen environment which make the microbial communities (bacteria, archaea, fungi and protozoa), as well as the virus populations associated with these microbes, distinct from those which establish in other environments.

The rumen environment is well adapted to the maintenance of the microbial population contained within it (Bryant 1959), consisting of a large intestinal chamber, with a constant temperature (approx. 39°C) and a low oxygen tension of the gaseous phage (anaerobic conditions). A steady supply of nutrients (feed, including easily fermentable sugars as well as complex carbohydrates) and water, combined with the effects of a heavily buffered saliva, maintains a relatively constant, near-neutral (slightly acidic) pH. There is absorption of fermentation products through the rumen wall into the blood stream and removal (outflow) into the lower digestive tract of smaller, digested feed particles, products of microbial fermentation, as well as the microbes themselves (Mackie et al. 2002). This keeps the rumen microbial populations actively growing, without desiccation events which may occur in the soil and with no effects of sunlight, as occurs in the upper layers of aquatic environments (Weinbauer et al. 1999; Weinbauer 2004). In this regard, the rumen is similar to other host-associated gut environments; however, the larger volume of the rumen, the act of rumination, relatively slow retention times and the intake of solely herbivorous feedstuffs which are not pre-exposed to an acid stomach (as in the human gut) contribute to the unique nature of rumen microbial populations.

Of the elements contributing to the rumen mobilome, viruses and plasmids are by far the most studied, and their impacts on rumen function can be hypothesised, if not experimentally proven. The roles of viruses and plasmids within the rumen microbial ecosystem include microbial cell lysis, HGT, modulation of rumen microbial populations, development of phenotypic traits and, potentially, effects on the animal host (e.g. immunity).

4.1 Cell lysis

It has been well established that viral lysis is a major cause of bacterial death in many different environments, and prokaryote viruses are considered to have a significant impact on nutrient cycling (Weinbauer and Höfle 1998; Weinbauer 2004; Clokie et al. 2011). In the context of the rumen, virus (phage) lysis has long been suggested as a cause of intra-ruminal, bacterial lysis (Jarvis 1968; Nolan and Leng 1972; Klieve et al. 1989). From the perspective of ruminant nutrition and protein efficiency, phage-mediated bacterial lysis represents a negative impact, with protein being recycled in the rumen rather than being passed into the lower intestine for utilisation by the animal (Leng and Nolan 1984). From a microbial ecology perspective, the opposite may be true, with phage-mediated bacterial lysis and intra-ruminal recycling of protein actually representing a positive, advantageous impact on the rumen microbiome. This type of positive contribution to the overall ecosystem carbon and nutrient cycling has previously been demonstrated in aquatic (e.g. marine) ecosystems (Fuhrman 1999; Roux and Brum 2019). Phage-mediated cell lysis may provide proteins (including enzymes), DNA and products of bacterial metabolism (simple sugars, degraded plant compounds) to other organisms within the rumen.

Blooms of lytic phage activity and changes in total phage numbers, presumably corresponding to episodes of bacterial lysis, have previously been observed in the rumen of sheep (Klieve and Swain 1993; Swain et al. 1996). A recent study suggested that viral predation may assist in interspecies cross-feeding (nutrient recycling) in the rumen (Solden et al. 2018), with viruses infecting and lysing key microbial populations involved in the breakdown of complex plant compounds. In addition, proteomics undertaken as part of the same study then showed that the most commonly expressed viral proteins were those relating to structural phage components (e.g. head proteins) (Solden et al. 2018), rather than phage-encoded enzymes involved in bacterial cell wall lysis (e.g. endoisopeptidases, lysins and holins). Further studies, utilising multiple techniques and analytical measures (e.g. proteomics, viral and microbial metagenomics and rumen ammonia levels), will be required to more completely understand the extent to which virus-mediated lysis impacts rumen microbial populations.

4.2 Horizontal gene transfer

In all microbial ecosystems, the mobilome enables the flow of genetic material between organisms (HGT), ensuring genome and phenotype plasticity, maintaining microbial diversity and providing a mechanism for adaptation (Koonin and Wolf 2008; Jørgensen et al. 2014; Hülter et al. 2017). The transfer

of toxin and antibiotic resistance genes between microbes has traditionally been a focus of HGT research, due to the serious clinical implications of these factors and the emergence of human pathogens (Aminov 2011; De Sordi et al. 2019; Dunivin et al. 2019). Toxin genes can be transferred to phage genomes, with a notable example being the transfer of neurotoxins by phages CEβ and DEβ into *Clostridium botulinum* (Eklund et al. 1971; Eklund et al. 1972). In addition, most of the regulators and cofactors required for toxin expression are also encoded by the phage; therefore, phage infection and integration of the phage genomic DNA into the bacterial chromosome to form a prophage (lysogenic conversion) is critical for 'toxin conversion' of susceptible *Clostridium* strains (Brussow et al. 2004).

The ability of mobilome elements to successfully undergo HGT by transferring to and forming a stable, heritable state can vary largely between different microbes. Some species, for example *Streptococcus pyogenes*, harbours a large number of MGEs, and up to 10% of the total genome of sequenced strains can be prophage related and are often described as polylysogenic (Brussow et al. 2004). In some instances, the HGT of multiple MGEs may be required for the development of pathogenicity. For example, while most *Escherichia coli* strains are benign, commensal gut bacteria, some strains have evolved via HGT into virulent pathogens. These strains are now classified on the basis of their virulence factors and the diseases they cause, such as enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), shiga-toxin producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC) and uropathogenic *E. coli* (UPEC) (reviewed by Kaper et al. 2004; Blount 2015). The HGT virulence factors associated with these strains include pathogenicity islands, transposons, plasmids and phages (Brussow et al. 2004). While phages encode the AB-type toxins (shiga-toxin genes *stx1* and *stx2*), which inhibit protein synthesis and cause severe illness, it requires a combination of pathogenicity factors to enable the virulent *E. coli* strains to colonise the gut wall and excrete the toxins, with the formation of an attaching and effacing lesion facilitated by the locus of enterocyte effacement (LEE) genomic island (Hazen et al. 2015). Plasmid-encoded genes, including α-haemolysin (hlyA), contribute to the virulence of strains such as O157:H7 (Johnson and Nolan 2009). In some *E. coli* strains, plasmid-associated genes also encode for the type IV pilus, which confer localised adherence and cytotoxicity to the surface of intestinal epithelial cells (Lang et al. 2018).

While one of the major roles of the rumen mobilome is undoubtedly HGT (Morrison 1996), and it can be anticipated that rumen microbes of many different genera undergo genetic exchange, resulting in strain variation, there is currently very little experimental evidence of this. This may be due, in part, to the phylogenetic complexity of the rumen microbial ecosystem. While phages, plasmids and extrachromosomal elements have been shown to exist

in several different rumen genera, for example *Streptococcus*, *Ruminococcus*, *Bacteroides* and *Methanobrevibacter* (Klieve et al. 1989; Attwood et al. 2008; Gilbert et al. 2017), whether the genes these MGE carry are found in other rumen microbes has not been investigated. Many different rumen organisms also carry closely related genes with the same functionality, for example enzymes for the breakdown of complex carbohydrates (Seshadri et al. 2018). This makes determining the flow of specific genes between organisms difficult to focus on experimentally.

HGT can also be studied using predictive networks based on shared sequences (Shapiro and Putonti 2018). Networks can be developed from rumen metagenomics data to connect viral and microbial genomes (Solden et al. 2018), giving an indication of genetic linkages and potentially historical gene flow, but cannot specifically determine recent transfer events of individual genes of interest. A recent study suggested that genes encoded by rumen viruses (glycoside hydrolases) augment the breakdown of complex carbohydrates in the rumen (Anderson et al. 2017), with this finding predicted from viral metagenomic data. In contrast, a viral and microbial metagenome study by Solden et al. (2018) found that genes involved in the breakdown of complex carbohydrates were not commonly associated with rumen viruses. Interestingly, studies into microbial and viral evolution have suggested that many smaller viruses (5–50 kb genome size) may only rarely capture or acquire genes from their hosts and tend not to carry non-essential, accessory genes within their genome (Forterre and Prangishvili 2013). More prokaryote viruses and extrachromosomal elements (plasmids and prophages) need to be sourced from the rumen, and their sequences deposited in genetic databases in order to develop a better understanding of the types of genes carried by and therefore potentially transferred by these genetic elements.

4.3 Modulation of microbial populations

Specific components of the rumen mobilome, such as plasmids, may convey genes which may give a beneficial, selective advantage to recipient microbes, therefore encouraging proliferation of carrier or host microbes (Teather 1982). Similarly, virus-derived extrachromosomal elements (prophages) may increase the competitiveness or environmental fitness of host microbes (Winstanley et al. 2009). This has been shown to occur not only in prokaryotes but also in microbial eukaryotes (fungi, protozoa); for example, yeasts and fungi (including *Saccharomyces*, *Hanseniaspora*, *Ustilago* and *Zygosaccharomyces*) infected by mycoviruses may have increased capacity to produce 'killer toxins' (Ghabrial et al. 2015), although the majority of mycoviruses cause apparently asymptomatic infections (Sato et al. 2018). The effect of viruses on either the anaerobic fungi or protozoa found in the rumen has not been reported.

The other way extrachromosomal elements, specifically phages, can modulate bacterial populations in microbial ecosystems is through large-scale host cell lysis events or blooms. This phenomenon, whereby phages control the most highly abundant or dominant microbial population, has previously described as 'kill-the-winner' (Weinbauer 2004) or 'top-down control' (Sieradzki et al. 2019) and serves to maintain population diversity, allowing less successful competitors to co-exist. This has been shown to occur most often in relatively nutrient-limited aquatic ecosystems where microbial blooms can periodically occur and may involve the activity of both lytic and lysogenic phages (Wommack and Colwell 2000; Suttle 2007; Howard-Varona et al. 2017).

In the rumen nutrient limitation is not usually as much of an issue as in freshwater and marine environments; however, phage numbers have been shown to fluctuate in response to feed intake (Swain et al. 1996). Previous studies have also shown that a significant proportion of dominant rumen bacterial genera may be infected by lysogenic phages, which can replicate upon prophage induction (Klieve et al. 1989); therefore, lysogenic phages may also play a role in regulating the relative abundance of rumen bacterial populations. For most rumen genera, however, there is a distinct lack of basic information regarding virus:host interactions, such as host range (the number of species a virus can infect), burst size (the number of viral particles released by an infected cell) and the rate of phage particle degradation in rumen conditions. This lack of knowledge makes it difficult to accurately demonstrate the rate at which new viral infections occur and to predict the true extent to which viruses may modulate rumen microbial populations.

4.4 Influence development of phenotypic traits and growth habits

To date there has been very little research undertaken on the impact of the mobilome on the development of phenotypic traits and growth habits of microbial populations in the rumen. However, in other microbial ecosystems, extracellular elements such as plasmids have been shown to confer traits such as antimicrobial resistance (Heuer and Smalla 2012; Hülter et al. 2017) and can directly impact and improve microbial survival and persistence. Phage infection has also been shown to result in bacterial toxin production (Kuhl et al. 2012; Penadés et al. 2015) and can contribute to photosynthesis and oxygen production by cyanobacteria (Sieradzki et al. 2019), as well as provide the bacterial host resistance to infection by other phages (phage superinfection) (Refardt 2011).

In the rumen bacteria *S. equinus* 2B, phage infection has been shown to result in the development of phage-resistant strains with an altered growth habit by the formation of thick polysaccharide capsules (Klieve and Bauchop 1991), presumably masking the phage receptors on the cell surface from

phage attachment and protecting the cell from phage attack. Interestingly, the ability of bacteria and archaea to form extracellular polysaccharide capsules often facilitates the formation of biofilms (Orell et al. 2013; Turnbull et al. 2016). The colonisation of plant material in the rumen results in the formation of bacterial biofilms, and the ways in which phage particles interact with or possibly contribute to the formation of these biofilms in the rumen may represent a new aspect for investigation. To date metagenomics appears to have difficulty in detecting the development of phage-resistant bacterial phenotypes via approaches such as the detection of mutations in the loci relating to phage receptors (De Sordi et al. 2019). Further *in vitro* studies using either simplified communities or individual microbial isolates are required in order to fully understand the extent of mobilome-mediated growth habits in the rumen.

4.5 Influence on the ruminant host

Given the intrinsic and sometimes inseparable association of the mobilome and microbes, it is highly likely that with the colonisation and establishment of microbial populations in the developing ruminant (Fonty et al. 1987; Dill-McFarland et al. 2017; Dias et al. 2018), the mobilome (e.g. viral populations) also becomes established during the early stages of rumen development. To date, any direct effects of the mobilome on the ruminant host is unknown.

Recent developments in human gut-associated microbial communities have shown that viruses, including bacteriophages, may permeate the gut lining and have effects on host immunity (reviewed by Barr 2019; De Sordi et al. 2019; Shkoporov and Hill 2019), and several forms of immunomodulation by phage particles, via interactions with immune cells (e.g. dendritic cells) associated with the human gut epithelium, have also been demonstrated to occur (Keen and Dantas 2018). In addition, the release of lipopolysaccharides (LPS) and other bacterial immunogens, following the phage-mediated lysis of bacteria, may stimulate antibacterial innate immunity (Duerkop and Hooper 2013). It has also been suggested that the ability of phage particles to attach to mucin glycoproteins, referred to as the BAM model (bacteriophage adherence to mucus), enables phage accumulation in the mucus layer of the gut and results in a phage-rich protective layer, which can reduce bacterial infiltration and colonisation of the gut lining (Barr et al. 2013).

These aspects of mobilome:host interaction can be considered an important, emerging field of research. Whether factors such as the relatively large rumen volume, proteolytic nature of the rumen fluid and physiological differences occurring between the rumen wall and the lining of the human large intestine reduce or preclude any possible effects on ruminant host immunity is currently unknown.

5 Conclusion and future trends

Research into the rumen mobilome has not traditionally been considered a high priority area. Although individual elements of the mobilome, such as rumen viruses, are recognised as a normal, commensal component of the rumen, to date the mobilome has generally not been considered a 'problem' requiring urgent investigation. As more research is undertaken, the extent to which the mobilome occurs is, however, both surprising and inspiring for new researchers to further investigate the potential impacts on the balance and functioning of the rumen microbiome (Kav et al. 2012; Anderson et al. 2017; Solden et al. 2018).

Future trends of research into rumen viruses and extracellular elements are therefore anticipated to revolve around two main foci: (1) finding answers to unknown questions, and (2) developing practical applications. Progress towards successfully achieving both of these hinges around the developments in technology. In this regard, with the current rate of developments in sequencing technologies, bioinformatics and proteomics, we are now better placed than ever before to characterise and model the impact of viruses and extrachromosomal elements in the rumen. When used in combination with culture-based techniques, these technologies are expected to provide information that will vastly exceed the amount of information that was obtained about the life-cycles and biology of rumen-associated viruses and extrachromosomal elements, in the pre-sequencing and bioinformatics era.

As the agricultural production sector moves away from the use of antibiotics, new approaches for the biological control of problematic bacterial populations are required. Phage-based therapies using either intact viruses (phage therapy) or virus-encoded enzymes (phage-based enzybiotics) have already been developed for the control of pathogens in the horticulture, poultry and aquaculture industries, and production issues and regulatory constraints are being increasingly addressed (Monk et al. 2010; Chan et al. 2013; Fernández et al. 2018; Seal et al. 2018). In the context of ruminant production systems, including the more intensive dairy industry, issues such as mastitis, shedding of pathogenic *E. coli* strains and enteric methane can potentially be addressed using phage-based therapies (Klieve and Hegarty 1999; Raya et al. 2011; Dias et al. 2013; Gilbert et al. 2015; Gutiérrez et al. 2019). The use of phage-encoded enzymes, which target the integrity of microbial cell walls (e.g. holins and lysins, endopeptidases and tail spike proteins), represents a powerful new avenue for the development of practical applications to reduce and control rumen microbial populations of concern.

Future research efforts for the rumen mobilome can be anticipated to rapidly expand, taking advantage of advances in technology. The increased presence of high-quality, annotated sequences for rumen-specific microbes,

viruses and plasmids in publicly available databases is essential to this expansion of knowledge. New reference sequence data will greatly enhance the currently limited capacity to confer relevant sequence homology to novel genetic elements. Only with the collective development of these resources will the full extent and impact of the mobilome on rumen function be revealed.

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7 Where to look for further information

An introduction to the subject of prokaryote viruses and extrachromosomal elements can be provided by the following publications: Brussow et al. (2004), Weinbauer (2004), Ackermann and Prangishvili (2012), Bellanger et al. (2014), Delavat et al. (2017), Keen and Dantas (2018), Roux and Brum (2019) and Shkoporov and Hill (2019). Additional information and links to bioinformatics resources are listed at: https://www.ncbi.nlm.nih.gov/genome/viruses/ and http://www.isvm.org/resources.html.

More specific information describing the rumen and its microbes, as well as rumen viruses and plasmids, is included in the following publications: Kav et al. (2012), Gilbert and Klieve (2015), Gilbert et al. (2017), Huws et al. (2018) and Seshadri et al. (2018). International research efforts, linkages and collaborative programs in rumen microbiology are also detailed at: https://globalresearchalliance.org/research/livestock/networks/rumen-microbial-genomics-network/.

8 References

Abedon, S. T. and Murray, K. L. 2013. Archaeal viruses, not archaeal phages: an archaeological dig. *Archaea* 2013, 1–10. doi:10.1155/2013/251245.

Ackermann, H. W. and Prangishvili, D. 2012. Prokaryote viruses studied by electron microscopy. *Arch. Virol.* 157(10), 1843–9. doi:10.1007/s00705-012-1383-y.

Adams, J. C., Gazaway, J. A., Brailsford, M. D., Hartman, P. A. and Jacobson, N. L. 1966. Isolation of bacteriophages from the bovine rumen. *Experientia* 22(11), 717–8. doi:10.1007/BF01901335.

Akhter, S., Aziz, R. K. and Edwards, R. A. 2012. PhiSpy: a novel algorithm for finding prophages in bacterial genomes that combines similarity- and composition-based strategies. *Nucleic Acids Res.* 40(16), e126. doi:10.1093/nar/gks406.

Altermann, E., Schofield, L. R., Ronimus, R. S., Beatty, A. K. and Reilly, K. 2018. Inhibition of rumen methanogens by a novel archaeal lytic enzyme displayed on tailored bionanoparticles. *Front. Mirobiol.* 9, 2378. doi:10.3389/fmicb.2018.02378.

Aminov, R. I. 2011. Horizontal gene exchange in environmental microbiota. *Front. Mirobiol.* 2, 158. doi:10.3389/fmicb.2011.00158.

Anderson, C. L., Sullivan, M. B. and Fernando, S. C. 2017. Dietary energy drives the dynamic response of bovine rumen viral communities. *Microbiome* 5(1), 155. doi:10.1186/s40168-017-0374-3.

Angly, F., Rodriguez-Brito, B., Bangor, D., McNairnie, P., Breitbart, M., Salamon, P., Felts, B., Nulton, J., Mahaffy, J. and Rohwer, F. 2005. PHACCS, an online tool for estimating the structure and diversity of uncultured viral communities using metagenomic information. *BMC Bioinformatics* 6, 41. doi:10.1186/1471-2105-6-41.

Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y. and Wishart, D. S. 2016. PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res.* 44(W1), W16–21. doi:10.1093/nar/gkw387.

Arntzen, M. Ø., Várnai, A., Mackie, R. I., Eijsink, V. G. H. and Pope, P. B. 2017. Outer membrane vesicles from *Fibrobacter succinogenes* S85 contain an array of carbohydrate-active enzymes with versatile polysaccharide-degrading capacity. *Environ. Microbiol.* 19(7), 2701–14. doi:10.1111/1462-2920.13770.

Asmundson, R. V. and Kelly, W. J. 1987. Isolation and characterization of plasmid DNA from *Ruminococcus*. *Curr. Microbiol.* 16(2), 97–100. doi:10.1007/BF01588178.

Attwood, G. T. and Brooker, J. D. 1992. Complete nucleotide sequence of a *Selenomonas ruminantium* plasmid and definition of a region necessary for its replication in *Escherichia coli*. *Plasmid* 28(2), 123–9. doi:10.1016/0147-619x(92)90043-a.

Attwood, G. T., Kelly, W. J., Altermann, E. H. and Leahy, S. C. 2008. Analysis of the *Methanobrevibacter ruminantium* draft genome: understanding methanogen biology to inhibit their action in the rumen. *Aust. J. Exp. Agric.* 48(2), 83–8. doi:10.1071/EA07269.

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G. D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A. and Zagnitko, O. 2008. The RAST server: rapid annotations using subsystems technology. *BMC Genomics* 9, 75. doi:10.1186/1471-2164-9-75.

Baresi, L. and Bertani, G. 1984. Isolation of a bacteriophage for a methanogenic bacterium. *Admin. Ann. Meet. Americ Soc. Microbiol.* 28, 133.

Barr, J. J. 2019. Missing a phage: unravelling tripartite symbioses within the human gut. *mSystems* 4(3). doi:10.1128/mSystems.00105-19.

Barr, J. J., Auro, R., Furlan, M., Whiteson, K. L., Erb, M. L., Pogliano, J., Stotland, A., Wolkowicz, R., Cutting, A. S., Doran, K. S., Salamon, P., Youle, M. and Rohwer, F. 2013. Bacteriophage adhering to mucus provide a non-host-derived immunity. *PNAS* 110(26), 10771–6. doi:10.1073/pnas.1305923110.

Barrangou, R. 2013. CRISPR-Cas systems and RNA-guided interference. *Wiley Interdisc. Rev. RNA* 4(3), 267–78. doi:10.1002/wrna.1159.

Belanche, A., Doreau, M., Edwards, J. E., Moorby, J. M., Pinloche, E. and Newbold, C. J. 2012. Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. *J. Nutr.* 142(9), 1684–92. doi:10.3945/jn.112.159574.

Bellanger, X., Payot, S., Leblond-Bourget, N. and Guedon, G. 2014. Conjugative and mobilizable genomic islands in bacteria: evolution and diversity. *FEMS Microbiol. Rev.* 38(4), 720–60. doi:10.1111/1574-6976.12058.

Berg Miller, M. E., Yeoman, C. J., Chia, N., Tringe, S. G., Angly, F. E., Edwards, R. A., Flint, H. J., Lamed, R., Bayer, E. A. and White, B. A. 2012. Phage–bacteria relationships and CRISPR elements revealed by a metagenomic survey of the rumen microbiome. *Environ. Microbiol.* 14(1), 207–27. doi:10.1111/j.1462-2920.2011.02593.x.

Blount, Z. D. 2015. The unexhausted potential of *E. coli*. *eLife* 4, e05826. doi:10.7554/eLife.05826.

Bobay, L. M., Touchon, M. and Rocha, E. P. C. 2014. Pervasive domestication of defective prophages by bacteria. *PNAS* 111(33), 12127–32. doi:10.1073/pnas.1405336111.

Bolduc, B., Shaughnessy, D. P., Wolf, Y. I., Koonin, E. V., Roberto, F. F. and Young, M. 2012. Identification of novel positive-strand RNA viruses by metagenomic analysis of archaea-dominated Yellowstone Hot Springs. *J. Virol.* 86(10), 5562–73. doi:10.1128/JVI.07196-11.

Brailsford, M. D. and Hartman, P. A. 1968. Characterisation of *Streptococcus durans* bacteriophages. *Can. J. Microbiol.* 14(4), 397–402. doi:10.1139/m68-063.

Brussow, H., Canchaya, C. and Hardt, W. D. 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiol. Mol. Biol. Rev.* 68(3), 560–602. doi:10.1128/MMBR.68.3.560-602.2004.

Bryant, M. P. 1959. Bacterial species of the rumen. *Bacteriol. Rev.* 23(3), 125–53.

Bunge, J., Woodard, L., Bohning, D., Foster, J. A., Connolly, S. and Allen, H. K. 2012. Estimating population diversity with CatchAll. *Bioinformatics* 28(7), 1045–7. doi:10.1093/bioinformatics/bts075.

Champion, K. M., Helaszek, C. T. and White, B. A. 1988. Analysis of antibiotic susceptibility and extrachromosomal DNA content of *Ruminococcus albus* and *Ruminococcus flavefaciens*. *Can. J. Microbiol.* 34(10), 1109–15. doi:10.1139/m88-196.

Chan, B. K., Abedon, S. T. and Loc-Carrillo, C. 2013. Phage cocktails and the future of phage therapy. *Future Microbiol.* 8(6), 769–83. doi:10.2217/fmb.13.47.

Clarke, A. J. 2018. The 'hole' story of predatory outer-membrane vesicles. *Can. J. Microbiol.* 64(9), 589–99. doi:10.1139/cjm-2017-0466.

Clokie, M. R. J., Millard, A. D., Letarov, A. V. and Heaphy, S. 2011. Phages in nature. *Bacteriophage* 1(1), 31–45. doi:10.4161/bact.1.1.14942.

Croucher, N. J., Mostowy, R., Wymant, C., Turner, P., Bentley, S. D. and Fraser, C. 2016. Horizontal DNA transfer mechanisms of bacteria as weapons of intragenomic conflict. *PLoS Biol.* 14(3), e1002394. doi:10.1371/journal.pbio.1002394.

Dassa, B., Borovok, I., Ruimy-Israeli, V., Lamed, R., Flint, H. J., Duncan, S. H., Henrissat, B., Coutinho, P., Morrison, M., Mosoni, P., Yeoman, C. J., White, B. A. and Bayer, E. A. 2014. Rumen cellulosomics: divergent fiber-degrading strategies revealed by comparative genome-wide analysis of six ruminococcal strains. *PLoS ONE* 9(7), e99221. doi:10.1371/journal.pone.0099221.

Deatherage, B. L. and Cookson, B. T. 2012. Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. *Infect. Immun.* 80(6), 1948–57. doi:10.1128/IAI.06014-11.

De Jonge, P. A., Nobrega, F. L., Brouns, S. J. J. and Dutilh, B. E. 2019. Molecular and evolutionary determinants of bacteriophage host range. *Trends Microbiol.* 27(1), 51–63. doi:10.1016/j.tim.2018.08.006.

Delavat, F., Miyazaki, R., Carraro, N., Pradervand, N. and van der Meer, J. R. 2017. The hidden life of integrative and conjugative elements. *FEMS Microbiol. Rev.* 41(4), 512–37. doi:10.1093/femsre/fux008.

De Sordi, L., Lourenço, M. and Debarbieux, L. 2019. The battle within: interactions of bacteriophages and bacteria in the gastrointestinal tract. *Cell Host Microbe* 25(2), 210–8. doi:10.1016/j.chom.2019.01.018.

Deutsch, D. R., Utter, B., Verratti, K. J., Sichtig, H., Tallon, L. J. and Fischetti, V. A. 2018. Extrachromosomal DNA sequencing reveals episomal prophages capable of impacting virulence factor expression in *Staphylococcus aureus*. *Front. Microbiol.* 9, 1406. doi:10.3389/fmicb.2018.01406.

Dias, R. S., Eller, M. R., Duarte, V. S., Pereira, ÂL., Silva, C. C., Mantovani, H. C., Oliveira, L. L., Silva, Ede A. and De Paula, S. O. 2013. Use of phages against antibiotic-resistant *Staphylococcus aureus* isolated from bovine mastitis. *J. Anim. Sci.* 91(8), 3930–9. doi:10.2527/jas.2012-5884.

Dias, J., Marcondes, M. I., Motta de Souza, S., Cardoso da Mata e Silva, B., Fontes Noronha, M., Tassinari Resende, R., Machado, F. S., Cuquetto Mantovani, H., Dill-McFarland, K. A. and Suen, G. 2018. Bacterial community dynamics across the gastrointestinal tracts of dairy calves during preweaning development. *Appl. Environ. Microbiol.* 84(9), e02675-17. doi:10.1128/AEM.02675-17.

Dill-McFarland, K. A., Breaker, J. D. and Suen, G. 2017. Microbial succession in the gastrointestinal tract of dairy cows from 2 weeks to first lactation. *Sci. Rep.* 7, 40864. doi:10.1038/srep40864.

Dinsdale, E. A., Edwards, R. A., Hall, D., Angly, F., Breitbart, M., Brulc, J. M., Furlan, M., Desnues, C., Haynes, M., Li, L. L., McDaniel, L., Moran, M. A., Nelson, K. E., Nilsson, C., Olson, R., Paul, J., Brito, B. R., Ruan, Y. J., Swan, B. K., Stevens, R., Valentine, D. L., Thurber, R. V., Wegley, L., White, B. A. and Rohwer, F. 2008. Functional metagenomic profiling of nine biomes. *Nature* 452(7187), 629–32. doi:10.1038/nature06810.

Duerkop, B. A. and Hooper, L. V. 2013. Resident viruses and their interaction with the immune system. *Nat. Immunol.* 14(7), 654–9. doi:10.1038/ni.2614.

Dunivin, T. K., Choi, J., Howe, A. and Shade, A. 2019. RefSoil+: a reference database for genes and traits of soil plasmids. *mSystems* 4(1), e00349-18. doi:10.1128/mSystems.00349-18.

Edwards, R. A. and Rohwer, F. 2005. Viral metagenomics. *Nat. Rev. Microbiol.* 3(6), 504–10. doi:10.1038/nrmicro1163.

Eklund, M. W., Poysky, F. T., Reed, S. M. and Smith, C. A. 1971. Bacteriophage and the toxigenicity of *Clostridium botulinum* type C. *Science* 172(3982), 480–2. doi:10.1126/science.172.3982.480.

Eklund, M. W., Poysky, F. T. and Reed, S. M. 1972. Bacteriophage and the toxigenicity of *Clostridium botulinum* type D. *Nat. New Biol.* 235(53), 16–7. doi:10.1038/newbio235016a0.

Ellis, T. N. and Kuehn, M. J. 2010. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol. Mol. Biol. Rev.* 74(1), 81–94. doi:10.1128/MMBR.00031-09.

Fernández, L., Gutiérrez, D., Rodríguez, A. and García, P. 2018. Application of bacteriophages in the agro-food sector: a long way toward approval. *Front. Cell. Infect. Microbiol.* 8, 296. doi:10.3389/fcimb.2018.00296.

Flint, H. J. 1997. The rumen microbial ecosystem—some recent developments. *Trends Microbiol.* 5(12), 483–8. doi:10.1016/S0966-842X(97)01159-1.

Flint, H. J., Thomson, A. M. and Bisset, J. 1988. Plasmid-associated transfer of tetracycline resistance in *Bacteroides ruminicola*. *Appl. Environ. Microbiol.* 54(4), 855–60.

Fonty, G., Gouet, P., Jouany, J. -P. and Senaud, J. 1987. Establishment of the microflora and anaerobic fungi in the rumen of lambs. *J. Gen. Microbiol.* 133(7), 1835–43. doi:10.1099/00221287-133-7-1835.

Forterre, P. and Prangishvili, D. 2013. The major role of viruses in cellular evolution: facts and hypotheses. *Curr. Opin. Virol.* 3(5), 558–65. doi:10.1016/j.coviro.2013.06.013.

Fuhrman, J. A. 1999. Marine viruses and their biogeochemical and ecological effects. *Nature* 399(6736), 541–8. doi:10.1038/21119.

Gaillard, B., Breton, A. and Bernalier, A. 1989. Study of the nuclear cycle of four species of strictly anaerobic rumen fungi by fluorescence microscopy. *Curr. Microbiol.* 19(2), 103–7. doi:10.1007/BF01570576.

Ghabrial, S. A., Caston, J. R., Jiang, D., Nibert, M. L. and Suzuki, N. 2015. 50-plus years of fungal viruses. *Virology* 479–480, 356–68. doi:10.1016/j.virol.2015.02.034.

Gilbert, R. A. and Klieve, A. V. 2015. Ruminal viruses (bacteriophages, Archaeaphages). In: Puniya, A. K., Singh, R. and Kamra, D. N. (Eds), *Rumen Microbiology: From Evolution to Revolution*. Springer, New Delhi, pp. 121–41. https://doi-org.ezproxy.library.uq.edu.au/10.1007/978-81-322-2401-3_9.

Gilbert, R. A., Ouwerkerk, D. and Klieve, A. V. 2015. Phage therapy in livestock methane amelioration. In: Malik, P. K., Bhatta, R., Takahashi, J., Kohn, R. A. and Prasad, C. S. (Eds), *Livestock Production and Climate Change*. CABI Publishing, Wallingford, Oxfordshire, UK, pp. 318–35.

Gilbert, R. A., Kelly, W. J., Altermann, E., Leahy, S. C., Minchin, C., Ouwerkerk, D. and Klieve, A. V. 2017. Toward understanding phage:host interactions in the rumen; complete genome sequences of lytic phages infecting rumen bacteria. *Front. Mirobiol*. 8, 2340. doi:10.3389/fmicb.2017.02340.

Gong, J. and Forsberg, C. W. 1993. Separation of outer and cytoplasmic membranes of *Fibrobacter succinogenes* and membrane and glycogen granule locations of glycanases and cellobiase. *J. Bacteriol.* 175(21), 6810–21. doi:10.1128/jb.175.21.6810-6821.1993.

Goodman, R. P., Ghabrial, S. A., Fichorova, R. N. and Nibert, M. L. 2011. *Trichomonasvirus*: a new genus of protozoan viruses in the family Totiviridae. *Arch. Virol.* 156(1), 171–9. doi:10.1007/s00705-010-0832-8.

Gregg, K., Kennedy, B. G. and Klieve, A. V. 1994. Cloning and DNA sequence analysis of the region containing *Att*P of the temperate phage φAR29 of *Prevotella ruminicola* AR29. *Microbiology (Reading, Engl.)* 140(8), 2109–14. doi:10.1099/13500872-140-8-2109.

Groleau, D. and Forsberg, C. W. 1981. Cellulolytic activity of the rumen bacterium *Bacteroides succinogenes*. *Can. J. Microbiol.* 27(5), 517–30. doi:10.1139/m81-077.

Grybchuk, D., Akopyants, N. S., Kostygov, A. Y., Konovalovas, A., Lye, L. F., Dobson, D. E., Zangger, H., Fasel, N., Butenko, A., Frolov, A. O., Votýpka, J., d'Avila-Levy, C. M., Kulich, P., Moravcová, J., Plevka, P., Rogozin, I. B., Serva, S., Lukeš, J., Beverley, S. M. and Yurchenko, V. 2018. Viral discovery and diversity in trypanosomatid protozoa with a focus on relatives of the human parasite *Leishmania*. *PNAS* 115(3), E506–15. doi:10.1073/pnas.1717806115.

Guedon, G., Libante, V., Coluzzi, C., Payot, S. and Leblond-Bourget, N. 2017. The obscure world of integrative and mobilizable elements, highly widespread elements

that pirate bacterial conjugative systems. *Genes (Basel)* 8(11), 337. doi:10.3390/genes8110337.

Gutiérrez, D., Fernández, L., Rodríguez, A. and García, P. 2019. Role of bacteriophages in the implementation of a sustainable dairy chain. *Front. Mirobiol.* 10, 12–. doi:10.3389/fmicb.2019.00012.

Harrison, P. W., Lower, R. P. J., Kim, N. K. D. and Young, J. P. W. 2010. Introducing the bacterial 'chromid': not a chromosome, not a plasmid. *Trends Microbiol.* 18(4), 141–8. doi:10.1016/j.tim.2009.12.010.

Hart, K. J., Yanez-Ruiz, D. R., Duval, S. M., McEwan, N. R. and Newbold, C. J. 2008. Plant extracts to manipulate rumen fermentation. *Anim. Feed Sci. Tech.* 147(1–3), 8–35. doi:10.1016/j.anifeedsci.2007.09.007.

Hazen, T. H., Kaper, J. B., Nataro, J. P. and Rasko, D. A. 2015. Comparative genomics provides insight into the diversity of the attaching and effacing *Escherichia coli* virulence plasmids. *Infect. Immun.* 83(10), 4103–17. doi:10.1128/IAI.00769-15.

Hefford, M. A., Teather, R. M. and Forster, R. J. 1993. The complete nucleotide sequence of a small cryptic plasmid from a rumen bacterium of the genus *Butyrivibrio*. *Plasmid* 29(1), 63–9. doi:10.1006/plas.1993.1008.

Heuer, H. and Smalla, K. 2012. Plasmids foster diversification and adaptation of bacterial populations in soil. *FEMS Microbiol. Rev.* 36(6), 1083–104. doi:10.1111/j.1574-6976.2012.00337.x.

Hobbs, Z. and Abedon, S. T. 2016. Diversity of phage infection types and associated terminology: the problem with 'lytic or lysogenic'. *FEMS Microbiol. Lett.* 363(7), fnw047. doi:10.1093/femsle/fnw047.

Hoogenraad, N. J. and Hird, F. J. R. 1970. Electron-microscopic investigation of the flora of sheep alimentary tract. *Aust. J. Biol. Sci.* 23(4), 793–808. doi:10.1071/bi9700793.

Hoogenraad, N. J., Hird, F. J. R., Holmes, I. and Millis, N. F. 1967. Bacteriophages in rumen contents of sheep. *J. Gen. Virol.* 1(4), 575–6. doi:10.1099/0022-1317-1-4-575.

Howard-Varona, C., Hargreaves, K. R., Abedon, S. T. and Sullivan, M. B. 2017. Lysogeny in nature: mechanisms, impact and ecology of temperate phages. *ISME J.* 11(7), 1511–20. doi:10.1038/ismej.2017.16.

Hülter, N., Ilhan, J., Wein, T., Kadibalban, A. S., Hammerschmidt, K. and Dagan, T. 2017. An evolutionary perspective on plasmid lifestyle modes. *Curr. Opin. Microbiol.* 38, 74–80. doi:10.1016/j.mib.2017.05.001.

Hungate, R. E. 1966. *The Rumen and Its Microbes*. Academic Press, New York.

Huws, S. A., Kim, E. J., Kingston-Smith, A. H., Lee, M. R., Muetzel, S. M., Cookson, A. R., Newbold, C. J., Wallace, R. J. and Scollan, N. D. 2009. Rumen protozoa are rich in polyunsaturated fatty acids due to the ingestion of chloroplasts. *FEMS Microbiol. Ecol.* 69(3), 461–71. doi:10.1111/j.1574-6941.2009.00717.x.

Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., Muñoz-Tamayo, R., Forano, E., Waters, S. M., Hess, M., Tapio, I., Smidt, H., Krizsan, S. J., Yáñez-Ruiz, D. R., Belanche, A., Guan, L., Gruninger, R. J., McAllister, T. A., Newbold, C. J., Roehe, R., Dewhurst, R. J., Snelling, T. J., Watson, M., Suen, G., Hart, E. H., Kingston-Smith, A. H., Scollan, N. D., do Prado, R. M., Pilau, E. J., Mantovani, H. C., Attwood, G. T., Edwards, J. E., McEwan, N. R., Morrisson, S., Mayorga, O. L., Elliott, C. and Morgavi, D. P. 2018. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Front. Mirobiol.* 9, 2161. doi:10.3389/fmicb.2018.02161.

Iverson, W. G. and Millis, N. F. 1976a. Characterisation of *Streptococcus bovis* bacteriophages. *Can. J. Microbiol.* 22(6), 847–52. doi:10.1139/m76-122.

Iverson, W. G. and Millis, N. F. 1976b. Lysogeny in *Streptococcus bovis*. *Can. J. Microbiol.* 22(6), 853–7. doi:10.1139/m76-123.

Jarvis, B. D. W. 1968. Lysis of viable rumen bacteria in bovine rumen fluid. *Appl. Microbiol.* 16(5), 714–23.

Johnson, C. M. and Grossman, A. D. 2015. Integrative and conjugative elements (ICEs): what they do and how they work. *Annu. Rev. Genet.* 49, 577–601. doi:10.1146/annurev-genet-112414-055018.

Johnson, T. J. and Nolan, L. K. 2009. Pathogenomics of the virulence plasmids of *Escherichia coli*. *Microbiol. Mol. Biol. Rev.* 73(4), 750–74. doi:10.1128/MMBR.00015-09.

Jørgensen, T. S., Kiil, A. S., Hansen, M. A., Sørensen, S. J. and Hansen, L. H. 2014. Current strategies for mobilome research. *Front. Mirobiol.* 5, 750. doi:10.3389/fmicb.2014.00750.

Jung, D. H., Chung, W. H., Seo, D. H., Nam, Y. D., Yoon, S. and Park, C. S. 2018. Complete genome sequence of *Bifidobacterium choerinum* FMB-1, a resistant starch-degrading bacterium. *J. Biotechnol.* 274, 28–32. doi:10.1016/j.jbiotec.2018.03.009.

Kaper, J. B., Nataro, J. P. and Mobley, H. L. T. 2004. Pathogenic *Escherichia coli*. *Nat. Rev. Microbiol.* 2(2), 123–40. doi:10.1038/nrmicro818.

Kav, A. B., Sasson, G., Jami, E., Doron-Faigenboim, A., Benhar, I. and Mizrahi, I. 2012. Insights into the bovine rumen plasmidome. *PNAS* 109, 5452–7. doi:10.1073/pnas.1116410109.

Keen, E. C. and Dantas, G. 2018. Close encounters of three kinds: bacteriophages, commensal bacteria, and host immunity. *Trends Microbiol.* 26(11), 943–54. doi:10.1016/j.tim.2018.05.009.

Kelly, W. J., Leahy, S. C., Altermann, E., Yeoman, C. J., Dunne, J. C., Kong, Z., Pacheco, D. M., Li, D., Noel, S. J., Moon, C. D., Cookson, A. L. and Attwood, G. T. 2010. The glycobiome of the rumen bacterium *Butyrivibrio proteoclasticus* B316(T) highlights adaptation to a polysaccharide-rich environment. *PLoS ONE* 5(8), e11942. doi:10.1371/journal.pone.0011942.

Kelly, W. J., Leahy, S. C., Li, D., Perry, R., Lambie, S. C., Attwood, G. T. and Altermann, E. 2014. The complete genome sequence of the rumen methanogen *Methanobacterium formicicum* BRM9. *Stand. Genom. Sci.* 9, 15. doi:10.1186/1944-3277-9-15.

Kim, Y. S., Singh, A. P., Wi, S. G., Myung, K. H., Karita, S. and Ohmiya, K. 2001. Cellulosome-like structures in ruminal cellulolytic bacterium *Ruminococcus albus* F-40 as revealed by electron microscopy. *Asian Australas. J. Anim. Sci* 14(10), 1429–33. doi:10.5713/ajas.2001.1429.

Klieve, A. V. and Bauchop, T. 1988. Morphological diversity or ruminal bacteriophages from sheep and cattle. *Appl. Environ. Microbiol.* 54(6), 1637–41.

Klieve, A. V. and Bauchop, T. 1991. Phage resistance and altered growth habit in a strain of *Streptococcus bovis*. *FEMS Microbiol. Lett.* 64(2–3), 155–9. doi:10.1016/0378-1097(91)90587-z.

Klieve, A. V. and Gilbert, R. A. 2005. Bacteriophage populations. In: Makkar, H. P. S. and McSweeney, C. S. (Eds), *Methods in Gut Microbial Ecology for Ruminants*. Springer Netherlands, Dordrecht, pp. 129–37. doi: 10.1007/1-4020-3791-0.

Klieve, A. V. and Hegarty, R. 1999. Opportunities for biological control of ruminal methanogenesis. *Aust. J. Agric. Res.* 50(8), 1315–9. doi:10.1071/AR99006.

Klieve, A. V. and Swain, R. A. 1993. Estimating ruminal bacteriophage numbers using pulsed field gel electrophoresis and laser densitometry. *Appl. Environ. Microbiol.* 59(7), 2299–303.

Klieve, A. V., Hudman, J. F. and Bauchop, T. 1989. Inducible bacteriophages from ruminal bacteria. *Appl. Environ. Microbiol.* 55(6), 1630–4.

Klieve, A. V., Gregg, K. and Bauchop, T. 1991. Isolation and characteristics of lytic phages from *Bacteroides ruminicola* ss *brevis*. *Curr. Microbiol.* 23(4), 183–7. doi:10.1007/BF02092277.

Klieve, A. V., Heck, G. L., Prance, M. A. and Shu, Q. 1999. Genetic homogeneity and phage susceptibility of ruminal strains of *Streptococcus bovis* isolated in Australia. *Lett. Appl. Microbiol.* 29(2), 108–12. doi:10.1046/j.1365-2672.1999.00596.x.

Klieve, A. V., Yokoyama, M. T., Forster, R. J., Ouwerkerk, D., Bain, P. A. and Mawhinney, E. L. 2005. Naturally occurring DNA transfer system associated with membrane vesicles in cellulolytic *Ruminococcus* spp. of ruminal origin. *Appl. Environ. Microbiol.* 71(8), 4248–53. doi:10.1128/AEM.71.8.4248-4253.2005.

Koonin, E. V. and Dolja, V. V. 2014. Virus world as an evolutionary network of viruses and capsidless selfish elements. *Microbiol. Mol. Biol. Rev.* 78(2), 278–303. doi:10.1128/MMBR.00049-13.

Koonin, E. V. and Wolf, Y. I. 2008. Genomics of bacteria and archaea: the emerging dynamic view of the prokaryotic world. *Nucleic Acids Res.* 36(21), 6688–719. doi:10.1093/nar/gkn668.

Koonin, E. V., Makarova, K. S. and Wolf, Y. I. 2017. Evolutionary genomics of defense systems in archaea and bacteria. *Annu. Rev. Microbiol.* 71, 233–61. doi:10.1146/annurev-micro-090816-093830.

Krupovic, M., Prangishvili, D., Hendrix, R. W. and Bamford, D. H. 2011. Genomics of bacterial and archaeal viruses: dynamics within the prokaryotic virosphere. *Microbiol. Mol. Biol. Rev.* 75(4), 610–35. doi:10.1128/MMBR.00011-11.

Krupovic, M., Makarova, K. S., Wolf, Y. I., Medvedeva, S., Prangishvili, D., Forterre, P. and Koonin, E. V. 2019. Integrated mobile genetic elements in Thaumarchaeota. *Environ. Microbiol.* 21(6), 2056–78. doi:10.1111/1462-2920.14564.

Kuhl, S., Abedon, S. T. and Hyman, P. 2012. Diseases caused by phages. In: Hyman, P. and Abedon, S. T. (Eds) *Bacteriophages in Health and Disease*. CAB International, Wallingford, Oxfordshire, UK, pp. 21–32.

Lang, C., Fruth, A., Holland, G., Laue, M., Muhlen, S., Dersch, P. and Flieger, A. 2018. Novel type of pilus associated with a Shiga-toxigenic *E. coli* hybrid pathovar conveys aggregative adherence and bacterial virulence. *Emerg. Microbes Infect.* 7(1), 203. doi:10.1038/s41426-018-0209-8.

Leahy, S. C., Kelly, W. J., Li, D., Li, Y., Altermann, E., Lambie, S. C., Cox, F. and Attwood, G. T. 2013. The complete genome sequence of *Methanobrevibacter* sp. AbM4. *STD Genomics Sci.* 8(2), 215–27. doi:10.4056/sigs.3977691.

Leedle, J. A. Z., Bryant, M. P. and Hespell, R. B. 1982. Diurnal variations in bacterial numbers and fluid parameters in ruminal contents of animals fed low- or high-forage diets. *Appl. Environ. Microbiol.* 44(2), 402–12.

Leng, R. A. and Nolan, J. V. 1984. Nitrogen metabolism in the rumen. *J. Dairy Sci.* 67(5), 1072–89. doi:10.3168/jds.S0022-0302(84)81409-5.

Letarov, A. V. 2012. Bacteriophages as a part of the human microbiome. In: Hyman, P. and Abedon, S. T. (Eds), *Bacteriophages in Health and Disease*. CAB International, Wallingford, Oxfordshire, UK, pp. 6–20.

Liu, M., Li, X., Xie, Y., Bi, D., Sun, J., Li, J., Tai, C., Deng, Z. and Ou, H. Y. 2019. ICEberg 2.0: an updated database of bacterial integrative and conjugative elements. *Nucleic Acids Res.* 47(D1), D660–5. doi:10.1093/nar/gky1123.

Mackie, R. I., McSweeney, C. S. and Klieve, A. V. 2002. Microbial ecology of the ovine rumen. In: Freer, M. and Dove, H. (Eds), *Sheep Nutrition*. CAB International, pp. 71–94.

May, T., Kocherginskaya, S. A., Mackie, R. I., Vercoe, P. E. and White, B. A. 1996. Complete nucleotide sequence of a cryptic plasmid, pbaw301, from the ruminai anaerobe *Ruminococcus flavefaciens* R13e2. *FEMS Microbiol. Lett.* 144(2–3), 221–7. doi:10.1111/j.1574-6968.1996.tb08534.x.

Meile, L., Jenal, U., Studer, D., Jordan, M. and Leisinger, T. 1989. Characterization of ψM1, a virulent phage of *Methanobacterium thermoautotrophicum* Marburg. *Arch. Microbiol.* 152(2), 105–10. doi:10.1007/BF00456085.

Monk, A. B., Rees, C. D., Barrow, P., Hagens, S. and Harper, D. R. 2010. Bacteriophage applications: where are we now? *Lett. Appl. Microbiol.* 51(4), 363–9. doi:10.1111/j.1472-765X.2010.02916.x.

Morrison, M. 1996. Do rumen bacteria exchange genetic material. *J. Dairy Sci.* 79(8), 1476–86. doi:10.3168/jds.S0022-0302(96)76507-4.

Namonyo, S., Wagacha, M., Maina, S., Wambua, L. and Agaba, M. 2018. A metagenomic study of the rumen virome in domestic caprids. *Arch. Virol.* 163(12), 3415–9. doi:10.1007/s00705-018-4022-4.

Nash, M. A., Smith, S. P., Fontes, C. M. and Bayer, E. A. 2016. Single versus dual-binding conformations in cellulosomal cohesin–dockerin complexes. *Curr. Opin. Struct. Biol.* 40, 89–96. doi:10.1016/j.sbi.2016.08.002.

Nibert, M. L., Woods, K. M., Upton, S. J. and Ghabrial, S. A. 2009. *Cryspovirus*: a new genus of protozoan viruses in the family Partitiviridae. *Arch. Virol.* 154(12), 1959–65. doi:10.1007/s00705-009-0513-7.

Nolan, J. V. and Leng, R. A. 1972. Dynamic aspects of ammonia and urea metabolism in sheep. *Br. J. Nutr.* 27(1), 177–94. doi:10.1079/bjn19720081.

Nölling, J., Groffen, A. and de Vos, W. M. 1993. φ F1 and φF3, two novel virulent, archaeal phages infecting different thermophilic strains of the genus *Methanobacterium*. *J. Gen. Microbiol.* 139(10), 2511–6. doi:10.1099/00221287-139-10-2511.

Ogata, K., Aminov, R. I., Nagamine, T., Benno, Y., Sekizaki, T., Mitsumori, M., Minato, H. and Itabashi, H. 1996. Structural organization of pRAM4, a cryptic plasmid from *Prevotella ruminicola*. *Plasmid* 35(2), 91–7. doi:10.1006/plas.1996.0011.

Ohara, H., Miyagi, T., Kaneichi, K., Karita, S., Kobayashi, Y., Kimura, T., Sakka, K. and Ohmiya, K. 1998. Structural analysis of a new cryptic plasmid pAR67 isolated from *Ruminococcus albus* AR67. *Plasmid* 39(1), 84–8. doi:10.1006/plas.1997.1324.

Orell, A., Frols, S. and Albers, S. V. 2013. Archaeal biofilms: the great unexplored. *Annu. Rev. Microbiol.* 67, 337–54. doi:10.1146/annurev-micro-092412-155616.

Orpin, C. G. and Munn, E. A. 1974. The occurrence of bacteriophages in the rumen and their influence on rumen bacterial populations. *Experientia* 30(9), 1018–20. doi:10.1007/bf01938983.

Overbeek, R., Begley, T., Butler, R. M., Choudhuri, J. V., Chuang, H. Y., Cohoon, M., de Crécy-Lagard, V., Diaz, N., Disz, T., Edwards, R., Fonstein, M., Frank, E. D., Gerdes, S., Glass, E. M., Goesmann, A., Hanson, A., Iwata-Reuyl, D., Jensen, R., Jamshidi, N., Krause, L., Kubal, M., Larsen, N., Linke, B., McHardy, A. C., Meyer, F., Neuweger, H., Olsen, G., Olson, R., Osterman, A., Portnoy, V., Pusch, G. D., Rodionov, D. A., Rückert, C., Steiner, J., Stevens, R., Thiele, I., Vassieva, O., Ye, Y., Zagnitko, O. and Vonstein, V.

2005. The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Res.* 33(17), 5691–702. doi:10.1093/nar/gki866.

Paez-Espino, D., Eloe-Fadrosh, E. A., Pavlopoulos, G. A., Thomas, A. D., Huntemann, M., Mikhailova, N., Rubin, E., Ivanova, N. N. and Kyrpides, N. C. 2016. Uncovering Earth's virome. *Nature* 536(7617), 425–30. doi:10.1038/nature19094.

Paez-Espino, D., Chen, I.-M. A., Palaniappan, K., Ratner, A., Chu, K., Szeto, E., Pillay, M., Huang, J., Markowitz, V. M., Nielsen, T., Huntemann, M., Reddy, T. B. K., Pavlopoulos, G. A., Sullivan, M. B., Campbell, B. J., Chen, F., McMahon, K., Hallam, S. J., Denef, V., Cavicchioli, R., Caffrey, S. M., Streit, W. R., Webster, J., Handley, K. M., Salekdeh, G. H., Tsesmetzis, N., Setubal, J. C., Pope, P. B., Liu, W.-T., Rivers, A. R., Ivanova, N. N. and Kyrpides, N. C. 2017. IMG/VR: a database of cultured and uncultured DNA Viruses and retroviruses. *Nucl. Acids Res.* 45(D1), D457–65. doi:10.1093/nar/gkw1030.

Palevich, N., Kelly, W. J., Leahy, S. C., Altermann, E., Rakonjac, J. and Attwood, G. T. 2017. The complete genome sequence of the rumen bacterium *Butyrivibrio hungatei* MB2003. *Stand. Genom. Sci.* 12, 72. doi:10.1186/s40793-017-0285-8.

Parmar, N. R., Jakhesara, S. J., Mohapatra, A. and Joshi, C. G. 2016. Rumen virome: an assessment of viral communities and their functions in the rumen of an Indian Buffalo. *Curr. Sci.* 111(5), 919–25. doi:10.18520/cs/v111/i5/919-925.

Paynter, M. J. B., Ewert, D. L. and Chalupa, W. 1969. Some morphological types of bacteriophages in bovine rumen contents. *Appl. Microbiol.* 18(5), 942–3.

Penadés, J. R., Chen, J., Quiles-Puchalt, N., Carpena, N. and Novick, R. P. 2015. Bacteriophage-mediated spread of bacterial virulence genes. *Curr. Opin. Microbiol.* 23, 171–8. doi:10.1016/j.mib.2014.11.019.

Piknova, M., Filova, M., Javorsky, P. and Pristas, P. 2004. Different restriction and modification phenotypes in ruminal lactate-utilizing bacteria. *FEMS Microbiol. Lett.* 236(1), 91–5. doi:10.1016/j.femsle.2004.05.024.

Pope, P. B., Totsika, M., Aguirre de Carcer, D., Schembri, M. A. and Morrison, M. 2011. Muramidases found in the foregut microbiome of the tammar wallaby can direct cell aggregation and biofilm formation. *ISME J.* 5(2), 341–50. doi:10.1038/ismej.2010.116.

Pristas, P. and Piknova, M. 2005. Underrepresentation of short palindromes in *Selenomonas ruminantium* DNA: evidence for horizontal gene transfer of restriction and modification systems? *Can. J. Microbiol.* 51(4), 315–8. doi:10.1139/w05-004.

Raya, R. R., Oot, R. A., Moore-Maley, B., Wieland, S., Callaway, T. R., Kutter, E. M. and Brabban, A. D. 2011. Naturally resident and exogenously applied T4-like and T5-like bacteriophages can reduce *Escherichia coli* O157:H7 levels in sheep guts. *Bacteriophage* 1(1), 15–24. doi:10.4161/bact.1.1.14175.

Refardt, D. 2011. Within-host competition determines reproductive success of temperate bacteriophages. *ISME J.* 5(9), 1451–60. doi:10.1038/ismej.2011.30.

Ritchie, A. E., Robinson, I. M. and Allison, M. J. 1970. Rumen bacteriophage: survey of morphological types. In: Favard, P. (Ed.), *Microscopie Electronique* (vol. 3). Societe Francaise de Microscopie Electronique, Paris, pp. 333–4.

Ross, E. M., Petrovski, S., Moate, P. J. and Hayes, B. J. 2013. Metagenomics of rumen bacteriophage from thirteen lactating dairy cattle. *BMC Microbiol.* 13, 242. doi:10.1186/1471-2180-13-242.

Roux, S. and Brum, J. R. 2019. A viral reckoning: viruses emerge as essential manipulators of global ecosystems. *Environ. Microbiol. Rep.* 11(1), 3–8. doi:10.1111/1758-2229.12700.

Rowan-Nash, A. D., Korry, B. J., Mylonakis, E. and Belenky, P. 2019. Cross-domain and viral interactions in the microbiome. *Microbiol. Mol. Biol. Rev.* 83(1), e00044-18. doi:10.1128/MMBR.00044-18.

Russell, J. B. and Rychlik, J. L. 2001. Factors that alter rumen microbial ecology. *Science* 292(5519), 1119–22. doi:10.1126/science.1058830.

Russell, J. B. and Wilson, D. B. 1988. Potential opportunities and problems for genetically altered rumen microorganisms. *J. Nutr.* 118(2), 271–9. doi:10.1093/jn/118.2.271.

Sato, Y., Caston, J. R. and Suzuki, N. 2018. The biological attributes, genome architecture and packaging of diverse multi-component fungal viruses. *Curr. Opin. Virol.* 33, 55–65. doi:10.1016/j.coviro.2018.07.009.

Schlegel, L., Grimont, F., Ageron, E., Grimont, P. A. D. and Bouvet, A. 2003. Reappraisal of the taxonomy of the *Streptococcus bovis*/*Streptococcus equinus* complex and related species: description of *Streptococcus gallolyticus* subsp. *gallolyticus* subsp. nov., *S. gallolyticus* subsp. *macedonicus* subsp. nov. and *S. gallolyticus* subsp. *pasteurianus* subsp. nov. *Int. J. Syst. Evol. Microbiol.* 53(3), 631–45. doi:10.1099/ijs.0.02361-0.

Seal, B. S., Drider, D., Oakley, B. B., Brussow, H., Bikard, D., Rich, J. O., Miller, S., Devillard, E., Kwan, J., Bertin, G., Reeves, S., Swift, S. M., Raicek, M. and Gay, C. G. 2018. Microbial-derived products as potential new antimicrobials. *Vet. Res.* 49(1), 66. doi:10.1186/s13567-018-0563-5.

Seshadri, R., Leahy, S. C., Attwood, G. T., Teh, K. H., Lambie, S. C., Cookson, A. L., Eloe-Fadrosh, E. A., Pavlopoulos, G. A., Hadjithomas, M., Varghese, N. J. and Paez-Espino, D., Perry, R., Henderson, G., Creevey, C. J., Terrapon, N., Lapebie, P., Drula, E., Lombard, V., Rubin, E., Kyrpides, N. C., Henrissat, B., Woyke, T., Ivanova, N. N., Kelly, W. J and Hungate Project Collaborators. 2018. Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nature Biotech.* 36, 359–67.

Shapiro, J. W. and Putonti, C. 2018. Gene co-occurrence networks reflect bacteriophage ecology and evolution. *mBio* 9(2), e01870-17. doi:10.1128/mBio.01870-17.

Shkoporov, A. N. and Hill, C. 2019. Bacteriophages of the human gut: the 'Known Unknown' of the microbiome. *Cell Host Microbe* 25(2), 195–209. doi:10.1016/j.chom.2019.01.017.

Sieradzki, E. T., Ignacio-Espinoza, J. C., Needham, D. M., Fichot, E. B. and Fuhrman, J. A. 2019. Dynamic marine viral infections and major contribution to photosynthetic processes shown by spatiotemporal picoplankton metatranscriptomes. *Nat. Comm.* 10(1), 1169. doi:10.1038/s41467-019-09106-z.

Solden, L. M., Naas, A. E., Roux, S., Daly, R. A., Collins, W. B., Nicora, C. D., Purvine, S. O., Hoyt, D. W., Schückel, J., Jørgensen, B., Willats, W., Spalinger, D. E., Firkins, J. L., Lipton, M. S., Sullivan, M. B., Pope, P. B. and Wrighton, K. C. 2018. Interspecies cross-feeding orchestrates carbon degradation in the rumen ecosystem. *Nat. Microbiol.* 3(11), 1274–84. doi:10.1038/s41564-018-0225-4.

Sorek, R., Kunin, V. and Hugenholtz, P. 2008. CRISPR – a widespread system that provides acquired resistance against phages in bacteria and archaea. *Nat. Rev. Microbiol.* 6(3), 181–6. doi:10.1038/nrmicro1793.

Srinivasiah, S., Bhavsar, J., Thapar, K., Liles, M., Schoenfeld, T. and Wommack, K. E. 2008. Phages across the biosphere: contrasts of viruses in soil and aquatic environments. *Res. Microbiol.* 159(5), 349–57. doi:10.1016/j.resmic.2008.04.010.

Stewart, R. D., Auffret, M. D., Warr, A., Wiser, A. H., Press, M. O., Langford, K. W., Liachko, I., Snelling, T. J., Dewhurst, R. J., Walker, A. W., Roehe, R. and Watson, M. 2018. Assembly of 913 microbial genomes from metagenomic sequencing of the cow rumen. *Nat. Commun.* 9(1), 870. doi:10.1038/s41467-018-03317-6.

Styriak, I., Kmet, V. and Spanova, A. 1989. Isolation and characterisation of two rumen *Streptococcus bovis* bacteriophages. *Microbiol.* 12, 317–22.

Styriak, I., Galfi, P. and Kmet, V. 1991. Preliminary observations of interaction between bacteriophages and *Streptococcus bovis* bacteria on ruminal epithelium primoculture. *Vet. Microbiol.* 29(3–4), 281–7. doi:10.1016/0378-1135(91)90135-3.

Styriak, I., Spanova, A., Montagova, H. and Kmet, V. 1994. Isolation and characterisation of a new ruminal bacteriophage lytic to *Streptococcus bovis*. *Curr. Microbiol.* 28(6), 355–8. doi:10.1007/BF01570201.

Styriak, I., Pristas, P. and Javorsky, P. 1998. Lack of surface receptors not restriction-modification system determines F4 phage resistance in *Streptococcus bovis* II/1. *Folia Microbiol.* 43, 35–8.

Suen, G., Stevenson, D. M., Bruce, D. C., Chertkov, O., Copeland, A., Cheng, J. F., Detter, C., Detter, J. C., Goodwin, L. A., Han, C. S., Hauser, L. J., Ivanova, N. N., Kyrpides, N. C., Land, M. L., Lapidus, A., Lucas, S., Ovchinnikova, G., Pitluck, S., Tapia, R., Woyke, T., Boyum, J., Mead, D. and Weimer, P. J. 2011. Complete genome of the cellulolytic ruminal bacterium *Ruminococcus albus* 7. *J. Bacteriol.* 193(19), 5574–5. doi:10.1128/JB.05621-11.

Suttle, C. A. 2007. Marine viruses--major players in the global ecosystem. *Nat. Rev. Microbiol.* 5(10), 801–12. doi:10.1038/nrmicro1750.

Swain, R. A. 1999. *Factors Affecting Bacteriophages of the Rumen Ecosystem*. Ph.D. Thesis. University of New England.

Swain, R. A., Nolan, J. V. and Klieve, A. V. 1996. Natural variability and diurnal fluctuations within the bacteriophage population of the rumen. *Appl. Environ. Microbiol.* 62(3), 994–7.

Tarakanov, B. V. 1972. The electron-microscopy examination of the microflora of reindeer rumen. *Microbiology* 41, 862–70 (translated from Mikrobiologiya 41, 862–70).

Tarakanov, B. V. 1974. Lysogenic cultures of *Streptococcus bovis* isolated from rumen of cattle and sheep. *Microbiology* 43, 375–7 (translated from Mikrobiologiya 43, 375–7).

Tarakanov, B. V. 1976. Biological properties of *Streptococcus bovis* bacteriophages isolated from lysogenic cultures and sheep rumen. *Microbiology* 45(4), 695–700 (translated from Mikrobiologiya 45, 695–700).

Tarakanov, B. V. 1994. Regulation of microbial processes in the rumen by bacteriophages of *Streptococcus bovis*. *Microbiology* 63, 373–8 (translated from Mikrobiologiya 63, 657–67).

Teather, R. M. 1982. Isolation of plasmid DNA from *Butyrivibrio fibrisolvens*. *Appl. Environ. Microbiol.* 43(2), 298–302.

Thomas, C. M., Thomson, N. R., Cerdeño-Tárraga, A. M., Brown, C. J., Top, E. M. and Frost, L. S. 2017. Annotation of plasmid genes. *Plasmid* 91, 61–7. doi:10.1016/j.plasmid.2017.03.006.

Thurber, R. V., Haynes, M., Breitbart, M., Wegley, L. and Rohwer, F. 2009. Laboratory procedures to generate viral metagenomes. *Nat. Protoc.* 4(4), 470–83. doi:10.1038/nprot.2009.10.

Toussaint, A. and Rice, P. A. 2017. Transposable phages, DNA reorganization and transfer. *Curr. Opin. Microbiol.* 38, 88–94. doi:10.1016/j.mib.2017.04.009.

Toyofuku, M., Nomura, N. and Eberl, L. 2019. Types and origins of bacterial membrane vesicles. *Nat. Rev. Microbiol.* 17(1), 13–24. doi:10.1038/s41579-018-0112-2.

Turnbull, L., Toyofuku, M., Hynen, A. L., Kurosawa, M., Pessi, G., Petty, N. K., Osvath, S. R., Carcamo-Oyarce, G., Gloag, E. S., Shimoni, R., Omasits, U., Ito, S., Yap, X., Monahan, L. G., Cavaliere, R., Ahrens, C. H., Charles, I. G., Nomura, N., Eberl, L. and Whitchurch, C. B. 2016. Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat. Comm.* 7, 11220. doi:10.1038/ncomms11220.

Volgers, C., Savelkoul, P. H. M. and Stassen, F. R. M. 2018. Gram-negative bacterial membrane vesicle release in response to the host-environment: different threats, same trick? *Crit. Rev. Microbiol.* 44(3), 258–73. doi:10.1080/1040841X.2017.1353949.

Wang, B., Ma, M. P., Diao, Q. Y. and Tu, Y. 2019. Saponin-induced shifts in the rumen microbiome and metabolome of young cattle. *Front. Microbiol.* 10, 356, doi:10.3389/fmicb.2019.00356.

Weidenbach, K., Nickel, L., Neve, H., Alkhnbashi, O. S., Künzel, S., Kupczok, A., Bauersachs, T., Cassidy, L., Tholey, A., Backofen, R. and Schmitz, R. A. 2017. *Methanosarcina* spherical virus, a novel archaeal lytic virus targeting *Methanosarcina* strains. *J. Virol.* 91(22), e00955-17. doi:10.1128/JVI.00955-17.

Weinbauer, M. G. 2004. Ecology of prokaryotic viruses. *FEMS Microbiol. Rev.* 28(2), 127–81. doi:10.1016/j.femsre.2003.08.001.

Weinbauer, M. G. and Höfle, M. G. 1998. Significance of viral lysis and flagellate grazing as factors controlling bacterioplankton production in a eutrophic lake. *Appl. Environ. Microbiol.* 64(2), 431–8.

Weinbauer, M. G., Wilhelm, S. W., Suttle, C. A., Pledger, R. J. and Mitchell, D. L. 1999. Sunlight-induced DNA damage and resistance in natural viral communities. *Aquat. Microb. Ecol.* 17, 111–20. doi:10.3354/ame017111.

Wells, J. E. and Russell, J. B. 1996. Why do many ruminal bacteria dies and lyse so quickly? *J. Dairy Sci.* 79(8), 1487–95. doi:10.3168/jds.S0022-0302(96)76508-6.

Willms, E., Johansson, H. J., Mäger, I., Lee, Y., Blomberg, K. E. M., Sadik, M., Alaarg, A., Smith, C. I. E., Lehtiö, J., El Andaloussi, S., Wood, M. J. A. and Vader, P. 2016. Cells release subpopulations of exosomes with distinct molecular and biological properties. *Sci. Rep.* 6, 22519. doi:10.1038/srep22519.

Willner, D., Thurber, R. V. and Rohwer, F. 2009. Metagenomic signatures of 86 microbial and viral metagenomes. *Environ. Microbiol.* 11(7), 1752–66. doi:10.1111/j.1462-2920.2009.01901.x.

Winstanley, C., Langille, M. G. I., Fothergill, J. L., Kukavica-Ibrulj, I., Paradis-Bleau, C., Sanschagrin, F., Thomson, N. R., Winsor, G. L., Quail, M. A., Lennard, N., Bignell, A., Clarke, L., Seeger, K., Saunders, D., Harris, D., Parkhill, J., Hancock, R. E. W., Brinkman, F. S. L. and Levesque, R. C. 2009. Newly introduced genomic prophage islands are critical determinants of *in vivo* competitiveness in the Liverpool Epidemic Strain of *Pseudomonas aeruginosa*. *Genome Res.* 19(1), 12–23. doi:10.1101/gr.086082.108.

Wolf, S., Fischer, M. A., Kupczok, A., Reetz, J., Kern, T., Schmitz, R. A. and Rother, M. 2019. Characterization of the lytic archaeal virus Drs3 infecting *Methanobacterium formicicum*. *Arch. Virol.* 164(3), 667–74. doi:10.1007/s00705-018-04120-w.

Wommack, K. E. and Colwell, R. R. 2000. Virioplankton: viruses in aquatic ecosystems. *Microbiol. Mol. Biol. Rev.* 64(1), 69–114. doi:10.1128/mmbr.64.1.69-114.2000.

Wommack, K. E., Hill, R. T., Muller, T. A. and Colwell, R. R. 1996. Effects of sunlight on bacteriophage viability and structure. *Appl. Environ. Microbiol.* 62(4), 1336–41.

Wong, C. M., Klieve, A. V., Hamdorf, B. J., Schafer, D. J., Brau, L., Seet, S. G. M. and Gregg, K. 2003. Family of shuttle vectors for ruminal *Bacteroides*. *J. Mol. Microbiol. Biotechnol.* 5(2), 123–32. doi:10.1159/000069982.

Yutin, N., Kapitonov, V. V. and Koonin, E. V. 2015. A new family of hybrid virophages from an animal gut metagenome. *Biol. Direct* 10, 19. doi:10.1186/s13062-015-0054-9.

Zabriskie, J. B. 1964. The role of temperate bacteriophage in the production of erythrogenic toxin by group A Streptococci. *J. Exp. Med.* 119, 761–80. doi:10.1084/jem.119.5.761.

Chapter 10

The rumen wall microbiota community

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1 Introduction

In the past few decades, with the help of the advances in nucleic acid sequencing methods, numerous studies have been conducted to examine microbial composition and microbial functions within the rumen. These have aimed to understand the role of the rumen microbiota in influencing host biology and to develop practical ways for manipulating microbial activity. The members of the rumen microbiota are usually divided into three major groups (Fig. 1):

- the planktonic group which is associated with rumen liquid (approximately 30%);
- the feed particle-associated group (approximately 70%); and
- the rumen wall-adherent group (also known as epimural microbiota; roughly 1–5%).

Although the epimural microbiota only accounts for a small fraction of the entire rumen microbiota, it represents an important niche that performs specific functions in maintaining host performance and health. The rumen wall is highly oxygenated and allows diffusion of urea from the blood into the rumen, so it is believed that a major function of the epimural microbiota is hydrolyzing urea and scavenging oxygen (Cheng et al., 1979; Cheng and

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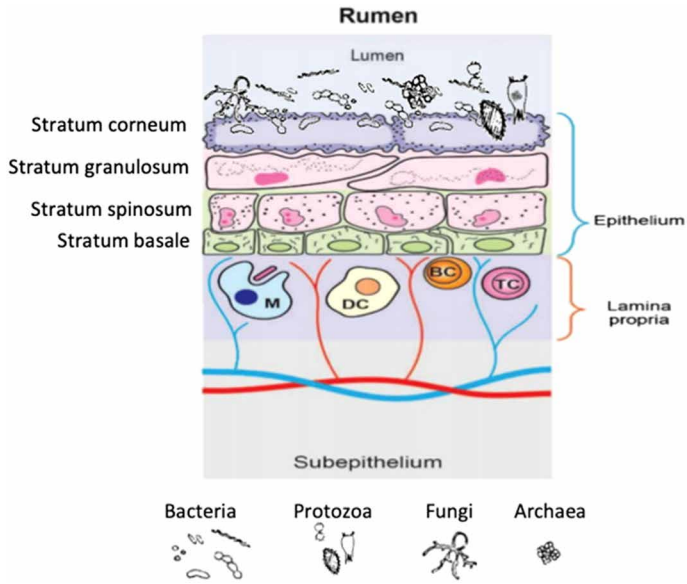

Figure 1 Illustration of rumen epimural microbiota. Source: adapted and modified from Garcia et al. (2017).

Wallace, 1979). The epimural microbiota digest the cells that become detached from the rumen epithelia and function in recycling rumen epithelial tissue. The epimural microbiota also function as the mediator communicating between the inner rumen environment and the host animal. There is, therefore, a need to better understand the role of epimural microbiota in host physiological traits. However, due to the technical difficulties, understanding of the epimural microbial community is limited compared to the other two fractions of the rumen microbiota. In the following sections, current knowledge related to the epimural microbiota will be described, and current challenges and future trends will be discussed.

2 The rumen wall microbial community

The major types of microorganisms attached to the rumen wall include bacteria and archaea, while a recent paper revealed the existence of protozoa and fungi within the epimural microbiota (Ishaq et al., 2017). The composition and diversity of the rumen wall microbial community can be affected by many factors, including breed, diet, health, and geographic location (e.g. Petri et al., 2013; Wetzels et al., 2017; Bi et al., 2018; Li et al., 2019a). Changes in the rumen wall community can potentially impact ruminant productivity in such areas as milk production and quality, feed efficiency and methane production (e.g. Kong, 2016; Mann et al., 2018; Li et al., 2019b; Neubauer et al., 2019).

Using nutritional strategies to manipulate the rumen wall community could contribute to the improvement of animal production efficiency. This chapter will summarize current research on rumen wall microbial community, including:

1 composition and function of the rumen wall microbiome;
2 development of the rumen wall microbiome;
3 factors affecting the rumen wall microbiome; and
4 impacts of the rumen wall microbial community on ruminant production.

These are discussed in the following sections.

2.1 Composition and function of rumen wall bacteria

Bacteria are the predominant community in rumen wall microbiome, and contribute about 90% of total microbial rRNA in rumen epithelia (Nagaraja, 2016). Early use of cultural methods showed that an average of 1.2×10^7 CFU bacteria were attached to each square centimeter of rumen wall in the dorsal rumen of sheep with *Butyrivibrio* and *Bacteroides* as the dominant genera (Dehority and Grubb, 1981). Wallace et al. (1979) reported that the rumen epithelia of hay-fed sheep had 4.4×10^7~2.2×10^8 CFU/g wet tissue weight. Mueller et al. (1984b) suggested the bacterial community attached to the rumen wall was not taxonomically different from the bacterial community of the contents of the rumen. However, Sadet et al. (2007) found that the rumen wall bacterial community was distinct from that of the rumen contents in 5-month-old lambs based on PCR-denaturing gradient gel electrophoresis (PCR-DGGE) profiles. By using 16S rDNA gene clone technology, Firmicutes, Bacteroidetes, and Proteobacteria were found to be the predominant phyla in the rumen of forage-fed wethers with a higher proportion of Proteobacteria (14%) attached to the rumen wall than in the rumen contents (Sadet-Bourgeteau et al., 2010). PCR-DGGE and quantitative real-time PCR technology analysis further confirmed that the phyla Firmicutes, Proteobacteria, and Bacteroidetes were predominant in the rumen wall community of beef heifers (Chen et al., 2011).

The more recent use of sequencing techniques in goats, beef cattle, and dairy cows has highlighted the fact that Firmicutes are the most abundant bacteria attached to the rumen wall, followed by Bacteroidetes and Proteobacteria (Petri et al., 2013; Mao et al., 2015; Liu et al., 2015, 2016). These studies also further confirmed that the relative abundance of Proteobacteria attached to the rumen wall is much higher than that in the rumen contents, which may be associated with their function in hydrolysis of urea, scavenging of oxygen, and recycling of epithelial tissue in ruminants. Using Illumina MiSeq sequencing technique, Wetzels et al. (2017) found, in contrast to other studies, that Proteobacteria (relative abundance of 45.2%) was the most dominant phylum, followed by

Firmicutes (33.7%) and Bacteroidetes (15.9%) in dairy cattle during adaptation to a subacute ruminal acidosis (SARA) challenge. At the genus levels, Petri et al. (2013) reported that *Atopobium*, *Desulfocurvus*, *Fervidicola*, *Lactobacillus,* and *Olsenella* were the most dominant genera in the rumen epithelia of beef cattle fed with a high-grain diet, based on 454 pyrosequencing techniques.

Liu et al. (2015) demonstrated that the rumen wall bacterial community were dominated by the genera *Butyrivibrio* (11.01% of the total sequences), *Desulfobulbus* (5.51%), *Mogibacterium* (4.33%), and *Prevotella* (4.01%) in goats, based on 454 pyrosequencing techniques. Using Illumina MiSeq sequencing technology, Liu et al. (2016) and Mao et al. (2015) showed that *Butyrivibrio* was the most predominant genus attached to the rumen wall of lactating dairy cattle. In 56-day-old lambs, *Prevotella* (22.65% of the total sequences), *Butyrivibrio* (13.86%), *Campylobacter* (6.91%), *Treponema* (6.16%), RC9 gut group (5.51%), and *Desulfobulbus* (2.99%) were the dominant genera in the rumen epithelia (Liu et al., 2017). Wetzels et al. (2017) reported that *Campylobacter* (15.5% relative abundance), *Kingella* (7.8%), *Desulfobulbus* (4.7%), and *Brachymonas* (4.2%) dominated the rumen wall of dairy cattle fed high-concentrate diet. These findings indicate that the composition of the rumen wall microbiome may be affected by variation in animal species/breeds, diets, and geographic location. It is also noticeable that different sequencing technologies may also lead to different results. More standardized research methods are needed for a more conclusive picture of the composition of microbiota and how they can be affected by diet, host, and environment.

Although early studies demonstrated that the bacteria attached to the rumen wall are involved in hydrolysis of urea, scavenging oxygen, and recycling rumen epithelial tissue (Cheng et al., 1979; Cheng and Wallace, 1979), the specific functions of rumen wall bacteria are largely unknown. Based on the composition and predicted functional genes of the rumen wall bacteria, it is believed that the bacteria attached to the rumen wall are more likely to be involved in modulating expression of genes related to VFA absorption (Chen et al., 2011), epithelium proliferation, diseases, protein metabolism, and energy metabolism (Mao et al., 2015; Liu et al., 2017; Lin et al., 2019). However, to date, there is lack of direct evidence to demonstrate the specific function of the rumen wall bacteria in ruminants. In the future, there will need to be more research to identify the function of rumen wall bacteria using culture methods or metagenomics and meta-transcriptomic techniques.

2.2 Composition and function of rumen wall archaea

Archaea is a specialized group of microorganisms inhabiting the rumen. *Methanobrevibacter* (61.6%), *Methanomicrobium* (14.9%), and *Methanomassiliicoccales* (previously referred to as rumen cluster C; 15.8%) are considered

the three dominant methanogens in the rumen contents (Nagaraja, 2016). Most species of methanogens use H_2 and CO_2 to produce CH_4, while methylotrophic methanogen (e.g. *Methanomassiliicoccales*) mainly use methyl compounds and H_2 to produce CH_4. Compared with bacterial ecosystems, there is limited information on archaea in the rumen epithelia. Janssen and Kirs (2008) reported that the archaeal community contributed 3–5% of total microbial rRNA in the rumen epithelia. Shin et al. (2004) identified the phylotypes of archaea in the rumen of Korean cows, with the Methanomicrobiaceae family (95% of clones) predominant in the rumen wall archaeal community and the Methanobacteriaceae family predominant in the rumen contents, based on 16S rDNA sequence technology. By using 16S rRNA gene sequences techniques, Pei et al. (2010) compared the diversity and abundance of methanogens found in rumen liquid, attached to feed particles and in the rumen wall in Chinese Jinnan (beef) cattle. They found that *Methanobrevibacter* was the predominant genus attached to feed particles (77.2%) and the rumen wall (77.8%). Using quantitative real-time PCR, they also reported that the rumen wall-associated methanogenic community had significantly higher density compared with that attached to feed particles, and suggested that this may be due to the slower turnover rates of rumen epithelia (Pei et al., 2010). However, other research suggests the archaeal community associated with the rumen epithelium in dairy cows is less diverse (De Mulder et al., 2017) and less abundant (Liu et al., 2016; Wang et al., 2017) than those in the rumen content and liquid fractions. The predominant archaeal phylotypes also differed based on host species. In Jinnan cattle, *Methanobrevibacter* was reported to be the major archaea of the epimural community (Pei et al., 2010). Shin et al. (2004) found that epimural archaeal sequences were mainly associated with *Methanomicrobium mobile* in Korean dairy cows. Wang et al. (2017) reported a higher proportion of *Methanosphaera* phylotypes (approximately 20%) in the rumen wall of black goats. It has been suggested that the *Mbb. gottschalkii*:*Mbb. ruminantium* ratio of the planktonic community may be associated with host methane emissions (Danielsson et al., 2017), but this ratio has not been identified as a key feature of the epimural archaeal community in other studies. The composition and function of archaea attached to the rumen wall still remains largely unknown.

3 The development of the rumen wall microbiome

As mentioned earlier, the rumen wall of adult ruminants possesses a complex microbial ecological system. The rumen is practically a sterile and germ-free organ at birth (Nagaraja, 2016). Together with the microbiota associated with the rumen contents, the colonization and establishment of the rumen wall microbial community is one of critical processes of rumen development in young ruminants (Yanez-Ruiz et al., 2015). Microbial colonization of the rumen

of newborn ruminants follows a typical succession in that bacteria proliferate in the rumen fluid phase immediately after birth and then colonize the ruminal wall within the next 36–48 h (Yanez-Ruiz et al., 2015). The colonized fluid-phase bacteria facilitate the subsequent sequential colonization of fungi, followed by protozoa in the rumen fluid (Yanez-Ruiz et al., 2015). This results in the establishment of a complex consortium that eventually develops in the fluid, on feed particles and on ruminal epithelial surfaces. Once established, content-associated microorganisms are generally stable and will only change when nutrients are changed (Stewart et al., 1988), while the diversity of rumen epithelial microbial community seems to change with age (Mueller et al., 1984a; Rieu et al., 1990). Early life is a key stage to manipulate the rumen microbiome, including microbiota attached to the rumen wall.

As stated previously, many studies show that the bacterial communities attached to the rumen wall differ from those associated with ruminal contents. Recent research suggests they may perform some specific functions and affect the development and immune function (mainly barrier function) of the rumen wall in young ruminants (Malmuthuge et al., 2012, 2014). Because of the special function of rumen wall microbiota in host metabolism, innate immunity, and rumen development, more attention should be paid to their colonization and establishment in the rumen wall in young ruminants.

By using scanning electron microscopy technique, Mueller et al. (1984a) identified 24 morphological types of bacteria associated with the rumen wall in 1- to 10-week-old lambs, but only seven types in both older lambs and adult sheep. Malmuthuge et al. (2014) reported that the bacterial community attached to the rumen wall was very different from those attached to the rumen contents in 3-week-old preweaned dairy male calves. They found lower abundances of *Prevotella* and higher abundances of *Bacteroidetes* attached to the rumen wall compared with those in the rumen contents. The development of the rumen wall bacteria community was strongly associated with the mRNA expression of some key genes involved in innate immunity in preweaned calves (Malmuthuge et al., 2014). This suggests that more attention should be paid to the colonization of the rumen wall microbial community in young ruminants.

Jiao et al. (2015) identified the changes in rumen wall bacterial composition and diversity during rumen development in goat kids (from day 0 to day 70) by MiSeq sequencing of 16S rRNA genes. They found that Firmicutes, Bacteroidetes, and Proteobacteria dominated the rumen wall bacterial community of goats during early rumen development, and that the proportions of Firmicutes and Bacteroidetes increased with age, while the proportion of Proteobacteria decreased with age. At the genus level, *Escherichia* (80.79%), which originates from the mother's vagina, skin, colostrum, or from the environment, is the dominant genus attached to the rumen wall of newborn goat kids. At 42 and 70 days, *Butyrivibrio* and *Campylobacter* became the

dominant genera attached to the rumen wall of goat kids, significantly higher than in the rumen contents, indicating these two genera might play critical roles in host function after introduction of solid feed. In addition, these authors also reported that the genera *Butyrivibrio*, *Campylobacter*, and *Desulfobulbus* may be involved in the morphology and functional development of the rumen in lambs. This study suggests that the provision of solid feed is a major factor affecting the establishment of the rumen wall microbial community in goat kids. More research needs to be done to understand the specific functions of these genera and how these three genera interact with the host.

Another study by Liu et al. (2015) investigated the effect of starter supplementation on the establishment of rumen epithelial bacterial community in Hu (breed) lambs and found that, compared with feeding only with ewe milk, starter supplementation changed the ruminal epithelial bacterial structure and composition significantly. It produced a higher diversity and greater relative abundance of *Desulfobulbus*, *Howardella*, *Bacteroides*, *Syntrophococcus*, *Pyramidobacter*, *Bifidobacterium*, *Megasphaera*, with a lower relative abundance of *Campylobacter* and *Snodgrassella*. All these studies indicate that the colonization and establishment of rumen wall bacteria is age-related (with establishment at 2 months). They also suggest that many factors (e.g. maternal influence, offspring reared in isolation, liquid/solid feed) could affect the colonization and establishment of the rumen wall microbial community. It is noticeable that the effect of age was affected by diet, and the establishment of rumen epithelial wall microbiota could be the result of interaction between diet and growth of animals. These are important factors to be considered for future early-life interventions.

4 Factors affecting the epimural microbiota

Diet has long been considered as one of the major driving forces in changing the ecology of the rumen content microbiota. A strong dietary impact on the rumen microbiota is often seen between animals offered a high-forage versus a high-grain diet. Such changes in the microbial community are believed to be the result of adaptation of the rumen microbiota to different feed components.

A number of recent studies have explored dietary effects on the epimural microbiota, with differing results. When dairy cows transitioned from a high-forage to a high-grain diet, AlZahal et al. (2017) reported a significant reduction in the diversity of the epimural community along with the reduction of *Fibrobacter* (from 5.3% to 1.3%) and *Ruminococcaceae* (from 7.5% to 2.3%) and an increase of *Prevotella* (from 16.1% to 19.2%). When gradually increasing the proportion of concentrates in the diet, Wetzels et al. (2017) found a significant decrease in the relative abundance of the two predominant bacterial genera *Campylobacter* (from 20.05% to 15.15%) and *Kingella* (from 12.46% to 7.31%).

Petri et al. (2018) fed dry Holstein cows with high-quality hay (HQH) to replace concentrates, and found that increasing HQH in the diet shifted the epimural microbiota from domination by *Firmicutes* to a predominance of *Proteobacteria* while reducing overall microbial diversity. They suggest that the change is due to the proliferation of *Campylobacter* (by approximately fivefold), which may be associated with the increasing protein and non-protein nitrogen content of HQH. Both these studies suggest the potentially important role of *Campylobacter* in the rumen epimural bacterial community, which warrants further investigation.

The rumen content- and liquid-associated microbiota are relatively stable regardless of differences in host species, geographical ranges, and diets (Henderson et al., 2015). However, the epimural microbiota vary according to host species. Beef cattle epimural microbiota was reported to be less diverse compared to that of the content microbiota (Li et al., 2012). This can be contrasted with dairy cow epimural microbiota which have been found to be more diverse than the content microbiota (Malmuthuge et al., 2012; AlZahal et al., 2017). AlZahal et al. (2017) have suggested that the more diverse epimural microbiota in dairy cows might suggest they participate in more complex biological processes.

Variation of epimural microbiota among different ruminant species exists in terms of the predominant phylotypes. At the phylum level, *Proteobacteria* has been found to be the predominant phylum in kid goats (Jiao et al., 2015) and beef cattle (Kong, 2016). *Bacteroidetes* was the predominant phylum in adult goats (Zhang et al., 2017a,b). Replacement of *Proteobacteria* by *Bacteroidetes* and *Firmicutes* was observed in dairy cows from birth to adulthood (Jami et al., 2013). Cross-individual variation has also been found when examining the rumen epimural microbiota. McCann et al. (2016) observed cross-individual variation in the epimural microbiota in response to SARA. The epimural microbiota of cattle suffering from SARA was found to have a community with reduced *Kingella* and *Azoarcus* and increased *Ruminobacter* (Wetzels et al., 2016). It has been suggested that such alteration in the epimural microbiota might have a negative impact on rumen function, leading to a greater decline in ruminant pH and increased severity of SARA. De Mulder et al. (2017) have also reported higher cross-individual variation in alpha diversity and beta diversity indices for epimural microbiota than those of lipid and solid phases in mid-lactating Holstein-Friesian dairy cows. These findings suggest that host genetic factors may have a stronger impact on the epimural microbiota closely associated with host tissue compared to microbiota associated with the rumen contents.

Studies of non-ruminant species suggest that different growth promoters or toxic growth inhibitors are a key factor in selecting microbes to colonize the epithelium (Schluter and Foster, 2012). However, this host effect is usually

masked by other factors (e.g. diet and environment) which also have an effect on the microbiota. To better isolate the host effect, one potential research direction would be linking the epimural microbiota to host genetic markers (such as SNPs identified from a genetically closed herd). Studies performed on twin/triplet ruminants may also be another option for clarifying host effects.

Based on the rumen census project, rumen content microbiota varies geographically (Henderson et al., 2015). The ecology of the rumen epimural microbiota of the same cattle species also differs according to geographical region. An example of such difference is the epimural microbiota found in Holstein cows. The study by Liu et al. (2016) found that *Firmicutes* accounted more than 80% of the community in China. However, a study by Petri et al. (2018) found *Firmicutes* accounted no more than 50% of the community in Austria. The overall microbial profiles also differed across these studies. While the host species were the same (Holstein dairy cows), animals were at similar lactating stages, and diets with similar dietary energy values were offered; such segregation on microbial profiles coincide with those reported for content microbiota (Henderson et al., 2015). The variation by geographical location may be attributed to complex combined effects such as temperature, surrounding environment, different dietary sources, and different housing managements. Additionally, the sampling techniques and sample processing differed among labs, which may also contribute to the distinctive results reported from multiple studies. The epimural microbial taxonomy observed from these studies is summarized in Table 1.

5 The impact of the epimural microbiota on ruminant production

5.1 Milk composition

Milk composition is associated with rumen fermentation and the available nutrients absorbed through the rumen epithelium (Matthews et al., 2019). Microbial nitrogen metabolism in the rumen is vital in providing microbial protein to the host for milk production (Tadele and Amha, 2015). VFAs also affect milk fat component (Hurtaud et al., 1993). Most of the studies investigating the association between rumen microbiota and milk composition have focused on the liquid and solid phase-associated microbiota (Jami et al., 2014; Bainbridge et al., 2016; Zhang et al., 2017a,b). They have concluded that modulation of the rumen microbiota to achieve a larger proportion of casein or whey relative to non-protein nitrogen (N) in the rumen can eventually improve milk protein yield (Tadele and Amha, 2015).

Some of the epimural bacteria (members of *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*) possess urease genes (Jin et al., 2017; Mann et al., 2018), and may play a role in the overall rumen N metabolism. The

Table 1 Summary of the rumen epimural microbiota revealed by different approaches through different studies

Study	Species	Diet(s)	Stage of life	Method(s)	Major phylotypes[a]
Chen et al. (2011)	Beef heifers	Corn: 97% hay, 3% concentrate Trt: transitioning from 60:40, 40:60, 25:75, 15:85, 8:92 forage-to-concentrate ratio	8 months old	16S rRNA PCR-DGGE	Bacteria: Proteobacteria, Firmicutes, Bacteroidetes
Petri et al. (2013)	Angus heifers	Forage: 95% grass hay, 5% supplement Mixed forage: 60% barley silage, 30% barley grain, 10% supplement High grain: 9% barley silage, 81% barley grain, 10% supplement	BW at 308 ± 35 kg	16S rRNA gene pyrosequencing; PCR-DGGE	Bacteria: Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria
Liu et al. (2015)	Boer × Yangtze River Delta White goats	Hay: 81% Chinese wildrye, 15% Alfalfa, HG: 30% Chinese wildrye, 45% corn meal, 20% wheat meal, 1.1% soybean	2–3 years	16S rRNA gene MiSeq sequencing	Bacteria: Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetae
Jiao et al. (2015)	Goat	1–40 days: 0.5 L goat milk, 0.04 kg DM/meal fresh grass, 0.12kg DM/meal starter concentrate 40–70 days: 0.06 kg DM fresh grass/meal, 0.17 kg starter concentrate/meal	1–70 days	16S rRNA gene MiSeq sequencing	Bacteria: Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria
Wetzels et al. (2017)	Holstein cows	Corn: 50% grass silage, 50% second-cut meadow hay Trt: 20% grass silage, 20% second-cut meadow hay, 60% concentrate	BW at 710 ± 118 kg	16S rRNA gene MiSeq sequencing	Bacteria: Proteobacteria, Firmicutes, Bacteroidetes, Synergistetes, Elusimicrobia
Abecia et al. (2014b)	Goats	Alfalfa hay *ad libitum* NAT: remained with dam ART: milk replacer	1, 3, 7, 14, 21, 28 days	16S rRNA pyrosequencing	Bacteria: Proteobacteria, Firmicutes, Bacteroidetes, Cyanobacteria

De Mulder et al. (2017)	Holstein-Friesian cows	70:30 forage-to-concentrate ratio	Mid-lactation	16S rRNA gene MiSeq sequencing	Bacteria: Firmicutes, Bacteroidetes, Fibrobacteres, Proteobacteria, Spirochaetes, TM7, Actinobacteria Archaea: *Methanobacteria*
AlZahal et al. (2017)	Holstein cows	High-forage: 77:23 forage-to-concentrate ratio (w/wo yeast) High-grain: 49:51 forage-to-concentrate ratio (w/wo yeast)	Lactating	16S rRNA gene MiSeq sequencing	Bacteria: Bacteroidetes, Firmicutes, Fibrobacteres, Proteobacteria, Tenericutes, Cyanobacteria, SR1, Spirochaetes
Mann et al. (2018)	Holstein cows	Corn: 50% grass silage, 50% second-cut meadow hay Trt: 60% concentrate, 40% forage	Non-lactating (3–4 parities)	Metatranscriptome	Bacteria: Proteobacteria, Firmicutes, Bacteroidetes, Spirochaetes, and Actinobacteria Archaea: *Methanocaldococcus Methanobrevibacter*
Frutos et al. (2018)	Merino lambs	43.3% barley, 15.0% corn, 23.7% soybean meal 44, 15.0% barley straw, 3.0% vitamin-mineral premix	Fattening period	qPCR	Bacteria: Only reported total bacteria, *Prevotella* sp., *Selenomonas ruminantium*, and methanogens
Li et al. (2019b)	Holstein bull calves	Corn: texturized, 35.3% starch, 25.3% NDF Trt: pelleted, 42.7% starch, 15.1% neutral detergent fiber, (NDF)	1–17 weeks	Metatranscriptome	Bacteria: Proteobacteria, Firmicutes, Fusobacteria, Actinobacteria, Bacteroidetes
Lin et al. (2019)	Hu sheep	Corn: breast milk only Trt: fed starter twice daily	10–56 days	rRNA gene MiSeq sequencing; TruSeq metagenome sequencing	Bacteria: Bacteroidetes, Firmicutes, Spirochaetae, Actinobacteria, Proteobacteria, Tenericutes Protozoa: *Entodinium*, *Polyplastron*, *unclassified Trichostomatia*, *Ophryoscolex*, *Diplodinium*, *Isotricha*
Neubauer et al. (2019)	Holstein cows	Corn: 50:50 hay:grass-silage mix Trt: 65% concentrate, 35% roughage	Non-lactating	16S rRNA gene MiSeq	Bacteria: Proteobacteria, Firmicutes, Synergistetes, Actinobacteria

[a] Phylotypes were listed according to their relative abundance in each study; only those with more than 1% were presented.

distinctive ureolytic bacterial composition and the urease genes observed from the epimural microbial community may indicate functional segregation from that of lumen microbial community (Jin et al., 2017). Currently, the exact functions of the epimural ureolytic bacteria, their urease activities, and to what extent the epimural microbiota influence N metabolism in the rumen, remain unclear. Advanced omics methods such as metagenomics and metatranscriptomics may help to reveal the functions of the epimural microbiota in overall nitrogen metabolism, but this depends on the accurately isolating epimural microbiota.

In addition to milk protein, milk fat is another economically important trait in milk production. The *de novo* synthesis of fatty acids in the udder relies on VFAs being transported from the rumen (Palmquist, 2006). VFA absorption and transportation is associated with aspects of rumen wall physiology such as epithelial surfaces and activity of transporters (O'Shea et al., 2016). There is evidence that rumen epithelial bacterial abundance is associated with total ruminal VFA concentration (Chen et al., 2012), suggesting that this community may play a role in regulating VFA absorption. It may be worth exploring the role of active transporters and the epimural microbiome using meta-transcriptomics to identify active transportation pathways for the corresponding VFAs and their relationship with active microbial groups in the rumen wall (Mann et al., 2018).

5.2 Feed efficiency

Epimural microbiota have also been reported to be a factor affecting host feed efficiency. Kong (2016) compared the epimural microbiota in beef cattle with different residue feed intake (RFI), a common measurement for cattle feed efficiency (where low RFI indicates higher efficiency). The study found that *Campylobacteraceae* and *Neisseriaceae* had significantly greater cell activity in the L-RFI epithelium than that in the H-RFI epithelium (5.8% vs 2.4%, 10.8% vs 1.0%, respectively). It was suggested that the higher oxidase activity enabled by these two phylotypes may lead to higher oxygen scavenging capacity in L-RFI animals, thus maintaining an anaerobic environment for better microbial anaerobic fermentation, resulting in higher energy efficiency. Liang et al. (2017) studied the epimural microbiota in finishing Hu breed lambs and found the proportion of *Butyrivibrio fibrisolvens* and *Escherichia coli* was higher in H-RFI animals. The greater abundance of *B. fibrisolvens* was reported to be associated with the lower propionate concentration and a greater acetate:propionate ratio, which coincided with lower feed efficiency. It was suggested that the higher *E. coli* population was the result of the greater fluctuation of the rumen pH and VFA concentrations within the rumen of H-RFI animals (Liang et al., 2017). It has been suggested that the effect of epimural microbiota on host feed efficiency which is unknown may rely on other functions rather than feed degradation. It would be useful to apply metagenome and/or metatranscriptome-based approaches

to identify the active pathway(s) to better define the exact mechanisms that may influence host feed efficiency and to what extent the epimural microbiota contribute to cattle feed efficiency.

5.3 Methane emissions

Oxygen concentration in the rumen epithelium has been found to be much higher than that in the rumen lumen. A high population of anaerobic archaea has commonly been identified within the epimural microbiota. The *Mbb. gottschalkii*:*Mbb. ruminantium* ratio of the planktonic community has been claimed to be positively associated with host methane emissions (Danielsson et al., 2017), but this is not supported by other studies.

Although there is no direct evidence that the epimural microbiota affect methane emissions, there are possible links worth investigating, such as the role of rumen epimural microbiota in regulating lumen [H] availability. If marker epimural microbes are found to be associated with lumen [H] concentration and/or indirectly associated with methanogenesis, we may be able to develop novel methane mitigation methods that focus on manipulating these epimural microbiota in addition to lumen microbiota.

There is indirect evidence of host effect on the archaeal community. In a study of rumen content transplantation, Zhou et al. (2018) found that the archaeal community was unaffected regardless by donor phenotype, and that the archaeal community of most of the animals returned to its original status. They argued that the rumen epimural community may be the innate driving force in re-establishment of microbiota, particularly the archaea. Host effect on methane emissions was reported by Roehe et al. (2016), who ranked the rumen archaeal population and host methane emissions based on host progeny groups. They have also suggested that that rumen archaeal abundance is under host genetic control. Difford et al. (2018) examined the factors related to methane emissions in dairy cows and reported that host genetics was the top factor followed by the bacteria and archaea contributing to this process. The authors have suggested the potential to modulate rumen microbiota by genetically selecting animals. These studies highlight the need to study epimural archaea individually to develop better methane mitigation strategies.

6 Challenges and future trends

6.1 Better illustration of microbial functions

Although recent studies have applied multi-omics-based methods to reveal the composition and functions of the rumen microbiota (e.g. Stewart et al., 2018; Li et al., 2019a), the knowledge of the epimural microbiota is still very

limited. Mann et al. (2018) employed a metatranscriptomic approach to study the gene expression and functional potentials of the rumen wall bacteria. They found that the genes involved in galactose, starch, sucrose, and energy metabolism were highly expressed by the epimural microbiota. This result showed the active roles of rumen epimural microbiota in providing host-relevant metabolites through cross-rumen-wall transportation. For the first time, they have reported nitrogen fixation by the cattle rumen epimural microbiota. High levels of expressions of the genes involved in oxidative stress were also observed. They also identified the active archaeal and fungal community within the epimural microbiome, though their functions are still not understood.

There is no doubt that more descriptive data on the rumen epimural microbiota will become available using multi-omics methods, but it is essential to perform accurate analyses to identify the 'TRUE and KEY' members of the microbial community. Factors such as sample collection methods (Paz et al., 2016), DNA/RNA extraction protocols (Villegas-Rivera et al., 2013; Henderson et al., 2015), sequencing methods, and bioinformatic modules for data processing (Neves et al., 2017) can all affect the outcome of identifying rumen epimural microbiota. In addition, the database used for data interpretation is also critical. The metagenome being assembled by Seshadri et al. (2018) was retrieved from the Hungate1000 collection, which is highly specialized for the rumen microbiota and claims to have covered approximately three quarters of the genus-level microbial taxa (JGI Hungate Collection). However, both compositional and functional segregation exist between rumen content microbiota and epimural microbiota. It is only possible to define the accurate microbial functions by properly assigning the sequence reads to the true phylotypes, but currently available tools cannot achieve this outcome yet.

Recently, Wilkins et al. (2019) developed a more efficient method for retrieving metagenome-assembled genomes (MAGs). Such methods offer the rumen microbiologist a new direction for defining the epimural MAGs that can be utilized as reference, thus allowing better resolution for future study. On top of identifying microbial composition, studying the metabolome of the microbiota may provide direct evidence on how microbial activities impact host performance. Although there is no metabolite-based study being performed on the epimural microbiota, a proteome-based study (Hart et al., 2018) on the rumen fluid microbiota has provided a direction for future examination of the epimural microbiota in terms of metabolites/enzymes/proteins, allowing us to obtain a complete description of their functions as well as their impact on the host. It should be noted, though, that microbial metabolites are dynamic over time. Whether the metabolome obtained from these samples is representative and sufficient to reflect the real conditions within the rumen environment remains an issue.

6.2 Better understanding of host-microbial interaction

Due to their proximity to the host, the epimural microbiota are considered to have close interaction with host animals. Chen et al. (2012) reported that the epimural bacterial population was positively correlated with TLR 4 expression in the rumen wall in acidosis resistant beef steers, but such a relationship was not seen in acidosis-susceptible animals. Liu et al. (2015) found that the expression of *TLR2* was associated with ten epimural bacterial taxa while *TLR4* was associated with one taxon belonging to *Anaerolineaceae*. These findings suggest that the abundance of epimural bacteria may stimulate host gene expression. Future studies should explore the association between host gene expression and the epimural microbiota, to better elucidate how microbiota colonization can influence host biology.

Lipopolysaccharide (LPS) is found in the outer layer of gram-negative bacteria (GNB; Wang and Quinn, 2010). The lysis of GNB in the rumen lumen is associated with increasing levels of rumen LPS (Nagaraja et al., 1978; Gozho et al., 2005, 2007). The LPS released from the epimural GNB is considered as endotoxin with strong pro-inflammatory potential, thus causing rumen tissue lesion (Steele et al., 2011), altering the expression of TLRs and the tight junction structure of the rumen epithelium (Liu et al., 2015; McCann et al., 2016).

Among these epimural-associated GNB, *Fusobacterium necrophorum* is one of the best understood species. *F. necrophorum* is an aerotolerant anaerobe commonly identified from the rumen wall, and has been found to be actively involved in feed degradation, metabolizing lactic acid and epithelial proteins (Li et al., 2019a). Under normal rumen conditions, *F. necrophorum* mainly functions as a feed digester. However, in animals suffering from SARA, it becomes an opportunistic pathogen (Berg and Scanlan, 1982), proliferating on the rumen wall where the mucosa are affected by parakeratosis and prolonged SARA conditions in the host (Okada et al., 1999; Takayama et al., 2000). It can also penetrate the rumen epithelium, translocate into the bloodstream, and invade cattle liver, causing abscesses (Nagaraja and Titgemeyer, 2007; Steele et al., 2009; Tadepalli et al., 2009). It is vital to understand the interaction between commensal epimural microbiota and the host, which could provide the basis for future strategies to prevent the pathogenesis of *F. necrophorum* under high-grain diets and ruminant metabolic dysfunctions.

Host-microbial interactions have been extensively studied in the case of content/liquid microbiota. These interactions help explain the variation in host feed efficiency for beef (Zhou et al., 2009; Hernandez-Sanabria et al., 2012; Myer et al., 2015) and dairy cattle (Jami et al., 2014; Jewell et al., 2015), in host methane emissions (Carberry et al., 2014; Shi et al., 2014) and in milk production (Jami et al., 2014; Jewell et al., 2015). However, the role of the epimural community in explaining host phenotypes is less well understood. It is only

recently that Mann et al. (2018) have examined and reported the expression of a wide range of microbial genes by the epimural microbiota. The products by these active genes may serve as the future targets for identifying their roles in influencing epithelium morphology, molecule transportation, signaling, and so forth, which provides direct evidence of microbial-host interactions.

6.3 More effective manipulation strategies

There have been many recent attempts to manipulate rumen microbiota to enhance fermentation efficiency and to create a healthier rumen environment. Such attempts to change rumen microbiota during adulthood have not shown consistent, long-term effectiveness. Abecia et al. (2014a,b) have proposed targeting the early-life rumen content microbiota for their long-term effect in reducing methane emission. As the epimural microbiota are exposed to stronger host-related selective pressures and are functionally diverse from the rumen microbes residing in other niches, future studies of host regulatory effects on the epimural microbiota may help to improve the chance for successful early-life interventions. There needs to be a meta-analysis of the existing multi-omics datasets generated from different studies to provide clues for novel targets such as the key phylotypes, key pathways, and key enzymes. We are currently working to properly allocate all the different types of data to a single analysis platform to better interpret the meta-omes from multiple studies and to provide more accurate comparisons among studies. Application of probiotics may also be a future direction for manipulating the epimural microbiota, which requires more experimental evidence to support this approach.

6.4 Improved sample collection and data handling

While multi-omics technologies can provide high-resolution analysis of nucleic acids (DNA and RNA) and metabolites within the collected samples, the major roadblock of studying the epimural community is identifying 'TRUE' epimural microbiota. Unlike the easy accessibility of rumen fluid and digesta samples either through tubing or rumen cannula methods, epithelial samples are comparatively difficult to obtain. van Niekerk et al. (2018) have developed a rumen tissue sampling method through endoscopy, which allows repeated sampling without rumen cannulation. This method allows us to collect rumen tissue samples from young and adult ruminants more efficiently and humanely, and also to study changes in the epimural microbiota within the same animals when they receive different treatments.

Removing the non-adherent microbes from the rumen epithelial tissue is also a technical issue for sample handling. An adequate rinse step to remove any non-adherent materials from the epithelium samples is necessary (Chen

et al., 2011). However, since the half-lives of microbial mRNAs can be short as seconds (Laalami et al., 2014), the rapid yet efficient way to remove the non-adherent microbes should be explored to ensure the integrity of the samples.

Contaminants such as reads identified from the blank control in commercial kits (Becker et al., 2016; Thoendel et al., 2017) and host reads identified from meta-omics data (e.g. Brown et al., 2019) should also be considered when interpreting the data to avoid false-positive results. Properly selecting the reaction kits as well as the data processing pipeline may help to limit the impact of contamination.

7 Conclusions

Current molecular-based methods have allowed detailed identification of epimural microbial composition and changes under different conditions. They provide the opportunity for better definition of their roles in coping with the planktonic and feed-associated microbiota for feed digestion and in interacting with host tissue for transferring nutrients and promoting immune function. Advanced multi-omics approaches that have been applied to dissect rumen contents microbiota should also be applied to research the epimural microbiome to understand its functions and contributions to host biology. Integrated analyses of the currently available dataset, development of proper data interpretation models, and involvement of larger sample sets may help to fill current knowledge gaps, and suggest future research for better rumen epithelial health and function.

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9 References

Abdel Rahman, S. 1966. Comparative study of the urease in the rumen wall and rumen content. *Nature* 209(5023), 618–9. doi:10.1038/209618b0.

Abdoun, K., Stumpff, F. and Martens, H. 2006. Ammonia and urea transport across the rumen epithelium: a review. *Anim. Health Res. Rev.* 7(1–2), 43–59. doi:10.1017/S1466252307001156.

Abecia, L., Waddams, K. E., Martínez-Fernandez, G., Martín-García, A. I., Ramos-Morales, E., Newbold, C. J. and Yáñez-Ruiz, D. R. 2014a. An antimethanogenic nutritional intervention in early life of ruminants modifies ruminal colonization by Archaea. *Archaea* 2014, 841463. doi:10.1155/2014/841463.

Abecia, L., Ramos-Morales, E., Martínez-Fernandez, G., Arco, A., Martín-García, A. I., Newbold, C. J. and Yáñez-Ruiz, D. R. 2014b. Feeding management in early life

influences microbial colonisation and fermentation in the rumen of newborn goat kids. *Anim. Prod. Sci.* 54(9), 1449–54. doi:10.1071/AN14337.

Adjei-Fremah, S., Ekwemalor, K., Worku, M. and Ibrahim, S. 2018. Probiotics and ruminant health. In: Enany, S. (Ed.), *Probiotics: Current Knowledge and Future Prospects*. IntechOpen, pp. 131–49. Chapter 8. doi:10.5772/intechopen.72846.

AlZahal, O., Li, F., Guan, L. L., Walker, N. D. and McBride, B. W. 2017. Factors influencing ruminal bacterial community diversity and composition and microbial fibrolytic enzyme abundance in lactating dairy cows with a focus on the role of active dry yeast. *J. Dairy Sci.* 100(6), 4377–93. doi:10.3168/jds.2016-11473.

Anders, S. and Huber, W. 2010. Differential expression analysis for sequence count data. *Genome Biol.* 11(10), R106. doi:10.1186/gb-2010-11-10-r106.

Bainbridge, M. L., Cersosimo, L. M., Wright, A. D. and Kraft, J. 2016. Rumen bacterial communities shift across a lactation in Holstein, Jersey and Holstein × Jersey dairy cows and correlate to rumen function, bacterial fatty acid composition and production parameters. *FEMS Microbiol. Ecol.* 92(5), fiw059. doi:10.1093/femsec/fiw059.

Becker, L., Steglich, M., Fuchs, S., Werner, G. and Nubel, U. 2016. Comparison of six commercial kits to extract bacterial chromosome and plasmid DNA for MiSeq sequencing. *Sci. Rep.* 6, 28063. doi:10.1038/srep28063.

Berg, J. N. and Scanlan, C. M. 1982. Studies of *Fusobacterium necrophorum* from bovine hepatic-abscesses – biotypes, quantitation, virulence, and antibiotic susceptibility. *Am. J. Vet. Res.* 43(9), 1580–6.

Bharanidharan, R., Arokiyaraj, S., Kim, E. B., Lee, C. H., Woo, Y. W., Na, Y., Kim, D. and Kim, K. H. 2018. Ruminal methane emissions, metabolic, and microbial profile of Holstein steers fed forage and concentrate, separately or as a total mixed ration. *PLoS ONE* 13(8), e0202446. doi:10.1371/journal.pone.0202446.

Bi, Y., Zeng, S., Zhang, R., Diao, Q. and Tu, Y. 2018. Effects of dietary energy levels on rumen bacterial community composition in Holstein heifers under the same forage to concentrate ratio condition. *BMC Microbiol.* 18(1), 69. doi:10.1186/s12866-018-1213-9.

Brown, S. M., Chen, H., Hao, Y., Laungani, B. P., Ali, T. A., Dong, C., Lijeron, C., Kim, B., Wultsch, C., Pei, Z. and Krampis, K. 2019. MGS-Fast: metagenomic shotgun data fast annotation suing microbial gene catalogs. *GIGA Sci.* 8(4), giz020. doi:10.1093/gigascience/giz020.

Carberry, C. A., Kenny, D. A., Kelly, A. K. and Waters, S. M. 2014. Quantitative analysis of ruminal methanogenic microbial populations in beef cattle divergent in phenotypic residual feed intake (RFI) offered contrasting diets. *J. Anim. Sci. Biotechnol.* 5(1), 41. doi:10.1186/2049-1891-5-41.

Chen, Y. H., Penner, G. B., Li, M. J., Oba, M. and Guan, L. L. 2011. Changes in bacterial diversity associated with epithelial tissue in the beef cow rumen during the transition to a high-grain diet. *Appl. Environ. Microbiol.* 77(16), 5770–81. doi:10.1128/AEM.00375-11.

Chen, Y., Oba, M. and Guan, L. L. 2012. Variation of bacterial communities and expression of Toll-like receptor genes in the rumen of steers differing in susceptibility to subacute ruminal acidosis. *Vet. Microbiol.* 159(3-4), 451–9. doi:10.1016/j.vetmic.2012.04.032.

Cheng, K. J. and Wallace, R. J. 1979. The mechanism of passage of endogenous urea through the rumen wall and the role of ureolytic epithelial bacteria in the urea flux. *Br. J. Nutr.* 42(3), 553–7. doi:10.1079/bjn19790147.

Cheng, K. J., Mccowan, R. P. and Costerton, J. W. 1979. Adherent epithelial bacteria in ruminants and their roles in digestive-tract function. *Am. J. Clin. Nutr.* 32(1), 139–48. doi:10.1093/ajcn/32.1.139.

Cho, S. J., Cho, K. M., Shin, E. C., Lim, W. J., Hong, S. Y., Choi, B. R., Kang, J. M., Lee, S. M., Kim, Y. H., Kim, H. and Yun, H. D. 2006. 16S rDNA analysis of bacterial diversity in three fractions of cow rumen. *J. Microbiol. Biotechn.* 16, 92–101.

Danielsson, R., Dicksved, J., Sun, L., Gonda, H., Muller, B., Schnurer, A. and Bertilsson, J. 2017. Methane production in dairy cows correlates with rumen methanogenic and bacterial community structure. *Front. Microbiol.* 8, 226. doi:10.3389/fmicb.2017.00226.

Dehority, B. A. and Grubb, J. A. 1981. Bacterial population adherent to the epithelium on the roof of the dorsal rumen of sheep. *Appl. Environ. Microbiol.* 41(6), 1424–7.

De Mulder, T., Goossens, K., Peiren, N., Vandaele, L., Haegeman, A., De Tender, C., Ruttink, T., de Wiele, T. V. and De Campeneere, S. 2017. Exploring the methanogen and bacterial communities of rumen environments: solid adherent, fluid and epimural. *FEMS Microbiol. Ecol.* 93(3), fiw251. doi:10.1093/femsec/fiw251.

Difford, G. F., Plichta, D. R., Lovendahl, P., Lassen, J., Noel, S. J., Hojberg, O., Wright, A. G., Zhu, Z., Kristensen, L., Nielsen, H. B., Guldbrandtsen, B. and Sahana, G. 2018. Host genetics and the rumen microbiome jointly associate with methane emissions in dairy cows. *PLoS Genet.* 14(10), e1007580. doi:10.1371/journal.pgen.1007580.

Frutos, J., Andres, S., Yanez-Ruiz, D., Benavides, J., López, S., Santos, A., Martínez-Valladares, M., Rozada, F. and Giráldez, F. J. 2018. Early feed restriction of lambs modifies ileal epimural microbiota and affects immunity parameters during the fattening period. *Animal* 12(10), 2115–22.

Garcia, M., Bradford, B. J. and Nagaraja, T. G. 2017. Invited review: ruminal microbes, microbial products, and systemic inflammation. *Prof. Anim. Sci.* 33(6), 635–50. doi:10.15232/pas.2017-01663.

Gloor, G. B., Macklaim, J. M., Pawlowsky-Glahn, V. and Egozcue, J. J. 2017. Microbiome dataset are compositional: and this is not option. *Front. Microbiol.* 8. doi:10.3389/fmicb.2017.02224.

Gozho, G. N., Plaizier, J. C., Krause, D. O., Kennedy, A. D. and Wittenberg, K. M. 2005. Subacute ruminal acidosis induces ruminal lipopolysaccharide endotoxin release and triggers an inflammatory response. *J. Dairy Sci.* 88(4), 1399–403. doi:10.3168/jds.S0022-0302(05)72807-1.

Gozho, G. N., Krause, D. O. and Plaizier, J. C. 2007. Ruminal lipopolysaccharide concentration and inflammatory response during grain-induced subacute ruminal acidosis in dairy cows. *J. Dairy Sci.* 90(2), 856–66. doi:10.3168/jds.S0022-0302(07)71569-2.

Granja-Salcedo, Y. T., Ramirez-Ui, R. A., Machado, E. G., Duarte Messana, J., Takeshi Kishi, L., Lino Dias, A. V. and Berchielli, T. T. 2017. Studies on bacterial community composition are affected by the time and storage method of the rumen content. *PLoS ONE* 12(4), e0176701. doi:10.1371/journal.pone.0176701.

Hart, E. H., Creevey, C. J., Hitch, T. and Kingston-Smith, A. H. 2018. Meta-proteomics of rumen microbiota indicates niche compartmentalization and functional dominance in a limited number of metabolic pathways between abundant bacteria. *Sci. Rep.* 8(1), 10504. doi:10.1038/s41598-018-28827-7.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Global Rumen Census Collaborators and Janssen, P. H. 2015 Rumen microbial community composition

varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5, 14567. doi:10.1038/srep14567.

Hernandez-Sanabria, E., Goonewardene, L. A., Wang, Z., Durunna, O. N., Moore, S. S. and Guan, L. L. 2012. Impact of feed efficiency and diet on adaptive variations in the bacterial community in the rumen fluid of cattle. *Appl. Environ. Microbiol.* 78(4), 1203–14. doi:10.1128/AEM.05114-11.

Hristov, A. N. and Ropp, J. K. 2003. Effect of dietary carbohydrate composition and availability on utilization of ruminal ammonia nitrogen for milk protein synthesis in dairy cows. *J. Dairy Sci.* 86(7), 2416–27. doi:10.3168/jds.S0022-0302(03)73836-3.

Hu, F., Xue, Y., Guo, C., Liu, J. and Mao, S. 2018. The response of ruminal fermentation, epithelium-associated microbiota, and epithelial barrier function to severe feed restriction in pregnant ewes. *J. Anim. Sci.* 96(10), 4293–305. doi:10.1093/jas/sky306.

Hurtaud, C., Rulquin, H. and Verite, R. 1993. Effect of infused volatile fatty acids and caseinate on milk composition and coagulation in dairy cows. *J. Dairy Sci.* 76(10), 3011–20. doi:10.3168/jds.S0022-0302(93)77640-7.

Ishaq, S. L., AlZahal, O., Walker, N. and McBride, B. 2017. An investigation into rumen fungal and protozoal diversity in three rumen fractions, during high-fiber or grain-induced sub-acute ruminal acidosis conditions, with or without active dry yeast supplementation. *Front. Microbiol.* 8, 1943. doi:10.3389/fmicb.2017.01943.

Jami, E., Israel, A., Kotser, A. and Mizrahi, I. 2013. Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7(6), 1069–79. doi:10.1038/ismej.2013.2.

Jami, E., White, B. A. and Mizrahi, I. 2014. Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. *PLoS ONE* 9(1), e85423. doi:10.1371/journal.pone.0085423.

Janssen, P. H. and Kirs, M. 2008. Structure of the archaeal community of the rumen. *Appl. Environ. Microbiol.* 74(12), 3619–25. doi:10.1128/AEM.02812-07.

Jewell, K. A., McCormick, C. A., Odt, C. L., Weimer, P. J. and Suen, G. 2015. Ruminal bacterial community composition in dairy cows is dynamic over the course of two lactations and correlates with feed efficiency. *Appl. Environ. Microbiol.* 81(14), 4697–710. doi:10.1128/AEM.00720-15.

Jiao, J., Huang, J., Zhou, C. and Tan, Z. 2015. Taxonomic identification of ruminal epithelial bacterial diversity during rumen development in goats. *Appl. Environ. Microbiol.* 81(10), 3502–9. doi:10.1128/AEM.00203-15.

Jin, D., Zhao, S. G., Zheng, N., Bu, D. P., Beckers, Y., Denman, S. E., McSweeney, C. S. and Wang, J. 2017. Differences in ureolytic bacterial composition between the rumen digesta and rumen wall based on ureC gene classification. *Front. Microbiol.* 8, 385. doi:10.3389/fmicb.2017.00385.

Kong, R. 2016. Contribution of the rumen epithelial transcriptome and microbial community to variation in beef cattle feed efficiency. MSc Thesis. doi:10.7939/R3833N87B.

Laalami, S., Zig, L. and Putzer, H. 2014. Initiation of mRNA decay in bacteria. *Cell. Mol. Life Sci.* 71(10), 1799–828. doi:10.1007/s00018-013-1472-4.

Li, M., Zhou, M., Adamowicz, E., Basarab, J. A. and Guan, L. L. 2012. Characterization of bovine ruminal epithelial bacterial communities using 16S rRNA sequencing, PCR-DGGE, and qRT-PCR analysis. *Vet. Microbiol.* 155(1), 72–80. doi:10.1016/j.vetmic.2011.08.007.

Li, F., Hitch, T. C. A., Chen, Y., Creevey, C. J. and Guan, L. L. 2019a. Comparative metagenomic and metatranscriptomic analyses reveal the breed effect on the rumen

microbiome and its associations with feed efficiency in beef cattle. *Microbiome* 7(1), 6. doi:10.1186/s40168-019-0618-5.

Li, W., Gelsinger, S., Edwards, A., Riehle, C. and Koch, D. 2019b. Transcriptome analysis of rumen epithelium and meta-transcriptome analysis of rumen epimural microbial community in young calves with feed induced acidosis. *Sci. Rep.* 9(1), 4744. doi:10.1038/s41598-019-40375-2.

Liang, Y. S., Li, G. Z., Li, X. Y., Lu, J. Y., Li, F. D., Tang, D. F., Li, F., Deng, Y., Zhang, H., Wang, Z. L. and Weng, X. X. 2017. Growth performance, rumen fermentation, bacteria composition, and gene expressions involved in intracellular pH regulation of rumen epithelium in finishing Hu lambs differing in residual feed intake phenotype. *J. Anim. Sci.* 95(4), 1727–38. doi:10.2527/jas.2016.1134.

Lin, L., Xie, F., Sun, D., Liu, J., Zhu, W. and Mao, S. Y. 2019. Ruminal microbiome-host crosstalk stimulates the development of the ruminal epithelium in a lamb model. *Microbiome* 7(1), 83. doi:10.1186/s40168-019-0701-y.

Liu, J. H., Bian, G. R., Zhu, W. Y. and Mao, S. Y. 2015. High-grain feeding causes strong shifts in ruminal epithelial bacterial community and expression of toll-like receptor genes in goats. *Front. Microbiol.* 6, 167. doi:10.3389/fmicb.2015.00167.

Liu, J. H., Zhang, M. L., Zhang, R. Y., Zhu, W. Y. and Mao, S. Y. 2016. Comparative studies of the composition of bacterial microbiota associated with the ruminal content, ruminal epithelium and in the faeces of lactating dairy cows. *Microb. Biotechnol.* 9(2), 257–68. doi:10.1111/1751-7915.12345.

Liu, J., Bian, G., Sun, D., Zhu, W. and Mao, S. 2017. Starter feeding altered ruminal epithelial bacterial communities and some key immune-related genes' expression before weaning in lambs. *J. Anim. Sci.* 95(2), 910–21. doi:10.2527/jas.2016.0985.

Lovell, D., Pawlowsky-Glahn, V., Egozcue, J. J., Marguerat, S. and Bähler, J. 2015. Proportionality: a valid alternative to correlation for relative data. *PLoS Comput. Biol.* 11(3), e1004075. doi:10.1371/journal.pcbi.1004075.

Malmuthuge, N., Li, M. J., Chen, Y. H., Fries, P., Griebel, P. J., Baurhoo, B., Zhao, X. and Guan, L. L. 2012. Distinct commensal bacteria associated with ingesta and mucosal epithelium in the gastrointestinal tracts of calves and chickens. *FEMS Microbiol. Ecol.* 79(2), 337–47. doi:10.1111/j.1574-6941.2011.01220.x.

Malmuthuge, N., Griebel, P. J. and le Guan, L. 2014. Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the gastrointestinal tracts of preweaned calves. *Appl. Environ. Microbiol.* 80(6), 2021–8. doi:10.1128/AEM.03864-13.

Mann, E., Wetzels, S. U., Wagner, M., Zebeli, Q. and Schmitz-Esser, S. 2018. Metatranscriptome sequencing reveals insights into the gene expression and functional potential of rumen wall bacteria. *Front. Microbiol.* 9, 43. doi:10.3389/fmicb.2018.00043.

Mao, S., Zhang, M., Liu, J. and Zhu, W. 2015. Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function. *Sci. Rep.* 5, 16116. doi:10.1038/srep16116.

Matthews, C., Rispie, F., Lewis, E., Reid, M., O'Toole, P. W. and Cotter, P. D. 2019. The rumen microbiome: a crucial consideration when optimizing milk and meat production and nitrogen utilization efficiency. *Gut Microbes* 19, 115–32.

McCann, J. C., Luan, S., Cardoso, F. C., Derakhshani, H., Khafipour, E. and Loor, J. J. 2016. Induction of subacute ruminal acidosis affects the ruminal microbiome and epithelium. *Front. Microbiol.* 7, 701. doi:10.3389/fmicb.2016.00701.

Mueller, R. E., Asplund, J. M. and Iannotti, E. L. 1984a. Successive changes in the epimural bacterial community of young lambs as revealed by scanning electron-microscopy. *Appl. Environ. Microbiol.* 47(4), 715–23.

Mueller, R. E., Iannotti, E. L. and Asplund, J. M. 1984b. Isolation and identification of adherent epimural bacteria during succession in young lambs. *Appl. Environ. Microbiol.* 47(4), 724–30.

Myer, P. R., Smith, T. P. L., Wells, J. E., Kuehn, L. A. and Freetly, H. C. 2015. Rumen microbiome from steers differing in feed efficiency. *PLoS ONE* 10(6), e0129174. doi:10.1371/journal.pone.0129174.

Nagaraja, T. G. 2016. Microbiology of the rumen. *Rumenology*, 39–61.

Nagaraja, T. G. and Titgemeyer, E. C. 2007. Ruminal acidosis in beef cattle: the current microbiological and nutritional outlook. *J. Dairy Sci.* 90 (Suppl. 1), E17–38. doi:10.3168/jds.2006-478.

Nagaraja, T. G., Bartley, E. E., Fina, L. R., Anthony, H. D. and Bechtle, R. M. 1978. Evidence of endotoxins in the rumen bacteria of cattle fed hay or grain. *J. Anim. Sci.* 47(1), 226–34. doi:10.2527/jas1978.471226x.

Neubauer, V., Humer, E., Mann, E., Kroger, I., Reisinger, N., Wagner, M., Zebeli, Q. and Petri, R. M. 2019. Effects clay mineral supplementation on particle-associated and epimural microbiota, and gene expression in the rumen of cows fed high-concentrate diet. *Anaerobe* 59, 38–48, doi:10.1016/j.anaerobe.2019.05.003.

Neves, A. L. A., Li, F., Ghoshal, B., McAllister, T. and Guan, L. L. 2017. Enhancing the resolution of rumen microbial classification from metatranscriptomic data using Kraken and Mothur. *Front. Microbiol.* 8, 2445. doi:10.3389/fmicb.2017.02445.

O'Hara, E., Neves, A. L. A., Song, Y. and Guan, L. L. 2019. The role of gut microbiome in cattle production and health: driver or passenger?

Okada, Y., Kanoe, M., Yaguchi, Y., Watanabe, T., Ohmi, H. and Okamoto, K. 1999. Adherence of *Fusobacterium necrophorum* subspecies necrophorum to different animal cells. *Microbios* 99(393), 95–104.

O'Shea, E., Waters, S. M., Keogh, K., Kelly, A. K. and Kenny, D. A. 2016. Examination of the molecular control of ruminal epithelial function in response to dietary restriction and subsequent compensatory growth in cattle. *J. Anim. Sci. Biotechnol.* 7, 53. doi:10.1186/s40104-016-0114-8.

Palmquist, D. L. 2006. Milk fat: origin of fatty acids and influence of nutritional factors thereon. In: Fox P. F. and McSweeney, P. L. H. (Eds), *Advanced Dairy Chemistry Volume 2 Lipids*. Springer, Boston, MA, pp. 43–92.

Paz, H. A., Anderson, C. L., Muller, M. J., Kononoff, P. J. and Fernando, S. C. 2016. Rumen bacterial community composition in holstein and jersey cows is different under same dietary condition and is not affected by sampling method. *Front. Microbiol.* 7, 1206. doi:10.3389/fmicb.2016.01206.

Pei, C. X., Mao, S. Y., Cheng, Y. F. and Zhu, W. Y. 2010. Diversity, abundance and novel 16S rRNA gene sequences of methanogens in rumen liquid, solid and epithelium fractions of Jinnan cattle. *Animal* 4(1), 20–9. doi:10.1017/S1751731109990681.

Petri, R. M., Schwaiger, T., Penner, G. B., Beauchemin, K. A., Forster, R. J., McKinnon, J. J. and McAllister, T. A. 2013. Changes in the rumen epimural bacterial diversity of beef cattle as affected by diet and induced ruminal acidosis. *Appl. Environ. Microbiol.* 79(12), 3744–55. doi:10.1128/AEM.03983-12.

Petri, R. M., Kleefisch, M. T., Metzler-Zebeli, B. U., Zebeli, Q. and Klevenhusen, F. 2018. Changes in the rumen epithelial microbiota of cattle and host gene expression

in response to alterations in dietary carbohydrate composition. *Appl. Environ. Microbiol.* 84(12). doi:10.1128/AEM.00384-18.

Rainard, P. and Foucras, G. 2018. A critical appraisal of probiotics for mastitis control. *Front. Vet. Sci.* 5, 251, doi:10.3389/fvets.2018.00251.

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O. and Monteil, V. 2014. Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. *J. Appl. Microbiol.* 116(2), 245–57. doi:10.1111/jam.12405.

Rieu, F., Fonty, G., Gaillard, B. and Gouet, P. 1990. Electron-microscopy study of the bacteria adherent to the rumen wall in young conventional lambs. *Can. J. Microbiol.* 36(2), 140–4. doi:10.1139/m90-025.

Roehe, R., Dewhurst, R. J., Duthie, C. A., Rooke, J. A., McKain, N., Ross, D. W., Hyslop, J. J., Waterhouse, A., Freeman, T. C., Watson, M. and Wallace, R. J. 2016. Bovine host genetic variation influences rumen microbial methane production with best selection criterion for low methane emitting and efficiently feed converting hosts based on metagenomic gene abundance. *PLoS Genet.* 12(2), e1005846. doi:10.1371/journal.pgen.1005846.

Sadet, S., Martin, C., Meunier, B. and Morgavi, D. P. 2007. PCR-DGGE analysis reveals a distinct diversity in the bacterial population attached to the rumen epithelium. *Animal* 1(7), 939–44. doi:10.1017/S1751731107000304.

Sadet-Bourgeteau, S., Martin, C. and Morgavi, D. P. 2010. Bacterial diversity dynamics in rumen epithelium of wethers fed forage and mixed concentrate forage diets. *Vet. Microbiol.* 146(1–2), 98–104. doi:10.1016/j.vetmic.2010.04.029.

Schluter, J. and Foster, K. R. 2012. The evolution of mutualism in gut microbiota via host epithelial selection. *PLoS Biol.* 10(11), e1001424, doi:10.1371/journal.pbio.1001424.

Seshadri, R., Leahy, S. C., Attwood, G. T., The, K. H., Lambie, S. C., Cookson, A. L., Eloe-Fadrosh, E. A., Pavlopoulos, G. A., Hadjithomas, M., Varghese, N. J., Paez-Espino, D., Hungate1000 project collaborators, Perry, R., Henderson, G., Creevey, C. J., Terrapon, N., Lapebie, P., Drula, E., Lombard, V., Rubin, E., Kyrpides, N. C., Henrissat, B., Woyke, T., Ivanova, N. N. and Kelly, W. J. 2018. Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nat. Biotechnol.* 36(4), 359–67. doi:10.1038/nbt.4110.

Shi, W., Moon, C. D., Leahy, S. C., Kang, D., Froula, J., Kittelmann, S., Fan, C., Deutsch, S., Gagic, D., Seedorf, H., Kelly, W. J., Atua, R., Sang, C., Soni, P., Li, D., Pinares-Patiño, C. S., McEwan, J. C., Janssen, P. H., Chen, F., Visel, A., Wang, Z., Attwood, G. T. and Rubin, E. M. 2014. Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. *Genome Res.* 24(9), 1517–25. doi:10.1101/gr.168245.113.

Shin, E. C., Choi, B. R., Lim, W. J., Hong, S. Y., An, C. L., Cho, K. M., Kim, Y. K., An, J. M., Kang, J. M., Lee, S. S., Kim, H. and Yun, H. D. 2004. Phylogenetic analysis of archaea in three fractions of cow rumen based on the 16S rDNA sequence. *Anaerobe* 10(6), 313–9. doi:10.1016/j.anaerobe.2004.08.002.

Steele, M. A., AlZahal, O., Hook, S. E., Croom, J. and McBride, B. W. 2009. Ruminal acidosis and the rapid onset of ruminal parakeratosis in a mature dairy cow: a case report. *Acta Vet. Scand.* 51, 39. doi:10.1186/1751-0147-51-39.

Steele, M. A., Croom, J., Kahler, M., AlZahal, O., Hook, S. E., Plaizier, K. and McBride, B. W. 2011. Bovine rumen epithelium undergoes rapid structural adaptations during grain-induced subacute ruminal acidosis. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 300(6), R1515–23. doi:10.1152/ajpregu.00120.2010.

Stewart, C. S., Fonty, G. and Gouet, P. 1988. The establishment of rumen microbial communities. *Anim. Feed Sci. Tech.* 21(2–4), 69–97. doi:10.1016/0377-8401(88)90093-4.

Stewart, R. D., Auffret, M. D., Warr, A., Wiser, A. H., Press, M. O., Langford, K. W., Liachko, I., Snelling, T. J., Dewhurst, R. J., Walker, A. W., Roehe, R. and Watson, M. 2018. Assembly of 913 microbial genomes from metagenomic sequencing of the cow rumen. *Nat. Commun.* 9(1), 870. doi:10.1038/s41467-018-03317-6.

Tadele, Y. and Amha, N. 2015. Use of different non protein nitrogen sources in ruminant nutrition: a review. *Adv. Life Sci. Technol.* 29, 100–6.

Tadepalli, S., Narayanan, S. K., Stewart, G. C., Chengappa, M. M. and Nagaraja, T. G. 2009. Fusobacterium necrophorum: a ruminal bacterium that invades liver to cause abscesses in cattle. *Anaerobe* 15(1–2), 36–43. doi:10.1016/j.anaerobe.2008.05.005.

Takayama, Y., Kanoe, M., Maeda, K., Okada, Y. and Kai, K. 2000. Adherence of *Fusobacterium necrophorum* subsp. *necrophorum* to ruminal cells derived from bovine rumenitis. *Lett. Appl. Microbiol.* 30(4), 308–11. doi:10.1046/j.1472-765x.2000.00722.x.

Thoendel, M., Jeraldo, P., Greenwood-Quaintance, K. E., Yao, J., Chia, N., Hanssen, A. D., Abdel, M. P. and Patel, R. 2017. Impact of contaminating DNA in whole-genome amplification kits used for metagenomic shotgun sequencing for infection diagnosis. *J. Clin. Microbiol.* 55(6), 1789–801, doi:10.1128/JCM.02402-16.

van Niekerk, J., Middeldorp, M. and Steele, M. 2018. Technical Note: the development of a methodology for ruminal and colon tissue biopsying of young Holstein dairy calves. *J. Dairy Sci.* 101(8), 7212–18.

Villegas-Rivera, G., Vargas-Cabrera, Y., Gonzalez-Silva, N., Aguilera-Garcia, F., Gutierrez-Vazquez, E., Bravo-Patino, A., Cajero-Juarez, M., Baizabal-Aguirre, V. M. and Valdez-Alarcon, J. J. 2013. Evaluation of DNA extraction methods of rumen microbial populations. *World J. Microbiol. Biotechnol.* 29(2), 301–7. doi:10.1007/s11274-012-1183-2.

Wallace, R. J., Cheng, K. J., Dinsdale, D. and Orskov, E. R. 1979. An independent microbial flora of the epithelium and its role in the ecomicrobiology of the rumen. *Nature* 279(5712), 424–6. doi:10.1038/279424a0.

Wang, X. and Quinn, P. J. 2010. Endotoxins: lipopolysaccharides of gram-negative bacteria. *Subcell. Biochem.* 53, 3–25. doi:10.1007/978-90-481-9078-2_1.

Wang, Z., Elekwachi, C. O., Jiao, J., Wang, M., Tang, S., Zhou, C., Tan, Z. and Forster, R. J. 2017. Investigation and manipulation of metabolically active methanogen community composition during rumen development in black goats. *Sci. Rept* 7(1), 422. doi:10.1038/s41598-017-00500-5.

Wetzels, S. U., Mann, E., Matzler-Zebeli, B. U., Pourazad, P., Qumar, M., Klevenhusen, F., Pinior, B., Wagner, M., Zebeli, Q. and Schmitz-Esser, S. 2016. Epimural indicator phylotypes of transiently-induced subacute ruminal acidosis in dairy cattle. *Front. Microbiol.* 7, 274. doi:10.3389/fmicb.2016.00274.

Wetzels, S. U., Mann, E., Pourazad, P., Qumar, M., Pinior, B., Metzler-Zebeli, B. U., Wagner, M., Schmitz-Esser, S. and Zebeli, Q. 2017. Epimural bacterial community structure in the rumen of Holstein cows with different responses to a long-term subacute ruminal acidosis diet challenge. *J. Dairy Sci.* 100(3), 1829–44. doi:10.3168/jds.2016-11620.

Wilkins, L., Ettinger, C., Josping, G. and Eisen, J. 2019. Metagenome-assembled genomes provide new insights into microbial diversity. *Sci. Rep.* 9(1), 3059–65.

Yanez-Ruiz, D. R., Abecia, L. and Newbold, C. J. 2015. Manipulating rumen microbiome and fermentation through interventions during early life: a review. *Front. Microbiol.* 6, 1133. doi:10.3389/fmicb.2015.01133.

Zhang, J., Xu, C., Huo, D., Hu, Q. and Peng, Q. 2017a. Comparative study of the gut microbiome potentially related to milk protein in Murrah buffaloes (*Bubalus bubalis*) and Chinese Holstein cattle. *Sci. Rep.* 7, 42189. doi:10.1038/srep42189.

Zhang, R., Ye, H., Liu, J. and Mao, S. 2017b. High-grain diets altered rumen fermentation and epithelial bacterial community and resulted in rumen epithelial injuries of goats. *Appl. Microbiol. Biotechnol.* 101(18), 6981–92. doi:10.1007/s00253-017-8427-x.

Zhou, M., Hernandez-Sanabria, E. and Guan, L. L. 2009. Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. *Appl. Environ. Microbiol.* 75(20), 6524–33. doi:10.1128/AEM.02815-08.

Zhou, M., Chen, Y., Griebel, P. J. and Guan, L. L. 2014. Methanogen prevalence throughout the gastrointestinal tract of pre-weaned dairy calves. *Gut Microbes* 5(5), 628–38. doi :10.4161/19490976.2014.969649.

Zhou, M., Peng, Y. J., Chen, Y., Klinger, C. M., Oba, M., Liu, J. X. and Guan, L. L. 2018. Assessment of microbiome changes after rumen transfaunation: implications on improving feed efficiency in beef cattle. *Microbiome* 6(1), 62. doi:10.1186/s40168-018-0447-y.

Part 3

Nutrient processing in the rumen and host interactions

Chapter 11

Ruminal fibre digestion

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1 Introduction

Herbivorous ruminants rely on a close symbiosis with their ruminal microbiota for the proficient conversion of plant biomass to microbial cell protein and volatile fatty acids (McCann et al., 2014). By breaking down the complex matrix of polymers that constitute the cell walls of lignocellulosic feedstuffs, the microorganisms fulfil their host's nutritional demands while thriving in a suitable environment where they are provided a constant influx of energy and relative environmental stability. These microbiomes utilize a prodigious catalogue of carbohydrate-active enzymes (CAZymes) to deconstruct the complex carbohydrates of the plant cell wall and access the large amounts of inherently stored energy that is otherwise extremely difficult to access. Cellulose in itself is highly recalcitrant to degradation, and in the plant cell wall, it is embedded in a matrix of complex hemicelluloses and lignin, making enzymatic access difficult. To overcome this challenge, the bacteria, ciliates and fungi colonizing the rumen have developed powerful strategies in which they utilize the CAZymes in various mechanisms to liberate and utilize the monomers from the lignocellulose.

Until recently, the study of cellulose degradation in the cow rumen was mostly attributed to a few cultured isolates from the phyla Firmicutes and Fibrobacteres, namely *Ruminococcus albus, R. flavefaciens* and *Fibrobacter*

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succinogenes (Hungate, 1950, 1960; Russell et al., 2009). The advent of culture-independent techniques has revealed that these well-studied isolates are often found in low abundance *in situ*, with the rumen dominated by uncultured Firmicutes and Bacteroidetes populations (Stevenson and Weimer, 2007; Konietzny et al., 2014). These uncharacterized phylotypes are a potential source of novel CAZymes and new knowledge into the saccharolytic mechanisms that are employed, which builds on classical views of biomass and cellulose degradation in the rumen. The known CAZyme configurations and mechanisms that have been described from well-known cultured microbes include cellulosomes, secreted free cellulases and polysaccharide utilization loci (PULs). In addition, the means of cellulose degradation in certain species is still elusive.

Considering that ruminants obtain most of their energy from their symbiotic microbiota, the efficiency of feed conversion and end-product meat and milk quality in bovines is tightly linked to the dynamics and function of the rumen microbiome. In this chapter, we will give an overview of the role of the microbiota in ruminal lignocellulose degradation, who is doing what and cover the mechanisms they utilize in the decomposition of biomass.

2 Lignocellulosic biomass

Via their anatomical structure and dietary actions, ruminants consume a plethora of plant glycans. In order to discuss the mechanisms of plant fibre degradation in the rumen, we will first briefly discuss the general composition of the plant cell wall (Fig. 1).

Plant cell walls comprise of a complex extracellular matrix of polysaccharides, lignin, lipids, minerals and glycoproteins, which provide both mechanical

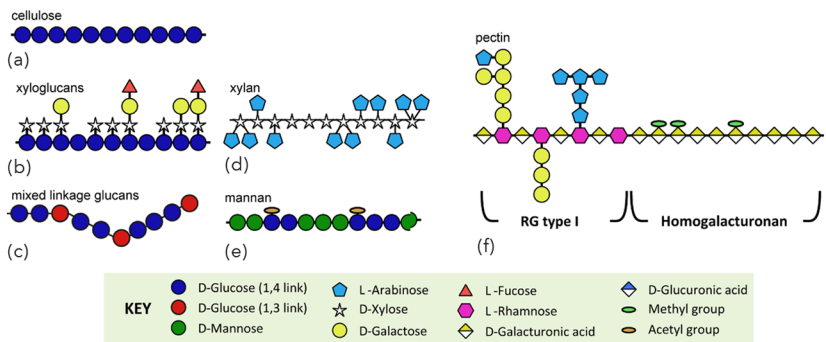

Figure 1 Structural and compositional variety of plant polysaccharides, including cellulose (a), hemicellulose (b–e) and pectin (f). Specific substrates include (a) cellulose, (b) xyloglucan, (c) mixed linkage glucans, (d) arabinoxylan, (e) glucomannan, (f) pectin (including rhamnogalacturon).

strength and protection for the cell. The cell wall is composed of distinct layers; the middle lamella, the primary cell wall and the secondary cell wall, which are all sequentially secreted by the cells' protoplasts (Gibson, 2012). The primary cell wall is composed of cellulose fibres that are embedded in a matrix of pectin and hemicelluloses such as xyloglucan, xylans and glucomannans (Scheller and Ulvskov, 2010). This layer is flexible, and allows for cell growth. When growth subsides, the secondary cell wall is produced either by modifying the primary cell wall, or by deposition of an additional second layer. The secondary cell wall is distinct from the primary by the incorporation of lignin, a matrix of cross-linked phenolic compounds which provide additional mechanical strength to the cell wall (Zhong and Ye, 2015). Vascular plants use cells with lignified cell wall as mechanical tissues, enabling them to grow tall and compete for sunlight. Although not technically part of the structural polysaccharides that make up the cell wall, starch is an important storage polysaccharide that consists of α-D-glucose units and is commonly ingested by ruminants.

2.1 Cellulose

The main component of the cell wall is cellulose, comprising 25–51.4% of typical lignocellulosic biomasses (Isikgor and Becer, 2015). Cellulose consists of chains of D-glucopyranoside (glucose) molecules linked by β-1,4-glycosidic bonds (Fig. 1a). The individual glucose molecules are rotated 180° relative to each other, making the repeating unit cellobiose. The innate molecular structure of cellobiose allows for a great hydrogen-bonding potential in longer oligomers, and with degrees of polymerization (DP) greater than seven, the affinity for other oligomers is so high that the molecules aggregate and cannot be solubilized in aqueous solvents (Brown, 2004). Native cellulose in the plant cell wall takes on a crystalline form called cellulose I_{β}, where 18–24 cellulose chains are produced by the cellulose synthase complex on the cell's plasma membrane, forming micro-fibrils that are stabilized by extensive hydrogen bonding between parallel aligned chains (Schneider et al., 2016). Elementary fibrils aggregate into larger structures called microfibrils, with crystalline and less crystalline (amorphous) regions, which again are interconnected to each other in the plant cell wall by hemicellulose and lignin.

2.2 Hemicellulose

'Hemicellulose' is a broad term that describes different heteropolymers that are embedded in the cell walls of plants, of which the detailed structure and abundance can vary depending on the plant species. Traditionally, after extracting pectins and lignin from the biomass, extraction with alkaline treatment was used to separate 'the rest' of the polysaccharides from cellulose,

which was subsequently termed 'hemicellulose' (Scheller and Ulvskov, 2010). More recently, the term 'hemicellulose' has been suggested by Scheller and Ulvskov (2010) to be redefined to include the cell wall polysaccharides that share a common equatorial β-1,4-glycosidic bond in their backbone. These include xyloglucans, mixed linkage β-(1,3, 1,4)-glucans, xylans, mannans, glucomannans and galactomannans.

Xyloglucan is found in all plant cell walls except Charophytes (Scheller and Ulvskov, 2010), and consists of a β-1,4-linked backbone of glucose with or without xylose substitutions (termed monomer X and G, respectively). The xylose residues can in turn be substituted with galactose (termed L) or in grasses, with fucosylated galactose (F) (Fig. 1b). The ratios and distribution of X and G in the backbone and the branching patterns vary between plant species, with common repeating units of XXXG for dicots, or XXGG for Solanaceous species, where the Xs can be un-substituted (X), or substituted (L or F) (Attia and Brumer, 2016). Another hemicellulose with a glucose backbone is mixed linkage β-glucan. It is found in the cell walls of grasses, and consist of trimers and tetramers of β-1,4-linked glucose, linked together *via* β-1,3-glycosidic bonds (Fig. 1c).

Xylans are hemicelluloses with β-1,4-linked xylose units in the backbone. They are often the dominating non-cellulose polysaccharide in secondary cell walls in dicots, and are usually found with α-1,2-linked glucuronosyl and 4-*O*-methyl glucuronosyl substitutions, and are called glucuronoxylans. In commelinid monocots, arabinoxylans are dominating in the primary cell wall, more heavily substituted with arabinose (Scheller and Ulvskov, 2010; Fig. 1d).

The third type of hemicelluloses contains a backbone comprised of mannose linked by β-1,4-glycosidic bonds, and is found in variable amounts in all cell walls. In mannans and galactomannans, the backbone contains only mannose, variously substituted with galactose units. Glucomannans have both mannose and glucose in their backbone, linked by β-1,4-bonds in nonrepeating patterns (Fig. 1e).

3 Carbohydrate-active enzymes

Lignocellulosic biomass is the most abundant biomass on Earth, and its complex structure makes it highly resistant to microbial attack. The structure of carbohydrates is enormously varied in nature, with one reducing hexameric sugar yielding 1012 possible linear and branched isomers (Laine, 1994). However, without recycling of fixated carbon within the carbohydrates of plant cell walls, heterotrophic organisms would not be able to acquire energy. Therefore, Nature has evolved a vast array of tools to overcome the complexity of carbohydrate breakdown.

Enzymes and accessory proteins that act on carbohydrates for both assembly and breakdown of polysaccharides are collectively designated

carbohydrate-active enzymes, otherwise referred to as CAZymes (Lombard et al., 2014). In 1999, the CAZy database (www.cazy.org) was launched to act as a central repository for CAZyme information, including sequence, 3D structures and biochemical data. The database currently holds protein families divided in the six classes based on their mode of action: glycoside hydrolases (GHs), carbohydrate esterases (CEs) and polysaccharide lyases (PLs) for deconstruction; glycosyltransferases (GTs) for synthesis; carbohydrate-binding modules (CBMs) that help targeting enzymes to their substrates; and auxiliary activity (AA) enzymes that cover redox-enzymes that act in concert with other CAZymes.

CAZymes are classified into families based on their amino acid sequence, which in turn reflects their three-dimensional structure and fold (Henrissat, 1991; Cantarel et al., 2009). As the number of carbohydrate substrates greatly exceeds the number of folds, the enzymes have evolved from common folds, and thus several enzyme specificities can exist within the same family. Likewise, the same enzyme specificities can be found in different families, exemplifying convergent evolution. CAZymes are often multi-modular and can contain several domains from different families, which allows one protein sequence to be classified into several families. The rest of this section will provide an overview of the functions of the various CAZyme classes.

3.1 Glycoside hydrolases

The largest class in terms of both the number of sequences and the number of families in the CAZy database is the class of GHs, with 166 families and over 700 000 sequences, reflecting the enormous variation in available carbohydrate substrates (Lombard et al., 2014). GHs catalyse the hydrolysis of glycosidic bonds, and the reaction can occur with two different mechanisms, where the anomeric configuration of the glycosidic bond is either retained or inverted (Koshland, 1953). The reaction is catalysed by two conserved amino acid residues in the enzyme, normally glutamic acid or aspartic acid (McCarter and Withers, 1994). These act as a general acid (proton donor) and a base, and the spatial position of their side chains correlates with the type of mechanism.

GHs have a wide variety of specificities, attacking the backbone of linear polysaccharides, targeting crystalline substrates or acting as debranching enzymes attacking only specific substitutions of a particular hemicellulose. GHs acting on polymers can be either endo- or exo-acting, referring to whether the enzyme attacks glycosidic bonds within the polysaccharide or at the chain-ends, respectively. Often, exo-acting enzymes processively perform several hydrolytic events without dissociation from their substrate, and they have specificities towards either the reducing- or non-reducing end of the polysaccharide (Davies and Henrissat, 1995; Barr et al., 1996). In cellulose

degradation, endo-cellulases (currently found in 14 GH families, but typically belonging to GH5, GH9 or GH45) and exo-cellulases (typically GH6, GH7, GH48, but some GH9s have been reported as processive endo-cellulases, releasing cellobiose) work synergistically to degrade the crystalline substrate (Wood and McCrae, 1979; Kostylev and Wilson, 2012). In this process, endo-cellulases cleave cellulose chains internally in amorphous regions, creating chain-ends for the processive exo-cellulases (cellobiohydrolases) that release cellobiose.

3.2 Carbohydrate-binding modules (CBMs)

Carbohydrate-binding modules (CBMs) are non-catalytic modules of CAZymes that help target the enzyme towards a carbohydrate (Boraston et al., 2004). The first characterized CBMs were cellulose-binding modules, found to facilitate binding to cellulose by *Trichoderma reesei* cellobiohydrolase I and II, and in two cellulases from *Cellulomonas fini* (Van Tilbeurgh et al., 1986; Tomme et al., 1988; Gilkes et al., 1988). Subsequently, the carbohydrate targets of CBMs have been shown to cover almost all known carbohydrates, including cellulose and hemicelluloses found in the plant cell wall (McCartney et al., 2004; Lombard et al., 2014). CBMs are classified into families based on their amino acid sequence, with 84 different families to date, and can be divided into three functional classes (Gilbert et al., 2013). Type A targets surfaces of crystalline polysaccharides, type B targets sites internally on carbohydrate chains and type C targets the termini of glycan chains.

CBMs are thought to contribute to the efficiency of the appended catalytic domain by increasing the concentration of the enzyme near the substrate (McCartney et al., 2004). By keeping the catalytic domain in closer proximity to its substrate, there is a greater probability for catalytic events to occur. This is more important in low substrate concentration conditions, as demonstrated by the observation that the presence of a cellulose-binding CBM in a cellulase had a lesser, or even negative effect on enzyme efficiency at high substrate concentrations (Várnai et al., 2013). Even though the CBM normally targets the substrate of the appended catalytic domain, there are examples of CBMs that bind to differing substrates enabling the catalysis of target polysaccharides located in the proximity of the bound glycan (Hervé et al., 2010).

3.3 Other CAZyme families

The AA group encompasses laccases, cellobiose dehydrogenases, copper radical oxygenases and various enzymes utilizing oxidative mechanisms on carbohydrates (Lombard et al., 2014). Lytic polysaccharide monooxygenases (LPMOs) were previously thought to be CBMs that helped disrupt the crystalline

structure of chitin, but the *Serratia marcescens* Cbp21 enzyme was shown to introduce chain-breaks, generating oxidized chain-ends on the crystalline chitin surface (Vaaje-Kolstad et al., 2010). Subsequent discovery of cellulose-active LPMOs from bacteria and fungi spurred the need for establishing a separate class in the CAZy database, namely the auxiliary activities (Forsberg et al., 2011; Quinlan et al., 2011; Levasseur et al., 2013). LPMOs are now classified into AA families 9, 10, 11 and 13, out of 13 AA families in total. These enzymes are currently receiving massive attention because they boost the activities of classical GHs and thus contribute to the overall efficiency of enzyme cocktails. Importantly, LPMOs may be crucial in solving the accessibility challenge discussed previously, because they are capable of breaking glycosidic bonds that are in a crystalline context, thus generating access for classical GHs. Despite a variety of rumen isolate genomes encoding predicted AA10 representatives (Seshadri et al., 2018), LPMO activity has not been detected in the rumen, and thus far has only been reported in aerobic microorganisms.

CEs function as debranching enzymes, and de-O- or de-N-acylate ester-based modifications of complex polysaccharides. In removing these ester-based modifications, they allow GHs easier access to their targets in complex polysaccharides (Cantarel et al., 2009). PLs utilize β-elimination to cleave the glycosidic bonds of uronic acid-containing polysaccharides. This leaves the sugar on the new non-reducing end unsaturated with a double bond between C4 and C5, while the new reducing end is saturated (Garron and Cygler, 2010). As the only anabolic members of the CAZy database, glycosyl transferases utilize sugar phosphates to form glycosidic linkages between the 'activated' sugar and other saccharides, lipids or proteins (Lairson et al., 2008). The glycosyl group is transferred to a nucleophilic group on the substrate in a retaining or inverting fashion.

4 Prokaryotic strategies for fibre digestion in the rumen

Decades of research on various digestive ecosystems (i.e. soil, marine, host-associated) have shown that all saccharolytic microbes rely on the actions of CAZymes; however, how these CAZymes are employed by their microbial host can vary considerably. In this section, we will describe the various mechanisms employed by the bacteria and anaerobic fungi of the rumen.

4.1 Cellulosomes

The cellulosome is the best-known cellulolytic mechanism and is one of the two main paradigms of microbial cellulose degradation, along with secreted free enzymes by aerobic fungi (Wilson, 2011). The cellulosome organization of cellulases is found in several anaerobic bacteria and anaerobic fungi, but

was first described in *Clostridium thermocellum*, an anaerobic thermophilic soil bacterium (Lamed et al., 1983; Bayer et al., 2008). The cellulosome is a multi-modular enzyme complex that enables the cell to adhere to crystalline cellulose, which is degraded by cellulases in the ultrastructure (Bayer and Lamed, 1986). The endo- and exo-cellulases along with hemicellulases of the cellulosome contain dockerin domains in addition to their catalytic domains, which facilitate docking to cohesin domains on the large non-catalytic scaffoldin subunit (Bayer et al., 1994; Yaron et al., 1995). The primary scaffoldin subunit of *C. thermocellum* (cipA) contains nine cohesin domains for the binding of dockerin-linked enzyme subunits, a CBM3 module that binds to crystalline cellulose, and a C-terminal dockerin domain that binds to type II cohesin in the cell-wall anchoring scaffoldins. The anchoring scaffoldins contain S-layer homology (SLH) domains that fix the cellulosome to the cell surface, enabling the cell to be in close proximity to the solubilized cellodextrins released by the cellulases (Bayer et al., 2008). Three different anchoring scaffoldins allow up to 63 different cellulosome components to be attached in a single complex in *C. thermocellum*. The modular structure of the cellulosome brings the endo- and exo-cellulases close together and binds them to the substrate, allowing for synergy in cellulose degradation (Krauss et al., 2012).

C. thermocellum is not found in ruminal environments; however, the known cellulose degraders *R. flavefaciens* and *R. albus* partly utilize cellulosomes to contribute to the plant biomass degradation of the rumen (Ohara et al., 2000; Ding et al., 2001). These differ from *C. thermocellum* in their modular composition of the cellulosome, where *R. flavefaciens* has a particularly elaborate system encoding a large amount of dockerin-encoding proteins, including novel CBMs (Dassa et al., 2014; Venditto et al., 2016). The cellulosomal proteins from *R. flavefaciens* have been shown to vary within strains, with genome analysis demonstrating that the number of dockerins can vary between 53 and 223 (Seshadri et al., 2018). Furthermore, the cohesin-dockerin interactions are for the most part strain specific (Israeli-Ruimy et al., 2017), while it has been demonstrated that up to 14 enzyme subunits from the *R. flavefaciens* strain FD-1 cellulosome are assembled across four distinct scaffoldins (Fig. 2a). In contrast to *R. flavefaciens*, *R. albus* contains a lower abundance of dockerin-encoding genes, and two of three sequenced strains contained only one cohesin-encoding gene, whereas the third contained no cohesin counterparts. This suggests the existence of a so-far undiscovered type of scaffoldin with a novel cohesin-like domain, or that the cellulolytic bacterium does not utilize a 'full' cellulosome mechanism (Dassa et al., 2014).

The cellulosomes found in anaerobic fungal genomes greatly differ from those found in anaerobic bacteria (Haitjema et al., 2017). Orthologues of a large scaffoldin protein (ScaA) with no sequence similarity to bacterial scaffoldins were found in all five sequenced genomes of anaerobic fungi. Interestingly,

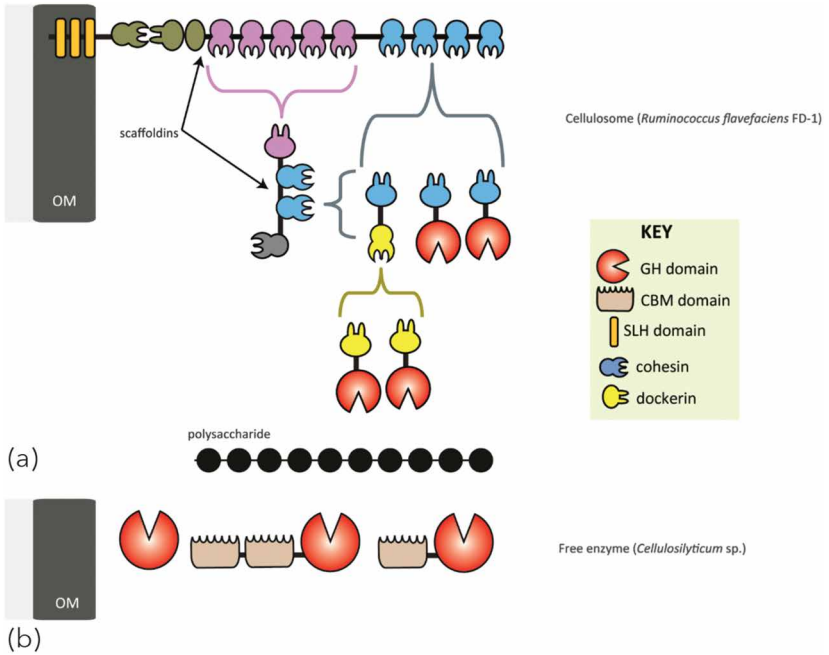

Figure 2 Schematic representation of cellulosome (a) and free-enzyme (b) strategies that are employed by rumen microorganisms. A specific example of the cellulosome characterized from *Ruminococcus flavefaciens* strain FD-1 is illustrated in (a). OM: outer membrane, GH: glycoside hydrolase, CBM: carbohydrate-binding module, SLH: S-layer homology.

the dockerin domains from three genera of gut fungi were able to bind to all combinations of ScaA fragments, containing cohesin-motifs. The authors, therefore, speculate that in their native environment, fungal cellulosomes may actually exist as composites of enzymes from different fungal species, unlike bacterial dockerin-cohesin interactions which are highly species-specific.

4.2 Secreted enzymes

The second most renowned paradigm of microbial cellulose degradation is that of secreted free cellulases, primarily in aerobic fungi and bacteria (Fig. 2b). This mechanism has been well studied in the mesophilic filamentous fungus *Trichoderma reesei*, which was originally isolated from rotting US Army equipment in the Solomon Islands during the Second World War, and is the dominant industrial cellulase-producing organism (Bischof et al., 2016; Reese, 1956).

T. reesei secretes large amounts of cellulase enzymes to the culture broth when grown on cellulose, enabling rapid degradation of cellulose to

glucose (Sheir-Neiss and Montenecourt, 1984). The secreted enzymes include endoglucanases (EG, Cel5A, Cel5B, Cel12A, Cel45A), non-reducing- and reducing end cellobiohydrolases (CBHI/GH6 and CBHII/Cel7A, respectively), β-glucosidase I (GH3) and AA9 LPMOs (formerly GH61; Saloheimo et al., 1997; Wilson, 2009; Westereng et al., 2011; Li et al., 2016). All these enzymes work in concert, where endoglucanases (with or without CBM1 domains) attack β-1,4 glycosidic bonds in the amorphous regions of cellulose, creating chain-ends for the processive CBHs that attack from both ends of the chains, disrupting the crystalline structure. Cellobiose released from the CBHs is degraded to glucose monomers which are taken up by the cell. The AA9 type LPMOs introduce oxidative breaks in the crystalline region of cellulose, creating more chain-ends for CBH, possibly acquiring reducing power from non-enzymatic donors such as lignin (Westereng et al., 2015). Other fungal cellulase systems utilize secreted enzymes as well, but these have not been studied to the same extent (Wilson, 2008). Several bacteria also use the secreted free-enzyme mechanism for cellulose degradation, in a similar manner to the fungi (Wilson, 2011). *Thermobifida fusca* is one well-studied example, utilizing GH5, GH6 and GH9 endo-cellulases, GH6 and GH48 exo-cellulases containing CBM2 cellulose-binding domains, and two AA10 type LPMOs (Gomez Del Pulgar and Saadeddin, 2013; Forsberg et al., 2014).

The role of secreted free enzymes in the rumen of herbivore is not well understood. For example, based on sequence prediction, it is believed that *R. flavefaciens* and rumen fungi also utilize secreted enzymes in addition to cellulosomes (Dassa et al., 2014). Several cellulose-degrading Firmicutes are predicted to use secreted exo- (GH48) and endo-cellulases (GH5, GH9) such as selected species affiliated to the *Lachnoclostrium*, *Cellulosilyticum* (Cai et al., 2010), *Ruminoclostridium* and *Ruminococcus* genera (Seshadri et al., 2018). Similarly, *Cellulomonas* sp. affiliated to the Actinobacteria phylum is also predicted to degrade cellulose via the actions of free exo- and endo-cellulases. Many populations are also predicted to degrade hemicellulose and starch using free enzymes such as the predominant rumen microbe *Butyrivibrio fibrisolvens* (Seshadri et al., 2018).

4.3 Polysaccharide utilization loci

A large fraction of the bacteria-colonizing gut microbial ecosystems are affiliated to the Bacteroidetes phylum (Tajima et al., 1999; Hold et al., 2002; Flint et al., 2008). These gram-negative bacteria are known to have broad carbohydrate degradation capabilities derived from numerous gene clusters termed PULs, first characterized in the human gut bacterium *Bacteroidetes thetaiotaomicron* (Bjursell et al., 2006; Martens et al., 2008). *B. thetaiotaomicron* encodes 101 susC/D pairs, enabling it to grow on a wide array of polysaccharides and

host-associated mucins. Moreover, transcriptomic analysis has demonstrated up-regulation of PULs specific to available substrates (Martens et al., 2011). Not limited to human gut bacteria, PULs have been shown to be prevalent in Bacteroidetes found in environmental and herbivorous gut microbiomes (including ruminants; McBride et al., 2009; Pope et al., 2010, 2012; Naas et al., 2014; Dodd et al., 2010; Terrapon et al., 2015; Accetto and Avguštin, 2015; Mackenzie et al., 2015; Güllert et al., 2016; Rosewarne et al., 2014). PULs typically target starches, hemicelluloses and pectins, and are encoded by a large number of the Prevotellaceae in the cow rumen (Stewart et al., 2018b; Solden et al., 2018), where they likely contribute to the liberation of cellulose from the hemicellulose matrix. In particular, a xylan-degrading PUL from *Prevotella bryantii* was one of the first to be characterized in detail outside of the human gut, using both culture- and omic-based methodologies (Dodd et al., 2010).

PULs are defined by the co-occurrence of SusC- and SusD-like genes, along with GHs and sugar transporters (Fig. 3a). SusC- and SusD-like proteins are named after their first description in the starch utilization system (Sus) (Reeves et al., 1996, 1997). The sus gene cluster contains eight genes, named *susRABCDEFG*, and contains all the enzymes required for the cell to degrade and import starch (Foley et al., 2016). SusR, an inner membrane trans-membrane protein, is the transcriptional regulator of the gene cluster, and can recognize

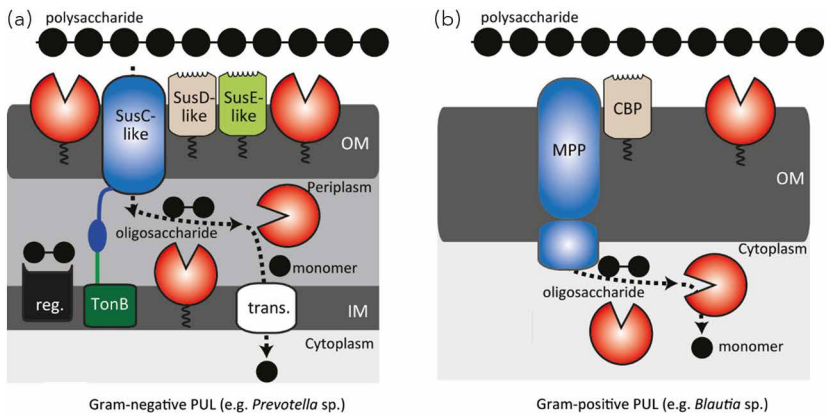

Figure 3 Schematic representation of polysaccharide utilization loci (PULs) that are found in gram-negative (a) and gram-positive (b) rumen microorganisms. Gram-negative PULs are typified by the genomic co-localization of SusC- and SusD-like lipoproteins and glycoside hydrolases, whereas in gram-positive microbiota, a carbohydrate-binding protein (CBP) and permeases (MPP) of an ABC transporter are encoded. Both strategies include extracellular (membrane attached) and intracellular CAZymes to take complex polysaccharides to monomeric sugars. OM: outer membrane, IM: inner membrane, reg.: transcriptional regulator, trans.: sugar transporter.

the starch degradation product maltose (D'Elia and Salyers, 1996b). This up-regulates the expression of the rest of the gene cluster, enabling the cell to respond to the presence of starch. The outer-membrane lipoproteins SusD, E and F facilitate binding of starch to the cell surface, bringing the outer-membrane lipo-anchored α-amylase SusG in close proximity of its substrate (Shipman et al., 1999, 2000; Koropatkin and Smith, 2010). SusD is essential for growth on maltooligosaccharides greater than DP4, and also greatly enhances the sensing of maltose compared to mutants where its single starch binding domain was disrupted (Cameron et al., 2014). The binding sites and expression of SusE and SusF were dispensable for activation of transcription, but enhanced the growth rate in a substrate-dependent manner. Maltooligosaccharides released by SusG are imported through the TonB-dependent outer-membrane porin SusC into the periplasmic space, where they are further hydrolysed by SusB α-glucosidase and SusA neopullunase (D'Elia and Salyers, 1996a; Reeves et al., 1996). The resulting glucose is further transported into the cell for fermentation.

Individual PULs are varied in the number of co-located CAZymes, which is correlated to the complexity of their predicted substrate. They can range from two enzymes in the predicted pectic galactan PUL of *B. thetaiotaomicron*, to 32 in the pectic rhamnogalacturonan II PUL (Cameron et al., 2012). Several PULs have been studied extensively through biochemical analyses of individual genes along with growth experiments, revealing complex enzyme interplays with sequential polysaccharide deconstruction activities on substrates including fructan, porphyran, xyloglucan, xylan and α-mannan (Sonnenburg and Zheng, 2010; Hehemann et al., 2012; Larsbrink et al., 2014; Cuskin et al., 2015; Rogowski et al., 2015). PULs usually contain enzymes to target one specific polysaccharide, but CAZymes characterized from a PUL encoded within an uncultured reindeer rumen Bacteroidetes species was shown to degrade mannans, xylans, xyloglucan and β-glucans (Mackenzie et al., 2015).

Until recently, Bacteroidetes PULs were thought to only target soluble glycans (Koropatkin et al., 2012). However, the soil Bacteroidetes *Flavobacterium johnsoniae* can degrade the crystalline cellulose homologue chitin efficiently via a PUL in cooperation with a secreted multi-modular chitinase (McBride et al., 2009; Larsbrink et al., 2016). In addition, PULs have also been linked with putative cellulases in several herbivore gut metagenomes (Pope et al., 2010, 2012; Dai et al., 2012; Naas et al., 2014); however, no isolated Bacteroidetes representative has been shown to degrade crystalline cellulose via the actions of a PUL.

4.4 PULs are in the gram positives as well

While gram-negative bacteria, in particular the Bacteroidetes, have long been associated with PULs, recent studies in Firmicutes have shown that similar

cell-wall enveloped plant biomass-degrading strategies exist in gram positives as well (La Rosa et al., 2019; Fig. 3b). Detailed genomic, transcriptomic and proteomic analysis of PULs from human gut *Roseburia* species illustrated that multi-gene loci encode and express the necessary cell-wall anchored CAZymes, binding proteins, ABC transporters, transcriptional regulators and cytoplasmic oligosaccharide-degrading CAZymes to convert complex hemicellulosic substrates into monomeric sugars. Moreover, detailed biochemical characterization of each component of the aforementioned PUL validated each of their predicted metabolic functions (La Rosa et al., 2019). To the best of our knowledge, such detailed examples of elucidated gram-positive PULs have not been described in ruminant microorganisms; however, perusal of the Hungate1000 genome collection quickly identifies potential examples of gram-positive PULs in various phyla including the Firmicutes (e.g. *Blautia schinkii* DSM 10518) and Actinobacteria (e.g. *Bifidobacterium longum* AGR2137; Seshadri et al., 2018).

4.5 Fibrobacter succinogenes *and outer-membrane vesicles (OMVs)*

One of the classically recognized cellulose-degrading species from the cow rumen, *Fibrobacter succinogenes*, utilizes a cellulolytic mechanism that does not conform to the classical views of cellulose degradation (Suen et al., 2011). It is regarded as one of the key fibrolytic populations in the rumen, and has demonstrated a greater ability to digest cellulose from forages than other species of rumen bacteria (Dehority and Scott, 1967). Despite this, its genome is devoid of the cellulosome components dockerin and cohesins, and it does not appear to encode any exo-cellulases, known to be required for both the secreted free enzymes and cellulosome mechanisms. *F. succinogenes* is equipped with a surprisingly high diversity of CAZymes and abilities, considering that the bacterium only utilizes cellulose as a carbon source. The 31 encoded endo-cellulases of *F. succinogenes* also do not contain CBMs associated with binding to cellulose, further discrediting secreted enzymes as a possible mechanism.

Several models of its cellulose degradation have been proposed, and some studies point towards cell attachment to cellulose using fibro-slime proteins and pili, bringing the substrate close to outer-membrane-bound endo-cellulases (Burnet et al., 2015). The bacterium also produces outer-membrane vesicles (OMVs) containing CAZymes, which are released from the cell to target plant biomass (Arntzen et al., 2017; Fig. 4a). OMVs have been observed in a wide range of gram-negative species found in various ecosystems (Kulp and Kuehn, 2010), and have been suggested to play wide-ranging roles including horizontal gene transfer, biofilm formation, communication and biomolecule delivery (Elhenawy et al., 2014; Roier et al., 2016). OMVs from *F. succinogenes*

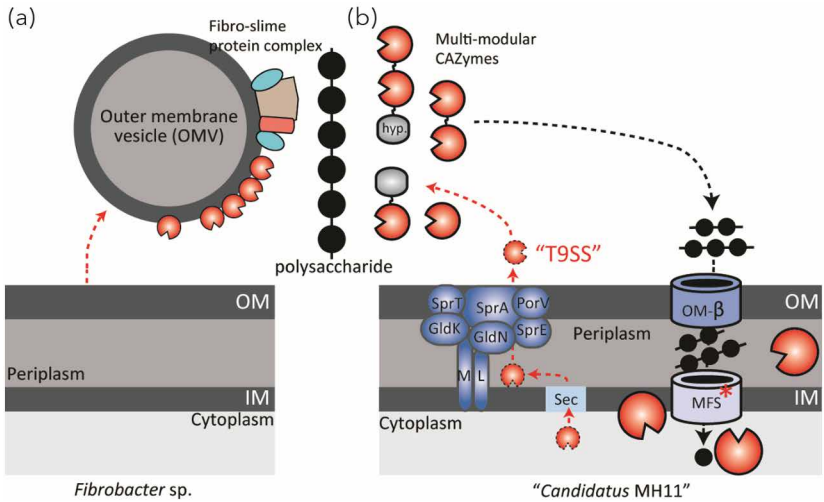

Figure 4 Hypothetical strategies predicted to occur in *Fibrobacter succinogenes* (a) and as-yet uncultured members of the Bacteroidetes family '*Candidatus* MH11' (b). *F. succinogenes* produces outer-membrane vesicles that contain multiple CAZymes and a fibro-slime protein complex that targets various cellulose, hemicellulose and pectin substrates. The Ca. MH11-affiliated '*Candidatus* Paraporphyromonas polyenzymogenes' encodes components of the Type IX secretion system (T9SS), which is predicted to facilitate secretion of multi-modular CAZymes. Whether or not TPSS CAZymes in Ca. MH11-affiliated populations are free or cell-wall attached is not known. OM: outer membrane, IM: inner membrane.

were found to contain fibro-slime proteins, cellulases and hemicellulases, and were able to degrade cellulose, pectin and hemicelluloses. The role of these OMVs in fibre digestion is debated, with studies claiming their production is due ageing of the cells (Gaudet and Gaillard, 1987). However, there is support they may have a biological role in cellulose degradation (Forsberg et al., 1981), with pre-treatment of switchgrass using the *F. succinogenes* OMVs showing a 2.4-fold increase in subsequent saccharification by a commercial cellulase cocktail (Arntzen et al., 2017). It has been illustrated that other fibre-digesting *Bacteroidetes* species from the human gut also produce fibrolytic OMVs (Elhenawy et al., 2014), and it is hypothesized that *F. succinogenes* OMVs could facilitate increased accessibility for the cell to cellulosic substrates by disrupting the complex structure of the lignocellulose matrix (Arntzen et al., 2017).

4.6 Bacteroidetes, the type IX secretion system and multi-modular CAZymes

A different non-classical mechanism is predicted to be utilized by deeply branched, as-yet uncultured members of the Bacteroidales order that are

inherent to the rumen (Naas et al., 2018). A novel family referred to as '*Candidatus* MH11' consists of populations that do not encode cellulosomes or PUL-like systems, and instead utilize multi-modular cellulases and hemicellulases that are secreted via the type IX secretion system (T9SS). The T9SS is essential for crystalline substrate degradation in both soil Bacteroidetes *Cytophaga hutchinsonii* (cellulose) and *F. johnsoniae* (chitin), which utilize T9SS secreted large cellulases and multi-modular chitinases, respectively. The importance of the T9SS for fibre digestion was first described as part of the aerobic gliding mechanism in *C. hutchinsonii*, which is cellulolytic but lacks dockerin and cohesin modules, as well as predicted exo-cellulases (Xie et al., 2007). The cells attach to, and move across, cellulose fibres utilizing its gliding motility, which is speculated to aid the cellulose deconstruction by locating more easily accessible substrate. The gliding motility of *Bacteroidetes* has been well studied in the distant relative *F. johnsoniae* and has been functionally linked to both crystalline substrate degradation and secretion through the T9SS (Sato et al., 2010; Ji et al., 2012; Zhu and McBride, 2014; McBride and Nakane, 2015).

In addition to the T9SS being important, multi-modular CAZymes have recently been shown to be widespread in bacterial genomes and many putatively target cellulose and the cellulose homologue chitin (Talamantes et al., 2016). For example, a multi-modular cellulase (*Cb*CelA: GH9/CBM3c/CBM3b/CBM3b/GH48) secreted by the thermophilic *Caldicellulosiruptor* species is predicted to play an important part in cellulose degradation due to its putative endo/exo synergistic activity, and its deletion was shown to greatly reduce growth on cellulose (Yi et al., 2013; Young et al., 2014). A study by Brunecky et. al. showed that purified *Cb*CelA from culture supernatant could outperform mixtures of commercial exo- and endo-cellulases, likely due to its inter-domain synergy (Brunecky et al., 2013). Within the Bacteroidetes phylum, the main chitinase of the chitin utilization locus (ChiUL) of *F. johnsoniae, Fj*ChiA, is similar to *Cb*CelA in that it has a flanking endo-/exo-acting pair of GH18 chitinases, with chitin/cellulose-binding domains in the middle region (Larsbrink et al., 2016). The high activities of these two similar enzymes on recalcitrant substrates suggest that multi-modularity in GHs is a well-functioning strategy that could possibly be found in other biomass-degrading organisms. *Fj*ChiA has also been shown to be secreted via the T9SS, whereas gene knock-out mutagenesis has demonstrated that the enzyme is vital for chitin metabolism and cell growth (Kharade and McBride, 2014).

Within the cow rumen, the Ca. MH11-affiliated, provisionally named '*Candidatus* Paraporphyromonas polyenzymogenes' is predicted to have cellulose-degrading capabilities, with over 100 CAZyme domains including 17 putative endo-cellulases and members of GH3 β-glucosidases and GH94 cellobiose phosphorylases (Naas et al., 2018). Notably, many of the encoded CAZymes were multi-modular and found to include a specific C-terminal domain

(CTD) known to target proteins for secretion through the T9SS (Fig. 4b). Similar cellulase-gene organizations were found in the six other representatives in Ca. MH11 affiliates, all native to the sheep rumen, suggesting that T9SS-secreted multi-modular enzymes play a part in ruminal cellulose and hemicellulose degradation. Moreover, biochemical and structural analysis of selected Ca. P. polyenzymogenes CAZymes has further supported the predicted cellulolytic phenotype of this hitherto uncultured bacterium, and has demonstrated activity towards linear polymers, such as amorphous and crystalline cellulose as well as mixed linkage β-glucans (Naas et al., 2018).

5 Current gaps in knowledge

5.1 The eukaryotes

While prokaryotes numerically dominate the rumen microbiome, it is also widely acknowledged that eukaryotic populations make important contributions towards fibre digestion. A detailed description regarding eukaryotic populations and their contributions to rumen function are outlined in detail within Parts 2.8 (Ruminal ciliated protozoa) and 2.9 (Ruminal anaerobic fungi). Rumen protozoa make up approximately 20% of the microbial biomass within the rumen due to their comparably large cell volume (Huws et al., 2018). Anaerobic rumen fungi were first reported back in the 1970s (Bauchop, 1979; Orpin, 1975), and have long been known to harness CAZymes to deconstruct the plant cell walls of ingested grasses (Borneman et al., 1989). However, for both rumen protozoa and fungi, there remains a dearth of genomic information, largely due to the difficultly with growing them axenically, and the ability to purify, sequence and annotate the genomes of both cultured and uncultured representatives. The first draft macronuclear genome sequence of a ruminal protozoa (*Entodinium caudatum* MZG-1) was released in 2018 (Park et al., 2018), and as of writing only representative fungal genomes from the genera *Anaeromyces, Neocallimastix, Orpinomyces* and *Piromyces* are available (Haitjema et al., 2017). New developments in culture-independent metagenomic data generation and the bioinformatic processing of this data are creating new possibilities to reconstruct representative genomes from uncultured fungal and protozoal populations. For example, long-read sequencing technologies (Oxford Nanopore, PacBio) are improving the assembly of eukaryotic genomes (Díaz-Viraqué et al., 2019), whereas genome binning software (EukRep) has recently been designed to enable reconstruction of eukaryotic genomes from complex microbial communities (West et al., 2018). Looking closer at eukaryotic function, the exact mechanisms by which rumen protozoa and fungi degrade plant fibre have not been detailed in the same manner as the rumen prokaryotes. Protozoa are believed to use multi-modular cellulases and hemicellulases,

whilst cellulosomes with similar structure to those produced by bacteria have been observed in rumen fungi (Steenbakkers et al., 2001; Fanutti et al., 1995; Ljungdahl, 2009), albeit with dockerin domains that share no sequence homology (Haitjema et al., 2017).

5.2 How much CAZyme and glycan diversity is there in the rumen?

Plant glycans present within animal feed are often broadly characterized as cellulose, hemicellulose, starch and/pectin. However, within several of these categories (i.e. hemicelluloses, pectins), there exists an extraordinary diversity of glycan structures, many of which remain unchartered. At a higher compositional level, common feed sources for pen-fed ruminants such as grasses, grains and legumes are dominated by cellulose, which accounts for between 15% and 50% depending on the plant cellular location (primary, secondary, etc.). Matrix polysaccharides, such as hemicellulose and pectin, are more structurally complex, and can be branched and/or substituted with methyl esterifications or acetylations. Grasses (and grains) are more commonly dominated by xylans (20–40%) and mixed linkage glucans (10–30%), whereas many dicots (such as legumes) contain higher levels of xyloglucan (20–25%), mannans (5–10%) and pectins (20–35%; Mertens, 2003; Vogel, 2008; Pattathil et al., 2015). In grazing animals, our understanding of the dietary fibre composition is much more deficient; however, plant microarray methods that attempt to estimate and/or map the 'carbon landscape' within a given plant sample have recently been used to characterize the diets of wild ruminants such as moose and reindeer (Mackenzie et al., 2015; Solden et al., 2018). These methods, which rely on monoclonal antibodies or CBMs (Moller et al., 2007), have reaffirmed that complex fibres dominate their cosmopolitan diets, and include varyingly structured mannans, xylans, xyloglucans, galactans, arabinans and pectins (Mackenzie et al., 2015; Solden et al., 2018). While many diverse fibres have been identified using these and other methods (Voiniciuc et al., 2018; Wood et al., 2017), many uncharacterized structural layers still exist within ruminant diets, requiring greater efforts to map this inherent glycan diversity.

Indigenous fibre degraders in the rumen have evolved to match this glycan diversity with an equally enormous inventory of CAZymes, which includes endo- and exo-acting enzymes that deconstruct the polysaccharide backbone as well as the auxiliary enzymes (CEs, PLs and other debranching enzymes). Just looking at the CAZyme profile of 964 available Bacteroidetes genomes alone (predominated by the human gut), over 13 500 PULs have been grouped and used to estimate that there exist approximately several thousand enzyme combinations for the breakdown of the various glycan structures found in nature (Lapébie et al., 2019). Analysis of individual rumen populations, such as *R. flavefaciens*, has illustrated an extraordinary

level of glycan recognition (Venditto et al., 2016); however, community-wide estimations have not yet been calculated for the hundreds of rumen fibrolytic species that exist, many of which are uncultured. It is, thus, apparent that much cataloguing of both glycan and CAZyme diversity remains for rumen microbiologists.

5.3 What do functional omic studies tell us?

Coinciding with rapidly improving DNA-based sequencing and bioinformatics techniques that are enabling rumen microbiologists to create thousands of microbial genomes from the rumen, functional RNA- and peptide-based expression studies have also experienced a transformation. Several chip-based microarrays have been developed over the years to quantify transcript levels from both bacterial as well as protozoan and fungal populations (Abot et al., 2016; Comtet-Marre et al., 2018). Overall, these methods have illustrated the high expression values of various cellulases and hemicellulases from both prokaryotic and eukaryotic origin, although the design of such chips on previously available CAZyme data prevents the detection of specific activities from individual populations that have not yet been genomically sampled.

Quantitative metatranscriptomic methods have been used to analyse gene expression patterns across the entire rumen microbiome, and have shown that similar taxa (Prevotellaceae, Succinivibrionaceae and Fibrobacteraceae) and CAZymes are prevalent in multiple studies (Söllinger et al., 2018; Comtet-Marre et al., 2017). Moreover, these studies have highlighted that anaerobic fungi and ciliates contribute an unexpectedly large share of transcripts for cellulose- and hemicellulose-degrading enzymes (Söllinger et al., 2018; Comtet-Marre et al., 2017; Qi et al., 2011). New multi-omic approaches that combine large metagenomic and metatranscriptomic or metaproteomic datasets are further improving resolution to specifically identify individual populations and the mechanisms they use to actively degrade fibre (Solden et al., 2018). For example, we used multi-omics to show that Ca. P. polyenzymogenes was detectable in metaproteomic data and was enriched in samples recovered from rumen-incubated plant biomass, thus indicating that active digestion of complex carbohydrates could be assigned to members of the novel Ca. MH11 family, which uses a non-conventional T9SS-based saccharolytic mechanism (Fig. 4b; Naas et al., 2018). Multi-omics have also reiterated that Bacteroidetes-affiliated PULs are critical for rumen fibre digestion, with genome-centric metaproteomic data recovered from Alaskan moose revealing greater than 90% of the detected CAZymes were expressed from PULs (Solden et al., 2018). While these initial studies illustrate that such methods can be used to create a deeper understanding regarding microbial plant fibre degradation, they have thus far only observed a fraction of the larger community dynamics that are in

play, which is constantly varying in response to time (i.e. such as before and after eating), as well as individuality factors of the animal host.

6 Improving the fibre digestion process

The rumen microbiome has long been viewed as a potential target for manipulation to increase fibre digestion, improve animal productivity and well-being, as well as to reduce methane emissions. For the most part, attempts to directly target specific fibrolytic populations via supplementation of actual live cultures (Chiquette et al., 2007; Præsteng et al., 2013; Krause et al., 2001), chemical agents (Chalupa, 1977) or exogenous CAZymes (Beauchemin et al., 2003) to boost ruminal fibre digestion have been unsuccessful (Moraïs and Mizrahi, 2019). While diet has long been assumed to play the main driver in shaping the gut microbiota of bilaterians (Spor et al., 2011), and especially for ruminants (Henderson et al., 2015), new evidence is validating that host genetics is also important. It has been highlighted that individual variation of rumen microbiota exists in both beef (Li and Guan, 2017) and dairy cattle (Jami and Mizrahi, 2012), even when animals were fed the same diet and managed under the same environment. More recently, genome-wide association studies (GWAS) have identified heritable rumen bacteria (Sasson et al., 2017; Li et al., 2019), and it has also been demonstrated that genetic variation in cows can lead to differences in microbial gene/taxa abundance, host feed efficiency and methane production (Roehe et al., 2016; Difford et al., 2018). Moreover, Li et al. (2019) recently showed that heritable microbiota are additionally associated with single-nucleotide polymorphisms located in the bovine genome that are known quantitative trait loci for feed efficiency in cattle, further highlighting that perhaps breeding strategies can be used to manipulate or select for beneficial microbiota.

This exciting new line of research thus proposes the tantalizing question: could we improve fibre digestion in ruminants by linking host genetics to microbiome function and beyond to specific glycan profiles in their diets? The ultimate idea being that we could theoretically customize diets for specific cattle breeds with specific glycan structures, which would match the enzymatic/mechanistic capabilities of their host-linked microbiota. Thus far, previous ruminant GWAS have yet to elucidate at a profound functional level how the *expressed metabolic enzymes or pathways* within (multiple) microbial populations are linked to host genotypes as well as specific glycan structures. Moreover, the majority of heritable populations identified are assigned to taxa for which no cultured isolate, genome or metabolic information is available. Therefore, we lack a deeper understanding of 'holobiont phenotypes', that is, how important interactions among cow and microbial genomes, and their *expressed* enzymes/metabolic pathways, affect variation in fibre digestion.

Tackling such a challenge has historically been 'out of bounds' both technically and economically; however, today's molecular toolkits are increasing the feasibility to create thousands of rumen microbial genomes (Stewart et al., 2018a; Seshadri et al., 2018), map the glycan structures consumed in animal feed and disentangle extremely complex interactions between feed, the 'gut microbiome' and host genetics. Thus, we hypothesize that it is becoming possible to study the high-dimensional multispecies molecular phenotype of animals and their residential microbiomes, that is, the holobiont. This includes the genomes, whose genes are expressed, and what these genes 'produce' in terms of enzymes and interacting biochemical reactions. Ultimately, such knowledge could be incorporated into commercial feed design and breeding programmes to optimize fibre digestion and animal production in general.

7 Summary and future trends

The symbiotic microbiota of the cow rumen are the backbone of the 'world's largest bioreactor' (Weimer et al., 2009), enabling the animal to efficiently utilize fibre-rich plant material for its energy needs. In a short space of time, advancements in culture-dependent and -independent techniques have rapidly advanced our understanding and appreciation of the genetic and metabolic diversity that exists within the rumen microbiome. Since 2010, our knowledge of the different strategies that rumen microbiota employ for fibre digestion has expanded from free enzymes and cellulosomes to PULs in both gram-negative and -positive populations, OMVs and T9SS-secreted multi-modular enzymes. Furthermore, new resources have recently been released that will enable further development in this discipline. For example, the sequencing and annotation of 410 rumen isolate genomes as part of the Hungate1000 has created an invaluable resource, whereby one can quickly examine the CAZyme profiles of each entry and predict their fibrolytic potential and strategies (Seshadri et al., 2018). Concurrently, in the space of 2 years, metagenomic approaches used to reconstruct population genomes from rumen microbiota have gone from hundreds (Stewart et al., 2018b; Solden et al., 2018) of recovered genomes to thousands (Stewart et al., 2018a), which releases an immense amount of genomic data and CAZymes to be mined. It is highly likely that closer examination of these resources will uncover CAZymes and fibrolytic strategies that vary from those outlined in this chapter.

Despite the potential value of these genomic resources, they do not currently address an elemental shortcoming that rumen microbiology still needs to overcome, which is that our knowledge is built from well-documented bacteria and archaea, whereas virtually nothing is known about the eukaryotic and viral populations. These under-represented facets of rumen microbiology are believed to contribute to digestion and enteric gas formation, but are poorly

understood due to their uncultivability and/or genome complexity. It is hoped that new technologies will help address the eukaryotic/viral challenge, such as long-read sequencing technology (Oxford nanopore (Stewart et al., 2018a)) and specific binning software VirSorter (Roux et al., 2015) and EukRep (West et al., 2018) that target uncultured virus (Emerson et al., 2018) and eukaryotes, respectively. In addition, there needs to be concerted efforts to incorporate axenic isolation and both biochemical and enzymological approaches into future efforts to deconvolute new fibrolytic mechanisms, as without hard biochemical evidence we cannot proceed beyond prediction and elucidate true metabolic function.

8 Where to look for further information

The Rumen microbial genomics (RMG) Network was formed in 2011 and provides a forum for a global collaborative network of researchers using microbial genomics approaches to understand the rumen microbiome (http://www.rmgnetwork.org), including fibre degradation. Conferences that focus on the interface between gastrointestinal microbiology and saccharolytic mechanisms include:

- Rowett-INRA joint symposium on gut microbiology organised by the Rowett Institute, University of Aberdeen and l'Institut National de la Recherche Agronomique (INRA), France.
- The Congress on Gastrointestinal Function (biannually held in Chicago, USA).
- The Gordon Research Conference on Carbohydrate-Active Enzymes for Glycan Conversions (biannually held in USA).
- The Carbohydrate Bioengineering Meeting (biannually held in Europe).

9 References

Abot, A., Arnal, G., Auer, L., Lazuka, A., Labourdette, D., Lamarre, S., Trouilh, L., Laville, E., Lombard, V., Potocki-Veronese, G., Henrissat, B., O'Donohue, M., Hernandez-Raquet, G., Dumon, C. and Leberre, V. A. 2016. CAZyChip: dynamic assessment of exploration of glycoside hydrolases in microbial ecosystems. *BMC Genomics* 17, 671. doi:10.1186/s12864-016-2988-4.

Accetto, T. and Avguštin, G. 2015. Polysaccharide utilization locus and CAZYme genome repertoires reveal diverse ecological adaptation of *Prevotella* species. *Syst. Appl. Microbiol.* 38(7), 453–61. doi:10.1016/j.syapm.2015.07.007.

Arntzen, M. Ø., Várnai, A., Mackie, R. I., Eijsink, V. G. H. and Pope, P. B. 2017. Outer membrane vesicles from *Fibrobacter succinogenes* S85 contain an array of Carbohydrate-Active Enzymes with versatile polysaccharide-degrading capacity. *Environ. Microbiol.* 19(7), 2701–14. doi:10.1111/1462-2920.13770.

Attia, M. A. and Brumer, H. 2016. Recent structural insights into the enzymology of the ubiquitous plant cell wall glycan xyloglucan. *Curr. Opin. Struct. Biol.* 40, 43–53. doi:10.1016/j.sbi.2016.07.005.

Barr, B. K., Hsieh, Y. L., Ganem, B. and Wilson, D. B. 1996. Identification of two functionally different classes of exocellulases. *Biochemistry* 35(2), 586–92. doi:10.1021/bi9520388.

Bauchop, T. 1979. Rumen anaerobic fungi of cattle and sheep. *Appl. Environ. Microbiol.* 38(1), 148–58.

Bayer, E. A. and Lamed, R. 1986. Ultrastructure of the cell surface cellulosome of *Clostridium thermocellum* and its interaction with cellulose. *J. Bacteriol.* 167(3), 828–36. doi:10.1128/jb.167.3.828-836.1986.

Bayer, E. A., Morag, E. and Lamed, R. 1994. The cellulosome – a treasure-trove for biotechnology. *Trends Biotechnol.* 12(9), 379–86. doi:10.1016/0167-7799(94)90039-6.

Bayer, E. A., Lamed, R., White, B. A. and Flint, H. J. 2008. From cellulosomes to cellulosomics. *Chem. Rec.* 8(6), 364–77. doi:10.1002/tcr.20160.

Beauchemin, K. A., Colombatto, D., Morgavi, D. P. and Yang, W. Z. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *J. Anim. Sci.* 81, E37–47.

Bischof, R. H., Ramoni, J. and Seiboth, B. 2016. Cellulases and beyond: the first 70 years of the enzyme producer *Trichoderma reesei*. *Microb. Cell Fact.* 15(1), 106. doi:10.1186/s12934-016-0507-6.

Bjursell, M. K., Martens, E. C. and Gordon, J. I. 2006. Functional genomic and metabolic studies of the adaptations of a prominent adult human gut symbiont, *Bacteroides thetaiotaomicron*, to the suckling period. *J. Biol. Chem.* 281(47), 36269–79. doi:10.1074/jbc.M606509200.

Boraston, A. B., Bolam, D. N., Gilbert, H. J. and Davies, G. J. 2004. Carbohydrate-binding modules: fine-tuning polysaccharide recognition. *Biochem. J.* 382(3), 769–81. doi:10.1042/BJ20040892.

Borneman, W. S., Akin, D. E. and Ljungdahl, L. G. 1989. Fermentation products and plant cell wall-degrading enzymes produced by monocentric and polycentric anaerobic ruminal fungi. *Appl. Environ. Microbiol.* 55(5), 1066–73.

Brown, R. M. 2004. Cellulose Structure and Biosynthesis: what is in Store for the 21st Century? *J. Polym. Sci. A Polym. Chem.* 42(3), 487–95. doi:10.1002/pola.10877.

Brunecky, R., Alahuhta, M., Xu, Q., Donohoe, B. S., Crowley, M. F., Kataeva, I. A., Yang, S. J., Resch, M. G., Adams, M. W., Lunin, V. V., Himmel, M. E. and Bomble, Y. J. 2013. Revealing nature's cellulase diversity: the digestion mechanism of *Caldicellulosiruptor bescii* CelA. *Science* 342(6165), 1513–6. doi:10.1126/science.1244273.

Burnet, M. C., Dohnalkova, A. C., Neumann, A. P., Lipton, M. S., Smith, R. D., Suen, G. and Callister, S. J. 2015. Evaluating models of cellulose degradation by *Fibrobacter succinogenes* S85. *PLoS ONE* 10(12), e0143809. doi:10.1371/journal.pone.0143809.

Cai, S., Li, J., Hu, F. Z., Zhang, K., Luo, Y., Janto, B., Boissy, R., Ehrlich, G. and Dong, X. 2010. Cellulosilyticum ruminicola, a newly described rumen bacterium that possesses redundant fibrolytic-protein-encoding genes and degrades lignocellulose with multiple carbohydrate- borne fibrolytic enzymes. *Appl. Environ. Microbiol.* 76(12), 3818–24. doi:10.1128/AEM.03124-09.

Cameron, E. A., Maynard, M. A., Smith, C. J., Smith, C. J., Koropatkin, N. M. and Martens, E. C. 2012. Multidomain carbohydrate-binding proteins involved in *Bacteroides thetaiotaomicron* starch metabolism. *J. Biol. Chem.* 287(41), 34614–25. doi:10.1074/jbc.M112.397380.

Cameron, E. A., Kwiatkowski, K. J., Lee, B. H., Hamaker, B. R., Koropatkin, N. M. and Martens, E. C. 2014. Multifunctional nutrient-binding proteins adapt human symbiotic bacteria for glycan competition in the gut by separately promoting enhanced sensing and catalysis. *mBio* 5(5), e01441–14. doi:10.1128/mBio.01441-14.

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V. and Henrissat, B. 2009. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic. Acids. Res.* 37, D233–8. doi:10.1093/nar/gkn663.

Chalupa, W. 1977. Manipulating rumen fermentation. *J. Anim. Sci.* 45, 585–99.

Chiquette, J., Talbot, G., Markwell, F., Nili, N. and Forster, R. J. 2007. Repeated ruminal dosing of *Ruminococcus flavefaciens* NJ along with a probiotic mixture in forage or concentrate-fed dairy cows: effect on ruminal fermentation, cellulolytic populations and in sacco digestibility. *Can. J. Anim. Sci.* 87(2), 237–49. doi:10.4141/A06-066.

Comtet-Marre, S., Parisot, N., Lepercq, P., Chaucheyras-Durand, F., Mosoni, P., Peyretaillade, E., Bayat, A. R., Shingfield, K. J., Peyret, P. and Forano, E. 2017. Metatranscriptomics reveals the active bacterial and eukaryotic fibrolytic communities in the rumen of dairy cow fed a mixed diet. *Front. Microbiol.* 8, 67. doi:10.3389/fmicb.2017.00067.

Comtet-Marre, S., Chaucheyras-Durand, F., Bouzid, O., Mosoni, P., Bayat, A. R., Peyret, P. and Forano, E. 2018. FibroChip, a functional DNA microarray to monitor cellulolytic and hemicellulolytic activities of rumen microbiota. *Front. Microbiol.* 9, 215. doi:10.3389/fmicb.2018.00215.

Cuskin, F., Lowe, E. C., Temple, M. J., Zhu, Y., Cameron, E., Pudlo, N. A., Porter, N. T., Urs, K., Thompson, A. J., Cartmell, A., Rogowski, A., Hamilton, B. S., Chen, R., Tolbert, T. J., Piens, K., Bracke, D., Vervecken, W., Hakki, Z., Speciale, G., Munōz-Munōz, J. L., Day, A., Peña, M. J., McLean, R., Suits, M. D., Boraston, A. B., Atherly, T., Ziemer, C. J., Williams, S. J., Davies, G. J., Abbott, D. W., Martens, E. C. and Gilbert, H. J. 2015. Human gut Bacteroidetes can utilize yeast mannan through a selfish mechanism. *Nature* 517(7533), 165–9. doi:10.1038/nature13995.

D'Elia, J. N. and Salyers, A. A. 1996a. Contribution of a neopullulanase, a pullulanase, and an alpha-glucosidase to growth of *Bacteroides thetaiotaomicron* on starch. *J. Bacteriol.* 178(24), 7173–9. doi:10.1128/jb.178.24.7173-7179.1996.

D'Elia, J. N. and Salyers, A. A. 1996b. Effect of regulatory protein levels on utilization of starch by *Bacteroides thetaiotaomicron*. *J. Bacteriol.* 178(24), 7180–6. doi:10.1128/jb.178.24.7180-7186.1996.

Dai, X., Zhu, Y., Luo, Y., Song, L., Liu, D., Liu, L., Chen, F., Wang, M., Li, J., Zeng, X., Dong, Z., Hu, S., Li, L., Xu, J., Huang, L. and Dong, X. 2012. Metagenomic insights into the fibrolytic microbiome in yak rumen. *PLoS ONE* 7(7), e40430. doi:10.1371/journal.pone.0040430.

Dassa, B., Borovok, I., Ruimy-Israeli, V., Lamed, R., Flint, H. J., Duncan, S. H., Henrissat, B., Coutinho, P., Morrison, M., Mosoni, P., Yeoman, C. J., White, B. A. and Bayer, E. A. 2014. Rumen cellulosomics: divergent fiber-degrading strategies revealed by comparative genome-wide analysis of six ruminococcal strains. *PLoS ONE* 9(7), e99221. doi:10.1371/journal.pone.0099221.

Davies, G. and Henrissat, B. 1995. Structures and mechanisms of glycosyl hydrolases. *Structure* 3(9), 853–9. doi:10.1016/S0969-2126(01)00220-9.

Dehority, B. A. and Scott, H. W. 1967. Extent of cellulose and hemicellulose digestion in various forages by pure cultures of rumen bacteria. *J. Dairy Sci.* 50(7), 1136–41. doi:10.3168/jds.S0022-0302(67)87579-9.

Díaz-Viraqué, F., Pita, S., Greif, G., de Souza, R. C. M., Iraola, G. and Robello, C. 2019. Nanopore sequencing significantly improves genome assembly of the eukaryotic protozoan parasite *Trypanosoma cruzi*. *Genome Biol. Evol.* 11(7), 1952–7. doi:10.1093/gbe/evz129.

Difford, G. F., Plichta, D. R., Løvendahl, P., Lassen, J., Noel, S. J., Højberg, O., Wright, A. G., Zhu, Z., Kristensen, L., Nielsen, H. B., Guldbrandtsen, B. and Sahana, G. 2018. Host genetics and the rumen microbiome jointly associate with methane emissions in dairy cows. *PLoS Genet.* 14(10), e1007580. doi:10.1371/journal.pgen.1007580.

Ding, S. Y., Rincon, M. T., Lamed, R., Martin, J. C., McCrae, S. I., Aurilia, V., Shoham, Y., Bayer, E. A. and Flint, H. J. 2001. Cellulosomal scaffoldin-like proteins from *Ruminococcus flavefaciens*. *J. Bacteriol.* 183(6), 1945–53. doi:10.1128/JB.183.6.1945-1953.2001.

Dodd, D., Moon, Y. H., Swaminathan, K., Mackie, R. I. and Cann, I. K. 2010. Transcriptomic analyses of xylan degradation by *Prevotella bryantii* and insights into energy acquisition by xylanolytic Bacteroidetes. *J. Biol. Chem.* 285(39), 30261–73. doi:10.1074/jbc.M110.141788.

Elhenawy, W., Debelyy, M. O. and Feldman, M. F. 2014. Preferential packing of acidic glycosidases and proteases into Bacteroides outer membrane vesicles. *mBio* 5(2), e00909–14. doi:10.1128/mBio.00909-14.

Emerson, J. B., Roux, S., Brum, J. R., Bolduc, B., Woodcroft, B. J., Jang, H. B., Singleton, C. M., Solden, L. M., Naas, A. E., Boyd, J. A., Hodgkins, S. B., Wilson, R. M., Trubl, G., Li, C., Frolking, S., Pope, P. B., Wrighton, K. C., Crill, P. M., Chanton, J. P., Saleska, S. R., Tyson, G. W., Rich, V. I. and Sullivan, M. B. 2018. Host-linked soil viral ecology along a permafrost thaw gradient. *Nat. Microbiol.* 3(8), 870–80. doi:10.1038/s41564-018-0190-y.

Fanutti, C. C., Ponyi, T. T., Black, G. W. G., Hazlewood, G. P. G. and Gilbert, H. J. 1995. The conserved noncatalytic 40-residue sequence in cellulases and hemicellulases from anaerobic fungi functions as a protein docking domain. *J. Biol. Chem.* 270(49), 29314–22. doi:10.1074/jbc.270.49.29314.

Flint, H. J., Bayer, E. A., Rincon, M. T., Lamed, R. and White, B. A. 2008. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat. Rev. Microbiol.* 6(2), 121–31. doi:10.1038/nrmicro1817.

Foley, M. H., Cockburn, D. W. and Koropatkin, N. M. 2016. The Sus operon: a model system for starch uptake by the human gut Bacteroidetes. *Cell. Mol. Life Sci.* 73(14), 2603–17. doi:10.1007/s00018-016-2242-x.

Forsberg, C. W., Beveridge, T. J. and Hellstrom, A. 1981. Cellulase and xylanase release from *Bacteroides succinogenes* and its importance in the rumen environment. *Appl. Environ. Microbiol.* 42(5), 886–96.

Forsberg, Z., Vaaje-Kolstad, G., Westereng, B., Bunæs, A. C., Stenstrøm, Y., MacKenzie, A., Sørlie, M., Horn, S. J. and Eijsink, V. G. 2011. Cleavage of cellulose by a CBM33 protein. *FEBS Lett.* 20(9), 1479–83. doi:10.1002/pro.689.

Forsberg, Z., Mackenzie, A. K., Sørlie, M., Røhr, Å. K., Helland, R., Arvai, A. S., Vaaje-Kolstad, G. and Eijsink, V. G. 2014. Structural and functional characterization of a conserved pair of bacterial cellulose-oxidizing lytic polysaccharide monooxygenases. *Proc. Natl Acad. Sci. U.S.A.* 111(23), 8446–51. doi:10.1073/pnas.1402771111.

Garron, M. L. and Cygler, M. 2010. Structural and mechanistic classification of uronic acid-containing polysaccharide lyases. *Glycobiology* 20(12), 1547–73. doi:10.1093/glycob/cwq122.

Gaudet, G. and Gaillard, B. 1987. Vesicle formation and cellulose degradation in *Bacteroides succinogenes* cultures: ultrastructural aspects. *Arch. Microbiol.* 148(2), 150–4. doi:10.1007/BF00425364.

Gibson, L. J. 2012. The hierarchical structure and mechanics of plant materials. *J. R. Soc. Interface* 9(76), 2749–66. doi:10.1098/rsif.2012.0341.

Gilbert, H. J., Knox, J. P. and Boraston, A. B. 2013. Advances in understanding the molecular basis of plant cell wall polysaccharide recognition by carbohydrate-binding modules. *Curr. Opin. Struct. Biol.* 23(5), 669–77. doi:10.1016/j.sbi.2013.05.005.

Gilkes, N. R., Warren, R. A., Miller, R. C. and Kilburn, D. G. 1988. Precise excision of the cellulose binding domains from two *Cellulomonas fimi* cellulases by a homologous protease and the effect on catalysis. *J. Biol. Chem.* 263(21), 10401–7.

Gomez Del Pulgar, E. M. and Saadeddin, A. 2013. The cellulolytic system of *Thermobifida fusca*. *Crit. Rev. Microbiol.* 7828, 1–12.

Güllert, S., Fischer, M. A., Turaev, D., Noebauer, B., Ilmberger, N., Wemheuer, B., Alawi, M., Rattei, T., Daniel, R., Schmitz, R. A., Grundhoff, A. and Streit, W. R. 2016. Deep metagenome and metatranscriptome analyses of microbial communities affiliated with an industrial biogas fermenter, a cow rumen, and elephant feces reveal major differences in carbohydrate hydrolysis strategies. *Biotechnol. Biofuels* 9, 121. doi:10.1186/s13068-016-0534-x.

Haitjema, C. H., Gilmore, S. P., Henske, J. K., Solomon, K. V., de Groot, R., Kuo, A., Mondo, S. J., Salamov, A. A., LaButti, K., Zhao, Z., Chiniquy, J., Barry, K., Brewer, H. M., Purvine, S. O., Wright, A. T., Hainaut, M., Boxma, B., van Alen, T., Hackstein, J. H. P., Henrissat, B., Baker, S. E., Grigoriev, I. V. and O'Malley, M. A. 2017. A parts list for fungal cellulosomes revealed by comparative genomics. *Nat. Microbiol.* 2, 17087. doi:10.1038/nmicrobiol.2017.87.

Hehemann, J. H., Kelly, A. G., Pudlo, N. A., Martens, E. C. and Boraston, A. B. 2012. Bacteria of the human gut microbiome catabolize red seaweed glycans with carbohydrate-active enzyme updates from extrinsic microbes. *Proc. Natl Acad. Sci. U. S. A.* 109(48), 19786–91. doi:10.1073/pnas.1211002109.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Global Rumen Census Collaborators and Janssen, P. H. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5, e14567. doi:10.1038/srep14567.

Henrissat, B. 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* 280(2), 309–16. doi:10.1042/bj2800309.

Hervé, C., Rogowski, A., Blake, A. W., Marcus, S. E., Gilbert, H. J. and Knox, J. P. 2010. Carbohydrate-binding modules promote the enzymatic deconstruction of intact plant cell walls by targeting and proximity effects. *Proc. Natl Acad. Sci. U.S.A.* 107(34), 15293–8. doi:10.1073/pnas.1005732107.

Hold, G. L., Pryde, S. E., Russell, V. J., Furrie, E. and Flint, H. J. 2002. Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. *FEMS Microbiol. Ecol.* 39(1), 33–9. doi:10.1111/j.1574-6941.2002.tb00904.x.

Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. *Bacteriol. Rev.* 14(1), 1–49.

Hungate, R. E. 1960. Symposium: selected topics in microbial ecology. I. Microbial ecology of the rumen. *Bacteriol. Rev.* 24(4), 353–64.

Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., Muñoz-Tamayo, R., Forano, E., Waters, S. M., Hess, M., Tapio, I., Smidt, H., Krizsan, S. J.,

Yáñez-Ruiz, D. R., Belanche, A., Guan, L., Gruninger, R. J., McAllister, T. A., Newbold, C. J., Roehe, R., Dewhurst, R. J., Snelling, T. J., Watson, M., Suen, G., Hart, E. H., Kingston-Smith, A. H., Scollan, N. D., do Prado, R. M., Pilau, E. J., Mantovani, H. C., Attwood, G. T., Edwards, J. E., McEwan, N. R., Morrisson, S., Mayorga, O. L., Elliott, C. and Morgavi, D. P. 2018. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Front. Microbiol.* 9, 2161. doi:10.3389/fmicb.2018.02161.

Isikgor, F. H. and Becer, C. R. 2015. Lignocellulosic biomass: a sustainable platform for production of bio-based chemicals and polymers. *Polym. Chem.* 6(25), 4497–559. doi:10.1039/C5PY00263J.

Israeli-Ruimy, V., Bule, P., Jindou, S., Dassa, B., Moraïs, S., Borovok, I., Barak, Y., Slutzki, M., Hamberg, Y., Cardoso, V., Alves, V. D., Najmudin, S., White, B. A., Flint, H. J., Gilbert, H. J., Lamed, R., Fontes, C. M. and Bayer, E. A. 2017. Complexity of the *Ruminococcus flavefaciens* FD-1 cellulosome reflects an expansion of family-related protein-protein interactions. *Sci. Rep.* 7, 42355. doi:10.1038/srep42355.

Jami, E. and Mizrahi, I. 2012. Composition and similarity of bovine rumen microbiota across individual animals. *PLoS One* 7(3), e33306. doi:10.1371/journal.pone.0033306.

Ji, X., Xu, Y., Zhang, C., Chen, N. and Lu, X. 2012. A new locus affects cell motility, cellulose binding, and degradation by *Cytophaga hutchinsonii*. *Appl. Microbiol. Biotechnol.* 96(1), 161–70. doi:10.1007/s00253-012-4051-y.

Kharade, S. S. and McBride, M. J. 2014. *Flavobacterium johnsoniae* chitinase ChiA is required for chitin utilization and is secreted by the type IX secretion system. *J. Bacteriol.* 196(5), 961–70. doi:10.1128/JB.01170-13.

Konietzny, S. G., Pope, P. B., Weimann, A. and McHardy, A. C. 2014. Inference of phenotype-defining functional modules of protein families for microbial plant biomass degraders. *Biotechnol. Biofuels* 7(1), 124. doi:10.1186/s13068-014-0124-8.

Koropatkin, N. M. and Smith, T. J. 2010. SusG: a unique cell-membrane-associated alpha-amylase from a prominent human gut symbiont targets complex starch molecules. *Structure* 18(2), 200–15. doi:10.1016/j.str.2009.12.010.

Koropatkin, N. M., Cameron, E. A. and Martens, E. C. 2012. How glycan metabolism shapes the human gut microbiota. *Nat. Rev. Microbiol.* 10(5), 323–35. doi:10.1038/nrmicro2746.

Koshland, D. E. 1953. Stereochemistry and the mechanism of enzymatic reactions. *Biol. Rev.* 28(4), 416–36. doi:10.1111/j.1469-185X.1953.tb01386.x.

Kostylev, M. and Wilson, D. B. 2012. Synergistic interactions in cellulose hydrolysis. *Biofuels* 3(1), 61–70. doi:10.4155/bfs.11.150.

Krause, D. O., Bunch, R. J., Conlan, L. L., Kennedy, P. M., Smith, W. J., Mackie, R. I. and McSweeney, C. S. 2001. Repeated ruminal dosing of *Ruminococcus* spp. does not result in persistence, but changes in other microbial populations occur that can be measured with quantitative 16S-rRNA-based probes. *Microbiology* 147(7), 1719–29. doi:10.1099/00221287-147-7-1719.

Krauss, J., Zverlov, V. V. and Schwarz, W. H. 2012. *In vitro* reconstitution of the complete *Clostridium thermocellum* cellulosome and synergistic activity on crystalline cellulose. *Appl. Environ. Microbiol.* 78(12), 4301–7. doi:10.1128/AEM.07959-11.

Kulp, A. and Kuehn, M. J. 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu. Rev. Microbiol.* 64, 163–84. doi:10.1146/annurev.micro.091208.073413.

Laine, R. A. 1994. A calculation of all possible oligosaccharide isomers both branched and linear yields 1.05 x 10(12) structures for a reducing hexasaccharide: the Isomer Barrier to development of single-method saccharide sequencing or synthesis systems. *Glycobiology* 4(6), 759–67. doi:10.1093/glycob/4.6.759.

Lairson, L. L., Henrissat, B., Davies, G. J. and Withers, S. G. 2008. Glycosyltransferases: structures, functions, and mechanisms. *Annu. Rev. Biochem.* 77, 521–55. doi:10.1146/annurev.biochem.76.061005.092322.

Lamed, R., Setter, E., Kenig, R. and Bayer, E. A. 1983. The cellulosome: a discrete cell surface organelle of *Clostridium thermocellum* which exhibits separate antigenic, cellulose-binding and various cellulolytic activities. *Biotechnol. Prog.* 13, 163–81.

Lapébie, P., Lombard, V., Drula, E., Terrapon, N. and Henrissat, B. 2019. Bacteroidetes use thousands of enzyme combinations to break down glycans. *Nat. Commun.* 10(1), 2043. doi:10.1038/s41467-019-10068-5.

La Rosa, S. L., Leth, M. L., Michalak, L., Hansen, M. E., Pudlo, N. A., Glowacki, R., Pereira, G., Workman, C. T., Arntzen, M. Ø, Pope, P. B., Martens, E. C., Hachem, M. A. and Westereng, B. 2019. The human gut Firmicute *Roseburia intestinalis* is a primary degrader of dietary β-mannans. *Nat. Commun.* 10(1), 905, doi:10.1038/s41467-019-08812-y.

Larsbrink, J., Rogers, T. E., Hemsworth, G. R., McKee, L. S., Tauzin, A. S., Spadiut, O., Klinter, S., Pudlo, N. A., Urs, K., Koropatkin, N. M., Creagh, A. L., Haynes, C. A., Kelly, A. G., Cederholm, S. N., Davies, G. J., Martens, E. C. and Brumer, H. 2014. A discrete genetic locus confers xyloglucan metabolism in select human gut Bacteroidetes. *Nature* 506(7489), 498–502. doi:10.1038/nature12907.

Larsbrink, J., Zhu, Y., Kharade, S. S., Kwiatkowski, K. J., Eijsink, V. G. H., Koropatkin, N. M., McBride, M. J. and Pope, P. B. 2016. A polysaccharide utilization locus from *Flavobacterium johnsoniae* enables conversion of recalcitrant chitin. *Biotechnol. Biofuels* 9, 260. doi:10.1186/s13068-016-0674-z.

Levasseur, A., Drula, E., Lombard, V., Coutinho, P. M. and Henrissat, B. 2013. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. *Biotechnol. Bioeng.* 6(1), 41. doi:10.1186/1754-6834-6-41.

Li, F. and Guan, L. L. 2017. Metatranscriptomic profiling reveals linkages between the active rumen microbiome and feed efficiency in beef cattle. *Appl. Environ. Microbiol.* 83(9), e00061-17. doi:10.1128/AEM.00061-17.

Li, C., Lin, F., Li, Y., Wei, W., Wang, H., Qin, L., Zhou, Z., Li, B., Wu, F. and Chen, Z. 2016. A β-glucosidase hyper-production *Trichoderma reesei* mutant reveals a potential role of cel3D in cellulase production. *Microb. Cell Fact.* 15(1), 151. doi:10.1186/s12934-016-0550-3.

Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., Fitzsimmons, C., Plastow, G. and Guan, L. L. 2019. Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. *Microbiome* 7(1), 92. doi:10.1186/s40168-019-0699-1.

Ljungdahl, L. G. 2009. A life with acetogens, thermophiles, and cellulolytic anaerobes. *Annu. Rev. Microbiol.* 63, 1–25. doi:10.1146/annurev.micro.091208.073617.

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P. M. and Henrissat, B. 2014. The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res.* 42, D490–5. doi:10.1093/nar/gkt1178.

Mackenzie, A. K., Naas, A. E., Kracun, S. K., Schückel, J., Fangel, J. U., Agger, J. W., Willats, W. G., Eijsink, V. G. and Pope, P. B. 2015. A polysaccharide utilization locus from an

uncultured Bacteroidetes phylotype suggests ecological adaptation and substrate versatility. *Appl. Environ. Microbiol.* 81(1), 187–95. doi:10.1128/AEM.02858-14.

Martens, E. C., Chiang, H. C. and Gordon, J. I. 2008. Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. *Cell Host Microbe* 4(5), 447–57. doi:10.1016/j.chom.2008.09.007.

Martens, E. C., Lowe, E. C., Chiang, H., Pudlo, N. A., Wu, M., McNulty, N. P., Abbott, D. W., Henrissat, B., Gilbert, H. J., Bolam, D. N. and Gordon, J. I. 2011. Recognition and degradation of plant cell wall polysaccharides by two human gut symbionts. *PLoS Biol.* 9(12), e1001221. doi:10.1371/journal.pbio.1001221.

McBride, M. J. and Nakane, D. 2015. *Flavobacterium* gliding motility and the type IX secretion system. *Curr. Opin. Microbiol.* 28, 72–7. doi:10.1016/j.mib.2015.07.016.

McBride, M. J., Xie, G., Martens, E. C., Lapidus, A., Henrissat, B., Rhodes, R. G., Goltsman, E., Wang, W., Xu, J., Hunnicutt, D. W., Staroscik, A. M., Hoover, T. R., Cheng, Y. Q. and Stein, J. L. 2009. Novel features of the polysaccharide-digesting gliding bacterium *Flavobacterium johnsoniae* as revealed by genome sequence analysis. *Appl. Environ. Microbiol.* 75(21), 6864–75. doi:10.1128/AEM.01495-09.

McCann, J. C., Wickersham, T. A. and Loor, J. J. 2014. High-throughput methods redefine the rumen microbiome and its relationship with nutrition and metabolism. *Bioinform. Biol. Insights* 8, 109–25. doi:10.4137/BBI.S15389.

McCarter, J. D. and Withers, S. G. 1994. Mechanisms of enzymatic glycoside hydrolysis. *Curr. Opin. Struct. Biol.* 4(6), 885–92. doi:10.1016/0959-440x(94)90271-2.

McCartney, L., Gilbert, H. J., Bolam, D. N., Boraston, A. B. and Knox, J. P. 2004. Glycoside hydrolase carbohydrate-binding modules as molecular probes for the analysis of plant cell wall polymers. *Anal. Biochem.* 326(1), 49–54. doi:10.1016/j.ab.2003.11.011.

Mertens, D. 2003. Nutritional implications of fiber and carbohydrate characteristics of corn silage and alfalfa hay. *Proceedings of the California Animal Nutrition Conference*, Fresno, CA, pp. 94–107.

Moller, I., Sørensen, I., Bernal, A. J., Blaukopf, C., Lee, K., Øbro, J., Pettolino, F., Roberts, A., Mikkelsen, J. D., Knox, J. P., Bacic, A. and Willats, W. G. 2007. High-throughput mapping of cell-wall polymers within and between plants using novel microarrays. *Plant J.* 50(6), 1118–28. doi:10.1111/j.1365-313X.2007.03114.x.

Moraïs, S. and Mizrahi, I. 2019. Islands in the stream: from individual to communal fiber degradation in the rumen ecosystem. *FEMS Microbiol. Rev.* 43(4), 362–79, doi:10.1093/femsre/fuz007.

Naas, A. E., Mackenzie, A. K., Mravec, J., Schückel, J., Willats, W. G., Eijsink, V. G. and Pope, P. B. 2014. Do rumen Bacteroidetes utilize an alternative mechanism for cellulose degradation? *mBio* 5(4), e01401–14. doi:10.1128/mBio.01401-14.

Naas, A. E., Solden, L. M., Norbeck, A. D., Brewer, H., Hagen, L. H., Heggenes, I. M., McHardy, A. C., Mackie, R. I., Paša-Tolić, L., Arntzen, M. Ø, Eijsink, V. G. H., Koropatkin, N. M., Hess, M., Wrighton, K. C. and Pope, P. B. 2018. 'Candidatus Paraporphyromonas polyenzymogenes' encodes multi-modular cellulases linked to the type IX secretion system. *Microbiome* 6(1), 44. doi:10.1186/s40168-018-0421-8.

Ohara, H., Karita, S., Kimura, T., Sakka, K. and Ohmiya, K. 2000. Characterization of the cellulolytic complex (cellulosome) from *Ruminococcus albus*. *Biosci. Biotechnol. Biochem.* 64(2), 254–60. doi:10.1271/bbb.64.254.

Orpin, C. G. 1975. Studies on the rumen flagellate *Neocallimastix frontalis*. *J. Gen. Microbiol.* 91(2), 249–62. doi:10.1099/00221287-91-2-249.

Park, T., Wijeratne, S., Meulia, T., Firkins, J. and Yu, Z. 2018. Draft macronuclear genome sequence of the ruminal ciliate *Entodinium caudatum*. *Microbiol. Resour Announc.* 7(1), e00826-18. doi:10.1128/MRA.00826-18.

Pattathil, S., Hahn, M. G., Dale, B. E. and Chundawat, S. P. 2015. Insights into plant cell wall structure, architecture, and integrity using glycome profiling of native and AFEXTM-pre-treated biomass. *J. Exp. Bot.* 66(14), 4279–94. doi:10.1093/jxb/erv107.

Pope, P. B., Denman, S. E., Jones, M., Tringe, S. G., Barry, K., Malfatti, S. A., McHardy, A. C., Cheng, J. F., Hugenholtz, P., McSweeney, C. S. and Morrison, M. 2010. Adaptation to herbivory by the Tammar wallaby includes bacterial and glycoside hydrolase profiles different to other herbivores. *Proc. Natl Acad. Sci. U.S.A.* 107(33), 14793–8. doi:10.1073/pnas.1005297107.

Pope, P. B., Mackenzie, A. K., Gregor, I., Smith, W., Sundset, M. A., McHardy, A. C., Morrison, M. and Eijsink, V. G. 2012. Metagenomics of the Svalbard reindeer rumen microbiome reveals abundance of polysaccharide *Utilization Loci*. *PLoS One* 7(6), e38571. doi:10.1371/journal.pone.0038571.

Præsteng, K. E., Pope, P. B., Cann, I. K., Mackie, R. I., Mathiesen, S. D., Folkow, L. P., Eijsink, V. G. and Sundset, M. A. 2013. Probiotic dosing of *Ruminococcus flavefaciens* affects rumen microbiome structure and function in reindeer. *Microb. Ecol.* 66(4), 840–9. doi:10.1007/s00248-013-0279-z.

Qi, M., Wang, P., O'Toole, N., Barboza, P. S., Ungerfeld, E., Leigh, M. B., Selinger, L. B., Butler, G., Tsang, A., McAllister, T. A. and Forster, R. J. 2011. Snapshot of the eukaryotic gene expression in muskoxen rumen—a metatranscriptomic approach. *PLoS One* 6(5), e20521. doi:10.1371/journal.pone.0020521.

Quinlan, R. J., Sweeney, M. D., Lo Leggio, L., Otten, H., Poulsen, J. C., Johansen, K. S., Krogh, K. B., Jørgensen, C. I., Tovborg, M., Anthonsen, A., Tryfona, T., Walter, C. P., Dupree, P., Xu, F., Davies, G. J. and Walton, P. H. 2011. Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. *Proc. Natl Acad. Sci. U.S.A.* 108(37), 15079–84. doi:10.1073/pnas.1105776108.

Reese, E. T. 1956. A microbiological process report; enzymatic hydrolysis of cellulose. *Appl. Microbiol.* 4(1), 39–45.

Reeves, A. R., D'elia, J. N., Frias, J. and Salyers, A. A. 1996. A *Bacteroides thetaiotaomicron* outer membrane protein that is essential for utilization of maltooligosaccharides and starch. *J. Bacteriol.* 178(3), 823–30. doi:10.1128/jb.178.3.823-830.1996.

Reeves, A. R., Wang, G. R. and Salyers, A. A. 1997. Characterization of four outer membrane proteins that play a role in utilization of starch by *Bacteroides thetaiotaomicron*. *J. Bacteriol.* 179(3), 643–9. doi:10.1128/jb.179.3.643-649.1997.

Roehe, R., Dewhurst, R. J., Duthie, C. A., Rooke, J. A., McKain, N., Ross, D. W., Hyslop, J. J., Waterhouse, A., Freeman, T. C., Watson, M. and Wallace, R. J. 2016. Bovine host genetic variation influences rumen microbial methane production with best selection criterion for low methane emitting and efficiently feed converting hosts based on metagenomic gene abundance. *PLoS Genet.* 12(2), e1005846. doi:10.1371/journal.pgen.1005846.

Rogowski, A., Briggs, J. A., Mortimer, J. C., Tryfona, T., Terrapon, N., Lowe, E. C., Baslé, A., Morland, C., Day, A. M., Zheng, H., Rogers, T. E., Thompson, P., Hawkins, A. R., Yadav, M. P., Henrissat, B., Martens, E. C., Dupree, P., Gilbert, H. J. and Bolam, D. N. 2015. Glycan complexity dictates microbial resource allocation in the large intestine. *Nat. Commun.* 6, 7481. doi:10.1038/ncomms8481.

Roier, S., Zingl, F. G., Cakar, F., Durakovic, S., Kohl, P., Eichmann, T. O., Klug, L., Gadermaier, B., Weinzerl, K., Prassl, R., Lass, A., Daum, G., Reidl, J., Feldman, M. F. and Schild, S. 2016. A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria. *Nat. Commun.* 7, 10515. doi:10.1038/ncomms10515.

Rosewarne, C. P., Pope, P. B., Cheung, J. L. and Morrison, M. 2014. Analysis of the bovine rumen microbiome reveals a diversity of Sus-like polysaccharide utilization loci from the bacterial phylum *Bacteroidetes*. *J. Ind. Microbiol. Biotechnol.* 41(3), 601–6. doi:10.1007/s10295-013-1395-y.

Roux, S., Enault, F., Hurwitz, B. L. and Sullivan, M. B. 2015. VirSorter: mining viral signal from microbial genomic data. *PeerJ* 3, e985. doi:10.7717/peerj.985.

Russell, J. B., Muck, R. E. and Weimer, P. J. 2009. Quantitative analysis of cellulose degradation and growth of cellulolytic bacteria in the rumen. *FEMS Microbiol. Ecol.* 67(2), 183–97. doi:10.1111/j.1574-6941.2008.00633.x.

Saloheimo, M., Nakari-SetaLa, T., Tenkanen, M. and Penttila, M. 1997. cDNA Cloning of a *Trichoderma reesei* cellulase and demonstration of endoglucanase activity by expression in yeast. *Eur. J. Biochem.* 249(2), 584–91. doi:10.1111/j.1432-1033.1997.00584.x.

Sasson, G., Kruger Ben-Shabat, S., Seroussi, E., Doron-Faigenboim, A., Shterzer, N., Yaacoby, S., Berg Miller, M. E., White, B. A., Halperin, E. and Mizrahi, I. 2017. Heritable bovine rumen bacteria are phylogenetically related and correlated with the cow's capacity to harvest energy from its feed. *mBio* 8(4), e00703-17. doi:10.1128/mBio.00703-17.

Sato, K., Naito, M., Yukitake, H., Hirakawa, H., Shoji, M., McBride, M. J., Rhodes, R. G. and Nakayama, K. 2010. A protein secretion system linked to bacteroidete gliding motility and pathogenesis. *Proc. Natl Acad. Sci. U.S.A.* 107(1), 276–81. doi:10.1073/pnas.0912010107.

Scheller, H. V. and Ulvskov, P. 2010. Hemicelluloses. *Annu. Rev. Plant Biol.* 61, 263–89. doi:10.1146/annurev-arplant-042809-112315.

Schneider, R., Hanak, T., Persson, S. and Voigt, C. A. 2016. Cellulose and callose synthesis and organization in focus, what's new? *Curr. Opin. Plant Biol.* 34, 9–16. doi:10.1016/j.pbi.2016.07.007.

Seshadri, R., Leahy, S. C., Attwood, G. T., Teh, K. H., Lambie, S. C., Cookson, A. L., Eloe-Fadrosh, E. A., Pavlopoulos, G. A., Hadjithomas, M., Varghese, N. J., Paez-Espino, D., Perry, R., Henderson, G., Creevey, C. J., Terrapon, N., Lapebie, P., Drula, E., Lombard, V., Rubin, E., Kyrpides, N. C., Henrissat, B., Woyke, T., Ivanova, N. N. and Kelly, W. J. 2018. Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nat. Biotechnol.* 36(4), 359–67. doi:10.1038/nbt.4110.

Sheir-Neiss, G. and Montenecourt, B. S. 1984. Characterization of the secreted cellulases of *Trichoderma reesei* wild type and mutants during controlled fermentations. *Appl. Microbiol. Biotechnol.* 20(1), 46–53. doi:10.1007/BF00254645.

Shipman, J. A., Cho, K. H., Siegel, H. A. and Salyers, A. A. 1999. Physiological characterization of SusG, an outer membrane protein essential for starch utilization by *Bacteroides thetaiotaomicron*. *J. Bacteriol.* 181(23), 7206–11.

Shipman, J. A., Berleman, J. E. and Salyers, A. A. 2000. Characterization of four outer membrane proteins involved in binding starch to the cell surface of *Bacteroides thetaiotaomicron*. *J. Bacteriol.* 182(19), 5365–72. doi:10.1128/jb.182.19.5365-5372.2000.

Solden, L. M., Naas, A. E., Roux, S., Daly, R. A., Collins, W. B., Nicora, C. D., Purvine, S. O., Hoyt, D. W., Schückel, J., Jørgensen, B., Willats, W., Spalinger, D. E., Firkins, J. L., Lipton, M. S., Sullivan, M. B., Pope, P. B. and Wrighton, K. C. 2018. Interspecies cross-feeding orchestrates carbon degradation in the rumen ecosystem. *Nat. Microbiol.* 3(11), 1274–84. doi:10.1038/s41564-018-0225-4.

Söllinger, A., Tveit, A. T., Poulsen, M., Noel, S. J., Bengtsson, M., Bernhardt, J., Frydendahl Hellwing, A. L., Lund, P., Riedel, K., Schleper, C., Højberg, O. and Urich, T. 2018. Holistic assessment of rumen microbiome dynamics through quantitative metatranscriptomics reveals multifunctional redundancy during key steps of anaerobic feed degradation. *mSystems* 3(4), e00038-18. doi:10.1128/mSystems.00038-18.

Sonnenburg, E. D., Zheng, H., Joglekar, P., Higginbottom, S. K., Firbank, S. J., Bolam, D. N. and Sonnenburg, J. L. 2010. Specificity of polysaccharide use in intestinal Bacteroides species determines diet-inducedmicrobiota alterations. *Cell* 141(7), 1241–52. doi:10.1016/j.cell.2010.05.005.

Spor, A., Koren, O. and Ley, R. 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. *Nat. Rev. Microbiol.* 9(4), 279–90. doi:10.1038/nrmicro2540.

Steenbakkers, P. J., Li, X. L., Ximenes, E. A., Arts, J. G., Chen, H., Ljungdahl, L. G. and Op Den Camp, H. J. 2001. Noncatalytic docking domains of cellulosomes of anaerobic fungi. *J. Bacteriol.* 183(18), 5325–33. doi:10.1128/jb.183.18.5325-5333.2001.

Stevenson, D. M. and Weimer, P. J. 2007. Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl. Microbiol. Biotechnol.* 75(1), 165–74. doi:10.1007/s00253-006-0802-y.

Stewart, R. D., Auffret, M. D., Warr, A., Walker, A. W., Roehe, R., Watson, M. 2018a. The genomic and proteomic landscape of the rumen microbiome revealed by comprehensive genome-resolved metagenomics. *BioRxiv*, doi:10.1101/489443.

Stewart, R. D., Auffret, M. D., Warr, A., Wiser, A. H., Press, M. O., Langford, K. W., Liachko, I., Snelling, T. J., Dewhurst, R. J., Walker, A. W., Roehe, R. and Watson, M. 2018b. Assembly of 913 microbial genomes from metagenomic sequencing of the cow rumen. *Nat. Commun.* 9(1), 870. doi:10.1038/s41467-018-03317-6.

Suen, G., Weimer, P. J., Stevenson, D. M., Aylward, F. O., Boyum, J., Deneke, J., Drinkwater, C., Ivanova, N. N., Mikhailova, N., Chertkov, O., Goodwin, L. A., Currie, C. R., Mead, D. and Brumm, P. J. 2011. The complete genome sequence of *Fibrobacter succinogenes* S85 reveals a cellulolytic and metabolic specialist. *PLoS ONE* 6(4), e18814. doi:10.1371/journal.pone.0018814.

Tajima, K., Aminov, R. I., Nagamine, T., Ogata, K., Nakamura, M., Matsui, H. and Benno, Y. 1999. Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. *FEMS Microbiol. Ecol.* 29(2), 159–69. doi:10.1111/j.1574-6941.1999.tb00607.x.

Talamantes, D., Biabini, N., Dang, H., Abdoun, K. and Berlemont, R. 2016. Natural diversity of cellulases, xylanases, and chitinases in bacteria. *Biotechnol. Biofuels* 9, 133. doi:10.1186/s13068-016-0538-6.

Terrapon, N., Lombard, V., Gilbert, H. J. and Henrissat, B. 2015. Automatic prediction of polysaccharide utilization loci in Bacteroidetes species. *Bioinformatics* 31(5), 647–55. doi:10.1093/bioinformatics/btu716.

Tomme, P., Van Tilbeurgh, H., Pettersson, G., Van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T. and Claeyssens, M. 1988. Studies of the cellulolytic system of *Trichoderma*

reesei QM 9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. *Eur. J. Biochem.* 170(3), 575–81. doi:10.1111/j.1432-1033.1988.tb13736.x.

Vaaje-Kolstad, G., Westereng, B., Horn, S. J., Liu, Z., Zhai, H., Sørlie, M. and Eijsink, V. G. 2010. An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. *Science* 330(6001), 219–22. doi:10.1126/science.1192231.

Van Tilbeurgh, H., Tomme, P., Claeyssens, M., Bhikhabhai, R. and Pettersson, G. 1986. Limited proteolysis of the cellobiohydrolase I from *Trichoderma reesei*. *FEBS Lett.* 204(2), 223–7. doi:10.1016/0014-5793(86)80816-X.

Várnai, A., Siika-Aho, M. and Viikari, L. 2013. Carbohydrate-binding modules (CBMs) revisited: reduced amount of water counterbalances the need for CBMs. *Biotechnol. Biofuels* 6(1), 30. doi:10.1186/1754-6834-6-30.

Venditto, I., Luis, A. S., Rydahl, M., Schückel, J., Fernandes, V. O., Vidal-Melgosa, S., Bule, P., Goyal, A., Pires, V. M., Dourado, C. G., Ferreira, L. M., Coutinho, P. M., Henrissat, B., Knox, J. P., Baslé, A., Najmudin, S., Gilbert, H. J., Willats, W. G. and Fontes, C. M. 2016. Complexity of the *Ruminococcus flavefaciens* cellulosome reflects an expansion in glycan recognition. *Proc. Natl Acad. Sci. U.S.A.* 113(26), 7136–41. doi:10.1073/pnas.1601558113.

Vogel, J. 2008. Unique aspects of the grass cell wall. *Curr. Opin. Plant. Biol.* 11(3), 301–7. doi:10.1016/j.pbi.2008.03.002.

Voiniciuc, C., Pauly, M. and Usadelb, B. 2018. Monitoring polysaccharide dynamics in the plant cell wall. *Plant Physiol.* 176(4), 2590–600. doi:10.1104/pp.17.01776.

Weimer, P. J., Russell, J. B. and Muck, R. E. 2009. Lessons from the cow: what the ruminant animal can teach us about consolidated bioprocessing of cellulosic biomass. *Bioresour. Technol.* 100(21), 5323–31. doi:10.1016/j.biortech.2009.04.075.

West, P. T., Probst, A. J., Grigoriev, I. V., Thomas, B. C. and Banfield, J. F. 2018. Genome-reconstruction for eukaryotes from complex natural microbial communities. *Genome Res.* 28(4), 569–80. doi:10.1101/gr.228429.117.

Westereng, B., Ishida, T., Vaaje-Kolstad, G., Wu, M., Eijsink, V. G. H., Igarashi, K., Samejima, M., Ståhlberg, J., Horn, S. J. and Sandgren, M. 2011. The putative endoglucanase PcGH61D from *Phanerochaete chrysosporium* is a metal-dependent oxidative enzyme that cleaves cellulose. *PLoS ONE* 6(11), e27807. doi:10.1371/journal.pone.0027807.

Westereng, B., Cannella, D., Wittrup Agger, J., Jørgensen, H., Larsen Andersen, M., Eijsink, V. G. and Felby, C. 2015. Enzymatic cellulose oxidation is linked to lignin by long-range electron transfer. *Sci. Rep.* 5, 18561. doi:10.1038/srep18561.

Wilson, D. B. 2008. Three microbial strategies for plant cell wall degradation. *Ann. N. Y. Acad. Sci.* 1125, 289–97. doi:10.1196/annals.1419.026.

Wilson, D. B. 2009. Aerobic microbial cellulase systems. In: Himmel, M. E. (Ed.), *Biomass Recalcitrance: Deconstructing the Plant Cell Wall for Bioenergy*. Blackwell Publishing Limited Company.

Wilson, D. B. 2011. Microbial diversity of cellulose hydrolysis. *Curr. Opin. Microbiol.* 14(3), 259–63. doi:10.1016/j.mib.2011.04.004.

Wood, T. M. and McCrae, S. I. 1979. Synergism between enzymes involved in the solubilization of native cellulose. *Adv. Chem.* 181, 181–209. doi:10.1021/ba-1979-0181.ch010.

Wood, I. P., Pearson, B. M., Garcia-Gutierrez, E., Havlickova, L., He, Z., Harper, A. L., Bancroft, I. and Waldron, K. W. 2017. Carbohydrate microarrays and their use for the

identification of molecular markers for plant cell wall composition. *Proc. Natl Acad. Sci. U.S.A.* 114(26), 6860–5. doi:10.1073/pnas.1619033114.

Xie, G., Bruce, D. C., Challacombe, J. F., Chertkov, O., Detter, J. C., Gilna, P., Han, C. S., Lucas, S., Misra, M., Myers, G. L., Richardson, P., Tapia, R., Thayer, N., Thompson, L. S., Brettin, T. S., Henrissat, B., Wilson, D. B. and McBride, M. J. 2007. Genome sequence of the cellulolytic gliding bacterium *Cytophaga hutchinsonii*. *Appl. Environ. Microbiol.* 73(11), 3536–46. doi:10.1128/AEM.00225-07.

Yaron, S., Morag, E., Bayer, E. A., Lamed, R. and Shoham, Y. 1995. Expression, purification and subunit-binding properties of cohesins 2 and 3 of the *Clostridium thermocellum* cellulosome. *FEBS Lett.* 360(2), 121–4. doi:10.1016/0014-5793(95)00074-j.

Yi, Z., Su, X., Revindran, V., Mackie, R. I. and Cann, I. 2013. Molecular and biochemical analyses of CbCel9A/Cel48A, a highly secreted multi-modular Cellulase by *Caldicellulosiruptor bescii* during growth on crystalline Cellulose. *PLoS One* 8(12), e84172. doi:10.1371/journal.pone.0084172.

Young, J., Chung, D., Bomble, Y. J., Himmel, M. E. and Westpheling, J. 2014. Deletion of *Caldicellulosiruptor bescii* CelA reveals its crucial role in the deconstruction of lignocellulosic biomass. *Biotechnol. Biofuels* 7(1), 142. doi:10.1186/s13068-014-0142-6.

Zhong, R. and Ye, Z. H. 2015. Secondary cell walls: biosynthesis, patterned deposition and transcriptional regulation. *Plant Cell Physiol.* 56(2), 195–214. doi:10.1093/pcp/pcu140.

Zhu, Y. and McBride, M. J. 2014. Deletion of the *Cytophaga hutchinsonii* type IX secretion system gene *sprP* results in defects in gliding motility and cellulose utilization. *Appl. Microbiol. Biotechnol.* 98(2), 763–75. doi:10.1007/s00253-013-5355-2.

Chapter 12

Ruminal protein breakdown and ammonia assimilation

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1 Introduction

Ruminal proteolysis has been studied for decades to optimize adequacy of nitrogenous precursors to support microbial protein production while minimizing excess proteolysis, which would be wasteful nutritionally, economically, and environmentally (Schwab and Broderick, 2017). Those authors underscored the need for adequate rumen-degraded protein (RDP) to optimize fiber degradability in the rumen and even the hindgut to ensure high feed intake, productivity, and feed efficiency. Excess N that is not captured in animal products also can lessen energetic efficiency of the animal (Reed et al., 2017). Despite these efforts, a better understanding of microbial processes (Huws et al., 2018; Reed et al., 2015) and management decisions (Foskolos and Moorby, 2018) limits further improvements in N efficiency in the ruminant livestock systems. Minimizing N excretion per unit of N intake is not necessarily a viable option if N excretion is simply lowered more than N intake; depressed feed intake or productivity will increase other managerial costs and, notably, increase both economic and dietary investment in replacement animals.

Microbial proteolysis (Walker et al., 2005), ammonia assimilation (Pengpeng and Tan, 2013), and biosynthesis of amino acids and nucleic acids (Morrison and Mackie, 1997; Walker et al., 2005) have been reviewed, with much of the information derived from model bacteria or isotopic fluxes from

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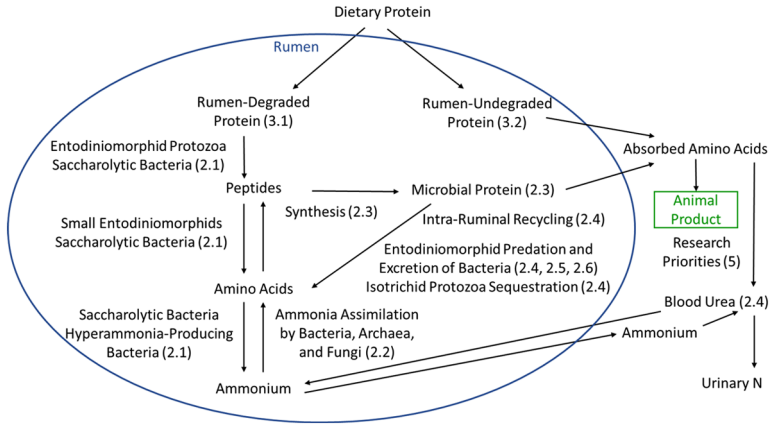


Figure 1 General schematic outlining ruminal nitrogen metabolism and its role in improving efficiency of conversion of dietary protein into animal product. Numbers in parentheses correspond with chapter section numbers in the text.

mixed cell cultures. Further information regarding metabolic feedback on amino acid biosynthesis has been described primarily in *E. coli* (Hackmann and Firkins, 2015b), but primary research in ruminal bacteria has lagged in the past 20 years. This review will follow the general outline depicted in Fig. 1 and will focus on newer information on N metabolism made available as a result of metagenomics while providing a perspective regarding how this information can build on ruminal N metabolism. A better mechanistic understanding of microbial proteolysis and capture of N as microbial protein hopefully then can be integrated into nutritional efforts to reduce variability and optimize ruminant animal productivity while minimizing its environmental impact.

2 Microbial nitrogen metabolism

2.1 Ruminal proteolysis and deamination

Walker et al. (2005) extensively reviewed the foundational microbiology literature on ruminal proteases, peptidases, and deaminases. Although the cellulolytic bacteria and archaea have low protease activity, other saccharolytic bacteria and protozoa have a variety of endoproteases that are both cell bound and secreted. Peptidases further the cascade toward dipeptides and amino acids. Dropping below a minimal ammonia-N concentration also poses risk for depressed ruminal digestibility, feed intake, and productivity in dairy cattle (Schwab and Broderick, 2017). However, adequacy of RDP (not just ammonia) also was stressed for beef cattle grazing poor quality pastures to stimulate efficiency of microbial protein synthesis (Bowen et al., 2017). Because ruminal concentration is a net of production and uptake, the statistical association of

ammonia concentration with efficiency of microbial protein synthesis also (in addition to RDP per se) reflects both carbohydrate fermentability and availability of growth factors such as branched-chain volatile fatty acids (Roman-Garcia et al., 2016). Clearly, ruminal proteolysis is not maximized compared with intestinal proteolysis because intestinal digestibility of rumen-undegraded protein (RUP) in feeds often exceeds 80% or even 90%. Exogenous proteases appear to improve microbial accessibility to fiber (Colombatto and Beauchemin, 2009) and starch (Ferraretto et al., 2015). Griswold et al. (2003) even documented that providing urea enhanced proteolysis of the same diet in continuous cultures.

Decreasing crude protein below 15% (and especially 12%) progressively increases the proportion of bacterial N assimilated by blood urea-N via the ruminal ammonia-N pool (Reynolds and Kristensen, 2008; Batista et al., 2017). Regulation of urea transport across the rumen epithelium helps provide ruminal ammonia when needed but reduces unnecessary and ineffective (ammonia absorption back into blood) urea recycling as ammonia-N concentration increases (Patra and Aschenbach, 2018). Urease activity in epimural bacteria (Mann et al., 2018) is likely more important for urea transport there, whereas urease activity in ruminal contents should be more important for salivary urea. Urease genes also are common in rumen bacteria in ruminal contents, including those from uncharacterized taxa (Jin et al., 2018). Those authors described urease systems but concluded that further research is needed to better understand how urease in ruminal bacteria is regulated.

For decades, research has designated a propensity for proteolysis among culturable bacteria. Reviews have summarized characterizations of microbial proteases (Walker et al., 2005) and associated those proteases with certain culturable bacteria, protozoa, and fungi (Hartinger et al., 2018). However, metagenomics approaches are now possible based on sequencing projects such as the Hungate 1000 (Seshadri et al., 2018) to lay the groundwork for subsequent studies assessing protease expression under different conditions. For example, normalized read abundance increased with extended time (1 h vs. 4 h) of incubation of perennial ryegrass for some but not all proteases, peptidases, and AA permeases in adherent bacteria (Mayorga et al., 2016). Fluorescent *in situ* hybridization detected signals from characterized proteolytic bacteria adhering to barley and corn grain, yet probes for serine proteases and metalloproteases also suggested important contributions from non-characterized proteolytic bacteria (Xia et al., 2016). Further research is needed to continue sorting out the functional role of proteolytic microbes that are predominantly adherent to particulate matter. Hence, ruminal sampling (particularly if from an oral tube) must obtain adequate particulate matter for a stronger inference to production situations (Paz et al., 2016).

Eukaryotic proteases are active in the rumen, ranging from plant and ciliate proteases (Walker et al., 2005) to fungal proteases (Hartinger et al., 2018). As

explained by the latter authors, particulate matter is required to represent both the adherent fungi and the protozoa that associate with particles (even if they only momentarily attach). Although research started more recently with fungi than protozoa, understanding of fungi is catching up in assessing their main niche: the degradation of recalcitrant fiber (Edwards et al., 2017). Pure cultures of fungi are being assessed under different conditions, but to our knowledge, the importance of their role in ruminal proteolysis is not well established.

In contrast with fungi, protozoa cannot be studied independent from bacteria competing with them in co-culture (Fondevila and Dehority, 2001), although some efforts are being renewed to understand why live bacteria are needed by protozoa (Park and Yu, 2018a). Hristov and Jouany (2005) reviewed studies assessing protozoal proteolytic capacity and noted that up to half of the predated bacterial protein can be excreted from protozoa, suggesting both a wasteful process and a potential benefit with extended time after feeding when these degraded fragments maintain bacterial growth between feedings. Cysteine and aspartate proteases were the main proteases in mixed protozoal digests (Forsberg et al., 1984). Those authors noted aminopeptidase activity being higher than deaminase activity, which could explain excreted fragments. Wallace and McPherson (1987) noted significant proteolysis but also deamination attributed to small entodinia. In contrast with the common notion of inexorable autolysis, Diaz et al. (2014) discussed that autolysis of isotrichids is exacerbated under certain culture conditions that also prevent their chemorepellance away from those lytic conditions. Clearly, there are major differences among ciliate species that deserve further research. Attributing all of the decrease in ammonia concentration to removal of proteolytic ciliates by defaunation also ignores that they rely on preformed amino and nucleic acids, whereas the protozoal void will be occupied by bacteria that extensively assimilate ammonia-N (Hackmann and Firkins, 2015b).

Proteolysis should be studied with viable ciliates because they maintain eukaryotic organelles and vesicle trafficking to support both digestion of ingested feed and normal endogenous protein turnover that need not lead to autolysis (Diaz et al., 2014). Ubiquitin-aided proteolysis appears to be conserved in ruminal ciliates as in environmental ciliates (Liu et al., 2013). Assuming a similar process as in the model non-rumen ciliates, *Paramecium* and *Tetrahymena*, ingested particles must undergo a maturation process in which phagosomes fuse with lysosomes for digestion followed by excretion of residual materials and recycling of membrane components (Diaz et al., 2014). When compared with lysed protozoal preparations, proteolysis in viable cells might be delayed by normal trafficking processes of digestive vacuoles (i.e. potentially is overestimated by lysed ciliate preparations) and might be more active with lower pH (i.e. inside lysosomes) that would inhibit cellular growth (i.e. potentially is underestimated at neutral pH).

Whether from plant or recycled microbial proteins, deamination is a normal process to provide ammonia and growth factors (such as branched-chain volatile fatty acids) for other ruminal microbes (Walker et al., 2005), particularly for those such as the cellulolytics that have limited deaminase activity and rely on cross-feeding in the microbial consortium (Firkins and Yu, 2015). Typical proteolytic bacterial isolates maintain cell numbers or grow slowly when provided protein as the sole substrate (Wallace et al., 1997). Fermentation of AA likely is of less value for ATP generation compared with carbohydrate and should be partitioned more toward protein synthesis (and should improve growth efficiency) when carbohydrate is provided (Hackmann and Firkins, 2015b). Examples of peptidases and amino acid transporters are described in the following section and integrated with ammonia assimilation.

The so-called hyperammonia-producing bacteria (HAB) were discovered in the 1980s and have been discussed as having a significant role in ruminal deamination (Walker et al., 2005). Those authors stated that their numbers and diversity are greater than previously thought. Even since that time, though, this HAB niche has been further expanded. For example, some asaccharolytic HAB rely more on small peptides than on free amino acids and require unique ways to dispose of reducing equivalents (Wallace et al., 2004; Leong et al., 2016), which might not be replicated in pure culture. Inhibition of methanogenesis *in vitro* did not increase hydrogen recovery in microbial amino acids (Ungerfeld et al., 2019). Therefore, the link between reducing equivalent disposal and deamination (Hino and Russell, 1985) has still not been fully clarified in the rumen. Although typically enriched with protein or peptide media when isolated, the majority of HAB also could use carbohydrate as substrate (Bento et al., 2015) and therefore likely cross over into the saccharolytic niche. Many, but (not all) HAB isolates with high deaminase activity had low protease activity and were sensitive to ionophores (Shen et al., 2018). The relative contribution to proteolysis is challenging to quantify in the rumen (Rychlik and Russell, 2000) but likely varies among animals fed the same diet (Firkins et al., 2007) or perhaps periodically fluctuates because of interactions with other saccharolytic microbes or methanogens.

2.2 Ammonia assimilation

Ammonia is the major end product of digestion of dietary protein and non-protein nitrogen (urea and amino acids), as well as the major source of nitrogen for protein synthesis by ruminal bacteria (Cotta and Russell, 1997; Morrison and Mackie, 1997; Wallace et al., 1997). Indeed, ammonia is the preferred nitrogen source for growth of many bacteria and archaea. Results over a wide range of feed and N intakes demonstrate that 60–80% of bacterial N is derived from ammonia as a precursor (Mackie and White, 1990) with the balance coming

from di and tri-peptides and amino acids. Rumen microbes approach 10% N in organic matter (Czerkawski, 1976; Fessenden et al., 2017) unless storage carbohydrate increases as a result of unbalanced (typically N-limited) growth (Hackmann and Firkins, 2015b). Therefore, bacterial protein synthesis and growth are greatly affected by the efficiency of ammonia assimilation. Despite its importance and central role as an intermediate in the degradation as well as assimilation of dietary nitrogen by intestinal bacteria, our understanding of the mechanisms and regulation of ammonia assimilation in ruminal bacteria remains superficial.

2.2.1 Ammonium transport

Ammonium transport (Amt) proteins form a ubiquitous family of integral membrane proteins that specifically shuttle ammonium across membranes. In bacteria and archaea, Amt's are used as environmental NH_4^+ scavengers for uptake and assimilation of nitrogen, and current dogma posits that bacteria are prepared to expend energy in the form of ATP to procure a critical nutrient under growth-limiting conditions. It is possible that under conditions of high ruminal ammonia concentrations that passive diffusion of ammonia can occur and satisfy N requirements of the cell. The protonated and positively charged ammonium can exist in a deprotonated and gaseous form, ammonia. The gaseous ammonia is able to diffuse through the membrane and become protonated ammonium. The pKa of ammonium is 8.95 at 35°C and at physiological pH (6.5–7.5) only 1% of the total ammonium/ammonia exist in the gaseous ammonia (Martinelle and Haggstrom, 1997). Thus, microbial cells require an ammonium translocation mechanism and indeed ammonia transport systems are ubiquitous among bacteria isolated from a variety of habitats including the rumen and human gut systems.

Depending on the total ammonium/ammonia concentration, gaseous ammonia diffusion is potentially responsible for ammonia transport across the cytoplasmic membrane. The known transport protein, AmtB, is not expressed unless the ammonium concentration is very low (Soupene et al., 1998, 2002; van Heeswijk et al., 1996; Winkler, 2006). Further evidence provided by organisms completely lacking ammonium transport facilitators, *Escherichia coli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Salmonella enterica*, and *Saccharomyces cerevisiae* are still able to grow optimally with high concentrations of ammonium/ammonia (Detsch and Stulke, 2003; Meier-Wagner et al., 2001; Soupene et al., 1998). In contrast, mixed ruminal microbes have an increased concentration gradient with 160 mg/L higher concentration in the cytoplasm compared to extracellular concentration, indicating active transport of ammonium (Russell and Strobel, 1987). When concentration of ammonium becomes too low, facilitated transport is required to move ammonium across

the cytoplasmic membrane. Ammonium analogs, like methyl ammonium and ethyl ammonium, have been used to study the transport of ammonium across the bacterial membrane. Although useful, the results should be interpreted with caution for two primary reasons: transporters are very selective of their ligands especially for substrates as small as ammonium, and the diffusion of the three different molecules are not equal and can bias results (Kleiner, 1982; Kleiner and Castorph, 1982; Stevenson and Silver, 1977).

Once ammonium enters the cytoplasm, glutamate and glutamine are the key metabolic intermediates central to intracellular nitrogen cycling. Glutamate is the most abundant metabolite in the cell, 96 mM in *E. coli*, and directly links nitrogen metabolism with carbon metabolism via α-ketoglutarate, onto which ammonium is appended during ammonium assimilation (Bennett et al., 2009). Bacterial cells primarily incorporate ammonium into glutamate and glutamine, irrespective of the nitrogen conditions in the environment, which are then used as nitrogenous building blocks for a wide range of N-containing metabolites, including amino acids, purines, pyrimidines, and other metabolites.

2.2.2 Enzymatic pathways of ammonium assimilation

Current knowledge of enteric ammonium assimilation and regulation is largely based on research on the model Proteobacteria (*E. coli*, *Klebsiella*, *Salmonella)*, and *Bacillus* (as a model for the Gram-positive type system), which does not necessarily reflect dominant gut microbes from Bacteroidetes and Firmicutes (Reitzer, 2003; Van Heeswijk et al., 2013). Research on *Bacillus subtilis* provides evidence for the divergence in regulation and mechanisms of the ammonium assimilation genes (Gunka and Commichau, 2012). The enteric paradigm based on the model Proteobacteria is structured around two competing pathways that are inversely regulated depending on the ammonium concentration and nitrogen status of the cell (Fig. 2). These two pathways are described as the high-affinity pathway and the low-affinity pathway. The first pathway is employed under limiting concentrations of ammonium and contains three functional enzymes including an ATP-dependent glutamine synthetase (GS), glutamate synthase (GOGAT), and an ammonium transporter (AmtB). In contrast, the low-affinity pathway is utilized under excess (non-limiting) concentrations of ammonium. This pathway consists of a NAD(P)H-oxidizing glutamate dehydrogenase (GDH). In *B. subtilis*, the GS/GOGAT system is solely responsible for the assimilation of ammonium, while the GDH enzymes run the reverse reaction for catabolism of glutamate.

In addition to the differences in energy expenditure and catalytic mechanisms, the regulation of these pathways has been characterized in detail. In *E. coli*, an elegant balance of transcriptional regulation and post-translational modification orchestrates the total contributions of both pathways.

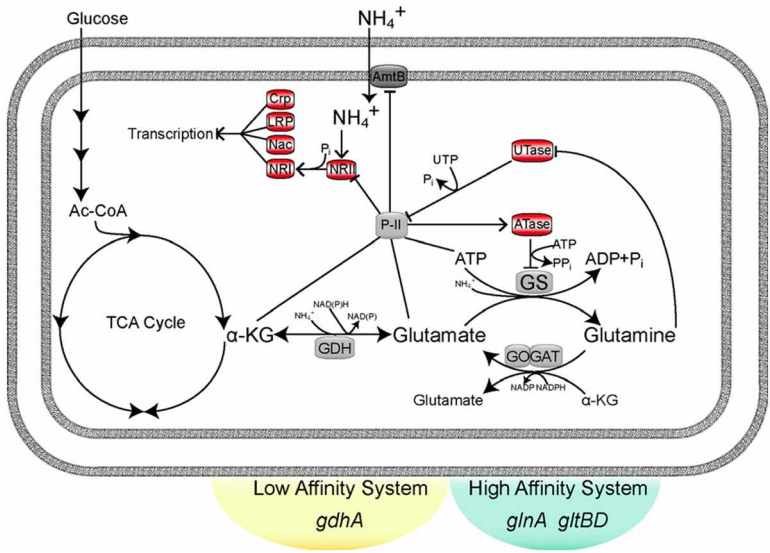


Figure 2 Enteric paradigm for ammonium assimilation based on *E. coli*. Enzymatic pathways include low-affinity and high-affinity pathways with the functional proteins glutamate dehydrogenase (GDH, *gdhA*), glutamine synthetase (GS, *glnA*), glutamate synthase (GOGAT, *gltB*), and the ammonium transporter (AmtB). The regulatory network includes UTase, ATase, and GlnB that modify the activity of functional proteins, and transcriptional regulators, NRI, NRII, and Nac, which regulate transcription of functional genes. Proteins encapsulated in red are not identified in Bacteroidetes by sequence homology. (PhD thesis, M. Iakiviak 2018).

The non-competitive binding of metabolic intermediates further modulates the differential regulation and function of the catalytic enzymes and regulatory proteins. Although helpful in understanding how an organism modulates enzymatic activity through transcriptional and posttranslational means, this paradigm fails to explain gut nitrogen utilization. The complicated machinery of the high-affinity pathway is more energy demanding than the low-affinity pathway. As such, a strong regulatory network is required to minimize the energy expenditure of the cell to optimize growth. Significant efforts have uncovered the intricate network of regulation that Proteobacteria have evolved to regulate the flux of metabolites through both pathways (Van Heeswijk et al., 2013). The model has been extended to include all gut organisms and is termed the enteric paradigm.

2.2.3 Mechanisms of regulation

An elegant model of regulation has been proposed through studies of model Gram-negative and Gram-positive bacteria (Van Heeswijk et al., 2013). Global

gene expression is also modulated primarily via NRI/NRII, a two-component system, and Nac, which is itself transcriptionally regulated by NRI/NRII. Rapid repression or activation of enzymatic occurs through the enzymatic modification of GS and GDH. The modulation of activity occurs through the regulatory proteins GlnB (also known as P-II), ATase (adenylyltransferase/adenylyl-removing enzyme), and UTase (uridylyltransferase/uridylyl-removing enzyme). In *B. subtilis*, the transcriptional regulators include TnrA and GlnR, which modulate transcription through protein–protein interaction with the functional enzymes (Fisher, 1999). Through very carefully fine-tuned enzymatic and regulatory pathways, organisms incorporate extracellular ammonium into intracellular α-ketoglutarate and glutamate to produce glutamate and glutamine, respectively.

In *E. coli*, the functional proteins are transcriptionally regulated by the aforementioned two-component system NRI/NRII, as well as several other regulators including Nac, CRP-cAMP, IHF, Lrp, and ArgR (Van Heeswijk et al., 2013). The sensing protein, NRII, binds to ammonium and undergoes autophosphorylation, subsequently transferring the phosphate to the response regulator NRI. The phosphorylated NRI goes on to increase transcription of *glnA*, *glnK*, *amtB*, *nac*, and other genes (Magasanik, 1989). Interestingly, Nac represses *gdhA* without a coeffector molecule or covalent modification. The bacterium responds to amino acid deficit through Lrp by increasing transcription of *gltBD*, and to energy (ATP) deficit through Crp-cAMP inhibiting *gltBD* expression and modulating a basal level of expression of *glnA* (Van Heeswijk et al., 2013).

In the model Firmicute, *B. subtilis*, three transcriptional regulators have been identified, GlnR, TnrA, and GltC (Fisher, 1999; Fisher and Wray, 2002; Schumacher et al., 2015; Wray et al., 2001). The transcriptional activity of GlnR is mediated by the binding of GS, stabilizing DNA interaction when bound, and is affected by pH. In contrast, TnrA is inactive when bound to glutamine synthetase. Additionally, TnrA can be titrated away from DNA by association with the membrane by interactions with GlnK and AmtB. Finally, GltC is responsible for the activation of transcription of glutamate synthase under increased glutamate demand during higher growth rates (Gunka and Commichau, 2012). The P-II proteins are central to regulation of protein activity as they incorporate signal from the intracellular metabolite pool and modulate enzymatic activity as well as transcription. A P-II (GlnB) and a P-II like protein (GlnK) are encoded by *glnB* and *glnK*, and *glnK* is commonly found adjacent to *amtB* (Arcondeguy et al., 2001; Blauwkamp and Ninfa, 2002; Detsch and Stulke, 2003; Forchhammer, 2008; Van Heeswijk et al., 1996). P-II proteins are homotrimers and possess binding sites for α-ketoglutarate and ATP, as well as uridylylation sites by which UTase acts as an efficient glutamine sensor. In addition, P-II proteins can undergo adenylylation in mycobacteria, phosphorylation in cyanobacteria, or remain unmodified (Forchhammer, 2008; Gunka and Commichau, 2012; Williams

et al., 2013). Several proteins directly interact with P-II including AmtB, ATase, NRII, and UTase in proteobacteria, as well as TnrA in *B. subtilis*. The transport of ammonium across AmtB is regulated by direct insertion of a P-II loop into the transport channel of the trimeric AmtB, preventing ammonium transport. This interaction is inhibited by UTase uridylylation of P-II at the loop (Reitzer, 2003).

Glutamine synthetase type 1 (GS-1) activity is regulated via covalent modification by the ATase in Proteobacteria. The ATase adenylylates a subunit of the homododecameric GS-1 and inactivates that subunit. Since a range of adenylylation states can exist (between 0-12), GS-1 can exist in a range of activities. ATase is also capable of activating GS-1 by the deadenylylation activity present within the same polypeptide. The regulation of adenylylation/deadenylylation is mediated by P-II interaction with the ATase. The regulatory activity of P-II toward ATase is dependent on its uridylylation state via the UTase's ability to uridylylate or deuridylylate P-II. The UTase uridylylation/deuridylylation activity is affected by glutamine and other small molecules (Fig. 3). Finally, transcription of ammonium assimilatory genes is also affected by P-II through its interaction with NRII. Interaction between P-II and NRII is affected by the metabolites ATP and α-ketoglutarate, resulting in decreased autophosphorylation under energy and nitrogen abundance. Extension of the enteric paradigm to representatives of the Bacteroidetes (*Prevotella*) and Firmicutes (*Ruminococcus*) phyla that are abundant in the rumen cannot be direct, as both genera lack homologs to the ammonium assimilation regulators, namely the two-component system (NRI/NRII), covalent modifiers (ATase/UTase), and the transcriptional regulators. In addition, conflicting reports exist concerning the dominant enzymatic activity that *Bacteroides* sp. exhibit under varying nitrogen availability.

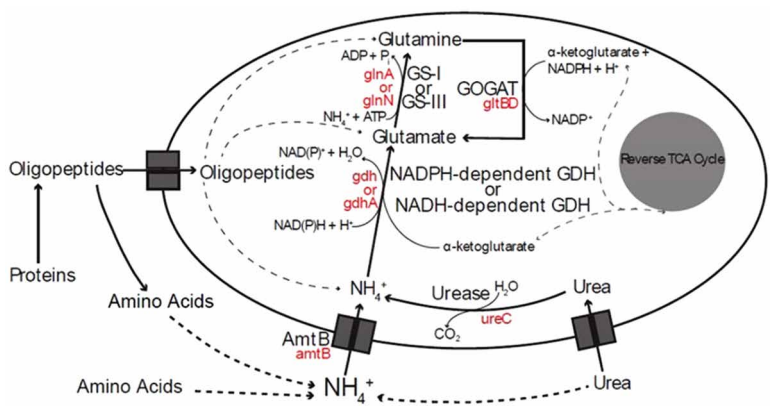


Figure 3 Nitrogen metabolism pathways identified in *Ruminococcus albus* 8 through bioinformatic analyses (Kim et al., 2014).

2.2.4 Ammonium assimilation in rumen bacteria

Three major discoveries from research on human colonic *Bacteroides* reveal a widely different nature of ammonium assimilation and regulation from the enteric model described above. This potential paradigm shift includes increased GDH activity under nitrogen limitation, increased GS transcription under ammonium excess, and an entirely new family of glutamine synthetases (GSIII) that are common in gut anaerobes (Kim et al., 2014, 2017). Features of regulation are critical to ascertain before a true enteric model can be applied to microbial ammonium assimilation within the human or animal gastrointestinal tract. In addition, the multiplicity of functional genes needs to be addressed to identify the roles for each putative glutamine synthetase and glutamine dehydrogenase encoded within gut Bacteroidales. Although investigations into ammonium assimilation within Bacteroidetes are limited, the breadth of the information primarily describes deviations from the classical enteric paradigm.

Recent genomic and transcriptomic research on two model rumen bacteria, *Ruminococcus albus* (a specialist plant cell wall degrading species with a Gram-positive cell wall type within the Firmicutes phylum; Kim et al., 2014) and *Prevotella ruminicola* (a metabolically versatile, Gram-negative cell wall type of bacterial species within the Bacteroidetes phylum; Kim et al., 2017) has recently been published and is summarized here to provide a case study relevant to ammonia assimilation and N metabolism in two representative rumen bacteria.

Bioinformatic analysis of the *Ruminococcus albus* 8 draft genome sequence (3.8 Mb) revealed a number of genes encoding enzymes critical for nitrogen metabolism and ammonia assimilation (Fig. 3). We identified genes for an NADH-dependent GDH (*gdh*), an NADPH-dependent GDH (*gdhA*), a high-affinity ammonium transporter (*amtB*), a regulatory protein (*glnK*), a putative urease, two different types of GS (type I GS [*glnA*] and type III GS [*glnN*]), and the two subunits of a typical bacterial GOGAT (large subunit [*gltB*] and small subunit [*gltD*]). *R. albus* 8 was grown with ammonia, urea, peptides, or amino acids as the nitrogen source, and transcript abundances and enzymatic activities of nitrogen metabolism proteins were analyzed to investigate nitrogen utilization and flux.

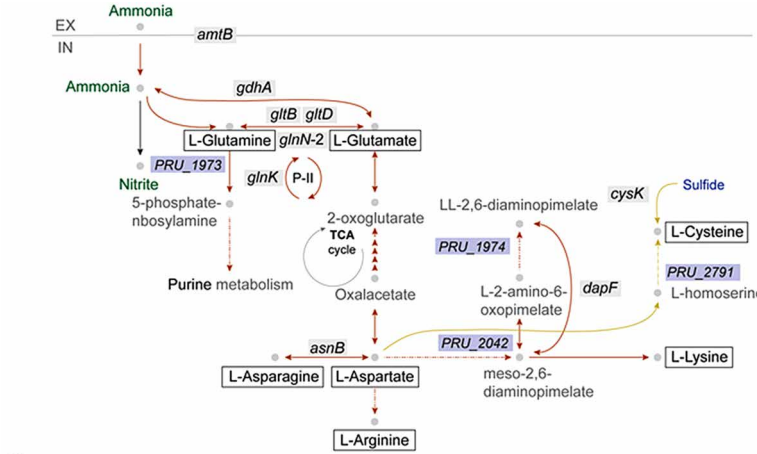
R. albus 8 utilized ammonia and urea and showed similar growth patterns on both substrates. *R. albus* 8 was also able to grow on peptides as a nitrogen source; although the growth yield was lower than that on preferred nitrogen sources (urea and ammonia), the maximum specific growth rate did not change. This is the first report of *R. albus* 8 using peptides as a nitrogen source. Bioinformatic analysis of the draft genome sequence revealed that *R. albus* 8 is also equipped with several peptide transporters and peptidases for uptake and

utilization of peptides, including three peptide ABC transporters, two dipeptide ABC transporters, and 25 genes involved in peptidase metabolism. This finding is also consistent with patterns of nitrogen utilization in the rumen, where free amino acids are rapidly deaminated to produce ammonia and amino acids are transported into bacterial cells in peptide form, enabling energy conservation at the transport level.

Prevotella ruminicola strain 23 is non-cellulolytic, but can efficiently degrade hemicellulose and pectin and could potentially degrade the proteoglycan of the host. Regarding nitrogen metabolism, *P. ruminicola* 23 can efficiently utilize both ammonia and peptides (preferentially larger peptides, up to 2 kDa) as a nitrogen source for growth. For breakdown of oligopeptides, *P. ruminicola* 23 harbors the greatest range and specific activity of dipeptidyl peptidases in comparison to species belonging to other abundant genera in the rumen, such as *Butyrivibrio*, *Ruminococcus*, and *Fibrobacter*. Whole genome transcriptional responses to environmental changes in the available nitrogen source and ammonium concentrations in *P. ruminicola* 23 were studied under defined medium and culture conditions, as well as proteome changes and the enzymatic activity of central enzymes in ammonium assimilation (Fig. 4).

Growth studies showed that *P. ruminicola* 23 can efficiently utilize peptides and ammonium as nitrogen sources for growth, but not amino acids (Kim et al., 2017). Shifts in its overall transcriptional profiles were shown when growth occurred on ammonium or peptides; specifically, ammonium assimilation pathways were not induced when the bacterium was grown on peptides. These results suggest that the bacterium utilizes peptides directly for protein synthesis after uptake and intracellular hydrolysis and entry into the intracellular amino acid pool. Interestingly, growth on ammonium sulfate was not observed in the absence of supplementation with methionine. Methionine is stimulatory and might be essential for growth of *P. ruminicola* 23, although this is likely not as a N source but as a methyl or methanethiol (CH_3S) donor in other biosynthetic reactions.

These results collectively show that *P. ruminicola* 23 responds to changes in environmental nitrogen differently from enteric species of Proteobacteria, such as *E. coli* or *Salmonella* spp (Kim et al., 2017). These responses reflect differential transcriptional regulation of genes involved in nitrogen metabolism and variations in related enzyme activities (Fig. 3). Previous studies had shown that *P. ruminicola* possesses both NADP- (anabolic) and NAD-dependent (catabolic) GDH activities. Correspondingly, higher transcript abundances of *gdhA* and elevated NADP-GDH activity were detected under excess concentrations of ammonium. The GS-GOGAT pathway constitutes a major ammonium assimilation pathway for enteric bacteria grown under ammonium-limiting conditions. Nevertheless, transcription of GS-GOGAT genes was highly induced in non-limiting concentrations of ammonium in *Prevotella*



Key

Gene/Locus tag	Gene product	Pathway
amtB	Ammonium transporter	
glnK	Nitrogen regulatory protein P-II	
gdhA	Glutamate dehydrogenase (NADP-dependent)	Glutamate and glutamine metabolism
glnN-2	Glutamine synthetase type III-2	Glutamate and glutamine metabolism
gltB	Glutamate synthase, large subunit	Glutamate and glutamine metabolism
gltD	Glutamate synthase, small subunit	Glutamate and glutamine metabolism
PRU_2791	O-acetylhomoserine aminocarboxypropyltransferase	Lysine biosynthesis
cysK	Cysteine synthase A	Cysteine biosynthesis
dapF	Diaminopimelate epimerase	Lysine biosynthesis
PRU_1974	Aminotransferase, homolog	Lysine biosynthesis
PRU_2042	Diaminopimelate dehydrogenase	Lysine biosynthesis
PRU_1973	Glutamine amidotransferase	Glutamate metabolism
asnB	Asparagine synthase	Asparagine biosynthesis

Figure 4 Metabolic networks for nitrogen utilization in *Prevotella ruminicola* 23 (Kim et al., 2017). The bacterium can utilize both ammonium and peptides for growth through activation of different biochemical pathways. In contrast to *E. coli* and *Salmonella* spp., growth on non-limiting ammonium conditions is maximized by both GDH and GS/GOGAT-dependent ammonium assimilation. Growth on peptides might rely on extracellular hydrolysis and transport of resulting amino acids, or intracellular deamination of imported oligopeptides. High induction of genes involved in cysteine synthesis could indicate generation of labile amino acids in the cell. Chemical/biochemical species are represented by grey circles. Nitrogen-containing species are represented in green, and sulfur-containing species are depicted in blue. EX and IN stand for extracellular and intracellular cell locations, respectively. Dashed arrows are used for clarity in order to represent that many steps are implicated in the generation of the displayed species. Red arrows represent pathways induced on ammonium and yellow arrows represent pathways induced on peptides.

ruminicola 23. In contrast to the enteric paradigm, our results demonstrate that *P. ruminicola* 23 utilizes the high substrate affinity GS-GOGAT enzymatic system to grow in non-limiting ammonium conditions, a pattern that has also been observed in *Ruminococcus albus* 8. To our knowledge, this is the first description of an organism that uses both GDH and GS-GOGAT pathways for ammonium assimilation when grown under non-limiting concentrations of ammonium. Even though the GS-GOGAT system requires ATP, and its down-regulation would prevent energy waste when ammonium assimilation relies on GDH, the observed behavior could reflect simultaneous utilization

of GDH and GS-GOGAT pathways to maintain the glutamate pool for the biosynthesis of amino acids. This may represent an evolutionary adaptation of strain 23 to rumen conditions, where nitrogen concentrations are rarely growth limiting (ammonium concentration typically ranges from 4 to 70 mM). This strategy would enable the organism to maintain an active growth in the natural environment, outcompeting other microbes in the utilization of ammonium as a nitrogen source, consistently agreeing with the observation that *Prevotella* constitutes the most abundant reported bacterial genus in the rumen.

Collective genomic and proteomic results provide strong evidence that GSIII-2 is the main enzyme implicated in ammonium assimilation when the nitrogen is non-limiting. Transcript abundances for the GOGAT genes were also higher than 22 fold on ammonium. Therefore, the GSIII-2–GOGAT coupling of enzymes is likely to play a major role in ammonium assimilation and ammonium recycling in *P. ruminicola* 23. An increased transcript abundance of the ammonium transporter coding gene (*amtB*) was shown during growth on non-limiting concentrations of ammonium, which suggests the ability of *P. ruminicola* 23 to detect and respond to fluctuations in the environmental ammonium concentration. However, when concentrations of ammonium became limiting, the bacterium induced peptide and polyamine ABC transporters, suggesting its capability to scavenge more complex and/or alternative available nitrogen sources. In addition, transporting peptides at the level of a di- or tri-peptide (1/2 or 1/3 ATP per amino acid transported) would enable the cell to conserve energy compared to the transport of a single or free amino acid (1 ATP per amino acid transported. Transcript abundances for the nitrogen regulatory protein P_{II} gene, *glnK*, were also higher on non-limiting ammonium. The most recent mechanistic insight into the signaling role of the P_{II} protein suggests that a post-translational change driven by changes in its observed ATPase activity under fluctuating nitrogen levels sensed by intracellular α-KG would facilitate or inhibit ammonium uptake through the ammonium transporter channel. The *glnK* gene can be located upstream or downstream of *amtB*. This genetic linkage is highly conserved, and both resulting proteins are functionally related.

In summary, *P. ruminicola* 23 utilizes both NADP-GDH and the GSIII-2–GOGAT pathways for ammonium assimilation when nitrogen is non-limiting for growth. This may reflect an adaptation of *P. ruminicola* 23 to a more stable concentration of ammonium in the rumen relative to other environments (e.g. human gut) by enhancing glutamate production and maintaining the intracellular glutamate/glutamine pool for amino acid, purine/pyrimidine, and cell wall biosynthesis. In contrast, under limiting concentrations of ammonium, *P. ruminicola* 23 may utilize basal levels of GDH or GS-GOGAT pathways to assimilate ammonium and synthesize amino acids.

2.2.5 Archael nitrogen metabolism

Much less is known about nitrogen metabolism in archaea than in bacteria. Methanogens in general use nitrogen in the biosynthesis of amino acids, purines, and pyrimidines and by employing most of the same reactions as Bacteria. In addition, ammonia assimilation in methanogens is similar to that seen in bacteria (DeMoll, 1993).

The availability of the complete genome sequences of several members of the euryarchaea has enabled new approaches to the understanding of methanogen physiology and biochemistry, including metabolic reactions involving nitrogen compounds in methanogens. *Methanobrevibacter smithii* is the dominant human-gut-associated hydrogenotrophic methanogen and uses ammonia as the preferred nitrogen source. The *M. smithii* proteome contains a transporter for ammonium (*AmtB*; *MSM0234*) plus two routes for its assimilation: (i) the ATP-dependent glutamine synthetase–glutamate synthase pathway, which has a high affinity for ammonium and thus is advantageous under nitrogen-limited conditions, and (ii) the ATP-independent glutamate dehydrogenase pathway, which has a lower affinity for ammonium (Samuel et al., 2007). Hansen et al. (2011) used RNA-Seq to perform expression profiles on five *M. smithii* isolates when grown on medium containing ammonia as a nitrogen source. Both pathways were expressed in all strains, with 0.4–1.21% of reads mapping to enzymes involved in assimilation of ammonia. The energy-dependent GlnA pathway was generally expressed at a much higher level than the low-affinity GDH pathway, although strain-specific differences in levels of expression were noted.

Methanococcus maripaludis and *Methanosarcina mazei* are both mesophilic methanogenic members of the Euryarchaeota, and studies have enriched and extended our knowledge of nitrogen regulation (Leigh and Dodsworth, 2007). Both species assimilate ammonia apparently by the GS/GOGAT pathway, and both can fix nitrogen. Both species contain a *nif* operon composed of homologs to bacterial dinitrogenase and dinitrogenase reductase genes and other *nif* genes involved in nitrogenase cofactor synthesis. *GlnA* and *nif* genes are regulated as expected by nitrogen conditions, and GS and nitrogenase activities are also regulated. Not surprisingly, studies in the Archaea have revealed new aspects of nitrogen regulation. Highlights from studies of *M. maripaludis* and *M. mazei* include the novel transcriptional repressor NrpR, direct regulation of GS by GlnK, and direct regulation of nitrogenase by NifI (Leigh and Dodsworth, 2007). Much further research is required on ruminal methanogens into the present research capabilities and promise.

2.2.6 Anaerobic rumen fungi

The nutritional requirements of ruminal fungi are relatively simple. Lowe et al. (1985) reported that they grow in medium lacking amino acids, implying that

ammonia can be used to synthesize all amino acids. Orpin and Greenwood (1986) also demonstrated that *Neocallimastix patriciarum* grew in a defined medium, and that growth was stimulated by amino acids, particularly glutamate, serine, and methionine. However, little is known concerning amino acid metabolism in ruminal fungi, or the extent to which fungi may incorporate amino acids (Theodorou et al., 1994). In order to evaluate the extent of de novo synthesis of individual amino acids in *Piromyces communis* and *Neocallimastix frontalis*, isotope enrichment in amino acids was determined during growth on $^{15}NH_4Cl$ in different media (Atasoglu and Wallace, 2002). Most amino acid N and hence cell N for *P. communis* and *N. frontalis* continued to be formed *de novo* from ammonia when 1 g l^{-1} trypticase was added to the medium; this concentration approximates the peak concentration of peptides in the rumen after feeding. Higher peptide/amino acid concentrations decreased de novo synthesis. Lysine was exceptional, in that its synthesis decreased much more than other amino acids when Trypticase or amino acids were added to the medium, suggesting that lysine synthesis might limit fungal growth in the rumen. These results need further study with more modern approaches to resolve which nitrogen sources are being utilized during rumen fermentation and metabolism.

2.2.7 Ciliate protozoa

The largest and most obvious protozoa in the rumen are the ciliates, which are divided into two main groups, the so-called isotrichid (Order: Vestibuliferida) and the entodiniomorphid (Order: Entodiniomorphida) protozoa that differ not only in morphology but also in their metabolism. The entodiniomorphid protozoa, although able to take up soluble compounds, feed principally by the engulfment of particulate matter. All entodiniomorphid protozoa, whether grown in vivo or in vitro, have bacteria in digestive vesicles in the cytoplasm and there is evidence that the species present reflect those in the surrounding medium and include methanogens (Williams and Coleman, 1997). Bacteria probably provide the most important source of nitrogenous compounds for protozoal growth, although plant protein and free amino acids also represent a valuable source with some species. After engulfment, which may be selective although this is not always consistent, bacteria are completely digested in a digestive vacuole. On incubation of ^{14}C labeled amino acids in *E. coli* with a suspension of entodiniomorphid protozoa some of the labeled amino acids are incorporated into protozoal protein, some may be incorporated as a related amino acid, and the remainder is released into the cell amino acid pool or the medium. Constituents of bacterial nucleic acid are incorporated into protozoal nucleic acid, with the transfer taking place at the nucleotide level (Williams and Coleman, 1997).

The isotrichid ciliate protozoa occurring in the rumen are mostly the genera *Isotricha* and *Dasytricha*, and are easily observed under the microscope

because of their size and motility compared to rumen bacteria. They are ellipsoid-shaped organisms with cilia covering the complete external surface of the cell. Both *Isotricha* and *Dasytricha* will ingest rumen bacteria and non-rumen bacteria, although some selectivity of bacteria being ingested has been observed. Following engulfment, the bacterial cells rapidly lose viability and are extensively degraded. Unchanged bacterial amino acids are directly incorporated into protozoal protein. The Isotrich protozoa obtain some of their nitrogen requirements from ingestion and digestion of bacteria, but they are also able to take up amino acids from the medium. These amino acids are assimilated directly and they are also capable of excretion of nitrogenous material (Williams and Coleman, 1997).

2.3 Microbial protein synthesis

Preformed amino acids can stimulate efficiency of microbial growth (Hackmann and Firkins, 2015b), but the majority of microbial protein in ruminants fed standard protein diets still is from amino acids synthesized using assimilated ammonia, which is underestimated when plant N contaminates bacterial samples (Ahvenjärvi et al., 2018). Microbial protein synthesis has been reviewed in efforts to improve our ability to improve both accuracy and precision of predicting microbial protein flow to the duodenum (Firkins et al., 2007; Hartinger et al., 2018). However, assessing microbial protein synthesis in vivo still has considerable variation that awaits explanation (White et al., 2016), so further improvements in accuracy and precision should improve mechanistic integration of RDP with ruminally available carbohydrate for beef (Galyean and Tedeschi, 2014) and dairy (Van Amburgh et al., 2015; White et al., 2017b) cattle. Ammonia concentration must be adequate to optimize microbial protein synthesis (Schwab and Broderick, 2017). However, ammonia concentration is hard to predict (Firkins et al., 2007) and clearly depends on adequacy for microbes (Ahvenjärvi and Huhtanen, 2018). Amino-N could be limiting the efficiency of microbial protein synthesis, particularly when there is high rumen availability of carbohydrate; increased availability of preformed AA could increase efficiency of growth enough to coincidentally increase assimilation of ammonia into AA (Firkins et al., 2007). Thus, there is a need to integrate N metabolism with ruminal carbohydrate availability. Assimilation of ammonia can increasingly rely on ATP as ammonia decreases (Section 2.2), and these reactions also might influence how unique ATP-yielding pathways can be used for advantage (Hackmann et al., 2017) compared with assimilation of central metabolites into cell constituents. Increasing the ratio of energy used for cell growth:maintenance functions needs further attention (Hackmann and Firkins, 2015b).

Microbial protein production typically is measured as a net value of synthesis and degradation. Protozoal outflow vs. retention and autolysis likely is increased

with faster passage rate from the rumen (Firkins et al., 2007) but has received limited research attention and particularly with respect to sequestration by isotrichids (also called holotrichs) but not entodiniomorphs (Diaz et al., 2014) and biomass:cell (Firkins and Yu, 2015). Previous models assumed no outflow of protozoal protein, which can cause significant error in predicting intestinal supply of microbial amino acids, particularly lysine (Sok et al., 2017; Fessenden et al., 2017). Those authors also noted differences in amino acid profile of the fluid- and particulate-phase bacteria. Moreover, increased bacterial protein flow was associated with the latter bacteria passing with rumen-undegraded fiber (Sauvant and Nozière, 2016). Consequently, passage versus recycling is intricately linked with proteolysis and ammonia assimilation and will therefore be discussed subsequently.

2.4 Intra-ruminal recycling of microbial protein

Normal turnover of microbial protein includes synthesis through cell growth to replace those cells that pass to the duodenum plus those that recycle within the rumen. Cell lysis is much more energetically wasteful than recycling of ammonia (Firkins et al., 2007). Protozoa have long been considered the main culprits contributing to excess proteolysis and recycling of previously synthesized microbial protein (Hristov and Jouany, 2005). However, recycling is challenging to assess in vivo because of incomplete mixing of $^{15}NH_4$ doses. Labeling feed fractions with ^{15}N fertilizer and an extensive sampling scheme allowed a model fit in dairy cattle (Ahvenjärvi et al., 2018). They noted that most of the protozoal protein was derived from bacterial protein. However, intra-ruminal N recycling was estimated at 22%, which is a value much lower than previous estimates primarily using sheep. Those authors suggested about 15% of the omasal microbial N was protozoal N, which agrees with other reports (Fessenden et al., 2019; Sok et al., 2017) and contrasts with models that assume no outflow (i.e. 100% of protozoal recycling via sequestration), yet they also noted about 40% selective retention of protozoa. As discussed previously, most of the selective retention would be expected to be from isotrichids, which might have been abundant in that study but were not enumerated.

Intra-ruminal recycling probably has been inflated by overestimating both 1) bacterial cells consumed through predation and 2) protozoal biomass, which also factors through an increased amount of bacteria ingested. Bacterial predation is overestimated when assessed with these cultures because protozoa typically were starved before being dosed only bacteria; omitting feed allows easier accounting for the counts of dosed bacteria, but it also would deprive normal substrate. As summarized by Williams and Coleman (1992), clearance of infused *E. coli* declined by 0, 0, 18, and 68% when mixed entodinia were fed 10, 52, 260, and 1670 rice grains per protozoan cell, respectively. Similar limitations

persist when comparing faunated versus defaunated (with vs. without protozoa) animals; that is, the difference between these observations ignores interactions by protozoa with prokaryotes and fungi. Clearly, these types of observations are very helpful (Newbold et al., 2015), but care must be exercised in interpretation.

The original estimations of protozoa up to 50% of biomass might have stemmed from papers that overestimated protozoal volume using geometric formulas that did not fit their shape (Wenner et al., 2018), did not account for bacterial contamination (Sylvester et al., 2005; Firkins et al., 2007), had marker problems (Sok et al., 2017), and deterministically discussed sequestration of all protozoa in the rumen, whereas only the isotrichids are highly sequestered (Diaz et al., 2014). Based on cell counts, Karnati et al. (2007) determined that generation time of protozoa (primarily entodiniomorphids) in lactating dairy cattle was approximated by the ruminal retention time of particulate matter. An elaborate model based on greater cell counts in the rumen versus omasum suggests greater sequestration and intra-ruminal recycling (Hook et al., 2017) but also ignores that omasal counts can be diluted by passage of unmixed drinking water and autolysis of cells resulting from backflow of abomasal acid can occur between the time of animal euthanization and sample collection (Firkins and Yu, 2006). Protozoa might be up to 50% of the microbial biomass for sheep fed at low intakes, but this 50% value should not necessarily be extrapolated to production situations (Firkins et al., 2007). Hence, 25% of the biomass is likely a more appropriate value, at least for dairy cattle (Ahvenjärvi et al., 2018).

Protozoa do consume bacteria, fungal zoospores, and (in lower amounts) smaller protozoa (Hartinger et al., 2018) and digest them incompletely (Hristov and Jouany, 2005), which lessens efficiency of microbial protein synthesis. Because of an increasing expectation for fungal involvement in fiber degradation (Edwards et al., 2017), the antagonism by protozoa against fungi is likely not beneficial to the host. Potential selective predation of certain bacteria, including fibrolytics (Park and Yu, 2018b), contrasts with the general expectation for protozoa to improve fiber digestibility (Newbold et al., 2015). A meta-transcriptomics screening of ruminal contents supported the importance of protozoa and fungi in fiber digestibility (Comtet-Marre et al., 2017). This work suggests revisiting the standard assumption of the main benefit from protozoa being indirect (e.g. O_2 consumption or rapid starch ingestion by protozoa). Perhaps, selective inhibition to limit the sequestering isotrichids (Newbold et al., 2015) would be beneficial, yet roadblocks in strategies such as vaccines still remain (Hartinger et al., 2018).

Approaches to selectively limit protozoa need to prevent oversimplification of results. For example, counts of protozoa were directly associated with the ratio of methane produced per unit of dry matter intake; however, meta-regression also documented that those counts also were inversely associated with fiber digestibility and dry matter intake (Guyader et al., 2014). Dry matter intake must be considered with methanogen-suppression approaches (Ungerfeld,

2018). Similarly, dietary protein (presumably through RDP) is positively associated with dry matter intake in dairy cattle (Zanton, 2016). Protozoa might outcompete bacteria (their protein sources) with decreasing dietary protein and more reliance on blood urea-N (Oelker et al., 2009). The advantage of urea-N diminishes with increasing concentration of dietary protein, particularly to dietary protein (and presumably RDP) concentrations needed to maintain milk production, as discussed in section 2.1.

Reliance on increasing blood urea-N to subsidize decreased dietary crude protein concentration is a questionable strategy for dairy cattle because of the potential depression of feed intake but also because typically the duodenal amino acid profile is more favorable for production compared with the original feed protein (Schwab and Broderick, 2017). Depressed microbial protein supply to the duodenum compared with its predicted supply either limits productivity or requires a safety factor for increased feeding of RUP; unfortunately, RUP is much more expensive than RDP, more variable in intestinal digestibility (even more so with respect to individual AA), and also contributes extensively to blood urea recycling to the gut and urinary N excretion (Batista et al., 2017). Those authors documented a strong potential for beef cattle to transfer blood urea-N transfer into bacterial protein.

2.5 Case study to advance understanding of protozoa-mediated proteolysis and intra-ruminal recycling of microbial protein

Williams and Coleman (1992) reported that single cultures of protozoa cleared bacteria 1.5- to 17.6-fold faster than did protozoa collected *ex vivo*. Thus, Belanche et al. (2012) set up an intricate *ex vivo* sampling protocol to quantitatively and mechanistically assess intra-ruminal recycling of microbial protein. Among the many conclusions outside of the current scope, the greater bacterial lysis in the protozoa recovered on the 20-μm sieve was from a combination of higher specific activity and higher abundance of biomass (Fig. 5). The authors noted that this fraction represents the more bacterivorous small entodinia such as *Entodinium caudatum*. These populations now can be assessed with an increasing repertoire of species-specific primers (Ishaq et al., 2017), which provide an alternative to traditional counting methods (Kittelmann et al., 2015). However, counts can be combined with microscopic measurements to estimate cell volume, with the assumption that volume is proportional to metabolic activity. Authors should consider real-time videography and the ellipsoid shape tool available in imaging software, which better capture width:depth ratios that vary among taxa but probably also by inhibitors against protozoa (Wenner et al., 2018).

Extending this approach, fractions could be split into a control that is starved during filtering and washing (as done in this study) to be compared to

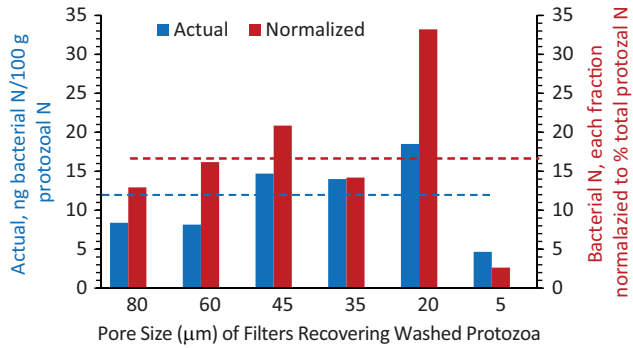


Figure 5 Lysis of bacterial N (determined by pre-enriching ruminal fluid-associated bacteria with ^{14}C) administered to protozoal fractions that were fractionated by successively smaller pore sizes of nylon mesh (Belanche et al., 2012). The activities are as reported (Actual; blue dashed line is the weighted mean of 12.0 ng bacterial N lysed per 100 g protozoal N/h) or were re-derived by the current authors by weighting the contribution of each fraction's recovered protozoal N as a percentage of the sum of all fractions (Normalized; red dashed line is mean of 16.7% = 100%/6 fractions).

the same protozoa also fed purified (N-free) cellulose, starch, and glucose (Ye et al., 2018). Would lysis of isotopically labeled bacteria be different if those labeled bacteria were dosed by themselves compared with being dosed at the same time as purified carbohydrates? Feeding substrate likely lessens predation of fluid-associated bacteria because of competition for intracellular space available for digestive vacuole formation (Diaz et al., 2014). Those authors used beads that mimic the size and cell wall charge of bacteria, which might be an alternative for researchers who cannot use radioisotopes to assess bacterial predation. In that report, increasing bead dosing rate increased bead uptake, but bead uptake was decreased when co-introduced with normal substrate. These findings also beg the question: does measured bacterial lysis increase with increasing dosage of bacteria? If so, what bacterial dosing rate represents in vivo conditions in which fluid-associated bacteria are in lower abundance and probably lesser functional importance than particulate-phase bacteria that are adherent to plant fragments that also are protozoal substrate?

Ex vivo approaches can advance our knowledge of bacterial lysis in the rumen of cattle differing in protozoal population abundance, community type, and activity per cell. Ciliate protozoa generally can be categorized into four community types based on generic distribution (Kittelmann et al., 2016) and are relatively static in community composition among cows and are more resilient after diet change compared with prokaryotes (Mizrahi and Jami, 2018). Even so, some diet changes can have remarkable shifts. For example, coconut oil profoundly inhibited most protozoa except genus *Epidinium* (Reveneau et al., 2012), whereas this greater tolerance has an unknown mechanism.

The predominant genus *Entodinium* varies considerably by cell size, niche, and bacterivorous activity (Williams and Coleman, 1992). Does the generic protozoal population structure (often estimated by generic counts) adequately reflect diet change?

2.6 Case study using sequencing approaches with protozoal in vitro or ex vivo cultures

Although single cultures of protozoa have to be qualified with respect to relevance to *in vivo* populations, they can provide very useful mechanistic information. Single cultures of *Entodinium caudatum* and *Epidinium caudatum* generally had recovered sequences of bacteria and archaea that were typical of the rumen but not necessarily in the same relative abundance compared with ruminal sequencing studies (Park and Yu, 2018a,b). Even so, relative sequence abundance of Proteobacteria was unusually high from bacteria recovered in close proximity to protozoa in single cultures; subsequent similar results from *ex vivo* cultures provide additional support for an important protozoal-associated role of this taxon. Although they could be preferred prey, Park and Yu (2018b) suggested that Proteobacteria might be able to resist proteolytic degradation by the protozoan and are likely endosymbionts. Do these endosymbionts contribute to 'protozoal' proteolysis or more general ecology in a significant and variable way?

Many questions could be refined with both *ex vivo* and *in vitro* approaches. Do ciliate protozoa help provide peptides and AA as substrates for HAB (increasing wasteful deamination) or, conversely, do protozoa selectively predate HAB (decreasing wasteful deamination by HAB) because those bacteria are more likely to be fluid associated than particulate associated (Firkins et al., 2007)? Current approaches inconsistently associate protozoal counts or 18S rRNA gene copies with feed efficiency (Delgado et al., 2019). However, those authors noted that ciliate protozoal interactions with bacteria and archaea are complex and probably only can be sorted out by combining transcriptomics with phylogenetic analyses. They also noted that gene databases lack protozoal representation, which should be rectified with emerging results from genomic sequencing (Park et al., 2018).

3 Opportunities to improve efficiency of ruminal nitrogen (N) metabolism

3.1 Measurement of rumen-degraded protein

Proteolysis of feed has been studied and used in all current metabolizable protein systems. Various *in vitro* and *in situ* approaches have been tested (Stern

et al., 1997; Schwab and Broderick, 2017), but all have limitations. For example, protease enzymes can be standardized to predict relative RDP values among feeds, whereas expression of proteases in the rumen probably also depends on diet. Other approaches rely on inocula from standardized conditions (preferably fed a diet similar to that of the intended feed's inclusion). Arguably the most critical factor in these approaches is that bacteria, which are very high in protein, increasingly colonize the feed while the feed's protein is continually degrading at a greater rate than is its fiber; the net result is an underestimated rate of degradation. This artifact typically is considered minor for high protein supplements. However, for fibrous feeds that support bacterial colonization and have relatively lower protein, a bacterial marker such as ^{15}N (Kamoun et al., 2014) and quantitative PCR (Paz et al., 2014) have been used successfully. Both markers rely on collection of a bacterial standard from that animal that represents the chemical and marker concentration of its ruminal bacterial community; perhaps the DNA extracted could be used in shotgun sequencing approaches to concomitantly relate variance in protein degradation among feeds to the proteolytic community composition. *Ex vivo* approaches suffer similar problems with bacterial contamination and add other potential caveats such as internal protein recycling, but using filter paper with smaller pore sizes than those in Dacron bags allows degradation kinetics for soluble proteins that pass through Dacron bags. Passage rates must be predicted with uncertain accuracy (Firkins et al., 1998), and no good approach exists to date than can effectively mark the specific passage of potentially degradable protein. For further details, see Hristov et al. (2019).

3.2 Ruminal proteolysis as affected by diet or additives

Dietary factors influence variability among feeds and probably interact with feeding conditions. Improved silage management, including wilting (Hartinger et al., 2018) and usage of inoculants (Muck et al., 2018), helps to prevent undesired proteolysis prior to actual feeding. Overheating increases ruminal escape of dietary protein but decreases its intestinal availability (Hartinger et al., 2018; Schwab and Broderick, 2017). Method of estimating RDP and intestinal digestibility of RUP can interact with feed type (Liebe et al., 2018). Furthermore, ruminal availability is not consistently similar for all individual AA within and among feed sources (White et al., 2017a). Hence, systematic research is still needed to improve accuracy and precision of estimating effects of ruminal proteolysis on intestinal protein supply.

Various dietary additives have been tested to limit ruminal proteolysis, including essential oils, tannins, and saponins (Hartinger et al., 2018). Some of the effects seem to be specific to proteases or deaminases, and some are related to shifting microbial populations from either toxicity or adaptation

to additives (Calsamiglia et al., 2007). Concentrations of active ingredients vary among batches, and bioactive ingredients potentially lose potency with increased storage time or ruminal adaptation, so an optimal dosage should be determined with long-term experiments. Essential oils have been assessed for anti-methanogenic effects (Benchaar and Greathead, 2011), but deamination activity should be assessed concomitantly because of overlapping inhibition of the HAB (McIntosh et al., 2003). Inhibition of methanogenesis was discussed previously, and this potential interaction has been discussed in continuous culture (Guyader et al., 2017) but appears to have inconsistent results based on inhibitor used and availability for other hydrogen sinks (Martinez-Fernandez et al., 2016). Clearly, deamination must be better integrated with other metabolic functions of the microbial community under conditions representative of the inferred animal situation.

Saponins and tannins also can decrease ruminal proteolysis, again potentially mediated through shifts in microbial populations. Saponins have been widely studied to inhibit protozoa (Newbold et al., 2015), but responses to saponins have not always been consistent (Patra and Saxena, 2009). Specific saponins or their derivatives might be more consistently efficacious as anti-protozoal agents (Ramos-Morales et al., 2017a,b). However, further research needs to address adaptation and efficacy testing in high producing animals, whereas too many studies were done with animals fed at low or restricted intakes. Anti-protozoal agents were not endorsed to limit enteric methane production because of inconsistency and potential negative effects of the protozoal suppression agent (Hristov et al., 2013). Tannins were tolerated by goats but decreased dry matter intake in sheep (Min and Solaiman, 2018). More selective browsers such as goats might have evolutionary adaptations to tolerate tannins. Tannins also can influence protein or NDF digestibility (Patra and Saxena, 2011). As with saponins, a variety of condensed tannins have been tested and offer some potential, but a more holistic evaluation is needed (Hartinger et al., 2018).

Ionophores were originally proposed to inhibit protozoa and subsequently to inhibit the HAB (Hartinger et al., 2018), thus lessening proteolysis. In general, protozoal counts are not altered in the rumen of animals that were adapted to ionophores (Schären et al., 2017). In contrast, the HAB are typically sensitive to ionophores (Bento et al., 2015; Shen et al., 2018) and appear to have much less adaptation compared with protozoa, although the latter adaptation time might slow protozal growth rate (Ye et al., 2018). Monensin generally is more inhibitory to Gram-positive bacteria, but adaptation of cell wall structure within the Firmicutes appears to maintain their abundance in animal studies (Schären et al., 2017). Those authors did not note a difference in ruminal ammonia concentration resulting from monensin, but ammonia concentration is affected by assimilation into microbial protein and urea transfer from blood in addition

to proteolysis and deamination. Ionophores have been proposed to be more efficacious in continuous feeding studies than in designs in which animals are rotated among treatments (Sauer et al., 1998), and long-term studies on microbial populations and proteolysis are limited (Hartinger et al., 2018). Firkins and Yu (2015) discussed the generally increased consistency in beef than in dairy studies, which might be mediated by effective dose (amount per unit of feed intake). Moreover, they discussed a potential role in modifying feed intake behavior and its potential role on the ruminal ecosystem.

Medium-chain and unsaturated fatty acids have been studied as anti-protozoal agents (Firkins and Yu, 2015). However, unsaturated fat can modify the microbiome in a broader way than just through lipolysis and biohydrogenation (Enjalbert et al., 2017). Therefore, while studying biohydrogenation, researchers should branch out to assess a potential role for proteolysis in the wider community beyond the butyrivibrios, which are well-known biohydrogenators (Hackmann and Firkins, 2015a). For example, hydrogenated palm oil increased *Prevotella bryantii* as assessed by quantitative PCR (Vargas-Bello-Pérez et al., 2016). Although *Prevotella* spp make a major contribution to proteolysis in the rumen, *P. bryantii* has high peptidase activity (Hartinger et al., 2018). Those authors also noted that fats sometimes decrease populations of protozoa, which have been discussed previously with respect to proteolysis. *Prevotella* and *Butyrivibrio* were among the dominant bacterial taxa associated with the rumen epithelium in dairy cattle (Mann et al., 2018), and they are well-described proteolytics (Walker et al., 2005). Although ureolysis is associated with this community, these recent studies suggest a much broader interaction of epimural bacteria with the bacteria in the rumen mat (Bickhart and Weimer, 2018). Meta-transcriptomic screening of the epimural community noted a high expression of amino acid metabolism genes, supporting an important proteolytic role for this community (Mann et al., 2018).

4 Conclusion

Proteolytic activity is extensive in the rumen. Efforts to restrict proteolysis to reduce N excretion need to consider that proteolysis removes structural impediments to starch and fiber and therefore is related to their ruminal degradability, microbial protein flow to the duodenum, and dry matter intake. Nutrition advisors can shift rumen-degraded protein with rumen-undegraded protein; thus, proteolysis associated with intra-ruminal recycling of microbial protein arguably provides the best opportunity to reduce wastage of dietary protein (the original source for microbial protein – even that which is recycled). Although some free amino acids are taken up, protozoa incorporate most of their amino acids from predating bacteria (or secondarily smaller protozoa or fungi). This uptake would not pose a major loss in efficiency of N capture into animal product except that a large proportion of the predated bacterial

protein is excreted back into the rumen and recycled. Some of this recycling is unavoidable and some helps to prevent an extremely low availability with extended time after feeding. However, excessive recycling needs to be reduced by filling knowledge gaps so that safety factors for dietary protein can be reduced. Ammonia assimilation typically is by glutamate dehydrogenase and glutamine synthetase–glutamate synthetase. Genome sequencing followed by growth studies has established how *R. albus* 8 and *P. ruminicola* 23 express these enzymes under differing ammonium concentrations in the rumen. Metagenomics examples also were provided for expressed proteases, peptidases, and transporters that could be integrated with nutrition studies. Similarly, ex vivo assessment of bacterial predation could be related to protozoal community structure and therefore diet. Case studies and research questions are provided to stimulate high priority research objectives with the expectation that microbial N metabolism can be better integrated with nutrition approaches to improve the efficiency of animal product produced per unit of dietary protein consumed.

5 Future trends

Historical measurements of ruminal proteolysis have provided an excellent foundation in the scientific literature. However, information derived from relatively few strains of bacteria needs to be expanded to broaden our understanding of the diversity of proteases that are expressed under differing conditions or post-prandially. Similarly, this information needs to expand from one or a few animals to multiple animals to help explain differences in ruminal digestibility, feed intake, and feed efficiency in animal systems. At the animal level, RDP is estimated empirically with different approaches that have limited accounting for bacterial contamination of residual proteins. Microbiological and nutritional approaches need further integration to help account for variability such that RDP safety factors can be lessened.

Proteolysis needs to be better distinguished between hydrolysis of feed protein and intra-ruminal recycling of microbial protein. We have highlighted the role of protozoa because of their importance in both activities but also because of challenges in assessing their activity independent from bacteria. In addition to defaunation approaches, we need more meta-analyses that combine protozoal counts adjusted for their volume and (or) relative activity in more comprehensive statistical models that include dietary variables and measured ruminal and total tract digestibilities. In addition to varying cell volume, protozoal taxa are not 'one-size-fits-all' with respect to predation, proteolysis, and sequestration in the rumen. How ciliates directly (predation) and indirectly (carbohydrate niche, oxygen scavenging, etc.) shape the resilience and function of the rumen microbiome still needs much more research

attention. Future research objectives should consider creative approaches, such as *ex vivo* culturing highlighted herein, combined with metagenomics and metatranscriptomics. Advanced understanding of microbial proteolysis, ammonia assimilation, and intra-ruminal N recycling needs to be integrated with model refinement to improve the efficiency of dietary protein conversion into animal product in ruminant nutrition studies and ultimately in livestock enterprises and systems.

6 Where to look for further information

Ruminal proteolysis has book chapters in the landmark book, 'The Rumen Microbial Ecosystem' by P. N. Hobson and C. S. Stewart. This work was updated for protein as cited herein (Walker et al., 2005). A compilation of highly relevant review articles is outlined by Ungerfeld and Newbold (doi: 10.3389/fmicb.2017.02627). Through the National Academies of Sciences, Engineering, and Medicine, there are several resources such as the nutrient requirements of beef, small ruminants, and dairy (to be updated soon) available through their website: https://www.nap.edu/topic/276/agriculture. In addition to feed tables and nutrient requirements, seminal components supporting those requirements are embedded therein. The Australian (CSIRO; ISBN 9780643092624), Dutch (DVE/OEB; doi:10.1017/S0021859610000912), French (INRA; doi.org/10.3920/978-90-8686-292-4), and Nordic (doi.org/10.3920/978-90-8686-718-9) countries are among those with similar resources. The Encyclopedia of Dairy Sciences (ISBN 978-0-12-374407-4) has a vast array of divergent chapters. Many proceedings from regional nutrition conferences across the U.S. and Canada are categorized by The Searchable Proceedings of Animal Conferences (https://spac.adsa.org/index.asp).

7 References

Ahvenjärvi, S. and Huhtanen, P. 2018. Effects of intraruminal urea-nitrogen infusions on feed intake, nitrogen utilization, and milk yield in dairy cows. *Journal of Dairy Science* 101(10), 9004–15. doi:10.3168/jds.2018-14617.

Ahvenjärvi, S., Vaga, M., Vanhatalo, A. and Huhtanen, P. 2018. Ruminal metabolism of grass silage soluble nitrogen fractions. *Journal of Dairy Science* 101(1), 279–94. doi:10.3168/jds.2016-12316.

Arcondeguy, T., Jack, R. and Merrick, M. 2001. P(II) signal transduction proteins, pivotal players in microbial nitrogen control. *Microbiology and Molecular Biology Reviews* 65(1), 80–105. doi:10.1128/MMBR.65.1.80-105.2001.

Atasoglu, C. and Wallace, R. J. 2002. *De novo* synthesis of amino acids by the ruminal anaerobic fungi, *Piromyces communis* and *Neocallimastix frontalis*. *FEMS Microbiology Letters* 212(2), 243–7. doi:10.1111/j.1574-6968.2002.tb11273.x.

Batista, E. D., Detmann, E., Valadares Filho, S. C., Titgemeyer, E. C. and Valadares, R. F. D. 2017. The effect of CP concentration in the diet on urea kinetics and microbial usage

of recycled urea in cattle: a meta-analysis. *Animal* 11(8), 1303–11. doi:10.1017/S1751731116002822.

Belanche, A., de la Fuente, G., Moorby, J. M. and Newbold, C. J. 2012. Bacterial protein degradation by different rumen protozoal groups. *Journal of Animal Science* 90(12), 4495–504. doi:10.2527/jas.2012-5118.

Benchaar, C. and Greathead, H. 2011. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. *Animal Feed Science and Technology* 166–167, 338–55. doi:10.1016/j.anifeedsci.2011.04.024.

Bennett, B. D., Kimball, E. H., Gao, M., Osterhout, R., Van Dien, S. J. and Rabinowitz, J. D. 2009. Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nature Chemical Biology* 5(8), 593–9. doi:10.1038/nchembio.186.

Bento, C. B. P., de Azevedo, A. C., Detmann, E. and Mantovani, H. C. 2015. Biochemical and genetic diversity of carbohydrate-fermenting and obligate amino acid-fermenting hyper-ammonia-producing bacteria from Nellore steers fed tropical forages and supplemented with casein. *BMC Microbiology* 15, 28. doi:10.1186/s12866-015-0369-9.

Bickhart, D. M. and Weimer, P. J. 2018. Symposium review: host-rumen microbe interactions may be leveraged to improve the productivity of dairy cows. *Journal of Dairy Science* 101(8), 7680–9. doi:10.3168/jds.2017-13328.

Blauwkamp, T. A. and Ninfa, A. J. 2002. Physiological role of the GlnK signal transduction protein of *Escherichia coli*: survival of nitrogen starvation. *Molecular Microbiology* 46(1), 203–14. doi:10.1046/j.1365-2958.2002.03153.x

Bowen, M. K., Poppi, D. P. and McLennan, S. R. 2017. Efficiency of rumen microbial protein synthesis in cattle grazing tropical pastures as estimated by a novel technique. *Animal Production Science* 57(8), 1702–12. doi:10.1071/AN15535.

Calsamiglia, S., Busquet, M., Cordozo, P. W., Castillejos, L. and Ferret, A. 2007. Invited review: essential oils as modifiers of rumen microbial fermentation. *Journal of Dairy Science* 90(6), 2580–95. doi:10.3168/jds.2006-644.

Colombatto, D. and Beauchemin, K. A. 2009. A protease additive increases fermentation of alfalfa diets by mixed ruminal microorganisms *in vitro*. *Journal of Animal Science* 87(3), 1097–105. doi:10.2527/jas.2008-1262.

Comtet-Marre, S., Parisot, N., Lepercq, P., Chaucheyras-Durand, F., Mosoni, P., Peyretaillade, E., Bayat, A. R., Shingfield, K. J., Peyret, P. and Forano, E. 2017. Metatranscriptomics reveals the active bacterial and eukaryotic fibrolytic communities in the rumen of dairy cow fed a mixed diet. *Frontiers in Microbiology* 8, 67. doi:10.3389/fmicb.2017.00067.

Cotta, M. A. and Russell, J. B. 1997. Digestion of nitrogen in the rumen: a model for metabolism of nitrogen compounds in gastrointestinal environments. In: Mackie, R. I. and White, B. A. (Eds), *Gastrointestinal Microbiology* (vol. 1). Springer, New York, pp. 380–412.

Czerkawski, J. W. 1976. Chemical composition of microbial matter in the rumen. *Journal of the Science of Food and Agriculture* 27(7), 621–32. doi:10.1002/jsfa.2740270707.

Delgado, B., Bach, A., Guasch, I., González, C., Elcoso, G., Pryce, J. E. and Gonzalez-Recio, O. 2019. Whole rumen metagenome sequencing allows classifying and predicting feed efficiency and intake levels in cattle. *Scientific Reports* 9(1), 11. doi:10.1038/s41598-018-36673-w.

DeMoll, E. 1993. Nitrogen and phosphorus metabolism in methanogens. In: Ferry, J. G. (Ed.), *Methanogenesis: Ecology, Physiology, Biochemistry and Genetics*. Chapman and Hall, New York, pp. 471–89.

Detsch, C. and Stulke, J. 2003. Ammonium utilization in *Bacillus subtilis*: transport and regulatory functions of NrgA and NrgB. *Microbiology* 149(11), 3289–97. doi:10.1099/mic.0.26512-0.

Diaz, H. L., Barr, K. N., Godden, K. R., Plank, J. E., Zapata, I., Schappacher, A. N., Wick, M. P. and Firkins, J. L. 2014. Eukaryotic inhibitors or activators elicit responses to chemosensory compounds by ruminal isotrichid and entodiniomorphid protozoa. *Journal of Dairy Science* 97(4), 2254–69. doi:10.3168/jds.2013-7698.

Edwards, J. E., Forster, R. J., Callaghan, T. M., Dollhofer, V., Dagar, S. S., Cheng, Y., Chang, J., Kittelmann, S., Fliegerova, K., Puniya, A. K., Henske, J. K., Gilmore, S. P., O'Malley, M. A., Griffith, G. W. and Smidt, H. 2017. PCR and omics based techniques to study the diversity, ecology and biology of anaerobic fungi: insights, challenges and opportunities. *Frontiers in Microbiology* 8, 1657. doi:10.3389/fmicb.2017.01657.

Enjalbert, F., Combes, S., Zened, A. and Meynadier, A. 2017. Rumen microbiota and dietary fat: a mutual shaping. *Journal of Applied Microbiology* 123(4), 782–97. doi:10.1111/jam.13501.

Ferraretto, L. F., Crump, P. M. and Shaver, R. D. 2015. Effect of ensiling time and exogenous protease addition to whole-plant corn silage of various hybrids, maturities, and chop lengths on nitrogen fractions and ruminal *in vitro* starch digestibility. *Journal of Dairy Science* 98(12), 8869–81. doi:10.3168/jds.2015-9511.

Fessenden, S. W., Hackmann, T. J., Ross, D. A., Foskolos, A. and Van Amburgh, M. E. 2017. Ruminal bacteria and protozoa composition, digestibility, and amino acid profile determined by multiple hydrolysis times. *Journal of Dairy Science* 100(9), 7211–26. doi:10.3168/jds.2016-12531.

Fessenden, S. W., Hackmann, T. J., Ross, D. A., Block, E., Foskolos, A. and Van Amburgh, M. E. 2019. Rumen digestion kinetics, microbial yield, and omasal flows of nonmicrobial, bacterial, and protozoal amino acids in lactating dairy cattle fed fermentation by-products or urea as a soluble nitrogen source. *Journal of Dairy Science* 102(4), 3036–52. doi:10.3168/jds.2018-15448.

Firkins, J. L. and Yu, Z. 2006. Characterisation and quantification of the microbial populations in the rumen. In: Sejrsen, K., Hvelplund, T. and Nielsen, M. O. (Eds), *Ruminant Physiology, Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress*. Wageningen Academic Publishers, Wageningen, The Netherlands, pp. 19–54.

Firkins, J. L. and Yu, Z. 2015. How to use data on the rumen microbiome to improve our understanding of ruminant nutrition. *Journal of Animal Science* 93(4), 1450–70. doi:10.2527/jas.2014-8754.

Firkins, J. L., Allen, M. S., Oldick, B. S. and St-Pierre, N. R. 1998. Modeling ruminal digestibility of carbohydrates and microbial protein flow to the duodenum. *Journal of Dairy Science* 81(12), 3350–69. doi:10.3168/jds.S0022-0302(98)75901-6.

Firkins, J. L., Yu, Z. and Morrison, M. 2007. Ruminal nitrogen metabolism: perspectives for integration of microbiology and nutrition for dairy. *Journal of Dairy Science* 90(E. Suppl.), E1–E16. doi:10.3168/jds.2006-518.

Fisher, S. H. 1999. Regulation of nitrogen metabolism in *Bacillus subtilis*: vive la difference! *Molecular Microbiology* 32(2), 223–32. doi:10.1046/j.1365-2958.1999.01333.x.

Fisher, S. H. and Wray, L. V. 2002. Mutations in the *Bacillus subtilis glnRA* operon that cause nitrogen source-dependent defects in regulation of TnrA activity. *Journal of Bacteriology* 184(16), 4636–9. doi:10.1128/jb.184.16.4636-4639.2002.

Fondevila, M. and Dehority, B. A. 2001. *In vitro* growth and starch digestion by *Entodinium exiguum* as influenced by the presence or absence of live bacteria. *Journal of Animal Science* 79(9), 2465–71. doi:10.2527/2001.7992465x.

Forchhammer, K. 2008. PII signal transducers: novel functional and structural insights. *Trends in Microbiology* 16(2), 65–72. doi:10.1016/j.tim.2007.11.004.

Forsberg, C. W., Lovelock, L. K. A., Krumholz, L. and Buchanan-Smith, J. G. 1984. Protease activities of rumen protozoa. *Applied and Environmental Microbiology* 47(1), 101–10.

Foskolos, A. and Moorby, J. M. 2018. Evaluating lifetime nitrogen use efficiency of dairy cattle: a modelling approach. *PLoS One* 13(8), e0201638. doi:10.1371/journal.pone.0201638.

Galyean, M. L. and Tedeschi, L. O. 2014. Predicting microbial protein synthesis in beef cattle: relationship to intakes of total digestible nutrients and crude protein. *Journal of Animal Science* 92(11), 5099–111. doi:10.2527/jas.2014-8098.

Griswold, K. E., Apgar, G. A., Bouton, J. and Firkins, J. L. 2003. Effects of urea infusion and ruminal degradable protein concentration on microbial growth, digestibility, and fermentation in continuous culture. *Journal of Animal Science* 81(1), 329–36. doi:10.2527/2003.811329x

Gunka, K. and Commichau, F. M. 2012. Control of glutamate homeostasis in *Bacillus subtilis*: a complex interplay between ammonium assimilation, glutamate biosynthesis and degradation. *Molecular Microbiology* 85(2), 213–24. doi:10.1111/j.1365-2958.2012.08105.x.

Guyader, J., Eugene, M., Noziere, P., Morgavi, D. P., Doreau, M. and Martin, C. 2014. Influence of rumen protozoa on methane emission in ruminants: a meta-analysis approach. *Animal* 8(11), 1816–25. doi:10.1017/S1751731114001852.

Guyader, J., Ungerfeld, E. M. and Beauchemin, K. A. 2017. Redirection of metabolic hydrogen by inhibiting methanogenesis in the rumen simulation technique (RUSITEC). *Frontiers in Microbiology* 8, 393. doi:10.3389/fmicb.2017.00393.

Hackmann, T. J. and Firkins, J. L. 2015a. Electron transport phosphorylation in rumen butyrivibrios: unprecedented ATP yield for glucose fermentation to butyrate. *Frontiers in Microbiology* 6, 622. doi:10.3389/fmicb.2015.00622.

Hackmann, T. J. and Firkins, J. L. 2015b. Maximizing efficiency of rumen microbial protein production. *Frontiers in Microbiology* 6, 465. doi:10.3389/fmicb.2015.00465.

Hackmann, T. J., Ngugi, D. K., Firkins, J. L. and Tao, J. 2017. Genomes of rumen bacteria encode atypical pathways for fermenting hexoses to short-chain fatty acids. *Environmental Microbiology* 19(11), 4670–83. doi:10.1111/1462-2920.13929.

Hansen, E. E., Lozupone, C. A., Rey, F. E., Wu, M., Guruge, J. L., Narra, A., Goodfellow, J., Zaneveld, J. R., McDonald, D. T., Goodrich, J. A., Heath, A. C., Knight, R. and Gordon, J. I. 2011. Pan-genome of the dominant human gut-associated archaeon, *Methanobrevibacter smithii*, studied in twins. *Proceedings of the National Academy of Sciences of the United States of America* 108, 4599–606. doi:10.1073/pnas.1000071108.

Hartinger, T., Gresner, N. and Südekum, K. H. 2018. Does intra-ruminal nitrogen recycling waste valuable resources? A review of major players and their manipulation. *Journal of Animal Science and Biotechnology* 9, 33. doi:10.1186/s40104-018-0249-x.

Hino, T. and Russell, J. B. 1985. Effect of reducing-equivalent disposal and NADH/NAD on deamination of amino acids by intact rumen microorganisms and their cell extracts. *Applied and Environmental Microbiology* 50(6), 1368–74.

Hook, S. E., France, J. and Dijkstra, J. 2017. Further assessment of the protozoal contribution to the nutrition of the ruminant animal. *Journal of Theoretical Biology* 416, 8–15. doi:10.1016/j.jtbi.2016.12.007.

Hristov, A. N. and Jouany, J.-P. 2005. Factors affecting the efficiency of nitrogen utilization in the rumen. In: Hristov, A. N. and Pfeffer, E. (Eds), *Nitrogen and Phosphorus Nutrition of Cattle and Environment*. CAB International, Wallingford, UK, pp. 117–66.

Hristov, A. N., Oh, J., Firkins, J. L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H. P., Adesogan, A. T., Yang, W., Lee, C., Gerber, P. J., Henderson, B. and Tricarico, J. M. 2013. Special topics—mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. *Journal of Animal Science* 91(11), 5045–69. doi:10.2527/jas.2013-6583.

Hristov, A. N., Bannink, A., Crompton, L. A., Huhtanen, P., Kreuzer, M., McGee, M., Nozière, P., Reynolds, C. K., Bayat, A. R., Yáñez-Ruiz, D. R., Dijkstra, J., Kebreab, E., Schwarm, A., Shingfield, K. J. and Yu, Z. 2019. Invited review: nitrogen in ruminant nutrition: a review of measurement techniques. *Journal of Dairy Science* 102(7), 5811–52. doi:10.3168/jds.2018-15829.

Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., Muñoz-Tamayo, R., Forano, E., Waters, S. M., Hess, M., Tapio, I., Smidt, H., Krizsan, S. J., Yáñez-Ruiz, D. R., Belanche, A., Guan, L., Gruninger, R. J., McAllister, T. A., Newbold, C. J., Roehe, R., Dewhurst, R. J., Snelling, T. J., Watson, M., Suen, G., Hart, E. H., Kingston-Smith, A. H., Scollan, N. D., do Prado, R. M., Pilau, E. J., Mantovani, H. C., Attwood, G. T., Edwards, J. E., McEwan, N. R., Morrisson, S., Mayorga, O. L., Elliott, C. and Morgavi, D. P. 2018. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Frontiers in Microbiology* 9, 2161. doi:10.3389/fmicb.2018.02161.

Iakiviak, M. 2018. Analysis of the ammonium assimilation pathways of the human colonic bacterium, *Bacteroides thetaiotaomicron*. PhD Thesis. University of Illinois, Urbana, IL.

Ishaq, S. L., AlZahal, O., Walker, N. and McBride, B. 2017. An investigation into rumen fungal and protozoal diversity in three rumen fractions, during high-fiber or grain-induced sub-acute ruminal acidosis conditions, with or without active dry yeast supplementation. *Frontiers in Microbiology* 8, 1943. doi:10.3389/fmicb.2017.01943.

Jin, D., Zhao, S., Zheng, N., Beckers, Y. and Wang, J. 2018. Urea metabolism and regulation by rumen bacterial urease in ruminants – a review. *Annals of Animal Science* 18(2), 303–18. doi:10.1515/aoas-2017-0028.

Kamoun, M., Ammar, H., Théwis, A., Beckers, Y., France, J. and López, S. 2014. Comparison of three 15N methods to correct for microbial contamination when assessing in situ protein degradability of fresh forages. *Journal of Animal Science* 92(11), 5053–62. doi:10.2527/jas.2014-7691.

Karnati, S. K. R., Sylvester, J. T., Noftsger, S. M., Yu, Z., St-Pierre, N. R. and Firkins, J. L. 2007. Assessment of ruminal bacterial populations and protozoal generation time in cows fed different methionine sources. *Journal of Dairy Science* 90(2), 798–809. doi:10.3168/jds.S0022-0302(07)71564-3.

Kim, J. N., Henriksen, E. D., Cann, I. K. and Mackie, R. I. 2014. Nitrogen utilization and metabolism in *Ruminococcus albus* 8. *Applied and Environmental Microbiology* 80(10), 3095–102. doi:10.1128/AEM.00029-14.

Kim, J. N., Mendez-Garcia, C., Geier, R. R., Iakiviak, M., Chang, J., Cann, I. and Mackie, R. I. 2017. Metabolic networks for nitrogen utilization in *Prevotella ruminicola* 23. *Scientific Reports* 7(1), 7851. doi:10.1038/s41598-017-08463-3.

Kittelmann, S., Devente, S. R., Kirk, M. R., Seedorf, H., Dehority, B. A. and Janssen, P. H. 2015. Phylogeny of the intestinal ciliates, including *Charonina ventriculi*, and comparison of microscopy and 18S rRNA gene pyrosequencing for rumen ciliate community structure analysis. *Applied and Environmental Microbiology* 81(7), 2433–44. doi:10.1128/AEM.03697-14.

Kittelmann, S., Pinares-Patino, C. S., Seedorf, H., Kirk, M. R., McEwan, J. C. and Janssen, P. H. 2016. Natural variation in methane emission of sheep fed on a lucerne pellet diet is unrelated to rumen ciliate community type. *Microbiology* 162(3), 459–65. doi:10.1099/mic.0.000245.

Kleiner, D. 1982. Ammonium (methylammonium) transport by *Klebsiella pneumoniae*. *Biochimica et Biophysica Acta* 688(3), 702–8. doi:10.1016/0005-2736(82)90282-6.

Kleiner, D. and Castorph, H. 1982. Inhibition of ammonium (methylammonium) transport in *Klebsiella pneumoniae* by glutamine and glutamine analogues. *FEBS Letters* 146(1), 201–3. doi:10.1016/0014-5793(82)80735-7.

Leigh, J. A. and Dodsworth, J. A. 2007. Nitrogen regulation in Bacteria and Archaea. *Annual Review of Microbiology* 61, 349–77. doi:10.1146/annurev.micro.61.080706.093409.

Leong, L. E. X., Denman, S. E., Hugenholtz, P. and McSweeney, C. S. 2016. Amino acid and peptide utilization profiles of the fluoroacetate-degrading bacterium Synergistetes Strain MFA1 under varying conditions. *Microbial Ecology* 71(2), 494–504. doi:10.1007/s00248-015-0641-4.

Liebe, D. M., Firkins, J. L., Tran, H., Kononoff, P. J. and White, R. R. 2018. Technical note: methodological and feed factors affecting measurement of protein A, B, and C fractions, degradation rate, and intestinal digestibility of rumen-undegraded protein. *Journal of Dairy Science* 101(9), 8046–53. doi:10.3168/jds.2018-14837.

Liu, X., Shi, F. and Gong, J. 2013. Variations and evolution of polyubiquitin genes from ciliates. *European Journal of Protistology* 49(1), 40–9. doi:10.1016/j.ejop.2012.05.001.

Lowe, S. E., Theodorou, M. K., Trinci, A. P. J. and Hespell, R. B. 1985. Growth of anaerobic rumen fungi on defined and semi-defined media lacking rumen fluid. *Journal of General Microbiology* 131, 2225– 9.

Mackie, R. I. and White, B. A. 1990. Recent advances in rumen microbial ecology and metabolism: potential impact on nutrient output. *Journal of Dairy Science* 73(10), 2971–95. doi:10.3168/jds.S0022-0302(90)78986-2.

Magasanik, B. 1989 Regulation of transcription of the glnALG operon of *Escherichia coli* by protein phosphorylation. *Biochimie* 71(9–10), 1005–12. doi:10.1016/0300-9084(89)90104-1.

Mann, E., Wetzels, S. U., Wagner, M., Zebeli, Q. and Schmitz-Esser, S. 2018. Metatranscriptome sequencing reveals insights into the gene expression and functional potential of rumen wall bacteria. *Frontiers in Microbiology* 9, 43. doi:10.3389/fmicb.2018.00043.

Martinelle, K. and Haggstrom, L. 1997. On the dissociation constant of ammonium: effects of using an incorrect pKa in calculations of the ammonia concentration in animal cell cultures. *Biotechnology Techniques* 11(8), 549–51. doi:10.1023/A:1018482520832.

Martinez-Fernandez, G., Denman, S. E., Yang, C., Cheung, J., Mitsumori, M. and McSweeney, C. S. 2016. Methane inhibition alters the microbial community, hydrogen flow, and fermentation response in the rumen of cattle. *Frontiers in Microbiology* 7, 1122. doi:10.3389/fmicb.2016.01122.

Mayorga, O. L., Kingston-Smith, A. H., Kim, E. J., Allison, G. G., Wilkinson, T. J., Hegarty, M. J., Theodorou, M. K., Newbold, C. J. and Huws, S. A. 2016. Temporal metagenomic and metabolomic characterization of fresh perennial ryegrass degradation by rumen bacteria. *Frontiers in Microbiology* 7, 1854. doi:10.3389/fmicb.2016.01854.

McIntosh, F. M., Williams, P., Losa, R., Wallace, R. J., Beever, D. A. and Newbold, C. J. 2003. Effects of essential oils on ruminal microorganisms and their protein metabolism. *Applied and Environmental Microbiology* 69(8), 5011–4. doi:10.1128/aem.69.8.5011-5014.2003.

Meier-Wagner, J., Nolden, L., Jakoby, M., Siewe, R., Kramer, R. and Burkovski, A. 2001. Multiplicity of ammonium uptake systems in *Corynebacterium glutamicum*: role of Amt and AmtB. *Microbiology* 147(1), 135–43. doi:10.1099/00221287-147-1-135.

Min, B. R. and Solaiman, S. 2018. Comparative aspects of plant tannins on digestive physiology, nutrition and microbial community changes in sheep and goats: a review. *Journal of Animal Physiology and Animal Nutrition* 102(5), 1181–93. doi:10.1111/jpn.12938.

Mizrahi, I. and Jami, E. 2018. Review: the compositional variation of the rumen microbiome and its effect on host performance and methane emission. *Animal* 12(s2), s220–32. doi:10.1017/S1751731118001957.

Morrison, M. and Mackie, R. I. 1997. Biosynthesis of nitrogen-containing compounds. In: Mackie, R. I. and White, B. A. (Eds), *Gastrointestinal Ecosystems and Fermentations* (vol. 1). Chapman and Hall, New York, NY, pp. 424–69.

Muck, R. E., Nadeau, E. M. G., McAllister, T. A., Contreras-Govea, F. E., Santos, M. C. and Kung, L. 2018. Silage review: recent advances and future uses of silage additives. *Journal of Dairy Science* 101(5), 3980–4000. doi:10.3168/jds.2017-13839.

Newbold, C. J., de la Fuente, G., Belanche, A., Ramos-Morales, E. and McEwan, N. R. 2015. The role of ciliate protozoa in the rumen. *Frontiers in Microbiology* 6, 1313. doi:10.3389/fmicb.2015.01313.

Oelker, E. R., Reveneau, C. and Firkins, J. L. 2009. Interaction of molasses and monensin in alfalfa hay- or corn silage-based diets on rumen fermentation, total tract digestibility, and milk production by Holstein cows. *Journal of Dairy Science* 92(1), 270–85. doi:10.3168/jds.2008-1432.

Orpin, C. G. and Greenwood, Y. 1986. Nutritional and germination requirements of the rumen chytridiomycete *Neocallimastix frontalis*. *Transactions – British Mycological Society* 86, 103–9.

Park, T. and Yu, Z. 2018a. Aerobic cultivation of anaerobic rumen protozoa, *Entodinium caudatum* and *Epidinium caudatum*. *Journal of Microbiological Methods* 152, 186–93. doi:10.1016/j.mimet.2018.08.006.

Park, T. and Yu, Z. 2018b. Do ruminal ciliates select their preys and prokaryotic symbionts? *Frontiers in Microbiology* 9, 1710. doi:10.3389/fmicb.2018.01710.

Park, T., Wijeratne, S., Meulia, T., Firkins, J. and Yu, Z. 2018. Draft macronuclear genome sequence of the ruminal ciliate *Entodinium caudatum*. *Microbiology Resource Announcements* 7(1), e00826–18. doi:10.1128/MRA.00826-18.

Patra, A. K. and Aschenbach, J. R. 2018. Ureases in the gastrointestinal tracts of ruminant and monogastric animals and their implication in urea-N/ammonia metabolism: a review. *Journal of Advanced Research* 13, 39–50. doi:10.1016/j.jare.2018.02.005.

Patra, A. K. and Saxena, J. 2009. The effect and mode of action of saponins on the microbial populations and fermentation in the rumen and ruminant production. *Nutrition Research Reviews* 22(2), 204–19. doi:10.1017/S0954422409990163.

Patra, A. K. and Saxena, J. 2011. Exploitation of dietary tannins to improve rumen metabolism and ruminant production. *Journal of the Science of Food and Agriculture* 91(1), 24–37. doi:10.1002/jsfa.4152.

Paz, H. A., Klopfenstein, T. J., Hostetler, D., Fernando, S. C., Castillo-Lopez, E. and Kononoff, P. J. 2014. Ruminal degradation and intestinal digestibility of protein and amino acids in high-protein feedstuffs commonly used in dairy diets. *Journal of Dairy Science* 97(10), 6485–98. doi:10.3168/jds.2014-8108.

Paz, H. A., Anderson, C. L., Muller, M. J., Kononoff, P. J. and Fernando, S. C. 2016. Rumen bacterial community composition in Holstein and Jersey cows is different under same dietary condition and is not affected by sampling method. *Frontiers in Microbiology* 7, 1206. doi:10.3389/fmicb.2016.01206.

Pengpeng, W. and Tan, Z. 2013. Ammonia assimilation in rumen bacteria: a review. *Animal Biotechnology* 24(2), 107–28. doi:10.1080/10495398.2012.756402.

Ramos-Morales, E., de la Fuente, G., Duval, S., Wehrli, C., Bouillon, M., Lahmann, M., Preskett, D., Braganca, R. and Newbold, C. J. 2017a. Antiprotozoal effect of saponins in the rumen can be enhanced by chemical modifications in their structure. *Frontiers in Microbiology* 8, 399. doi:10.3389/fmicb.2017.00399.

Ramos-Morales, E., de la Fuente, G., Nash, R. J., Braganca, R., Duval, S., Bouillon, M. E., Lahmann, M. and Newbold, C. J. 2017b. Improving the antiprotozoal effect of saponins in the rumen by combination with glycosidase inhibiting iminosugars or by modification of their chemical structure. *PLoS One* 12(9), e0184517. doi:10.1371/journal.pone.0184517.

Reed, K. F., Casper, D. P., France, J. and Kebreab, E. 2015. Prediction of nitrogen efficiency in dairy cattle: a review. *CAB Reviews* 10(1), 1. doi:10.1079/PAVSNNR201510001.

Reed, K. F., Bonfá, H. C., Dijkstra, J., Casper, D. P. and Kebreab, E. 2017. Estimating the energetic cost of feeding excess dietary nitrogen to dairy cows. *Journal of Dairy Science* 100(9), 7116–26. doi:10.3168/jds.2017-12584.

Reitzer, L. 2003. Nitrogen assimilation and global regulation in *Escherichia coli*. *Annual Review of Microbiology* 57, 155–76. doi:10.1146/annurev.micro.57.030502.090820.

Reveneau, C., Karnati, S. K. R., Oelker, E. R. and Firkins, J. L. 2012. Interaction of unsaturated fat or coconut oil with monensin in lactating dairy cows fed twelve times daily. I. Protozoal abundance, nutrient digestibility, and microbial protein flow to the omasum. *Journal of Dairy Science* 95(4), 2046–60. doi:10.3168/jds.2011-4887.

Reynolds, C. K. and Kristensen, N. B. 2008. Nitrogen recycling through the gut and the nitrogen economy of ruminants: an asynchronous symbiosis. *Journal of Animal Science* 86(E. Suppl.), E293–305. doi:10.2527/jas.2007-0475.

Roman-Garcia, Y., White, R. R. and Firkins, J. L. 2016. Meta-analysis of postruminal microbial nitrogen flows in dairy cattle. I. Derivation of equations. *Journal of Dairy Science* 99(10), 7918–31. doi:10.3168/jds.2015-10661.

Russell, J. B. and Strobel, H. J. 1987. Concentration of ammonia across cell membranes of mixed rumen bacteria. *Journal of Dairy Science* 70(5), 970–6. doi:10.3168/jds.S0022-0302(87)80101-7.

Rychlik, J. L. and Russell, J. B. 2000. Mathematical estimations of hyper-ammonia producing ruminal bacteria and evidencefor bacterial antagonism that decreases ruminal ammonia production. *FEMS Microbiology Ecology* 32(2), 121–8. doi:10.1111/j.1574-6941.2000.tb00706.x.

Samuel, B. S., Hansen, E. E., Manchester, J. K., Coutinho, P. M., Henrissat, B., Fulton, R., Latreille, P., Kim, K., Wilson, R. K. and Gordon, J. I. 2007. Genomic and metabolic adaptations of *Methanobrevibacter smithii* to the human gut. *Proceedings of the National Academy of Sciences of the United States of America* 104(25), 10643–8. doi:10.1073/pnas.0704189104.

Sauer, F. D., Fellner, V., Kinsman, R., Kramer, J. K., Jackson, H. A., Lee, A. J. and Chen, S. 1998. Methane output and lactation response in Holstein cattle with monensin or unsaturated fat added to the diet. *Journal of Animal Science* 76(3), 906–14. doi:10.2527/1998.763906x.

Sauvant, D. and Nozière, P. 2016. Quantification of the main digestive processes in ruminants: the equations involved in the renewed energy and protein feed evaluation systems. *Animal* 10(5), 755–70. doi:10.1017/S1751731115002670.

Schären, M., Drong, C., Kiri, K., Riede, S., Gardener, M., Meyer, U., Hummel, J., Urich, T., Breves, G. and Dänicke, S. 2017. Differential effects of monensin and a blend of essential oils on rumen microbiota composition of transition dairy cows. *Journal of Dairy Science* 100(4), 2765–83. doi:10.3168/jds.2016-11994.

Schumacher, M. A., Chinnam, N. B., Cuthbert, B., Tonthat, N. K. and Whitfill, T. 2015. Structures of regulatory machinery reveal novel molecular mechanisms controlling *B. subtilis* nitrogen homeostasis. *Genes and Development* 29(4), 451–64. doi:10.1101/gad.254714.114.

Schwab, C. G. and Broderick, G. A. 2017. A 100-year Review: protein and amino acid nutrition in dairy cows. *Journal of Dairy Science* 100(12), 10094–112. doi:10.3168/jds.2017-13320.

Seshadri, R., Leahy, S. C., Attwood, G. T., Teh, K. H., Lambie, S. C., Cookson, A. L., Eloe-Fadrosh, E. A., Pavlopoulos, G. A., Hadjithomas, M., Varghese, N. J., Paez-Espino, D. Hungate project c, Palevich, N., Janssen, P. H., Ronimus, R. S., Noel, S., Soni, P., Reilly, K., Atherly, T., Ziemer, C., Wright, A-D., Ishaq, S., Cotta, M., Thompson, S., Crosley, K., McKain, N., Wallace, R. J., Flint, H. J., Martin, J. C., Forster, R. J., Gruninger, R. J., McAllister, T., Gilbert, R., Ouwerkerk, D., Klieve, A., Jassim, R. A., Denman, S., McSweeney, C., Rosewarne, C., Koike, S., Kobayashi, Y., Mitsumori, M., Shinkai, T., Cravero, S., Cucchi, M. C., Perry, R., Henderson, G., Creevey, C. J., Terrapon, N., Lapebie, P., Drula, E., Lombard, V., Rubin, E., Kyrpides, N. C., Henrissat, B., Woyke, T., Ivanova, N. N. and Kelly, W. J. 2018. Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nature Biotechnology* 36, 359.

Shen, J., Yu, Z. and Zhu, W. 2018. Insights into the populations of proteolytic and amino acid-fermenting bacteria from microbiota analysis using in vitro enrichment cultures. *Current Microbiology* 75(11), 1543–50. doi:10.1007/s00284-018-1558-1.

Sok, M., Ouellet, D. R., Firkins, J. L., Pellerin, D. and Lapierre, H. 2017. Amino acid composition of rumen bacteria and protozoa in cattle. *Journal of Dairy Science* 100(7), 5241–9. doi:10.3168/jds.2016-12447.

Soupene, E., He, L., Yan, D. and Kustu, S. 1998. Ammonia acquisition in enteric bacteria: physiological role of the ammonium/methylammonium transport B (AmtB) protein. *Proceedings of the National Academy of Sciences of the United States of America* 95(12), 7030–4. doi:10.1073/pnas.95.12.7030.

Soupene, E., Lee, H. and Kustu, S. 2002. Ammonium/methylammonium transport (Amt) proteins facilitate diffusion of NH3 bidirectionally. *Proceedings of the National Academy of Sciences of the United States of America* 99(6), 3926–31. doi:10.1073/pnas.062043799.

Stern, M. D., Bach, A. and Calsamiglia, S. 1997. Alternative techniques for measuring nutrient digestion in ruminants. *Journal of Animal Science* 75(8), 2256–76. doi:10.2527/1997.7582256x.

Stevenson, R. and Silver, S. 1977. Methylammonium uptake by *Escherichia coli*: evidence for a bacterial NH4+ transport system. *Biochemical and Biophysical Research Communications* 75(4), 1133–9. doi:10.1016/0006-291x(77)91501-7.

Sylvester, J. T., Karnati, S. K., Yu, Z., Newbold, C. J. and Firkins, J. L. 2005 Evaluation of a real-time PCR assay quantifying the ruminal pool size and duodenal flow of protozoal nitrogen. *Journal of Dairy Science* 88(6), 2083–95. doi:10.3168/jds.S0022-0302(05)72885-X.

Theodorou, M. K., Davies, D. R. and Orpin, C. G. 1994. Nutrition and survival of anaerobic fungi. In: Mountfort, D. O. and Orpin, C. G. (Eds), *Anaerobic Fungi: Biology, Ecology and Function*. Marcel Dekker, New York, pp. 107–28.

Ungerfeld, E. M. 2018. Inhibition of rumen methanogenesis and ruminant productivity: a meta-analysis. *Frontiers in Veterinary Science* 5, 113. doi:10.3389/fvets.2018.00113.

Ungerfeld, E. M., Aedo, M. F., Martínez, E. D. and Saldivia, M. 2019. Inhibiting methanogenesis in rumen batch cultures did not increase the recovery of metabolic hydrogen in microbial amino acids. *Microorganisms* 7(5), 115. doi:10.3390/microorganisms7050115.

Van Amburgh, M. E., Collao-Saenz, E. A., Higgs, R. J., Ross, D. A., Recktenwald, E. B., Raffrenato, E., Chase, L. E., Overton, T. R., Mills, J. K. and Foskolos, A. 2015. The Cornell Net carbohydrate and Protein System: updates to the model and evaluation of version 6.5. *Journal of Dairy Science* 98(9), 6361–80. doi:10.3168/jds.2015-9378.

Van Heeswijk, W. C., Hoving, S., Molenaar, D., Stegeman, B., Kahn, D. and Westerhoff, H. V. 1996. An alternative PII protein in the regulation of glutamine synthetase in *Escherichia coli*. *Molecular Microbiology* 21(1), 133–46. doi:10.1046/j.1365-2958.1996.6281349.x.

Van Heeswijk, W. C., Westerhoff, H. V. and Boogerd, F. C. 2013. Nitrogen assimilation in *Escherichia coli*: putting molecular data into a systems perspective. *Microbiology and Molecular Biology Reviews* 77(4), 628–95. doi:10.1128/MMBR.00025-13.

Vargas-Bello-Pérez, E., Cancino-Padilla, N., Romero, J. and Garnsworthy, P. C. 2016. Quantitative analysis of ruminal bacterial populations involved in lipid metabolism in dairy cows fed different vegetable oils. *Animal* 10(11), 1821–8. doi:10.1017/S1751731116000756.

Walker, N. D., Newbold, C. J. and Wallace, R. J. 2005. Nitrogen metabolism in the rumen. In: Pfeffer, E. and Hristov, A. (Eds), *Nitrogen and Phosphorus Nutrition of Cattle*. CABI Publishing, Cambridge, MA, pp. 71–115.

Wallace, R. J. and McPherson, C. A. 1987. Factors affecting the rate of breakdown of bacterial protein in rumen fluid. *The British Journal of Nutrition* 58(2), 313–23. doi:10.1079/bjn19870098.

Wallace, R. J., Onodera, R. and Cotta, M. A. 1997. Metabolism of nitrogen-containing compounds. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem* (2nd edn.). Chapman and Hall, New York, NY, pp. 283–328.

Wallace, R. J., Chaudhary, L. C., Miyagawa, E., McKain, N. and Walker, N. D. 2004. Metabolic properties of *Eubacterium pyruvativorans*, a ruminal 'hyper-ammonia-producing' anaerobe with metabolic properties analogous to those of *Clostridium kluyveri*. *Microbiology* 150(9), 2921–30. doi:10.1099/mic.0.27190-0.

Wenner, B. A., Wagner, B. K. and Firkins, J. L. 2018. Using video microscopy to improve quantitative estimates of protozoal motility and cell volume. *Journal of Dairy Science* 101(2), 1060–73. doi:10.3168/jds.2017-13513.

White, R. R., Roman-Garcia, Y. and Firkins, J. L. 2016. Meta-analysis of postruminal microbial nitrogen flows in dairy cattle. II. Approaches to and implications of more mechanistic prediction. *Journal of Dairy Science* 99(10), 7932–44. doi:10.3168/jds.2015-10662.

White, R. R., Kononoff, P. J. and Firkins, J. L. 2017a. Technical note: methodological and feed factors affecting prediction of ruminal degradability and intestinal digestibility of essential amino acids. *Journal of Dairy Science* 100(3), 1946–50. doi:10.3168/jds.2016-12008.

White, R. R., Roman-Garcia, Y., Firkins, J. L., Kononoff, P. J., VandeHaar, M. J., Tran, H., McGill, T., Garnett, R. and Hanigan, M. D. 2017b. Evaluation of the 2001 dairy NRC and derivation of new prediction equations. 2. Rumen degradable and undegradable protein. *Journal of Dairy Science* 100(5), 3611–27. doi:10.3168/jds.2015-10801.

Williams, A. G. and Coleman, G. S. 1992. *The Rumen Protozoa*. Springer, New York.

Williams, A. G. and Coleman, G. S. 1997. The rumen protozoa. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem* (2nd edn.). Blackie Academic and Professional, London, pp. 73–139.

Williams, K. J., Bennett, M. H., Barton, G. R., Jenkins, V. A. and Robertson, B. D. 2013. Adenylylation of mycobacterial Glnk (PII) protein is induced by nitrogen limitation. *Tuberculosis* 93(2), 198–206. doi:10.1016/j.tube.2012.12.003.

Winkler, F. K. 2006. Amt/MEP/Rh proteins conduct ammonia. *Pflugers Archiv* 451(6), 701–7. doi:10.1007/s00424-005-1511-6.

Wray, L. V., Zalieckas, J. M. and Fisher, S. H. 2001. *Bacillus subtilis* glutamine synthetase controls gene expression through a protein-protein interaction with transcription factor TnrA. *Cell* 107(4), 427–35. doi:10.1016/s0092-8674(01)00572-4.

Xia, Y., Kong, Y., Huang, H., Yang, H. E., Forster, R. and McAllister, T. A. 2016. In situ identification and quantification of protein-hydrolyzing ruminal bacteria associated with the digestion of barley and corn grain. *Canadian Journal of Microbiology* 62(12), 1063–7. doi:10.1139/cjm-2016-0293.

Ye, D., Karnati, S. K. R., Wagner, B., Firkins, J. L., Eastridge, M. L. and Aldrich, J. M. 2018. Essential oil and monensin affect ruminal fermentation and the protozoal population in continuous culture. *Journal of Dairy Science* 101(6), 5069–81. doi:10.3168/jds.2017-13646.

Zanton, G. I. 2016. Analysis of production responses to changing crude protein levels in lactating dairy cow diets when evaluated in continuous or change-over experimental designs. *Journal of Dairy Science* 99(6), 4398–410. doi:10.3168/jds.2015-10438.

Chapter 13

Factors influencing the efficiency of rumen energy metabolism

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1 Introduction

Ruminants have been associated with humans since the Neolithic as providers of meat, milk, wool, and traction. A key aspect that promoted their domestication is their ability to utilize fiber and non-protein nitrogen (N) unavailable to humans. Fundamental to this nutritional flexibility is digestion and fermentation of ingested feed by the complex microbial community of bacteria, protozoa, fungi, methanogens, and bacteriophages that inhabits the rumen. Volatile fatty acids (VFA) resulting from feed fermentation are absorbed and used by the host animal as sources of energy, glucose, and fat. Microbial cells flowing out of the rumen are digested in more distal portions of the gastrointestinal tract providing the main source of amino acids for the ruminant host animal.

While the nutritional flexibility of ruminants has allowed them to play a central role in the provision of useful products to humans, this comes at the cost of inefficiencies in energy and N utilization. The manipulation of the rumen microbial community can have several objectives, related to energy, N or fatty acid metabolism, or detoxification (Nagaraja et al., 1997; Lourenco et al., 2010). In this chapter, we discuss several aspects that affect the efficiency of energy utilization in the rumen and propose research directions toward the improvement of rumen energy efficiency.

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2 Main pathways of rumen fermentation

2.1 Production of volatile fatty acids

Digestion of carbohydrates in the rumen is followed by microbial energy transactions. Microbial catabolism (fermentation) provides energy as ATP for driving the otherwise unfeasible processes of microbial anabolism (growth). Fermentation can be defined as an incomplete oxidation in which the ultimate electron acceptors are carbon compounds formed in the process itself. In the rumen, simple sugars released from the hydrolysis of complex carbohydrates such as cellulose and starch are fermented to VFA, mainly acetate, propionate and butyrate, and gases, mainly carbon dioxide (CO_2) and methane (CH_4, Fig. 1). Fermentation intermediates such as dihydrogen (H_2), formate, succinate, and lactate are produced but do not accumulate as they are rapidly metabolized to end products of fermentation. From an energetic viewpoint, fermentation is associated with a negative Gibbs energy change (ΔG), part of which can be conserved by microbial cells, mainly as ATP generation and used in anabolic reactions of cell growth and maintenance (Czerkawski, 1986; Russell and Wallace, 1997).

Historically, the Embden-Meyerhof-Parnas pathway (EMP, glycolysis) has been thought to be the main pathway of glucose metabolism in rumen fermentation (Russell and Wallace, 1997). The initial experiments by Wallnöfer et al. (1966) incubating labeled glucose in vitro and labeled cotton cellulose in situ followed by an in vitro cellulose incubation indicated that glycolysis was the main catabolic pathway for glucose. Later results with rumen cell free extract also supported glycolysis as the main metabolic pathway for glucose and glycolytic intermediates (Hamar and Borchers, 1967).

Recently, an examination of genomes of 48 rumen bacteria that were 99.5% complete showed that 27% did not encode the glycolytic pathway or it was incomplete (Hackmann et al., 2017). Furthermore, 11% of bacteria encoded multiple pathways for catabolizing glucose apart from glycolysis. Evidently, the quantitative importance of each pathway of glucose catabolism depends not only on the presence in microbial genomes of the genes encoding the necessary enzymes but also on the microbial community composition, gene expression, and the flow of carbon through each catabolic pathway with active enzymes. That said, biochemical experiments have shown that some rumen bacteria are missing certain enzymes of glycolysis (Scardovi, 1965; Kelly et al., 2010).

Given the paucity in research on carbohydrate metabolism, research with more complex and representative substrates actually utilized in animal feeding, as opposed to pure carbohydrates, is recommended. For example, in the experiments conducted by Wallnöfer et al. (1966) with pure substrates, only about 10% of cellulose was degraded, and it was fermented in vitro to a relatively low acetate to propionate ratio, while fermentation of roughages

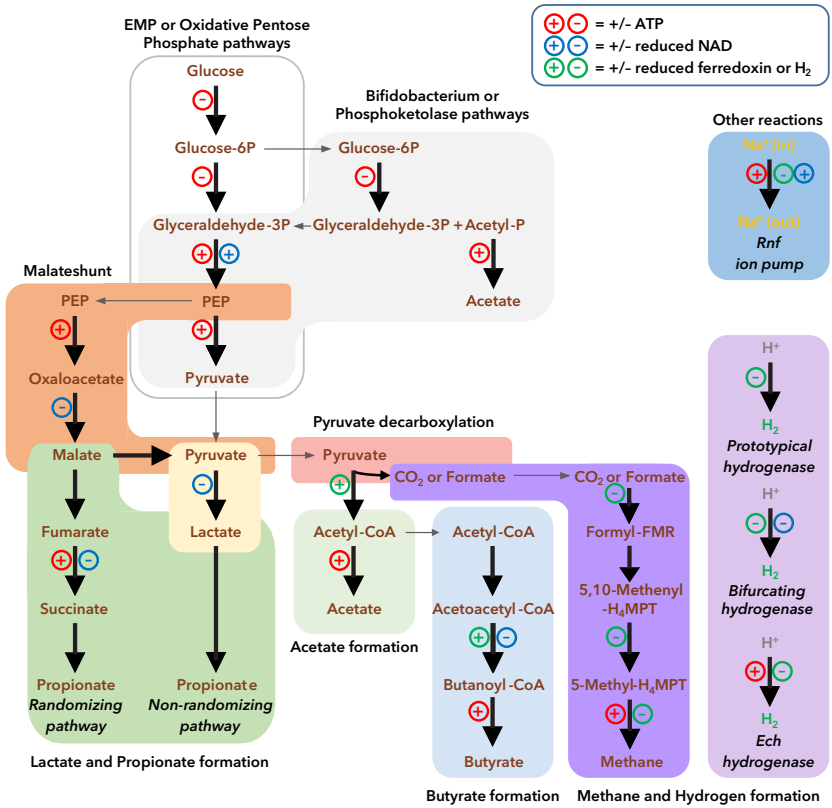
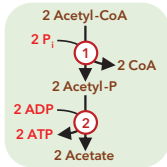


Figure 1 Overview of fermentation pathways in rumen bacteria and methanogens. Pathways have been simplified, and each arrow may represent multiple reactions. See Hackmann et al. (2017) for details. Generation or hydrolysis of ATP (red) and reduction or oxidation of NAD (blue) or ferredoxin or dihydrogen (H_2) formation or incorporation (green) are indicated for the reactions in which they are involved. The pathway for methane formation is encoded by six rumen methanogens (*Methanobrevibacter boviskoreani* JH-1, *Methanobrevibacter millerae* ZA-10, *Methanobrevibacter olleyae* 1H5-1P, *Methanobrevibacter ruminantium* M1, *Methanomicrobium mobile* 1, *Methanosarcina* sp. Ms 97). One methanogen (*Methanosarcina* sp. Ms 97) does not encode a pathway for forming methane from formate. Abbreviations: -P = phosphate, -3P = 3-phosphate, -6P = 6-phosphate, CoA = coenzyme A, CoM = coenzyme M, H_4MPT = tetrahydromethanopterin or tetrahydrosarcinapterin, MFR = methanofuran, and PEP = phosphoenolpyruvate.

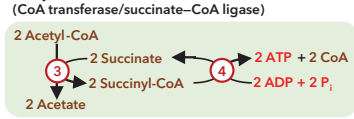
fed to ruminants typically results in a high acetate to propionate ratio (Janssen, 2010). It is possible that catabolism at different rates of complex mixtures of mono- and oligosaccharides released from the degradation of carbohydrates in animal feeds involves a more diverse range of catabolic pathways compared with pure substrates, as the discovery by Hackmann et al. (2017) of the various pathways encoded by rumen bacteria could suggest. The partition of carbon

Acetate formation

Acetyl-CoA → Acetate I (Acetate kinase)

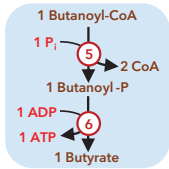


Acetyl-CoA → Acetate II
(CoA transferase/succinate–CoA ligase)

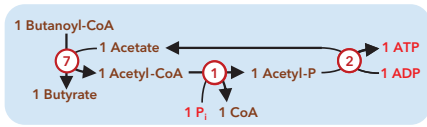


Butyrate formation

Butanoyl-CoA → Butyrate I (Butyrate kinase)

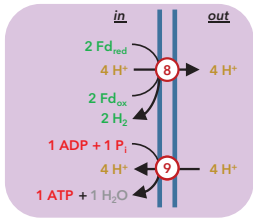


Butanoyl-CoA → Butyrate II (CoA transferase/acetate kinase)



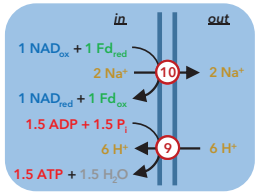
Hydrogen formation

Ech hydrogenase



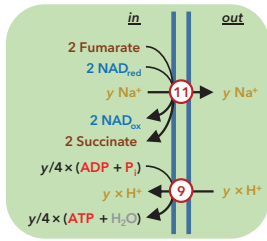
Other reactions

Rnf ion pump



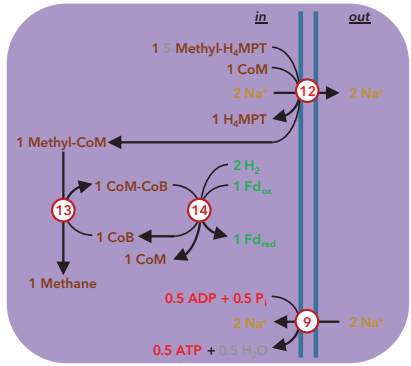
Propionate formation (randomising pathway)

Fumarate → Succinate



Methane formation

5-Methyl-H_4MPT → Methane I (without cytochromes)



5-Methyl-H_4MPT → Methane II (with cytochromes)

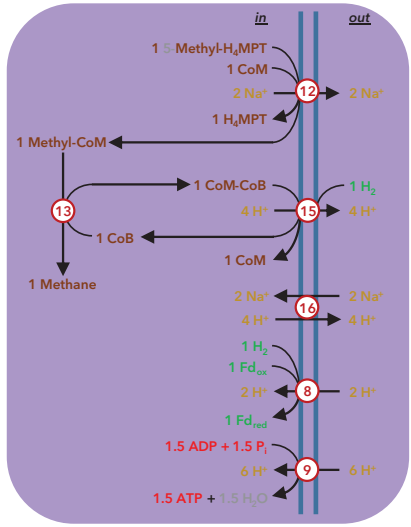


Figure 2 Several reactions generate ATP during fermentation. Reactions in the EMP pathway and malate shunt are not shown. Coefficient *y* in propionate formation is 4 for *Prevotella* (which encode Nqr NADH dehydrogenase) and 0 for *Selenomonas* (which encode Ndh NADH dehydrogenase). The reactions for propionate formation assume that: i) Pyruvate catabolism forms only reduced ferredoxin (not formate) and ii) One mole of acetate is formed for every 2 moles of propionate. The reactions for butyrate formation assume that: i) Pyruvate catabolism forms only reduced ferredoxin (not formate) and ii) No acetate is formed. If not shown, it is assumed that another reaction (e.g. one catalysed by an antiporter) balances Na^+ and H^+. See text and Hackmann et al. (2017) for details. The reactions for methane formation are those encoded by six rumen methanogens (*Methanobrevibacter boviskoreani* JH-1, *Methanobrevibacter millerae* ZA-10, *Methanobrevibacter olleyae* 1H5-1P, *Methanobrevibacter ruminantium* M1, *Methanomicrobium mobile* 1, *Methanosarcina* sp. Ms 97). The database IDs searched are those in Hackmann et al. (2017) and the following: K00577 to K00584 and pfam04210 for Mtr methyltransferase; K00399 to K00402 and K03421 to K03422 for Mcr reductase; K03388 to K03390 for HdrABC reductase; K14126 to K14128 for Mvh hydrogenase; K08264 and K08265 for HdrDE hydrogenase; COG0374, COG2864, and COG1740 for Vho hydrogenase; and pfam00999 for Nha antiporter. Those reactions are as defined by KEGG (Kanehisa et al., 2017) and Thauer et al. (2008). *Methanomicrobium mobile* 1 does not encode reaction 14, and it is not clear what reaction substitutes. Reactions: 1, phosphate acetyltransferase; 2, acetate kinase; 3, succinyl-CoA:acetate CoA-transferase; 4, succinate–CoA ligase (ADP forming); 5, phosphate butyryltransferase; 6, butyrate kinase; 7, butyryl-CoA:acetate-CoA transferase; 8, Ech hydrogenase; 9, ATP synthase; 10, Rnf ferredoxin-NAD^+ oxidoreductase; 11, NADH dehydrogenase and fumarate reductase/succinate dehydrogenase; 12, Mtr methyltransferase; 13, Mcr reductase; 14, HdrABC reductase/Mvh hydrogenase; 15, HdrDE reductase/Vho hydrogenase; and 16, Nha antiporter. Abbreviations: -P = phosphate, CoA = coenzyme A, CoB = coenzyme B, CoM = coenzyme M, Fd_{ox} = oxidized ferredoxin, Fd_{red} = reduced ferredoxin, H_4MPT = tetrahydromethanopterin or tetrahydrosarcinapterin, NAD_{ox} = oxidized NAD, NAD_{red} = reduced NAD, and P_i = inorganic phosphate.

through different pathways could be influenced by the release rates of the various mono- and oligosaccharides, pH, and other variables.

Pyruvate formed in glycolysis is a central branching point where the pathways leading toward the formation of acetate, propionate, and butyrate diverge (Russell and Wallace, 1997). In the first step of acetate and butyrate formation, pyruvate formed in glycolysis is oxidatively decarboxylated to acetyl-CoA, CO_2, and reduced ferredoxin in a reaction catalyzed by pyruvate oxidoreductases (Fig. 1). Alternatively, it can be decarboxylated to acetyl-CoA, CO_2, and NADH if catalyzed by pyruvate dehydrogenases (Hackmann et al., 2017). Another possibility is decarboxylation to formate and acetyl-CoA if the reaction is catalyzed by pyruvate-formate lyases (Asanuma et al., 1999b) (Fig. 1).

Acetyl-CoA is then converted to acetate via phosphotransacetylase and acetate kinase (Russell and Wallace, 1997) (Fig. 2). Certain bacteria (*Selenomonas* and *Mitsuokella*) do not encode phosphotransacetylase and

acetate kinase. Instead, they may use succinyl-CoA:acetate CoA-transferase and succinate–CoA ligase (Fig. 2). Rumen bacteria can also potentially produce acetate through the bifidobacterium pathway (Hackmann et al., 2017) (Fig. 1).

Acetyl-CoA can also be converted to butyrate (Russell and Wallace, 1997) (Fig. 1). First there is a condensation of two molecules of acetyl-CoA to yield acetoacetyl-CoA, which is then reduced to β-hydroxybutanoyl-CoA. β-hydroxybutanoyl-CoA is then dehydrated to crotonyl-CoA, which is reduced to butanoyl-CoA. In the final steps of the pathway, butanoyl-CoA is converted to butanoylphosphate and finally to butyrate (Fig. 2). Butyrate is also produced by transfer of CoA from butanoyl-CoA to acetate (Miller and Jenesel, 1979; Asanuma et al., 2003; Hackmann et al., 2017) (Fig. 2).

Pyruvate and phosphoenolpyruvate can be metabolized to propionate through two different pathways that have succinate or lactate as intermediates (Fig. 1). They are distinguished from each other by the randomization of carbon in pyruvate (Russell and Wallace, 1997). In the randomizing (succinate) pathway, pyruvate or phosphoenolpyruvate are carboxylated to oxaloacetate. Oxaloacetate is then reduced to malate, and malate dehydrated to fumarate. Fumarate is then reduced to succinate, with NADH or reduced ferredoxin as the intracellular electron donors (Hackmann et al., 2017). External H_2 can be taken up to reduce NAD^+ or oxidized ferredoxin to donate metabolic hydrogen ([H]) to reduce fumarate to succinate (Henderson, 1980; Asanuma and Hino, 2000). Succinate then undergoes an activation to succinyl-CoA and a chain rearrangement and decarboxylation to produce propionate (Russell and Wallace, 1997). The activation of succinate to succinyl-CoA is energetically silent as it can be coupled to propionyl-CoA conversion to propionate in the final step of the pathway. Some microorganisms release succinate as a final product, which is then taken up by succinate utilizers and converted to propionate.

In propionate non-randomizing, direct reductive pathway, pyruvate is reduced to lactate (Fig. 1). Lactate is then activated to lactyl-CoA, which is dehydrated to acrylyl-CoA. Acrylyl-CoA is then reduced to propionyl-CoA and finally propionate is produced from propionyl-CoA hydrolysis (Russell and Wallace, 1997). Lactate can be released as a final product of fermentation by some microorganisms and is taken up by lactate utilizers that convert it to propionate and acetate and smaller amounts of butyrate (Satter and Esdale, 1968; Gill et al., 1986). Lactate can accumulate as an end product of fermentation and decrease rumen pH in non-adapted animals fed rapidly fermentable diets, causing lactic acidosis (Nagaraja and Titgemeyer, 2007).

Hemicellulose and pectins are chemically heterogeneous polysaccharides that also form part of plant structural carbohydrates and contain pentose monomers such as xylose and arabinose (Scheller and Ulvskov, 2010). Xylose was shown to be metabolized by mixed rumen cultures by the transketolase and transaldolase pathways to a hexose intermediate and then through

glycolysis to VFA and to a lesser extent through phosphoketolase cleavage (Pazur et al., 1958; Wallnöfer et al., 1966) (Fig. 1) yielding glyceraldehyde-3-phosphate and acetyl phosphate. *Prevotella ruminicola* strains $B_1$4 and S23, *Selenomonas ruminantium* strain D, and *Fibrobacter succinogenes* S85 had transketolase activity, but only *S. ruminantium* D and *P. ruminicola* S23 possessed phosphoketolase activity (Matte et al., 1992). Phosphoketolase activity was found in some, but not all, strains of the hemicellulolytic bacterium *Butyrivibrio fibrisolvens* (Marounek and Petr, 1995). *Ruminococcus albus* had constitutive transketolase activity, while phosphoketolase activity was not found (Thurston et al., 1994). The same as with hexoses, metabolism of pentoses produces intermediates that are used in microbial anabolism as precursors of amino acids and nucleic acids (Marounek and Petr, 1995).

In the rumen, much of the amino acids resulting from proteolysis are deaminated, rather than incorporated directly into microbial protein, a process carried out both by abundant bacteria with low deaminating activity and by bacteria present in low numbers but possessing a high deaminating activity, and by protozoa (Wallace, 1996; Wallace et al., 1997; Hartinger et al., 2018). Although energy is presumably produced in the metabolism of the carbon skeletons released from deamination and also from Stickland-like reactions of coupled oxidation and reduction between pairs of amino acids, amino acids fermentation by pure or mixed cultures of rumen bacteria seems to yield little energy for microbial growth (Russell and Wallace, 1997; Wallace et al., 1997).

Acyl fatty acids are hydrolyzed by microbial lipases in the rumen, yielding fatty acids and glycerol, and sugars and phosphate in the case of glycolipids and phospholipids, respectively (Harfoot and Hazlewood, 1997). Glycerol so released can yield energy by entering glycolysis, and is finally metabolized mostly to propionate (Bergner et al., 1995; Krueger et al., 2010; Avila et al., 2011). Saturated fatty acids are largely inert in the rumen. Microbial biohydrogenation of unsaturated fatty acids has so far not been demonstrated to yield energy, although the reductase of *B. fibrisolvens* seemed to be membrane associated (Jenkins et al., 2008).

Most rumen methane (CH_4) is thought to be formed from dihydrogen (H_2) reduction of CO_2 (Hungate, 1967), followed in the second place of importance by formate (Hungate et al., 1970) (Fig. 1). Dihydrogen and formate are both produced in the fermentation of carbohydrates (see 2.3. Disposal of metabolic hydrogen). In hydrogenotrophic methanogenesis, CO_2 is transferred to a carrier, and then the carbon is progressively reduced to CH_4. The first carrier is methanofuran (MFR), and later carriers are tetrahydromethanopterin (in methanogens without cytochromes) or tetrahydrosarcinapterin (in methanogens with cytochromes) and coenzyme M. Methane is not directly formed from formate; rather, formate is oxidized to CO_2 before being reduced to CH_4 (Thauer et al., 2008). Formate oxidation can be carried out by

methanogens themselves or by bacteria. Methylotrophic methanogens can use methanol released from pectins and methylamines, as well as methylated sulfur compounds, as precursors of CH_4 (Enzmann et al., 2018). Recent work suggests that methylotrophic methanogens can be quantitatively important in CH_4 generation in the rumen (Söllinger et al., 2018); this may be related to the diet containing rapeseed cake used in that experiment, as rapeseed cake contains pectin (Jeong et al., 2014), which undergoes a hydrolysis of methyl esters in the rumen, yielding methanol (Pol and Demeyer, 1988).

2.2 Generation of adenosine triphosphate (ATP)

The negative ΔG of various fermentation reactions is coupled to ATP generation (Table 1). Glycolysis generates 2 net moles of ATP per mole of glucose (Czerkawski, 1986), the same as in aerobic metabolism. When acetate is formed, additional 2 ATP are generated by substrate-level phosphorylation in the conversion of acetyl-CoA to acetate (Fig. 2), resulting in a total of 4 moles of ATP per mole of glucose fermented (Table 1).

When propionate (or succinate) is formed via randomizing pathway, additional ATP are generated during the reduction of fumarate to succinate (Fig. 2). This ATP is formed by electron-transport phosphorylation (ETP). The approximate number formed is 1.5 moles of ATP per mole of glucose (i.e.

Table 1 Approximate number of ATP generated (mol/mol glucose) per fermentation product (columns) in various reactions (rows)

	Acetate	Propionate	Butyrate	Lactate	Methane
Glucose → Glucose-6P	−1	−1	−1	−1	0
Glucose-6P → Glyceraldehyde-3P	−1	−1	−1	−1	0
Glyceraldehyde-3P → PEP	+2	+2	+2	+2	0
PEP → Pyruvate	+2	0	+2	+2	0
PEP → Oxaloacetate	0	+2	0	0	0
Acetyl-CoA → Acetate	+2	0	0	0	0
Fumarate → Succinate	0	+1	0	0	0
Butanoyl-CoA → Butyrate	0	0	+1	0	0
Rnf ion pump	0	+0.5	+0.5	0	0
Ech hydrogenase	0	0	+1	0	0
5-Methyl-H_4MPT→ Methane	0	0	0	0	+0.5
Total	+4	+3.5	+4.5	+2	+0.5

See text for details. Abbreviations: $1,3P_2$ = 1, 3-bisphosphate; $1,6P_2$ = 1, 6-bisphosphate; 3P = 3-phosphate; 6P = 6-phosphate; CoA = coenzyme A, H_4MPT = tetrahydromethanopterin, and PEP = phosphoenolpyruvate.

0.75 moles of ATP per mole of propionate) for *Prevotella* and 0.5 ATP for *Selenomonas* (Hackmann et al., 2017). If 1.5 ATP are generated by ETP, then the total yield is 3.5 ATP per glucose (Table 1). Those stoichiometries of ATP generation have not been experimentally determined. Instead, they are the expected stoichiometries given the types of ion pumps (NADH dehydrogenase and Rnf; Fig. 3; see also 2.3. Disposal of metabolic hydrogen) encoded by the genomes of those rumen bacteria. They are also consistent with experimental measurements with *Wolinella succinogenes*. In this rumen bacterium, the yield was approximately 1 mole of ATP per 2 moles of fumarate reduced to succinate (resulting from the stoichiometry of ~1 H^+/1 e^-, 2 e^-/fumarate, and assuming 4 H^+/ATP) (Kröger et al., 2002).

If lactate is produced, or if propionate is formed by the direct reductive pathway, no additional ATP appears to be generated by ETP (Thauer et al., 1977; Seeliger et al., 2002). Thus, the total yield is two moles of ATP per mole of glucose fermented.

When butyrate is formed, additional ATP are generated in two ways. First, 1 mole of ATP per mole of glucose fermented (or butyrate produced) is generated by substrate level phosphorylation in the conversion of butanoyl-CoA to butyrate (Fig. 2). Second, in most *Butyrivibrio* and *Pseudobutyrivibrio*, up to 1.5 ATP are generated by ETP (Fig. 2). In other bacteria, only 0.5 ATP are generated because one ion pump (Ech) is not encoded. The number of ATP generated has not been experimentally determined, and again is that expected from the types of ion pumps possessed by these bacteria (Hackmann and Firkins, 2015a; Hackmann et al., 2017; Schoelmerich et al., 2020). If 1.5 ATP are generated by ETP, then the total yield is 4.5 moles of ATP per mole of glucose (Table 1).

Methanogenesis generates ATP by ETP only (Fig. 2). Approximately 0.5 moles of ATP per mole of CH_4 is generated by methanogens without cytochromes, which include *Methanobrevibacter* and *Methanomicrobium*. Approximately 1.5 moles of ATP per mole of CH_4 is generated by methanogens with cytochromes (Thauer et al., 2008), which include the *Methanosarcina*. Because most rumen methanogens belong to *Methanobrevibacter* (Henderson et al., 2015), the ATP yield in the rumen is probably closer to 0.5 per mole of CH_4 (Table 1).

Yields of ATP per mole of hexose fermented have seldom been experimentally determined in rumen bacteria, but can be estimated (Table 1). Table 1 summarizes an approximation, and it should be considered that the yield of ATP per mole of hexose fermented is affected by numerous factors, such as the ratio of propionate produced through the randomizing vs. the non-randomizing pathway (Russell and Wallace, 1997), the stoichiometry of H^+ or Na^+ extrusion by ion pumps per pair of electrons (Schuchmann and Müller, 2014), the stoichiometry of ATP synthases, variations in biochemical pathways such as pyruvate carboxylation in propionate randomizing pathway (Hackmann

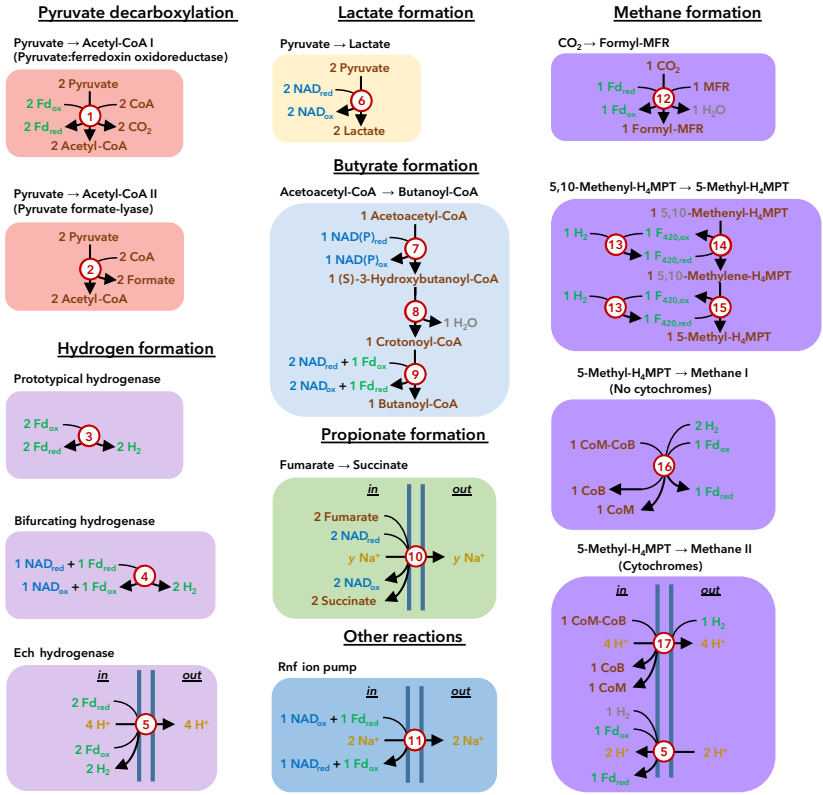


Figure 3 Several reactions generate or consume metabolic hydrogen ([H]) during fermentation. Reactions in the EMP pathway and malate shunt are not shown. See text, Fig. 2, and Hackmann et al. (2017) for details. Reactions for hydrogenases are from Sondergaard et al. (2016) and Greening et al. (2019). The reactions for methane formation are those encoded by six rumen methanogens as described in Fig. 2. The database IDs searched are those in Hackmann et al. (2017), Fig. 2, and the following: K00200 to K00203, K00205, K11260, and K11261 for formyl-MFR dehydrogenase; K00440 to K00443 for coenzyme F_{420} hydrogenase; K00319 for 5,10-methylene-H_4MPT dehydrogenase; and K00320 for 5,10-methylene-H_4MPT reductase. Those reactions are as defined by KEGG (Kanehisa et al., 2017) and Thauer et al. (2008). Reactions: 1, pyruvate:ferredoxin oxidoreductase; 2, pyruvate formate-lyase; 3, prototypical hydrogenase; 4, bifurcating hydrogenase; 5, Ech hydrogenase; 6, lactate dehydrogenase; 7, 3-hydroxybutyryl-CoA dehydrogenase; 8, enoyl-CoA hydratase; 9, butyryl-CoA dehydrogenase; 10, NADH dehydrogenase and fumarate reductase/succinate dehydrogenase; 11, Rnf ferredoxin-NAD^+ oxidoreductase; 12, formyl-MFR dehydrogenase; 13, coenzyme F_{420} hydrogenase; 14, 5,10-methylene-H_4MPT dehydrogenase; 15, 5,10-methylene-H_4MPT reductase; 16, HdrABC reductase/Mvh hydrogenase; and 17, HdrDE reductase/Vho hydrogenase. Abbreviations: CoA = coenzyme A, CoB = coenzyme B, CoM = coenzyme M, $F_{420,ox}$ = oxidized coenzyme F_{420}, $F_{420,red}$ = reduced coenzyme F_{420}, Fd_{ox} = oxidized ferredoxin, Fd_{red} = reduced ferredoxin, H_4MPT = tetrahydromethanopterin (in methanogens without cytochromes) or tetrahydrosarcinapterin (in methanogens with cytochromes), MFR = methanofuran, $NAD(P)_{ox}$ = oxidized NAD(P), and $NAD(P)_{red}$ = reduced NAD(P).

et al., 2017), pyruvate decarboxylation to acetyl-CoA (Hackmann and Firkins, 2015a), phosphorylation with pyrophosphate (Roberton and Glucina, 1982; Petzel et al., 1989), type of transport of carbohydrates into the cell (Russell and Wallace, 1997), and the utilization of fermentation intermediates for microbial anabolism.

Generation of ATP is of foremost importance to the production of microbial protein, the most important source of amino acids for ruminants (Wallace et al., 1997). That said, it is questionable that maximizing ATP generation per mole of substrate (e.g. glucose) will always maximize microbial biomass production in the rumen. As a greater proportion of the negative ΔG of reactions of fermentation is coupled to ATP generation, the net ΔG decreases in absolute value and reactions can approach equilibrium. If that happens, the rate of the reverse reaction approaches the rate of the forward reaction and the rate of the net reaction trends to zero, slowing fermentation and eventually halting it. An example of this concept are methanogens with and without cytochromes. Methanogens with cytochromes generate more ATP per mole of CH_4, but their methanogenesis must proceed with lower ΔG to generate more ATP. This means that their H_2 threshold is higher and their growth is thermodynamically unfeasible in environments with very low H_2 pressure (Thauer et al., 2008).

The number of moles of ATP generated per mole of monosaccharide fermented decreases if some fermentation intermediates, such as lactate, accumulate as final products (Kohn and Boston, 2000). Although the ATP obtained per unit of substrate fermented to lactate are lower compared to VFA production, it should be noted that lactate production is not necessarily always disadvantageous for lactate producers. With rapidly fermentable diets, lactate production is faster compared to VFA production, so that more ATP is produced per unit of time. Conversely, when energy sources are scarce, the best strategy becomes maximizing ATP generation per mole of substrate fermented by producing acetate and propionate (Russell and Wallace, 1997).

2.3 Disposal of metabolic hydrogen

In glycolysis and in pyruvate oxidative decarboxylation to acetyl-CoA, [H] is transferred to oxidized co-factors such as NAD^+ and oxidized ferredoxin (Fig. 1, Fig. 3). Catabolism of carbohydrates requires that reduced co-factors be re-oxidized and [H] disposed (Wolin et al., 1997). In the rumen and in other anaerobic microbial ecosystems, electrons can be transferred by hydrogenases from reduced co-factors to protons (H^+) to form H_2 (Czerkawski, 1986; Thiele and Zeikus, 1988) (Fig. 3). The central role of H_2 as an intercellular intermediate in the flow of [H] in rumen fermentation is evidenced by the fact that two thirds of bacterial and archaeal genomes encode hydrogenases (Greening et al., 2019).

Dihydrogen is utilized by methanogenic archaea to reduce CO_2 to CH_4 (Stewart et al., 1997; Wolin et al., 1997) (Fig. 3). Methanogenesis keeps a low concentration of H_2 in the rumen, which allows the re-oxidation of reduced co-factors and thermodynamically favors H_2-producing pathways (Janssen, 2010; van Lingen et al., 2016). Very important to interspecies H_2 transfer is the close proximity between hydrogenogens and hydrogenotrophs (Leng, 2014; Wolf et al., 2016).

Various experiments comparing fermentation products and microbial growth of monocultures of H_2 producers with co-cultures of the same H_2 producer and hydrogenotrophs, including methanogens and succinate or propionate producers, have depicted the profound role played by interspecies H_2 transfer in rumen fermentation. In the presence of a hydrogenotroph, H_2 producers favor the production of more oxidized fermentation products, and fermentation and microbial growth are stimulated (Chung, 1976; Chen and Wolin, 1977; Bauchop and Mountfort, 1981; Marvin-Sikkema et al., 1990; Pavlostathis et al., 1990). Co-culturing *R. albus* with H_2-utilizing *W. succinogenes* diminished the transcription of the ferredoxin-dependent hydrogenase by about two orders of magnitude compared to *R. albus* in monoculture (Geier et al., 2016; Greening et al., 2019).

Flows of [H] can be coupled to energy conservation through the generation of transmembrane electrochemical gradients. The oxidation of reduced ferredoxins by NAD^+ catalyzed by Rnf ion pumps allows the generation of a transmembrane electrochemical gradient of H^+ or sodium cations (Na^+) which can drive ATP generation through ATPases (Fig. 3; also see Subsection 2.2). Likewise, the oxidation of reduced ferredoxins by H^+ catalyzed by ferredoxin:H^+ reductases (Ech hydrogenases) forms H_2 and also allows the generation of a transmembrane electrochemical gradient of H^+ or Na^+ (Fig. 3). The latter reaction needs a low H_2 pressure to be thermodynamically feasible, which is generally the case in anaerobic environments in which hydrogenotrophs are present (Buckel and Thauer, 2013).

Ferredoxins have very low standard reducing potentials ($Eh°'$) and are generally more than 90% reduced, with which they can be electron donors in reactions with $Eh°'$ as low as −500 mV. The regeneration of oxidised ferredoxin with NADH or H_2 is endergonic if not coupled to exergonic reactions using appropriate combinations of electron donors such as NADH, NADPH, H_2, formate, pyruvate, or reduced F_{420} in methanogenic archaea, and electron acceptors such as NAD^+, $NADP^+$, crotonyl-CoA, acetyl-CoA, or the heterodisulphide CoM-CoB in methanogenic archaea. These mechanisms of ferredoxin reduction are known as flavin-based electron bifurcation as they are catalyzed by cytoplasmic enzyme complexes containing flavin adenine dinucleotide (FAD) or mononucleotide (FMN) (Buckel and Thauer, 2013). The coupling of an endergonic and an exergonic redox reaction thermodynamically

allows splitting an electron pair into one electron with greater *Eh°´* and a second one with lower *Eh°´* than the electron pair, thus increasing the reducing potential of the latter at the expense of the former (Buckel and Thauer, 2018b). In the reverse reaction, called confurcation, the high and low *Eh´* reduced co-factors donate electrons to reduce the bifurcating co-factor (Buckel and Thauer, 2018a).

The rumen bacterium *Megasphaera elsdenii* bifurcates two electrons from NADH to oxidised ferredoxin (endergonic reaction) and crotonyl-CoA (exergonic reaction) to yield reduced ferredoxin and butanoyl-CoA (Chowdhury et al., 2015). The rumen bacterium *R. albus* possesses an electron-bifurcating ferredoxin/NAD-hydrogenase and a ferredoxin-dependent hydrogenase, with their relative activities depending on H_2 pressure. The *hydS* gene, which is co-transcribed with the ferredoxin-dependent hydrogenase, harbors both a H-cluster to which H_2 can bind and a PAS domain which is thought to trigger signal transferring to sense H_2 pressure (Zheng et al., 2014).

Greening et al. (2019) reported that the most abundant hydrogenases in 501 genomes of cultured rumen bacteria were confurcating group A3[FeFe]-hydrogenases, which oxidize NADH and reduced ferredoxin to H_2. In agreement, another important finding was that 54% of hydrogenases transcripts in sheep rumens corresponded to group A3[FeFe]-hydrogenases, most of which were assigned to genomes of H_2-producing Clostridia (Greening et al., 2019). Similar findings with regard to the abundance of hydrogenotrophic electron bifurcating hydrogenases had been previously reported for the human colon (Wolf et al., 2016). The Greening et al. (2019) study strongly suggests a central role of electron bifurcation in [H] dynamics in the rumen. Proteomic experiments could be useful to confirm the translation of transcripts belonging to the different groups of hydrogenases in the rumen.

Metabolic hydrogen in reduced intracellular co-factors and H_2 and formate can also be transferred to electron acceptors other than CO_2, such as oxaloacetate and fumarate in propionate formation or acetoacetyl-CoA and crotonyl-CoA in butyrate formation (Henderson, 1980; Asanuma et al., 1999a; Asanuma and Hino, 2000; Greening et al., 2019). Apart from being reduced intermediates of propionate formation, succinate and lactate are also intercellular electron carriers (Stewart et al., 1997; Asanuma et al., 1999a). Metabolic hydrogen can also be donated to inorganic electron acceptors such as nitrate and sulfate. Reduction of nitrate and sulfate is thermodynamically more favorable than methanogenesis, but the incorporation of [H] into these pathways is usually limited by the availability of those substrates (Ungerfeld and Kohn, 2006).

Acetate and butyrate production result in net production of [H] (Table 2). Propionate, lactate, and methane formation on the other hand result in net consumption of [H]. In in vitro batch and continuous cultures with functional

Table 2 Metabolic hydrogen ([H]) generated during fermentation (mol/mol glucose)

	Acetate	Propionate	Butyrate	Lactate	Methane
A. Reduced NAD					
Glyceraldehyde-3P → PEP	+2	+2	+2	+2	0
Oxaloacetate → Malate	0	−2	0	0	0
Pyruvate → Acetyl-CoA	0	0	0	0	0
Pyruvate → Lactate	0	0	0	−2	0
Fumarate → Succinate	0	−2	0	0	0
Acetoacetyl-CoA → Butanoyl-CoA	0	0	−3	0	0
Rnfion pump	0	+1	+1	0	0
CO2 → Formyl-MFR	0	0	0	0	0
5,10-Methenyl-H_4MPT → 5-Methyl-H_4MPT	0	0	0	0	0
5-Methyl-H_4MPT→ Methane	0	0	0	0	0
Total	+2	−1	0	0	0
B. Reduced ferredoxin or H_2					
Glyceraldehyde-3P → PEP	0	0	0	0	0
Oxaloacetate → Malate	0	0	0	0	0
Pyruvate → Acetyl-CoA	+2	0	+2	0	0
Pyruvate → Lactate	0	0	0	0	0
Fumarate → Succinate	0	0	0	0	0
Acetoacetyl-CoA → Butanoyl-CoA	0	0	+1	0	0
Rnfion pump	0	−1	−1	0	0
CO2 → 5-Methyl-H_4MPT	0	0	0	0	−1
5,10-Methenyl-H_4MPT → 5-Methyl-H_4MPT	0	0	0	0	−2
5-Methyl-H_4MPT→ Methane	0	0	0	0	−1
Total	+2	−1	+2	0	−4

See text for details. Abbreviations: 1,3P_2 = 1, 3-bisphosphate; CoA = coenzyme A, H_4MPT = tetrahydromethanopterin or tetrahydrosarcinapterin, and MFR = methanofuran.

methanogenesis, CH_4 appears to be the main [H] sink, with propionate coming in the second place of importance (Ungerfeld, 2015b). Strictly speaking, it has not been determined whether or not this is true in in vivo, for there are no published in vivo experiments in which the production of gases and the actual flows of production of volatile fatty acids have been simultaneously determined so as to conduct a balance of reducing equivalents ([2H]) produced and incorporated. However, a quick estimation based on a meta-analysis of published experiments with dairy cows (Cabezas-Garcia et al., 2017) leads one to estimate that CH_4 must surpass propionate as a [H] sink, at least with those animals and diets (calculation not shown).

3 Methane

3.1 Significance

Methane formed in the rumen is released to the atmosphere through eructation and exhalation. The heat of combustion present in CH_4 is therefore not incorporated into compounds that the animal can absorb and utilize and represents a loss of between 2 and 12% of ingested gross energy (Johnson and Johnson, 1995). The energy loss as CH_4 was identified early on by ruminant nutrition scientists as an inefficiency of rumen fermentation. It was proposed that if [H] in CH_4 could be redirected toward useful sinks, animal productivity might improve (Czerkawski and Breckenridge, 1975; Davies et al., 1982; Martin and Macy, 1985). As a consequence, research on the inhibition of rumen methanogenesis in vitro (Bauchop, 1967) and in vivo (Clapperton, 1974; Cole and McCroskey, 1975; Czerkawski and Breckenridge, 1975) has been conducted for several decades.

More recently, the interest in controlling CH_4 formation in the rumen and the emissions of enteric CH_4 to the atmosphere has been re-enforced by the increasing concerns about climate change (Moss et al., 2000). The increase in atmospheric CH_4 accounts for about 20% of the increase in the total emissions of greenhouse gases as expressed as CO_2-eq (the sum of the amounts of each greenhouse gas weighted by its warming potential) since the industrial revolution. About 30% of the anthropogenic emissions of CH_4 are estimated to originate from livestock enteric fermentation and manure decomposition (Saunois et al., 2016a). A simple arithmetic calculation would then result that, on a global scale, enteric CH_4 accounts for a relatively minor 6% or less of total anthropogenic emissions of greenhouse gases as CO_2-eq. However, there are reasons for why an abatement of CH_4 emissions is strategic to the amelioration of the emissions of greenhouses gases: i) The increase in the concentration of atmospheric CH_4 in the last decade has been much more rapid than that of CO_2, and might be primarily from agricultural origin (although there are uncertainties regarding changes in the main sources and sinks (Saunois et al., 2016b); ii) The 28-fold greater global warming potential relative to CO_2 implies that decreasing the emissions of CH_4 would have a much greater impact compared to an equivalent amount of CO_2; iii) The much shorter lifetime of CH_4 in the atmosphere relative to CO_2 (9 vs. 30 years) makes CH_4 an attractive target for achieving shorter term impacts of amelioration of climate change (Montzka et al., 2011; Saunois et al., 2016b).

In view of the above, there is an interest in understanding and controlling CH_4 production in the rumen. The readers are referred to excellent reviews discussing past and current research to control CH_4 emissions from ruminants (Beauchemin et al., 2008; Eckard et al., 2010; Martin et al., 2010; Morgavi et al., 2010). Herein we will focus on the biochemistry of rumen fermentation and

methanogenesis, and the consequences of inhibiting CH_4 production in the rumen on microbial metabolism.

3.2 Methane production and the VFA profile

The pathways of formation of the main VFA determine that acetate, and to a lesser extent butyrate, production, are associated with a net release of [H]. If acetate is formed through the *Bifidobacterium* shunt along with lactate no [H] is released, although the proportion of rumen acetate formed through this pathway is unknown. Propionate production on the other hand requires a net incorporation of [H], which results in propionate competing with [H] with methanogenesis and associating negatively with CH_4 production (Janssen, 2010). This results in the profile of VFA formed in rumen fermentation being closely associated with the amount of CH_4 produced per unit of organic matter (OM) fermented. Wolin et al. (1997) summarized the theoretically expected moles of CH_4 per mole of fermented hexose for various example cases of acetate, propionate, and butyrate molar percentages.

In theory, the stoichiometries of production of the three main VFA, acetate, propionate, and butyrate allow for an ample range of VFA profiles, some of which could be associated with no CH_4 formation. For example, in theory, a 2:1 molar production ratio of propionate to butyrate, and no acetate being formed (and thus an acetate to propionate molar ratio equal to zero), would result in a theoretical 'CH_4-less' fermentation profile. On the other extreme, a theoretical fermentation profile with acetate as only VFA and all [H] incorporated into CH_4 would have an acetate to propionate molar production ratio of virtually $+\infty$. In reality however, in vivo molar concentration ratios of acetate to propionate in 73 different experiments varied within a much narrower range of between 4 and 1.5:1 (Ungerfeld, 2013).

Therefore, although the stoichiometries of VFA production would allow an ample range of VFA profiles, there seems to exist a tight control of fermentation. In in vivo experiments in which labeled VFA were used to determine flows of VFA production, the great majority of measurements of opposite flows of VFA interconversion between acetate and propionate, and acetate and butyrate, were relatively similar, suggesting closeness to thermodynamic equilibrium between VFA and thermodynamic control as an explanation for the relatively narrow range observed in the rumen VFA profile (Ungerfeld and Kohn, 2006).

The theory of thermodynamic control of the VFA profile can offer a possible explanation for the relatively narrow range observed in VFA ratios relative to what their stoichiometries of formation would theoretically allow. Yet, thermodynamic equilibria alone do not explain the changes in VFA ratios that occur with dietary changes and that are associated to the extent of CH_4

formation. Janssen (2010) proposed a model based on rumen H_2 concentration and methanogens growth rate based on the Monod function, a mathematical model of microbial growth, to explain how high concentrate diets result in a fermentation shift from acetate to propionate. The model was developed exemplifying how the growth of methanogens is affected by rumen outflow rate, pH, and methanogenesis inhibitors. Changes in methanogen growth rate affect in turn H_2 concentration according to the Monod relationship. The model also considers the diurnal variation in H_2 production (Janssen, 2010). How changes in rumen outflow rate, rumen pH, and methanogenesis inhibitors, can affect CH_4 production and the VFA profile through methanogens growth rate and H_2 concentration will be discussed below.

High concentrate diets increase rumen outflow rates, which imposes greater growth rates to methanogens. Based on the Monod function, when methanogens growing on H_2 as energy source grow faster, H_2 concentration increases. In turn, and according to the stoichiometries of production of the different VFA, increased H_2 concentration thermodynamically favors a shift from acetate to propionate production (Janssen, 2010). In agreement with the predictions of this theory, increasing passage rate in a chemostat resulted in a shift of fermentation toward more propionate and less CH_4, although butyrate decreased more than acetate with increasing outflow rate in that experiment (Isaacson et al., 1975). Similar results were reported for the recovery of [H] in CH_4, H_2, and VFA at low and high dilution rates in continuous cultures (Stanier and Davies, 1981).

Similarly, methanogens are sensitive to pH drops, and a decrease in rumen pH would decrease their maximum growth rates. This would result in an increased H_2 concentration to maintain the same actual growth rate according to the Monod function. Increased H_2 concentration would again favor a fermentation shift toward H_2-incorporating pathways such as propionate production. Methanogenesis inhibitors are likewise postulated to elevate H_2 concentration and shift fermentation toward propionate production through a similar mechanism (Janssen, 2010). In agreement with the predicted effects of methanogenesis inhibitors, elevated H_2 accumulation has been summarized for methanogenesis-inhibited batch and continuous cultures (Ungerfeld, 2015b) and in vivo (Ungerfeld, 2018), although a fermentation shift toward propionate was reported for methanogenesis-inhibited batch but not continuous cultures (Ungerfeld, 2015b).

An in vitro batch cultures experiment found an effect of the roughage to concentrate ratio on the acetate to propionate ratio that was independent of pH (Russell, 1998). Janssen (2010) also discussed how the substrate fermentability per se could affect H_2 production and concentration, and CH_4 production and the VFA profile. The spatial and temporal heterogeneity in the rumen environment results in some niches H_2 production and methanogenesis being tightly coupled and H_2 concentration kept low, while in others rapid fermentation could result in transient and local high concentration of H_2.

A particularly important example of temporal variation are the high rates of fermentation and H_2 production right after feeding (van Lingen et al., 2017; Söllinger et al., 2018), which would cause an increase in H_2 concentration and temporarily inhibit H_2-releasing processes and stimulate propionate production. The uncoupling between H_2 production and utilization has been explained as rapid H_2 evolution exceeding the capacity of methanogens to incorporate all the H_2 produced (Rooke et al., 2014). An interesting experiment by Walker and Monk (1971) seems to support this proposition: those investigators found that increasing the size of a dose of labeled glucose in in vitro rumen batch cultures by about 30 000-fold and caused a decrease in the acetate to propionate ratio from 24.3 to 2.42. pH was not reported in that work so it cannot be confirmed that the change in the acetate to propionate ratio was only the result of greater rate of fermentation and a drop in pH did not have any role.

These short-term changes in the fermentation profile are more likely due to relative changes in the activities of the different microorganisms within the microbial community and the different fermentation pathways within microbial cells, rather than changes in the microbial community composition. On the other hand, longer term shifts in the fermentation profile resulting from a dietary change are more likely to be predominantly caused by changes in the composition of the microbial community (Janssen, 2010).

Models integrating kinetics and thermodynamics to represent and predict flows of [H] in the rumen (Kohn and Boston, 2000; Offner and Sauvant, 2006; van Lingen et al., 2016, 2019) or depicting the thermodynamic feasibility of particular [H] sinks in the methanogenesis inhibited rumen fermentation (Ungerfeld, 2015a) have been developed. Further progress in understanding the control of [H] flows would likely necessitate in vivo measurements of co-factor oxidation states, particularly the NADH/NAD^+ pair and ferredoxins, the activities of the different types of hydrogenases, and the magnitude of dissolved H_2 gradients. It would be important to conduct determinations in animals on different diets and at different times after feeding. Reports on intracellular pH and concentrations of reduced and oxidized co-factors in rumen microorganisms are scarce and have been limited to the NADH/NAD^+ pair in in vitro cultures (Hino and Russell, 1985). These determinations would evidently be experimentally challenging. Better representation of animal and dietary factors such as rumen outflow rates and particles size is also proposed to contribute to improve existing models (van Lingen et al., 2019).

3.3 The methanogenesis-inhibited rumen fermentation

It is attractive to propose inhibiting the production of CH_4 in the rumen as an opportunity to ameliorate the impact of ruminant production on the environment and simultaneously improve the efficiency of energy utilization by ruminants.

With regard to the latter aspect, however, a meta-analysis of experiments in which rumen methanogenesis was targeted with specific chemical inhibitors, in the absence of changes in the diet or animal, revealed that inhibiting rumen methanogenesis did not consistently improve the efficiency of milk production or of growth and fattening (Ungerfeld, 2018).

Because an improvement in animal productivity would help the adoption of tools to control rumen methanogenesis, it is important to understand why decreasing energy losses as CH_4 did not always translate into gains in productivity:

1. With a hypothetical diet with 70% DM digestibility, and based on gross energy losses between 2 and 12% GE (Johnson and Johnson, 1995), energy losses as CH_4 would represent between approximately 3 and 17% of ingested digestible energy (DE). In most experiments, the inhibition of CH_4 production has been moderate, e.g. 30%, which could represent an increase of between 1 and 5% in the ingestion of DE. Ignoring urine energy losses, and considering metabolizable to net energy conversion factors of 0.6 for maintenance and milk production, and 0.2 for growth and fattening, the maximum gain in net energy obtained by inhibiting CH_4 production by 30% could be of about 3% available to increase productivity. It is thought that few experiments would have the power to detect such differences.
2. A consistent finding in [2H] balances from in vitro experiments is a severe decrease in [2H] recovery in VFA, CH_4 and H_2. Apart from H_2, it is possible that other atypical [H] sinks that are intermediates in the rumen fermentation with functional methanogenesis and do not have a nutritional value, such as formate, accumulate when methanogenesis is inhibited (Ungerfeld, 2015b). Accumulation of H_2 can represent a considerable proportion of the energy saved in CH_4 that is not formed. The response of H_2 accumulation to inhibiting methanogenesis has been highly variable among in vivo experiments even with the same inhibitor and similar diets and animals (Fig. 4).
3. More research about the consequences of inhibiting methanogenesis on the amount of OM digested and fermented is needed. Dihydrogen accumulation can indicate a disruption of the disposal of [H] and co-factors re-oxidation, which can inhibit fermentation (Wolin et al., 1997). There are indications of an inhibition of fermentation when methanogenesis was inhibited in vitro (Ungerfeld, 2015b), which agrees with an increase observed in the ratio of NADH to NAD^+ in a batch cultures experiment (Hino and Russell, 1985).

   Inhibiting methanogenesis in vivo has not affected in situ DM apparent digestibility in some (Nolan et al., 2010; Martínez-Fernández et al.,

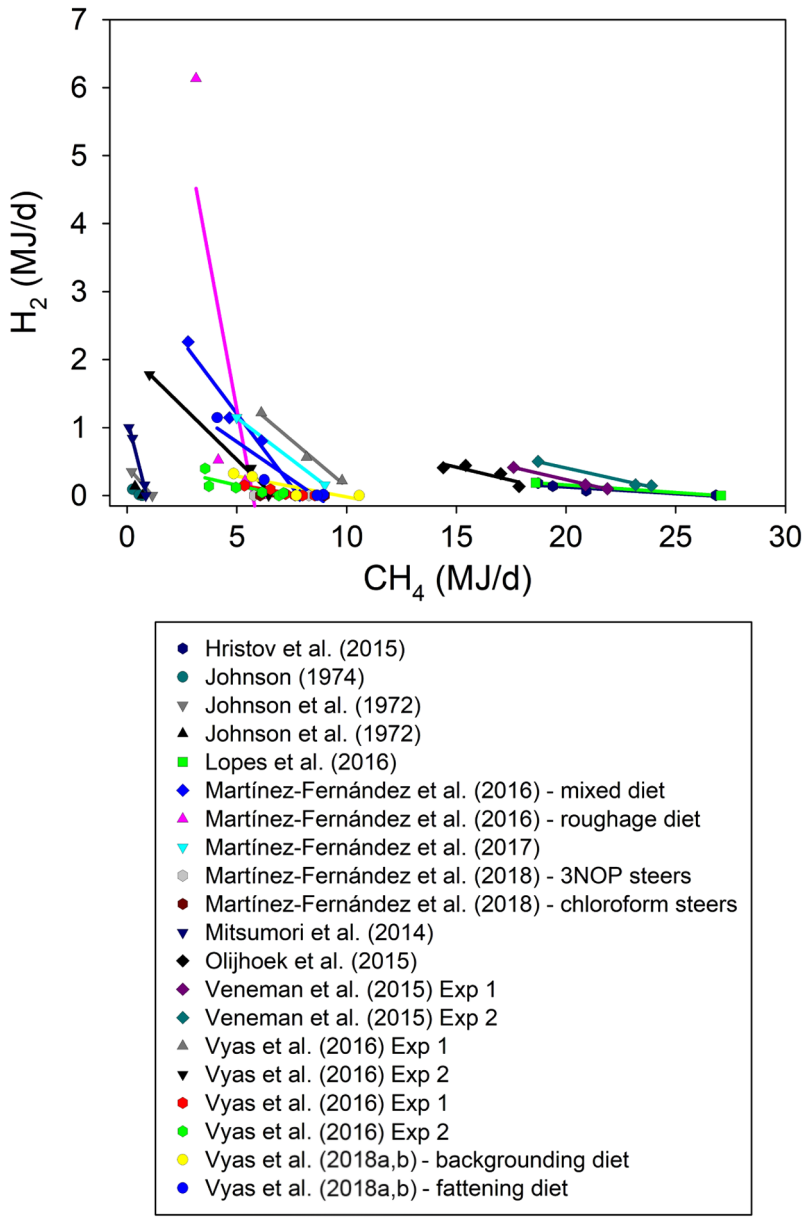


Figure 4 Response in energy losses in dihydrogen (H_2) as methane (CH_4) production decreases in 13 studies including 20 experiments or diets: H_2 (MJ) = 1.62 (±0.42; $P < 0.001$) + *exp* ($P = 0.081$)– 0.30 (±0.17; $P = 0.085$) CH_4 (MJ) + *exp* × CH_4 ($P = 0.004$); N = 67, $R^2 = 0.81$ ($P = 0.002$). Source: adapted from Hristov et al. (2015), Johnson et al. (1972, 1974), Lopes et al. (2016), Martinez-Fernandez et al. (2016, 2017, 2018), Mitsumori et al. (2014), Olijhoek et al. (2016), Veneman et al. (2015), Vyas et al. (2016, 2018).

2014) but not all (Martinez-Fernandez et al., 2018) experiments and was negatively related to total VFA concentration (Ungerfeld, 2018). Neither the measurements of in situ apparent digestibility nor VFA concentration might accurately reflect digestion and fermentation, as on the one hand apparent digestibility does not consider microbial biomass, and on the other hand VFA concentration is affected not only by VFA production but also by VFA absorption, passage, incorporation into microbial biomass and also changes in rumen volume (Dijkstra et al., 1993; Storm et al., 2012; Hall et al., 2015). Whilst it is clear that a disruption of [H] flows must halt fermentation, the question is to what extent the oxidation states of co-factors can be altered without hindering fermentation, and which alternative pathways in combination might incorporate [H] at the same rates as methanogenesis.

4 Inhibiting rumen methanogenesis could cause changes in rumen fermentation that may not always match animal requirements. For example, inhibiting methanogenesis is predicted to displace rumen fermentation from acetate to propionate (Janssen, 2010), which has been confirmed in batch culture experiments (Ungerfeld, 2015b). If inhibiting methanogenesis also increases propionate production in vivo, it is possible that animals not deficient in glucogenic precursors do not respond to extra propionate, unless the diet is made more ketogenic to adjust it to the methanogenesis inhibition intervention. Greater supply of energy and glucose may not translate into greater productivity in animals limited by the supply of other nutrients. Moreover, propionate acts as a satiety signal (Allen et al., 2009), which seems consistent with the decrease in intake associated to the inhibition of methanogenesis (Ungerfeld, 2018).

It is clear that for obtaining responses in productivity it is not enough to inhibit CH_4 production and [H] must be redirected toward the fermentation products that limit the productivity of each type of animal. In turn this demands a thorough understanding of the control of [H] flows of production and utilization both in the rumen with functional methanogenesis and in the methanogenesis-inhibited rumen fermentation.

Partial success in incorporating [H] into useful sinks, as reflected by decreased H_2 accumulation and increased concentration of some products of interest, has been illustrated by some in vitro batch culture experiments. The addition of malate or fumarate, which are intermediates of the randomizing pathway of propionate production, could partially alleviate H_2 accumulation when methanogenesis was inhibited, and sometimes increase final propionate concentration (Mohammed et al., 2004; Tatsuoka et al., 2008; Ebrahimi et al., 2011). Other work has shown enhancement of propionate, succinate, or

butyrate production, and less CH_4, in batch cultures supplemented with live succinate- or propionate-producing organisms (Mamuad et al., 2014; Kim et al., 2016). Addition of reductive acetogens to methanogenesis-inhibited batch cultures decreased H_2 accumulation (Nollet et al., 1997; Le Van et al., 1998; Lopez et al., 1999). In contrast, in vivo dosage with Propionibacteria did not affect total CH_4 emissions (Vyas et al., 2014a,b, 2015). In an in vivo experiment, Martinez-Fernandez et al. (2017) achieved a decrease in H_2 emissions in steers whose methanogenesis had been inhibited with chloroform through the addition of phloroglucinol. It was apparent that phloroglucinol incorporated H_2 in its reduction to acetate.

4 Factors influencing the efficiency of microbial growth

Microbes use ATP generated in fermentation to provide energy for growth, but their growth efficiency is far from perfect. There are high stakes in increasing this efficiency, as microbes growing in the rumen account for 70% of the protein available to animal digestion (Broderick et al., 2010). Theoretically, microbes should grow with high and nearly constant efficiency. From biochemical pathways, one can calculate how much ATP is required to synthesize macromolecules in microbial cells. These calculations, first done in the 1970s, showed that microbes should grow with an efficiency, or Y_{ATP}, of ~30 g dry matter/mol ATP (Stouthamer, 1973). Calculated efficiency was little affected by changing some of the assumptions in the calculation (e.g. whether protein is synthesized from glucose and ammonia or from preformed amino acids).

In reality, rumen microbes grow with low and variable efficiency. In fact, it appears that they grow from ⅓ to ⅔ of the theoretical efficiency (Fig. 5). Most studies showing low efficiency were conducted with pure cultures (Russell and Wallace, 1997). However, available evidence suggests efficiency is just as low for mixed cultures (Isaacson et al., 1975) and in vivo (Kennedy and Milligan, 1978). Importantly, growth efficiency was not only lower than theoretical but considerably variable, even within experiments (Fig. 5). ATP generation was not measured in these and other experiments reporting Y_{ATP} (Bauchop and Elsden, 1960; de Vries et al., 1973), but estimated assuming constant stoichiometries of ATP generation per each VFA produced. Therefore, reported values of Y_{ATP} actually correspond to ratios of microbial biomass to different linear combinations of VFA and CH_4 production. As discussed in Subsection 2.2, generation of ATP per mole of each VFA produced is affected by numerous factors, which likely differ among experiments and treatments, thus, actual variation in Y_{ATP} could be even higher than the reported one. The assessment of Y_{ATP} is probably more accurate for acetate, lactate, and ethanol production,

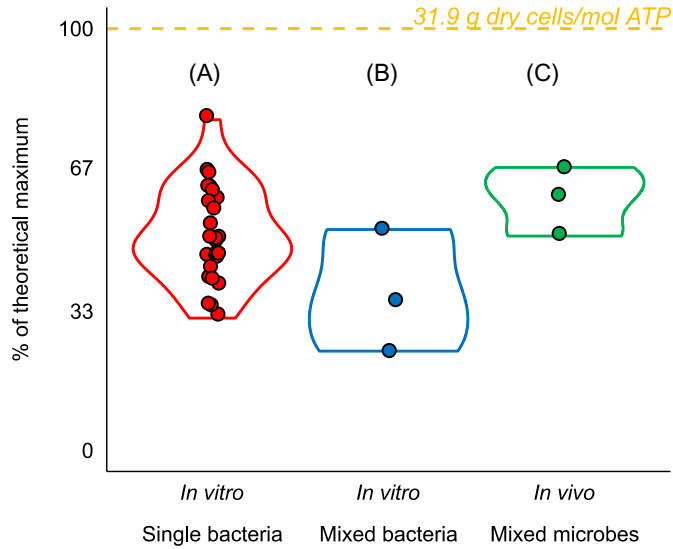


Figure 5 Efficiency of growth in rumen microbes. Values are the actual growth efficiency (g dry cells/mol ATP) expressed as a percentage of a theoretical maximum efficiency [31.9 g dry cells/mol ATP; (Stouthamer, 1973)]. Data for single bacteria in vitro (A) are summarized from Russell and Wallace (1997). Data for mixed bacteria in vitro (B) are from Isaacson et al. (1975). Data from mixed microbes *in vivo* (C) are from Kennedy and Milligan (1978).

in which ATP is generated by substrate-level phosphorylation, but deviations might be important for propionate, butyrate, and CH_4, in which ETP also occurs.

4.1 Energy sinks

Although it has been long recognized that microbes grow with low efficiency, the underlying causes have been difficult to identify. We can infer that low efficiency results from microbes directing ATP away from growth and toward other energy sinks (Fig. 6). Although several energy sinks could exist, recent work has suggested that three of them are important for mixed rumen microbes (Hackmann et al., 2013a; Teixeira et al., 2017). These energy sinks are maintenance, glycogen accumulation, and energy spilling (Fig. 6).

4.1.1 Maintenance

Maintenance refers to cellular 'housekeeping,' including mostly maintaining ion balances across the cell membrane and to a lesser extent turnover of macromolecules. Motility is another component of maintenance, although it is a minor one (Russell and Cook, 1995). Even though it is required for microbial

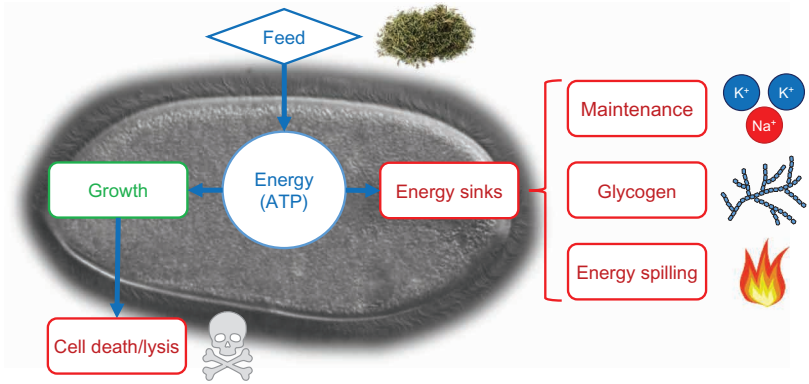


Figure 6 Factors that cause microbes to grow inefficiently. Some ATP released from fermentation of feed is directed towards growth, but large amounts are directed towards energy sinks such as maintenance, glycogen accumulation and energy spilling. Death and lysis of cells also decreases efficiency.

survival, maintenance is wasteful from the cow's and ruminant production perspective, because its net product is heat (rather than microbial cells).

Maintenance is important to influence growth efficiency, especially when microbial growth rates are low. At a low growth rate of 5%/h, maintenance accounts for more than 30% total energy expended by mixed rumen bacteria. At high growth rate of 20%/h, by comparison, it accounts for only 10% (Russell, 2007a). This is because maintenance is a fixed cost, and as in economics, fixed costs are proportionally more important when productivity is low. Protozoa have a greater generation time than bacteria, which implies proportionally higher maintenance energy usage in total energy expenditure (Newbold et al., 2015). Although maintenance energy is a non-growth, fixed cost, there is evidence that maintenance requirements are not constant (Russell and Cook, 1995). For example, maintenance may be higher on a rapidly fermentable substrate (glucose) than a more slowly fermentable one (cellobiose) (Thurston et al., 1993). A greater understanding of those mechanisms, and if possible, the design of means to decrease maintenance costs could have a positive impact in extensive ruminant production systems in which microorganisms grow at low rates.

4.1.2 Glycogen accumulation

Microbes accumulate glycogen when the cell has excess carbohydrate (Preiss and Romeo, 1990). In mixed rumen microbial cultures, small amounts of glucose and maltose are also accumulated (Hackmann et al., 2013b). Accumulating glycogen may appear an economical way to store extra carbohydrate. However, the cell loses ATP in the process, unless the cell outflows the rumen without

hydrolyzing its glycogen, and glycogen is digested in the duodenum and absorbed as glucose. During glycogen synthesis, the cell spends two moles of ATP per mole of glucose incorporated. If the cell later degrades the glycogen, it recovers only one mole of ATP equivalent per mole glucose (in the form of glucose-1-phosphate) (Nelson and Cox, 2017). Because glycolysis generates as little as two moles of ATP per mole of glucose (Table 1), the amount of ATP spent on glycogen accumulation could be important.

4.1.3 Energy spilling

Energy spilling indicates an uncoupling between catabolism and anabolism. During energy spilling, microbial cells dissipate excess energy as heat. The cell spills energy when catabolism generates more ATP than it is spent on growth, maintenance, and glycogen accumulation. Microbial cells spill energy in response to excess fermentable carbohydrates or when nutrients necessary for microbial growth, such as N, become limiting (Russell, 2002; Hackmann and Firkins, 2015b).

Energy spilling is accomplished by futile cycles of ions, glycogen, or trehalose (Russell and Cook, 1995; Hackmann and Firkins, 2015b). In *S. bovis*, for example, H^+ are cycled across the membrane (Russell, 2002). Energy spilling in *S. bovis* can be triggered by excess glucose in the presence of ammonium (NH_4^+) as the only N source. The intracellular messenger that activates energy spilling in this bacterium seems to be ATP. The permeability of cell membrane decreases, and this allows the futile cycle of H^+ (Russell and Cook, 1995). The mechanisms of energy spilling in mixed rumen microbes have not been determined. Generating more knowledge with mixed cultures of the possible magnitude and mechanisms of energy spilling with different substrates would be important for understanding the extent to which rumen microbes can spill energy in vivo, and under which conditions.

4.1.4 Comparison of sinks

Maintenance, glycogen accumulation, and energy spilling were shown to be important in batch culture experiments (Hackmann et al., 2013a; Teixeira et al., 2017). The most recent of these experiments compared sinks in bacteria and protozoa (Teixeira et al., 2017), which are the two largest groups making up mixed microbes. When given glucose, protozoa responded by accumulating large amounts of glycogen (Fig. 7). This was apparent when i) measuring glycogen and ii) calculating how much heat production could be accounted by its synthesis. Bacteria, in contrast, synthesized comparatively less glycogen, but energy spilling was important. In both groups, maintenance also was important (Fig. 7).

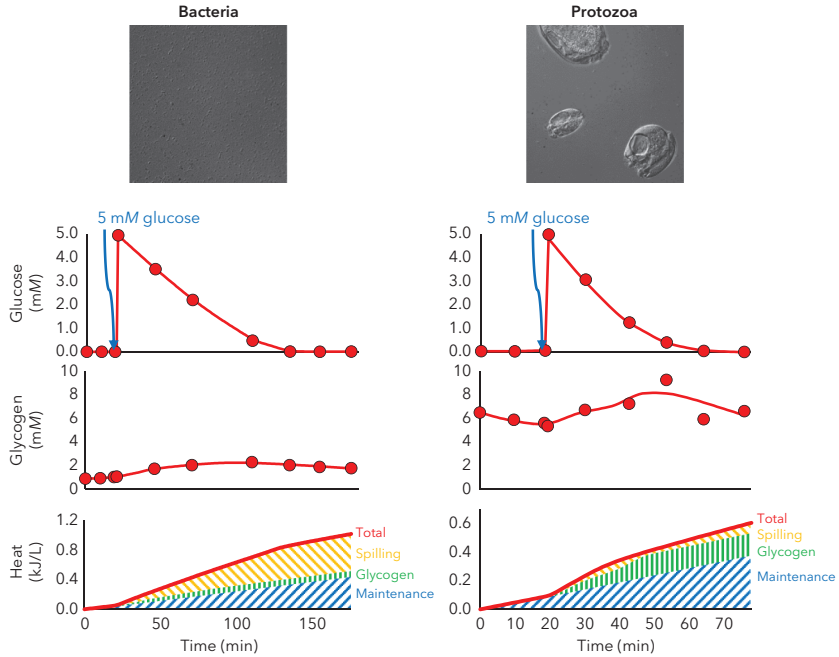


Figure 7 The importance of different energy sinks in rumen bacteria vs. protozoa. Bacteria and protozoa were separated and grown with glucose (5 mM). Energy spilling and maintenance were important in bacteria, whereas glycogen and maintenance was important in protozoa. Source: adapted from Teixeira et al. (2017).

From a thermodynamic point of view, maintenance, glycogen accumulation, energy spilling, and microbial growth all involve physicochemical processes that dissipate heat. It is tempting to think that heat production might increase with growth rate, as more chemical reactions may be expected to occur per unit of time and volume in cells growing faster. This was not the case however for *S. ruminantium* and *P.* (*Bacteroides*) *ruminicola* growing in continuous culture at varying growth rates with limiting glucose, except when growth rates were sufficiently high to cause glucose accumulation. With limiting glucose, it seemed that heat production was associated with maintenance rather than with growth (Russell, 1986).

4.2 Other factors influencing growth efficiency

4.2.1 Cell composition

Stouthamer (1973) calculated that the greatest demand for cell growth in *Escherichia coli* corresponded to polymerization of amino acids to proteins.

The high energy cost for the cell of synthesizing protein results both from the high demand for ATP to assemble amino acids to build proteins and from the high content of proteins in microbial cells. Polysaccharides and RNA are also quantitatively important components of the cell whose proportions vary considerably depending on the stage of growth; yet, the ATP cost of protein synthesis was still estimated to be greater in *E. coli* (Russell and Cook, 1995). However, and as discussed, in mixed rumen bacteria and protozoa, the accumulation of glycogen and the energy cost of its synthesis can also be considerable (Hackmann and Firkins, 2015b).

4.2.2 Cell lysis

Though non-growth energy sinks are important, another factor that causes low efficiency is cell death and lysis, resulting in microbial biomass turnover (Fig. 5). Protozoal predation, autolysis, and bacteriophages are all causes of cell lysis. In the rumen, the magnitude of lysis is high, and as much as 50% of microbial protein can be degraded to non-protein N (Wells and Russell, 1996; Oldick et al., 2000). Only part of the amino acids and non-protein N originated from the degraded microbial proteins will be re-incorporated into newly synthesized microbial protein, which implies an inefficiency in N utilization. Furthermore, microbial protein degradation in the rumen also implies an energy utilization inefficiency, even for the amino acids resulting from protein breakdown that are not degraded and are utilized to synthesize new microbial proteins, as ATP has to be spent in protein re-synthesis (Czerkawski, 1986).

Defaunation has been proposed as a means to decrease cell lysis, as initial experiments with low N diets seemed to point out toward an improved production of microbial protein and efficiency of N utilization. However, research with high-producing animals has been less clear in showing the benefits of defaunation on the production of microbial N (Firkins et al., 2007). In addition, the composition of the protozoal community may influence bacterial predation and cell lysis, as holotrichs have a lower bacterial predatory activity than entodiniomorphids. Currently, several approaches to control protozoa are being investigated, but a defaunation method that is practical and safe to apply at a production setting is not available (Newbold et al., 2015). Also, increasing rumen outflow rate has been proposed as a mechanism to increase the proportion of cells that can pass out of the rumen before they lyse (Wells and Russell, 1996), although it should be taken into account that greater passage rates can result in lower digestibility (Løvendahl et al., 2018).

Another factor that causes low efficiency could be release of intracellular metabolites (Bond et al., 1998), cellodextrins (Wells et al., 1995), and maltodextrins (Matulova et al., 2001; Nouaille et al., 2005). Although this release

has been observed for pure cultures, its importance for mixed microbes in the rumen is unknown.

4.2.3 Utilization of energy sources as carbon for cell growth

The proportion of carbon in the energy substrate that is incorporated into cell biomass largely depends on ATP generation in fermentation. If ATP generation per mole of energy substrate fermented is relatively low, most of the energy source will be used for energy production, but if ATP generation is high, much of the carbon in the energy source can be assimilated into cell components (Russell and Cook, 1995). Mathematically though, changes in the partition of carbon between fermentation products and microbial anabolism caused in turn by changes in ATP generation would affect more microbial biomass produced per gram of OM degraded than microbial biomass per mole of ATP (Y_{ATP}), because microbial biomass formed and ATP generated would change in the same direction.

4.3 Future work on strategies for increasing the efficiency of microbial growth

We need to characterize the relative importance of each energy sink in more complex experimental models than batch cultures. Experiments with continuous cultures growing on glucose have already confirmed that maintenance is important for mixed bacteria (Isaacson et al., 1975). Future experiments with other carbohydrates, such as starch or fiber, are needed to expand this knowledge toward the live animal. Only batch cultures have been used to measure energy spilling in mixed microbes (Hackmann and Firkins, 2015b). More experiments are needed to evaluate and quantify the importance of energy spilling in the rumen.

In vivo experiments have measured glycogen in mixed bacteria (Jouany and Thivend, 1972b; McAllan and Smith, 1974; Leedle et al., 1982) and protozoa (Jouany and Thivend, 1972a). These experiments confirm that glycogen is accumulated by mixed microbes. However, those experiments have not established how important is the amount accumulated. To answer this question, future experiments could feed different isotopically labeled carbohydrates under different conditions, and measure what proportion is incorporated into glycogen.

One way to increase microbial efficiency is by improving models for evaluating dairy cattle diets. Despite their wide use by nutritionists, aspects of these models are still crude. The Dairy NRC (2001), for example, assumes microbial efficiency is a constant value (adjusted for ruminally degraded N). This leaves applied nutritionists with little guidance for improving efficiency.

With recent measurements of energy sinks, better models can be constructed. These improved models will help nutritionists formulate diets that increase efficiency.

5 Interactions between rumen energy and nitrogen metabolism

Energy and N metabolism in the rumen are intimately linked (Nocek and Russell, 1988). Synthesis of nitrogenous compounds such as proteins and nucleic acids requires the utilization of negative ΔG generated in catabolism, and in turn catabolic reactions require the participation of enzymes whose synthesis is part of N metabolism. In this section, we will discuss three different aspects of the interaction between energy and N metabolism in the rumen: i) The effects of methanogenesis inhibition on the synthesis of microbial amino acids and protein; ii) The effects of N source on energy spilling; iii) Synchrony in the supply of energy and N sources.

5.1 Consequences of inhibiting rumen methanogenesis on microbial synthesis of amino acids

The mixed rumen microbiota incorporates NH_4^+ from the medium into carbon chains to synthesize amino acids that can be used for protein synthesis. Amination requires the incorporation of one mole of [2H], usually provided by NADH or NADPH, per mole of NH_4^+ incorporated. The opposite reaction, deamination, releases [2H] as NADH or NADPH (Wallace et al., 1997). Inhibiting rumen methanogenesis disrupts electron relocation, as evidenced by H_2 accumulation and decreased reducing potential (*Eh*) (Czerkawski, 1986; Sauer and Teather, 1987). It has been speculated that inhibiting methanogenesis could stimulate the disposal of [H] into amination and increase microbial protein production (Ungerfeld et al., 2007). In agreement, inhibiting methanogenesis in rumen batch cultures increased the NADH/NAD^+ ratio and inhibited amino acids fermentation (Russell and Jeraci, 1984; Hino and Russell, 1985). In cell extracts of rumen bacteria and protozoa, deamination of the more reduced amino acids was inhibited by NADH and stimulated by methylene blue, an oxidizing agent (Hino and Russell, 1985).

Inhibiting rumen methanogenesis in batch and continuous cultures consistently decreases the recovery of [2H] in the main VFA and gases, and it was proposed that the unaccounted [2H] incorporation could be partially explained by an increase in amination (Ungerfeld, 2015b). More recent results, however, could not demonstrate a net increase of [2H] incorporation in the synthesis of microbial amino acids in rumen batch cultures (Ungerfeld et al., 2019). The effects of inhibiting methanogenesis in mixed rumen batch

cultures on the production of microbial N and other cell components has been inconsistent, with some increases (Ungerfeld et al., 2007; Guo et al., 2009) and decreases in other studies (Russell and Martin, 1984).

In vivo results of the effects of inhibiting rumen methanogenesis on microbial protein production in the rumen are scarce. Nolan et al. (2010) observed a numerical increase in microbial protein output estimated using purine derivatives in urine when CH_4 production was moderately inhibited using nitrate. Also, when CH_4 production was moderately inhibited using nitrate, Wang et al. (2018) observed an increase in microbial N in rumen contents.

The effects of inhibiting rumen methanogenesis in vivo on the metabolism of amino acids was studied by Martinez-Fernandez et al. (2016), who found that inhibiting CH_4 production with chloroform increased the concentrations of several amino acids in rumen fluid of steers fed a mixed diet, and the concentrations of almost all amino acids with a hay diet. Their results suggest an increase in proteolysis (Martinez-Fernandez et al., 2016), as the alternative possibility of an inhibition in the deamination of free amino acids would not seem to agree with the increase in branched chain fatty acids observed with both diets when methanogenesis was inhibited. Martinez-Fernandez et al. (2016) also found increases in rumen fluid concentration of purine metabolites inosine and hypoxanthine when methanogenesis was inhibited in steers on the hay diet, which might result from greater degradation of feed nucleic acids and/or greater lysis of microbial cells. An increase in the concentration of free amino acids may thus also result from decreased incorporation of amino acids into microbial protein and/or greater lysis of microbial cells.

Martinez-Fernandez et al. (2018) showed an increase in the concentration of methylamines and other methylated compounds when rumen methanogenesis was inhibited by chloroform or 3-nitrooxypropanol (3NOP). Although methylamines can be formed from decarboxylation of amino acids, it seems more likely that the increase in their concentration resulted from an inhibition in methylamines utilization by methylotrophic methanogens. This agrees with the decrease they observed in Methanomassiliicoccaceae with 3NOP, although Methanomassiliicoccaceae were not affected by chloroform (Martinez-Fernandez et al., 2018). In vitro and in vivo results so far do not support amination being a consistent alternative [H] sink to methanogenesis, but more research, especially in vivo experiments, is needed.

5.2 Effect of nitrogen on energy spilling

Both cellulolytic (Atasoglu et al., 2001) and non-cellulolytic rumen bacteria (Atasoglu et al., 1998) responded to the supplementation with amino acids

and peptides by decreasing the proportion of bacterial protein derived from NH_4^+. *S. bovis* fermenting excess glucose grew faster and produced more protein per mole of glucose utilized when supplemented amino acids than when growing only on NH_4^+. Supplementation with amino acids decreased heat production per milligram of cell protein produced, which was interpreted as a decrease in energy spilling resulting from a closer coupling between catabolism and anabolism (Russell, 1993). Similarly, Bond and Russell (1996) estimated that *S. bovis* growing on glucose and on NH_4^+ as only N source spilled about twice more energy as heat compared to cells growing on glucose and amino acids. The absence of amino acidic N in rumen cultures growing on a source of rapidly available carbohydrates triggers energy spilling through ATP hydrolysis uncoupled from the synthesis of cell components (Russell and Cook, 1995; Van Kessel and Russell, 1996; Russell, 2007b). It remains to be elucidated how important could this phenomenon be in vivo with different animal diets.

5.3 Synchrony

Deficiencies in the availability of N in the rumen can affect the metabolism of carbohydrates and vice versa. Low availability of N can impair digestion of carbohydrates, and insufficient availability of carbohydrates can decrease the incorporation of NH_4^+ into microbial protein. Furthermore, amino acids can be fermented as an energy source instead of being incorporated into proteins if the provision of energy from carbohydrates is limited (Nocek and Russell, 1988). Asynchrony in the supply of carbohydrates and N was examined in mixed rumen batch cultures by Newbold and Rust (1992), who concluded that cultures recovered quickly after the N and energy imbalances stopped. Valkeners et al. (2006) fed the same formulated diet to bulls but created three levels of imbalance of rumen degradable OM and N by changing the proportions of the ingredients in the morning and afternoon meals while keeping the same overall formula. The peak of rumen NH_4^+ concentration after the morning meal was greater when the diet offered in the morning and afternoon meals was kept balanced, and conversely, NH_4^+ remained at very low concentrations with the greatest imbalance treatment. However, microbial N flow at the duodenum, rumen NDF degradation and N excretion in urine were unaffected.

Synchronizing nutrient availability through diet formulation has generally not improved microbial protein production or animal performance (Cole and Todd, 2008; Hall and Huntington, 2008). The animal has mechanisms to ameliorate nutrient imbalances in the rumen caused by the diet. The host animal contributes N to the rumen through urea recycling and endogenous protein. Therefore, animal factors have to be considered in addition to

the diet as influencing the balance among nutrients (Hall and Huntington, 2008).

In pastoral systems, additional challenges to balance energy and N are the measurement of forage intake and knowledge of chemical composition of consumed forage and its spatial and temporal variation (Hersom, 2008). In extensive pastoral systems in which animals are supplemented once daily or less frequently, responses to nutrient synchronization in terms of more frequent supplementation might be thought to occur. However, there was no advantage of daily supplementation of cottonseed meal to cows compared to supplementing once per week (Huston et al., 1999). Supplementation of a low-quality forage-based diet of corn stalk with soybean meal or corn gluten meal every 24 or 48 h could not demonstrate consistent advantages of the more frequent protein supplementation (Collins and Pritchard, 1992). Farmer et al. (2004) reported that the frequency of supplementation interacted with the protein content and composition of the supplement in cattle grazing low-quality grass. It was possible to decrease the frequency of supplementation to 3 days per week with supplements containing less than 30% N from urea without detrimental effects on performance. Overall, there seem to be few situations in which synchronizing the supply of energy and N can help improve rumen function or animal performance.

6 Conclusion and future trends

Research in rumen energy metabolism is needed to fill knowledge gaps in aspects important to ruminant nutrition such as the control of the VFA profile and CH_4 production in the rumen, and the efficiency of microbial protein production. Progress in these areas can allow improving mechanistic nutritional models to optimize animal diets. Greater understanding of rumen fermentation can also help optimizing the effects of specific interventions, such as methanogenesis inhibition, or design new interventions to improve rumen function. We should regard the rumen and its microbial community and the host animal as one system with interacting compartments. Research in different compartments of microbial metabolism and ecology, and the host physiology, can be treated separately for the purpose of study, but should not be regarded as distinct and isolated. An integrative approach is necessary as it is expected that most interventions designed to affect one particular aspect of rumen metabolism will affect other parts of the microbial or animal system.

There is also a need of fundamental research on the quantification of carbon and [H] flows through the main metabolic pathways and their physicochemical control. The growing range of powerful –omics techniques coupled with isotopic studies should be powerful tools for future progress in rumen metabolism.

7 Where to look for further information

- Congress on Gastrointestinal Function: https://www.congressgastrofunction.org/.
- Czerkawski, J. W. *An Introduction to Rumen Studies*. Pergamon Press. Exeter, UK.
- Greenhouse Gases and Animal Agriculture Conference: http://www.ggaa2019.org/.
- International Symposium on Ruminant Physiology: https://www.isrp2019.com/.
- Rumen Microbial Genomics Network: http://www.rmgnetwork.org/.
- RuminOmics project: http://www.ruminomics.eu/.
- Russell, J. B. 2002. *Rumen Microbiology and Its Role in Ruminant Nutrition*. James B. Russell, Ithaca, NY. https://www.ars.usda.gov/research/software/download/?softwareid=409
- Russell, J. B. and Wallace, R. J. 1997. Energy-yielding and energy-consuming reactions. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem* (2nd edn.). Blackie Academic & Professional, London, UK.
- Rowett-INRA Conference: https://fems-microbiology.org/opportunities/11th-rowett-inra-conference-gut-microbiology-no-longer-forgotten-organ-11-14-june-2018-uk/.

8 References

Allen, M. S., Bradford, B. J. and Oba, M. 2009. Board-invited review: the hepatic oxidation theory of the control of feed intake and its application to ruminants. *Journal of Animal Science* 87(10), 3317–34. doi:10.2527/jas.2009-1779.

Asanuma, N. and Hino, T. 2000. Activity and properties of fumarate reductase in ruminal bacteria. *Journal of General and Applied Microbiology* 46(3), 119–25. doi:10.2323/jgam.46.119.

Asanuma, N., Iwamoto, M. and Hino, T. 1999a. Effect of the addition of fumarate on methane production by ruminal microorganisms *in vitro*. *Journal of Dairy Science* 82(4), 780–7. doi:10.3168/jds.S0022-0302(99)75296-3.

Asanuma, N., Iwamoto, M. and Hino, T. 1999b. The production of formate, a substrate for methanogenesis, from compounds related with the glyoxylate cycle by mixed ruminal microbes. *Animal Science Journal* 70(2), 67–73. doi:10.2508/chikusan.70.67.

Asanuma, N., Kawato, M., Ohkawara, S. and Hino, T. 2003. Characterization and transcription of the genes encoding enzymes involved in butyrate production in *Butyrivibrio fibrisolvens*. *Current Microbiology* 47(3), 203–7. doi:10.1007/s00284-002-3976-2.

Atasoglu, C., Valdes, C., Walker, N. D., Newbold, C. J. and Wallace, R. J. 1998. *De novo* synthesis of amino acids by the ruminal bacteria *Prevotella bryantii* B14, *Selenomonas ruminantium* HD4, and *Streptococcus bovis* ES1. *Applied and Environmental Microbiology* 64(8), 2836–43. doi:10.1128/AEM.64.8.2836-2843.1998.

Atasoglu, C., Newbold, C. J. and Wallace, R. J. 2001. Incorporation of [^{15}N] ammonia by the cellulolytic ruminal bacteria *Fibrobacter succinogenes* BL2, *Ruminococcus albus* SY3, and *Ruminococcus flavefaciens* 17. *Applied and Environmental Microbiology* 67(6), 2819–22. doi:10.1128/AEM.67.6.2819-2822.2001.

Avila, J. S., Chaves, A. V., Hernandez-Calva, M., Beauchemin, K. A., McGinn, S. M., Wang, Y., Harstad, O. M. and McAllister, T. A. 2011. Effects of replacing barley grain in feedlot diets with increasing levels of glycerol on *in vitro* fermentation and methane production. *Animal Feed Science and Technology* 166–167, 265–8. doi:10.1016/j.anifeedsci.2011.04.016.

Bauchop, T. 1967. Inhibition of rumen methanogenesis by methane analogues. *Journal of Bacteriology* 94(1), 171–5. doi:10.1128/JB.94.1.171-175.1967.

Bauchop, T. and Elsden, S. R. 1960. The growth of micro-organisms in relation to their energy supply. *Journal of General Microbiology* 23, 457–69. doi:10.1099/00221287-23-3-457.

Bauchop, T. and Mountfort, D. O. 1981. Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. *Applied and Environmental Microbiology* 42(6), 1103–10. doi:10.1128/AEM.42.6.1103-1110.1981.

Beauchemin, K. A., Kreuzer, M., O'Mara, F. and McAllister, T. A. 2008. Nutritional management for enteric methane abatement: a review. *Australian Journal of Experimental Agriculture* 48(2), 21–7. doi:10.1071/EA07199.

Bergner, H., Kijora, C., Ceresnakova, Z. and Szakacs, J. 1995. *In vitro* studies on glycerol transformation by rumen microorganisms. *Archiv fur Tierernahrung* 48(3), 245–56. doi:10.1080/17450399509381845.

Bond, D. R. and Russell, J. B. 1996. A role for fructose 1,6-diphosphate in the ATPase-mediated energy-spilling reaction of *Streptococcus bovis*. *Applied and Environmental Microbiology* 62(6), 2095–9. doi:10.1128/AEM.62.6.2095-2099.1996.

Bond, D. R., Tsai, B. M. and Russell, J. B. 1998. The diversion of lactose carbon through the tagatose pathway reduces the intracellular fructose 1,6-bisphosphate and growth rate of *Streptococcus bovis*. *Applied Microbiology and Biotechnology* 49(5), 600–5. doi:10.1007/s002530051220.

Broderick, G. A., Huhtanen, P., Ahvenjärvi, S., Reynal, S. M. and Shingfield, K. J. 2010. Quantifying ruminal nitrogen metabolism using the omasal sampling technique in cattle–a meta-analysis. *Journal of Dairy Science* 93(7), 3216–30. doi:10.3168/jds.2009-2989.

Buckel, W. and Thauer, R. K. 2013. Energy conservation via electron bifurcating ferredoxin reduction and proton/Na(+) translocating ferredoxin oxidation. *Biochimica et Biophysica Acta* 1827(2), 94–113. doi:10.1016/j.bbabio.2012.07.002.

Buckel, W. and Thauer, R. K. 2018a. Flavin-based electron bifurcation, a new mechanism of biological energy coupling. *Chemical Reviews* 118(7), 3862–86. doi:10.1021/acs.chemrev.7b00707.

Buckel, W. and Thauer, R. K. 2018b. Flavin-based electron bifurcation, ferredoxin, flavodoxin, and anaerobic respiration with protons (Ech) or NAD^+ (Rnf) as electron acceptors: a historical review. *Frontiers in Microbiology* 9, 401. doi:10.3389/fmicb.2018.00401.

Cabezas-Garcia, E. H., Krizsan, S. J., Shingfield, K. J. and Huhtanen, P. 2017. Between-cow variation in digestion and rumen fermentation variables associated with methane production. *Journal of Dairy Science* 100(6), 4409–24. doi:10.3168/jds.2016-12206.

Clapperton, J. L. 1974. The effect of trichloroacetamide, chloroform and linseed oil given into the rumen of sheep on some of the end-products of rumen digestion. *British Journal of Nutrition* 32(1), 155–61. doi:10.1079/bjn19740065.

Cole, N. A. and McCroskey, J. E. 1975. Effects of hemiacetal of chloral and starch on the performance of beef steers. *Journal of Animal Science* 41(6), 1735–41. doi:10.2527/jas1975.4161735x.

Cole, N. A. and Todd, R. W. 2008. Opportunities to enhance performance and efficiency through nutrient synchrony in concentrate-fed ruminants. *Journal of Animal Science* 86(14 Suppl.), E318–33. doi:10.2527/jas.2007-0444.

Collins, R. M. and Pritchard, R. H. 1992. Alternate day supplementation of corn stalk diets with soybean meal or corn gluten meal fed to ruminants. *Journal of Animal Science* 70(12), 3899–908. doi:10.2527/1992.70123899x.

Czerkawski, J. W. 1986. *An Introduction to Rumen Studies*. Pergamon Press, Exeter, UK.

Czerkawski, J. W. and Breckenridge, G. 1975. New inhibitors of methane production by rumen micro- organisms. Experiments with animals and other practical possibilities. *British Journal of Nutrition* 34(3), 447–57. doi:10.1017/s0007114575000505.

Chen, M. and Wolin, M. J. 1977. Influence of CH_4 production by *Methanobacterium ruminantium* on the fermentation of glucose and lactate by *Selenomonas ruminantium*. *Applied and Environmental Microbiology* 34(6), 756–9. doi:10.1128/AEM.34.6.756-759.1977.

Chowdhury, N. P., Kahnt, J. and Buckel, W. 2015. Reduction of ferredoxin or oxygen by flavin-based electron bifurcation in *Megasphaera elsdenii*. *The FEBS Journal* 282(16), 3149–60. doi:10.1111/febs.13308.

Chung, K. T. 1976. Inhibitory effects of H_2 on growth of *Clostridium cellobioparum*. *Applied and Environmental Microbiology* 31(3), 342–8. doi:10.1128/AEM.31.3.342-348.1976.

Davies, A., Nwaonu, H. N., Stanier, G. and Boyle, F. T. 1982. Properties of a novel series of inhibitors of rumen methanogenesis; *in vitro* and *in vivo* experiments including growth trials on 2,4-bis (trichloromethyl)-benzo [1, 3]dioxin-6-carboxylic acid. *The British Journal of Nutrition* 47(3), 565–76. doi:10.1079/bjn19820068.

De Vries, W., Van Wyck-Kapteyn, W. M. and Stouthamer, A. H. 1973. Generation of ATP during cytochrome-linked anaerobic electron transport in propionic acid bacteria. *Journal of General Microbiology* 76(1), 31–41. doi:10.1099/00221287-76-1-31.

Dijkstra, J., Boer, H., Van Bruchem, J., Bruining, M. and Tamminga, S. 1993. Absorption of volatile fatty acids from the rumen of lactating dairy cows as influenced by volatile fatty acid concentration, pH and rumen liquid volume. *The British Journal of Nutrition* 69(2), 385–96. doi:10.1079/bjn19930041.

Ebrahimi, S. H., Mohinia, M., Singhala, K. K., Miria, V. H. and Tyagi, A. K. 2011. Evaluation of complementary effects of 9,10-anthraquinone and fumaric acid on methanogenesis and ruminal fermentation *in vitro*. *Archives of Animal Nutrition* 65(4), 267–77. doi:10.1080/1745039x.2011.594345.

Eckard, R. J., Grainger, C. and De Klein, C. A. M. 2010. Options for the abatement of methane and nitrous oxide from ruminant production: a review. *Livestock Science* 130(1–3), 47–56. doi:10.1016/j.livsci.2010.02.010.

Enzmann, F., Mayer, F., Rother, M. and Holtmann, D. 2018. Methanogens: biochemical background and biotechnological applications. *AMB Express* 8(1), 1–. doi:10.1186/s13568-017-0531-x.

Farmer, C. G., Woods, B. C., Cochran, R. C., Heldt, J. S., Mathis, C. P., Olson, K. C., Titgemeyer, E. C. and Wickersham, T. A. 2004. Effect of supplementation frequency

and supplemental urea level on dormant tallgrass-prairie hay intake and digestion by beef steers and prepartum performance of beef cows grazing dormant tallgrass-prairie. *Journal of Animal Science* 82(3), 884–94. doi:10.2527/2004.823884x.

Firkins, J. L., Yu, Z. and Morrison, M. 2007. Ruminal nitrogen metabolism: perspectives for integration of microbiology and nutrition for dairy. *Journal of Dairy Science* 90(Suppl. 1), E1–E16. doi:10.3168/jds.2006-518.

Geier, R. R., Kwon, I. H., Cann, I. K. and Mackie, R. I. 2016. Interspecies hydrogen transfer and its effects on global transcript abundance in *Ruminococcus albus*, a predominant fiber-degrading species in the rumen. *The FASEB Journal* 30, 1102.1–.

Gill, M., Siddons, R. C., Beever, D. E. and Rowe, J. B. 1986. Metabolism of lactic acid isomers in the rumen of silage-fed sheep. *The British Journal of Nutrition* 55(2), 399–407. doi:10.1079/bjn19860046.

Greening, C., Geier, R., Wang, C., Woods, L. C., Morales, S. E., Mcdonald, M. J., Rushton-Green, R., Morgan, X. C., Koike, S., Leahy, S. C., Kelly, W. J., Cann, I., Attwood, G. T., Cook, G. M. and Mackie, R. I. 2019. Diverse hydrogen production and consumption pathways influence methane production in ruminants. *The ISME Journal* 13(10), 2617–32. doi:10.1038/s41396-019-0464-2.

Guo, W. S., Schaefer, D. M., Guo, X. X., Ren, L. P. and Meng, Q. X. 2009. Use of nitrate-nitrogen as a sole dietary nitrogen source to inhibit ruminal methanogenesis and to improve microbial nitrogen synthesis *in vitro*. *Asian-Australasian Journal of Animal Sciences* 22(4), 542–9. doi:10.5713/ajas.2009.80361.

Hackmann, T. J. and Firkins, J. L. 2015a. Electron transport phosphorylation in rumen butyrivibrios: unprecedented ATP yield for glucose fermentation to butyrate. *Frontiers in Microbiology* 6, 622. doi:10.3389/fmicb.2015.00622.

Hackmann, T. J. and Firkins, J. L. 2015b. Maximizing efficiency of rumen microbial protein production. *Frontiers in Microbiology* 6, 465. doi:10.3389/fmicb.2015.00465.

Hackmann, T. J., Diese, L. E. and Firkins, J. L. 2013a. Quantifying the responses of mixed rumen microbes to excess carbohydrate. *Applied and Environmental Microbiology* 79(12), 3786–95. doi:10.1128/AEM.00482-13.

Hackmann, T. J., Keyser, B. L. and Firkins, J. L. 2013b. Evaluation of methods to detect changes in reserve carbohydrate for mixed rumen microbes. *Journal of Microbiological Methods* 93(3), 284–91. doi:10.1016/j.mimet.2013.03.025.

Hackmann, T. J., Ngugi, D. K., Firkins, J. L. and Tao, J. 2017. Genomes of rumen bacteria encode atypical pathways for fermenting hexoses to short-chain fatty acids. *Environmental Microbiology* 19(11), 4670–83. doi:10.1111/1462-2920.13929.

Hall, M. B. and Huntington, G. B. 2008. Nutrient synchrony: sound in theory, elusive in practice. *Journal of Animal Science* 86(14) (Suppl.), E287–92. doi:10.2527/jas.2007-0516.

Hall, M. B., Nennich, T. D., Doane, P. H. and Brink, G. E. 2015. Total volatile fatty acid concentrations are unreliable estimators of treatment effects on ruminal fermentation *in vivo*. *Journal of Dairy Science* 98(6), 3988–99. doi:10.3168/jds.2014-8854.

Hamar, D. and Borchers, R. 1967. Glycolytic pathway in rumen microorganisms. *Journal of Animal Science* 26(3), 654–7. doi:10.2527/jas1967.263654x.

Harfoot, C. G. and Hazlewood, G. P. 1997. Lipid metabolism in the rumen. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem*. Blackie Academic & Professional, London, UK, pp. 382–426.

Hartinger, T., Gresner, N. and Sudekum, K. H. 2018. Does intra-ruminal nitrogen recycling waste valuable resources? A review of major players and their manipulation. *Journal of Animal Science and Biotechnology* 9, 33. doi:10.1186/s40104-018-0249-x.

Henderson, C. 1980. The influence of extracellular hydrogen on the metabolism of *Bacteroides rurninicola*, *Anaerovibrio lipolytica* and *Selenomonas ruminantiurn*. *Journal of General Microbiology* 119(2), 485–91. doi:10.1099/00221287-119-2-485.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Global Rumen Census, C., Abecia, L., Angarita, E., Aravena, P., Nora Arenas, G., Ariza, C., Attwood, G. T., Mauricio Avila, J., Avila-Stagno, J., Bannink, A., Barahona, R., Batistotti, M., Bertelsen, M. F., Brown-Kav, A., Carvajal, A. M., Cersosimo, L., Vieira Chaves, A., Church, J., Clipson, N., Cobos-Peralta, M. A., Cookson, A. L., Cravero, S., Cristobal Carballo, O., Crosley, K., Cruz, G., Cerón Cucchi, M., De La Barra, R., De Menezes, A. B., Detmann, E., Dieho, K., Dijkstra, J., Dos Reis, W. L. S., Dugan, M. E. R., Hadi Ebrahimi, S., Eythórsdóttir, E., Nde Fon, F., Fraga, M., Franco, F., Friedeman, C., Fukuma, N., Gagić, D., Gangnat, I., Javier Grilli, D., Guan, L. L., Heidarian Miri, V., Hernandez-Sanabria, E., Gomez, A. X. I., Isah, O. A., Ishaq, S., Jami, E., Jelincic, J., Kantanen, J., Kelly, W. J., Kim, S.-H., Klieve, A., Kobayashi, Y., Koike, S., Kopecny, J., Nygaard Kristensen, T., Julie Krizsan, S., Lachance, H., Lachman, M., Lamberson, W. R., Lambie, S., Lassen, J., Leahy, S. C., Lee, S.-S., Leiber, F., Lewis, E., Lin, B., Lira, R., Lund, P., Macipe, E., Mamuad, L. L., Cuquetto Mantovani, H., Marcoppido, G. A., Márquez, C., Martin, C., Martinez, G., Eugenia Martinez, M., Lucía Mayorga, O., Mcallister, T. A., McSweeney, C., Mestre, L., Minnee, E., Mitsumori, M., Mizrahi, I., Molina, I., Muenger, A., Muñoz, C., Murovec, B., Newbold, J., Nsereko, V., O'Donovan, M., Okunade, S., et al. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific Reports* 5, 14567.

Hersom, M. J. 2008. Opportunities to enhance performance and efficiency through nutrient synchrony in forage-fed ruminants. *Journal of Animal Science* 86(14 Suppl.), E306–17. doi:10.2527/jas.2007-0463.

Hino, T. and Russell, J. B. 1985. Effect of reducing-equivalent disposal and NADH/NAD on deamination of amino acids by intact rumen microorganisms and their cell extracts. *Applied and Environmental Microbiology* 50(6), 1368–74. doi:10.1128/AEM.50.6.1368-1374.1985.

Hristov, A. N., Oh, J., Giallongo, F., Frederick, T. W., Harper, M. T., Weeks, H. L., Branco, A. F., Moate, P. J., Deighton, M. H., Williams, S. R., Kindermann, M. and Duval, S. 2015. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. *Proceedings of the National Academy of Sciences of the United States of America*. Proceedings of the National Acadademy of Sciences of the United States of America 112(34), 10663–8. doi:10.1073/pnas.1504124112.

Hungate, R. E. 1967. Hydrogen as an intermediate in the rumen fermentation. *Archiv für Mikrobiologie* 59(1), 158–64. doi:10.1007/bf00406327.

Hungate, R. E., Smith, W., Bauchop, T., Yu, I. and Rabinowitz, J. C. 1970. Formate as an intermediate in the bovine rumen fermentation. *Journal of Bacteriology* 102(2), 389–97. doi:10.1128/JB.102.2.389-397.1970.

Huston, J. E., Lippke, H., Forbes, T. D. A., Holloway, J. W. and Machen, R. V. 1999. Effects of supplemental feeding interval on adult cows in western Texas. *Journal of Animal Science* 77(11), 3057–67. doi:10.2527/1999.77113057x.

Isaacson, H. R., Hinds, F. C., Bryant, M. P. and Owens, F. N. 1975. Efficiency of energy utilization by mixed rumen bacteria in continuous culture. *Journal of Dairy Science* 58(11), 1645–59. doi:10.3168/jds.S0022-0302(75)84763-1.

Janssen, P. H. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Animal Feed Science and Technology* 160(1–2), 1–22. doi:10.1016/j.anifeedsci.2010.07.002.

Jenkins, T. C., Wallace, R. J., Moate, P. J. and Mosley, E. E. 2008. Board-invited review: recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. *Journal of Animal Science* 86(2), 397–412. doi:10.2527/jas.2007-0588.

Jeong, H. S., Kim, H. Y., Ahn, S. H., Oh, S. C., Yang, I. and Choi, I. G. 2014. Optimization of enzymatic hydrolysis conditions for extraction of pectin from rapeseed cake (*Brassica napus* L.) using commercial enzymes. *Food Chemistry* 157, 332–8. doi:10.1016/j.foodchem.2014.02.040.

Johnson, D. E. 1972. Effects of a hemiacetal of chloral and starch on methane production and energy balance of sheep fed a pelleted diet. *Journal of Animal Science* 35(5), 1064–8. doi:10.2527/jas1972.3551064x.

Johnson, D. E. 1974. Adaptational responses in nitrogen and energy balance of lambs fed a methane inhibitor. *Journal of Animal Science* 38(1), 154–7. doi:10.2527/jas1974.381154x.

Johnson, K. A. and Johnson, D. E. 1995. Methane emissions from cattle. *Journal of Animal Science* 73(8), 2483–92. doi:10.2527/1995.7382483x.

Jouany, J.-P. and Thivend, P. 1972a. Évolution postprandiale de la composition glucidique des corps microbiens du rumen en fonction de la nature des glucides du régime. I. – les protozoaires. *Annales de Biologie Animale Biochimie Biophysique* 12(4), 673–7. doi:10.1051/rnd:19720414.

Jouany, J.-P. and Thivend, P. 1972b. Évolution postprandiale de la composition glucidique des corps microbiens du rumen en fonction de la nature des glucides du régime. II. – les bactéries. *Annales de Biologie Animale Biochimie Biophysique* 12(4), 679–83. doi:10.1051/rnd:19720415.

Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y. and Morishima, K. 2017. KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Research* 45(D1), D353–61. doi:10.1093/nar/gkw1092.

Kelly, W. J., Leahy, S. C., Altermann, E., Yeoman, C. J., Dunne, J. C., Kong, Z., Pacheco, D. M., Li, D., Noel, S. J., Moon, C. D., Cookson, A. L. and Attwood, G. T. 2010. The glycobiome of the rumen bacterium *Butyrivibrio proteoclasticus* B316(T) highlights adaptation to a polysaccharide-rich environment. *PLoS ONE* 5(8), e11942. doi:10.1371/journal.pone.0011942.

Kennedy, P. M. and Milligan, L. P. 1978. Effects of cold exposure on digestion, microbial synthesis and nitrogen transformations in sheep. *The British Journal of Nutrition* 39(1), 105–17. doi:10.1079/bjn19780017.

Kim, S. H., Mamuad, L. L., Kim, D. W., Kim, S. K. and Lee, S. S. 2016. Fumarate reductase-producing enterococci reduce methane production in *in vitro* rumen fermentation. *Journal of Microbiology and Biotechnology* 26(3), 558–66. doi:10.4014/jmb.1512.12008.

Kohn, R. and Boston, R. 2000. The role of thermodynamics in controlling rumen metabolism. In: McNamara, J. P., France, J. and Beever, D. E. (Eds), *Modelling Nutrient Utilization in Farm Animals*. CABI, Wallingford, UK, pp. 11–24.

Kröger, A., Biel, S., Simon, J., Gross, R., Unden, G. and Lancaster, C. R. D. 2002. Fumarate respiration of *Wolinella succinogenes*: enzymology, energetics and coupling mechanism. *Biochimica et Biophysica Acta* 1553(1–2), 23–38. doi:10.1016/s0005-2728(01)00234-1.

Krueger, N. A., Anderson, R. C., Tedeschi, L. O., Callaway, T. R., Edrington, T. S. and Nisbet, D. J. 2010. Evaluation of feeding glycerol on free-fatty acid production and fermentation kinetics of mixed ruminal microbes *in vitro*. *Bioresource Technology* 101(21), 8469–72. doi:10.1016/j.biortech.2010.06.010.

Leedle, J. A., Bryant, M. P. and Hespell, R. B. 1982. Diurnal variations in bacterial numbers and fluid parameters in ruminal contents of animals fed low- or high-forage diets. *Applied and Environmental Microbiology* 44(2), 402–12. doi:10.1128/AEM.44.2.402-412.1982.

Leng, R. A. 2014. Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation. *Animal Production Science* 54(5), 519–43. doi:10.1071/AN13381.

Le Van, T. D., Robinson, J. A., Ralph, J., Greening, R. C., Smolenski, W. J., Leedle, J. A. Z. and Schaefer, D. M. 1998. Assessment of reductive acetogenesis with indigenous ruminal bacterium populations and *Acetitomaculum ruminis*. *Applied and Environmental Microbiology* 64(9), 3429–36. doi:10.1128/AEM.64.9.3429-3436.1998.

Lopes, J. C., de Matos, L. F., Harper, M. T., Giallongo, F., Oh, J., Gruen, D., Ono, S., Kindermann, M., Duval, S. and Hristov, A. N. 2016. Effect of 3-nitrooxypropanol on methane and hydrogen emissions, methane isotopic signature, and ruminal fermentation in dairy cows. *Journal of Dairy Science* 99(7), 5335–44. doi:10.3168/jds.2015-10832.

Lopez, S., McIntosh, F. M., Wallace, R. J. and Newbold, C. J. 1999. Effect of adding acetogenic bacteria on methane production by mixed rumen microorganisms. *Animal Feed Science and Technology* 78(1–2), 1–9. doi:10.1016/S0377-8401(98)00273-9.

Lourenco, M., Ramos-Morales, E. and Wallace, R. J. 2010. The role of microbes in rumen lipolysis and biohydrogenation and their manipulation. *Animal* 4(7), 1008–23. doi:10.1017/S175173111000042X.

Løvendahl, P., Difford, G. F., Li, B., Chagunda, M. G. G., Huhtanen, P., Lidauer, M. H., Lassen, J. and Lund, P. 2018. Review: Selecting for improved feed efficiency and reduced methane emissions in dairy cattle. *Animal* 12(s2), s336–49. doi:10.1017/S1751731118002276.

Mamuad, L., Kim, S. H., Jeong, C. D., Choi, Y. J., Jeon, C. O. and Lee, S. S. 2014. Effect of fumarate reducing bacteria on *in vitro* rumen fermentation, methane mitigation and microbial diversity. *Journal of Microbiology* 52(2), 120–8. doi:10.1007/s12275-014-3518-1.

Marounek, M. and Petr, O. 1995. Fermentation of glucose and xylose in ruminal strains of *Butyrivibrio fibrisolvens*. *Letters in Applied Microbiology* 21(4), 272–6. doi:10.1111/j.1472-765x.1995.tb01058.x.

Martin, S. A. and Macy, J. M. 1985. Effects of monensin, pyromellitic diimide and 2-bromoethanesulfonic acid on rumen fermentation *in vitro*. *Journal of Animal Science* 60(2), 544–50. doi:10.2527/jas1985.602544x.

Martin, C., Morgavi, D. P. and Doreau, M. 2010. Methane mitigation in ruminants: from microbe to the farm scale. *Animal* 4(3), 351–65. doi:10.1017/S1751731109990620.

Martínez-Fernández, G., Abecia, L., Arco, A., Cantalapiedra-Hijar, G., Martín-García, A. I., Molina-Alcaide, E., Kindermann, M., Duval, S. and Yáñez-Ruiz, D. R. 2014. Effects

of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal fermentation, microbial abundance, and methane emissions in sheep. *Journal of Dairy Science* 97(6), 3790–9. doi:10.3168/jds.2013-7398.

Martinez-Fernandez, G., Denman, S. E., Yang, C., Cheung, J., Mitsumori, M. and McSweeney, C. S. 2016. Methane inhibition alters the microbial community, hydrogen flow, and fermentation response in the rumen of cattle. *Frontiers in Microbiology* 7, 1122. doi:10.3389/fmicb.2016.01122.

Martinez-Fernandez, G., Denman, S. E., Cheung, J. and McSweeney, C. S. 2017. Phloroglucinol degradation in the rumen promotes the capture of excess hydrogen generated from methanogenesis inhibition. *Frontiers in Microbiology* 8, 1871. doi:10.3389/fmicb.2017.01871.

Martinez-Fernandez, G., Duval, S., Kindermann, M., Schirra, H. J., Denman, S. E. and McSweeney, C. S. 2018. 3-NOP vs. Halogenated compound: methane production, ruminal fermentation and microbial community response in forage fed cattle. *Frontiers in Microbiology* 9, 1582. doi:10.3389/fmicb.2018.01582.

Marvin-Sikkema, F. D., Richardson, A. J., Stewart, C. S., Gottschal, J. C. and Prins, R. A. 1990. Influence of hydrogen-consuming bacteria on cellulose degradation by anaerobic fungi. *Applied and Environmental Microbiology* 56(12), 3793–7. doi:10.1128/AEM.56.12.3793-3797.1990.

Matte, A., Forsberg, C. W. and Gibbins, A. M. V. 1992. Enzymes associated with metabolism of xylose and other pentoses by *Prevotella* (*Bacteroides*) *ruminicola* strains, *Selenomonas ruminantium* D, and *Fibrobacter succinogenes* S85. *Canadian Journal of Microbiology* 38(5), 370–6. doi:10.1139/m92-063.

Matulova, M., Delort, A. M., Nouaille, R., Gaudet, G. and Forano, E. 2001. Concurrent maltodextrin and cellodextrin synthesis by *Fibrobacter succinogenes* S85 as identified by 2D NMR spectroscopy. *European Journal of Biochemistry* 268(14), 3907–15. doi:10.1046/j.1432-1327.2001.02300.x.

McAllan, A. B. and Smith, R. H. 1974. Carbohydrate metabolism in the ruminant. Bacterial carbohydrates formed in the rumen and their contribution to digesta entering the duodenum. *The British Journal of Nutrition* 31(1), 77–88. doi:10.1079/bjn19740010.

Miller, T. L. and Jenesel, S. E. 1979. Enzymology of butyrate formation by *Butyrivibrio fibrisolvens*. *Journal of Bacteriology* 138(1), 99–104. doi:10.1128/JB.138.1.99-104.1979.

Mitsumori, M., Matsui, H., Tajima, K., Shinkai, T., Takenaka, A., Denman, S. E. and McSweeney, C. S. 2014. Effect of bromochloromethane and fumarate on phylogenetic diversity of the formyltetrahydrofolate synthetase gene in bovine rumen. *Animal Science Journal = Nihon Chikusan Gakkaiho* 85(1), 25–31. doi:10.1111/asj.12072.

Mohammed, N., Lila, Z. A., Ajisaka, N., Hara, K., Mikuni, K., Hara, K., Kanda, S. and Itabashi, H. 2004. Inhibition of ruminal microbial methane production by β-cyclodextrin iodopropane, malate and their combination *in vitro*. *Journal of Animal Physiology and Animal Nutrition* 88(5–6), 188–95. doi:10.1111/j.1439-0396.2004.00456.x.

Montzka, S. A., Dlugokencky, E. J. and Butler, J. H. 2011. Non-CO_2 greenhouse gases and climate change. *Nature* 476(7358), 43–50. doi:10.1038/nature10322.

Morgavi, D. P., Forano, E., Martin, C. and Newbold, C. J. 2010. Microbial ecosystem and methanogenesis in ruminants. *Animal* 4(7), 1024–36. doi:10.1017/S1751731110000546.

Moss, A. R., Jouany, J.-P. and Newbold, J. 2000. Methane production by ruminants: its contribution to global warming. *Annales de Zootechnie* 49(3), 231–53. doi:10.1051/animres:2000119.

Nagaraja, T. G. and Titgemeyer, E. C. 2007. Ruminal acidosis in beef cattle: the current microbiological and nutritional outlook. *Journal of Dairy Science* 90(Suppl. 1), E17–38. doi:10.3168/jds.2006-478.

Nagaraja, T. G., Newbold, C. J., Van Nevel, C. J. and Demeyer, D. I. 1997. Manipulation of rumen fermentation. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem* (2nd edn.). Blackie Academic & Professional, London, UK, pp. 524–632.

Nelson, D. L. and Cox, M. M. 2017. *Lehninger Principles of Biochemistry*. W.H. Freeman, Macmillan Learning, New York.

Newbold, J. R. and Rust, S. R. 1992. Effect of asynchronous nitrogen and energy supply on growth of ruminal bacteria in batch culture. *Journal of Animal Science* 70(2), 538–46. doi:10.2527/1992.702538x.

Newbold, C. J., De La Fuente, G., Belanche, A., Ramos-Morales, E. and Mcewan, N. R. 2015. The role of ciliate protozoa in the rumen. *Frontiers in Microbiology* 6, 1313. doi:10.3389/fmicb.2015.01313.

Nocek, J. E. and Russell, J. B. 1988. Protein and energy as an integrated system. Relationship of ruminal protein and carbohydrate availability to microbial synthesis and milk production. *Journal of Dairy Science* 71(8), 2070–107. doi:10.3168/jds.S0022-0302(88)79782-9.

Nolan, J. V., Hegarty, R. S., Hegarty, J., Godwin, I. R. and Woodgate, R. 2010. Effects of dietary nitrate on fermentation, methane production and digesta kinetics in sheep. *Animal Production Science* 50(8), 801–6. doi:10.1071/AN09211.

Nollet, L., Demeyer, D. I. and Verstraete, W. 1997. Effect of 2-bromoethanesulfonic acid and *Peptostreptococcus productus* ATCC 35244 addition on stimulation of reductive acetogenesis in the ruminal ecosystem by selective inhibition of methanogenesis. *Applied and Environmental Microbiology* 63(1), 194–200. doi:10.1128/AEM.63.1.194-200.1997.

Nouaille, R., Matulova, M., Delort, A. M. and Forano, E. 2005. Oligosaccharide synthesis in *Fibrobacter succinogenes* S85 and its modulation by the substrate. *The FEBS Journal* 272(10), 2416–27. doi:10.1111/j.1742-4658.2005.04662.x.

NRC. 2001. *Nutrient Requirements of Dairy Cattle*. National Research Council, Washington DC.

Offner, A. and Sauvant, D. 2006. Thermodynamic modeling of ruminal fermentations. *Animal Research* 55(5), 343–65. doi:10.1051/animres:2006021.

Oldick, B. S., Firkins, J. L. and Kohn, R. A. 2000. Compartmental modeling with nitrogen-15 to determine effects of degree of fat saturation on intraruminal N recycling. *Journal of Animal Science* 78(9), 2421–30. doi:10.2527/2000.7892421x.

Olijhoek, D. W., Hellwing, A. L. F., Brask, M., Weisbjerg, M. R., Hojberg, O., Larsen, M. K., Dijkstra, J., Erlandsen, E. J. and Lund, P. 2016. Effect of dietary nitrate level on enteric methane production, hydrogen emission, rumen fermentation, and nutrient digestibility in dairy cows. *Journal of Dairy Science* 99(8), 6191–205. doi:10.3168/jds.2015-10691.

Pavlostathis, S. G., Miller, T. L. and Wolin, M. J. 1990. Cellulose fermentation by continuous cultures of *Ruminococcus albus* and *Methanobrevibacter smithii*. *Applied Microbiology and Biotechnology* 33(1), 109–16. doi:10.1007/BF00170581.

Pazur, J. H., Shuey, E. W. and Georgi, C. E. 1958. The conversion of d-xylose into volatile organic acids by rumen bacteria. *Archives of Biochemistry and Biophysics* 77(2), 387–94. doi:10.1016/0003-9861(58)90086-9.

Petzel, J. P., Mcelwain, M. C., Desantis, D., Manolukas, J., Williams, M. V., Hartman, P. A., Allison, M. J. and Pollack, J. D. 1989. Enzymic activities of carbohydrate, purine, and pyrimidine metabolism in the *Anaeroplasmataceae* (class *Mollicutes*). *Archives of Microbiology* 152(4), 309–16. doi:10.1007/bf00425166.

Pol, A. and Demeyer, D. I. 1988. Fermentation of methanol in the sheep rumen. *Applied and Environmental Microbiology* 54(3), 832–4. doi:10.1128/AEM.54.3.832-834.1988.

Preiss, J. and Romeo, T. 1990. Physiology, biochemistry and genetics of bacterial glycogen synthesis. In: Rose, A. H. and Tempest, D. W. (Eds), *Advances in Microbial Physiology* (vol. 30). Academic Press, pp. 183–238. doi:10.1016/s0065-2911(08)60113-7.

Roberton, A. M. and Glucina, P. G. 1982. Fructose 6-phosphate phosphorylation in *Bacteroides* species. *Journal of Bacteriology* 150(3), 1056–60. doi:10.1128/JB.150.3.1056-1060.1982.

Rooke, J. A., Wallace, R. J., Duthie, C. A., McKain, N., De Souza, S. M., Hyslop, J. J., Ross, D. W., Waterhouse, T. and Roehe, R. 2014. Hydrogen and methane emissions from beef cattle and their rumen microbial community vary with diet, time after feeding and genotype. *The British Journal of Nutrition* 112(3), 398–407. doi:10.1017/S0007114514000932.

Russell, J. B. 1986. Heat production by ruminal bacteria in continuous culture and its relationship to maintenance energy. *Journal of Bacteriology* 168(2), 694–701. doi:10.1128/jb.168.2.694-701.1986.

Russell, J. B. 1993. Effect of amino acids on the heat production and growth efficiency of Streptococcus bovis: balance of anabolic and catabolic rates. *Applied and Environmental Microbiology* 59(6), 1747–51. doi:10.1128/AEM.59.6.1747-1751.1993.

Russell, J. B. 1998. The importance of pH in the regulation of ruminal acetate to propionate ratio and methane production in vitro. *Journal of Dairy Science* 81(12), 3222–30. doi:10.3168/jds.S0022-0302(98)75886-2.

Russell, J. B. 2002. *Rumen Microbiology and Its Role in Ruminant Nutrition*. James B. Russell, Ithaca, NY.

Russell, J. B. 2007a. Can the heat of ruminal fermentation be manipulated to decrease heat stress? *Proceedings of the Southwest Nutrition Conference*, pp. 109–15.

Russell, J. B. 2007b. The energy spilling reactions of bacteria and other organisms. *Journal of Molecular Microbiology and Biotechnology* 13(1–3), 1–11. doi:10.1159/000103591.

Russell, J. B. and Cook, G. M. 1995. Energetics of bacterial growth: balance of anabolic and catabolic reactions. *Microbiological Reviews* 59(1), 48–62. doi:10.1128/MMBR.59.1.48-62.1995.

Russell, J. B. and Jeraci, J. L. 1984. Effect of carbon monoxide on fermentation of fiber, starch, and amino acids by mixed rumen microorganisms *in vitro*. *Applied and Environmental Microbiology* 48(1), 211–7. doi:10.1128/AEM.48.1.211-217.1984.

Russell, J. B. and Martin, S. A. 1984. Effects of various methane inhibitors on the fementation of amino acids by mixed rumen microorganisms *in vitro*. *Journal of Animal Science* 59(5), 1329–38. doi:10.2527/jas1984.5951329x.

Russell, J. B. and Wallace, R. J. 1997. Energy-yielding and energy-consuming reactions. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem* (2nd edn.). Blackie Academic & Professional, London, UK, pp. 246–82.

Satter, L. D. and Esdale, W. J. 1968. *In vitro* lactate metabolism by ruminal ingesta. *Applied Microbiology* 16(5), 680–8. doi:10.1128/AEM.16.5.680-688.1968.

Sauer, F. D. and Teather, R. M. 1987. Changes in oxidation reduction potentials and volatile fatty acids production by rumen bacteria when methane synthesis

is inhibited. *Journal of Dairy Science* 70(9), 1835–40. doi:10.3168/jds.S0022-0302(87)80222-9.

Saunois, M., Bousquet, P., Poulter, B., Peregon, A., Ciais, P., Canadell, J. G., Dlugokencky, E. J., Etiope, G., Bastviken, D., Houweling, S., Janssens-Maenhout, G., Tubiello, F. N., Castaldi, S., Jackson, R. B., Alexe, M., Arora, V. K., Beerling, D. J., Bergamaschi, P., Blake, D. R., Brailsford, G., Brovkin, V., Bruhwiler, L., Crevoisier, C., Crill, P., Covey, K., Curry, C., Frankenberg, C., Gedney, N., Höglund-Isaksson, L., Ishizawa, M., Ito, A., Joos, F., Kim, H. S., Kleinen, T., Krummel, P., Lamarque, J. F., Langenfelds, R., Locatelli, R., Machida, T., Maksyutov, S., Mcdonald, K. C., Marshall, J., Melton, J. R., Morino, I., Naik, V., O'Doherty, S., Parmentier, F. W., Patra, P. K., Peng, C., Peng, S., Peters, G. P., Pison, I., Prigent, C., Prinn, R., Ramonet, M., Riley, W. J., Saito, M., Santini, M., Schroeder, R., Simpson, I. J., Spahni, R., Steele, P., Takizawa, A., Thornton, B. F., Tian, H., Tohjima, Y., Viovy, N., Voulgarakis, A., Van Weele, M., Van Der Werf, G. R., Weiss, R., Wiedinmyer, C., Wilton, D. J., Wiltshire, A., Worthy, D., Wunch, D., Xu, X., Yoshida, Y., Zhang, B., Zhang, Z. and Zhu, Q. 2016a. The global methane budget 2000–2012. *Earth System Science Data* 8(2), 697–751. doi:10.5194/essd-8-697-2016.

Saunois, M., Jackson, R. B., Bousquet, P., Poulter, B. and Canadell, J. G. 2016b. The growing role of methane in anthropogenic climate change. *Environmental Research Letters* 11(12). doi:10.1088/1748-9326/11/12/120207.

Scardovi, V. 1965. The fructose-6-phosphate shunt as a peculiar pattern of hexose degradation in the genus *Bifidobacterium*. *Annals of Microbiology and Enzymology* 15, 19–29.

Scheller, H. V. and Ulvskov, P. 2010. Hemicelluloses. *Annual Review of Plant Biology* 61, 263–89. doi:10.1146/annurev-arplant-042809-112315.

Schoelmerich, M. C., Katsyv, A., Dönig, J., Hackmann, T. J., and Müller, V. 2019. Energy conservation involving 2 respiratory circuits. *Proceedings of the National Academy of Sciences of the United States of America* 117, 1167–73. doi:10.1073/pnas.1914939117.

Schuchmann, K. and Müller, V. 2014. Autotrophy at the thermodynamic limit of life: a model for energy conservation in acetogenic bacteria. *Nature Reviews. Microbiology* 12(12), 809–21. doi:10.1038/nrmicro3365.

Seeliger, S., Janssen, P. H. and Schink, B. 2002. Energetics and kinetics of lactate fermentation to acetate and propionate via methylmalonyl-CoA or acrylyl-CoA. *FEMS Microbiology Letters* 211(1), 65–70. doi:10.1111/j.1574-6968.2002.tb11204.x.

Söllinger, A., Tveit, A. T., Poulsen, M., Noel, S. J., Bengtsson, M., Bernhardt, J., Frydendahl Hellwing, A. L., Lund, P., Riedel, K., Schleper, C., Højberg, O. and Urich, T. 2018. Holistic assessment of rumen microbiome dynamics through quantitative metatranscriptomics reveals multifunctional redundancy during key steps of anaerobic feed degradation. *mSystems* 3(4). doi:10.1128/mSystems.00038-18.

Sondergaard, D., Pedersen, C. N. and Greening, C. 2016. HydDB: A web tool for hydrogenase classification and analysis. *Scientific Reports* 6, 34212. doi:10.1038/srep34212.

Stanier, G. and Davies, A. 1981. Effects of the antibiotic monensin and an inhibitor of methanogenesis on *in vitro* continuous rumen fermentations. *The British Journal of Nutrition* 45(3), 567–78. doi:10.1079/bjn19810135.

Stewart, C. S., Flint, H. J. and Bryant, M. P. 1997. The rumen bacteria. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem* (2nd edn.). Blackie Academic & Professional, London, UK, pp. 10–72.

Storm, A. C., Kristensen, N. B. and Hanigan, M. D. 2012. A model of ruminal volatile fatty acid absorption kinetics and rumen epithelial blood flow in lactating Holstein cows. *Journal of Dairy Science* 95(6), 2919–34. doi:10.3168/jds.2011-4239.

Stouthamer, A. H. 1973. A theoretical study on the amount of ATP required for synthesis of microbial cell material. *Antonie van Leeuwenhoek* 39(3), 545–65. doi:10.1007/bf02578899.

Tatsuoka, N., Hara, K., Mikuni, K., Hara, K., Hashimoto, H. and Itabashi, H. 2008. Effects of the essential oil cyclodextrin complexes on ruminal methane production *in vitro*. *Animal Science Journal* 79(1), 68–75. doi:10.1111/j.1740-0929.2007.00499.x.

Teixeira, C. R. V., Lana, R. P., Tao, J. and Hackmann, T. J. 2017. Comparing the responses of rumen ciliate protozoa and bacteria to excess carbohydrate. *FEMS Microbiology Ecology* 93(6). doi:10.1093/femsec/fix060.

Thauer, R. K., Jungermann, K. and Decker, K. 1977. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriological Reviews* 41(1), 100–80. doi:10.1128/MMBR.41.1.100-180.1977.

Thauer, R. K., Kaster, A. K., Seedorf, H., Buckel, W. and Hedderich, R. 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. *Nature Reviews. Microbiology* 6(8), 579–91. doi:10.1038/nrmicro1931.

Thiele, J. H. and Zeikus, J. G. 1988. Control of interspecies electron flow during anaerobic digestion: significance of formate transfer versus hydrogen transfer during syntrophic methanogenesis in flocs. *Applied and Environmental Microbiology* 54(1), 20–9. doi:10.1128/AEM.54.1.20-29.1988.

Thurston, B., Dawson, K. A. and Strobel, H. J. 1993. Cellobiose versus glucose utilization by the ruminal bacterium *Ruminococcus albus*. *Applied and Environmental Microbiology* 59(8), 2631–7. doi:10.1128/AEM.59.8.2631-2637.1993.

Thurston, B., Dawson, K. A. and Strobel, H. J. 1994. Pentose utilization by the ruminal bacterium *Ruminococcus albus*. *Applied and Environmental Microbiology* 60(4), 1087–92. doi:10.1128/AEM.60.4.1087-1092.1994.

Ungerfeld, E. M. 2013. A theoretical comparison between two ruminal electron sinks. *Frontiers in Microbiology* 4, 319. doi:10.3389/fmicb.2013.00319.

Ungerfeld, E. M. 2015a. Limits to dihydrogen incorporation into electron sinks alternative to methanogenesis in ruminal fermentation. *Frontiers in Microbiology* 6, 1272. doi:10.3389/fmicb.2015.01272.

Ungerfeld, E. M. 2015b. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. *Frontiers in Microbiology* 6, 37. doi:10.3389/fmicb.2015.00037.

Ungerfeld, E. M. 2018. Inhibition of rumen methanogenesis and ruminant productivity: a meta-analysis. *Frontiers in Veterinary Science* 5, 113. doi:10.3389/fvets.2018.00113.

Ungerfeld, E. M. and Kohn, R. A. 2006. The role of thermodynamics in the control of ruminal fermentation. In: Sejrsen, K., Hvelplund, T. and Nielsen, M. O. (Eds), *Ruminant Physiology*. Wageningen Academic Publishers, Wageningen, The Netherlands, pp. 55–85.

Ungerfeld, E. M., Rust, S. R. and Burnett, R. 2007. Increases in microbial nitrogen production and efficiency in vitro with three inhibitors of ruminal methanogenesis. *Canadian Journal of Microbiology* 53(4), 496–503. doi:10.1139/W07-008.

Ungerfeld, E. M., Aedo, M. F., Martínez, E. D. and Saldivia, M. 2019. Inhibiting methanogenesis in rumen batch cultures did not increase the recovery of metabolic

hydrogen in microbial amino acids. *Microorganisms* 7(5), 115. doi:10.3390/microorganisms7050115.

Valkeners, D., Théwis, A., Amant, S. and Beckers, Y. 2006. Effect of various levels of imbalance between energy and nitrogen release in the rumen on microbial protein synthesis and nitrogen metabolism in growing double-muscled Belgian Blue bulls fed a corn silage-based diet. *Journal of Animal Science* 84(4), 877–85. doi:10.2527/2006.844877x.

Van Kessel, J. S. and Russell, J. B. 1996. The effect of amino nitrogen on the energetics of ruminal bacteria and its impact on energy spilling. *Journal of Dairy Science* 79(7), 1237–43. doi:10.3168/jds.S0022-0302(96)76476-7.

Van Lingen, H. J., Plugge, C. M., Fadel, J. G., Kebreab, E., Bannink, A. and Dijkstra, J. 2016. Thermodynamic driving force of hydrogen on rumen microbial metabolism: a theoretical investigation. *PLoS ONE* 11(10), e0161362. doi:10.1371/journal.pone.0161362.

Van Lingen, H. J., Edwards, J. E., Vaidya, J. D., Van Gastelen, S., Saccenti, E., Van Den Bogert, B., Bannink, A., Smidt, H., Plugge, C. M. and Dijkstra, J. 2017. Diurnal dynamics of gaseous and dissolved metabolites and microbiota composition in the bovine rumen. *Frontiers in Microbiology* 8, 425. doi:10.3389/fmicb.2017.00425.

Van Lingen, H. J., Fadel, J. G., Moraes, L. E., Bannink, A. and Dijkstra, J. 2019. Bayesian mechanistic modeling of thermodynamically controlled volatile fatty acid, hydrogen and methane production in the bovine rumen. *Journal of Theoretical Biology* 480, 150–65. doi:10.1016/j.jtbi.2019.08.008.

Veneman, J. B., Muetzel, S., Hart, K. J., Faulkner, C. L., Moorby, J. M., Perdok, H. B. and Newbold, C. J. 2015. Does dietary mitigation of enteric methane production affect rumen function and animal productivity in dairy cows? *PLoS ONE* 10(10), e0140282. doi:10.1371/journal.pone.0140282.

Vyas, D., McGeough, E. J., Mcginn, S. M., McAllister, T. A. and Beauchemin, K. A. 2014a. Effect of *Propionibacterium* spp. on ruminal fermentation, nutrient digestibility, and methane emissions in beef heifers fed a high-forage diet. *Journal of Animal Science* 92(5), 2192–201. doi:10.2527/jas.2013-7492.

Vyas, D., McGeough, E. J., Mohammed, R., McGinn, S. M., McAllister, T. A. and Beauchemin, K. A. 2014b. Effects of *Propionibacterium* strains on ruminal fermentation, nutrient digestibility and methane emissions in beef cattle fed a corn grain finishing diet. *Animal* 8(11), 1807–15. doi:10.1017/S1751731114001657.

Vyas, D., Alazezeh, A., McGnn, S. M., McAllister, T. A., Harstad, O. M., Holo, H. and Beauchemin, K. A. 2015. Enteric methane emissions in response to ruminal inoculation of *Propionibacterium* strains in beef cattle fed a mixed diet. *Animal Production Science* 56(7). doi:10.1071/AN14801.

Vyas, D., McGinn, S. M., Duval, S. M., Kindermann, M. and Beauchemin, K. A. 2016. Effects of sustained reduction of enteric methane emissions with dietary supplementation of 3-nitrooxypropanol on growth performance of growing and finishing beef cattle. *Journal of Animal Science* 94(5), 2024–34. doi:10.2527/jas.2015-0268.

Vyas, D., Alemu, A. W., McGinn, S. M., Duval, S. M., Kindermann, M. and Beauchemin, K. A. 2018a. The combined effects of supplementing monensin and 3-nitrooxypropanol on methane emissions, growth rate, and feed conversion efficiency in beef cattle fed high-forage and high-grain diets. *Journal of Animal Science* 96(7), 2923–38. doi:10.1093/jas/sky174.

Vyas, D., McGinn, S. M., Duval, S. M., Kindermann, M. K. and Beauchemin, K. A. 2018b. Optimal dose of 3-nitrooxypropanol for decreasing enteric methane emissions from beef cattle fed high-forage and high-grain diets. *Animal Production Science* 58(6) doi:10.1071/AN15705.

Walker, D. J. and Monk, P. R. 1971. Fate of carbon passing through the glucose pool of rumen digesta. *Applied Microbiology* 22(5), 741–7. doi:10.1128/AEM.22.5.741-747.1971.

Wallace, R. J. 1996. Ruminal microbial metabolism of peptides and amino acids. *The Journal of Nutrition* 126(4 Suppl.), 1326S–34S. doi:10.1093/jn/126.suppl_4.1326S.

Wallace, R. J., Onodera, R. and Cotta, M. A. 1997. Metabolism of nitrogen-containing compounds. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem* (2nd edn.). Blackie Academic & Professional, London, UK, pp. 283–328.

Wallnöfer, P., Baldwin, R. L. and Stagno, E. 1966. Conversion of C-labeled substrates to volatile fatty acids by the rumen microbiota. *Applied Microbiology* 14(6), 1004–10. doi:10.1128/AEM.14.6.1004-1010.1966.

Wang, R., Wang, M., Ungerfeld, E. M., Zhang, X. M., Long, D. L., Mao, H. X., Deng, J. P., Bannink, A. and Tan, Z. L. 2018. Nitrate improves ammonia incorporation into rumen microbial protein in lactating dairy cows fed a low-protein diet. *Journal of Dairy Science* 101(11), 9789–99. doi:10.3168/jds.2018-14904.

Wells, J. E. and Russell, J. B. 1996. Why do many ruminal bacteria die and lyse so quickly? *Journal of Dairy Science* 79(8), 1487–95. doi:10.3168/jds.S0022-0302(96)76508-6.

Wells, J. E., Russell, J. B., Shi, Y. and Weimer, P. J. 1995. Cellodextrin efflux by the cellulolytic ruminal bacterium *Fibrobacter succinogenes* and its potential role in the growth of nonadherent bacteria. *Applied and Environmental Microbiology* 61(5), 1757–62. doi:10.1128/AEM.61.5.1757-1762.1995.

Wolf, P. G., Biswas, A., Morales, S. E., Greening, C. and Gaskins, H. R. 2016. H_2 metabolism is widespread and diverse among human colonic microbes. *Gut Microbes* 7(3), 235–45. doi:10.1080/19490976.2016.1182288.

Wolin, M. J., Miller, T. L. and Stewart, C. S. 1997. Microbe-microbe interactions. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem* (2nd edn.). Blackie Academic & Professional, London, UK, pp. 467–91.

Zheng, Y., Kahnt, J., Kwon, I. H., Mackie, R. I. and Thauer, R. K. 2014. Hydrogen formation and its regulation in *Ruminococcus albus*: involvement of an electron-bifurcating [FeFe]-hydrogenase, of a non-electron-bifurcating [FeFe]-hydrogenase, and of a putative hydrogen-sensing [FeFe]-hydrogenase. *Journal of Bacteriology* 196(22), 3840–52. doi:10.1128/JB.02070-14.

Chapter 14

Understanding rumen lipid metabolism to optimize dairy products for enhanced human health and to monitor animal health

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1 Introduction

Bovine milk contains approximately 3.5–5.0% milk fat in the form of milk fat globules dispersed in an aqueous phase. The fat globules consist of a relatively large core of triacylglycerides (TAG) enveloped by the milk fat globule membrane, a thin tri-layer membrane of, mainly, phospholipids (PL) (Bernard et al., 2018). As a result, milk fat mainly consists of TAG (97.5–99%), a minority of diglycerides (<1%), PL, non-esterified fatty acids (NEFA), cholesterol and monoglycerides, and nil or only trace amounts (<0.1%) of cholesterol esters (CE) (Castro-Gómez et al., 2014; Glasser et al., 2007).

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Table 1 Fatty acid composition of German summer and winter milk lipids (wt %)[1]

Fatty acid	Summer	Winter
4:0	3.79	3.85
5:0	0.02	0.02
6:0	2.10	2.37
7:0	0.02	0.03
8:0	1.19	1.39
9:0	0.02	0.04
10:0	2.44	3.03
10:1	0.27	0.27
11:0	0.04	0.06
12:0	2.98	3.57
12:1	0.08	0.09
13:0 *iso*	0.14	0.13
13:0 *anteiso*	0.02	–
13:0	0.08	0.10
14:0 *iso*	0.16	0.10
14:0	9.75	11.1
14:1	1.08	1.07
15:0 *iso*	0.43	0.29
15:0 *anteiso*	0.74	0.50
15:0	1.35	1.17
16:0 *iso*	0.30	0.22
16:0	23.5	30.3
16:1	2.00	2.03
17:0 *iso*	0.65	0.55
17:0 *anteiso*	0.55	0.52
17:0	0.72	0.64
17:1	0.39	0.36
18:0 *iso*	0.05	0.08
18:0	10.6	9.42
trans-4 18:1	0.02	0.01
trans-5 18:1	0.02	0.01
trans-6/7/8 18:1	0.26	0.21
trans-9 18:1	0.27	0.17
trans-10 18:1	0.29	0.25
trans-11 18:1	3.82	1.11
trans-12 18:1	0.33	0.27
trans-13/14 18:1	0.34	0.24
trans-15 18:1	0.51	0.38

Fatty acid	Summer	Winter
trans-16 18:1	0.26	0.19
trans (total) 18:1	6.12	2.84
cis-9 18:1	19.4	17.3
cis-11 18:1	0.55	0.53
cis-12 18:1	0.10	0.14
cis-13 18:1	0.06	0.08
cis-15 18:1	0.02	0.03
18:2 (total)	2.17	2.18
18:3 *n*-3	0.61	0.42
19:0	0.09	0.05
20:0	0.16	0.15
20:1	0.31	0.22
20:4	0.09	–
22:0	0.07	0.06
22:1	0.03	–
24:0	0.04	0.04

[1]Adapted from Jensen (2002).

As a result of the microbial activity in the rumen and of the mammary gland metabolism, bovine milk is characterized by a high variety of milk fatty acids (FA) (Jensen, 2002). Typical bovine milk FA profiles are shown in Table 1. Odd- and branched-chain FA (OBCFA) mainly are the result of microbial *de novo* synthesis of FA in the rumen, while a series of *trans* and *cis* unsaturated FA (UFA) are formed from incomplete biohydrogenation of (poly)-unsaturated FA (PUFA), resulting in milk fat that is high (~70%) in saturated FA (SFA). Both aspects of ruminal lipid metabolism and their implications are elaborately discussed in the current chapter.

For a better understanding of the origin of fatty acids in dairy products, the first sections address the ruminal metabolism, intestinal digestion, transfer to the mammary gland and mammary fatty acid metabolism. The chapter then addresses the potential to improve the fatty acid composition of dairy products for enhanced human health. Here, technologies to protect unsaturated fatty acids from rumen biohydrogenation are particularly emphasized as non-protected lipid supplements that already have been extensively reviewed in earlier studies (e.g. Kliem and Shingfield, 2016; Gebreyowhans et al., 2019). Finally, the chapter discusses the use of variation in the milk fatty acid profile, induced by changes in the ruminal lipid metabolism, for monitoring animal health.

2 Ruminal metabolism of dietary lipids and *de novo* fatty acid synthesis

2.1 Lipolysis and biohydrogenation of dietary lipids

The diet of lactating dairy cows typically contains 4–5% crude fat (on DM basis). Primary sources of lipid in the ruminant diet are forages and concentrates, which mainly contain 18-carbon UFA (i.e. α-linolenic acid, 18:3*n*-3; linoleic acid, 18:2*n*-6; and oleic acid, *cis*-9 18:1) (Ferlay et al., 2017). However, the lipid content can be increased by the use of fat supplements. The major lipid class of forages is glycolipids, whereas the majority of lipids in concentrates are present in the form of TAG. Following ingestion, dietary lipids are hydrolyzed and the NEFA are released into the rumen. 18:3*n*-3, 18:2*n*-6 and *cis*-9 18:1 are then converted by the rumen microbial community to SFA first via a *cis-trans* isomerization to *trans* FA intermediates followed by hydrogenation of the double bonds (Harfoot and Hazlewood, 1997; Shingfield and Wallace, 2014). This process is called biohydrogenation.

The main members of the rumen microbial community are (per mL of live liquor) anaerobic bacteria (10^{10}), ciliate protozoa (10^{7}) and anaerobic fungi (10^{6}) (Jenkins et al., 2008; Buccioni et al., 2012). Bacteria, either or not living in symbiosis with protozoa, are known to be mainly responsible for rumen biohydrogenation, while the contribution of protozoa and fungi is negligible (Lourenço et al., 2010; Buccioni et al., 2012). Based on in vitro research, *Butyrivibrio* spp. seems to be the predominant biohydrogenating bacteria (e.g. Kepler et al., 1966; Maia et al., 2007; Wallace et al., 2007). Nevertheless, in several in vivo studies, a correlation between intermediate or end products of the biohydrogenation process and abundance of *Butyrivibrio* spp. in the rumen or at the entrance of the omasal canal was virtually absent (e.g. Zened et al., 2016; Zhu et al., 2016; Kairenius et al., 2017). This suggests potentially that also other – as yet uncultured – species might be involved in ruminal biohydrogenation (Huws et al., 2011; Toral et al., 2016). From such association studies, it is suggested that uncultured bacteria phylogenetically classified as Prevotella, *Lachnospiraceae incertae sedis* and unclassified Bacteroidales, Clostridiales and Ruminococcaceae may play a role in rumen biohydrogenation. The main reason for biohydrogenation of PUFA by bacteria is thought to be reduction of the toxicity of those PUFA (Maia et al., 2007, 2010; Fukuda et al., 2009). The mode of action of PUFA antimicrobial activities is not yet clear, but the prime target seems to be the bacterial cell membrane and the various essential processes that occur within and at the membrane. Bacteria prefer SFA for their membrane synthesis as the double bonds present in UFA alter the shape of the molecule and disrupt the lipid bilayer structure (Keweloh and Heipieper, 1996).

Numerous in vivo and in vitro studies have enabled several ruminal biohydrogenation pathways of 18:2*n*-6, 18:3*n*-3 and *cis*-9 18:1 to be elucidated. Under normal rumen conditions, 18:2*n*-6 is mainly isomerized to *cis*-9, *trans*-11 conjugated linoleic acid (CLA), which is further hydrogenated to *trans*-11 18:1, and ultimately to 18:0 (Fig. 1, solid line arrows). The major biohydrogenation pathway of 18:3*n*-3 involves *cis*-9, *trans*-11, *cis*-15 conjugated linolenic acid (CLnA), *trans*-11, *cis*-15 18:2 and *trans*-11 18:1 as intermediates (Fig. 2, solid line arrows), whereas the major part of *cis*-9 18:1 is directly hydrogenated to 18:0 in the rumen (Fig. 3, solid line arrow). However, ruminal biohydrogenation of 18:2*n*-6, 18:3*n*-3 and *cis*-9 18:1 might also result in the formation of several other minor FA intermediates, for example, *trans*-9, *trans*-11 CLA, *trans*-10 18:1 and *cis*-12 18:1 (Figs. 1–3, dashed line arrows). Some of these alternative pathways might become more important under certain rumen conditions

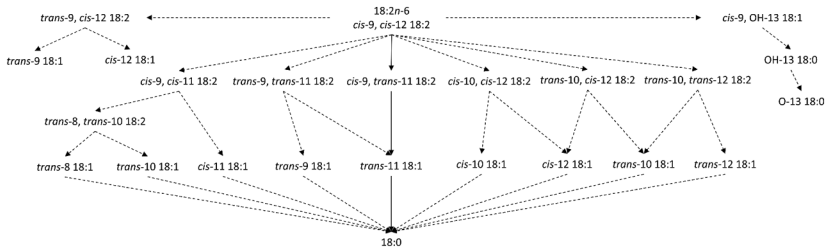

Figure 1 Pathways of ruminal 18:2*n*-6 metabolism (adapted from Shingfield and Wallace, 2014). Arrows with solid lines highlight the major biohydrogenation pathway while arrows with dashed lines describe the formation of minor intermediates, under physiologically normal conditions in the rumen.

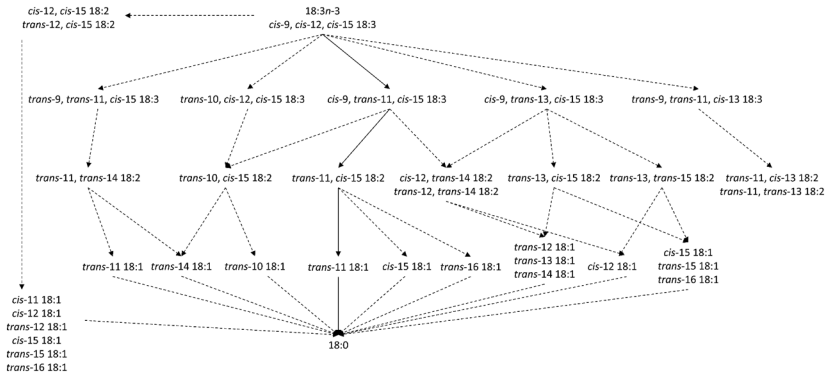

Figure 2 Pathways of ruminal 18:3*n*-3 metabolism (adapted from Ferlay et al., 2017). Arrows with solid lines highlight the major biohydrogenation pathway while arrows with dashed lines describe the formation of minor intermediates, under physiologically normal conditions in the rumen.

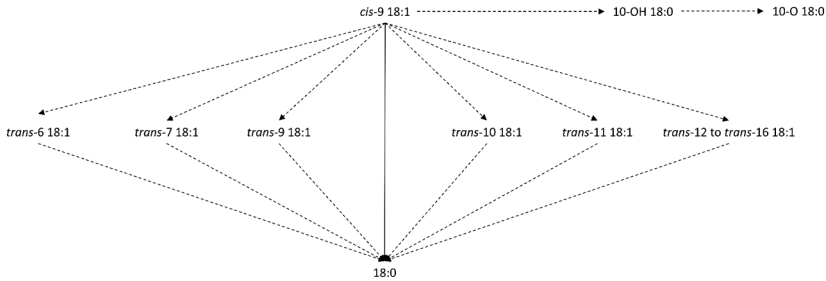

Figure 3 Pathways of ruminal *cis*-9 18:1 metabolism (adapted from Shingfield and Wallace, 2014). Arrows with solid lines highlight the major biohydrogenation pathway while arrows with dashed lines describe the formation of minor intermediates, under physiologically normal conditions in the rumen.

and might be of interest as indicators to monitor these rumen conditions. For example, when high-starch diets are fed, 18:3*n*-3 and 18:2*n*-6 might also be converted via an alternative pathway, resulting in an increased accumulation of *trans*-10 intermediates in the rumen, particularly of *trans*-10 18:1 (Shingfield and Griinari, 2007). However, these shifts may vary among ruminant species. While a high-starch diet supplemented with sunflower oil resulted in a *trans*-11 to *trans*-10 shift in cows, this shift tends to be less in goats (Toral et al., 2016). On the other hand, marine lipids, rich in very-long-chain *n*-3 PUFA, efficiently inhibit the last step of C18 FA biohydrogenation in the bovine, ovine and caprine, increasing the outflow from the rumen of *trans*-11 18:1, *cis*-9, *trans*-11 CLA and *trans*-10 18:1 (Toral et al., 2016). In addition to the pathways shown in Figs. 1–3, other alternative pathways might also exist, as supported by the identification of numerous additional biohydrogenation intermediates in recent in vitro experiments using stable isotopes (deuterium oxide and ^{13}C-labelled FA; Honkanen et al., 2016; Toral et al., 2018a, 2019).

2.2 De novo *fatty acid synthesis by rumen bacteria*

Bacterial *de novo* synthesis of SFA is mediated by two types of FA synthases, being straight-chain and branched-chain FA synthase (Kaneda, 1991). *De novo* synthesis of straight-chain FA with an even number of carbons is achieved by repeated condensation of malonyl-coenzyme A (CoA) with acetyl-CoA as primer, yielding palmitic acid as the dominant end product (Fulco, 1983). Linear odd-chain FA are formed when propionyl-CoA, instead of acetyl-CoA, is used as primer (Fulco, 1983; Kaneda, 1991). Branched-chain FA can be distinguished in three series: even *iso* acids (e.g. *iso* 14:0, *iso* 16:0), odd *iso* acids (e.g. *iso* 15:0, *iso* 17:0) and odd *anteiso* acids (e.g. *anteiso* 15:0, *anteiso* 17:0) (Kaneda, 1977), and their primers are isobutyryl-CoA, *iso*valeryl-CoA and 2-methylbutyryl-CoA, respectively (Kaneda, 1977; Annous et al., 1997). Bacterial *de novo* synthesis of

OBCFA is of particular interest as only trace levels occur in most plants (Diedrich and Henschel, 1990) and their presence in animal tissues is particularly limited to those animals with important symbiotic fermentation (Keeney et al., 1962). More importantly, rumen bacteria show a distinct OBCFA profile which seems largely determined by the substrate specificity of the acetyl-CoA acyl-carrier-protein transacylase (Kaneda, 1991). As a result, variations in the profile of ruminal OBCFA are mainly a reflection of changes in the relative abundance of specific bacterial populations in the rumen.

3 Digestion and transfer of dietary and rumen fatty acids to the mammary gland and fatty acid metabolism in the mammary gland

Dietary FA bypassing rumen metabolism as well as rumen FA flows to the duodenum. The apparent recovery of these duodenal FA in milk fat depends on various factors, including digestibility, FA metabolism (both synthesis and oxidation) in the cow's tissues, the cow's physiological status (positive vs. negative energy balance), the blood lipid classes in which FA are transported and the duodenal FA flow, with higher transfer efficiencies at lower intestinal flows (Chilliard et al., 2000). Finally, some FA are also partially transformed in the mammary gland.

3.1 Intestinal digestion of fatty acids

About 70% of the short-chain fatty acids are absorbed in the rumen, while uptake of medium- and long-chain fatty acids before reaching the small intestine is negligible (Noble, 1978). Fatty acids reaching the small intestine are absorbed from the jejunum after solubilization in a micellar phase. Although in ruminants – due to lipolysis in the rumen – mainly free FA flow to the small intestine, these FA still need to be "released" to a certain extent. Indeed, because of their hydrophobic nature, free FA are attached to small particles of the digesta. To make these hydrophobic FA "soluble" in the aqueous environment, micelle formation must take place. In both ruminants and monogastric animals, the efficiency of micelle formation is the limiting factor in the absorption of FA in the small intestine. Micelle formation of long-chain, SFA (such as e.g. stearic acid) is generally less efficient than of short-chain and UFA. Ruminants have therefore developed a number of important differences and characteristics in FA absorption as compared to non-ruminants that allow efficient absorption of SFA (Bauman and Lock, 2006). These include differences in both bile salt composition (more taurine-conjugated bile salts than glycine-conjugated) and the predominant amphiphilic substances involved in micelle formation. Lysolecithin is the most important amphiphilic substance in the small intestine of ruminants, while monoglycerides and bile salts primarily perform this function

in monogastric animals. Compared to these two components, lysolecithin is a much better emulsifier of stearic acid. Lysolecithin (lysophosphatidylcholine) is formed in the duodenum from lecithin (a phospholipid) through the action of phospholipases (produced in the pancreas). In addition, the supply to the small intestine is characterized by a slow and continuous release of relatively small amounts of FA (due to the continuous feed intake and passage from the rumen). Consequently, in general, the ability of ruminants to absorb SFA is much higher than that of non-ruminants (Bauman and Lock, 2006). The average apparent intestinal absorption of FA is 0.83, with a tendency for higher absorptions of microbial FA (Schmidely et al., 2008).

Nevertheless, recently, abomasal infusion of exogenous emulsifiers has been shown to improve FA absorption, which implies that solubilization of FA is the primary limiting step for the transfer of FA from the intestinal lumen to the blood stream (Prom and Lock, 2019). Moreover, excessive supplementation of dietary fat will suppress the FA digestibility, and this effect is much greater with increased uptake (or outflow from the rumen) of 18:0 than 16:0 (Boerman et al., 2015, 2017; Rico et al., 2017).

3.2 Fatty acid transport in blood and transfer to the mammary gland

After absorption, free FA are esterified into TAG and PL in intestinal epithelial cells and transported, first in the lymph and afterwards in the blood. They predominantly occur as chylomicrons (Demeyer and Doreau, 1999), and to a lesser extent in very-low-density lipoproteins (VLDL). Both lipoproteins predominantly consist of TAG, besides PL, CE and NEFA (in decreasing order of importance) (Bruss, 1997). Lipid classes largely differ in composition, due to selective incorporation of specific FA, as well as in effectiveness to deliver FA to the mammary gland. Indeed, FA concentrated in CE and PL are poorly transferred to milk fat, given the low affinity of the mammary gland lipoprotein-lipase for these fractions (Annison et al., 1967; Shennan and Peaker, 2000). Such distribution differences in lipid classes – at least partially – could explain difficulties in enriching milk fat with PUFA as, for example, 18:3*n*-3, which is predominantly incorporated in blood plasma CE (Loor et al., 2002b; Tyburczy et al., 2009), while, for example, 20:5*n*-3 (eicosapentaenoic acid, EPA) and 22:6*n*-3 (docosahexaenoic acid, DHA) are predominantly present in PL. Additionally, mobilization of adipose tissue might contribute to modification of the milk FA profile compared with the duodenal FA profile (Jorjong et al., 2014), particularly because adipose tissue is rich in FA of longer chain length. For the OBCFA, this results in a preferential incorporation during anabolism of OBCFA with a chain length of 17 carbon atoms compared with OBCFA with chain lengths of 13–15 carbon atoms, as supported by the inverse ratio of 17:0

to 15:0 in adipose tissue (2:1) as compared to milk (1:2) (Craninx et al., 2008). As a result, during the first weeks of lactation when the cow is mobilizing fat, milk secretion of OBCFA with 17 carbon atoms was increased. Inversely, shorter OBCFA (with chain lengths of 14–15 carbon atoms) were diluted in milk fat, similarly to other short- and medium-chain FA (Craninx et al., 2008).

4 Endogenous fatty acid metabolism in the mammary gland

Endogenous FA metabolism in the mammary gland include *de novo* synthesis of short and medium-chain FA, desaturation and, to a limited extent, chain elongation of FA with two carbon atoms. The current section is limited to general principles and some recent findings on endogenous milk fatty acid synthesis that enhances human health (particularly UFA) and allows to monitor animal health (odd- and branched-chain fatty acids and *trans* fatty acids). As major mammary lipogenic pathways seem to differ between species it is worth mentioning that these are bovine results; for example, the abundance of mRNA encoding for genes involved in lipid metabolism, as well as enzyme activities related to fatty acid and lipid synthesis in the mammary gland of the caprine and the bovine, shows strong species specificities (Bernard et al., 2017).

4.1 Endogenous mammary metabolism of odd and branched-chain fatty acids

The *de novo* synthesis of FA in the mammary gland is mainly catalyzed by FA synthase (FASN) and acetyl-CoA carboxylase (ACACA). In this process, malonyl-CoA, generated from acetyl-CoA, is the elongation substrate used to produce short and medium even-chain FA with acetyl-CoA as a primer. Replacing acetyl-CoA by propionyl-CoA as a precursor in this process explains the occurrence in milk of (trace amounts of) 5:0, 7:0, 9:0, 11:0 and 13:0, although traces of the latter FA are also detected in duodenal contents (Dodds et al., 1981; Massart-Leën et al., 1983). Moreover, ruminal infusion of propionate increased the amount of not only the former odd-chain FA in milk fat, but also 15:0 and 17:0 (Rigout et al., 2003; Maxin et al., 2011; French et al., 2012). As such, this process adds to 15:0 and 17:0 transferred from the duodenum, similar to the dual origin of 16:0 in milk fat. Where *de novo* synthesis in the mammary gland has been suggested to contribute to about half of the secretion in milk of 16:0, this process also has been suggested to contribute to at least one-third of the 15:0 and 17:0 secretions in milk (Vlaeminck et al., 2015).

On the other hand, mammary FASN does not seem to elongate isovaleryl-CoA, 2-methylbutyryl-CoA and isobutyryl-CoA to *iso* odd chain, *anteiso* odd chain or *iso* even chain FA, as suggested from infusion studies with (labelled) isovalerate (Verbeke et al., 1959; French et al., 2012) and 2-methylbutyrate

(French et al., 2012). Nevertheless, recoveries above 1.0 were observed for *iso* 17:0 (e.g. Dewhurst et al., 2007) and *anteiso* 17:0 (e.g. Vlaeminck et al., 2006). Although FA elongation with two carbon atoms to generate FA beyond 16 carbons does not seem relevant in milk FA synthesis of linear even and odd chain FA, Vlaeminck et al. (2015) provided evidence of a post-ruminal 2-carbon elongation of contributing to the secretion of *iso* 17:0 and *anteiso* 17:0. However, the partial conversion of *iso* 15:0 and *anteiso* 15:0 to their C17 counterparts also seems to occur prior to the mammary gland (e.g. in duodenal epithelial cells), as suggested from the enrichment of *iso* 17:0 and *anteiso* 17:0 in plasma TAG as compared to duodenal contents (Vlaeminck et al., 2015).

Finally, odd chain FA can further be metabolized by Δ^9-desaturase activity, but only the conversion of 17:0 to 17:1 seems of quantitative importance (Fievez et al., 2003).

4.2 Endogenous mammary metabolism of saturated and mono-unsaturated fatty acids

Stearoyl-CoA desaturase (SCD) is an important enzyme in the bovine mammary gland. It converts SFA into mono-unsaturated FA (MUFA) by introducing a double bond between carbon atoms 9 and 10 in the saturated carbon chain, for example, desaturation of 18:0 to *cis*-9 18:1 (Annison et al., 1967). However, it can also catalyze the desaturation of MUFA, particularly of *trans*-11 18:1 to generate *cis*-9, *trans*-11 CLA (Griinari et al., 2000).

4.2.1 Endogenous mammary metabolism of poly-unsaturated fatty acids

Fatty acid elongation with two carbon atoms to generate FA with chain lengths beyond 16 carbons is a very common process in various ruminant tissues, except for the mammary gland (Moore and Christie, 1981).

5 Impact of ruminant fatty acids on human health

According to a cohort study in 13 European countries, dairy products were estimated to contribute from 14% (Spain) to 40% (Germany) of the total dietary fat intake (Hulshof et al., 1999), indicating the significance of ruminant FA in the Western diet. The FA profile of ruminant animal products is mainly saturated, as biohydrogenation is extensive, resulting in 18:0 being the major FA leaving the rumen (Shingfield and Wallace, 2014). As 18:0 is a neutral FA regarding human health, biohydrogenation to 18:0 is neither negative nor positive for human health. However, the reduction of unsaturated 18-carbon FA to 18:0 in the rumen is incomplete and a series of 18:1, 18:2 and 18:3 intermediates accumulate with double bonds of different positions and configurations.

Among this plethora of UFA, some might positively or negatively impact human health depending on the FA structure.

5.1 Impact of ruminant trans *fatty acids on human health*

Under normal conditions, the major *trans* FA present in milk or dairy products is *trans*-11 18:1 (25–75% of total 18:1 isomers) (Lock and Bauman, 2004). According to Kuhnt et al. (2006), 19–24% of dietary *trans*-11 18:1 is endogenously converted to *cis*-9, *trans*-11 CLA in humans by Δ^9-desaturase, and this CLA isomer has been shown to have anticarcinogenic and anti-atherogenic effects (Lock and Bauman, 2004). However, the remaining *trans*-11 18:1 which is not converted might increase cancer risk as suggested by human epidemiological studies (odds ratio ranging from 0.30 to 3.69; Field et al., 2009). Nevertheless, this is not supported by animal and in vitro studies which have been performed (Field et al., 2009). Furthermore, in human T cells, *trans*-11 18:1 has been shown to have a cytokine reducing effect (interleukin-2 and tumor necrosis factor-α), which was independent of *cis*-9, *trans*-11 CLA as no bioconversion occurred (Jaudszus et al., 2012). This indicates that *trans*-11 18:1 might also have health-promoting effects beyond those associated with *cis*-9, *trans*-11 CLA (Field et al., 2009). In their state-of-the-art summary, Kuhnt et al. (2016) summarized several human intervention studies related to ruminant *trans* FA, in which three- and ten-fold amounts of ruminant *trans* FA were compared to levels consumed by the average population. They concluded that with the average amounts ingested, no negative effects on human health were observed. The amount of ruminant *trans* FA that induced negative effects on blood lipids was extremely high and not realistic (11–12 g/day, for example, corresponding to a daily combined consumption of about 490 g cheese, 1200 mL milk, 430 g yoghurt and 80 g butter as calculated from data reported by Kuhnt et al., 2016).

When high-starch diets are fed to dairy cows, a shift might occur in milk-fat profile from mainly *trans*-11 18:1 towards increased proportions of *trans*-10 18:1 (e.g. Conte et al., 2018). Individual *trans* isomers of 18:1 might have specific properties (Ferlay et al., 2017). Epidemiological studies showed a negative impact of industrial *trans* FA on serum cholesterol and lipoprotein metabolism, thereby increasing the risk for coronary heart disease (Kuhnt et al., 2016). As such, since industrial fatty acids are particularly rich in *trans*-10 18:1, milk rich in *trans*-10 18:1 might have a negative effect on human health. Nevertheless, with *trans*-10 18:1 containing milk (products), no human intervention studies have been performed yet and only two animal studies compared *trans*-10 18:1 containing milk or butter with *trans*-11 18:1 containing milk or butter. Roy et al. (2007) observed increased total cholesterol and LDL cholesterol concentrations in plasma and increased lipid deposition in the aorta of rabbits supplemented with *trans*-10 18:1 compared to *trans*-11 18:1 enriched butter. Furthermore,

in rats, treatment with *trans*-10 18:1-enriched milk fat tended to increase TAG concentration, whereas treatment with *trans*-11 18:1 and *cis*-9, *trans*-11 CLA containing milk fat tended to reduce it (Anadón et al., 2010). Although those animal studies showed a potential negative risk of *trans*-10 18:1 containing milk (products) for human health, extrapolation of findings from animal studies to humans has to be made with caution.

5.2 Impact of conjugated linoleic acids and n-3 fatty acids on human health

A wide range of CLA isomers with double bond positions from 7 to 9 and from 12 to 14 in different combinations of geometrical configurations have been identified in dairy products, with *cis*-9, *trans*-11 CLA being the predominant isomer (~90%) (Ferlay et al., 2017). The health effects of CLA have been extensively studied, showing a variety of positive effects on health, such as anticarcinogenic, anti-obesity and anti-inflammatory effects (Dhiman et al., 2005; Ferlay et al., 2017). However, almost all studies were performed using a synthetic mixture of CLA isomers, containing equal amounts of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomer (80–95%) and other minor CLA isomers. Recently, a few studies showed beneficial effects of *trans*, *trans* CLA isomers (either *trans*-9, *trans*-11 or a mix) on health, such as anticarcinogenic, anti-inflammatory, antiplatelet aggregation and hypocholesterolemia effects as well as prevention of fatty liver (Kim et al., 2016). Overall, CLA consumption has been shown to improve human health (Dhiman et al., 2005). Nevertheless, more research is needed to elucidate the metabolic role of individual CLA isomers and their interaction.

Considering PUFA, a differentiation is made between the *n*-3 and *n*-6 series of long-chain FA (LCFA), which are formed by elongation and desaturation of the essential linolenic (18:3*n*-3) and linoleic (18:2*n*-6) acids, respectively, and can be further converted to eicosanoids. *n*-6 eicosanoids, derived from arachidonic acid (20:4*n*-6, AA), are known to have pro-inflammatory properties, while *n*-3 eicosanoids, derived from EPA have an anti-inflammatory effect (Wall et al., 2010). As the formation of the *n*-3 and *n*-6 series of LCFA from its precursors are catalyzed by the same set of enzymes, competition exists. As a result, a low *n*-6 to *n*-3 ratio (between 1:1 and 4:1) is recommended (Wall et al., 2010). In comparison, in Western diets (high in 18:3*n*-3), this ratio is estimated between 15:1 and 20:1 (Simopoulos, 2001). Even though dairy fat is highly saturated, a Dutch study estimated that milk fat contributes significantly to the intake of the essential *n*-3 PUFA 18:3*n*-3 (5.3–15.7%), but to a lesser extent to the intake of the essential *n*-6 PUFA 18:2*n*-6 (1.8 to 3.6%) (van Valenberg et al., 2013). Several very-long-chain *n*-3 and *n*-6 PUFA are consumed mainly via the intake of milk fat (van Valenberg et al., 2013), which shows an *n*-6 to

n-3 ratio of 2.28, within the desired range. The only very long-chain PUFA for which recommendations of intake have been formulated are EPA and DHA. The contribution of milk fat in the intake of DHA is marginal, while, in contrast to previous reports (Meyer et al., 2003; Sioen et al., 2006; Astorg et al., 2004), 10–15% of the daily EPA intake was estimated to originate from milk fat (van Valenberg et al., 2013).

6 Rumen bypass polyunsaturated fatty acid (PUFA) products to improve the milk fatty acid profile

Even though several health benefits can be ascribed to the consumption of milk FA (Gómez-Cortés et al., 2018), the general perception of dairy fat is negative, as it is high in SFA associated with an increased risk of cardiovascular diseases. Grass-based production systems are an effective dietary strategy to enhance the milk FA profile, with grass as a cheap and easily accessible *n*-3 FA source for the farmer (Gebreyowhans et al., 2019). As such, grass-based production methods were shown to increase the content of the health-promoting CLA and *n*-3 FA in milk (Elgersma, 2015). Indeed, the intake of CLA – including human bioconversion from *trans*-11 18:1 – via milk and dairy products was shown to increase from 350–550 mg per day to 707–1107 mg per day when grass-based production methods (maximum 50% grain-based feed) were applied (van Wijlen and Colombani, 2010). However, often such grass-based production systems are not feasible, because of limited availability of grass land or suboptimal climate.

Supplementation of *n*-3 or *n*-6 PUFA in the form of oil or oilseeds could be considered as an alternative, either or not in combination with supplements to modulate the ruminal lipid metabolism. Addition of such modulation agents which could be considered as dietary supplementation of unprotected oils or oilseeds only results in a limited PUFA enrichment of milk due to the extensive ruminal biohydrogenation in the rumen (Gebreyowhans et al., 2019; Shingfield et al., 2013). As such, plant secondary compounds and direct-fed microbials have been examined in an attempt to enhance the concentration of putative desirable PUFA in ruminal digesta, milk and intramuscular fat.

The compiled information in the recent review by Toral et al. (2018b) indicates only a limited potential of such additives to change the milk PUFA composition in a repeatable manner. Diversity in active components and doses (e.g. a wide variety of plant secondary compounds, including, for example, tannins, essential oils, oxygenated fatty acids and saponins, has been examined) and differences in ruminant species, basal diet composition and timing of treatments are some factors which might have contributed to these inconsistent results. Accordingly, the most efficient way to transfer PUFA towards milk would be to bypass the rumen, through the use of rumen protection technologies.

Given the recent excellent review by Toral et al. (2018b) on the modulation of the ruminal metabolism of unprotected lipid supplements, this chapter will further focus on rumen protection technologies. Two classes of such protection technologies could be determined (Jenkins and Bridges, 2007):

1 modification of the FA structure to inhibit bacterial isomerases
2 encapsulation in a protective matrix

An overview of the most common technologies is presented in Table 2 (adapted from Gadeyne et al., 2017), with the corresponding transfer efficiencies of PUFA from the diet to the milk. By comparison, in post-ruminal infusion studies transfer efficiencies of 18:2*n*-6 and 18:3*n*-3 could reach 49% and 50% (Shingfield et al., 2013), and for *trans*-10, *cis*-12 CLA and 22:6*n*-3, transfer efficiencies as high as 22% (de Veth et al., 2004) and 25% (Shingfield et al., 2013) were reported, respectively.

7 Rumen lipid protection technologies

This section is based on Gadeyne et al. (2017),where sections 7.1 to 7.5 have been literally cited from Gadeyne et al. (2017), while section 7.6 was adapted from Gadeyne et al. (2017).

7.1 Alterations of the fatty acid structure: calcium salts

Calcium salts of LCFA are soaps formed by the creation of an ionic bond between the free carboxyl group of the FA and calcium ions. The possibility to protect calcium salts of FA against ruminal biohydrogenation was first proposed by Palmquist and Jenkins (1987) and suggested that it could be caused by the insoluble character of the calcium salts, permitting an efficient bypass across the rumen without disturbing the rumen microorganisms. As dissociation constants (pKa) of calcium salts range between 4.5 and 6 (Sukhija and Palmquist, 1990), salts dissociate again in the acid environment of the abomasum, which makes the FA available for absorption in the small intestine.

However, a major disadvantage of this technology is that dissociation might already occur, if the pH decreases beneath 6.3 (Chalupa et al., 1986; Van Nevel and Demeyer, 1996), making the FA accessible to bacterial isomerases. Indeed, pKa depends on the unsaturation of the FA in the soap (Sukhija and Palmquist, 1990), meaning more dissociation will occur with increasing concentrations of UFA at a given rumen pH. Finally, a major (economic) disadvantage is that the production of calcium salts requires free FA as precursors. Generally, very inconsistent results are found in literature dealing with calcium salts of UFA, but most of them reported an incomplete protection (Table 2).

Table 2 Protective mechanism, possible disadvantages and literature-extracted transfer of polyunsaturated fatty acids (PUFA) from intake to dairy cow's milk for the most described or promising rumen lipid bypass technologies

Protection technique	Protective mechanism (+) Disadvantages (−)	Lipid source	Evaluated PUFA[a]	*Transfer*[b] (%)		Reference
				Protected supplement	Unprotected supplement (control)	
Calcium salt	+Blocking free FA carboxyl end	Linseed oil	18:3*n-3*	0.67	–	Chouinard et al. (1998)
		Linseed oil	18:3*n-3*	1.2	–	Sultana et al. (2008)
	−Protection impaired by dissociation	Linseed oil	18:3*n-3*	1.9	1.5[c]	Cortes et al. (2010)
		Fish oil	22:6*n-3*	6.0	3.3[d]	Castaneda-Gutierrez et al. (2007b)
	−Limited protection of PUFA	High-PUFA palm oil	18:2*n-6*	13.2	–	Theurer et al. (2009)
		CLA oil	*t*10 *c*12 18:2	1.9–7.2	–	de Veth et al. (2005)[e]
	−Free FA needed	CLA oil	*t*10 *c*12 18:2	3.2	0.0[f]	de Veth et al. (2005)
		Soybean oil	18:2*n-6*	6.5	6.9	Lundy et al. (2004)
Fatty acyl amide	+ Blocking carboxyl end	Canola oil	18:2*n-6*	17	18	Loor et al. (2002a)
		Soybean oil	18:2*n-6*	5.5	6.9	Lundy et al. (2004)
		CLA oil	*t*10 *c*12 18:2	7.1	0.0[f]	Perfield et al. (2004)
Formaldehyde	+ Encapsulation within formaldehyde-protein matrix	Canola/soybean	18:2*n-6*	25–44	–	Gulati et al. (2005)[e]
		Cottonseed	18:2*n-6*	43	–	Gulati et al. (2005)[e]
		Soybean/linseed	18:3*n-3*	19–24	–	Gulati et al. (2005)[e]
	−Toxic product needed	Soybean/fish oil	22:6*n-3*	10–14	–	Gulati et al. (2005)[e]
	−Untargeted reaction	Linseed oil	18:3*n-3*	13	3.0[g]	Sterk et al. (2012)
	−High cost	CLA oil	*t*10 *c*12 18:2	7.0	0.0[f]	de Veth et al. (2005)
		CLA oil	*t*10 *c*12 18:2	6.9–8.6	–	Gulati et al. (2006)

(*Continued*)

Table 2 (*Continued*)

Protection technique	Protective mechanism (+) Disadvantages (−)	Lipid source	Evaluated PUFA[a]	*Transfer*[b] (%) Protected supplement	*Transfer*[b] (%) Unprotected supplement (control)	Reference
Lipid composite gels	+ Encapsulation within gelled protein matrix	Soybean oil	18:2*n-6*	46–69 (16–30)[i]	22–37	Carroll et al. (2006)
		Soybean/linseed oil	18:3*n-3*	81–225[j] (9–43)[i]	21	Heguy et al. (2006)
	−contains large volume of waters	Soybean/linseed oil	18:3*n-3*	13–19	–	van Vuuren et al. (2010)
		Rapeseed oil	18:2*n-6*	11–15	13	Kliem et al. (2016)
Encapsulation within lipid	+ Encapsulation within higher-melting point lipid matrix	CLA oil	*t*10 *c*12 18:2	7.9	0.0[f]	Perfield et al. (2004)
		CLA oil	*t*10 *c*12 18:2	5.1	0.0[f]	Castaneda-Gutierrez et al. (2007a)
		CLA oil	*t*10 *c*12 18:2	4.8	–	Moallem et al. (2010)
	−low payloads	CLA oil	*t*10 *c*12 18:2	6.3	0.0[f]	Odens et al. (2007)
	−low post-ruminal release	CLA oil	*t*10 *c*12 18:2	2.4–5.8	–	Pappritz et al. (2011)
		CLA oil	*t*10 *c*12 18:2	4.9	–	Schwarz et al. (2009)
		Algal oil	22:6*n-3*	1.0	–	Stamey et al. (2012)
		Algal biomass	22:6*n-3*	2.0–3.4	–	Stamey et al. (2012)
		Echium oil	18:4*n-3*	3.2–3.4	–	Bainbridge et al. (2015)
Tyrosinase cross-linking	+ Encapsulation in phenol-protein matrix	Linseed oil	*t*10 *c*12 18:2	4.0	–	Gadeyne et al. (2016)
	−Protein extraction					
	−Phenolic mediator needed					

[a]The most prominent PUFA within the oil was used for evaluation; [b]Transfer was calculated as (g PUFA in milk)/(g PUFA in diet)×100, whereby fat was assumed to contain 90% (w/w) FA; [c]Whole linseed; [d]Ruminal infusion of fish oil, no statistical difference with the treatment; [e]Summary of earlier studies; [f]No *t*10*c*12 18:2 measured in milk of control treatment; [g]Extruded whole linseed; [h]No scientific literature describing in vivo milk data available; [i]Net transfer efficiency as reported in reference; [j]Calculation with data from reference results in an unrealistic high transfer.
Source: adapted from Gadeyne et al. (2017).

7.2 Alterations of the fatty acid structure: fatty acyl amides

Fatty acyl amides consist of a FA chemically linked through an amide bound to an amine. This approach was proven potentially useful to protect UFA against ruminal biohydrogenation, using either amino acids (Fotouhi and Jenkins, 1992), non-acidic primary amines, such as aliphatic amines containing 1–30 carbon atoms (Jenkins, 1996), or ammonia (Cummings and Forrest, 1997). As for the Ca salts, the production process of simple amide-protected supplements requires free FA as precursor. However, fatty acyl amides do not seem to be more effective in transferring dietary PUFA to milk than pure oil (Table 2).

7.3 Encapsulation in a microbe-resistant shell: aldehyde treatment

In 1975, Scott and Hills (1975) proposed a method to protect UFA by encapsulation within a protein aldehyde reaction product. Prior to aldehyde addition, lipids first need to be emulsified using proteins such as casein, gelatin or other plant, fish, meat or oilseed proteins to ensure a homogeneous distribution of the lipid within the protein, and can further be processed using spray-drying to obtain a coated particulate solid. However, formaldehyde is a noxious product and its use in the European Union is subject to strict regulations (2011/391/EU). Although formaldehyde treatment is considered to be the most effective technique so far, its application remains limited nowadays due to its high cost, the bad image of chemical treatments of feedstuffs and possible residues in the final animal products (Doreau et al., 2015; Palmquist and Jenkins, 2017). The method proposed by Scott and Hills (1975) effectively prevented ruminal biohydrogenation, both in vitro and in vivo (Table 2).

7.4 Encapsulation in a microbe-resistant shell: encapsulation within lipids

A more recent method describes the potential of composite gels containing amino acids and lipids to bypass the rumen. Rosenberg and DePeters (2010) claimed that dispersions of lipid droplets in an aqueous protein phase can be protected against ruminal degradation by heat-induced gelatinization. In contrast with the aldehyde treatment, the cross-linking of protein is not induced by a divalent linker such as formaldehyde, but by gelation of proteins. Reducing sugars can be present in the matrix to additionally cross-link the proteins by a Maillard reaction. Embedding lipids in a protein matrix of whey or blood proteins has the advantage of upgrading such side streams, while creating rumen bypass lipid. However, it could be assumed that gelled

emulsions have a low shelf life as they contain generally large volumes of water, which may cause deterioration of the gels and the enclosed lipids during storage (van Vuuren et al., 2010). Nevertheless, as gels are prepared at elevated temperatures, others consider composite gel's shelf-stable (Weinstein et al., 2015). Carroll et al. (2006) were the first to report on the efficacy of whey protein gel complexes to increase the PUFA content of bovine milk fat (Table 2). Similarly, Heguy et al. (2006) found that feeding whey protein isolate gel complexes of soybean/linseed oil successfully increased the PUFA content and decreased *trans* FA of plasma and milk lipids. More recent research demonstrated the persistent effect of long-term administration (10 weeks) (van Vuuren et al., 2010).

7.5 Encapsulation in a microbe-resistant shell: lipid composite gels

Technologies described in the former sections relied on different types of protein-cross-linking to achieve rumen protection. In other formulations active compounds are protected in a microcapsule of lipids according to either one of two basic concepts: active compounds are either embedded in a lipid matrix or are formulated in small spheres, which then are coated with lipid (Desai and Jin Park, 2005; Wu and Papas, 1997). Generally, coatings are comprised of fats with a high melting point, that is, at least higher than the matrix it envelops (Lorenzon, 2015; Meade et al., 1999). Despite this common overall principle, the composition of the outer coating particularly differs between described methods, which results in varying protection efficiencies (Table 2).

7.6 Encapsulation in a microbe-resistant shell: tyrosinase cross-linking of emulsions[1]

Another recently explored lipid protection technology involved enzymatic cross-linking of protein-stabilized emulsions by tyrosinase (Gadeyne et al., 2015). The transfer of *trans*-10, *cis*-12 CLA, encapsulated in tyrosinase-cross-linked emulsions, from the diet to the milk was evidenced in vivo (Gadeyne et al., 2016). However, transfer efficiencies were not higher than those reached for *trans*-10, *cis*-12 CLA in commercially available rumen-protected products (Table 2). Therefore, further research is needed to optimize the current enzyme-based method, which is particularly of interest because of the possibility of solvent-free processing, at ambient temperatures and pressure, and the potential to valorize industrial agri-food side streams.

1 Current paragraph was adapted from Gadeyne et al., 2017, while other parts of section 7 were cited without modification to the original.

8 Milk fatty acids originating from the rumen as biomarkers to monitor animal health

Changes in the ruminal metabolism of dietary lipids and *de novo* bacterial FA synthesis might indicate changes and even disturbances in the ruminal microbial population which sometimes initiate a chain of events that eventually might lead to (subacute) ruminal acidosis (SARA). Disturbed rumen (pH) conditions have been correlated with inflammation, as measured by increased acute-phase proteins in the blood (e.g. Gozho et al., 2005, 2007), which further might result in feed-intake depression and production losses (Plaizier et al., 2008). Reduced rumen pH might also result in reduced fibre digestion (Plaizier et al., 2008) and/or in milk fat depression (Dewanckele et al., 2019). Other consequences of SARA are diarrhoea, laminitis and lameness, and liver abscesses.

Particularly specific *trans* FA, notably *trans*-10 intermediates, odd chain and iso-fatty acids have been suggested as candidates for the (early) detection of SARA. Here, we first summarize the most important changes in *trans* FA and OBCFA associated with shifts in the rumen microbial population. Further, these FA in milk are evaluated as candidates to identify SARA (risk).

8.1 Rumen bacteria related to the accumulation of trans*-10 intermediates*

Situations with greater *trans*-10 accumulation are often associated with increased ruminal abundance of the lactate-utilizing bacterium *Megasphaera elsdenii* (Weimer et al., 2010; Dewanckele et al., 2018). Furthermore, this rumen species was increased during grain-induced SARA both in vitro (Mickdam et al., 2016) and in vivo (Khafipour et al., 2009; Fernando et al., 2010; Plaizier et al., 2017), which is probably linked to the higher lactate production in those situations.

Correlation analysis based on ruminal bacterial populations and milk (Pitta et al., 2018; Dewanckele et al., 2019) or rumen FA profiles (Zened et al., 2016; Dewanckele et al., 2018) revealed positive correlations of *trans*-10 intermediates with *Acidaminococcus* spp., *Bulleidia* spp., *Bifidobacterium* spp., *Carnobacterium* spp., *Desulfovibrio* spp., *Dialister* spp., *Eubacterium* spp., *Lactobacillus* spp., *Olsenella* spp., *Sharpea* spp., *Syntrophococcus* spp. and unclassified Coriobacteriaceae, Lachnospiraceae and Ruminococcaceae. Some of those genera were also increased during a SARA challenge, for example, *Bifidobacterium* (Mao et al., 2013), *Lactobacillus* (Petri et al., 2013; Mickdam et al., 2016; Plaizier et al., 2017), *Olsenella* (Petri et al., 2013; Zened et al., 2013), *Sharpea* (Petri et al., 2013; Plaizier et al., 2017) and *Syntrophococcus* (Petri et al., 2013). Additionally, the genera *Lactobacillus* and *Sharpea* are important lactate

producers in the rumen (Sharpe et al., 1973; Kamke et al., 2016), which might explain their association with SARA.

Devillard et al. (2007) and McIntosh et al. (2009) observed the formation of *trans*-10, *cis*-12 CLA from 18:2*n*-6 by *Propionibacterium freudenreichii*, a bacterial species isolated from the human intestine. In line with this, Wallace et al. (2007) found that *Propionibacterium acnes*, which was isolated from sheep rumen, converts 18:2*n*-6 to *trans*-10, *cis*-12 CLA as an end product (McKain et al., 2010; Dewanckele et al., 2017). In our laboratory, a human strain of *P. acnes* isomerized 18:3*n*-3 to *trans*-10, *cis*-12, *cis*-15 CLnA (L. Dewanckele, J. Jeyanathan, B. Vlaeminck, V. Fievez, unpublished data). *Propionibacteria* produce propionic acid, whereas some strains additionally ferment lactate (Bryant, 1959). High-grain diets might induce a shift from mainly the production of acetic acid towards increased production of propionic acid and lactate (Balch and Rowland, 1957), which might support a link between *Propionibacteria* and SARA. Nevertheless, no studies reported on an increased abundance of this genus upon a SARA challenge, which might be due to the rather low abundance of this genus in ruminal content (Kim et al., 2002; Shingfield et al., 2012).

8.2 Rumen bacteria and their odd- and branched-chain fatty acids

Microbial formation of OBCFA has been outlined in detail by Vlaeminck et al. (2006). In summary, odd-chain fatty acids (15:0 and 17:0) are formed through elongation of propionate or valerate, whereas precursors of branched-chain fatty acids (*iso* 13:0, *iso* 14:0, *iso* 15:0, *iso* 16:0, *iso* 17:0, *iso* 18:0, *anteiso* 13:0, *anteiso* 15:0, *anteiso* 17:0) are branched-chain amino acids (valine, leucine and isoleucine) and their corresponding branched short-chain carboxylic acids (*iso*butyric, *iso*valeric and 2-methyl butyric acids). The OBCFA profile of the rumen bacteria seems to be largely determined by the fatty acid synthase activity of the microorganism rather than by the precursor availability (Vlaeminck et al., 2006). Hence, variation in the OBCFA profile leaving the rumen is expected to reflect changes in the relative abundance of specific bacterial populations in the rumen rather than an altered bacterial fatty acid synthesis. Accordingly, higher proportions of *iso*-fatty acids in solid-associated bacteria were suggested to reflect their enrichment in cellulolytic bacteria, whereas higher proportions of *anteiso* 15:0 in liquid-associated bacteria might indicate their enrichment in pectin and sugar-fermenting bacteria, which seem particularly rich in this fatty acid (Table 3) (Vlaeminck et al., 2006; Bessa et al., 2009).

Detailed information on the OBCFA profile of different rumen bacteria as well as their main fermentation substrates and end products are shown in Table 3. The OBCFA profile of *Ruminococcus albus*, *Butyrivibrio fibrisolvens* and

Ruminococcus flavefaciens, which are considered to be representative rumen cellulolytic bacteria, contains relatively high proportions of even and/or odd-*iso*-fatty acids (Table 3). These fibre-digesting bacteria mainly ferment cellulose, hemicellulose and pectin to acetate, butyrate, hydrogen (H_2) and carbon dioxide (CO_2). On the other hand, the OBCFA profile of *Ruminobacter amylophilus*, *Selenomonas ruminantium*, *Streptococcus bovis* and *Succinomonas amylolytica,* considered as representative amylolytic bacteria, shows low proportions of branched-chain fatty acids, particularly *iso*-branched chains and are rich in linear odd-chain fatty acids and/or *anteiso*-branched-chain fatty acids (Table 3). The latter fatty acids seem to be of particular importance in sugar or pectin fermenting bacteria such as *Prevotella* spp., *Lachnospira multiparus* and *Succinovibrio dextrinosolvens* (Table 3). Amylolytic or starch and sugar-digesting bacteria ferment sugar, starch and peptides to propionate, butyrate, acetate, lactate, H_2 and CO_2. *Eubacterium ruminantium* and *Streptococcus bovis* are lactate producers and do not synthesize acetate. These bacterial species play an important role in the onset of acidosis. Their most important OBCFA are 15:0 and *anteiso* C15:0. Also *Megashpaera elsdennii* plays an important role (Nagaraja and Lechtenberg, 2007) with the main FA in its cell wall 15:0 (Table 3).

8.3 Rumen bacteria associated with the accumulation of trans*-10 intermediates, rich in odd-chain fatty acids and poor in iso-fatty acids linked to inflammation?*

Many of the bacteria associated with the accumulation of trans-10 intermediates, rich in odd-chain fatty acids and poor in iso-fatty acids, for example, of the genera *Acidaminococcus*, *Desulfovibrio*, *Dialister* and *Syntrophococcus* as well as the *Succinivibrionaceae* family, including the genera *Succinivibrio*, *Succinimonas* and *Ruminobacter* are gram-negative. It is well known that gram-negative bacteria contain endotoxins such as lipopolysaccharide (LPS) in the outer membrane of their cell wall, which act as immunogenic compounds in their free form (Hurley, 1995). These endotoxins are extensively shed during the logarithmic and stationary phases of bacterial growth and also released following cell disintegration and lysis (Nagaraja et al., 1978a,b; Hurley, 1995; Plaizier et al., 2012). Nevertheless, the LPS potency varies among gram-negative species, and as such, the proinflammatory response of LPS may differ between species (Ghaffari et al., 2017). Furthermore, *M. elsdenii* produces material which has many biological and chemical characteristics common to enterobacterial endotoxins (Nagaraja et al., 1979). Although its endotoxic potency is rather low (Nagaraja et al., 1979), a higher rumen concentration upon increased *M. elsenii* abundance might partially contribute to the onset of an immune response (Plaizier et al., 2008). Nevertheless, further research is required to confirm this hypothesis.

Table 3 Predominant substrate (from Harfoot and Hazlewood, 1997), fermentation end products (from Russell and Rychlik, 2001) and OBCFA profile (g/100 g fatty acids; original references in Vlaeminck et al., 2006) of some major bacteria involved in rumen carbohydrate fermentation

	Ferm. prod.[d]	*Anteiso* C13:0	*Anteiso* C15:0	*Anteiso* C17:0	*Iso* C13:0	*Iso* C15:0	*Iso* C17:0	*Iso* C14:0	*Iso* C16:0	C13:0	C15:0	C17:0	C17:1
Ruminococcus albus[a]	A	–	9.4	1.3	–	–	0.7	20.6	11.0	–	10.3	1.4	–
Butyrivibrio fibrisolvens[a]	A, B, F	6.4	16.2	8.6	6.8	10.4	5.7	10.8	11.1	2.9	7.8	4.3	3.5
Ruminococcus flavefaciens[a]	A, S	–	2.3	2.9	–	35.7	5.2	2.5	7.3	0.1	3.2	0.5	–
Succinimonas amylolytica[b] N6	A, P	–	–	–	–	52.6	10.8	1.6	5.3	1.6	5	–	–
Succinimonas amylolytica[b] B24	A, P	–	–	–	–	0.1	0.3	–	0.6	1.4	3.3	1.3	0.6
Prevotella[bc]	A, S	1.2	36.7	4.2	3.0	14.7	2.3	3.3	3.0	1.2	12.1	2.1	–
Lachnospira multiparus[bc]	A, L, F	–	4.0	2.6	–	1.1	1.1	1.2	1.8	0.3	2.9	0.8	0.1
Succinivibrio dextrinosolvens[c]	A, S	0.8	3.6	1.0	–	0.1	–	0.6	1.5	0.5	4.0	0.7	–
Ruminobacter amylophilus[b]	A, S, F	–	1.1	–	–	–	–	–	–	0.5	1.1	0.3	0.1
Fibrobacter succinogenes[a]	A, S	3.9	7.7	1.2	–	0.1	0.2	3.6	3.4	9.0	30.2	2.1	–
Streptococcus bovis[b]	L	–	0.9	–	–	–	–	0.4	0.2	0.6	1.7	1.2	0.2
Megasphaera elsdenii[c]	A, P, B	–	2.8	–	0.1	0.2	0.2	1.5	0.5	1.5	6.0	4.5	3.0
Eubacterium ruminantium[b] B1C23	B, L, F	–	–	–	–	17.7	1.4	–	–	5.4	49.0	1.5	–
Eubacterium ruminantium[b] GA195	B, L, F	–	30.1	1.7	–	0.4	0.2	6.1	3.7	0.4	6.5	0.4	–
Selenomonas ruminantium[b]	A, P, L	–	0.1	–	–	0.2	–	0.3	0.1	1.3	6.0	2.9	2.6

[a] Bacteria fermenting cellulose and hemicellulose.
[b] Bacteria fermenting starch.
[c] Bacteria fermenting sugar and pectin.
[d] Fermentation products: A: Acetate; S: Succinate; B: Butyrate; F: Formate; P: Propionate; L: Lactate.
Predominant OBCFA is underlined. Indication of main substrate is given by superscript letter.

8.4 Trans *and odd- and branched-chain fatty acids in milk fat to identify subacute ruminal acidosis (risk)*

Traditionally, subacute ruminal acidosis has been defined as a rumen condition associated with a depressed pH for several hours per day (Plaizier et al., 2008). Hence, the frequency of observations below an arbitrary pH threshold is used, for example, time spent below pH < 5.6 or 5.8 and pH < 6.0 (when using rumen pH boli residing in the reticulum). Alternatively, the area under the curve (drop below pH-threshold multiplied with the duration of this drop), either or not normalized for feed intake level to obtain an acidosis index (Gao and Oba, 2014), is used as an improved indicator of SARA severity. Some applied signal processing on the raw pH of the daily kinetic data in order to calculate relative pH indicators, which correct for inter-individual variability, sensor drift and sensor noise (Villot et al., 2018). Such relative pH indicators monitor intra-individual changes in pH values. Denwood et al. (2018) also proposed to monitor deviation, but they assessed the deviation from a predictable pattern, describing the temporal variation in rumen pH. In research from our group (Colman et al., 2012), it was proposed to describe diurnal pH variation by fitting a logistic curve, which is described by β1 (reflecting average rumen pH) and β0 (representing the rumen pH range). *Trans* and OBCFA in milk fat have been explored as biomarkers to diagnose SARA and identify cows at risk for SARA development (early warning and inter-animal susceptibility). For this, identification of SARA cases most often have been based on the daily time below a pH threshold and to a lesser extent by the acidosis index and the parameters of the logistic curve. Milk FA have not yet been linked to deviations in rumen pH from a kinetic pattern nor to SARA-related inflammatory responses.

Vlaeminck et al. (2006) suggested higher proportions of *iso*-fatty acids in milk to reflect a rumen condition associated with a lower risk for SARA, while higher proportions of *anteiso* 15:0, 15:0 and 17:0 might indicate a situation of higher risk for SARA. Indeed, increasing levels of milk fat 15:0 have been reported during SARA development (Enjalbert et al., 2008; Colman et al., 2010). This is in accordance with the recent meta-analysis of Prado et al. (2019) who reported that dietary starch and ruminal pH are associated with a positive and negative slope, respectively, in equations predicting milk yield of 15:0 (g/d) as well as 17:0 (g/d). Also *anteiso* 17:0 (g/d) was negatively correlated with rumen pH, but the determination coefficient of this equation was smaller. *Iso*-even chain fatty acids were not reported in this meta-analysis. In the study of Colman et al. (2015), combining data of six acidosis induction experiments with rumen-fistulated dairy cows, reduced amounts of *iso*-branched chain fatty acids seemed more determining than increased concentrations of 15:0 and 17:0 to distinguish SARA cases, while the inverse was true in the study of Jing et al. (2018). Nevertheless, in both papers, *trans*-10 18:1 and/or the ratio of *trans*-10

18:1 to *trans*-11 18:1 were associated with SARA (development) and/or cows at risk for SARA, although in some models of Colman et al. (2015), *trans*-10 18:1 was replaced by *cis*-9 *trans*-11 18:2 as a major discriminating variable. Furthermore, Jing et al. (2018) monitored SARA-indicative milk FA (*iso* even and odd chain FA, *anteiso* odd chain FA as well as *trans*-FA) over a 4-week period of concentrate build-up during early lactation. Next to *trans*-10 18:1 and 15:0, Δ *trans*-11 18:1, defined as the maximum decrease in the proportion of this *trans*-isomer over the 4-week period, was proposed as an additional parameter to determine inter-animal variability in SARA susceptibility. Indeed, in the work by Jing et al. (2018), milk OBCFA and trans-FA not only showed the potential to diagnose the occurrence of SARA during a SARA-induction experiment, but also to distinguish cows with relatively high or low susceptibility for SARA development within the same herd.

Further, Colman et al. (2012) attempted to distinguish milk FA associated with rumen pH level from those associated with rumen pH fluctuations as described by the logistic curve-parameters β1 and β, respectively. Based on the two experiments described in this article, milk fat proportions of *trans*-10 18:1 were suggested to be associated with situations of both low and largely fluctuating pH, whereas situations with low, stable pH did not induce a shift from the formation of mainly *trans*-11 intermediates towards increased formation of *trans*-10 intermediates. On the other hand, *trans*-11 18:1 and *cis*-9, *trans*-11 18:2 were only influenced by pH variation and not by the average pH, whereas *iso* FA depended on the average pH and were not influenced by diurnal pH variation. The relation between the *iso* FA (positive) and *trans*-10 18:1 (negative) with the average rumen pH was confirmed in the extended database of six SARA-induction experiments described in Colman's PhD dissertation (Colman, 2012). In all experiments, the rumen pH range related to the specific milk OBCFA and *trans*-FA, but the relations were equivocal, which might be related to the large variation in amplitude of the pH range between the six experiments.

A multivariate and robust model should be developed in the future to identify cows at risk for SARA. Furthermore, to be of practical relevance, these diagnostic milk FA should be determined routinely, which is currently not possible. Earlier research by our group indicated Raman spectroscopy showed the potential for the determination of individual and grouped *trans*-(mono-) UFA in milk fat (Stefanov et al., 2011), but further investment in the development of such a routine technology is required.

9 Conclusion

Particularly *trans* and odd- and branched-chain fatty acids that are generated during the ruminal metabolism are interesting potential biomarkers to diagnose impaired rumen function, to identify cows at risk for development of subacute

ruminal acidosis at an early stage, and to differentiate between animals with relatively higher or lower susceptibility for ruminal disorders. On the other hand, prevention of rumen metabolism might facilitate the production of dairy products enriched in unsaturated fatty acids for enhanced human health. Up till now, encapsulation technologies, either in a formaldehyde-protein, gelled protein or solid fat matrix, resulted in the highest diet-to-milk transfer efficiencies.

10 Where to look for further information

Some scientific literature that might be of interest to provide basic knowledge on the topics discussed above is listed here:

- Palmquist and Jenkins (2017) give a comprehensive overview on how the role of dietary fat evolved from a high-energy source to support milk fat synthesis towards the supplementation of specific LCFA to improve reproductive performance.
- The rumen lipid metabolism with emphasis on microbial lipid biosynthesis, ruminal lipolysis and biohydrogenation of dietary lipids is extensively discussed by Harfoot and Hazlewood (1997).
- The review by Toral et al. (2018b) gives an excellent overview of the recent advances on the modulation of the ruminal lipid metabolism for a healthier fatty acid profile of milk and meat.
- The potential of milk odd- and branched-chain FA as a diagnostic tool for rumen function is elaborately demonstrated in the reviews by Vlaeminck et al. (2006) and Fievez et al. (2012).

The research groups listed below are currently involved in research on rumen lipid metabolism:

- At the French National Research Institute for Agriculture, Food and Environment (INRAE), Anne Ferlay and Pierre Nozière, from the UMR Herbivore unit in Clermont-Ferrand are investigating (dietary) strategies to optimise milk FA composition in relation to human health, and are modelling digestion of dietary FA, their intermediary metabolism and transfer to milk. The latter also is done in collaboration with Philippe Schmidely from the AgroParisTech department of INRAE in Paris.
- Annabelle Meynadier and Francis Enjalbert at The National Veterinary School of Toulouse (ENVT) of INRAE are mainly involved in studying rumen lipid microbial conversions, also in relation to SARA.
- The group of Pilar de Frutos Fernandez, including Pablo Toral (Instituto de Ganaderia de Montaña, CSIC, Spain) is currently one of the leading

groups studying and elucidating the interaction between ruminal fatty acid metabolism and milk fat content and milk fatty acid composition.

- Rachel Gervais and Yvan Chouinard from the Department of Animal Sciences at the University of Laval are working on the use of milk FA composition as a biomarker and on the transfer of FA to the milk.
- The group of Adam Lock at the Dairy Lipids Nutrition Program and Laboratory of Michigan State University is well-known for its numerous studies on fatty acid digestibility and efforts on improved milk fat synthesis.
- Our group, under supervision of Veerle Fievez, has a number of publications on the potential of milk FA composition as a diagnostic tool, and is currently involved in a number of projects related to the evaluation of milk FA composition as a non-invasive biomarker for early detection of health disorders.

11 References

Anadón, A., Martínez-Larrañaga, M. R., Martínez, M. A., Ares, I., Ramos, E., Gómez-Cortés, P., Juárez, M. and de la Fuente, M. A. 2010. Acute oral safety study of dairy fat rich in *trans*-10 C18:1 versus vaccenic plus conjugated linoleic acid in rats. *Food Chem. Toxicol.* 48:591–8.

Annison, E. F., Linzell, J. L., Fazakerley, S. and Nichols, B. W. 1967. The oxidation and utilization of palmitate, stearate, oleate and acetate by the mammary gland of the fed goat in relation to their overall metabolism, and the role of plasma phospholipids and neutral lipids in milk-fat synthesis. *Biochem. J.* 102:637–47.

Annous, B. A., Becker, L. A., Bayles, D. O., Labeda, D. P. and Wilkinson, B. J. 1997. Critical role of *anteiso*-C15:0 fatty acid in the growth of *Listeria* monocytogenes at low temperatures. *Appl. Environ. Microbiol.* 63:3387–894.

Astorg, P., Arnault, N., Czernichow, S., Noisette, N., Galan, P. and Hercberg, S. 2004. Dietary intakes and food sources of *n*-6 and *n*-3 PUFA in French adult men and women. *Lipids* 39:527–35.

Bainbridge, M. L., Lock, A. L. and Kraft, J. 2015. Lipid-encapsulated echium oil (*Echium plantagineum*) increases the content of stearidonic acid in plasma lipid fractions and milk fat of dairy cows. *J. Agric. Food Chem.* 63:4827–35.

Balch, D. A. and Rowland, S. J. 1957. Volatile fatty acids and lactic acid in the rumen of dairy cows receiving a variety of diets. *Br. J. Nutr.* 11:288–98.

Bauman, D. E. and Lock, A. L. 2006. Concepts in lipid digestion and metabolism in dairy cows. *Proc. Tri-State Dairy Nutr. Conf.*, pp. 1–14.

Bernard, L., Toral, P.G. and Chilliard, Y. 2017. Comparison of mammary lipid metabolism in dairy cows and goats fed diets supplemented with starch, plant oil, or fish oil. *J. Dairy Sci.* 100:9338–51.

Bernard, L., Bonnet, M., Delavaud, C., Delosière, M., Ferlay, A., Fougère, H. and Graulet, B. 2018. Milk fat globule in ruminant: major and minor compounds, nutritional regulation and differences among species. *Eur. J. Lipid Sci. Technol.* 120:201700039.

Bessa, R. J. B., Maia, M. R. G., Jeronimo, E., Belo, A. T., Cabrita, A. R. J., Dewhurst, R. J. and Fonseca, A. J. M. 2009. Using microbial fatty acids to improve understanding of the

contriubution of solid associated bacteria to microbial mass in the rumen. *Anim. Feed Sci. Technol.* 150:197–206.

Boerman, P., Firkins, J. L., St-Pierre, N. R. and Lock, A. L. 2015. Intestinal digestibility of long-chain fatty acids in lactating dairy cows: A meta-analysis and meta-regression. *J. Dairy Sci.* 98:8889–903.

Boerman, J. P., de Souza, J. and Lock, A. L. 2017. Milk production and nutrient digestibility responses to increasing levels of stearic acid supplementation of dairy cows. *J. Dairy Sci.* 100:2729–38.

Bruss, M. L. 1997. Lipids and ketones. In: Kaneko, J. J., Harvey, J. W. and Bruss, M. L. (Eds), *Clinical Biochemistry of Domestic Animals* (5th edn.). Academic Press, San Diego, CA, pp. 83–115. Chapter 4.

Bryant, M. P. 1959. Bacterial species of the rumen. *Bacterial. Rev.* 23:125–53.

Buccioni, A., Decandia, M., Minieri, S., Molle, G. and Cabiddu, A. 2012. Lipid metabolism in the rumen: new insights on lipolysis and biohydrogenation with an emphasis on the role of endogenous plant factors. *Anim. Feed Sci. Tech.* 174:1–25.

Carroll, S. M., DePeters, E. J. and Rosenberg, M. 2006. Efficacy of a novel whey protein gel complex to increase the unsaturated fatty acid composition of bovine milk fat. *J. Dairy Sci.* 89:640–50.

Castaneda-Gutierrez, E., Benefield, B. C., de Veth, M. J., Santos, N. R., Gilbert, R. O., Butler, W. R. and Bauman, D. E. 2007a. Evaluation of the mechanism of action of conjugated linoleic acid isomers on reproduction in dairy cows. *J. Dairy Sci.* 90:4253–64.

Castaneda-Gutierrez, E., de Veth, M. J., Lock, A. L., Dwyer, D. A., Murphy, K. D. and Bauman, D. E. 2007b. Effect of supplementation with calcium salts of fish oil on *n*-3 fatty acids in milk fat. *J. Dairy Sci.* 90:4149–56.

Castro-Gómez, M. P., Rodriguez-Alcalá, L. M., Calvo, M. V., Romero, J., Mendiola, J. A., Ibañez, E. and Fontecha, J. 2014. Total milk fat extraction and quantification of polar and neutral lipids of cow, goat, and ewe milk by using a pressurized liquid system and chromatographic techniques. *J. Dairy Sci.* 97:6719–28.

Chalupa, W., Vecchiarelli, B., Elser, A. E., Kronfeld, D. S., Sklan, D. and Palmquist, D. L. 1986. Ruminal fermentation *in vivo* as influenced by long-chain fatty-acids. *J. Dairy Sci.* 69:1293–301.

Chilliard, Y., Ferlay, A., Mansbridge, R. M. and Doreau, M. 2000. Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated, *trans* and conjugated fatty acids. *Ann. Zootech.* 49:181–205.

Chouinard, P. Y., Girard, V. and Brisson, G. J. 1998. Fatty acid profile and physical properties of milk fat from cows fed calcium salts of fatty acids with varying unsaturation. *J. Dairy Sci.* 81:471–81.

Colman, E. 2012. Milk fatty acids as biomarkers of subacute ruminal acidosis in dairy cows. PhD dissertation, Ghent University, Belgium, 278p. ISBN 978-90-5989-553-9.

Colman, E., Fokkink, W. B., Craninx, M., Newbold, J.R., De Baets, B. and Fievez, V. 2010. Effect of induction of sub-acute ruminal acidosis (SARA) on milk fat profile and rumen parameters. *J. Dairy Sci.* 93:4759–73.

Colman, E., Tas, B. M., Waegeman, W., De Baets, B. and Fievez, V. 2012. The logistic curve as a tool to describe the daily ruminal pH pattern and its link with milk fatty acids. *J. Dairy Sci.* 95:5845–65.

Colman, E., Waegeman, W., De Baets, B. and Fievez, V. 2015. Prediction of subacute ruminal acidosis based on milk fatty acids: a comparison of linear discriminant and

support vector machine approaches for model development. *Comp. Electron. Agric.* 111:179–85.

Conte, G., Dimauro, C., Serra, A., Macciotta, N. P. P. and Mele, M. 2018. A canonical discriminant analysis to study the association between milk fatty acids of ruminal origin and milk fat depression in dairy cows. *J. Dairy Sci.* 101:6497–510.

Cortes, C., da Silva-Kazama, D. C., Kazama, R., Gagnon, N., Benchaar, C., Santos, G. T. D., Zeoula, L. M. and Petit, H. V. 2010. Milk composition, milk fatty acid profile, digestion, and ruminal fermentation in dairy cows fed whole flaxseed and calcium salts of flaxseed oil. *J. Dairy Sci.* 93:3146–57.

Craninx, M., Steen, A., Van Laar, H., Van Nespen, T., Martin-Tereso, J., De Baets, B. and Fievez, V. 2008. Effect of lactation stage on the odd- and branched-chain milk fatty acids of dairy cattle under grazing and indoor conditions. *J. Dairy Sci.* 91:2662–77.

Cummings, K. R. and Forrest, R. L. 1997. Aliphatic amide feed supplement for ruminants. US patent 5670191 A.

Demeyer, D. and Doreau, M. 1999. Target and procedures for altering ruminant meat and milk lipids. *P. Nutr. Soc.* 58:593–607.

Denwood, M. J., Kleen, J. L., Jensen, D. B. and Jonsson, N. N. 2018. Describing temporal variation in reticulo-ruminal pH using continuous monitoring data. *J. Dairy Sci.* 101:233–45.

Desai, K. G. and Jin Park, H. 2005. Recent developments in microencapsulation of food ingredients. *Drying Technol*. 23:1361–94.

de Veth, M. J., Griinari, J. M., Pfeiffer, A. M. and Baumann, D. E. 2004. Effect of CLA on milk fat synthesis in dairy cows: comparison of inhibition by methyl esters and free fatty acids, and relationships among studies. *Lipids* 39:365–72.

de Veth, M. J., Gulati, S. K., Luchini, N. D. and Bauman, D. E. 2005. Comparison of calcium salts and formaldehyde-protected conjugated linoleic acid in inducing milk fat depression. *J. Dairy Sci.* 88:1685–93.

Devillard, E., McIntosh, F. M., Duncan, S. H. and Wallace, R. J. 2007. Metabolism of linoleic acid by human gut bacteria: different routes for biosynthesis of conjugated linoleic acid. *J. Bacteriol.* 189:2566–70.

Dewanckele, L., Vlaeminck, B., Jeyanathan, J. and Fievez, V. 2017. Effect of pH and 22:6*n*−3 on *in vitro* biohydrogenation of 18:2*n*−6 by different ratios of *Butyrivibrio fibrisolvens* to *Propionibacterium acnes*. *J. Dairy Sci.* 100(Suppl. 2):221 (Abstract).

Dewanckele, L., Vlaeminck, B., Hernandez-Sanabria, E., Ruiz-González, A., Debruyne, S., Jeyanathan, J. and Fievez, V. 2018. Rumen biohydrogenation and microbial community changes upon early life supplementation of 22:6*n*−3 enriched microalgae to goats. *Front. Microbiol.* 9:573.

Dewanckele, L., Jing, L., Stefańska, B., Vlaeminck, B., Jeyanathan, J., Van Straalen, W. M., Koopmans, A. and Fievez, V. 2019. Distinct blood and milk 18-carbon fatty acid proportions and buccal bacterial populations in dairy cows differing in reticulorumen pH response to dietary supplementation to rapidly fermentable carbohydrates. *J. Dairy Sci.* 102:4025–40.

Dewhurst, R. J., Moorby, J. M., Vlaeminck, B., Van Nespen, T. and Fievez, V. 2007. Apparent recovery of duodenal odd- and branched-chain fatty acids in milk. *J. Dairy Sci.* 90:1775–80.

Dhiman, T. R., Nam, S. H. and Ure, A. L. 2005. Factors affecting conjugated linoleic acid content in milk and meat. *Crit. Rev. Food Sci. Nutr.* 45:463–82.

Diedrich, M. and Henschel, K. P. 1990. The natural occurrence of unusual fatty acids. Part 1. Odd numbered fatty acids. *Nahrung* 34:935–43.

Dodds, P. F., Guadalupe, M., Guzman, F., Chalberg, S. C. anderson, G. J. and Kumar, S. 1981. Acetoacetyl-CoA reductase activity of lactating bovine mammary fatty acid synthase. *J. Biol. Chem.* 256:6282–90.

Doreau, M., Troegeler-Meynadier, A., Fievez, V. and Ferlay, A. 2015. Ruminal metabolism of fatty acids: modulation of polyunsaturated, conjugated, and trans fatty acids in meat and milk. In: Watson, R. R. and De Meester, F. (Eds), *Handbook of Lipids in Human Function: Fatty Acids*. AOCS Press, San Diego, CA, pp. 521–42. Chapter 19.

Elgersma, A. 2015. Grazing increases the unsaturated fatty acid concentration of milk from grass-fed cows: a review of the contributing factors, challenges and future perspectives. *Eur. J. Lipid Sci. Technol.* 117:1345–69.

Enjalbert, F., Videau, Y., Nicot, M.C., Troegeler-Meynadier, A. 2008. Effects of induced subacute ruminal acidosis on milk fat content and milk fatty acid profile. *J. Anim. Physiol. An. N.* 92, 284–91.

Ferlay, A., Bernard, L., Meynadier, A. and Malpuech-Brugère, C. 2017. Production of *trans* and conjugated fatty acids in dairy ruminants and their putative effects on human health: a review. *Biochimie* 141:107–20.

Fernando, S. C., Purvis, H. T., Najar, F. Z., Sukharnikov, L. O., Krehbiel, C. R., Nagaraja, T. G., Roe, B. A. and DeSilva, U. 2010. Rumen microbial population dynamics during adaptation to a high-grain diet. *Appl. Environ. Microbiol.* 76:7482–90.

Field, C. J., Blewett, H. H., Proctor, S. and Vine, D. 2009. Human health benefits of vaccenic acid. *Appl. Physiol. Nutr. Metabol.* 34:979–91.

Fievez, V., Vlaeminck, B., Dhanoa, M. S. and Dewhurst, R. J. 2003. Use of principal component analysis to investigate the origin of heptadecenoic and conjugated linoleic acids in milk. *J. Dairy Sci.* 86:4047–53.

Fievez, V., Colman, E., Castro-Montoya, J.M., Stefanov, I. and Vlaeminck, B. 2012. Milk odd and branched chain fatty acids as biomarkers of rumen function – an update. *Anim. Feed Sci. Technol.* 172, 51-65.

Fotouhi, N. and Jenkins, T. C. 1992. Resistance of fatty acyl amides to degradation and hydrogenation by ruminal microorganisms. *J. Dairy Sci.* 75:1527–32.

French, E. A., Bertics, S. J. and Armentano, L. E. 2012. Rumen and milk odd- and branched-chain fatty acid proportions are minimally influenced by ruminal volatile fatty acid infusions. *J. Dairy Sci.* 95:2015–26.

Fukuda, S., Nakanishi, Y., Chikayama, E., Ohno, H., Hino, T. and Kikuchi, J. 2009. Evaluation and characterization of bacterial metabolic dynamics with a novel profiling technique, real-time metabolotyping. *PLoS ONE* 4:e4893.

Fulco, A. J. 1983. Fatty acid metabolism in bacteria. *Prog. Lipid Res.* 22:133–60.

Gadeyne, F., Van Ranst, G., Vlaeminck, B., Vossen, E., Van der Meeren, P. and Fievez, V. 2015. Protection of polyunsaturated oils against ruminal biohydrogenation and oxidation during storage using a polyphenol oxidase containing extract from red clover. *Food Chem*. 171:241–50.

Gadeyne, F., De Neve, N., Vlaeminck, B., Van Der Meeren, P. and Fievez V. 2016. Transfer to the milk of rumen bypass CLA emulsions created by potato tuber peel polyphenol oxidase. Book of abstracts *14th Euro Fed Lipid Congress*, 18–21 September, Ghent, Belgium.

Gadeyne, F., De Neve, N., Vlaeminck, B. and Fievez, V. 2017. State of the art in rumen lipid protection technologies and emerging interfacial protein cross-linking methods. *Eur. J. Lipid Sci. Technol.* 119:1600345.

Gao, X. and Oba, M. 2014. Relationship of severity of subacute ruminal acidosis to rumen fermentation, chewing activities, sorting behavior, and milk production in lactating dairy cows fed a high-grain diet. *J. Dairy Sci.* 97: 3006–16.

Gebreyowhans, S., Lu, J., Zhang, S., Pang, X., Lv, J. 2019. Dietary enrichment of milk and dairy products with n-3 fatty acids: a review. *Int. Dairy J.* 97:158–66.

Ghaffari, M. H., Khafipour, E. and Steele, M. A. 2017. Systems biology and ruminal acidosis. In: Ametaj, B. (Ed.), *Periparturient Diseases of Dairy Cows*. Springer, Cham, Switzerland, pp. 51–69.

Glasser, F., Doreau, M., Ferlay, A. and Chilliard, Y. 2007. Technical Note: Estimation of milk fatty acid yield from milk fat data. *J. Dairy Sci.* 90:2302–4.

Gómez-Cortés, P., Juárez, M. and de la Fuente, M. A. 2018. Milk fatty acids and potential health benefits: an updated vision. *Trends Food Sci. Tech.* 81:1–9.

Gozho, G. N., Plaizier, J. C., Krause, D. O., Kennedy, A. D. and Wittenberg, K. M. 2005. Subacute ruminal acidosis induces ruminal lipopolysaccharide endotoxin release and triggers an inflammatory response. *J. Dairy Sci.* 88:1399–403.

Gozho, G. N., Krause, D. O. and Plaizier, J. C. 2007. Ruminal lipopolysaccharide concentration and inflammatory response during grain induced subacute ruminal acidosis in dairy cows. *J. Dairy Sci.* 90:856–66.

Griinari, J. M., Corl, B. A., Lacy, S. H., Chouinard, P. Y., Nurmela, K. V. V. and Bauman, D. E. 2000. Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Δ9-desaturase. *J. Nutr.* 130:2285–91.

Gulati, S. K., Garg, M. R. and Scott, T. W. 2005. Rumen protected protein and fat produced from oilseeds and/or meals by formaldehyde treatment; their role in ruminant production and product quality: a review. *Aust. J. Exp. Agr.* 45:1189–203.

Gulati, S. K., McGrath, S., Wynn, P. C., Thomson, P. C. and Scott, T. W. 2006. Rumen protected fat reverses the conjugated linoleic acid induced low milk fat content in dairy cows. *Canadian J. Anim. Sci.* 86:63–70.

Harfoot, C. G. and Hazlewood, G. P. 1997. Lipid metabolism in the rumen. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem*. Springer, Dordrecht, the Netherlands, pp. 382–426. Chapter 9.

Heguy, J. M., Juchem, S. O., DePeters, E. J., Rosenberg, M., Santos, J. E. P. and Taylor, S. J. 2006. Whey protein gel composites of soybean and linseed oils as a dietary method to modify the unsaturated fatty acid composition of milk lipids. *Anim. Feed Sci. Tech.* 131:370–88.

Honkanen, A. M., Leskinen, H., Toivonen, V., McKain, N., Wallace, R. J. and Shingfield, K. J. 2016. Metabolism of α-linolenic acid during incubations with strained bovine rumen contents: products and mechanisms. *Br. J. Nutr.* 115:2093–105.

Hulshof, K. F. A. M., van Erp-Baart, M. A., Anttolainen, M., Becker, W., Church, S. M., Couet, C., Hermann-Kunz, E., Kesteloot, H., Leth, T., Martins, I., Moreiras, O., Moschandreas, J., Pizzoferrato, L., Rimestad, A. H., Thorgeirsdottir, H., van Amelsvoort, J. M. N., Aro, A., Kafatos, A. G., Lanzmann-Petithory, D. and van Poppel, G. 1999. Intake of fatty acids in Western Europe with emphasis on *trans* fatty acids: the TRANSFAIR study. *Eur. J. Clin. Nutr.* 53:143–57.

Hurley, J. C. 1995. Endotoxemia: methods of detection and clinical correlates. *Clin. Microbial. Rev.* 8:268–92.

Huws, S. A., Kim, E. J., Lee, M. R. F., Scott, M. B., Tweed, J. K. S., Pinloche, E., Wallace, R. J. and Scollan, N. D. 2011. As yet uncultured bacteria phylogenetically classified as *Prevotella, Lachnospiraceae* incertae sedis and unclassified *Bacteroidales, Clostridiales* and *Ruminococcaceae* may play a predominant role in ruminal biohydrogenation. *Environ. Microbiol.* 13:1500–12.

Jaudszus, A., Jahreis, G., Schlörmann W., Fischer J., Kramer R., Degen, C., Rohrer C., Roth A., Gabriel H., Barz D. and Gruen, G. 2012. Vaccenic acid-mediated reduction in cytokine production is independent of *c*9,*t*11-CLA in human peripheral blood mononuclear cells. *Biochim. Biophys. Acta.* 1821:1316–22.

Jenkins, T. C. 1996. Feed supplements for ruminants and method for using same. US patent 5547686 A.

Jenkins, T. C. and Bridges, W. C. 2007. Protection of fatty acids against ruminal biohydrogenation in cattle. *Eur. J. Lipid Sci. Tech.* 109:778–89.

Jenkins, T. C., Wallace, R. J., Moate, P. J. and Mosley, E. E. 2008. Board-invited review: Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. *J. Anim. Sci.* 86:397–412.

Jensen, R. G. 2002. The composition of bovine milk lipids: January 1995 to December 2000. *J. Dairy Sci.* 85:295–350.

Jing, L., Dewanckele, L., Vlaeminck, B., Van Straalen, W. M., Koopmans, A. and Fievez, V. 2018. Susceptibility of dairy cows to subacute ruminal acidosis is reflected in milk fatty acid proportions, with C18:1 *trans*-10 as primary and C15:0 and C18:1 *trans*-11 as secondary indicators. *J. Dairy Sci.* 101:1–14.

Jorjong, S., van Knegsel, A. T. M., Verwaeren, J., Val Lahoz, M., Bruckmaier, R. M., De Baets, B., Kemp, B. and Fievez, V. 2014. Milk fatty acids as possible biomarkers to early diagnose elevated concentrations of blood plasma nonesterified fatty acids in dairy cows. *J. Dairy Sci.* 97:7054–64.

Kairenius, P., Leskinen, H., Toivonen, V., Muetzel, S., Ahvenjärvi, S., Vanhatalo, A., Huhtanen, P., Wallace, R. J. and Shingfield, K. J. 2017. Effect of dietary fish oil supplements alone or in combination with sunflower and linseed oil on ruminal lipid metabolism and bacterial populations in lactating cows. *J. Dairy Sci.* 101:3021–35.

Kamke, J., Kittelmann, S., Soni, P., Li, Y., Tavendale, M., Ganesh, S., Janssen, P. H., Shi, W., Froula, J., Rubin, E. M. and Attwood, G. T. 2016. Rumen metagenome and metatranscriptome analysis of low methane yield sheep reveals a *Sharpea*-enriched microbiome characterised by lactic acid formation and utilisation. *Microbiome* 4:56.

Kaneda, T. 1977. Fatty acids of the genus *Bacillus*: an example of branched-chain preference. *Bacteriol. Rev.* 41:391–418.

Kaneda, T. 1991. *Iso*- and *anteiso*-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. *Microbiol. Rev.* 55:288–302.

Keeney, M., Katz, I. and Allison, M. J. 1962. On the probable origin of some milk fat acids in rumen microbial lipids. *J. Am. Oil Chem. Soc.* 39:198–201.

Kepler, C. R., Hirons, K. P., McNeill, J. J. and Tove, S. B. 1966. Intermediates and products of the biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*. *J. Biol. Chem.* 241:1350–4.

Keweloh, H. and Heipieper, H. J. 1996. *Trans* unsaturated fatty acids in bacteria. *Lipids* 31:129–37.

Khafipour, E., Li, S., Plaizier, J. C. and Krause, D. O. 2009. Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. *Appl. Environ. Microbiol.* 75:7115–24.

Kim, Y. J., Liu, R. H., Rychlik, J. L. and Russell, J. B. 2002. The enrichment of a ruminal bacterium (*Megasphaera elsdenii* YJ-4) that produces the *trans*-10, *cis*-12 isomer of conjugated linoleic acid. *J. Appl. Microbiol.* 92:976–82.

Kim, J. H., Kim, Y., Kim, Y. J. and Park, Y. 2016. Conjugated linoleic acid: potential health benefits as a functional food ingredient. *Annu. Rev. Food Sci. Technol.* 7:221–44.

Kliem, K. E. and Shingfield, K. J. 2016. Manipulation of milk fatty acid composition in lactating cows: Opportunities and challenges. *Eur. J. Lipid Sci. Technol.* 118:1661–83.

Kliem, K. E., Humphries, D. J., Grandison, A. S., Morgan, R., Livingstone, K. M., Givens, D. I. and Reynolds C. K. 2016. Effect of whey protein and rapeseed oil gel feed supplement on milk fatty acid composition of Holstein cows. *J. Dairy Sci.* 102:288–300.

Kuhnt, K., Kraft, J., Moeckel, P. and Jahreis, G. 2006. *Trans*-11-18:1 is effectively Δ9-desaturated compared with *trans*-12-18:1 in humans. *Br. J. Nutr.* 95:752–61.

Kuhnt, K., Degen, C. and Jahreis, G. 2016. Evaluation of the impact of ruminant *trans* fatty acids on human health: Important aspects to consider. *Crit. Rev. Food Sci. Nutr.* 56:1964–80.

Lock, A. L. and Bauman, D. E. 2004. Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. *Lipids* 39:12.

Loor, J. J., Herbein, J. H. and Jenkins, T. C. 2002a. Nutrient digestion, biohydrogenation, and fatty acid profiles in blood plasma and milk fat from lactating Holstein cows fed canola oil or canolamide. *Anim. Feed Sci. Tech.* 97:65–82.

Loor, J. J., Quinlan, L. E. and Herbein, J. H. 2002b. Distribution of *trans*-vaccenic acid and *cis*9, *trans*11-conjugated linoleic acid (rumenic acid) in blood plasma lipid fractions and secretion into milk of Jersey cows fed canola or soybean oil. *Anim. Res.* 51:119–34.

Lorenzon, M. 2015. Product based on conjugated linoleic acid and a method for the manufacture thereof. US patent 9034385 B2.

Lourenço, M., Ramos-Morales, E. and Wallace, R. J. 2010. The role of microbes in rumen lipolysis and biohydrogenation and their manipulation. *Animal* 4:1008–23.

Lundy, F. P., Block, E., Bridges, W. C., Bertrand, J. A. and Jenkins, T. C. 2004. Ruminal biohydrogenation in Holstein cows fed soybean fatty acids as amides or calcium salts. *J. Dairy Sci.* 87:1038–46.

Maia, M. R. G., Chaudhary, L. C., Figueres, L. and Wallace, R. J. 2007. Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen. *Anton. Leeuw.* 91:303–14.

Maia, M. R. G., Chaudhary, L. C., Bestwick, C. S., Richardson, A. J., McKain, N., Larson, T. R., Graham, I. A. and Wallace, R. J. 2010. Toxicity of unsaturated fatty acids to the biohydrogenating ruminal bacterium, *Butyrivibrio fibrisolvens*. *BMC Microbiol*. 10:52.

Mao, S. Y., Zhang, R. Y., Wang, D. S. and Zhu, W. Y. 2013. Impact of subacute ruminal acidosis (SARA) adaptation on rumen microbiota in dairy cattle using pyrosequencing. *Anaerobe* 24:12–19.

Massart-Leën, A. M., Roets, E., Peeters, G. and Verbeke, R. 1983. Propionate for fatty acid synthesis by the mammary gland of the lactating goat. *J. Dairy Sci.* 66:1445–54.

Maxin, G., Glasser, F., Hurtaud, C., Peyraud, J. L. and Rulquin, H. 2011. Combined effects of *trans*-10,*cis*-12 conjugated linoleic acid, propionate, and acetate on milk fat yield and composition in dairy cows. *J. Dairy Sci.* 94:2051–9.

McIntosh, F. M., Shingfield, K. J., Devillard, E., Russell, W. R. and Wallace, R. J. 2009. Mechanism of conjugated linoleic acid and vaccenic acid formation in human faecal suspensions and pure cultures of intestinal bacteria. *Microbiol*. 155:285–94.

McKain, N., Shingfield, K. J. and Wallace, R. J. 2010. Metabolism of conjugated linoleic acids and 18:1 fatty acids by ruminal bacteria: products and mechanisms. *Microbiol*. 156:579–88.

Meade, T. L., Plakias, R. C. and Auth, J. C. 1999. Rumen by-pass feed supplement. US patent 5928687 A.

Meyer, B. J., Mann, N. J., Lewis, J. L., Milligan, G. C., Sinclair, A. J. and Howe, P. R. C. 2003. Dietary intakes and food sources of omega-6 and omega-3 polyunsaturated fatty acids. *Lipids* 38:391–8.

Mickdam, E., Khiaosa-ard, R., Metzler-Zebeli, B. U., Klevenhusen, F., Chizzola, R. and Zebeli, Q. 2016. Rumen microbial abundance and fermentation profile during severe subacute ruminal acidosis and its modulation by plant derived alkaloids *in vitro*. *Anaerobe* 39:4–13.

Moallem, U., Lehrer, H., Zachut, M., Livshitz, L. and Yacoby, S. 2010. Production performance and pattern of milk fat depression of high-yielding dairy cows supplemented with encapsulated conjugated linoleic acid. *Animal* 4:641–52.

Moore, J. H. and Christie, W. W. 1981. Lipid metabolism in the mammary gland of ruminant animals. In: Christie, W.W. (Ed.), *Lipid Metabolism in Ruminant Animals*. Pergamon Press, Oxford, UK, pp. 227–77.

Nagaraja, T. G. and Lechtenberg, K. F. 2007. Acidosis in feedlot cattle. *Vet. Clin. Food Anim.* 23:333–50.

Nagaraja, T. G., Bartley, E. E., Fina, L. R., Anthony, H. D. and Bechtle, R. M. 1978a. Evidence of endotoxins in the rumen bacteria of cattle fed hay or grain. *J. Anim. Sci.* 47:226–34.

Nagaraja, T. G., Bartley, E. E., Fina, L. R., Anthony, H. D., Dennis, S. M. and Bechtle, R. M. 1978b. Quantitation of endotoxin in cell-free rumen fluid of cattle. *J. Anim. Sci.* 46:759–1767.

Nagaraja, T. G., Fina, L. R., Lassman, B. A., Bartley, E. E., Anthony, H. D., Sapienza, D. A. and Brent, B. E. 1979. Characterization of endotoxin from the rumen bacterium *Megasphaera elsdenii*. *Am. J. Vet. Res.* 40:35–9.

Noble, R. C. 1978. Digestion, absorption and transport of lipids in ruminant animals. *Prog. Lipid Res.* 17:55–91.

Odens, L. J., Burgos, R., Innocenti, M., VanBaale, M. J. and Baumgard, L. H. 2007. Effects of varying doses of supplemental conjugated linoleic acid on production and energetic variables during the transition period. *J. Dairy Sci.* 90:293–305.

Palmquist, D. L. and Jenkins, T. C. 1987. Process for feeding ruminant animals and composition for use therein. US patent 4642317 A.

Palmquist, D. L. and Jenkins, T. C. 2017. A 100-year review: fat feeding of dairy cows. *J. Dairy Sci.* 100:10061–77.

Prado, L. A., Schmidely, Ph., Nozière, P. and Ferlay, A. 2019. Milk saturated fatty acids, odd- and branched-chain fatty acids, and isomers of C18:1, C18:2, and C18:3n -3 according to their duodenal flows in dairy cows: a meta-analysis approach. *J. Dairy Sci.* 102:3053–70.

Pappritz, J., Lebzien, P., Meyer, U., Jahreis, G., Kramer, R., Flachowsky, G. and Danicke, S. 2011. Duodenal availability of conjugated linoleic acids after supplementation to dairy cow diets. *Eur. J. Lipid Sci. Technol.* 113:1443–55.

Perfield, J. W., Lock, A. L., Pfeiffer, A. M. and Bauman, D. E. 2004. Effects of amide-protected and lipid-encapsulated conjugated linoleic acid (CLA) supplements on milk fat synthesis. *J. Dairy Sci.* 87:3010–16.

Petri, R. M., Schwaiger, T., Penner, G. B., Beauchemin, K. A., Forster, R. J., McKinnon, J. J. and McAllister, T. A. 2013. Changes in the rumen epimural bacterial diversity of beef cattle as affected by diet and induced ruminal acidosis. *Appl. Environ. Microbiol.* 79:3744–55.

Pitta, D. W., Indugu, N., Vecchiarelli, B., Rico, D. E. and Harvatine, K. J. 2018. Alterations in ruminal bacterial populations at induction and recovery from diet-induced milk fat depression in dairy cows. *J. Dairy Sci.* 101:295–309.

Plaizier, J. C., Krause, D. O., Gozho, G. N. and McBride, B. W. 2008. Subacute ruminal acidosis in dairy cows: the physiological causes, incidence and consequences. *Vet. J.* 176:21–31.

Plaizier, J. C., Khafipour, E., Li, S., Gozho, G. N. and Krause, D. O. 2012. Subacute ruminal acidosis (SARA), endotoxins and health consequences. *Anim. Feed Sci. Tech.* 172: 9–21.

Plaizier, J. C., Li, S., Tun, H. M. and Khafipour, E. 2017. Nutritional models of experimentally-induced subacute ruminal acidosis (SARA) differ in their impact on rumen and hindgut bacterial communities in dairy cows. *Front. Microbiol.* 7:2128.

Prom, C. M. and Lock, A. L. 2019. Abomasal infusion of different exogenous emulsifiers alters fatty acid digestibility and milk fat yield of lactating dairy cows. *J. Dairy Sci.* 102(Suppl. 1):318 (Abstract).

Rico, J. E., de Souza, J., Allen, M. S. and Lock, A. L. 2017. Nutrient digestibility and milk production responses to increasing levels of palmitic acid supplementation vary in cows receiving diets with or without whole cottonseed. *J. Anim. Sci.* 95:436–46.

Rigout, S., Hurtaud, C., Lemosquet, S., Bach, A. and Rulquin, H. 2003. Lactational effect of propionic acid and duodenal glucose in cows. *J. Dairy Sci.* 86:243–53.

Rosenberg, M. and DePeters, E. J. 2010. Method and compositions for preparing and delivering rumen protected lipids, other nutrients and medicaments. US patent 7700127 B2.

Roy, A., Chardigny, J. M., Bauchart, D., Ferlay, A., Lorenz, S., Durand, D., Gruffat, D., Faulconnier, Y., Sébédio, J. L. and Chilliard, Y. 2007. Butters rich either in *trans*-10-C18:1 or in *trans*-11-C18:1 plus *cis*-9, *trans*-11 CLA differentially affect plasma lipids and aortic fatty streak in experimental atherosclerosis in rabbits. *Animal* 1:467–76.

Russell, J. B. and Rychlik, J. L. 2001. Factors That Alter Rumen Microbial Ecology. *Science* 292:1119–22. https://doi.org/10.1126/science.1058830.

Schmidely, P. Glasser, F., Doreau, M. and Sauvant, D. 2008. Digestion of fatty acids in ruminants: a meta-analysis of flows and variation factors. 1. Total fatty acids. *Animal* 2:677–90.

Schwarz, F. J., Lierman, T., Möcker, P., Pfeiffer, A. M. and Jahreis, G. 2009. Performance, metabolic parameters and fatty acid composition of milk fat due to dietary CLA and rumen protected fat of dairy cows. Book of abstracts *No. 15 of the 60th Annual Meeting of the European Association for Animal Production*, 24–27 August 2009, Wageningen Academic Pubishers, Barcelona, Spain, p. 351.

Scott, T. W. and Hills, G. D. L. 1975. Feed supplements for ruminants comprising lipid encapsulated with protein-aldehyde reaction product. US patent 3925560 A.

Sharp, M. E., Latham, M. J., Garvie, E. I., Zirngibl, J. and Kandler, O. 1973. Two new species of *Lactobacillus* isolated from the bovine rumen, *Lactobacillus ruminis* sp.nov. and *Lactobacillus vitulinus* sp. nov. *J. Gen. Microbiol.* 77:37–49.

Shennan, D. B. and Peaker, M. 2000. Transport of milk constituents by the mammary gland. *Physiol. Rev.* 80:925–51.

Shingfield, K. J. and Griinari, J. M. 2007. Role of biohydrogenation intermediates in milk fat depression. *Eur. J. Lipid Sci. Technol.* 109:799–816.

Shingfield, K. J. and Wallace, R. J. 2014. Synthesis of conjugated linoleic acid in ruminants and humans. In: Sels, B. and Philippaerts, A. (Eds), *Conjugated Linoleic Acids and Conjugated Vegetable Oils*. Royal Society of Chemistry (RSC) Publishing, pp. 1–65. Chapter 1.

Shingfield, K. J., Kairenius, P., Ärölä, A., Paillard, D., Muetzel, S., Ahvenjärvi, S., Vanhatalo, A., Huhtanen, P., Toivonen, V., Griinari, J. M. and Wallace, R. J. 2012. Dietary fish oil supplements modify ruminal biohydrogenation, alter the flow of fatty acids at the omasum, and induce changes in the ruminal *Butyrivibrio* population in lactating cows. *J. Nutr.* 142:1437–48.

Shingfield, K. J., Bonnet, M. and Scollan, N. D. 2013. Recent developments in altering the fatty acid composition of ruminant-derived foods. *Animal* 7:132–62.

Simopoulos, A. P. 2001. *n*−3 fatty acids and human health: Defining strategies for public policy. *Lipids* 36:S83–9.

Sioen, I. A., Pynaert, H., Matthys, C., De Backer, G., Van Camp, J. and De Henauw, S. 2006. Dietary intakes and food sources of fatty acids for Belgian women, focused on *n*-6 and *n*-3 polyunsaturated fatty acids. *Lipids* 41:415.

Stamey, J. A., Shepherd, D. M., de Veth, M. J. and Corl, B. A. 2012. Use of algae or algal oil rich in *n*-3 fatty acids as a feed supplement for dairy cattle. *J. Dairy Sci.* 95:5269–75.

Stefanov, I., Baeten, V., Abbas, O., Colman, E., Vlaeminck, B., De Baets, B. and Fievez, V. 2011. Determining milk isolated and conjugated *trans* unsaturated fatty acids using Fourier transform Raman spectroscopy. *J. Agric. Food Chem.* 59:12771–83.

Sterk, A., Vlaeminck, B., van Vuuren, A. M., Hendriks, W. H. and Dijkstra, J. 2012. Effects of feeding different linseed sources on omasal fatty acid flows and fatty acid profiles of plasma and milk fat in lactating dairy cows. *J. Dairy Sci.* 95:3149–65.

Sukhija, P. S. and Palmquist, D. L. 1990. Dissociation of calcium soaps of long-chain fatty-acids in rumen fluid. *J. Dairy Sci.* 73:1784–7.

Sultana, H., Ishida, T., Shintaku, T., Kanda, S. and Itabashi, H. 2008 Effect of feeding Ca-salts of fatty acids from soybean oil and linseed oil on *c*9,*t*11-CLA production in ruminal fluid and milk of Holstein dairy cows. *Asian Austral. J. Anim.* 21:1262–70.

Theurer, M. L., Block, E., Sanchez, W. K. and McGuire, M. A. 2009. Calcium salts of polyunsaturated fatty acids deliver more essential fatty acids to the lactating dairy cow. *J. Dairy Sci.* 92:2051–6.

Toral, P. G., Bernard, L., Belenguer, A., Rouel, J., Hervás, G., Chilliard, Y. and Frutos, P. 2016. Comparison of ruminal lipid metabolism in dairy cows and goats fed diets supplemented with starch, plant oil, or fish oil. *J. Dairy Sci.* 99:301–16.

Toral, P. G., Hervás, G., Peiró, V. and Frutos, P. 2018a. Conditions associated with marine lipid-induced milk fat depression in sheep cause shifts in the *in vitro* ruminal metabolism of 1-^{13}C oleic acid. *Animals* 8:196.

Toral, P. G., Monahan, F. J., Hervas, G., Frutos, P. and Moloney, A. P. 2018b. Review: Modulating ruminal lipid metabolism to improve the fatty acid composition of meat and milk. challenges and opportunities. *Animal* 12:S272–81.

Toral, P. G., Hervás, G. and Frutos, P. 2019. *In vitro* biohydrogenation of ^{13}C-labeled α-linolenic acid in response to ruminal alterations associated with diet-induced milk fat depression in ewes. *J. Dairy Sci.* 102:1213–23.

Tyburczy, C., Major, C., Lock, A. L., Destaillats, F., Lawrence, P., Brenna, J. T., Salter, A. M. and Bauman, D. E. 2009. Individual *trans* octadecenoic acids and partially hydrogenated vegetable oil differentially affect hepatic lipid and lipoprotein metabolism in golden Syrian hamsters. *J. Nutr.* 139:257–63.

Van Nevel, C. J. and Demeyer, D. I. 1996. Effect of pH on biohydrogenation of polyunsaturated fatty acids and their Ca-salts by rumen microorganisms *in vitro*. *Arch. Tierernahr.* 49:151–7.

van Valenberg, H. J. F., Hettinga, K. A., Dijkstra, J., Bovenhuis, H. and Feskens, E. J. M. 2013. Concentrations of *n*-3 and *n*-6 fatty acids in Dutch bovine milk fat and their contribution to human dietary intake. *J. Dairy Sci.* 96:4173–81.

van Vuuren, A. M., van Wikselaar, P. G., van Riel, J. W., Klop, A. and Bastiaans, J. A. H. P. 2010. Persistency of the effect of long-term administration of a whey protein gel composite of soybean and linseed oils on performance and milk fatty acid composition of dairy cows. *Livest. Sci.* 129:213–22.

van Wijlen, R. P. J. and Colombani, P. C. 2010. Grass-based ruminant production methods and human bioconversion of vaccenic acid with estimations of maximal dietary intake of conjugated linoleic acids. *Int. Dairy J.* 20:433–48.

Verbeke, R., Lauryssens, M., Peeters, G. and James, A. T. 1959. Incorporation of DL-[1-^{14}C] leucine and [1-^{14}C] *iso*valeric acid into milk constituents by the perfused cow's udder. *Biochem. J.* 73:24–9.

Villot, C., Meunier, B. Bodin, J. Martin, C. and Silberberg, M. 2018. Relative reticulo rumen pH indicators for subacute ruminal acidosis detection in dairy cows. *Animal* 12: 481–90.

Vlaeminck, B., Fievez, V., Cabrita, A. R. J., Fonseca, A. J. M. and Dewhurst, R. J. 2006. Factors affecting odd- and branched-chain fatty acids in milk: a review. *Anim. Feed Sci. Technol.* 131:389–417.

Vlaeminck, B., Gervais, R., Rahman, M. M., Gadeyne, F., Gorniak, M., Doreau, M. and Fievez, V. 2015. Postruminal synthesis modifies the odd- and branched chain fatty acid profile from the duodenum to milk. *J. Dairy Sci.* 98:4829–40.

Wall, R., Ross, R. P., Fitzgerald, G. F. and Stanton, C. 2010. Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. *Nutr. Rev.* 68:280–9.

Wallace, R. J., McKain, N., Shingfield, K. J. and Devillard, E. 2007. Isomers of conjugated linoleic acids are synthesized via different mechanisms in ruminal digesta and bacteria. *J. Lipid Res.* 48:2247–54.

Weimer, P. J., Stevenson, D. M. and Mertens, D. R. 2010. Shifts in bacterial community composition in the rumen of lactating dairy cows under milk fat-depressing conditions. *J. Dairy Sci.* 93:265–78.

Weinstein, J. A., Taylor, S. J., Rosenberg, M. and DePeters, E. J. 2015. Whey protein gel composites in the diet of goats increased the omega-3 and omega-6 content of milk fat. *J. Anim. Physiol. An. N.* 100:789–800.

Wu, S. H. W. and Papas, A. 1997. Rumen-stable delivery systems. *Adv. Drug Deliver. Rev.* 28:323–34.

Zened, A., Combes, S., Cauquil, L., Mariette, J., Klopp, C., Bouchez, O., Troegeler-Meynadier, A. and Enjalbert, F. 2013. Microbial ecology of the rumen evaluated by

454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets. *FEMS Microbiol. Ecol.* 83:504–14.

Zened, A., Meynadier, A., Cauquil, L., Mariette, J., Klopp, C., Dejean, S., Gonzalez, I., Bouchez, O., Enjalbert, F. and Combes, S. 2016. *Trans*-11 to *trans*-10 shift of ruminal biohydrogenation of fatty acids is linked to changes in rumen microbiota. Abstr. P-202 (p. 150). *Gut Microbiology 2016: Proceedings of the 10th Joint Symposium on Gut Microbiology*, 20–23 June 2016, Clermont-Ferrand, France.

Zhu, H., Fievez, V., Mao, S., He, W. and Zhu, W. 2016. Dose and time response of ruminally infused algae on rumen fermentation characteristics, biohydrogenation and *Butyrivibrio* group bacteria in goats. *J. Anim. Sci. Biotechnol.* 7:22.

Chapter 15

Nutritional factors affecting greenhouse gas production from ruminants: implications for enteric and manure emissions

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1 Introduction

Animal agriculture has been identified as one of the major sources of greenhouse gases (GHGs), accounting for approximately 40% of total agricultural-related emissions (IPCC, 2006) (Fig. 1). Animal production and manure management comprises 26.8% and 31.0%, respectively, of the 7.1 Gt of CO_2-eq that the livestock sector is estimated to produce annually (Gerber et al., 2013). The two central GHGs emitted directly from animal agriculture include methane (CH_4) and nitrous oxide (N_2O) which have 28 and 298 times the global warming potential of CO_2, respectively (Gerber et al., 2013). Livestock CH_4 and N_2O emissions have been estimated to contribute 40% and 48% of livestock sector emissions and ruminants account for 80% of the total livestock sector's emissions (Opio et al., 2013). Enteric fermentation and manure-related CH_4 contribute 82% and 18% of CH_4 related to livestock production, respectively. The main sources of N_2O emissions arise from chemical fertilisers, applied

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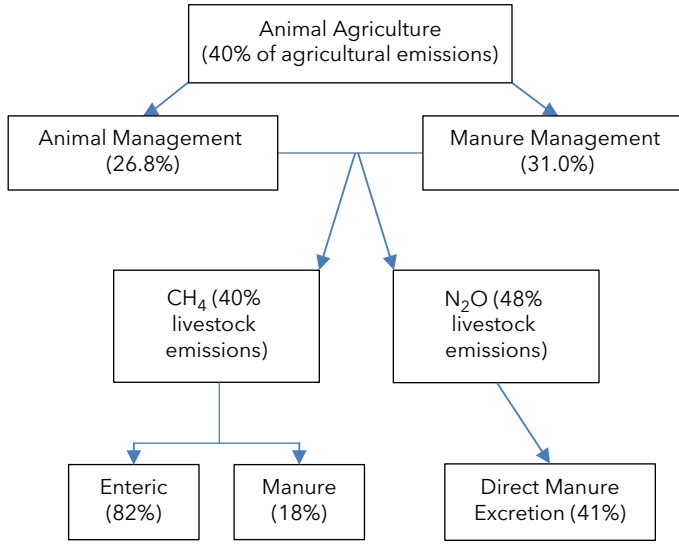

Figure 1 Greenhouse gases associated with animal agriculture.

manure and N deposition from housed animals and manure storage (Adler et al., 2015).

1.1 Greenhouse gas production

Ruminants produce CH_4 as a natural by-product of microbial fermentation, with the biochemical pathways being well documented (Huws et al., 2018). Starches, cell wall polymers and proteins are fermented by a consortium of rumen microbiota into simple sugars and carbon skeletons. Both primary and secondary fermenters convert these products, under anaerobic conditions, into volatile fatty acids (VFA), CO_2 and metabolic hydrogen [H]. Rumen ciliates and anaerobic fungi are two groups of eukaryotes which produce large volumes of [H] and share a commensal relationship with archaea (Guyader et al., 2014). Both protozoa and fungi contain hydrogenosomes which are specialised organelles that are responsible for the conversion of the intermediates of monosaccharide fermentation into [H]. Methanogens play an important role in maintaining a low partial pressure of [H], favouring hydrogenase activity within hydrogenosomes. During fermentation, the reduced co-factors NADH, NADPH and FADH are oxidised and the released [H] is transferred to methanogenic archaea through a series of biochemical pathways to reduce CO_2 to CH_4 (Ungerfeld, 2015b).

Manure CH_4, like enteric CH_4, is produced during anaerobic decomposition of organic matter (OM). Manure is also a significant emitter of N_2O, ammonia

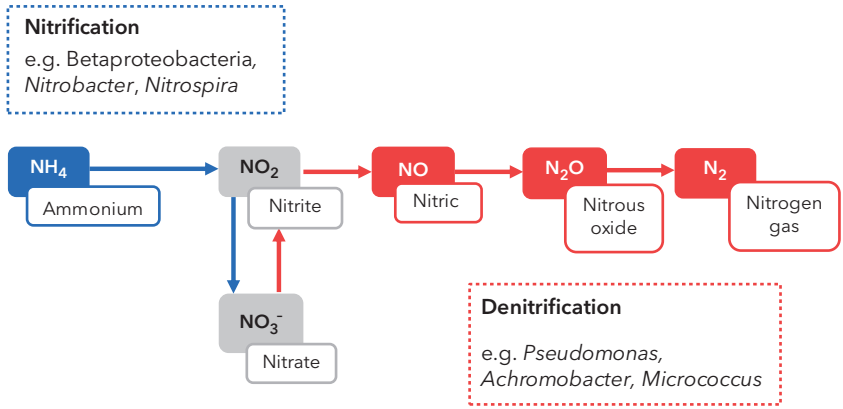

Figure 2 Process of nitrification and denitrification via nitrite pathway. N_2O is a greenhouse gas with a global warming potential that is 298 times that of CO_2.

(NH_3) and nitrous oxides (NO_x). These gases may act as direct or indirect sources of GHGs and environmental pollutants. Factors which influence the concentration of N_2O, NH_3 and NO_x include (i) the type of feed, (ii) manure nutrient profile and (iii) the handling and storage of manure. The conversion of N into gases occurs through the simultaneous nitrification and denitrification process (Fig. 2). Nitrification occurs by both NH_3 oxidising bacteria (i.e. Betaproteobacteria or *Thaumarchaeota* NH_3-oxidizing archaea) and nitrite (NO_2^-) oxidising bacteria (i.e. Alphaproteobacteria *Nitrobacter* and *Nitrospira*). Denitrifying bacteria are phylogenetically diverse and have specific genes coding for their catalytic enzymes (Maeda et al., 2011).

Although not a direct source of GHG, NH_3 emissions from manure should also be considered when assessing the impact of feed additives on air quality. Ammonia arises from the rapid hydrolysis of urea in urine and can also be a precursor to N_2O. Ammonia is highly volatile and can have serious implications for human health when threshold limits of 25–35 ppm are exceeded (National Research Council, 2008). Additionally, dry or wet NH_3 deposition may contribute to soil acidity and eutrophication of surface water (Hünerberg et al., 2013a). Shifting the excretion of N from urine to faeces may be more environmentally beneficial as faecal N is considered a slow-release form of N that is more likely to be captured by soil flora.

1.2 Balancing enteric methane production and manure emissions

Significant interrelationships exist between enteric CH_4 production and both CH_4 and N_2O manure emissions (Knapp et al., 2014). Balancing net emissions produced directly from the rumen or indirectly from manure is challenging.

	ENTERIC							MANURE					OVERALL	
DIETARY CHANGES	Fermentation	Fibre Digestion	N Digestion	Starch Digestion	pH	CH_4 PRODUCTION DAILY	CH_4 INTENSITY	Starch	Urine N	Faecal N	CH_4 EMISSIONS	N_2O EMISSIONS	DECREASE IN GHG PRODUCTION	DECREASE IN GHG INTENSITY
Concentrate/Forage														
Increased Concentrate/Forage	↑	↓	NA	↑	↓	~	↓	↑	NA	NA	↑	↑	~	✓
Acidosis	↑	↓	NA	↑	↓	~	-	~	NA	NA	~	~	~	~
High Forage	↓	↑	NA	↓	↑	↑	↑	↓	NA	NA	↓	↓	~	×
Nitrogen														
DDGS	~	↑	↑	~	~	↓	↓	↑	↑	↑	↑	↑	×	×
Fat														
< 6%	~	-	NA	-	~	↓	↓	-	NA	NA	-	-	✓	✓
> 6%	↓	↓	NA	~	↑	↓	-	↑	NA	NA	↑	~	✓	×
Inhibitors														
Nitrate	~	-	-	~	-	↓	↓	~	~	~	~	~	✓	✓
3NOP	~	-	-	-	~	↓	↓	-	~	~	-	-	✓	✓
PSC														
Tannins	↓	↓	↓	NA	NA	~	~	~	↓	↑	↓	↓	✓	~

Figure 3 Consequences of dietary manipulation on enteric production and greenhouse gas emissions. Symbols indicate: ↑ = increase, ↓ = decrease, - = no change, NA = not applicable, ~ = variable/unknown.

Another important consideration when evaluating dietary manipulation strategies is accounting for the difference in GHG production and GHG intensity. Although global GHG emissions from ruminants have decreased as a factor of animal product (intensity), the total production of GHG has increased, and will continue to do so as the world's domestic ruminant population is projected to increase from 3.2 to 5.3 billion by 2050 (Turk, 2016).

A standard feedlot diet fed to cattle may result in a higher CH_4 production (g/d) and a reduced CH_4 intensity (g/kg consumable product) than those grazing on pasture (low CH_4 production and high CH_4 intensity). However, due to low dietary energy content, pasture-raised ruminants produce manure with half the CH_4 yield potential of those raised in feedlots (Koneswaran and Nierenberg, 2008), as pasture-raised animals have much lower starch levels in manure (Hales et al., 2013). Additionally, dietary alterations which result in a shift of fermentation from the rumen to the hindgut may decrease enteric CH_4 production, but not change overall net GHG emissions. This concept is also known as pollution swapping, in which an alteration in the production of one GHG results in an upstream or downstream change in the emissions of the same or another GHG (Hristov et al., 2013). Nutritional strategies that alter diet digestibility through increasing dietary fermentable carbohydrates, N and fat content can all result in pollution swapping (Fig. 3).

1.2.1 Diet digestibility and fermentable carbohydrates

Diet digestibility is intrinsically linked to enteric and manure GHG production. The more readily a diet is fermented, the lower the nutrient wastage and GHG emission intensity. Factors such as forage quality, forage-to-concentrate ratio and type of concentrate/forage can all contribute to the microbial efficiency of feed digestion. For example, increasing the quantity of concentrates in the diet can reduce enteric CH_4 production through a greater proportion of easily fermentable carbohydrates. This can shift fermentation from acetate which produces [H], towards propionate which utilises [H] in its synthesis and consequently, decreases the availability of [H] for methanogenesis. Increasing fermentable carbohydrates within the diet can also increase digestibility and passage rate. This can both improve productive performance and decrease the amount of OM excreted in the manure. Less OM in manure reduces the amount of substrate available for decomposition and thus the supply of [H] to methanogens, decreasing manure CH_4. Alternatively, increasing the digestibility of the diet can also increase enteric CH_4 on a g/d basis as more substrates are fermented in the rumen and the production of reducing equivalents increases. For example, lactating dairy cows grazing pasture have shown to have an increase in CH_4 production (g/d) and milk yield (van Wyngaard et al., 2018; Muñoz et al., 2015) when supplemented with increasing concentrates (0–8 kg/d).

Increasing concentrate in the diet can increase the protozoa, *Entodinium* in the rumen, a known non-fibrous carbohydrate degrader. Whereas microbes associated with cellulolytic degradation including *Fibrobacter*, *Polyplastron* and *Ostracodinium* decrease as the level of concentrate increases in the diet (Zhang et al., 2017). The diversity and richness of fungal communities were similar in high-forage versus high-concentrate diets; however, the relative abundance of the fungal phyla *Ascomycota*, *Basidiomycota*, *Cercozoa* and *Chytridiomycota* increased, and *Neocallimastigomycota* decreased with increasing proportions of concentrate (Zhao et al., 2018). Other studies also report that fungal communities have been enriched as the forage proportion of the diet increases (Kumar et al., 2015), reflecting their role in the degradation of complex fibre. Changes in eukaryote abundance and diversity are likely to impact methanogen abundance and diversity as eukaryotes produce the [H] required for methanogenesis.

A high-concentrate diet decreased the overall abundance (richness) of the archaeal population, but did not change the range of microbial species (diversity) (Zhang et al., 2017; Mao et al., 2016). Despite their role in methanogenesis, archaeal communities have been reported to show less variation and diversity as a ruminant adapts from a high-forage to a high-concentrate diet (Henderson et al., 2015; Kumar et al., 2015). This may be due to their low density and

their less-diverse metabolic capabilities (Kumar et al., 2015; Henderson et al., 2015). However, *Methanomicrobium* (Methanomicrobiales order) and *Methanomicrococcus* (Methanosarcinales order) are reported to be sensitive to dietary changes with both these taxa being absent in high-grain diets and only detected in forage diets (Friedman et al., 2017a). The absence of *Methanomicrococcus* (Methanosarcinales order) in high-grain diets is likely related to an increase in redox potential associated with a lower rumen pH (Friedman et al., 2017a).

Diets composed of highly fermentable substrates can result in conditions where organic acid production by the microbial population exceeds the buffering capacity of the rumen, leading to a prolonged reduction in rumen pH. Ruminal acidosis is characterised by a reduction in microbial diversity and rumen malfunction including decreased feed intake and feed digestibility. Abundance and diversity of bacteria were decreased in sheep ruminal fluid (Li et al., 2017) and ruminal fluid and faeces in dairy cows (Plaizier et al., 2017) with induced sub-acute ruminal acidosis. High-grain diets usually result in an increase in starch and lactate utilisers as well as propionate producers (*Prevotella*, *Selenomonas*, *Megasphaera*, *Streptococcus*) (Plaizier et al., 2017; Zhu et al., 2018). Specifically, *Prevotella* and *Succinivibrionaceae* dominate the rumen of ruminants-fed high-grain diets (Henderson et al., 2015). In contrast, fibrolytic bacteria including *Butyrivibrio*, *Ruminococcus* and *Fibrobacter* are vulnerable to low pH and usually decrease in abundance in high-grain diets (Zhu et al., 2018). Whilst other studies report a decrease in fungal diversity (Kumar et al., 2015; Tapio et al., 2017a), Ishaq et al. (2017) found that abundance and diversity of rare fungal taxa was increased with diet-induced sub-acute acidosis, including those associated with lactic acid utilisation (*Pichia* and *Candida*). The abundance of the archaeal population has been shown to not change with increasing concentrate in the diet and decreased ruminal pH (Hook et al., 2011). This suggests that they are resilient to changes in pH and only their functional activity is suppressed under conditions of low pH. This may explain why there is a poor relationship between methanogen abundance and CH_4 production (Firkins and Yu, 2015).

Altering the ability of ruminal microbes to degrade feed can increase nutrient loss in the faeces, increasing the amount of OM available for CH_4 production from manure. Although increasing the concentration of ruminal escape starch in the diet can modulate fermentation and potentially reduce methanogenesis, starch digestion can also be limited (<60%) in the lower digestive tract (Haque, 2018). This results in more starch in the faeces, potentially increasing CH_4 emissions during decomposition of the manure. *Ruminococcaceae* was more abundant in manure of cattle-fed processed grain and forage-fed diets, whereas *Prevotella* dominated in manure from cattle-fed unprocessed grain (Shanks et al., 2011). *Bacteroidetes*, as a reflection of

their role in the digestion of complex carbohydrates, increased and *Firmicutes* decreased with increased levels of starch in the faeces. The concentration of starch in faeces from cattle-fed unprocessed grains and processed grains was 98.4% and 66.9% higher, respectively, than in those fed forage, suggesting that inadequate grain processing could increase CH_4 emissions from manure (Shanks et al., 2011).

Improving diet digestibility and thereby the potential of the ruminant to obtain nutrients from feed improves overall growth efficiency. This may lead to an increase or decrease in total GHG emissions depending on the types of gases produced and the balance between enteric and manure emissions. Regardless, if there is an actual decrease in GHG when production from the animal is improved (i.e. less days on feed), GHG emission intensity as a proportion of usable product (i.e. meat, milk, wool, etc.) is reduced (Hristov et al., 2013).

1.2.2 Nitrogen content

Ruminants are a significant contributor to the global N budget. In ruminants, nitrogen cycles through a series of complex biogeochemical interactions involving inorganic- and organic-N in feed, manure and soils (Fig. 4; Robertson and Vitousek, 2009). Plants and animals utilise N throughout this cycle; however, they are limited in their ability to deposit it as a product. For example, the conversion of dietary N into consumable protein (i.e. milk, meat) by ruminants is very low (20–30%) and fertiliser-N recovery by cereal crops seldom exceeds 50% of applied N (Fageria and Baligar, 2005). Excess N from animal agriculture results in the release of a large surplus of reactive N into the environment, mainly via NH_3 and N_2O emissions and/or nitrate (NO_3-N) leaching (Galloway et al., 2004; Powell et al., 2011). Pollution of groundwater by NO_3-N, widespread eutrophication and global warming through N_2O emissions

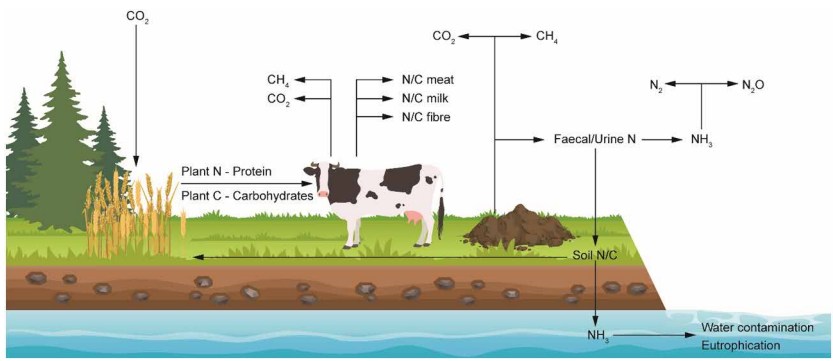

Figure 4 Nitrogen and carbon cycle within ruminant agriculture.

are some well-documented contributions of agricultural-anthropogenic N (Erisman et al., 2013).

The cycle of N within ruminant systems is mainly defined by the transformation of feed-N into milk or meat products, with the remaining N excreted in urine or manure. The concentration of N in urine and faeces depends on the crude protein (CP) content of the diet (Dijkstra et al., 2013). Feeding ruminants to the level of metabolisable protein requirements ensures the best utilisation and the least loss of nutrients (Broderick, 2003). The amount and type of N fed in ruminant diets also has several implications for how it is utilised and excreted by ruminants. Dietary protein supplies both rumen-degradable and -undegradable protein. Rumen-degradable protein is composed of true protein and non-protein N, which when broken down can be utilised for microbial protein synthesis and growth (Bach et al., 2005). Requirements for dietary protein and energy are intrinsically linked, as high-energy diets will stimulate microbial synthesis, enhancing the need for rumen-degradable protein (Broderick, 2003). Whilst changing the CP content in the diet has no obvious direct effect on enteric CH_4, its replacement by carbohydrates can influence emissions.

Replacing protein supply with fermentable carbohydrates is an effective way to reduce urinary N excretion, increase microbial N capture and decrease NH_3 production (Dijkstra et al., 2013). However, replacing dietary CP with fermentable carbohydrates can exacerbate enteric CH_4 production (Sauvant et al., 2011) as increased substrates are available for ruminal methanogenesis. Dijkstra et al. (2013) estimated that higher enteric CH_4 fluxes from increased carbohydrates in the diet are frequently offset by a decrease in N_2O emissions from manure.

Prevotella is a predominant genus within ruminants around the globe (Henderson et al., 2015) and participates in both carbohydrate and N metabolism. Specifically, *Prevotella ruminicola* strain 23 can efficiently degrade hemicellulose and pectin, utilising both NH_3 and peptides as a N source for growth as opposed to amino acids (Kim et al., 2017). Their importance in N metabolism was supported by an observed decline in *Prevotella bryantii* in dairy cattle fed a low-protein diet (Belanche et al., 2012). In this study, the relative abundance of *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes* and *Butyrivibrio fibrisolvens* all declined, potentially highlighting the vulnerability of cellulolytic bacteria to N shortages. In contrast, non-cellulolytic bacteria including *Prevotella ruminicola, Selenomonas ruminantium*, *Streptococcus bovis*, *Megasphaera elsdenii* and *Aliiglaciecola lipolytica* were not affected by a N shortage which may reflect their low NH_3 requirements for growth (Belanche et al., 2012). Niu et al. (2016) evaluated the effect of two different concentrations of CP (15.2% vs 18.5%) in dairy cows and demonstrated that the low-protein diet decreased total tract digestibility of OM, N and starch

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compared to the high protein diet (18.5%). Belanche et al. (2012) indicated that ruminal concentrations of protozoa and methanogens declined with the low-protein diet, whereas Niu et al. (2016) observed no differences in CH_4 emissions as a result of differing protein content in the diet.

Degradation of dietary protein and its assimilation into microbial protein can also contribute to a decrease in available [H] for methanogenesis as this process can both utilise and produce reducing equivalents (Knapp et al., 2014). For example, the synthesis of amino acids can increase with a decrease in methanogenesis, as they act as a [H] sink (Ungerfeld, 2015b). An increase in amino acid production could be related to an increase in the relative abundance of *Bacteroidetes* and *Prevotella* species, both associated with increased proteolytic activity (Martinez-Fernandez et al., 2016). Increasing the proportion of soluble carbohydrates in diets has been related to decreases in branched-chain fatty acids, which are needed for de novo synthesis of amino acids by ruminal microbes. Decreasing availability of branched-chain fatty acids could decrease microbial protein synthesis and microbial growth (Hall and Huntington, 2008) and thereby reduce the extent to which this process acts as an alternative [H] sink to CH_4 production.

Potential interactions between dietary protein content and ruminal methanogenesis remain unclear. Addition of protein to high-fibre diets could increase the efficiency of microbial protein synthesis and reduce the intensity of enteric CH_4 emissions, while increasing N excretion and N_2O missions from manure. Increasing the efficiency of microbial protein synthesis could redirect [H] away from methanogenesis towards the formation of microbial cells and increase the productivity of the ruminants.

1.2.3 Dietary fat

Dietary additives which target microbes involved in methanogenesis may be superior at decreasing enteric CH_4 production without affecting manure emissions. For example, dietary fats may decrease enteric CH_4 production by (i) having a toxic effect on methanogens and protozoa, (ii) replacing fermentable carbohydrates or (iii) providing an alternative [H] sink via biohydrogenation (Beauchemin et al., 2008; Knapp et al., 2014).

The effects of dietary lipids on the rumen are largely dependent on fat composition, concentration and source. Consequently, the impact on the rumen microbial populations varies depending on the nature of the oil. *Methanobrevibacter ruminantium* is the most abundant species of rumen methanogen and was found to be reduced by saturated fatty acids and oleic acid (Henderson et al., 2015; Enjalbert et al., 2017). Addition of linseed and coconut oil decreased CH_4 production, but this was not correlated with changes in the abundance or diversity of the archaeal population (Patra and Yu, 2013; Martin

et al., 2016). Similarly, lambs fed linseed oil had a higher relative abundance of *Succinivibrionaceae* (succinate producers) and *Veillonellaceae* (propionate producers) and a decreased abundance of *Ruminococcaceae* (Lyons et al., 2017). An increased abundance of *Succinivibrionaceae* and *Ruminococcaceae* has been associated with low and high CH_4 emitters, respectively (Wallace et al., 2015). *Succinivibrionaceae* produce succinate via utilisation of [H], whereas *Ruminococcaceae* are known hydrogen producers (Wallace et al., 2015). Lambs supplemented with linseed oil had a 19.5% decrease in the relative abundance of *Methanobrevibacter* and a 34.7% increase in *Methanosphaera*, although CH_4 emissions were not measured in this study.

The growth of the rumen fungus, *Neocallimastix frontalis*, was impeded by soybean oil (Boots et al., 2013) and others have found that the fibrolytic bacteria, *Fibrobacter* and *Ruminococcus* are also inhibited by lipids (Enjalbert et al., 2017). Increasing the degree of unsaturated fatty acids in the diet may correlate with decreased protozoal counts (Oldick and Firkins, 2000), and as a result of disrupting the close relationship between protozoa and archaea, change the diversity of archaeal communities (Hristov et al., 2012).

Alteration of the ruminal microbiome due to the type of fat may prompt a high variation in physiological responses including an inhibition of fibre digestion which may decrease CH_4 production. It is well documented that increasing fat content in ruminant diet above 6–7% dry matter (DM) can reduce the digestibility of fibre (Johnson and Johnson, 1995). In continuous culture, an oil (Tucumã) high in oleic acid inhibited CH_4 at 1% (v/v) through suppression of *Fibrobacter* with no alteration in methanogens (Ramos et al., 2018). Decreasing fibre digestibility can result in an increase in manure C, providing substrate for CH_4 emissions. Although, Gautam et al. (2016) found that varying sources of dried distillers grain plus solubles (DDGS) with corn oil (dietary fat of 3–5.5% dietary DM) had no effect on nutrient composition or GHG emissions from manure.

Dietary fat supplementation is an effective enteric CH_4 mitigation strategy. Depending on their fatty acid composition, dietary fats may decrease both daily and the intensity of CH_4 production. However, the main constraint with dietary fat is that the amount that can be supplemented without inhibiting fibre digestion is restricted to ≈6% of dietary DM, so the CH_4 mitigation potential is limited to 10–15%.

2 Case study: Dried distillers' grains plus solubles (DDGS)

A primary example of the importance of a holistic approach to investigating the impact of feeding a dietary additive is shown by research evaluating DDGS. DDGS is a by-product of ethanol production capable of replacing cereal grains in ruminant diets due to its high energy and fat content. Corn

(*Zea mays* L.) and wheat distillers' grains have been shown to decrease CH_4 production as a result of their high oil content. Depending on market demands, DDGS may at times be more economical in least-cost diets than cereal grains.

A meta-analysis by Griffin et al. (2012) found that when DDGS replaced forage, it increased the average daily gain and final body weight of backgrounding cattle. Triticale DDGS could be substituted for barley (*Hordeum vulgare* L.) silage and barley grain in finishing diets with no effect on growth performance or carcass quality in finishing beef steers.

Due to its high fat content, DGGS can successfully decrease enteric CH_4 emissions. This was verified when 35% of barley grain (DM basis) was replaced with corn DDGS and enteric CH_4 production was decreased by 16.4% (% DM intake) in beef cattle fed a barley silage-based diet (McGinn et al., 2009). Similarly, replacing 35% of the barley grain and 5% of the canola meal (*Brassica napus* L.) in a diet with corn DDGS decreased CH_4 production by 15.1% (% DM intake; Hünerberg et al., 2013a). Methane emissions on an intake basis were also decreased (18.0%) in cattle fed a finishing high-grain diet with corn DDGS replacing 40% of barley grain (Hünerberg et al., 2013b). Despite the observed reduction of CH_4 production, these studies reported that including DDGS dramatically increased both N intake and excretion. Additionally, corn DDGS has been shown to decrease the digestibility of starch (Castillo-Lopez et al., 2014), increasing its availability for CH_4 production from manure.

Fibrobacteres and *Bacteroidetes* have been shown to decrease in abundance with inclusion of DDGS in the diet (Castillo-Lopez et al., 2017; Castillo-Lopez et al., 2014). Castillo-Lopez et al. (2017) reported that *Bacteroidetes* were decreased and *Tenericutes* were increased by 20% DDGS (diet DM). Interestingly, the abundance of *Ruminococcaceae* increased with DDGS (Castillo-Lopez et al., 2017), even though it has been suggested that this family is associated with increased CH_4 production (Wallace et al., 2015). Including DDGS at 50% (DM basis) decreased *Succinivibrio* and increased *Bacteroides* and *Prevotella* by −75.4%, 61.0% and 34.6%, respectively.

Though originally seen as an effective GHG mitigation strategy, using a life cycle approach revealed that feeding corn or wheat-based DDGS resulted in a 6.2% and 9.3% increase, respectively, in total GHG emissions compared to a control barley-based diet (Hünerberg et al., 2014). Increased N_2O emissions from manure were found to be a leading factor contributing to total GHG emissions even though manure-related CH_4 emissions were reduced. The increase in N_2O emissions was a result of the increased CP content of the diet and greater N excretion in the urine and the faeces.

For DDGS to be suitable as a GHG mitigation strategy, it needs to be fed at a level that does not exceed the protein requirement of ruminants. However, lower amounts of DDGS in the diet are unlikely to increase fat concentrations

to a level that is sufficient to lower enteric CH_4 emissions (Castillo-Lopez et al., 2017; Judy et al., 2016; Hales et al., 2013).

3 Nitro-based compounds

Nitro-based compounds have been examined for their enteric CH_4 mitigation potential in ruminants. These compounds reduce CH_4 via action as an alternative electron sink or possibly as a direct inhibitor of methanogens. Two dietary feed additives which have received attention for their ability to consistently reduce enteric CH_4 emissions include nitrates and 3-nitrooxypropanol (3-NOP).

3.1 Nitrate

Nitrate is a form of N which is found naturally within feed, with variable concentrations occurring in different types of forages. Nitrate's potential for reducing enteric CH_4 arises from its ability to act as a [H] sink within the rumen. Both NO_3^- and CO_2 are available as alterative electron acceptors in the rumen and their reduction results in an energy release (ΔG) of 371 and 67 KJ, respectively. Nitrate acts as an alternative electron acceptor, utilising [H] in the reduction of NO_2^- to NH_3 and this reaction is thermodynamically more favourable than the reduction of CO_2 to CH_4 by methanogens (Ungerfeld and Kohn, 2006). Accumulation of NO_2^- within the rumen may occur if the reduction of NO_3^- to NO_2^- takes place at a rate faster than the conversion of NO_2^- to NH_3 (Latham et al., 2016). This accumulation can result in NO_2^- toxicity, reducing the ability of red blood cells to carry oxygen (Lee and Beauchemin, 2014). However, various studies have concluded that provided the rumen microbes are gradually acclimatised to NO_3^-, the risk of toxicity is negligible (Cottle et al., 2011; Olijhoek et al., 2016).

3.1.1 Ruminant studies

Studies have reported a reduction of 16–35% in CH_4 per unit of DM intake across a range of ruminants consuming different diets supplemented with NO_3^-. An increase in total VFA (El-Zaiat et al., 2014; Nolan et al., 2010; van Zijderveld et al., 2010) and NH_3 concentrations (Olijhoek et al., 2016) have also been reported. Though CH_4 production is decreased, no studies have reported an increase in either milk or meat production, despite a predicted increase in the availability of metabolisable energy as a result of a reduction in CH_4 emissions (Johnson and Johnson, 1995). Several studies have reported an accumulation of H_2 within the rumen (Lee et al., 2015c; Olijhoek et al., 2016), a possible outcome of H_2 not being used in the reduction of either CO_2 or NO_3^-. Emission of H_2 also represents a loss of metabolic energy.

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The primary factor in the dominance of NO_3^- reduction is related to it being more thermodynamically favourable than the reduction of CO_2 to CH_4; however, NO_3^- also has variable effects on methanogen abundance (Zhou et al., 2014; Liu et al., 2017). Zhao et al. (2018) found that although NO_3^- decreased *in vitro* CH_4 production, rumen fluid collected from donor steers fed NO_3^- exhibited no change in the total methanogen population. However, the relative abundances of *Methanomicrobiales* decreased and *Methanosarcinales* increased with increasing NO_3^- (0%, 1%, 2% DM basis NO_3^-). *Methanosphaera* and *Methanimicrococcus* increased and *Methanoplanus* decreased as a result of inclusion of NO_3^- in the diet. In contrast, Asanuma et al. (2015) found that the populations of methanogens, protozoa and fungi were all drastically decreased when goats were administered 6 or 9 g/d of NO_3^- per day. *Streptococcus bovis and Selenomonas ruminantium* were also increased in this study, possibly suggesting that they may play a role in NO_3^- metabolism (Asanuma et al., 2015).

Though a reduction in enteric CH_4 is consistent with NO_3^- supplementation, this is not often accompanied by a reduction in ruminal methanogen populations. This leads to the conclusion that NO_3^- inhibits that activity of methanogens rather than their growth. There are a large number of bacterial species that may be involved in NO_3^- metabolism. Bacteria identified as potential denitrifiers include *Pseudomonas aeruginosa* and members of *Propionibacterium, Butyrivibrio, Clostridium, Peptostreptococcus, Nitrosomona, Desulfovibrio* and *Enterobacteriaceae* (Latham et al., 2016). In culture, *Selenomonas ruminantium* was tolerant of NO_3^- with some strains exhibiting the ability to reduce NO_3^- to NO_2^-. *Veillonella parvula* and *Wolinella succinogenes* decreased in cultures that lacked NO_3^- and increased with the addition of 5 mM nitrate (Iwamoto et al., 2002).

Nitrite as the intermediate in the reduction of NO_3^- to NH_3 has been shown to cause reductions in cellulolytic bacteria (Iwamoto et al., 2002; Asanuma et al., 2015). However, NO_3^- addition has also been reported to cause no change or an increase in *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus* as well as total protozoa (Patra and Yu, 2014; Zhao et al., 2015). Protozoal numbers have also been reported to decline with NO_3^- (Nolan et al., 2010; El-Zaiat et al., 2014), which may also contribute to an indirect reduction in CH_4 production as a result of inhibition of commensal methanogens.

3.1.2 Manure

In soils, nitrification is performed by NH_3 oxidising bacteria and archaea, heterotrophic nitrifiers and fungi (Norton, 2008), whereas denitrification is carried out by heterotrophic denitrifiers, denitrifying fungi and autotrophic/heterotrophic nitrifiers (Coyne, 2008). Betaproteobacterial NH_3-oxidising

bacteria, *Thaumarchaeota* NH_3-oxidizing archaea and fungi including *Fusarium* and *Trichoderma* (Maeda et al., 2015) are capable of denitrification of NO_3^- to produce N_2O (Maeda et al., 2011).

Compared to a 55% forage and a 45% concentrate diet with encapsulated urea, inclusion of encapsulated slow-release NO_3^- at up to 3% DM linearly decreased total urinary N excretion despite both of the diets being isonitrogenous (Lee et al., 2015a). However, NO_3^-N excretion in urine and faeces was increased and urea-N in the urine was decreased. Decreasing urinary urea-N and increasing urinary NO_3-N excretion may decrease NH_3 emissions as urea is the primary source of volatile NH_3. However, this could potentially increase direct N_2O emissions as a result of denitrification of NO_3-N by soil microbes. van Zijderveld et al. (2011) and Li et al. (2012) found that there was no difference in N excretion in dairy cows or sheep when NO_3^- was used to replace urea in the diet. Lee et al. (2015b) established that DM, OM and starch digestibility increased with NO_3^- supplementation, potentially decreasing the amount of substrate that would be available for CH_4 production from manure.

3.2 3-nitrooxypropanol (3-NOP)

Three nitrooxypropanol has been shown to decrease enteric CH_4 emissions by up to 80% (DM intake basis) in beef steers fed a high-grain diet (Vyas et al., 2016). 3-NOP is thought to impede methanogenesis via the inhibition of methyl-coenzyme, which is involved in the transfer of a methyl group to methyl-coenzyme M reductase, the terminal reaction in methanogenesis (Duin et al., 2016).

3.2.1 Ruminant studies

3-NOP has been found to result in a 7.7–80.7% reduction in enteric CH_4 emissions in sheep (Martínez-Fernández et al., 2014), dairy cows (Haisan et al., 2014, 2017; Hristov et al., 2015; Lopes et al., 2016; Reynolds et al., 2014) and beef cattle (Romero-Perez et al., 2014, 2015; Vyas et al., 2016, 2018b). Increasing concentrations of 3-NOP (0.75–4.5 mg NOP/kg BW) have been shown to linearly decrease CH_4 production (from 6.49 to 4.34 CH_4, % gross energy intake) (Romero-Perez et al., 2014).

The energetic saving as a result of reduced CH_4 production has been reported to result in an increase in the body weight of dairy (Haisan et al., 2014; Hristov et al., 2015) and beef steers (Martinez-Fernandez et al., 2018). Others have reported a decrease in the acetate-to-propionate ratio in rumen fluid (Haisan et al., 2014; Romero-Perez et al., 2014, 2015; Martínez-Fernández et al., 2014; Lopes et al., 2016; Haisan et al., 2017), as well as increase in DM digestibility (Haisan et al., 2017) and milk protein (Reynolds et al., 2014).

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Though Vyas et al. (2016) found that 3-NOP resulted in a 37.6% decrease in CH_4 and tended to increase the gain-to-feed ratio of beef cattle fed a barley silage-based diet, there was also a tendency for a decrease in average daily gain when these cattle were fed a high-grain finishing diet. Alternatively, Vyas et al. (2018a) found that CH_4 yield decreased and feed conversion efficiency was improved by 3-NOP in cattle-fed both high-forage and high-grain diets.

In pure culture, 3-NOP has also been shown to inhibit the growth of methanogens without affecting other bacteria within the rumen (Duin et al., 2016). The growth and production of CH_4 by *Methanothermobacter marburgensis* was inhibited by both 1 and 10 μM of 3-NOP. However, methanogenesis in the cultures resumed 5 h after administration, suggesting that 3-NOP concentration may have been insufficient to completely inhibit the growth of methanogens. Similarly, growth and methanogenesis by *Methanobrevibacter ruminantium, Methanobacterium smithii, Methanobrevibacter millerae*, *Methanobacterium bryantii*, *Methanothermobacter wolfeii*, *Methanosphaera stadtmanae*, *Methanomicrobium mobile* and *Methanosarcina barkeri* were all inhibited at 3-NOP concentrations from 0.25 to 10 μM in pure culture (Duin et al., 2016). Not all *in vivo* trials have exhibited a change in the copy number of methanogens as a result of 3-NOP (Romero-Perez et al., 2014; Lopes et al., 2016). Alternatively, Haisan et al. (2014), Romero-Perez et al. (2015) and Martinez-Fernandez et al. (2018) reported a 56.6–64.7% decrease in the abundance of methanogens.

Methanobrevibacter spp and the *Methanomassiliicoccaceae* were reduced by 5.6 and 4.0 fold in Brahman steers fed 2.5 g 3-NOP per day, resulting in a 38% reduction in CH_4 (g/kg DMI). The decrease in *Methanomassiliicoccaceae* may have resulted in the increase of trimethylamines and dimethylamines in the rumen as this family utilises these compounds as substrates for methanogenesis (Martinez-Fernandez et al., 2018). Though originally thought to play a minor role in ruminal methanogenesis, methylotrophic methanogens (*Methanoplasmatales* and *Methanosphaera* spp.) account for 20% of methanogens in the rumen as compared to the 77% that are hydrogenotrophic (mostly *Methanobacteriales*) (Henderson et al., 2015). In beef steers, 3-NOP decreased the ratio of hydrogenotrophic to methylotrophic methanogens, suggesting that this additive has a greater inhibitory effect on hydrogenotrophic methanogens (Martinez-Fernandez et al., 2018).

In sacco, Martinez-Fernandez et al. (2018) found that DM degradability at 24 h was decreased by 7.1%, though DM, OM and neutral detergent fibre (NDF) digestibility was increased in lactating Holstein cows receiving 3-NOP (Haisan et al., 2017). This would imply that the electron carriers produced during fermentation are being used to reduce alternative electron acceptors in the rumen. A 4.9–26.0% increase in propionate production would account for some of this [H] utilization, but increases in propionate have not been observed in all 3-NOP studies (Guyader et al., 2017). In backgrounding beef

steers provided with 200 mg/kg BW of 3-NOP, a 37.6% decrease in CH_4 (g/d) coincided with an 89.1% increase in H_2 emissions. Similarly, during the finishing phase, an 84.3% decrease in CH_4 resulted in a 99.8% increase in H_2 (Vyas et al., 2016). Hristov et al. (2015) found a 64-fold increase in H_2 emissions by dairy cows supplemented with 3-NOP, but this accounted for only 3% of the [H] that was spared from methanogenesis (Latham et al., 2016). Alternatively, administration of 3-NOP to steers fed a high-forage diet decreased CH_4 production by 30.2% with no change in H_2 emissions. Propionate concentration was also unchanged; however, the concentration of butyrate was decreased and acetate, isobutyrate, iso-valerate concentrations were increased (Martinez-Fernandez et al., 2018). This suggests that some [H] might have been redirected to microbial cell mass, reflected by the increases in NH_3 and branched-chain VFA.

3.2.2 Manure

Inclusion of 3-NOP resulted in increased faecal DM, NDF and acid detergent fibre (ADF) (Reynolds et al., 2014) and decreased *in sacco* DM digestibility (Martinez-Fernandez et al., 2018). However, it has also been shown to increase DM and NDF digestibility (Haisan et al., 2017). 3-NOP has not been shown to have an impact on CP digestibility or reduce N excretion. This suggests that the relatively small amount of N in 3-NOP is not having an appreciable impact of N metabolism in ruminants. 3-NOP has been reported to rapidly decompose in the environment (Owens et al., unpublished), so it may not be biologically active within manure. Owens et al. (unpublished) investigated the effects of cattle-fed 3-NOP on GHG emissions from composted and stockpiled manure. They concluded that 3-NOP did not affect CO_2, CH_4, N_2O or NH_3 emissions.

Nitrate and 3-NOP have both been shown to suppress enteric CH_4 emissions; however, limited studies have assessed their effects on GHG emissions from a life cycle perspective, including emissions from manure. Concern over NO_2^- poisoning with NO_3 administration has limited its implementation within industry, in spite of studies showing that the reduction of NO_2^- to NH_3 in the rumen can be accelerated through microbial adaptation. Several studies have shown that 3-NOP can dramatically lower enteric CH_4 emissions. However, the magnitude and consistency of this response appears to depend on the forage-to-concentration ratio of the diet. There is a need for appropriate dosage rates to be identified for ruminants-fed different diets so that the appropriate regulatory approval for the use of 3-NOP in ruminants can be obtained. Additionally, determining whether NO_3^- and 3-NOP consistently improve feed efficiency in ruminants is needed to determine if this can serve as an economic incentive for producers to adopt these technologies. Both of these additives also need to be linked with methodologies that enable them to

be administered to ruminants in extensive grazing systems, where the intensity of GHG emissions is typically higher than intensive production systems.

4 Plant secondary compounds

A large variety of plant secondary compounds from a diverse range of plants have been explored for their potential to mitigate enteric CH_4 emissions. Secondary metabolic compounds commonly employed as feed additives include essential oils, saponins and tannins. However, over 200 000 defined phytochemicals have been identified (Hartmann, 2007). Tannins are aromatic, recalcitrant compounds with the ability to form complexes with carbohydrates, proteins and minerals that are either reversible or irreversible in nature. They can be classified as hydrolysable or condensed with the most distinguishing feature between these groups being some hydrolysable tannins can be metabolised by rumen microorganisms, whereas condensed tannins (CT) resist biodegradation (Aboagye et al., 2018).

4.1 Tannins

There is considerable evidence that there is an array of tannin-rich forages or tannin extracts which can reduce enteric CH_4 emissions from cattle (Waghorn, 2008; Hess et al., 2006; Grainger et al., 2009; Alves et al., 2017). However, the actual mode of action whereby tannins decrease CH_4 is not well understood. Some hypotheses include a depression of methanogenic archaea, interference with the symbiotic relationship between methanogens and protozoa, inhibition of rumen ciliates (Bhatta et al., 2015), a reduction in fermentable substrates, binding to microbial enzymes (Gonçalves et al., 2011) or acting as a [H] sink (Naumann et al., 2017). CT have a higher binding capacity for dietary proteins and can increase the flow of metabolisable protein to the small intestine, a trait that could decrease microbial protein synthesis. In comparison, hydrolysable tannins exhibit a lower affinity for proteins and bacterial degradation of these tannins may result in the formation of low-molecular-weight metabolites including phenolics which may be toxic to ruminants (Patra and Saxena, 2011).

4.1.1 Ruminant studies

The impact of tannins on ruminal CH_4 emissions has been inconsistent. Grainger et al. (2009) found up to a 30% decrease in CH_4 production when lactating cows were fed up to 266 g of *Acacia mearnsii* per day. Alves et al. (2017) also reported that *Acacia mearnsii* decreased CH_4 emissions (g/kg milk yield) by up to 32% in Holstein dairy cows grazing tropical pastures, with no adverse effects on milk production (yield, fat %, protein %). Koenig et al. (2018) found that addition of

CT from *Acacia mearnsii* to a 40% corn DDGS decreased OM, NDF, ADF, N and gross energy digestibility, with an increase in faecal N (32.4%), and a decrease in urinary N (17.5%). Rumen NH_3-N was also decreased as was VFA concentration and the acetate-to-propionate ratio, but CH_4 production was not measured in this study. Carulla et al. (2005) fed 41 g/kg of a CT extract from *Acacia mearnsii* to growing Swiss White Hill wethers and found that apparent digestibility of OM, CP, NDF and ADF were decreased (1.89–10.6%). Ammonia-N was also decreased but the acetate-to-propionate ratio was increased with propionate increasing by 6.3%. Methane production was decreased as per cent of DM and OM intake, most likely as a result of inhibition of fibre digestibility.

No difference has been observed in performance (feed conversion efficiency) when beef cattle and sheep were fed a diet containing *Acacia mearnsii* CT (Koenig et al., 2018; Carulla et al., 2005). Woodward et al. (2004) found that feeding *Lotus corniculatus*, a forage with CT reduced CH_4 production (dry intake basis) by up to 16%. The CT extract from quebracho trees (*Schinopsis quebracho*) had no effect on CH_4 production and CP digestibility was decreased (Beauchemin et al., 2007). Aboagye et al. (2018) found that a hydrolysable chestnut tannin with and without a CT (quebracho) had no effect on ADG or G:F over 12 weeks in beef cattle. There was only a tendency for a decrease in CH_4 production (g/kg DMI), although digestibility was not reported. These studies highlight the inconsistency of feeding dietary tannins on enteric CH_4 emissions, possibly a result of (i) the tannin-derived species, (ii) phytochemical composition, (iii) administration method and (iv) concentration in the diet.

The ability of tannins to form complexes with rumen microorganisms involves polyphenic reactivity with the cell wall and secreted extracellular enzymes (McSweeney et al., 2001). Bacteria including *Fibrobacter succinogenes*, *Butyrivibrio fibrosolvens*, *Ruminobacter amylophilus* and *Streptococcus bovis* have all been shown to have a high affinity for a diverse range of tannins. McSweeney et al. (1998) and McAllister et al. (1994) suggested that proteolytic bacteria and fungi are less susceptible to tannins than cellulolytic bacteria. Tan et al. (2011) indicated that tannins altered the diversity of methanogens within the rumen when *Leucaena leucocephala* was fed as it decreased the proportion of *Methanomicrobiales* (15.1%) and *Methanobacteriales* (6.8%). In contrast, *Thermoplasmatales* were increased by 21.9% and the overall diversity of the archaeal population was reduced.

The most common response to feeding dietary tannins is a reduction in ruminal CP digestibility. CT form complexes with proteins through H bonds and hydrophobic interactions (Koenig and Beauchemin, 2018). This reduces the availability of dietary protein for microbial degradation within the rumen. It has also been proposed that whilst the protein is bound within the rumen, these complexes dissociate in the abomasum enabling the protein to be digested and the amino acids absorbed in the lower digestive tract. However, this is not

a consistent effect as not all complexes dissociate, often resulting in a reduction in CP digestibility and a shift in N excretion from urine to faeces (Patra and Saxena, 2011).

4.1.2 Manure

Unlike other dietary additives, the effects of including tannins in ruminant diets on manure have been extensively examined (Koenig and Beauchemin, 2018; Powell et al., 2011; Halvorson et al., 2017; Jordan et al., 2015). An advantage of feeding dietary tannins with regard to GHG emissions from manure is that they shift the site of N excretion from urine to faeces. Faecal N in the form of CT-protein complexes is much more stable in manure and less likely to contribute to high NH_3 emissions than urea in urine. Slow-release faecal N is also more likely to be captured by the plant and used for the synthesis of plant proteins.

The inhibitory effect of tannins on urease activity has also been linked to the formation of substrate-tannins complexes (Powell et al., 2011). Tannins may also have the ability to reduce N_2O emissions due to their ability to form complexes with proteins, resulting in insoluble and unavailable forms of N (Powell et al., 2011). Powell et al. (2011) used ventilated chambers to assess the inclusion of dietary tannins on lactating Holstein dairy cow's manure. Cattle were fed a red quebracho (*Schinopsis lorentaii*) and chestnut (*Castanea sativa*) tree mixture at four increasing concentrations (0, 4.5, 9.0 and 18.0 g/kg DMI). Cumulative NH_3 emissions from the tannin slurries were up to 27% lower than the slurry from control animals. Moreover, 54% and 66% of the applied urea was emitted as NH_3 from tannin fed and control slurries, respectively.

As an external application, CT of quebracho were added separately to both composted manure from goats, and N and phosphorus (P) poor soils at 4% (w/w). Tannins decreased cumulative C emissions by 40% and N emissions by 36% in the compost. Tannins applied directly to soil also reduced N_2O emissions by 17%, and reduced NH_3 release by 51% as compared to soil that did not receive tannins (Jordan et al., 2015). Tannins are known to reduce inorganic-N availability by sequestering organic-N sources through complex interactions. Similarly, tannins may act as labile-C sources leading to increased N immobilisation (Kraus et al., 2004). Koenig et al. (2018) fed black wattle at 2.5% with a 40% distillers grain diet to finishing beef steers. An integrated horizontal flux technique with passive NH_3 samplers was used to assess NH_3 emissions from pens. Though measurements were limited, NH_3-N emissions were 23% lower in cattle-fed the diet containing tannins.

Pseudomonas citronellolis and *Pseudomonas plecoglossicidda* were identified as two bacteria capable of utilising tannic (hydrolysable) and gallic acid (phenolic hydrolysate of tannin acid), respectively, within tannery soils.

Bending and Read (1996) found that a hydrolysable polyphenol–protein could be degraded by ectomycorrhizal fungi (*Hysterangium setchellii*, *Lactarius affinis*, *Lactarius controversus*), ericoid mycorrhizal (*Hymenoscyphus ericae*) and wood decomposing fungi (*Hypholoma fasciculare* and *Phanerochaete velutina*) from a soluble tannin acid in forest soils (Mutabaruka et al., 2007). Mutabaruka et al. (2007) found that the ratio of fungi to bacteria increased in systems with high amounts of CT complexes with acidic soils. Though chemically distinct, similar microbes may inhabit tannin manure composts, breaking down tannic complexes during composting or after land application.

Including tannins in ruminant diets has generated varying results on ruminant metabolism and CH_4 emissions. It seems that the CT extracted from black wattle (*Acacia mearnsii*) has been the most consistent at lowering enteric CH_4 emissions. From a manure GHG mitigation perspective, most CT consistently decrease both NH_3 and N_2O emissions. However, tannins can also reduce the digestibility of CP, constituting a loss of N from the animal. Although this could have a negative impact on the productivity of ruminants, it may improve the nutrient composition of manure as a fertiliser and soil amendment.

5 Carbon-derived materials

The cycling of C is defined by the conversion of atmospheric CO_2 to plant biomass-C through photosynthesis. In ruminants, consumed C is partitioned into metabolic animal by-products and waste products, including CO_2 from respiration and the decomposition of manure (Fig. 4). Deposited faeces may decompose and release labile-C, increasing OM levels in surface soil (Sharma et al., 2017). Humic substances (HS) and biochar originally received attention for their ability to increase and sequester soil C. However, more recently, biochar and HS have been assessed for their potential to mitigate enteric CH_4 emissions when they are included directly in the diet of ruminants.

5.1 Humic substances

OM within the soil is a complex heterogeneous mixture of plant- and animal-derived precursors at varying stages of oxidation and decay (Masoom et al., 2016). HS are mainly comprised of polymerized molecules exhibiting strong resistance to biodegradation (Stevenson, 1995). HS can be classified into three operational fractions based on their solubility in alkaline or acidic solutions. Accordingly, fulvic acids are small-sized aliphatic compounds soluble in both alkali and acid, whereas humic acids are high-molecular-weight materials extracted by dilute alkali, that precipitate at a pH of 2 with humins representing the insoluble proportion of HS (Stevenson, 1995; Lamar et al., 2014). The chemical nature of HS may differ widely in terms of functional group structure,

composition and reactivity, and consequently influencing their impact on rumen function (Stevenson, 1995).

HS have been postulated to reduce enteric CH_4 production as well as influence zoonotic pathogens. Existing literature has examined the impact of various HS derivatives on both *in vitro* fermentation (Sheng et al., 2017; Terry et al., 2018a; Varadyova et al., 2009) and *in vivo* metabolism (Terry et al., 2018b; El-Zaiat et al., 2018; Ponce et al., 2016). However, there is little consistency between studies, possibly because the concentration and types of HS have not been well characterised.

5.1.1 Ruminant studies

The ability of HS to imitate ionophores was shown when the average daily gain, DM intake and feed efficiency of cattle administered monensin did not differ from those supplemented with HS (McMurphy et al., 2009). However, as no negative control was utilised in this study, results need to be interpreted with caution. Saanen goats fed diets containing humic acids at up to 3% diet DM had higher milk yields and lower levels of blood cholesterol (Degirmenci, 2012). Agazzi et al. (2007) found that milk consumption and average daily gain in newborn kids fed HS were higher than the control group. This study hypothesised that it was the antibiotic properties of HS that improved cell-mediated immunity, decreasing the risk of digestive orders and diarrhoea in young kids.

Recently, HS have been evaluated for their ability to inhibit ruminal methanogenesis. Whilst an *in vitro* batch culture showed that HS resulted in a consistent decrease in CH_4 production (Sheng et al., 2017), this was not verified in continuous culture using the rumen simulation technique (Rusitec) (Terry et al., 2018a). Further to this, a study conducted to investigate the effect of HS on beef heifers fed a barely silage-based diet found that there was no effect on enteric CH_4 production (Terry et al., 2018b). However, there was an increase in total N retention when heifers were fed up to 300 mg/kg live BW of HS, suggesting an improvement in protein utilisation.

Both Terry et al. (2018a,b) examined the rumen microbial population using 16s rRNA sequencing. In the Rusitec, *Fibrobacter* and *Christensenellaceae* R-7 were reduced by HS in solid-associated samples. Although the *Methanobacteria* were not changed by HS, *Methanobacterium* was increased and *Methanobrevibacter* and *Methanosphaera* were decreased. *In vivo*, the relative abundance of *Proteobacteria*, *Synergistetes* and *Euryarchaeota* were decreased by HS.

5.1.2 Manure

Information concerning the incorporation of HS into manure mixtures is scarce. In this regard, HS were shown to decrease total N excretion when fed to beef

heifers. This may imply that HS could have the potential to reduce NH_3 emissions from manure. This is in agreement with the findings of Shi et al. (2001) who investigated the effects of black and brown humates on NH_3 emissions after their inclusion in a soil-faeces-urine mixture (1.7% of total mixture mass). Results indicated that NH_3 emissions decreased by 39.8% compared to the manure-soil mixture with no humates. However, in this experiment HS were added to the manure after excretion, so results may differ from when HS are included directly in the diet.

Various studies have reported that HS decrease (Miller et al., 2015; Blodau and Deppe, 2012; Tan et al., 2018) CH_4 emissions under soil anoxic environments. This is thought to be induced by functional structures within HS that can act as electron acceptors (i.e. hydrogen during methanogenesis) (Martinez et al., 2013; Terry et al., 2018b). However, there is also contradictory evidence which found that HS facilitated CH_4 production in anoxic paddy soils (Zhou et al., 2014).

5.2 Biochar

Biochar, a pyrolysed thermal degraded form of black-C, is obtained by heating (350–600°C) plant biomass residues under oxygen-limited conditions (Cha et al., 2016). Biochar is mostly comprised of recalcitrant-C, but also contains an array of inorganic nutrients (Joseph et al., 2018). In general, biochar is characterised by a porous structure, large surface area and high mineral content (Cha et al., 2016), characteristics which depend on the original biomass or feedstock source. Similarly, other reports have found biochar to exhibit high ion exchange and absorption properties (Yuan et al., 2017) that make it suitable as a soil amendment, water and air scrubber and a detoxifying agent (Tawheed and Baowei, 2017). The ability of biochar to reduce N_2O and CH_4 emissions in cultivated fields (Karhu et al., 2011; Cayuela et al., 2014) and act as a detoxifying agent has sparked interest in their use as a feed additive for ruminants.

5.2.1 Ruminant studies

The original proposal for using biochar as a dietary CH_4 mitigation tool comes from Leng et al. (2012), who hypothesised that, due to its porous nature, biochar may promote the formation of biofilms or induce interspecies electron transfer within the rumen. Additionally, biochar has been hypothesised to increase the population of CH_4-oxidising bacteria, methanotrophs, within the rumen; even though it has been shown *in vitro* that methanotrophs account for a small proportion of microbial activity (Kajikawa et al., 2003). Absorption of CH_4 by biochar was also thought to play a significant role in the reduction of CH_4 production. However, as described by Saleem et al. (2018), it seems

unlikely that biochar (typically added at 0.5–2% of diet DM) would absorb the large volume of CH_4 that is typically produced by rumen methanogens.

Leng et al. (2012) used 12 'yellow' calves from Laos to investigate the effect of both nitrate and biochar produced from rice husks on CH_4 production and growth performance. Methane production was reduced by 22% with biochar and live weight gain was increased by an almost unbelievable 25%. However, these results need to be interpreted with caution as they did not use continuous calorimetry for CH_4 measurements. Saleem et al. (2018) examined a pine-based biochar using the Rusitec and found that CH_4 (g/g DM digested) was decreased by 22.4% when biochar was fed at up to 2.0% of diet DM. Similarly, total VFA, NH_3-N and nutrient disappearance of DM, OM, CP, NDF and ADF were all improved. In contrast, using the same product, Terry et al. (2019) found that VFA, CH_4 production and apparent digestibility were not affected by biochar when it comprised up to 2.0% of dietary DM of a barley silage-based diet fed to beef heifers.

5.2.2 Manure

Joseph et al. (2015) found that the manure of cows fed a mixture of molasses (0.1 kg day^{-1}) and high temperature jarrah wood (*Eucalyptus marginata*) biochar (0.33 kg day^{-1}) improved soil properties and increased OM sequestration (0–40 cm) in an Australian Chromosol. This response was attributed to enhanced biochar N and P adsorption from the cow's gut and limited transformation of recalcitrant-C upon digestion, increasing stable C which enhanced soil fertility.

Yuan et al. (2017) employed rice (Oryza sativa) husk-derived biochar as co-compositing element for chicken manure. Compared to compost, biochar reduced soil CO_2 and N_2O emissions by 35% and 27%, respectively. They hypothesised that biochar increased OM stabilisation through the soil profile and impacted denitrifying bacterial populations as evidenced by an increase in the archaeal genes encoding for enzymes related to bacterial nitrification. The abundance of 16S rRNA was decreased in biochar amended manure. Similarly, biochar has been shown to effectively retain NH_3 and N_2O in co-composted poultry litter (Steiner et al., 2010). Jia et al. (2016) found rice hull biochar decreased the peak rate of N_2O emissions by 60% compared to pure compost when it was used as a bulking agent.

From available research (Atkinson et al., 2010; Jia et al., 2016; Steiner et al., 2010; Yuan et al., 2017), it seems that biochar amended compost is an effective mitigation strategy for composted chicken manure. More research is required to evaluate whether the same responses occur with composted beef cattle manure. It will also be important to evaluate how feeding biochar to ruminants alters post-excretion GHG emissions from manure.

From current research, it can be concluded that HS and biochar are largely ineffective at mitigating ruminal CH_4 production. Although HS did not mitigate CH_4 production in ruminants, there may still be associated health or metabolic benefits as shown by an increase in average daily gain in goats (Agazzi et al., 2007) and N retention in beef heifers (Terry et al., 2018b). However, assessment using a performance trial is needed to support this evaluation. Although biochar has been shown to mitigate enteric CH_4 *in vitro*, this response has not been confirmed *in vivo*. The current mechanisms proposed as to how biochar may mitigate enteric CH_4 are not supported by current *in vivo* findings, and further assessment of how it may alter the rumen microbiome is required. Limited studies have suggested that both HS and biochar are successful at mitigating manure emissions, and perhaps at defined levels, feeding these additives will improve the nutrient composition of manure as a fertilizer without negative impacts on ruminant performance.

6 Microbial hydrogen utilisation

It is generally accepted that an increase in the partial pressure of [H] within the rumen will result in an inhibition of fermentation through reduced re-oxidisation of co-factors (Ungerfeld, 2015b). Additionally, stoichiometry shows that a decrease in enteric CH_4 production from ruminants should result in more energy for maintenance and production (Johnson and Johnson, 1995). However, these two concepts are not always observed as responses to a reduction in enteric CH_4 production.

Hydrogenotrophic archaea (*Methanobrevibacter, Methanobacterium*) are the predominant archaea (Henderson et al., 2015) and can utilise [H] or to a much lesser extent formate, as sources of electrons to reduce CO_2 to CH_4 (Richards et al., 2016). The contribution of formate to CH_4 production is estimated at 18% of CH_4 produced in the rumen (Tapio et al., 2017b). Methylotrophic methanogens (i.e. *Methanosarcinales, Methanosphaera* and *Methanomassiliicoccaceae*) are less abundant and can utilise methanol and methylamines to produce CH_4 (Huws et al., 2018). The acetoclastic pathway can also result in the formation of CH_4 from acetate, but the *Methanosarcinales* spp. which utilise this pathway have a slow growth rate and are not prominent within the mature rumen (Friedman et al., 2017b). Whilst the majority of [H] is utilised by archaea, several other means of [H] disposal may occur in the rumen, including the use of reducing equivalents to reduce sulphate and nitrate (NO_3^-) as well as reductive acetogenesis, propionogenesis and the synthesis of microbial biomass.

Sulphate and NO_3^- reduction reactions are more thermodynamically favourable than the reduction of CO_2 (Morgavi et al., 2010; Haque, 2018) and sulphate and NO_3^- reducing bacteria have been shown to outcompete methanogens in anoxic environments (Scheid et al., 2003). Reductive

acetogenesis is not as thermodynamically favourable as methanogenesis and it is unlikely that the rumen would establish a dominant and sustained microbial population capable of this process (Fonty et al., 2007; Friedman et al., 2017b). However, if a reductive acetogen was developed that could exist naturally in the rumen and utilise only [H], rather than sugars (obligate hydrogenotroph), it could potentially act as a successful [H] sink and suppress methanogenesis (Ungerfeld, 2015a).

Feed additives which redirect [H] towards an alternative metabolic sink represent a new avenue for investigation. Martinez-Fernandez et al. (2017) supplemented Brahman steers with the antimethanogenic compound, chloroform. Half of the steers were also administered phloroglucinol, an intermediate metabolite of flavonoid degradation which through the utilisation [H] and formate can form acetate. Phloroglucinol resulted in an increase in acetate production, *Prevotella*, *Ruminococcus* and *Fibrobacter* abundance as well as a decrease in H_2 and formate production. This study was the first *in vivo* trial to demonstrate that [H] can be redirected towards the reduction phloroglucinol as a means of inhibiting methanogenesis. Further studies examining the redirection of [H] during the inhibition of methanogenesis, and how this alters the rumen microbiome are needed. For example, 3-NOP can successfully decrease methanogenesis; however, excess [H] is not captured through other reductive process and the H_2 emission is increased. Redirection of this [H] into a usable metabolite would result in a further reduction of CH_4 production and CH_4 intensity, potentially without the loss of energy in the form of H_2.

It has been suggested that, as within other anaerobic environments, there is a balance between methane producing and methane utilising microbes. Methanotrophs are specific archaea or bacteria which can metabolise CH_4 in the presence of oxygen (Leng, 2014). However, CH_4 can also be anaerobically oxidised utilising existing oxygen within sulphate, metal oxides and nitrate (Joye, 2012). Their presence or importance in the ruminal environment is a matter of debate.

In an artificial rumen system, it was found that only 0.2–0.5% of CH_4 produced was oxidised by coupling with sulphate reduction (Kajikawa et al., 2003). However, it is theorised that methanotrophs are more likely to colonise the rumen wall due to diffusion of oxygen from the bloodstream. A metagenomic analysis of the microbial populations in beef cattle rumen did not detect methanotrophs (Wallace et al., 2015). Using methanotroph-specific primers, Mitsumori et al. (2002) suggested that methanotrophs exist in both ruminal fluid and the biofilm attached to the rumen wall, but only type I methanotrophs were detected. Type I methanotrophs include *Methylomonas*, *Methylobacter*, *Methylomicrobium* and *Methylococcus* which utilise the ribulose monophosphate pathway to assimilate carbon. Type II includes *Methylocystis* and *Methylosinus*, which use the serine pathway to assimilate carbon (Mitsumori et al., 2002). Jin et al.

(2017) found that the *Methylococcaceae* family was dominant in solid, liquid and rumen wall-associated populations. In rumen batch culture, Liu et al. (2017) found that the addition of NO_3^- decreased methanogenesis and increased the phylum NC10. The NC10 bacteria are the only known bacteria that are capable of anaerobic oxidisation of CH_4. The bacterium *Methoxymirabilis oxyfera* converts NO_2^- to nitric oxide and then dismutates nitric oxide into nitrogen and oxygen, using the resulting O_2 to support CH_4 oxidation (He et al., 2016; Joye, 2012). All other microbes with the ability to anaerobically oxidise CH_4 are archaea. Klieve et al. (2012) identified that rumen contents of cattle had mcrA gene sequences relating to CH_4 oxidising archaea, represented by archaea which have been shown to anaerobically catabolise methane using sulphate reduction in sediments from the Gulf of Mexico (Lloyd et al., 2006).

Parmar et al. (2015) established that Type I methanotrophs were more abundant in 50:50 forage-to-concentrate diets, whereas Type II increased in a complete forage diet. The enzyme formate dehydrogenase, which oxidises formate, was increased within the high-forage diet, a finding consistent with an increase in Type II methanotrophs. Auffret et al. (2018) also identified the presence of three methanotrophic bacteria including *Methylobacterium*, *Methylomonas* and *Methylomicrobium* in low abundance (0.1 ± 0.01%) in the rumen of beef steers. The *Methylomonas* were more abundant and negatively correlated with CH_4 emissions. The overall diversity of methanotrophs was greater in high CH_4 emitters as compared to low emitters. The inconsistency in the identification of methanotrophs in the rumen could reflect the lack of sequencing depth and breadth for these rare populations.

The importance of methanotrophs in a nutrient-rich environment like the rumen is questionable. However, the use of CH_4 by methanotrophs may partially account for [H] that is not stoichiometrically accounted for when CH_4 inhibitors such as NO_3^- and 3-NOP are included in ruminant diets. Methanotrophs are important in other anaerobic environments such as sediments, the oceanic seafloor and both freshwater and saline water systems (He et al., 2016), oxidising over 80% of the emitted CH_4 before it reaches the atmosphere (Cai et al., 2016). However, these are relatively stable environments, and do not experience the same passage rate or daily variation in substrate availability as within the rumen. As sequencing technologies improve our ability to delve deeper into the ruminal microbiome, a more detailed identification of methanotrophs should enhance our understanding of [H] balance in the rumen.

7 Future trends and conclusion

Presently, 3-NOP and NO_3^- can mitigate enteric CH_4 production while having little effects on GHG emissions from manure; however, the excess [H] is not completely captured in the form of reduced substrates (Table 1). Likewise,

Table 1 Summary of dietary additives and their implications for GHG mitigation from ruminant production

Dietary additive	Enteric emissions	Improvement in product	Manure emissions	Improvement in product	Interaction	Recommend
Nitro compounds						
Nitrate	↓CH_4 ↑H_2	No	N/A[a], may ↑N_2O, ↓NH_3	N/A	N/A, Likely	Yes[a]
3 – NOP	↓CH_4 ↑H_2	Inconsistent	N/A	N/A	N/A, Unlikely	Yes[a]
Secondary compounds						
Tannins	Variable, may ↓CH_4	Inconsistent, may ↓DMI	↓NO_2 ↓NH_3	Yes	Yes	Too variable
Organic carbon						
Humic substances	No	N/A	N/A[a], may ↓NH_3	N/A[a], may ↑ stable C	No	N/A
Biochar	No	N/A	N/A[a] may ↓NO_2, ↓NH_3	N/A[a], may ↑ stable C	N/A	N/A

[a] Based on limited research.
N/A = information not available.

tannins can reduce GHG emissions from manure, whereas their effect on enteric CH_4 emissions from ruminants is highly variable. Organic C additives may have potential for mitigation of manure GHG, but there is limited research to support their ability to reduce enteric CH_4 emissions.

Dietary manipulation as a mitigation strategy is thought to be the most viable method for reducing GHG emissions from ruminants. However, as highlighted, there is a balance to be met towards ensuring disrupting rumen metabolism does not cause unintended increases in GHG from manure. There are also additional considerations for how dietary changes alter the rumen microbiome and how long these changes are sustained. Investigations regarding the effects of dietary additives on both enteric and manure CH_4 emissions have reinforced the complexity of the dynamics between enteric- and manure-CH_4. Though enteric CH_4 production has a much larger CO_2-equivalent contribution to total GHG emissions, N_2O produced by manure is a more potent GHG. Therefore, when recommending GHG mitigation strategies from ruminants, it is important to validate its efficiency at a whole-farm level.

8 Where to look for further information

8.1 Further reading

Extensive review of GHG mitigation strategies from livestock production: 'Mitigation of Greenhouse gas emissions in livestock production – FAO Animal

production and Health'. Available at: http://www.fao.org/docrep/018/i3288e/i3288e00.htm.

Extensive characterisation of livestock production by region and associated GHG production: 'Assessment of greenhouse gas emissions and mitigation potential' – FAO Global Livestock Environmental Assessment Model (GLEAM). Available at: http://www.fao.org/gleam/results/en/.

A review on agricultural N cycle: Robertson and Vitousek (2009).

8.2 Key journals/conferences

The Greenhouse Gas and Animal Agriculture (GGAA) Conference is an international conference held every 3 years.

8.3 Major international research projects

Biochar Project: Assessment of the potential for adding biochar to beef cattle diets to reduce GHG emissions in agriculture: http://www.agr.gc.ca/eng/programs-and-services/agricultural-greenhouse-gases-program/approved-projects/?id=1508423883267.

9 References

Aboagye, I. A., Oba, M., Castillo, A. R., Koenig, K. M., Iwaasa, A. D. and Beauchemin, K. A. 2018. Effects of hydrolyzable tannin with or without condensed tannin on methane emissions, nitrogen use, and performance of beef cattle fed a high-forage diet. *J. Anim. Sci.* 96(12), 5276–86. doi:10.1093/jas/sky352.

Adler, A. A., Doole, G. J., Romera, A. J. and Beukes, P. C. 2015. Managing greenhouse gas emissions in two major dairy regions of New Zealand: a system-level evaluation. *Agric. Sys.* 135, 1–9. doi:10.1016/j.agsy.2014.11.007.

Agazzi, A., Cigalino, G., Mancin, G., Savoini, G. and Dell'Orto, V. 2007. Effects of dietary humates on growth and an aspect of cell-mediated immune response in newborn kids. *Small Rumin. Res.* 72(2–3), 242–5. doi:10.1016/j.smallrumres.2006.10.020.

Alves, T. P., Dall-Orsoletta, A. C. and Ribeiro-Filho, H. M. N. 2017. The effects of supplementing *Acacia mearnsii* tannin extract on dairy cow dry matter intake, milk production, and methane emission in a tropical pasture. *Trop. Anim. Health Prod.* 49(8), 1663–8. doi:10.1007/s11250-017-1374-9.

Asanuma, N., Yokoyama, S. and Hino, T. 2015. Effects of nitrate addition to a diet on fermentation and microbial populations in the rumen of goats, with special reference to *Selenomonas ruminantium* having the ability to reduce nitrate and nitrite. *Anim. Sci. J.* 86(4), 378–84. doi:10.1111/asj.12307.

Atkinson, C. J., Fitzgerald, J. D. and Hipps, N. A. 2010. Potential mechanisms for achieving agricultural benefits from biochar application to temperate soils: a review. *Plant Soil* 337(1–2), 1–18. doi:10.1007/s11104-010-0464-5.

Auffret, M. D., Stewart, R., Dewhurst, R. J., Duthie, C. A., Rooke, J. A., Wallace, R. J., Freeman, T. C., Snelling, T. J., Watson, M. and Roehe, R. 2018. Identification, comparison, and

validation of robust rumen microbial biomarkers for methane emissions using diverse *Bos taurus* breeds and basal diets. *Front. Microbiol.* 8, 2642. doi:10.3389/fmicb.2017.02642.

Bach, A., Calsamiglia, S. and Stern, M. D. 2005. Nitrogen metabolism in the rumen. *J. Dairy Sci.* 88 (Suppl. 1), E9–21. doi:10.3168/jds.S0022-0302(05)73133-7.

Beauchemin, K. A., McGinn, S. M., Martinez, T. F. and McAllister, T. A. 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. *J. Anim. Sci.* 85(8), 1990–6. doi:10.2527/jas.2006-686.

Beauchemin, K. A., Kreuzer, M., O'Mara, F. and McAllister, T. A. 2008. Nutritional management for enteric methane abatement: a review. *Aust. J. Exp. Agric.* 48(2), 21–7. doi:10.1071/EA07199.

Belanche, A., Doreau, M., Edwards, J. E., Moorby, J. M., Pinloche, E. and Newbold, C. J. 2012. Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. *J. Nutr.* 142(9), 1684–92. doi:10.3945/jn.112.159574.

Bending, G. D. and Read, D. J. 1996. Nitrogen mobilization from protein-polyphenol complex by ericoid and ectomycorrhizal fungi. *Soil Biol. Biochem.* 28(12), 1603–12. doi:10.1016/S0038-0717(96)00258-1.

Bhatta, R., Saravanan, M., Baruah, L. and Prasad, C. S. 2015. Effects of graded levels of tannin-containing tropical tree leaves on *in vitro* rumen fermentation, total protozoa and methane production. *J. Appl. Microbiol.* 118(3), 557–64. doi:10.1111/jam.12723.

Blodau, C. and Deppe, M. 2012. Humic acid addition lowers methane release in peats of the Mer Bleue bog, Canada. *Soil Biol. Biochem.* 52, 96–8. doi:10.1016/j.soilbio.2012.04.023.

Boots, B., Lillis, L., Clipson, N., Petrie, K., Kenny, D. A., Boland, T. M. and Doyle, E. 2013. Responses of anaerobic rumen fungal diversity (phylum Neocallimastigomycota) to changes in bovine diet. *J. Appl. Microbiol.* 114(3), 626–35. doi:10.1111/jam.12067.

Broderick, G. A. 2003. Effects of varying dietary protein and energy levels on the production of lactating dairy cows. *J. Dairy Sci.* 86(4), 1370–81. doi:10.3168/jds.S0022-0302(03)73721-7.

Cai, Y., Zheng, Y., Bodelier, P. L. E., Conrad, R. and Jia, Z. 2016. Conventional methanotrophs are responsible for atmospheric methane oxidation in paddy soils. *Nat. Commun.* 7, 11728. doi:10.1038/ncomms11728.

Carulla, J. E., Kreuzer, M., Machmüller, A. and Hess, H. D. 2005. Supplementation of *Acacia mearnsii* tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. *Aust. J. Agric. Res.* 56(9), 961–70. doi:10.1071/AR05022.

Castillo-Lopez, E., Ramirez Ramirez, H. A., Klopfenstein, T. J., Anderson, C. L., Aluthge, N. D., Fernando, S. C., Jenkins, T. and Kononoff, P. J. 2014. Effect of feeding dried distillers grains with solubles on ruminal biohydrogenation, intestinal fatty acid profile, and gut microbial diversity evaluated through DNA pyro-sequencing. *J. Anim. Sci.* 92(2), 733–43. doi:10.2527/jas.2013-7223.

Castillo-Lopez, E., Jenkins, C. J. R., Aluthge, N. D., Tom, W., Kononoff, P. J. and Fernando, S. C. 2017. The effect of regular or reduced-fat distillers grains with solubles on rumen methanogenesis and the rumen bacterial community. *J. Appl. Microbiol.* 123(6), 1381–95. doi:10.1111/jam.13583.

Cayuela, M. L., van Zwieten, L., Singh, B. P., Jeffery, S., Roig, A. and Sánchez-Monedero, M. A. 2014. Biochar's role in mitigating soil nitrous oxide emissions: a review and meta-analysis. *Agric. Ecosyst. Environ.* 191, 5–16. doi:10.1016/j.agee.2013.10.009.

Cha, J. S., Park, S. H., Jung, S.-C., Ryu, C., Jeon, J.-K., Shin, M. and Park, Y. 2016. Production and utilization of biochar: a review. *J. Ind. Eng. Chem.* 40, 1–15. doi:10.1016/j.jiec.2016.06.002.

Cottle, D. J., Nolan, J. V. and Wiedemann, S. G. 2011. Ruminant enteric methane mitigation: a review. *Anim. Prod. Sci.* 51(6), 491–514. doi:10.1071/AN10163.

Coyne, M. S. 2008. Biological denitrification. In: Schepers, J. S. and Raun, W. R. (Eds), *Nitrogen in Agricultural Systems*. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI, pp. 201–53.

Degirmenci, T. 2012. Effects of diets containing humic acid on the milk yield, milk composition and blood metabolites in Saanen goats. *Res. J. Anim. Sci.* 6(1), 4–7. doi:10.3923/rjnasci.2012.4.7.

Dijkstra, J., Oenema, O., van Groenigen, J. W., Spek, J. W., van Vuuren, A. M. and Bannink, A. 2013. Diet effects on urine composition of cattle and N_2O emissions. *Animal* 7, 292–302. doi:10.1017/S1751731113000578.

Duin, E. C., Wagner, T., Shima, S., Prakash, D., Cronin, B., Yáñez-Ruiz, D. R., Duval, S., Rümbeli, R., Stemmler, R. T., Thauer, R. K. and Kindermann, M. 2016. Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol. *Proc. Natl. Acad. Sci. U.S.A.* 113(22), 6172–7. doi:10.1073/pnas.1600298113.

El-Zaiat, H. M., Araujo, R. C., Soltan, Y. A., Morsy, A. S., Louvandini, H., Pires, A. V., Patino, H. O., Correa, P. S. and Abdalla, A. L. 2014. Encapsulated nitrate and cashew nut shell liquid on blood and rumen constituents, methane emission, and growth performance of lambs. *J. Anim. Sci.* 92(5), 2214–24. doi:10.2527/jas.2013-7084.

El-Zaiat, H. M., Morsy, A. S., El-Wakeel, E. A., Anwer, M. M. and Sallam, S. M. 2018. Impact of humic acid as an organic additive on ruminal fermentation constituents, blood parameters and milk production in goats and their kids growth rate. *J. Anim. Feed Sci.* 27(2), 105–13. doi:10.22358/jafs/92074/2018.

Enjalbert, F., Combes, S., Zened, A. and Meynadier, A. 2017. Rumen microbiota and dietary fat: a mutual shaping. *J. Appl. Microbiol.* 123(4), 782–97. doi:10.1111/jam.13501.

Erisman, J. W., Galloway, J. N., Seitzinger, S., Bleeker, A., Dise, N. B., Petrescu, A. M., Leach, A. M. and de Vries, W. 2013. Consequences of human modification of the global nitrogen cycle. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* 368(1621), 20130116. doi:10.1098/rstb.2013.0116.

Fageria, N. K. and Baligar, V. C. 2005. Enhancing nitrogen use efficiency in crop plants. In: *Advances in Agronomy*. Academic Press, pp. 97–185. doi:10.1016/S0065-2113(05)88004-6.

Firkins, J. L. and Yu, Z. 2015. RUMINANT NUTRITION SYMPOSIUM: how to use data on the rumen microbiome to improve our understanding of ruminant nutrition. *J. Anim. Sci.* 93(4), 1450–70. doi:10.2527/jas.2014-8754.

Fonty, G., Joblin, K., Chavarot, M., Roux, R., Naylor, G. and Michallon, F. 2007. Establishment and development of ruminal hydrogenotrophs in methanogen-free lambs. *Appl. Environ. Microbiol.* 73(20), 6391–403. doi:10.1128/AEM.00181-07.

Friedman, N., Shriker, E., Gold, B., Durman, T., Zarecki, R., Ruppin, E. and Mizrahi, I. 2017a. Diet-induced changes of redox potential underlie compositional shifts in the rumen archaeal community. *Environ. Microbiol.* 19(1), 174–84. doi:10.1111/1462-2920.13551.

Friedman, N., Jami, E. and Mizrahi, I. 2017b. Compositional and functional dynamics of the bovine rumen methanogenic community across difrerent developmental stages. *Environ. Microbiol.* 19(8), 3365–73. doi:10.1111/1462-2920.13846.

Galloway, J. N., Dentener, F. J., Capone, D. G., Boyer, E. W., Howarth, R. W., Seitzinger, S. P., Asner, G. P., Cleveland, C. C., Green, P. A., Holland, E. A., Karl, D. M., Michaels, A. F., Porter, J. H., Townsend, A. R. and Vöosmarty, C. J. 2004. Nitrogen cycles: past, present, and future. *Biogeochemistry* 70(2), 153–226. doi:10.1007/s10533-004-0370-0.

Gautam, D. P., Rahman, S., Borhan, M. S. and Engel, C. 2016. The effect of feeding high fat diet to beef cattle on manure composition and gaseous emission from a feedlot pen surface. *J. Anim. Sci. Technol.* 58, 22–. doi:10.1186/s40781-016-0104-6.

Gerber, P. J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A. and Tempio, G. 2013. *Tackling Climate Change through Livestock: a Global Assessment of Emissions and Mitigation Opportunities*. Food and Agriculture Organization of the United Nations, Rome.

Gonçalves, R., Mateus, N. and de Freitas, V. 2011. Inhibition of α-amylase activity by condensed tannins. *Food Chem.* 125(2), 665–72. doi:10.1016/j.foodchem.2010.09.061.

Grainger, C., Clarke, T., Auldist, M. J., Beauchemin, K. A., McGinn, S. M., Waghorn, G. C. and Eckard, R. J. 2009. Potential use of *Acacia mearnsii* condensed tannins to reduce methane emissions and nitrogen excretion from grazing dairy cows. *Can. J. Anim. Sci.* 89(2), 241–51. doi:10.4141/CJAS08110.

Griffin, W. A., Bremer, V. R., Klopfenstein, T. J., Stalker, L. A., Lomas, L. W., Moyer, J. L. and Erickson, G. E. 2012. A meta-analysis evaluation of supplementing dried distillers grains plus solubles to cattle consuming forage-based diets 1. *Prof. Anim. Sci.* 28(3), 306–12. doi:10.15232/S1080-7446(15)30360-0.

Guyader, J., Eugene, M., Noziere, P., Morgavi, D. P., Doreau, M. and Martin, C. 2014. Influence of rumen protozoa on methane emission in ruminants: a meta-analysis approach. *Animal* 8(11), 1816–25. doi:10.1017/S1751731114001852.

Guyader, J., Ungerfeld, E. M. and Beauchemin, K. A. 2017. Redirection of metabolic hydrogen by inhibiting methanogenesis in the rumen simulation technique (RUSITEC). *Front. Microbiol.* 8, 393. doi:10.3389/fmicb.2017.00393.

Haisan, J., Sun, Y., Guan, L. L., Beauchemin, K. A., Iwaasa, A., Duval, S., Barreda, D. R. and Oba, M. 2014. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. *J. Dairy Sci.* 97(5), 3110–9. doi:10.3168/jds.2013-7834.

Haisan, J., Sun, Y., Guan, L., Beauchemin, K. A., Iwaasa, A., Duval, S., Kindermann, M., Barreda, D. R. and Oba, M. 2017. The effects of feeding 3-nitrooxypropanol at two doses on milk production, rumen fermentation, plasma metabolites, nutrient digestibility, and methane emissions in lactating Holstein cows. *Anim. Prod. Sci.* 57(2), 282–9. doi:10.1071/AN15219.

Hales, K. E., Cole, N. A. and Macdonald, J. C. 2013. Effects of increasing concentrations of wet distillers grains with solubles in steam-flaked, corn-based diets on energy metabolism, carbon-nitrogen balance, and methane emissions of cattle. *J. Anim. Sci.* 91(2), 819–28. doi:10.2527/jas.2012-5418.

Hall, M. B. and Huntington, G. B. 2008. Nutrient synchrony: sound in theory, elusive in practice. *J. Anim. Sci.* 86(14 Suppl), E287–92. doi:10.2527/jas.2007-0516.

Halvorson, J. J., Kronberg, S. L. and Hagerman, A. E. 2017. Effects of dietary tannins on total and extractable nutrients from manure. *J. Anim. Sci.* 95(8), 3654–65. doi:10.2527/jas.2016.1129.

Haque, M. N. 2018. Dietary manipulation: a sustainable way to mitigate methane emissions from ruminants. *J. Anim. Sci. Technol.* 60, 15–. doi:10.1186/s40781-018-0175-7.

Hartmann, T. 2007. From waste products to ecochemicals: fifty years research of plant secondary metabolism. *Phytochemistry* 68(22–24), 2831–46. doi:10.1016/j.phytochem.2007.09.017.

He, Z., Cai, C., Wang, J., Xu, X., Zheng, P., Jetten, M. S. and Hu, B. 2016. A novel denitrifying methanotroph of the NC10 phylum and its microcolony. *Sci. Rep.* 6, 32241. doi:10.1038/srep32241.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Global Rumen Census Collaborators and Janssen, P. H. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5, 14567. doi:10.1038/srep14567.

Hess, H. D., Tiemann, T. T., Noto, F., Carulla, J. E. and Kreuzer, M. 2006. Strategic use of tannins as means to limit methane emission from ruminant livestock. *Int. Congr. Ser.* 1293, 164–7. doi:10.1016/j.ics.2006.01.010.

Hook, S. E., Steele, M. A., Northwood, K. S., Wright, A. D. and McBride, B. W. 2011. Impact of high-concentrate feeding and low ruminal pH on methanogens and protozoa in the rumen of dairy cows. *Microb. Ecol.* 62(1), 94–105. doi:10.1007/s00248-011-9881-0.

Hristov, A. N., Callaway, T. R., Lee, C. and Dowd, S. E. 2012. Rumen bacterial, archaeal, and fungal diversity of dairy cows in response to ingestion of lauric or myristic acid. *J. Anim. Sci.* 90(12), 4449–57. doi:10.2527/jas.2011-4624.

Hristov, A. N., Oh, J., Lee, C., Meinen, R., Montes, F., Ott, T., Firkins, J., Rotz, A., Dell, C., Adesogan, A., Yang, W., Tricarico, J., Kebreab, E., Waghorn, G., Dijkstra, J. and Oosting, S. 2013. *Mitigation of Greenhouse Gas Emissions in Livestock Production – A Review of Technical Options for non-CO2 Emissions*. Gerber, P. J., Henderson, B. and Makkar, H. P. S. (Eds). Food and Agriculture Organization, Rome, Italy.

Hristov, A. N., Oh, J., Giallongo, F., Frederick, T. W., Harper, M. T., Weeks, H. L., Branco, A. F., Moate, P. J., Deighton, M. H., Williams, S. R., Kindermann, M. and Duval, S. 2015. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. *Proc. Natl. Acad. Sci. U.S.A.* 112(34), 10663–8. doi:10.1073/pnas.1504124112.

Hünerberg, M., McGinn, S. M., Beauchemin, K. A., Okine, E. K., Harstad, O. M. and McAllister, T. A. 2013a. Effect of dried distillers grains plus solubles on enteric methane emissions and nitrogen excretion from growing beef cattle1. *J. Anim. Sci.* 91(6), 2846–57. doi:10.2527/jas.2012-5564.

Hünerberg, M., McGinn, S. M., Beauchemin, K. A., Okine, E. K., Harstad, O. M. and McAllister, T. A. 2013b. Effect of dried distillers' grains with solubles on enteric methane emissions and nitrogen excretion from finishing beef cattle. *Can. J. Anim. Sci.* 93(3), 373–85. doi:10.4141/cjas2012-151.

Hünerberg, M., Little, S. M., Beauchemin, K. A., McGinn, S. M., O'Connor, D., Okine, E. K., Harstad, O. M., Kröbel, R. and McAllister, T. A. 2014. Feeding high concentrations of corn dried distillers' grains decreases methane, but increases nitrous oxide emissions from beef cattle production. *Agric. Sys.* 127, 19–27. doi:10.1016/j.agsy.2014.01.005.

Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., Muñoz-Tamayo, R., Forano, E., Waters, S. M., Hess, M., Tapio, I., Smidt, H., Krizsan, S. J., Yáñez-Ruiz, D. R., Belanche, A., Guan, L., Gruninger, R. J., McAllister, T. A., Newbold, C. J., Roehe, R., Dewhurst, R. J., Snelling, T. J., Watson, M., Suen, G., Hart, E. H., Kingston-Smith, A. H., Scollan, N. D., do Prado, R. M., Pilau, E. J., Mantovani, H. C., Attwood, G. T., Edwards, J. E., McEwan, N. R., Morrisson, S., Mayorga, O. L., Elliott, C. and Morgavi, D. P. 2018. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Front. Microbiol.* 9, 2161. doi:10.3389/fmicb.2018.02161.

IPCC. 2006. *IPCC Guidelines for National Greenhouse Gas Inventories – A Primer*. Institute for Global Environmental Strategies, Japan.

Ishaq, S. L., AlZahal, O., Walker, N. and McBride, B. 2017. An investigation into rumen fungal and protozoal diversity in three rumen fractions, during high-fiber or grain-induced sub-acute ruminal acidosis conditions, with or without active dry yeast supplementation. *Front. Microbiol.* 8, 1943. doi:10.3389/fmicb.2017.01943.

Iwamoto, M., Asanuma, N. and Hino, T. 2002. Ability of *Selenomonas ruminantium*, *Veillonella parvula*, and *Wolinella succinogenes* to reduce nitrate and nitrite with special reference to the suppression of ruminal methanogenesis. *Anaerobe* 8(4), 209–15. doi:10.1006/anae.2002.0428.

Jia, X., Wang, M., Yuan, W., Shah, S., Shi, W., Meng, X., Ju, X. and Yang, B. 2016. N_2O emission and nitrogen transformation in chicken manure and biochar co-composting. *Trans. ASABE* 59(5), 1277–83. doi:10.13031/trans.59.11685.

Jin, D., Zhao, S., Zheng, N., Bu, D., Beckers, Y., Denman, S. E., McSweeney, C. S. and Wang, J. 2017. Differences in ureolytic bacterial composition between the rumen digesta and rumen wall based on ureC gene classification. *Front. Microbiol.* 8, 385–. doi:10.3389/fmicb.2017.00385.

Johnson, K. A. and Johnson, D. E. 1995. Methane emissions from cattle. *J. Anim. Sci.* 73(8), 2483–92. doi:10.2527/1995.7382483x.

Jordan, G., Predotova, M., Ingold, M., Goenster, S., Dietz, H., Joergensen, R. G. and Buerkert, A. 2015. Effects of activated charcoal and tannin added to compost and to soil on carbon dioxide, nitrous oxide and ammonia volatilization. *J. Plant Nutr. Soil Sci.* 178(2), 218–28. doi::doi:10.1002/jpln.201400233.

Joseph, S., Pow, D., Dawson, K., Mitchell, D. R. G., Rawal, A., Hook, J., Taherymoosavi, S., Van Zwieten, L., Rust, J., Donne, S., Munroe, P., Pace, B., Graber, E., Thomas, T., Nielsen, S., Ye, J., Lin, Y., Pan, G., Li, L. and Solaiman, Z. M. 2015. Feeding biochar to cows: an innovative solution for improving soil fertility and farm productivity. *Pedosphere* 25(5), 666–79. doi:10.1016/S1002-0160(15)30047-3.

Joseph, S., Kammann, C. I., Shepherd, J. G., Conte, P., Schmidt, H. P., Hagemann, N., Rich, A. M., Marjo, C. E., Allen, J., Munroe, P., Mitchell, D. R. G., Donne, S., Spokas, K. and Graber, E. R. 2018. Microstructural and associated chemical changes during the composting of a high temperature biochar: mechanisms for nitrate, phosphate and other nutrient retention and release. *Sci. Total Environ.* 618, 1210–23. doi:10.1016/j.scitotenv.2017.09.200.

Joye, S. B. 2012. Microbiology: a piece of the methane puzzle. *Nature* 491(7425), 538–9. doi:10.1038/nature11749.

Judy, J. V., Brown-Brandl, T. M., Fernando, S. C. and Kononoff, P. J. 2016. 1454 Manipulation of lactating dairy cows diets using reduced-fat distillers' grains, corn oil, and calcium

sulfate to reduce methane production measured by indirect calorimetry. *J. Anim. Sci.* 94(suppl_5), 706–. doi:10.2527/jam2016-1454.

Kajikawa, H., Valdes, C., Hillman, K., Wallace, R. J. and J Newbold, C. 2003. Methane oxidation and its coupled electron-sink reactions in ruminal fluid. *Lett. Appl. Microbiol.* 36(6), 354–7. doi:10.1046/j.1472-765X.2003.01317.x.

Karhu, K., Mattila, T., Bergström, I. and Regina, K. 2011. Biochar addition to agricultural soil increased CH_4 uptake and water holding capacity – results from a short-term pilot field study. *Agric. Ecosyst. Environ.* 140(1–2), 309–13. doi:10.1016/j.agee.2010.12.005.

Kim, J. N., Méndez-García, C., Geier, R. R., Iakiviak, M., Chang, J., Cann, I. and Mackie, R. I. 2017. Metabolic networks for nitrogen utilization in Prevotella ruminicola 23. *Sci. Rep.* 7(1), 7851. doi:10.1038/s41598-017-08463-3.

Klieve, A. V., Ouwerkerk, D. and Maguire, A. J. 2012. Archaea in the foregut of macropod marsupials: PCR and amplicon sequence-based observations. *J. Appl. Microbiol.* 113(5), 1065–75. doi::doi:10.1111/j.1365-2672.2012.05428.x.

Knapp, J. R., Laur, G. L., Vadas, P. A., Weiss, W. P. and Tricarico, J. M. 2014. Invited review: enteric methane in dairy cattle production: quantifying the opportunities and impact of reducing emissions. *J. Dairy Sci.* 97(6), 3231–61. doi:10.3168/jds.2013-7234.

Koenig, K. M. and Beauchemin, K. A. 2018. Effect of feeding condensed tannins in high protein finishing diets containing corn distillers grains on ruminal fermentation, nutrient digestibility, and route of nitrogen excretion in beef cattle. *J. Anim. Sci.* 96(10), 4398–413. doi:10.1093/jas/sky273.

Koenig, K. M., Beauchemin, K. A. and McGinn, S. M. 2018. Feeding condensed tannins to mitigate ammonia emissions from beef feedlot cattle fed high-protein finishing diets containing distillers grains. *J. Anim. Sci.* 96(10), 4414–30. doi:10.1093/jas/sky274.

Koneswaran, G. and Nierenberg, D. 2008. Global farm animal production and global warming: impacting and mitigating climate change. *Environ. Health Perspect.* 116(5), 578–82. doi:10.1289/ehp.11034.

Kraus, T. E. C., Zasoski, R. J., Dahlgren, R. A., Horwath, W. R. and Preston, C. M. 2004. Carbon and nitrogen dynamics in a forest soil amended with purified tannins from different plant species. *Soil Biol. Biochem.* 36(2), 309–21. doi:10.1016/j.soilbio.2003.10.006.

Kumar, S., Indugu, N., Vecchiarelli, B. and Pitta, D. W. 2015. Associative patterns among anaerobic fungi, methanogenic archaea, and bacterial communities in response to changes in diet and age in the rumen of dairy cows. *Front. Microbiol.* 6, 781–. doi:10.3389/fmicb.2015.00781.

Lamar, R. T., Olk, D. C., Mayhew, L. and Bloom, P. R. 2014. A new standardized method for quantification of humic and fulvic acids in humic ores and commercial products. *J. AOAC Int.* 97(3), 721–30. doi:10.5740/jaoacint.13-393.

Latham, E. A., Anderson, R. C., Pinchak, W. E. and Nisbet, D. J. 2016. Insights on alterations to the rumen ecosystem by nitrate and nitrocompounds. *Front. Microbiol.* 7, 228. doi:10.3389/fmicb.2016.00228.

Lee, C. and Beauchemin, K. A. 2014. A review of feeding supplementary nitrate to ruminant animals: nitrate toxicity, methane emissions, and production performance. *Can. J. Anim. Sci.* 94(4), 557–70. doi:10.4141/cjas-2014-069.

Lee, C., Araujo, R. C., Koenig, K. M. and Beauchemin, K. A. 2015a. Effects of encapsulated nitrate on eating behavior, rumen fermentation, and blood profile of beef heifers fed restrictively or ad libitum. *J. Anim. Sci.* 93(5), 2405–18. doi:10.2527/jas.2014-8851.

Lee, C., Araujo, R. C., Koenig, K. M. and Beauchemin, K. A. 2015b. Effects of encapsulated nitrate on enteric methane production and nitrogen and energy utilization in beef heifers. *J. Anim. Sci.* 93(5), 2391–404. doi:10.2527/jas.2014-8845.

Leng, R. A. 2014. Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation. *Anim. Prod. Sci.* 54(5), 519–43. doi:10.1071/AN13381.

Leng, R., Preston, T. and Inthapanya, S. 2012. Biochar reduces enteric methane and improves growth and feed conversion in local "Yellow" cattle fed cassava root chips and fresh cassava foliage. *Livest. Res. Rural Dev.* 24. Available at: http://www.lrrd.org/lrrd24/11/leng24199.htm.

Li, L., Davis, J., Nolan, J. and Hegarty, R. 2012. An initial investigation on rumen fermentation pattern and methane emission of sheep offered diets containing urea or nitrate as the nitrogen source. *Anim. Prod. Sci.* 52(7), 653–8. doi:10.1071/AN11254.

Li, F., Wang, Z., Dong, C., Li, F., Wang, W., Yuan, Z., Mo, F. and Weng, X. 2017. Rumen bacteria communities and performances of fattening lambs with a lower or greater subacute ruminal acidosis risk. *Front. Microbiol.* 8, 2506. doi:10.3389/fmicb.2017.02506.

Liu, L., Xu, X., Cao, Y., Cai, C., Cui, H. and Yao, J. 2017. Nitrate decreases methane production also by increasing methane oxidation through stimulating NC10 population in ruminal culture. *AMB Express* 7(1), 76–. doi:10.1186/s13568-017-0377-2.

Lloyd, K. G., Lapham, L. and Teske, A. 2006. An anaerobic methane-oxidizing community of ANME-1b archaea in hypersaline Gulf of Mexico sediments. *Appl. Environ. Microbiol.* 72(11), 7218–30. doi:10.1128/AEM.00886-06.

Lopes, J. C., de Matos, L. F., Harper, M. T., Giallongo, F., Oh, J., Gruen, D., Ono, S., Kindermann, M., Duval, S. and Hristov, A. N. 2016. Effect of 3-nitrooxypropanol on methane and hydrogen emissions, methane isotopic signature, and ruminal fermentation in dairy cows. *J. Dairy Sci.* 99(7), 5335–44. doi:10.3168/jds.2015-10832.

Lyons, T., Boland, T., Storey, S. and Doyle, E. 2017. Linseed oil supplementation of lambs' diet in early life leads to persistent changes in rumen microbiome structure. *Front. Microbiol.* 8, 1656. doi:10.3389/fmicb.2017.01656.

Maeda, K., Hanajima, D., Toyoda, S., Yoshida, N., Morioka, R. and Osada, T. 2011. Microbiology of nitrogen cycle in animal manure compost. *Microb. Biotechnol.* 4(6), 700–9. doi:10.1111/j.1751-7915.2010.00236.x.

Maeda, K., Spor, A., Edel-Hermann, V., Heraud, C., Breuil, M. C., Bizouard, F., Toyoda, S., Yoshida, N., Steinberg, C. and Philippot, L. 2015. N_2O production, a widespread trait in fungi. *Sci. Rep.* 5, 9697–. doi:10.1038/srep09697.

Mao, S. Y., Huo, W. J. and Zhu, W. Y. 2016. Microbiome-metabolome analysis reveals unhealthy alterations in the composition and metabolism of ruminal microbiota with increasing dietary grain in a goat model. *Environ. Microbiol.* 18(2), 525–41. doi:10.1111/1462-2920.12724.

Martin, C., Ferlay, A., Mosoni, P., Rochette, Y., Chilliard, Y. and Doreau, M. 2016. Increasing linseed supply in dairy cow diets based on hay or corn silage: effect on enteric methane emission, rumen microbial fermentation, and digestion. *J. Dairy Sci.* 99(5), 3445–56. doi:10.3168/jds.2015-10110.

Martinez, C. M., Alvarez, L. H., Celis, L. B. and Cervantes, F. J. 2013. Humus-reducing microorganisms and their valuable contribution in environmental processes. *Appl. Microbiol. Biotechnol.* 97(24), 10293–308. doi:10.1007/s00253-013-5350-7.

Martínez-Fernández, G., Abecia, L., Arco, A., Cantalapiedra-Hijar, G., Martín-García, A. I., Molina-Alcaide, E., Kindermann, M., Duval, S. and Yáñez-Ruiz, D. R. 2014. Effects of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal fermentation, microbial abundance, and methane emissions in sheep. *J. Dairy Sci.* 97(6), 3790–9. doi:10.3168/jds.2013-7398.

Martinez-Fernandez, G., Denman, S. E., Yang, C., Cheung, J., Mitsumori, M. and McSweeney, C. S. 2016. Methane inhibition alters the microbial community, hydrogen flow, and fermentation response in the rumen of cattle. *Front. Microbiol.* 7, 1122–. doi:10.3389/fmicb.2016.01122.

Martinez-Fernandez, G., Denman, S. E., Cheung, J. and McSweeney, C. S. 2017. Phloroglucinol degradation in the rumen promotes the capture of excess hydrogen generated from methanogenesis inhibition. *Front. Microbiol.* 8, 1871. doi:10.3389/fmicb.2017.0187.

Martinez-Fernandez, G., Duval, S., Kindermann, M., Schirra, H. J., Denman, S. E. and McSweeney, C. S. 2018. 3-NOP vs. halogenated compound: methane production, ruminal fermentation and microbial community response in forage fed cattle. *Front. Microbiol.* 9, 1582. doi:10.3389/fmicb.2018.01582.

Masoom, H., Courtier-Murias, D., Farooq, H., Soong, R., Kelleher, B. P., Zhang, C., Maas, W. E., Fey, M., Kumar, R., Monette, M., Stronks, H. J., Simpson, M. J. and Simpson, A. J. 2016. Soil organic matter in its native state: unravelling the most complex biomaterial on earth. *Environ. Sci. Technol.* 50(4), 1670–80. doi:10.1021/acs.est.5b03410.

McAllister, T. A., Bae, H. D., Yanke, L. J., Cheng, K. J. and Muir, A. 1994. Effect of condensed tannins from birdsfoot trefoil on endoglucanase activity and the digestion of cellulose filter paper by ruminal fungi. *Can. J. Microbiol.* 40(4), 298–305. doi:10.1139/m94-048.

McGinn, S. M., Chung, Y.-H., Beauchemin, K. A., Iwaasa, A. D. and Grainger, C. 2009. Use of corn distillers' dried grains to reduce enteric methane loss from beef cattle. *Can. J. Anim. Sci.* 89(3), 409–13. doi:10.4141/CJAS08133.

McMurphy, C. P., Duff, G. C., Harris, M. A., Sanders, S. R., Chirase, N. K., Bailey, C. R. and Ibrahim, R. M. 2009. Effect of humic/fulvic acid in beef cattle finishing diets on animal performance, ruminal ammonia and serum urea nitrogen concentration. *J. Appl. Anim. Res.* 35(2), 97–100. doi:10.1080/09712119.2009.9706995.

McSweeney, C., Palmer, B., Kennedy, P. and Krause, D. 1998. Effect of *Calliandra tannins* on rumen microbial function. *Anim. Prod. Aust* 22, 289–.

McSweeney, C. S., Palmer, B., McNeill, D. M. and Krause, D. O. 2001. Microbial interactions with tannins: nutritional consequences for ruminants. *Anim. Feed Sci. Technol.* 91(1–2), 83–93. doi:10.1016/S0377-8401(01)00232-2.

Miller, K. E., Lai, C.-T., Friedman, E. S., Angenent, L. T. and Lipson, D. A. 2015. Methane suppression by iron and humic acids in soils of the Arctic Coastal Plain. *Soil Biol. Biochem.* 83, 176–83. doi:10.1016/j.soilbio.2015.01.022.

Mitsumori, M., Ajisaka, N., Tajima, K., Kajikawa, H. and Kurihara, M. 2002. Detection of Proteobacteria from the rumen by PCR using methanotroph-specific primers. *Lett. Appl. Microbiol.* 35(3), 251–5. doi::doi:10.1046/j.1472-765X.2002.01172.x.

Morgavi, D. P., Forano, E., Martin, C. and Newbold, C. J. 2010. Microbial ecosystem and methanogenesis in ruminants. *Animal* 4(7), 1024–36. doi:10.1017/S1751731110000546.

Muñoz, C., Hube, S., Morales, J. M., Yan, T. and Ungerfeld, E. M. 2015. Effects of concentrate supplementation on enteric methane emissions and milk production of grazing dairy cows. *Livest. Sci.* 175, 37–46. doi:10.1016/j.livsci.2015.02.001.

Mutabaruka, R., Hairiah, K. and Cadisch, G. 2007. Microbial degradation of hydrolysable and condensed tannin polyphenol–protein complexes in soils from different land-use histories. *Soil Biol. Biochem.* 39(7), 1479–92. doi:10.1016/j.soilbio.2006.12.036.

National Research Council. 2008. *Acute Exposure Guideline Levels for Selected Airborne Chemicals: Volume 6*. Available at: https://www.ncbi.nlm.nih.gov/books/NBK207883/.

Naumann, H. D., Tedeschi, L. O., Zeller, W. E. and Huntley, N. F. 2017. The role of condensed tannins in ruminant animal production: advances, limitations and future directions. *Rev. Bras. Zootec.* 46(12), 929–49. doi:10.1590/s1806-92902017001200009.

Niu, M., Appuhamy, J. A. D. R. N., Leytem, A. B., Dungan, R. S. and Kebreab, E. 2016. Effect of dietary crude protein and forage contents on enteric methane emissions and nitrogen excretion from dairy cows simultaneously. *Anim. Prod. Sci.* 56(3). doi:10.1071/AN15498.

Nolan, J. V., Hegarty, R. S., Hegarty, J., Godwin, I. R. and Woodgate, R. 2010. Effects of dietary nitrate on fermentation, methane production and digesta kinetics in sheep. *Anim. Prod. Sci.* 50(8), 801–6. doi:10.1071/AN09211.

Norton, J. M. 2008. Nitrification in agricultural soils. In: Schepers, J. S. and Raun, W. R. (Eds), *Nitrogen in Agricultural Systems*. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI, pp. 173–99.

Oldick, B. S. and Firkins, J. L. 2000. Effects of degree of fat saturation on fiber digestion and microbial protein synthesis when diets are fed twelve times daily. *J. Anim. Sci.* 78(9), 2412–20. doi:10.2527/2000.7892412x.

Olijhoek, D. W., Hellwing, A. L. F., Brask, M., Weisbjerg, M. R., Højberg, O., Larsen, M. K., Dijkstra, J., Erlandsen, E. J. and Lund, P. 2016. Effect of dietary nitrate level on enteric methane production, hydrogen emission, rumen fermentation, and nutrient digestibility in dairy cows. *J. Dairy Sci.* 99(8), 6191–205. doi:10.3168/jds.2015-10691.

Opio, C., Gerber, P., Mottet, A., Falcucci, A., Tempio, G., MacLeod, M., Vellinga, T., Henderson, B. and Steinfeld, H. 2013. *Greenhouse Gas Emissions from Ruminant Supply Chains–A Global Life Cycle Assessment*. Food and Agriculture Organization of the United Nations (FAO), Rome.

Parmar, N. R., Nirmal Kumar, J. I. and Joshi, C. G. 2015. Exploring diet-dependent shifts in methanogen and methanotroph diversity in the rumen of Mehsani buffalo by a metagenomics approach. *Front. Life Sci.* 8(4), 371–8. doi:10.1080/21553769.2015.1063550.

Patra, A. K. and Saxena, J. 2011. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. *J. Sci. Food Agric.* 91(1), 24–37. doi:10.1002/jsfa.4152.

Patra, A. K. and Yu, Z. 2013. Effects of coconut and fish oils on ruminal methanogenesis, fermentation, and abundance and diversity of microbial populations *in vitro*. *J. Dairy Sci.* 96(3), 1782–92. doi:10.3168/jds.2012-6159.

Patra, A. K. and Yu, Z. 2014. Combinations of nitrate, saponin, and sulfate additively reduce methane production by rumen cultures in vitro while not adversely affecting feed digestion, fermentation or microbial communities. *Bioresour. Technol.* 155, 129–35. doi:10.1016/j.biortech.2013.12.099.

Plaizier, J. C., Li, S., Tun, H. M. and Khafipour, E. 2017. Nutritional models of experimentally-induced subacute ruminal acidosis (SARA) differ in their impact on rumen and hindgut bacterial communities in dairy cows. *Front. Microbiol.* 7, 2128. doi:10.3389/fmicb.2016.02128.

Ponce, C. H., Arteaga, C. and Flores, A. 2016. Effects of humic acid supplementation on pig growth performance, nitrogen digestibility, odor, and ammonia emission. *J. Anim. Sci.* 94(suppl_5), 486. doi:10.2527/jam2016-1016.

Powell, J. M., Aguerre, M. J. and Wattiaux, M. A. 2011. Dietary crude protein and tannin impact dairy manure chemistry and ammonia emissions from incubated soils. *J. Environ. Qual.* 40(6), 1767–74. doi:10.2134/jeq2011.0085.

Ramos, A. F. O., Terry, S. A., Holman, D. B., Breves, G., Pereira, L. G. R., Silva, A. G. M. and Chaves, A. V. 2018. Tucumã oil shifted ruminal fermentation, reducing methane production and altering the microbiome but decreased substrate digestibility within a RUSITEC fed a mixed hay – concentrate diet. *Front. Microbiol.* 9, 1647–. doi:10.3389/fmicb.2018.01647.

Reynolds, C. K., Humphries, D. J., Kirton, P., Kindermann, M., Duval, S. and Steinberg, W. 2014. Effects of 3-nitrooxypropanol on methane emission, digestion, and energy and nitrogen balance of lactating dairy cows. *J. Dairy Sci.* 97(6), 3777–89. doi:10.3168/jds.2013-7397.

Richards, M. A., Lie, T. J., Zhang, J., Ragsdale, S. W., Leigh, J. A. and Price, N. D. 2016. Exploring hydrogenotrophic methanogenesis: a genome scale metabolic reconstruction of Methanococcus maripaludis. *J. Bacteriol.* 198, 3379–90. doi:10.1128/JB.00571-16.

Robertson, G. P. and Vitousek, P. M. 2009. Nitrogen in agriculture: balancing the cost of an essential resource. *Annu. Rev. Environ. Resour.* 34(1), 97–125. doi:10.1146/annurev.environ.032108.105046.

Romero-Perez, A., Okine, E. K., McGinn, S. M., Guan, L. L., Oba, M., Duval, S. M., Kindermann, M. and Beauchemin, K. A. 2014. The potential of 3-nitrooxypropanol to lower enteric methane emissions from beef cattle1. *J. Anim. Sci.* 92(10), 4682–93. doi:10.2527/jas.2014-7573.

Romero-Perez, A., Okine, E. K., McGinn, S. M., Guan, L. L., Oba, M., Duval, S. M., Kindermann, M. and Beauchemin, K. A. 2015. Sustained reduction in methane production from long-term addition of 3-nitrooxypropanol to a beef cattle diet. *J. Anim. Sci.* 93(4), 1780–91. doi:10.2527/jas.2014-8726.

Saleem, A. M., Ribeiro, G. O., Yang, W. Z., Ran, T., Beauchemin, K. A., McGeough, E. J., Ominski, K. H., Okine, E. K. and McAllister, T. A. 2018. Effect of engineered biocarbon on rumen fermentation, microbial protein synthesis, and methane production in an artificial rumen (RUSITEC) fed a high forage diet. *J. Anim. Sci.* 96(8), 3121–30. doi:10.1093/jas/sky204.

Sauvant, D., Giger-Reverdin, S., Serment, A. and Broudiscou, L. 2011. Influences des régimes et de leur fermentation dans le rumen sur la production de méthane par les ruminants. *INRA Product. Anim.* 24, 433–46.

Scheid, D., Stubner, S. and Conrad, R. 2003. Effects of nitrate- and sulfate-amendment on the methanogenic populations in rice root incubations. *FEMS Microbiol. Ecol.* 43(3), 309–15. doi:10.1111/j.1574-6941.2003.tb01071.x.

Shanks, O. C., Kelty, C. A., Archibeque, S., Jenkins, M., Newton, R. J., McLellan, S. L., Huse, S. M. and Sogin, M. L. 2011. Community structures of fecal bacteria in cattle from different animal feeding operations. *Appl. Environ. Microbiol.* 77(9), 2992–3001. doi:10.1128/AEM.02988-10.

Sharma, P., Laor, Y., Raviv, M., Medina, S., Saadi, I., Krasnovsky, A., Vager, M., Levy, G. J., Bar-Tal, A. and Borisover, M. 2017. Compositional characteristics of organic matter and its water-extractable components across a profile of organically managed soil. *Geoderma* 286, 73–82. doi:10.1016/j.geoderma.2016.10.014.

Sheng, P., Ribeiro, G. O., Wang, Y. and McAllister, T. A. 2017. Humic substances supplementation reduces ruminal methane production and increases the efficiency of microbial protein synthesis in vitro. *J. Anim. Sci.* 95(suppl_4), 300. doi:10.2527/asasann.2017.613.

Shi, Y., Parker, D., Cole, N., Auvermann, B. and Mehlhorn, J. 2001. Surface amendments to minimize ammonia emissions from beef cattle feedlots. *Transactions of the ASAE* 44(3), 677–82. doi:10.13031/2013.6105.

Steiner, C., Das, K. C., Melear, N. and Lakly, D. 2010. Reducing nitrogen loss during poultry litter composting using biochar. *J. Environ. Qual.* 39(4), 1236–42. doi:10.2134/jeq2009.0337.

Stevenson, F. J. 1995. Humus Chemistry: Genesis, Composition, Reactions, Second Edition. *J. Chem. Educ.* 72, A93. doi:10.1021/ed072pA93.6.

Tan, H. Y., Sieo, C. C., Lee, C. M., Abdullah, N., Liang, J. B. and Ho, Y. W. 2011. Diversity of bovine rumen methanogens *in vitro* in the presence of condensed tannins, as determined by sequence analysis of 16S rRNA gene library. *J. Microbiol.* 49(3), 492–8. doi:10.1007/s12275-011-0319-7.

Tan, W., Jia, Y., Huang, C., Zhang, H., Li, D., Zhao, X., Wang, G., Jiang, J. and Xi, B. 2018. Increased suppression of methane production by humic substances in response to warming in anoxic environments. *J. Environ. Manage.* 206, 602–6. doi:10.1016/j.jenvman.2017.11.012.

Tapio, I., Fischer, D., Blasco, L., Tapio, M., Wallace, R. J., Bayat, A. R., Ventto, L., Kahala, M., Negussie, E., Shingfield, K. J. and Vilkki, J. 2017a. Taxon abundance, diversity, co-occurrence and network analysis of the ruminal microbiota in response to dietary changes in dairy cows. *PLoS ONE* 12(7), e0180260. doi:10.1371/journal.pone.0180260.

Tapio, I., Snelling, T. J., Strozzi, F. and Wallace, R. J. 2017b. The ruminal microbiome associated with methane emissions from ruminant livestock. *J. Anim. Sci. Biotechnol.* 8, 7. doi:10.1186/s40104-017-0141-0.

Tawheed, M. E. S. and Baowei, Z. 2017. Review paper: the fundamentals of biochar as a soil amendment tool and management in agriculture scope: an overview for farmers and gardeners. *J. Agric. Chem. Environ.* 6, 38–61. doi:10.4236/jacen.2017.61003.

Terry, S. A., Ramos, A. F. O., Holman, D. B., McAllister, T. A., Breves, G. and Chaves, A. V. 2018a. Humic substances alter ammonia production and the microbial populations within a RUSITEC fed a mixed hay – concentrate diet. *Front. Microbiol.* 9, 1410. doi:10.3389/fmicb.2018.01410.

Terry, S. A., Ribeiro, G. O., Gruninger, R. J., Hunerberg, M., Ping, S., Chaves, A. V., Burlet, J., Beauchemin, K. A. and McAllister, T. A. 2018b. Effect of humic substances on rumen fermentation, nutrient digestibility, methane emissions, and rumen microbiota in beef heifers. *J. Anim. Sci.* 96, 3863–77. doi:10.1093/jas/sky265.

Terry, S. A., Ribeiro, G. O., Gruninger, R. J., Chaves, A. V., Beauchemin, K. A., Okine, E. and McAllister, T. A. 2019. A pine enhanced biochar does not decrease enteric CH4 emissions, but alters the rumen microbiota. *Front. Vet. Sci.* 6, 308. doi:10.3389/fvets.2019.00308.

Turk, J. 2016. Meeting projected food demands by 2050: understanding and enhancing the role of grazing ruminants. *J. Anim. Sci.* 94(suppl_6), 53–62. doi:10.2527/jas.2016-0547.

Ungerfeld, E. M. 2015a. Limits to dihydrogen incorporation into electron sinks alternative to methanogenesis in ruminal fermentation. *Front. Microbiol.* 6, 1272. doi:10.3389/fmicb.2015.01272.

Ungerfeld, E. M. 2015b. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. *Front. Microbiol.* 6, 37–. doi:10.3389/fmicb.2015.00037.

Ungerfeld, E. and Kohn, R. 2006. The role of thermodynamics in the control of ruminal fermentation. In: Sejrsen, K., Hvelplund, T. and Nielsen, M. O. (Eds), *Ruminant Physiology: Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress*. Wageningen Academic Publishers, Wageningen, Netherlands, pp. 55–85.

van Wyngaard, J. D. V., Meeske, R. and Erasmus, L. J. 2018. Effect of concentrate feeding level on methane emissions, production performance and rumen fermentation of Jersey cows grazing ryegrass pasture during spring. *Anim. Feed Sci. Technol.* 241, 121–32. doi:10.1016/j.anifeedsci.2018.04.025.

van Zijderveld, S. M., Gerrits, W. J. J., Apajalahti, J. A., Newbold, J. R., Dijkstra, J., Leng, R. A. and Perdok, H. B. 2010. Nitrate and sulfate: effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. *J. Dairy Sci.* 93(12), 5856–66. doi:10.3168/jds.2010-3281.

van Zijderveld, S. M., Gerrits, W. J. J., Dijkstra, J., Newbold, J. R., Hulshof, R. B. A. and Perdok, H. B. 2011. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. *J. Dairy Sci.* 94(8), 4028–38. doi:10.3168/jds.2011-4236.

Varadyova, Z., Kisidayová, S. and Jalc, D. 2009. Effect of humic acid on fermentation and ciliate protozoan population in rumen fluid of sheep *in vitro*. *J. Sci. Food Agric.* 89(11), 1936–41. doi:10.1002/jsfa.3675.

Vyas, D., McGinn, S. M., Duval, S. M., Kindermann, M. and Beauchemin, K. A. 2016. Effects of sustained reduction of enteric methane emissions with dietary supplementation of 3-nitrooxypropanol on growth performance of growing and finishing beef cattle1. *J. Anim. Sci.* 94(5), 2024–34. doi:10.2527/jas.2015-0268.

Vyas, D., Alemu, A. W., McGinn, S. M., Duval, S. M., Kindermann, M. and Beauchemin, K. A. 2018a. The combined effects of supplementing monensin and 3-nitrooxypropanol on methane emissions, growth rate, and feed conversion efficiency in beef cattle fed high-forage and high-grain diets. *J. Anim. Sci.* 96(7), 2923–38. doi:10.1093/jas/sky174.

Vyas, D., McGinn, S. M., Duval, S. M., Kindermann, M. K. and Beauchemin, K. A. 2018b. Optimal dose of 3-nitrooxypropanol for decreasing enteric methane emissions from

beef cattle fed high-forage and high-grain diets. *Anim. Prod. Sci.* 58(6), 1049–55. doi:10.1071/AN15705.

Waghorn, G. 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production–progress and challenges. *Anim. Feed Sci. Technol.* 147(1–3), 116–39. doi:10.1016/j.anifeedsci.2007.09.013.

Wallace, R. J., Rooke, J. A., McKain, N., Duthie, C. A., Hyslop, J. J., Ross, D. W., Waterhouse, A., Watson, M. and Roehe, R. 2015. The rumen microbial metagenome associated with high methane production in cattle. *BMC Genomics* 16, 839. doi:10.1186/s12864-015-2032-0.

Woodward, S. L., Waghorn, G. C. and Laboyrie, P. G. 2004. Condensed tannins in birdsfoot trefoil (*Lotus corniculatus*) reduce methane emissions from dairy cows. In: *Proceedings of the New Zealand Society of Animal Production*. New Zealand Society of Animal Production, Hamilton, New Zealand, pp. 160–4.

Yuan, Y., Chen, H., Yuan, W., Williams, D., Walker, J. T. and Shi, W. 2017. Is biochar-manure co-compost a better solution for soil health improvement and N_2O emissions mitigation? *Soil Biol. Biochem.* 113, 14–25. doi:10.1016/j.soilbio.2017.05.025.

Zhang, J., Shi, H., Wang, Y., Li, S., Cao, Z., Ji, S., He, Y. and Zhang, H. 2017. Effect of dietary forage to concentrate ratios on dynamic profile changes and interactions of ruminal microbiota and metabolites in Holstein heifers. *Front. Microbiol.* 8, 2206. doi:10.3389/fmicb.2017.02206.

Zhao, L., Meng, Q., Ren, L., Liu, W., Zhang, X., Huo, Y. and Zhou, Z. 2015. Effects of nitrate addition on rumen fermentation, bacterial biodiversity and abundance. *Asian-Australas. J. Anim. Sci.* 28(10), 1433–41. doi:10.5713/ajas.15.0091.

Zhao, L., Meng, Q., Li, Y., Wu, H., Huo, Y., Zhang, X. and Zhou, Z. 2018. Nitrate decreases ruminal methane production with slight changes to ruminal methanogen composition of nitrate-adapted steers. *BMC Microbiol.* 18(1), 21. doi:10.1186/s12866-018-1164-1.

Zhou, S., Xu, J., Yang, G. and Zhuang, L. 2014. Methanogenesis affected by the co-occurrence of iron(III) oxides and humic substances. *FEMS Microbiol. Ecol.* 88(1), 107–20. doi:10.1111/1574-6941.12274.

Zhu, Z., Kristensen, L., Difford, G. F., Poulsen, M., Noel, S. J., Abu Al-Soud, W., Sørensen, S. J., Lassen, J., Løvendahl, P. and Højberg, O. 2018. Changes in rumen bacterial and archaeal communities over the transition period in primiparous Holstein dairy cows. *J. Dairy Sci.* 101(11), 9847–62. doi:10.3168/jds.2017-14366.

Chapter 16

Host-rumen microbiome interactions and influences on feed conversion efficiency (FCE), methane production and other productivity traits

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1 Introduction

The rumen microbiome has the important task of supplying ruminants with most of their dietary requirements and is responsible for providing them with up to 70% of their metabolic needs and protein supply (Siciliano-Jones and Murphy, 1989; Bergman, 1990). This tremendous feat is possible due to the large diversity of microorganisms in the rumen, operating on different trophic levels (Flint et al., 2008; Moraïs and Mizrahi, 2019a,b). As such, ruminants are considered the hallmark of obligatory host-microbe interactions. The notion that differences in microbial composition could affect the animals' physiology, efficiency and waste output, has been suggested for the past 70 years of ruminant research, much earlier than the development of high throughput technology, which allowed researchers in the past decade to establish such a link (Krause et al., 2003). Hungate, in his seminal book *The Rumen and Its Microbes* (Hungate, 2013), suggested that a modulation of the microbial community toward improving fiber digestion may represent an avenue for increased productivity. Indeed the early works regarding the rumen microbiome largely focused on a subset of cultivable bacteria for which improved function was

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thought to increase animal productivity (Krause et al., 2003). However, these early attempts at modifying the rumen microbial composition toward improved efficiency have mostly failed to sustain a desired phenotype (Attwood et al., 1988; Flint et al., 1989; Wallace and Walker, 1993; Miyagi et al., 1995; Krause et al., 1999, 2003). It has been pointed out that, in order to achieve this goal, one has to first understand the role of each component of the microbiome and its effect on the overall microbial community and the host.

Today, with our ability to assess the composition of the rumen microbial community as a whole, a new holistic view of the microbiome has emerged, whereby application of basic ecological principles on the overall microbiome structure and the physiological response of the host can be studied. This will lead us to an increased understanding of the role of the microbiome and its components on production efficiency, health and waste emissions such as methane. This chapter focuses on the recent discovery about the role of the ruminant microbiome on energy harvest, methane emission and the potential genetic factors determining its microbial composition and selection.

2 Core community, resilience and natural variation in rumen microbiome composition

2.1 Core community

Identifying common microbial features can lead to an understanding of the more basic requirements of the rumen ecosystem as they likely serve key functions in rumen metabolism. Several independent studies recognize the existence of a core microbial community in the rumen shared between and within ruminant lineages (Jami and Mizrahi, 2012a; Henderson et al., 2015). A comprehensive analysis of the microbiome of 32 ruminant and pseudo-ruminant species (Henderson et al., 2015) emphasized the shared and divergent nature of the rumen microbiome composition across a wide geographical range, animal lineages and management conditions (Henderson et al., 2015). The authors identified a core community of taxa at the genus and species level, shared across different ruminant animal lineages. These include *Prevotella,* the most dominant genus in the rumen, *Butyrivibrio* and *Ruminococcus*, which harbor the main cellulolytic species in the rumen, as well as unclassified Lachnospiraceae, Ruminococcaceae, Clostridiales (all Firmicutes) and Bacteroidales (Bacteroidetes) for bacteria. The bovine rumen was also shown to be particularly enriched with Fibrobacter, an important cellulolytic species, shown to be most abundant in cattle-fed high-forage diet (Henderson et al., 2015). The members of the *Methanobrevibacter gottschalkii* and *Methanobrevibacter ruminantium* clades represent the core methanogenic community observed in the rumen (Henderson et al., 2015). This shared presence of bacterial taxa across different foregut animal lineages suggests

that the core taxa have a key role in rumen metabolism and function (Shade and Handelsman, 2012). This suggestion is reinforced by the recent observation that species defined as core are more associated with physiological traits of the host in dairy cattle (Li et al., 2019; Wallace et al., 2019).

2.2 Variation

In all model animals tested, including ruminants, inter-individual variation in both microbial composition and abundance exists between animals despite stringent account for external factors such as housing, management and diet (Brulc et al., 2009; Jami and Mizrahi, 2012a; Henderson et al., 2015). Brulc et al. (2009) conducted a pioneering study characterizing the rumen microbial composition using shotgun metagenomics sequencing and showed broad differences in the community composition of three steers. One of the steers exhibited a different composition compared with the other two steers despite being under the same diet. Similarly, the characterization of 16 dairy cows under the same diet and management condition exhibited a 0.51 average pairwise similarity using the Bray-Curtis index, which takes into account the presence, and abundance of taxa. Some genera appeared to be relatively stable in terms of presence and abundance while others can exhibit up to two orders of magnitude differences in abundance between the cows (Jami and Mizrahi, 2012a,b).

2.3 Resilience

While studies have consistently pointed out that samples taken from different host animals can exhibit high variation in composition (Brulc et al., 2009; Li et al., 2009; Jami and Mizrahi, 2012a), microbiome assessment across different sampling time points within the same cow reveals a remarkable stability (Li et al., 2009; Welkie et al., 2009). In a study using automated ribosomal intergenic spacer analysis (ARISA) to examine the changes in ruminal bacterial communities during the feeding cycle, similar observations were made, emphasizing both the stability of the rumen microbial community when established within a cow and the large differences in composition between different cows (Welkie et al., 2009). In steers, long-term temporal assessment of the changes in microbiome composition after dietary change revealed that, after 25 days on a new diet, the microbiome shows little variation hereafter (Snelling et al., 2019). The microbiome is resilient to such an extent that even large perturbations, such as transfaunation, where the rumen fluid of one cow is almost completely replaced with the rumen fluid of another, showed that within just a few weeks, rumen microbiome content reverted to a composition more closely resembling the original (Weimer et al., 2017). This stresses that host factors may strongly influence microbial assembly. Recent studies show a connection between the individual animals' genetics and its respective

microbiome, as well as heritability of some rumen microbiome components (Roehe et al., 2016; Li et al., 2016; Sasson et al., 2017; Wallace et al., 2019). A larger experiment showed that following transfaunation, each individual cow exhibited unique patterns of reestablishment further strengthening the possibility of large host effect on the microbial community (Zhou et al., 2018).

3 Microbiome-dependent traits

The fermentation products of rumen microbial activity serve as the main source of energy for the animal, contributing to up to 70% of their metabolic requirements (Wolin, 1979; Bergman, 1990). Plant feed ingested undergoes a cascade of degradation, from complex polymers to intermediate molecules, reaching end products which are either absorbed by the animal or emitted to the environment. Several methods and indexes were developed to account for the level of efficiency in which feed is being converted into usable products by the animal for growth and production, including feed conversion ratio (FCR), residual feed intake (RFI), energy corrected milk/dry matter intake (ECM/DMI), and the more recent residual intake and gain (RGI) (NRC, 2001; Berry and Crowley, 2012). Although each of these indexes calculate efficiency differently and encompass different observed physiological parameters of the ruminant, the overall rationale behind them remains similar in their attempt to measure the overall ratio between the energetic value of the feed or diet ingested compared with the energy absorbed by the ruminant for maintenance and production (Mizrahi, 2012). These global traits are supplemented by more specific assessment, depending on the research question, such as protein, carbohydrate, and lipid content in milk (Jami et al., 2014), health parameters of the host (Jewell et al., 2015), or rumen-specific metabolites linked to energy uptake such as VFA composition and quantification, or energy loss, such as methane (Hernandez-Sanabria et al., 2010; Shabat et al., 2016; Tapio et al., 2017). The proposed effects of microbiome composition and microbial gene expression on animal physiology, with emphasis on performance and methane emission, have been the focus of many studies in the past decade, summarized in Table 1. As mentioned above, the fundamental microbial functions of the plant fiber degradation and fermentation processes are similar between microbiomes across host animals (Moraïs and Mizrahi, 2019a). However, the variation in composition and abundance of specific microbial taxa and their gene expression were linked to methane emissions as well as specific rumen metabolites which have considerable impact on host traits such as milk composition and energy-harvest efficiency from the feed (Shi et al., 2014; Shabat et al., 2016; Kamke et al., 2016; Li and Guan, 2017). In cattle, species of the *Prevotella* genus have been implicated in both increased and decreased productivity and milk composition parameters (Carberry et al., 2012; Jami et al.,

Table 1 Summary list of studies assessing the link between rumen microbiome composition and animal physiology

Animal	Method	Main findings	Reference
Beef cattle	PCR-DGGE	Feed efficiency phenotype and specific bacteria linked to SCFA profile	Guan et al. (2008)
Beef cattle	PCR-DGGE	Differential prevalence of *M.stadtmanae* and specific *Methanobrevibacter* strains between high and low RFI. Overall higher methanogens taxonomic diversity in high RFI animals	Zhou et al. (2009)
Beef cattle	PCR-DGGE, qPCR	Specific bacterial and archaeal OTUs associated with specific VFAs and with different RFI phenotypes under low-energy diet	Hernandez-Sanabria et al. (2010)
Beef cattle	PCR-DGGE, qPCR	*Succinovibrio spp.* associated with low-methane emission. Acetate higher in high-efficient animals	Hernandez-Sanabria et al. (2012)
Beef cattle	PCR-DGGE and qPCR	High *Prevotella* abundance in low-efficiency animals. Link between bacterial profile and feed efficiency is different between diets.	Carberry et al. (2012)
Beef cattle	Clone library and 16S amplicon sequencing	*Methanobrevibacter* species differ in abundance between high and low RFI animals	Carberry et al. (2014)
Sheep	Shotgun metagenomics and metatranscriptomics	Higher gene expression related to the hydrogenotrophic methanogenesis pathway in high methane-emitting sheep	Shi et al. (2014)
Dairy cattle	16S amplicon sequencing	Correlations between bacterial genera and production parameters. *Prevotella* negatively correlates with milk fat yield	Jami et al. (2014)
Beef cattle	16S amplicon sequencing	High abundance of *Prevotella* linked to inefficient phenotype	McCann et al. (2014)
Sheep	16S amplicon sequencing	Identification of three different 'ruminotypes' associated with high- and low-methane emission	Kittelmann et al. (2014)

(*Continued*)

Table 1 (*Continued*)

Animal	Method	Main findings	Reference
Dairy cattle	16S amplicon sequencing	Core OTUs associated with either efficient (*Prevotella spp.*) or inefficient cows (*Prevotella* genus, *Butyrivibrio*)	Jewell et al. (2015)
Dairy cattle	16S Amplicon sequencing, shotgun metagenomics, metabolomics	*Megasphaera elsdenii* and the acrylate pathway involved in increased efficiency to high efficiency. Efficient cows exhibit lower bacterial diversity	Shabat et al. (2016)
Sheep	Metatrancriptomics	Low methane emission phenotype in sheep enriched in *Sharpea azabuensis* and *Megasphaera spp.* and the acrylate pathway	Kamke et al. (2016)
Dairy cattle	16S amplicon sequencing	*Prevotella*, S24-7 and Succinivibrionaceae lineages positively correlated with milk yield	Indugu et al. (2017)
Dairy cattle	16S amplicon sequencing	*M. ruminantium* and *M. gottschalkii* associated with low methane emission	Danielsson et al. (2017)
Beef cattle	Metatranscriptomics	Low feed-efficiency animals express a higher diversity of gene pathways	Li and Guan (2017)
Dairy cattle	Shotgun metagenomics	Higher abundance of Bacteroidetes and lower abundance of methanogens in efficient cows	Delgado et al. (2019)
Beef cattle	Shotgun metagenomics	Microbial genes related to cell wall biosynthesis, hemicellulose and cellulose degradation host-microbiome cross talk associated with FCR. Vitamin B12 biosynthesis, environmental information processing and bacterial mobility genes associated with RFI	Lima et al. (2019)
Dairy cattle	16S amplicon sequencing, metabolomics, host genotyping	Core microbes more associated and predictive of a wide range of host traits, including feed efficiency, methane emission and VFAs	Wallace et al. (2019)

DGGE = denaturing gradient gel electrophoresis; SCFA = short-chain fatty acids; RFI = residual feed intake; qPCR = quantitative PCR; OTUs = operational taxonomic units; VFA = volatile fatty acids; FCR = feed conversion ratio.

2014; McCann et al., 2014; Jewell et al., 2015; Shabat et al., 2016; Indugu et al., 2017). In a study assessing the correlation between taxa abundance of the microbiome components and physiological parameters of 15 cows, a positive correlation was observed between the ratio of Firmicutes/Bacteroidetes and daily fat production in the milk (Jami et al., 2014). The difference in ratio was mostly driven by the vast difference in the abundance of the *Prevotella* genus (Bacteroidetes), which negatively correlated with milk fat yield (Jami et al., 2014). These findings can be mirrored to observations from mice and human microbiome studies, in which a lower abundance of Bacteroidetes correlated with increased blood and tissue adiposity in mice (Turnbaugh et al., 2006). *Prevotella* and its species were also found to be linked to feed efficiency in a study investigating the dynamics of the microbial population in the cow's rumen throughout two lactation periods (Jewell et al., 2015). This study also found a negative correlation between specific operational taxonomic units (OTUs; at 97% similarity defined as species) associated with the *Prevotella* genus and production efficiency. However, the same study also identified various *Prevotella* species associated with higher feed efficiency (Jewell et al., 2015), along with another study showing that *Prevotella* was linked to increased milk production yield in dairy cattle (Indugu et al., 2017). It is likely that different *Prevotella* species affect rumen physiology differently as this genus is highly diverse in the rumen (Ley, 2016). Our functional understanding of this genus is restricted to a number of cultivated species, limiting our understanding of their full functional scope. The recent availability of a compendium of ~5000 genomes from the rumen using deep metagenomic sequencing may shed light on the diverse functions carried by the species belonging to the *Prevotella* genus (Stewart et al., 2019). In a series of studies performed on steers grouped according to their RFI values (low/high RFI), the bacterial and archaeal rumen community was assessed using PCR-DGGE fingerprinting. The authors showed that specific bacterial taxa had a higher likelihood of being present depending on the respective groups along with VFA production patterns (Guan et al., 2008; Hernandez-Sanabria et al., 2010). Furthermore, species diversity was lower in the low RFI steers, when methanogenic populations were compared using 16s rDNA clone libraries, and a correlation between the composition of methanogens and host efficiency could be observed (Zhou and Hernandez-Sanabria, 2009; Zhou et al., 2010). In a subsequent study, the ruminal methanogenic and bacterial populations of 58 steers which differed in feed efficiency and diet were analyzed (Hernandez-Sanabria et al., 2012). The authors found that *Succinivibrio* spp. and *Eubacterium* spp. were correlated with increased efficiency in steers. The authors suggested that the higher abundance of *Succinivibrio* in low RFI steers, along with the higher acetate and lower isovalerate production, shifts rumen metabolism away from methanogenesis and toward propionate production (Hernandez-Sanabria

et al., 2012). Interestingly, the family Succinivibrionaceae and its species have been recurrently implicated in a low methane phenotype in sheep and tammar wallabies (Pope et al., 2011; Wallace et al., 2015b). A microbiome-dependent steering of metabolism away from methanogenesis and toward VFA production was also demonstrated in dairy cattle (Shabat et al., 2016). A study was conducted examining 78 cows with extreme RFI phenotype (38 inefficient, 40 efficient), bacterial taxonomic composition, bacterial gene composition and ecological features, which were significantly correlated and predictive of the feed-efficiency phenotype (Shabat et al., 2016). The authors showed that the microbiome of efficient cows had a lower diversity than that of inefficient cows, and a gene composition steered toward production of usable end products for the cows such as propionate. The study identified *Megasphaera elsdenii* and *Coprococcus catus*, and the acrylate pathway encoded by these species, as a central pathway linked to higher efficiency. The acrylate pathway uses the intermediate product lactate to produce propionate. This study proposed a model whereby an interplay exists between the acrylate pathway and VFA production and methanogenesis, suggesting that both phenotypes, though not necessarily converging, link to feed efficiency. These findings may further relate to the previously mentioned observations of increased Succinivibrionaceae taxa associated with a different VFA profile, lower methane emission and higher efficiency (Wallace et al., 2015b). *Succinivibrio dextrinosolvens*, a known resident of the rumen, is shown to increase its lactate production under specific growth conditions (O'Herrin and Kenealy, 1993). Integrated together, these results point toward a specific cascade of fermentation, with lactate as intermediate, being central in determining efficiency in ruminants. A caveat to these observation remains that link between physiological parameters and the microbiome might be diet specific and that different links can be observed across different diets (Carberry et al., 2012).

4 Methane production

Methane production is exclusively performed by methanogenic archaea (methanogens) inhabiting the rumen. Methanogens serve as electron sinks for the entire rumen ecosystem driving the directionality of the fermentation process, which would be otherwise inhibited by the H_2 produced (McAllister and Newbold, 2008; van Lingen et al., 2016). This is mostly performed by the hydrogenotrophic methanogenesis pathway, the main methanogenesis pathway in the rumen, in which CO_2 and H_2 products of fermentation are converted to methane. Although necessary, methane production constitutes an energy loss for the animal, ranging between 2and 12%, in addition to the detrimental effect of methane on the environment (Johnson and Johnson, 1995). Thus, studies on the microbiome effect on methane production are

intimately connected to those assessing its effect on production efficiency (Shabat et al., 2016). While being an integral part of the rumen ecosystem, ruminants can exhibit a wide variation in methane emission. Several studies have shown that higher methane emission is weak, or not at all linked to the absolute abundance of methanogens present in the rumen of cattle and sheep (Shi et al., 2014; Kittelmann et al., 2014; Tapio et al., 2017). However, a recent study using whole-genome sequencing of the rumen of steer found that the ratio of archaea bacteria is predictive of methane emission with a correlation of 0.49 (Wallace et al., 2015a).

Differential composition of methanogens and methanogenic gene expression has been more consistently implicated in the different methane emission phenotypes observed in cattle and sheep, and several methanogenic taxa have been linked to increased methane emission. It is generally agreed upon that the most abundant methanogenic genus in the mature rumen is the *Methanobrevibacter* (Janssen and Kirs, 2008; Friedman et al., 2017; Tapio et al., 2017). Several studies categorized this genus into two clades – SMGT (*smithii-gottschalkii-millerae-thaurei*) and RO (*ruminantium-olleyae*) (King et al., 2011) – with the high presence of SMGT clade being associated with higher methanogenesis potential (Danielsson et al., 2012, 2017; Shi et al., 2014). Using metatranscriptomics, Shi et al. (2014) observed that *Methanobrevibacter gottschalkii* abundance is increased in high methane-yielding sheep. The authors also noted that methanogenic transcript abundance of genes encoding to CO_2/H_2 pathway was significantly increased in high methane-emitting sheep while no difference was observed in terms of methanogen abundance.

Although methanogens are sole producers of methane, they rely on upstream fermentation processes and outputs by other microbial taxa, which were shown to affect its production. These include fermentation by bacteria, protozoa and fungi. Kittelmann et al. (2014) identified three different bacterial assemblies, termed 'ruminotypes', linked to different methane production phenotypes in sheep (Kittelmann et al., 2014). Ruminotypes associated with high methane emission exhibited an enrichment in H_2-producing bacteria, such as Ruminococcaceae, Lachnospiraceae, Catabacteriaceae, *Coprococcus*, other Clostridiales, *Prevotella*, Bacteroidales and Alphaproteobacteria. The ruminotypes associated with low methane emission was associated with either the propionate-producing *Quinella ovalis*, or with lactate and succinate producers such as *Fibrobacter* spp., *Kandleria vitulina*, *Olsenella* spp., *Prevotella bryantii* and *Sharpea azabuensis* (Kittelmann et al., 2014). As mentioned in the context of feed efficiency, lactate and succinate producers such as *Succinivibrio dextrinosolvens* have been suggested as being responsible for the low methane production observed in tammar wallabies and associated with high-feed efficiency in cattle (Pope et al., 2011; Wallace et al., 2015b). Another study similarly identified the lactate producer, *Sharpeae albenenzis*,

enriched in the rumen of low methane-emitting sheep (Kamke et al., 2016). The author suggested that lactate production by this species diverts electrons in the form of H_2 from methanogenesis toward production of lactate, which in turn is converted to propionate through the acrylate pathway of *Megasphaera elsdenii*. These findings mirror the observed higher abundance of lactate-utilizing bacteria and genes observed in high efficiency, low CH_4-emitting dairy cows (Shabat et al., 2016). The overall aggregated conclusions of these findings suggest that differences in electron transfer between microbial species across the rumen trophic network result in differences in community states (Moraïs and Mizrahi, 2019a), leading to different outcomes in terms of output end products. The alternative community state model suggests that, for yet unresolved reasons, one rumen ecosystem may favor a metabolism steered toward H_2 production leading to higher methane production while another would favor lactate production and utilization toward VFA production (Moraïs and Mizrahi, 2019a).

In addition to the observed link between the bacterial domain and methanogenesis, rumen ciliate protozoa have been attributed many roles in enhancing methane emission through mutualistic associations with rumen methanogens (Newbold et al., 2015). This research topic has been the focus of many studies in the past three decades. As opposed to bacteria, ciliate protozoa are not essential to the proper functioning of the rumen, giving the opportunity to assess protozoa effect on the rumen ecosystem and host physiology using defaunation – the removal of protozoa from the rumen by various means. A recent meta-analyses summarizing 30 years of experimental defaunation studies presented significant physiological differences between faunated and defaunated ruminants (sheep and cattle), including protein availability to methane emission (Guyader et al., 2014; Newbold et al., 2015). Specifically, the meta-analyses revealed that defaunation consistently leads to a significant decrease in methane emission of up to 11% from the animal in vivo. In vitro studies further suggested that within the protozoa community, holotrichous protozoa are more involved in methane production (Belanche et al., 2015). Furthermore, an additional meta-analysis which compared methane emission to the protozoa cell abundance in 79 studies showed a strong linear correlation between protozoa cell numbers in vivo and methane emission (Guyader et al., 2014). As most protozoa in the rumen are known to produce high quantities of H_2, it has been suggested that the nature of this mutualism stems from the rich H_2 environment provided by protozoa favored by hydrogenotrophic methanogens (Newbold et al., 2015). Additional evidence of such a syntrophic mechanism was observed in a co-cultivation experiment between the protozoa *Polyplastron multivesiculatum* and the methanogen *Methanosarcina barkeri*, in which a decrease of H_2 in the culture coincided with an increase in methane (Ushida et al., 1997).

Using in situ hybridization, studies have shown that protozoa are largely colonized by methanogens, thereby strengthening the hypothesis of a strong mutualistic relationship between protozoa and methanogens (Finlay et al., 1994; Lloyd et al., 1996). Lloyd et al. (1996) showed that methanogens can be found attached to the outer pellicle of protozoa as well as inside the protozoa, but outside of the food vacuole, suggesting that they are not serving as food to the predatory protozoa, but rather as symbionts. Furthermore, protozoa were shown to carry a higher methanogens/bacteria ratio than the free-living fraction of the rumen, suggesting a specific tropism between methanogens and protozoa (Levy and Jami, 2018). However, when correcting for protozoa size, Belanche et al. (2014) did not observe such enrichment; therefore the question remains as to whether methanogens accumulate in a higher proportion than bacteria in and around protozoa (Belanche et al., 2014). While this question remains open, several studies have shown that the composition of methanogens associated with protozoa differs when compared to the free-living prokaryotic population (Tymensen et al., 2012; Tymensen and McAllister, 2012; Belanche et al., 2014; Levy and Jami, 2018). Taxa belonging to the *Methanobrevibacter* genus were overrepresented in the protozoa-associated fractions (Görtz, 2006; Tymensen et al., 2012; Belanche et al., 2014; Levy and Jami, 2018). This would support the notion that the methanogens-protozoa relationship is based on interspecies electron transfer via H_2, as species from the M*ethanobrevibacter* genus are commonly associated with *hydrogenotrophic* methanogenesis (Janssen and Kirs, 2008). Furthermore, following the observation that no difference in methanogens abundance could be observed following defaunation, it was hypothesized that the methanogens associated with protozoa are more active in terms of methane production. Levy and Jami (2018) showed that, when separated by size, large protozoa exhibited a higher relative abundance of OTUs associated with the SGMT group of *Methanobrevibacter*, previously linked to high methane emission in cattle (Danielsson et al., 2017), supporting this hypothesis.

5 Nitrogen compounds: utilization and emission

Nitrogen cycling in the rumen ecosystem could potentially affect community composition and host attributes. In a study linking feed efficiency to the rumen microbiome and its functional capacities, it was shown that inefficient cows were enriched in functions related to protein digestion and amino acid biosynthesis (Shabat et al., 2016). Additionally, ciliate protozoa were shown to negatively affect N availability to the animal through increased predation of bacteria (Newbold et al., 2015). Specifically, *Entodinium* spp. were shown to decrease microbial protein availability to the host animal, and their elimination through defaunation was shown to increase protein supply by 30% (Newbold et al., 2015).

Proteolytic activity of microbes in the rumen can vary greatly between animals, with excess proteolytic activity, such as deamination, regarded as detrimental to efficient N utilization (Hartinger et al., 2018). Inefficient N utilization thus carries a negative effect on host production as well as the environment, contributing a large proportion of anthropogenic nitrous oxide released into the atmosphere (Huws et al., 2018). The differential abundance of specific community members such as hyper-ammonia-producing bacteria (HAB) may result in different utilization efficiency of N, and therefore control over such populations could improve nitrogen utilization by the animal (Firkins et al., 2007; Hartinger et al., 2018). This topic represents an important avenue for future studies in order to better understand the potential connection between the bacterial community composition and N cycling efficiency.

6 Microbiome and host genetics

While important strides were made in understanding the link between the microbiome and host physiology and productivity, the issue regarding the potential control of the host genetics on microbiome composition and selection remains vastly understudied, particularly compared with the plethora of studies on the topic in human microbiome research (Rothschild et al., 2018). The question whether host genetics affect the microbiome composition and subsequently microbial features related to energy harvest and methane emission, can have vast impact on our ability to rationally select genotypes in order to obtain favored phenotypes through selective breeding (Myer, 2019). In humans and mice, where arguably more data is available on the topic, several studies reported that the microbial community composition is partly dictated by the genetic makeup of the host (Benson et al., 2010; Goodrich et al., 2014, 2016; Bonder et al., 2016; Turpin et al., 2016). In contrast, a recent human study put into question the degree of influence that host genetics has on microbial composition and abundance (Rothschild et al., 2018). A first indication for a host-genetic influence on the composition of the microbial community and physiological parameters was derived by comparing the microbial composition of different cattle breeds and their hybrids (Guan et al., 2008; Hernandez-Sanabria et al., 2013; Paz et al., 2016). One such study performed on two different sire breeds showed a link between host genetics, microbial composition, specifically archaea:bacteria ratio, and methane, which would allow selection of animals based on genetics (Roehe et al., 2016). The authors proposed several mechanisms by which the host animal could control microbiome composition and subsequently its output, such as pH buffering by saliva production, or feed retention time in the rumen, the latter shown to be a heritable trait (Roehe et al., 2016). By combining microbial abundance data with the genomic profile of 47 dairy cows differing in efficiency, Sasson et al.

(2017) identified 22 OTUs associated with rumen-metabolic traits and host-physiological traits, which showed measurable heritability (Sasson et al., 2017). This study also shows that these heritable OTUs were more closely connected to host physiology and rumen metabolites than other rumen microbes. Recently, three large-scale studies examining hundreds of individual animals for establishing the connection of the host genetics to microbial composition using genome wide association (GWAS) approaches shared several similarities in their results (Difford et al., 2018; Li et al., 2019; Wallace et al., 2019). Difford et al. (2018), using a cohort of 750 dairy cattle, identified that the abundance of 6% of the bacterial community and 12% of archaeal community at the species level were heritable ($h^2 > 0.15$), that is, controlled by host genome, and that methane emission was associated with both microbiome features and host genetics, although both associations were largely independent (Difford et al., 2018). The independent nature of host genetic influence on methane emission and the microbiome suggests methane variation between animals is likely not a result of host genetics on the microbiome. The authors thus suggested that two parallel avenues should be considered in order to decrease methane emission: one related to breeding selection of low methane-associated traits within the animal genome and the second related to microbiome modulation toward mitigating methane emission (Difford et al., 2018). In contrast, in a cohort of 669 steers, a study showed that animal genetics contributes to the abundance of 59 microbial taxa, 56 bacteria and 3 archaea, which were also associated with host-feed efficiency traits and rumen-metabolic output (Li et al., 2019). The authors revealed 19 single nucleotide polymorphisms (SNPs), five of which are located in loci linked to feed efficiency, associated with 12 microbial taxa. Thus, this study suggests that host control of a subset of microbes in the rumen may have a direct effect on production efficiency. Furthermore, four of the observed heritable taxa were shown, using interaction network inference, to interact with a large number of taxa. In a large-scale GWAS performed throughout Europe, 1000 dairy cattle across four European countries were genotyped and the rumen microbiome composition was sequenced along with a large range of metabolic and physiological parameters. This study showed that taxa considered 'core', that is, recurrent in at least 50% of the cows within a specific farm, were connected to variation in host genetics, whereby their abundance can be explained to a significant extent by host genetics (Wallace et al., 2019). Within this core microbiome, 39 taxa were found to have measured heritability of up to $h^2 = 0.6$. Furthermore, using network inference against phenotypic traits, this study showed that those heritable core microbes are more linked to productivity parameters than to the non-core taxa. Moreover, in agreement with the study by Li et al. (2019), this study showed that these heritable microbes were central to the microbiome interaction networks, suggesting them as keystone species. These findings suggest that by controlling a subset

of microbes, the host could have a larger indirect control over a broader range of microbes and therefore on rumen physiology. These observations validate the notion that the core microbial community is selected, and carry a large proportional role in defining animals' physiological traits. Although seemingly contradictory to the latest research on humans, some similarities in the results can be seen, while the differences in experimental design may explain the discrepancies. Firstly, as within humans, only a relatively small subset of taxa could be associated with host genetics (1.9%) (Rothschild et al., 2018), which is also true for cattle (0.25% of the OTUs) (Wallace et al., 2019). However, in terms of abundance, these taxa represented up to 60% of the microbiome in cattle while in humans the heritable taxa represent around 6% of the microbiome. Furthermore, animal studies exert a tighter control over many physiological and management parameters, as opposed to human studies, in which differences in diet between people for instance cannot be absolutely ascertained and controlled. Achieving homogeneity in environmental factors may indeed reveal the stronger impact of host genetics in determining microbiome structure. It is however clear that environmental factors such as diet likely exert the largest effect on microbiome compositions, but the heritability of output parameters such as methane emission suggests that host genetics may influence the fate of the alternative stable community states in each individual animal. This would lead to the possibility of devising novel criteria for selection based on the genetics of the ruminant in order to steer the microbiome toward an agriculturally favorable phenotype.

7 References

Attwood, G. T., Lockington, R. A., Xue, G. P. and Brooker, J. D. 1988. Use of a unique gene sequence as a probe to enumerate a strain of *Bacteroides ruminicola* introduced into the rumen. *Applied and Environmental Microbiology* 54, 534–9.

Belanche, A., de la Fuente, G. and Newbold, C. J. 2014. Study of methanogen communities associated with different rumen protozoal populations. *FEMS Microbiology Ecology* 90, 663–77.

Belanche, A., de la Fuente, G. and Newbold, C. J. 2015. Effect of progressive inoculation of fauna-free sheep with holotrich protozoa and total-fauna on rumen fermentation, microbial diversity and methane emissions. *FEMS Microbiology Ecology* 91(3).

Benson, A. K., Kelly, S. A., Legge, R., Ma, F., Low, S. J., Kim, J., Zhang, M., Oh, P. L., Nehrenberg, D., Hua, K., Kachman, S. D., Moriyama, E. N., Walter, J., Peterson, D. A. and Pomp, D. 2010. Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proceedings of the National Academy of Sciences of the United States of America* 107, 18933–8.

Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiological Reviews* 70, 567–90.

Berry, D. P. and Crowley, J. J. 2012. Residual intake and body weight gain: a new measure of efficiency in growing cattle. *Journal of Animal Science* 90, 109–15.

Bonder, M. J., Kurilshikov, A., Tigchelaar, E. F., Mujagic, Z., Imhann, F., Vila, A. V., Deelen, P., Vatanen, T., Schirmer, M., Smeekens, S. P., Zhernakova, D. V., Jankipersadsing, S. A., Jaeger, M., Oosting, M., Cenit, M. C., Masclee, A. A. M., Swertz, M. A., Li, Y., Kumar, V., Joosten, L., Harmsen, H., Weersma, R. K., Franke, L., Hofker, M. H., Xavier, R. J., Jonkers, D., Netea, M. G., Wijmenga, C., Fu, J. and Zhernakova, A. 2016. The effect of host genetics on the gut microbiome. *Nature Genetics* 48, 1407–12.

Brulc, J. M., Antonopoulos, D. A., Miller, M. E., Wilson, M. K., Yannarell, A. C., Dinsdale, E. A., Edwards, R. E., Frank, E. D., Emerson, J. B., Wacklin, P., Coutinho, P. M., Henrissat, B., Nelson, K. E. and White, B. A. 2009. Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. *Proceedings of the National Academy of Sciences of the United States of America* 106, 1948–53.

Carberry, C. A., Kenny, D. A., Han, S., McCabe, M. S. and Waters, S. M. 2012. Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. *Applied and Environmental Microbiology* 78, 4949–58.

Carberry, C. A., Waters, S. M., Waters, S. M., Kenny, D. A. and Creevey, C. J. 2014. Rumen methanogenic genotypes differ in abundance according to host residual feed intake phenotype and diet type. *Applied and Environmental Microbiology* 80, 586–94.

Danielsson, R., Schnürer, A. and Arthurson, V. 2012. Methanogenic population and CH_4 production in Swedish dairy cows fed different levels of forage. *Applied and Environmental Microbiology* 78(17), 6172–9.

Danielsson, R., Dicksved, J., Sun, L., Gonda, H., Müller, B., Schnürer, A. and Bertilsson, J. 2017. Methane production in dairy cows correlates with rumen methanogenic and bacterial community structure. *Frontiers in Microbiology* 8, 226.

Delgado, B., Bach, A., Guasch, I., González, C., Elcoso, G., Pryce, J. E. and Gonzalez-Recio, O. 2019. Whole rumen metagenome sequencing allows classifying and predicting feed efficiency and intake levels in cattle. *Scientific Reports* 9, 11.

Difford, G. F., Plichta, D. R., Løvendahl, P., Lassen, J., Noel, S. J., Højberg, O., Wright, A-D. G., Zhu, Z., Kristensen, L., Nielsen, H. B., Guldbrandtsen, B. and Sahana, G. 2018. Host genetics and the rumen microbiome jointly associate with methane emissions in dairy cows. *PLoS Genetics* 14, e1007580.

Finlay, B. J., Esteban, G., Clarke, K. J., Williams, A. G., Embley, T. M. and Hirt, R. P. 1994. Some rumen ciliates have endosymbiotic methanogens. *FEMS Microbiology Letters* 117, 157–61.

Firkins, J. L., Yu, Z. and Morrison, M. 2007. Ruminal nitrogen metabolism: perspectives for integration of microbiology and nutrition for dairy. *Journal of Dairy Science* 90 Suppl. 1, E1–16.

Flint, H. J., Bisset, J. and Webb, J. 1989. Use of antibiotic resistance mutations to track strains of obligately anaerobic bacteria introduced into the rumen of sheep. *The Journal of Applied Bacteriology* 67, 177–83.

Flint, H. J., Bayer, E. A., Rincon, M. T., Lamed, R. and White, B. A. 2008. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nature Reviews. Microbiology* 6, 121–31.

Friedman, N., Jami, E. and Mizrahi, I. 2017. Compositional and functional dynamics of the bovine rumen methanogenic community across different developmental stages. *Environmental Microbiology* 19(8), 3365–73.

Goodrich, J. K., Waters, J. L., Poole, A. C., Sutter, J. L., Koren, O., Blekhman, R., Beaumont, M., Van Treuren, W., Knight, R., Bell, J. T., Spector, T. D., Clark, A. G. and Ley, R. E. 2014. Human genetics shape the gut microbiome. *Cell* 159, 789–99.

Goodrich, J. K., Davenport, E. R., Beaumont, M., Jackson, M. A., Knight, R., Ober, C., Spector, T. D., Bell, J. T., Clark, A. G. and Ley, R. E. 2016. Genetic determinants of the gut microbiome in UK twins. *Cell Host & Microbe* 19, 731–43.

Görtz, H.-D. 2006. Symbiotic associations between ciliates and prokaryotes. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H. and Stackebrandt, E. (Eds), *The Prokaryotes: Volume 1: Symbiotic Associations, Biotechnology, Applied Microbiology*. Springer New York, New York, NY, pp. 364–402.

Guan, L. L., Nkrumah, J. D., Basarab, J. A. and Moore, S. S. 2008. Linkage of microbial ecology to phenotype: correlation of rumen microbial ecology to cattle's feed efficiency. *FEMS Microbiology Letters* 288, 85–91.

Guyader, J., Eugène, M., Nozière, P., Morgavi, D. P., Doreau, M. and Martin, C. 2014. Influence of rumen protozoa on methane emission in ruminants: a meta-analysis approach. *Animal* 8, 1816–25.

Hartinger, T., Gresner, N. and Südekum, K.-H. 2018. Does intra-ruminal nitrogen recycling waste valuable resources? A review of major players and their manipulation. *Journal of Animal Science and Biotechnology* 9, 33.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Global Rumen Census Collaborators and Janssen, P. H. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific Reports* 5.

Hernandez-Sanabria, E., Guan, L. L., Goonewardene, L. A., Li, M., Mujibi, D. F., Stothard, P., Moore, S. S. and Leon-Quintero, M. C. 2010. Correlation of particular bacterial PCR-denaturing gradient gel electrophoresis patterns with bovine ruminal fermentation parameters and feed efficiency traits. *Applied and Environmental Microbiology* 76, 6338–50.

Hernandez-Sanabria, E., Goonewardene, L. A., Wang, Z., Durunna, O. N., Moore, S. S. and Guan, L. L. 2012. Impact of feed efficiency and diet on adaptive variations in the bacterial community in the rumen fluid of cattle. *Applied and Environmental Microbiology* 78, 1203–14.

Hernandez-Sanabria, E., Goonewardene, L. A., Wang, Z., Zhou, M., Moore, S. S. and Guan, L. L. 2013. Influence of sire breed on the interplay among rumen microbial populations inhabiting the rumen liquid of the progeny in beef cattle. *PLoS ONE* 8, e58461.

Hungate, R. E. 2013. *The Rumen and Its Microbes*. Elsevier.

Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., Muñoz-Tamayo, R., Forano, E., Waters, S. M., Hess, M., Tapio, I., Smidt, H., Krizsan, S. J., Yáñez-Ruiz, D. R., Belanche, A., Guan, L., Gruninger, R. J., McAllister, T. A., Newbold, C. J., Roehe, R., Dewhurst, R. J., Snelling, T. J., Watson, M., Suen, G., Hart, E. H., Kingston-Smith, A. H., Scollan, N. D., do Prado, R. M., Pilau, E. J., Mantovani, H. C., Attwood, G. T., Edwards, J. E., McEwan, N. R., Morrisson, S., Mayorga, O. L., Elliott, C. and Morgavi, D. P. 2018. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Frontiers in Microbiology* 9, 2161.

Indugu, N., Vecchiarelli, B., Baker, L. D., Ferguson, J. D., Vanamala, J. K. P. and Pitta, D. W. 2017. Comparison of rumen bacterial communities in dairy herds of different production. *BMC Microbiology* 17, 190.

Jami, E. and Mizrahi, I. 2012a. Composition and similarity of bovine rumen microbiota across individual animals. *PLoS ONE* 7, e33306.

Jami, E. and Mizrahi, I. 2012b. Similarity of the ruminal bacteria across individual lactating cows. *Anaerobe* 18, 338–43.

Jami, E., White, B. A. and Mizrahi, I. 2014. Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. *PLoS ONE* 9, e85423.

Janssen, P. H. and Kirs, M. 2008. Structure of the archaeal community of the rumen. *Applied and Environmental Microbiology* 74, 3619–25.

Jewell, K. A., McCormick, C. A., Odt, C. L., Weimer, P. J. and Suen, G. 2015. Ruminal bacterial community composition in dairy cows is dynamic over the course of two lactations and correlates with feed efficiency. *Applied and Environmental Microbiology* 81, 4697–710.

Johnson, K. A. and Johnson, D. E. 1995. Methane emissions from cattle. *Journal of Animal Science* 73, 2483–92.

Kamke, J., Kittelmann, S., Soni, P., Li, Y., Tavendale, M., Ganesh, S., Janssen, P. H., Shi, W., Froula, J., Rubin, E. M. and Attwood, G. T. 2016. Rumen metagenome and metatranscriptome analyses of low methane yield sheep reveals a Sharpea-enriched microbiome characterised by lactic acid formation and utilisation. *Microbiome* 4, 56.

King, E. E., Smith, R. P., St-Pierre, B. and Wright, A.-D. G. 2011. Differences in the rumen methanogen populations of lactating Jersey and Holstein dairy cows under the same diet regimen. *Applied and Environmental Microbiology* 77, 5682–7.

Kittelmann, S., Pinares-Patiño, C. S., Seedorf, H., Kirk, M. R., Ganesh, S., McEwan, J. C. and Janssen, P. H. 2014. Two different bacterial community types are linked with the low-methane emission trait in sheep. *PLoS ONE* 9, e103171.

Krause, D. O., Smith, W. J., Ryan, F. M., Mackie, R. I. and McSweeney, C. S. 1999. Use of 16S-rRNA based techniques to investigate the ecological succession of microbial populations in the immature lamb rumen: tracking of a specific strain of inoculated ruminococcus and interactions with other microbial populations *in vivo*. *Microbial Ecology* 38, 365–76.

Krause, D. O., Denman, S. E., Mackie, R. I., Morrison, M., Rae, A. L., Attwood, G. T. and McSweeney, C. S. 2003. Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiology Reviews* 27, 663–93.

Levy, B. and Jami, E. 2018. Exploring the prokaryotic community associated within the rumen ciliate protozoa population. *Frontiers in Microbiology* 9, 2526.

Ley, R. E. 2016. Gut microbiota in 2015: prevotella in the gut: choose carefully. *Nature Reviews. Gastroenterology and Hepatology* 13, 69–70.

Li, F. and Guan, L. L. 2017. Metatranscriptomic profiling reveals linkages between the active rumen microbiome and feed efficiency in beef cattle. *Applied and Environmental Microbiology* 83(9).

Li, M., Penner, G. B., Hernandez-Sanabria, E., Oba, M. and Guan, L. L. 2009. Effects of sampling location and time, and host animal on assessment of bacterial diversity and fermentation parameters in the bovine rumen. *Journal of Applied Microbiology* 107, 1924–34.

Li, Z., Wright, A.-D. G., Si, H., Wang, X., Qian, W., Zhang, Z. and Li, G. 2016. Changes in the rumen microbiome and metabolites reveal the effect of host genetics on hybrid crosses. *Environmental Microbiology Reports* 8, 1016–23.

Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., Fitzsimmons, C., Plastow, G. and Guan, L. L. 2019. Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. *Microbiome* 7, 92.

Lima, J., Auffret, M. D., Stewart, R. D., Dewhurst, R. J., Duthie, C.-A., Snelling, T. J., Walker, A. W., Freeman, T. C., Watson, M. and Roehe, R. 2019. Identification of rumen microbial genes involved in pathways linked to appetite, growth, and feed conversion efficiency in cattle. *Frontiers in Genetics* 10, 701.

Lloyd, D., Williams, A. G., Amann, R., Hayes, A. J., Durrant, L. and Ralphs, J. R. 1996. Intracellular prokaryotes in rumen ciliate protozoa: detection by confocal laser scanning microscopy after in situ hybridization with fluorescent 16S rRNA probes. *European Journal of Protistology* 32, 523–31.

McAllister, T. A. and Newbold, C. J. 2008. Redirecting rumen fermentation to reduce methanogenesis. *Australian Journal of Experimental Agriculture* 48, 7–13.

McCann, J. C., Wiley, L. M., Forbes, T. D., Rouquette Jr., F. M. and, Tedeschi, L. O. 2014. Relationship between the rumen microbiome and residual feed intake-efficiency of brahman bulls stocked on bermudagrass pastures. *PLoS ONE* 9, e91864.

Miyagi, T., Kaneichi, K., Aminov, R. I., Kobayashi, Y., Sakka, K., Hoshino, S. and Ohmiya, K. 1995. Enumeration of transconjugated *Ruminococcus albus* and its survival in the goat rumen microcosm. *Applied and Environmental Microbiology* 61, 2030–2.

Mizrahi, I. 2012. The role of the rumen microbiota in determining the feed efficiency of dairy cows. In: *Beneficial Microorganisms in Multicellular Life Forms*, pp. 203–10. Springer.

Moraïs, S. and Mizrahi, I. 2019a. The road not taken: the rumen microbiome, functional groups, and community states. *Trends in Microbiology* 27, 538–49.

Moraïs, S. and Mizrahi, I. 2019b. Islands in the stream: from individual to communal fiber degradation in the rumen ecosystem. *FEMS Microbiology Reviews* 43, 362–79.

Myer, P. R. 2019. Bovine genome-microbiome interactions: metagenomic frontier for the selection of efficient productivity in cattle systems. *mSystems* 4(3).

National Research Council. 2001. *Nutrient Requirements of Dairy Cattle*. National Academy Press, Washington DC.

Newbold, C. J., de la, Fuente, G., Belanche, A., Ramos-Morales, E. and McEwan, N. R. 2015. The role of ciliate protozoa in the rumen. *Frontiers in Microbiology* 6.

O'Herrin, S. M. and Kenealy, W. R. 1993. Glucose and carbon dioxide metabolism by *Succinivibrio dextrinosolvens*. *Applied and Environmental Microbiology* 59, 748–55.

Paz, H. A., Anderson, C. L., Muller, M. J., Kononoff, P. J. and Fernando, S. C. 2016. Rumen bacterial community composition in holstein and jersey cows is different under same dietary condition and is not affected by sampling method. *Frontiers in Microbiology* 7, 1206.

Pope, P. B., Smith, W., Denman, S. E., Tringe, S. G., Barry, K., Hugenholtz, P., McSweeney, C. S., McHardy, A. C. and Morrison, M. 2011. Isolation of *Succinivibrionaceae* implicated in low methane emissions from tammar wallabies. *Science* 333, 646–8.

Roehe, R., Dewhurst, RJ, Duthie, C-A, Rooke, JA, McKain, N., Ross, DW, Hyslop, JJ, Waterhouse, A., Freeman, T. C., Watson, M. and Wallace, R. J. 2016. Bovine host genetic variation influences rumen microbial methane production with best selection criterion for low methane emitting and efficiently feed converting hosts based on metagenomic gene abundance. *PLoS Genetics* 12, e1005846.

Rothschild, D., Weissbrod, O., Barkan, E., Kurilshikov, A., Korem, T., Zeevi, D., Costea, P. I., Godneva, A., Kalka, I. N., Bar, N., Shilo, S., Lador, D., Vila, A. V., Zmora, N., Pevsner-Fischer, M., Israeli, D., Kosower, N., Malka, G., Wolf, B. C., Avnit-Sagi, T., Lotan-Pompan, M., Weinberger, A., Halpern, Z., Carmi, S., Fu, J., Wijmenga, C., Zhernakova, A., Elinav,

E. and Segal, E. 2018. Environment dominates over host genetics in shaping human gut microbiota. *Nature* 555, 210–15.

Sasson, G., Kruger, B-S. S., Seroussi, E., Doron-Faigenboim, A., Shterzer, N., Yaacoby, S., Berg, M. M. E., White, B. A., Halperin, E. and Mizrahi, I. 2017. Heritable bovine rumen bacteria are phylogenetically related and correlated with the cow's capacity to harvest energy from its feed. *mBio* 8.

Shabat, S. K. B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg, Miller, M. E., White, B. A., Shterzer, N. and Mizrahi, I. 2016. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *The ISME Journal* 10, 2958.

Shade, A. and Handelsman, J. 2012. Beyond the Venn diagram: the hunt for a core microbiome. *Environmental Microbiology* 14, 4–12.

Shi, W., Moon, C. D., Leahy, S. C., Kang, D., Froula, J., Kittelmann, S., Fan, C., Deutsch, S., Gagic, D. and Seedorf, H. 2014. Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. *Genome Research* 24, 1517–25.

Siciliano-Jones, J. and Murphy, M. R. 1989. Production of volatile fatty acids in the rumen and cecum-colon of steers as affected by forage: concentrate and forage physical form. *Journal of Dairy Science* 72, 485–92.

Snelling, T. J., Auffret, M. D., Duthie, C-A, Stewart, R. D., Watson, M., Dewhurst, R. J., Roehe, R. and Walker, A. W. 2019. Temporal stability of the rumen microbiota in beef cattle, and response to diet and supplements. *Animal Microbiome* 1, 16.

Stewart, R. D., Auffret, M. D., Warr, A., Walker, A. W., Roehe, R. and Watson, M. 2019. Compendium of 4,941 rumen metagenome-assembled genomes for rumen microbiome biology and enzyme discovery. *Nature Biotechnology* 37, 953–61.

Tapio, I., Snelling, T. J., Strozzi, F. and Wallace, R. J. 2017. The ruminal microbiome associated with methane emissions from ruminant livestock. *Journal of Animal Science and Biotechnology* 8, 7.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R. and Gordon, J. I. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444, 1027–31.

Turpin, W., Espin-Garcia, O., Xu, W., Silverberg, M. S., Kevans, D., Smith, M. I., Guttman, D. S., Griffiths, A., Panaccione, R., Otley, A., Xu, L., Shestopaloff, K., Moreno-Hagelsieb G, G. E. M. Project Research Consortium, Paterson, A. D. and Croitoru, K. 2016. Association of host genome with intestinal microbial composition in a large healthy cohort. *Nature Genetics* 48, 1413–17.

Tymensen, L. D. and McAllister, T. A. 2012. Community structure analysis of methanogens associated with rumen protozoa reveals bias in universal archaeal primers. *Applied and Environmental Microbiology* 78, 4051–6.

Tymensen, L. D., Beauchemin, K. A. and McAllister, T. A. 2012. Structures of free-living and protozoa-associated methanogen communities in the bovine rumen differ according to comparative analysis of 16S rRNA and mcrA genes. *Microbiology* 158, 1808–17.

Ushida, K., Newbold, C. J. and Jouany, J.-P. 1997. Interspecies hydrogen transfer between the rumen ciliate *Polyplastron multivesiculatum* and, Methanosarcina barkeri. *The Journal of General and Applied Microbiology* 43, 129–31.

van Lingen, H. J., Plugge, C. M., Fadel, J. G., Kebreab, E., Bannink, A. and Dijkstra, J. 2016. Thermodynamic driving force of hydrogen on rumen microbial metabolism: a theoretical investigation. *PLoS ONE* 11, e0161362.

Wallace, R. J. and Walker, N. D. 1993. Isolation and attempted introduction of sugar alcohol-utilizing bacteria in the sheep rumen. *The Journal of Applied Bacteriology* 74, 353–9.

Wallace, R. J., John, Wallace, R., Rooke, J. A., Duthie, C-A, Hyslop, J. J., Ross, D. W., McKain, N., de, Souza, S. M., Snelling, T. J., Waterhouse, A. and Roehe, R. 2015a. Archaeal abundance in post-mortem ruminal digesta may help predict methane emissions from beef cattle. *Scientific Reports* 4.

Wallace, R. J., Rooke, J. A., McKain, N., Duthie, C.-A., Hyslop, J. J., Ross, D. W., Waterhouse, A., Watson, M. and Roehe, R. 2015b. The rumen microbial metagenome associated with high methane production in cattle. *BMC Genomics* 16, 839.

Wallace, R. J., Sasson, G., Garnsworthy, P. C., Tapio, I., Gregson, E., Bani, P., Huhtanen, P., Bayat, A. R., Strozzi, F., Biscarini, F., Snelling, T. J., Saunders, N., Potterton, S. L., Craigon, J., Minuti, A., Trevisi, E., Callegari, M. L., Cappelli, F. P., Cabezas-Garcia, E. H., Vilkki, J., Pinares-Patino, C., Fliegerová K. O., Mrázek, J., Sechovcová H, Kopečný J, Bonin, A., Boyer, F., Taberlet, P., Kokou, F., Halperin, E., Williams, J. L., Shingfield, K. J. and Mizrahi, I. 2019. A heritable subset of the core rumen microbiome dictates dairy cow productivity and emissions. *Science Advances* 5, eaav8391.

Weimer, P. J., Cox, M. S., de, Paula, T. V., Lin, M., Hall, M. B. and Suen, G. 2017. Transient changes in milk production efficiency and bacterial community composition resulting from near-total exchange of ruminal contents between high-and low-efficiency Holstein cows. *Journal of Dairy Science* 100, 7165–82.

Welkie, D. G., Stevenson, D. M. and Weimer, P. J. 2009. ARISA analysis of ruminal bacterial community dynamics in lactating dairy cows during the feeding cycle. *Anaerobe* 16, 94–100.

Wolin, M. J. 1979. The rumen fermentation: a model for microbial interactions in anaerobic ecosystems. In: Alexander, M. (Ed.), *Advances in Microbial Ecology* (vol. 3). Springer US, Boston, MA, pp. 49–77.

Zhou, M. I. and Hernandez-Sanabria, E. 2009. Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. *Applied and Environmental Microbiology* 75, 6524–33.

Zhou, M., Hernandez Sanabria, E. and Guan, L. 2009. Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. *Applied and Environmental Microbiology* 75, 6524–33. doi:10.1128/AEM.02815-081.

Zhou, M., Hernandez-Sanabria, E. and Guan, L. L. 2010. Characterization of variation in rumen methanogenic communities under different dietary and host feed efficiency conditions, as determined by PCR-denaturing gradient gel electrophoresis analysis. *Applied and Environmental Microbiology* 76, 3776–86.

Zhou, M., Peng, Y.-J., Chen, Y., Klinger, C. M., Oba, M., Liu, J.-X. and Guan, L. L. 2018. Assessment of microbiome changes after rumen transfaunation: implications on improving feed efficiency in beef cattle. *Microbiome* 6, 62.

Chapter 17

The rumen as a modulator of immune function in cattle

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1 Introduction

Modern dairy cows are typically fed grain-rich diets to fulfill their high energy requirements. Nonetheless, this feeding practice impairs chewing activity and production of buffering substances via saliva, while increasing volatile fatty acid (VFA) production in the rumen. This leads to subacute ruminal acidosis (SARA) (Plaizier et al., 2008; Kleen and Cannizzo, 2012). SARA is described as an intermittent drop of ruminal pH below 5.6 for longer than 3 hours/day (Gozho et al., 2005; Plaizier et al., 2008) or below 5.8 for longer than 5–6 hours/day (Zebeli et al., 2008). Although the bovine rumen can adapt to grain-rich diets by increasing the ruminal papilla epithelium surface, the rate of cellular aging can decrease to a level that induces parakeratosis and hyperkeratosis during sustained high-grain feeding. This compromises VFA absorption, reducing the pH and risking the onset of SARA (Zebeli and Metzler-Zebeli, 2012).

In a healthy rumen, the squamous multilayer epithelium acts as the main site for the absorption of key nutrients (i.e. VFA and electrolytes), and is highly selective to prevent simultaneous entry of microbes and luminal toxins into systemic circulation (Plaizier et al., 2018; Aschenbach et al., 2019). However, SARA can lead to a failure in the selective rumen epithelium barrier function, thereby enabling luminal immunogens to translocate into the blood supply

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and lymphatic system (Wu et al., 2016). More specifically, various luminal toxins such as endotoxins and biogenic amines seem to interfere with the epithelial constraint function by altering the structure and function of the tight junction barrier, thereby disrupting the integrity of epithelial cells and enabling their translocation changing cellular pathways (Berkes et al., 2003).

There is a growing body of evidence that indicates that SARA leads to enhanced growth and lysis of Gram-negative bacteria (GNB) followed by the release of large amounts of cell-free lipopolysaccharides (LPS) (Nagaraja et al., 1978; Beutler and Rietschel, 2003; Plaizier et al., 2012) and biogenic amines (BA) such as ethanolamine and histamine (Dong et al., 2011). While some BA are considered as local inflammatory agents (Gozho et al., 2005), the gastrointestinal LPS, which is part of the outer membrane of GNB's cell wall, is an abundant and potentially pro-inflammatory molecule which has been investigated extensively in relation to immunity and disease (Emmanuel et al., 2008; Li et al., 2012a; Plaizier et al., 2012). Once gastrointestinal LPS enters into circulation, a pro-inflammatory cascade is triggered which is commonly known as a low-degree acute phase response (APR). This is characterized by moderate elevation of serum acute phase proteins (APP) with lipopolysaccharide-binding protein (LBP), serum amyloid A (SAA) and haptoglobin (Hp; Ceciliani et al., 2012) being the main markers of the ruminal LPS translocation in cattle (Iqbal, 2013). The SAA promotes clearance of LPS that enters circulation through LPS-SAA-lipoprotein complexes in hepatocytes and the bile (Ametaj et al., 2010a; Zebeli and Metzler-Zebeli, 2012).

On the other hand, LBP transports LPS to immune cells such as macrophages, monocytes and neutrophils to be detoxified (Gallay et al., 1994; Schumann et al., 1994). More specifically, since LBP carries LPS to macrophages, the membrane receptor-CD14 at the macrophage surface interacts with TLR-4 and myeloid differentiation factor 2 (MD-2) which signal activation of macrophages (Chow et al., 1999; da Silva Correia et al., 2001). These signals are recognized by the myeloid differentiation primary response gene 88 (MyD88) activating nuclear factor κ-B (NFκ-B) and pro-inflammatory cytokines, for example, tumor-necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-8 (Erridge et al., 2002; Emmanuel et al., 2008; Ceciliani et al., 2012; Plaizier et al., 2012). These pro-inflammatory cytokines activate inflammation and trigger fever, stress, low feed intake, lipolysis and other metabolic changes in host cattle (Zebeli and Metzler-Zebeli, 2012; Abaker et al., 2017).

Besides the systemic inflammatory responses caused by LPS, a higher nutrient requirement to support immune responses lowers the nutrients available for the synthesis of milk components (Dong et al., 2011). Furthermore, when LPS is transported to the mammary gland via systemic circulation, the bacterial toxins may have harmful effects on the epithelial cell functions in the mammary gland (Dong et al., 2011). Indeed, it has been reported that dairy cows

which fed high-grain/low-forage diets showed increased LPS-concentrations (determined using the limulus amebocyte lysate test) in mammary blood and epigenetic changes in the mammary tissues (Dong et al., 2014) as well as increased LBP concentrations in milk (Khafipour et al., 2009a). The impact of SARA and the LPS in the gastrointestinal tract of dairy cows on immunity and metabolism has recently attracted attention with a number of comprehensive review articles (Plaizier et al., 2008, 2012; Dong et al., 2011; Kleen and Cannizzo, 2012). However, current knowledge about the relationships between SARA, LPS and its resulting inflammation, systemic metabolism and the mammary immune system still needs to be summarized and reviewed. This review aims to update and synthesize recent research regarding the effect of SARA and the resulting increase in free rumen LPS on metabolism and health in cattle, with a special emphasis on the mammary gland of dairy cows.

2 Prevalence of subacute ruminal acidosis (SARA) in dairy herds

Subacute ruminal acidosis (SARA) is commonly viewed as a severe and prevalent health disorder of cattle, which particularly occurs during early and mid-lactating periods and causes substantial economic losses to the dairy industry (Garrett et al., 1997; Plaizier et al., 2008). Previous studies showed a prevalence of SARA of about 11–27% in earlylactation and of 18–27% during mid-lactation (Garrett et al., 1997; Kleen et al., 2004; Tajik et al., 2009). Early lactating cows are predisposed to SARA when, during the transition period, the diet changes too abruptly from dry cow diets rich in forage to a lactating cow diet rich in starch (a transition which should take at least 4–5 weeks (Enemark, 2008; Humer et al., 2018a). Short adaptation to a grain-rich diet also increases the risk of SARA (Pourazad et al., 2016). Another factor that may increase the risk of SARA in mid-lactating cows is high feed consumption, especially when diets are deficient in physicallyeffective neutral detergent fiber (Nordlund et al., 1995; Stone, 2004). Parity also seems to play a role, with primiparous cows having a greater risk of developing SARA when compared with multiparous cows (Humer et al., 2015). This might be due to a less well-adapted rumen epithelium and microbiome, differences in feeding behavior and body weight and less experience on how to self-regulate the ruminal pH (Humer et al., 2018a).

According to field studies conducted in several countries, the overall prevalence of SARA in dairy farms ranges between 8–33% (Table 1). This difference might be due to several factors such as less intensive feeding in pasture-based systems predominating in Ireland and Australia, for instance, as well as differences in feeding and herd management. Higher SARA risks in larger herds may be due to less intensive animal observation (Kleen et al., 2013). In general, SARA reduces the productivity of cattle operations (Nagaraja and

Table 1 Prevalence of subacute ruminal acidosis (SARA) in dairy cows of some countries

Prevalence (%)	Countries	References
33	Italy	Morgante et al. (2007)
28	Iran	Tajik et al. (2009)
26	United Kingdom	Atkinson (2013)
22	Denmark	Enemark and Jørgensen (2001)
20	Germany	Kleen et al. (2013)
16	Greece	Kitkas et al. (2013)
14	The Netherlands	Kleen et al. (2009)
11	Ireland	O'Grady et al. (2008)
8	Australia	Bramley et al. (2008)

Lechtenberg, 2007; Plaizier et al., 2008). It does so by reducing body condition, feed intake, milk production and milk energy efficiency (Yang and Beauchemin, 2006). Overall, losses due to SARA are reported to reach US$1.12/day per affected cow (Stone, 1999).

To minimize these economic losses, early detection of this disorder is crucial. Although SARA is considered as a sub-clinical disorder, SARA-affected cows might be recognized by para-clinical signs, such as reduced cud-chewing (Zebeli et al., 2010), milk fat depression (Zebeli and Ametaj, 2009), diarrhea, foamy feces (Nordlund and Garrett, 1994; Kleen et al., 2003), and presence of undigested grain in feces (Enemark, 2008). On the other hand, the onset of SARA might also be related to other metabolic disorders (Aditya et al., 2017), such as sudden death syndrome, fatty liver (Ametaj et al., 2005) and laminitis (Nocek, 1997). The reasons why SARA increases the risk of developing metabolic disorders in cattle have not yet been fully established. However, alterations to the rumen ecosystem, such as increase in GNB, and the immunological changes which result, have recently mentioned as playing a role (Plaizier et al., 2008, 2012; Dong et al., 2011; Kleen and Cannizzo, 2012; Zebeli and Metzler-Zebeli, 2012).

3 Rumen health, metabolic activity and disorders

Although activation of the APR is essential to eliminate agent(s) that cause inflammation and to reestablish homeostasis, prolonged inflammatory processes are associated with negative consequences for the host (Morris and Li, 2012; Lacetera, 2016). For instance, the higher energy requirements associated with activation of the APR result in lowered feed efficiency and might aggravate the negative energy balance of dairy cows, particularly during early lactation (Zebeli and Metzler-Zebeli, 2012; Lacetera, 2016). The APR can lead to changes in energy and lipid metabolism in different body tissues, including the

mammary gland (Kushibiki et al., 2002; Khovidhunkit et al., 2004). Furthermore, it has been suggested that LPS and the activated APR may be involved in the development of multiple metabolic diseases, including displaced abomasum, fatty liver, liver abscesses, laminitis and downer cow syndrome (Nocek, 1997; Kleen et al., 2003; Plaizier et al., 2008; Zebeli et al., 2015). There is, however, currently limited information about the interaction between rumen health and the systemic metabolism in dairy cows. There has been little research into the consequences of increased concentrations of potentially toxic compounds in the rumen, for example, LPS and BA, and possible changes of metabolic pathways in systemic circulation in dairy cows.

Most studies investigating the effect of rumen fermentation disorders on systemic metabolism have focused on single metabolites, such as non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHBA), cholesterol and liver enzymes indicating liver tissue damage (Zebeli et al., 2011; Marchesini et al., 2013). Several studies report an association between rumen fermentation disorders and increased activity of liver enzymes (Humer et al., 2018b; Kröger et al., 2019). This corresponds with increased ruminal LPS and BA loads during high-grain feeding (Humer et al., 2018b). This suggests that these changes are caused by an enhanced clearance rate of LPS and other circulating toxins in the Kupffer cells of the liver (Lechowski, 1997; Marchesini et al., 2013). Studies have shown that, even when the ruminal pH increased, liver enzymes continued to increase during high-grain feeding of the cows for 2 weeks (Fig. 1). Thus, it can be assumed that cows require more time to restore liver health than to regulate ruminal pH, which can return to healthy levels almost within a few days after

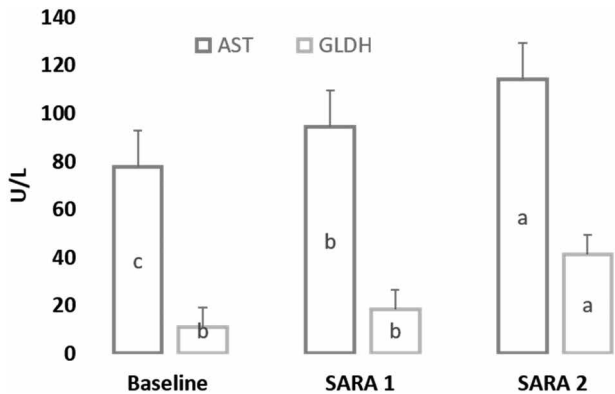

Figure 1 Concentrations of liver enzymes (aspartate aminotransferase (AST); glutamate dehydrogenase (GLDH)) in the blood of dairy cows that fed either a pure forage diet (Baseline) or a 65% concentrated diet causing subacute ruminal acidosis (SARA) during two consecutive weeks (SARA 1 and SARA 2). Different letters indicate differences among baseline, SARA 1 and SARA 2 at $P < 0.05$. Source: adapted from Humer et al.(2018b).

a high-grain diet (Kröger et al., 2017; Khiaosa-ard et al., 2018). An increased activity of liver enzymes due to rumen fermentation disorders suggests a negative and potentially accumulative effect of high-grain feeding on LPS load and liver health, which might cause an impairment of overall health.

In recent years, metabolomic technologies have enabled the detection of multiple classes of metabolites reflecting changes in key metabolic pathways. These help to improve the understanding of interactions between nutrition, metabolism and health. Metabolomics is the quantitative analysis of all metabolites in an organism under specific conditions. Metabolites represent intermediates and end products of metabolic pathways. They are able to reflect physiological dysfunctions more rapidly than current biomarkers (such as APP or liver enzymes). Metabolomics may highlight earlier stages of metabolic disorders, helping to identify biomarkers of important cow diseases such as rumen fermentation disorders (Ametaj, 2010b).

Metabolomic technologies have been applied to rumen fluid samples in cows fed increasing levels of grain levels (Ametaj et al., 2010b; Saleem et al., 2012). More recently a metabolomics approach was also used to analyze blood samples taken from dairy cows that fed either a pure forage diet or receiving a high-grain diet (51% grains) which caused SARA (Humer et al., 2018b). Potentially toxic compounds, for example, BA and LPS, were analyzed in the rumen content and linked to changes in patterns of blood metabolic profiles using different data mining approaches (Humer et al., 2018b). Multivariate analyses indicated that cows experiencing SARA had elevated concentrations of ruminal LPS and BA (i.e. histamine, ethanolamine, isopropylamine, pyrrolidine, putrescine, cadaverine, spermidine), which accompanied significant changes in the blood metabolome. Decreases in phosphatidylcholines (PC; Fig. 2), lysophosphatidylcholines (lysoPC) and sphingomyelins were observed in particular. A decrease in PC has also been reported in the rumen fluid of cows that fed high-grain diets containing up to 45% barley grain (Saleem et al., 2012). This might be due to a decline in protozoa during rumen pH depression, which are the main source of PC in the rumen fluid (Jouany et al., 1988; Goad et al., 1998; Khafipour et al., 2009). The decrease in PC probably also caused the decrease of lysoPC, because lysoPC are a hydrolysis product of PC (Hailemariam et al., 2014a). A decrease in plasma lysoPC has also been reported in cows receiving LPS from *Escherichia coli* (O26:B6) intramammarily or experiencing diseases such as mastitis, metritis, retained placenta and laminitis (Hailemariam et al., 2014a; Humer et al., 2018c).

A further explanation for the decrease in PC might be the decrease in cholesterol, as PC are generally associated with cholesterol and triacylglycerols (Gruffat et al., 1996). As high levels of rapidlyfermentable carbohydrates are generally associated with a decreased production of precursors for cholesterol synthesis in ruminants (i.e. acetate; Liepa et al., 1978; Neubauer et al., 2018),

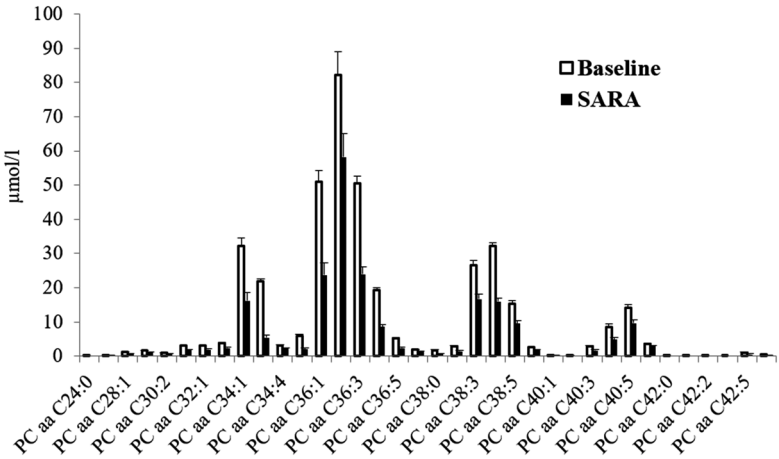

Figure 2 Concentrations of phosphatidylcholines (PC) with diacyl-residues (aa) in the blood of dairy cows that fed either a pure forage diet (Baseline) or a 65% concentrated diet causing subacute ruminal acidosis (SARA).

decreasing PC and lysoPC might be a common indicator in cows receiving high-grain diets. Sphingomyelins belong to the group of bovine phospholipids (Nilsson and Duan, 2006). This is supported by a strong positive association of PC, sphingomyelins and cholesterol, which were identified in one subcluster through multivariate and correlation analysis.

Figure 3 shows the effects on amino acids (AA). Decreases in arginine, citrulline, isoleucine, methionine, phenylalanine, tryptophan and tyrosine were observed while concentrations of glycine and serine increased. The decreasing effect of SARA-related feeding on AA concurs with previous studies reporting a pronounced decrease in cows receiving either an external *E. Coli*-LPS-challenge (Humer et al., 2018c) or experiencing one or several periparturient diseases (Hailemariam et al., 2014b). The underlying mechanism might be increased protein catabolism driven by the requirement for AA for immune cells, causing enhanced consumption of AA such as arginine or tryptophan (Le Floćh et al., 2004; Hailemariam et al., 2014b). Only glycine and serine were enhanced in dairy cows experiencing SARA. An enhanced production of glycine and its precursor serine might be a way for cows to counterbalance SARA-associated inflammation and oxidative stress, as glycine has been reported to be protective against injuries and diseases due its antioxidant, anti-inflammatory, cytoprotective and immunomodulatory properties (Razak et al., 2017).

Multivariate analysis suggests a negative relationship between the concentration of several deleterious compounds in the rumen fluid (i.e. LPS, histamine, ethanolamine, pyrrolidine, and spermidine) and the concentration of AA, PC, lysoPC and sphingomyelins in the blood. This suggests that enhanced

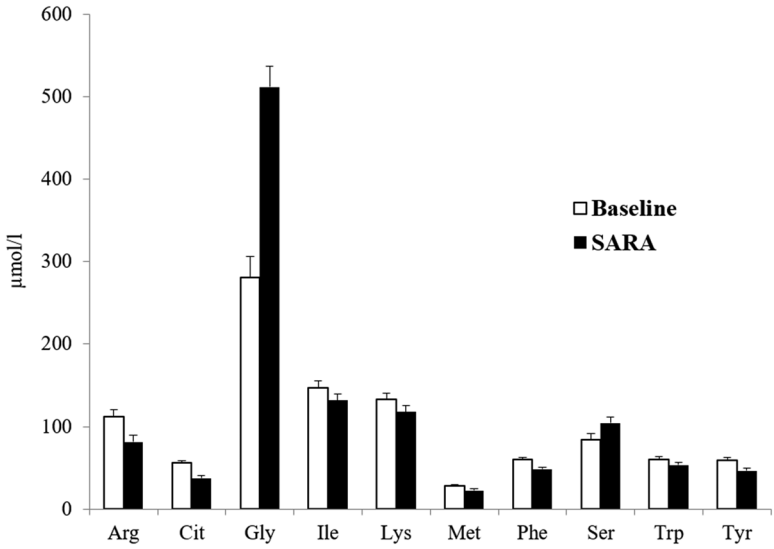

Figure 3 Concentrations of amino acids in the blood of dairy cows that fed either a pure forage diet (Baseline) or a 65% concentrated diet causing subacute ruminal acidosis (SARA).

release of toxic substances in the rumen in cows experiencing grain-induced SARA affect metabolic pathways for AA and lipid metabolism.

4 Rumen health and the mammary immune system

A common symptom of SARA is lower milk fat (Zebeli and Ametaj, 2009). Dairy cattle scientists have long recognized the effect of rumen fermentation disorders (i.e. SARA) on mammary gland metabolism. New data suggest that endogenous LPS derived from the gastrointestinal tract can invade the mammary gland after breaking the milk-blood barrier (Kim et al., 2013), thereby eliciting a local immune response (Dong et al., 2011). Higher amounts of LBP in milk (Khafipour et al., 2009) and pro-inflammatory cytokines in mammary blood (Zhou et al., 2014), in cows experiencing SARA episodes, have also been reported in the literature. These results are similar to the effects caused by exogenous LPS-induced mastitis in cows, which also results in higher milk-LBP (Bannerman et al., 2003) and pro-inflammatory cytokines (Lee et al., 2003; Wellnitz et al., 2011). It is likely that endogenous LPS (deriving from the gastrointestinal bacteria) as well as exogenous LPS (deriving from the environment) are able to destroy the blood-milk barrier (Wall et al., 2016; Zhang et al., 2016). Humer et al. (2018b) found that cows experiencing SARA had higher concentrations of milk amyloid A (MAA) compared with non-SARA cows when they were intramammarily challenged with exogenous LPS (Fig. 4). It is likely that SARA episodes amplify

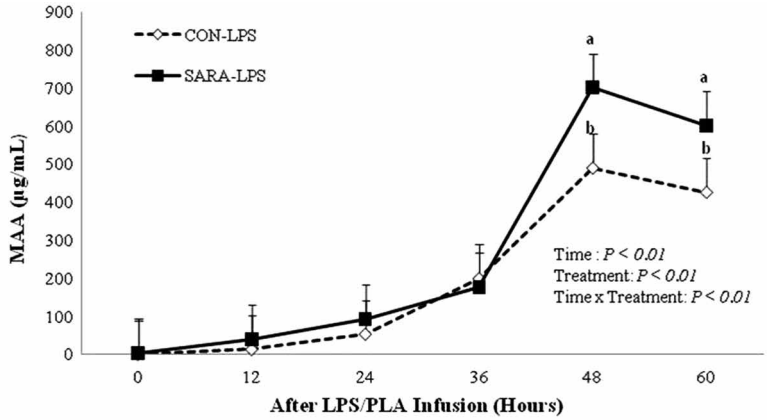

Figure 4 The concentration of milk amyloid A (MAA) in cows receiving LPS infusion subjected SARA (SARA-LPS) and non-SARA (CON-LPS) conditions.

the inflammatory response to infectious external stimuli like exogenous LPS (Aditya et al., 2017).

Contrasting effects were observed in a study by Gott et al. (2015). They found higher MAA concentrations and somatic cell counts in cows fed a control diet compared to a high starch diet (formulated to induce chronic ruminal pH depression) after an intramammary LPS challenge, thus suggesting a certain LPS tolerance. One explanation for the observed differences might be a stronger SARA and thus a chronic exposure to higher LPS loads in the study conducted by Gott et al. (2015). One possible explanation for the stronger response in SARA cows in the study by Humer et al. (2018b) might be the assumed long-term exposure to low dosages of LPS related to mild SARA and generally low concentrations of APP, for example, LBP. Studies in humans have reported that low doses of LPS induce a state of tolerance to subsequent toxic doses of LPS, while very low doses can even have an opposite effect (Morris and Li, 2012). However, as no information regarding ruminal pH dynamics and LPS or LBP concentrations has been reported by Gott et al. (2015), this explanation remains hypothetical. There is clearly a need for further research to elucidate the responsiveness of dairy cows to external infectious agents after experiencing rumen fermentation disorders.

It has been recently reported that LPS is able to disrupt the blood-milk barrier by modifying claudins in the alveolar tight junctions (TJ) of mammary epithelial cells (Kobayashi et al., 2013). Claudins are the most important proteins in the control of the TJ barrier function (Beeman et al., 2012; Schlingmann et al., 2016). Alterations of their composition might enable LPS to destroy the blood-milk barrier. Interestingly, the activation of NF-κB pathways via LPS/TLR-4 signaling has been assumed to induce changes in TJ permeability (Kobayashi

et al., 2013). Once LPS breaks this barrier, bovine mammary epithelial cells would be the further line of defense (Strandberg et al., 2005; Zbinden et al., 2014).

Most of the defense mechanisms of the mammary gland act as non-specific immune responses via leukocytes and soluble immune components such as inflammatory markers and antimicrobial factors (Schmitz et al., 2004). Antimicrobial defense proteins in milk, such as lactoferrin and lysozyme, are typically augmented during acute mastitis (Carlsson et al., 1989). Jin et al. (2016) observed an enhanced expression of defensins, such as lingual antimicrobial peptide, in the mammary epithelial cells of dairy cows experiencing SARA. These authors hypothesized the activation of the NF-κB signaling pathway as one of the underlying mechanisms. The activities of other bactericidal proteins reflecting inflammation and oxidative stress (i.e. β-N-acetyl glucosaminidase and myeloperoxidase) have been upregulated in cows experiencing SARA, indicating infection in the bovine mammary glands.

When LPS invades the mammary gland, excessive pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α are produced, inducing local inflammatory events via LPS/TLR4 signaling pathways (Akira et al., 2006; Ingman et al., 2014). In general, bovine mammary epithelial cells contribute to the innate immune response to intramammary infections by recognizing pathogens through specialized pattern recognition receptors, such as TLR4, which binds and is activated by the LPS-LBP complex (Ibeagha-Awemu et al., 2008). Overall, about 10 bovine TLRs have been identified, each recognizing specific ligands or pathogen-associated molecular patterns (McGuire et al., 2006). However, some pathogens have been reported to be able to activate more than one TLR (Swanson et al., 2004). In this regard, TLR4 mainly recognizes LPS, whereas TLR2 is commonly triggered by other cell wall components including those found on Gram-positive bacteria (GPB), such as peptidoglycan and lipoteichoic acid (Eckel and Ametaj, 2016). Incubation of bovine mammary epithelial cells with increasing LPS-concentrations has been shown to upregulate the expression of TLR4 as well as TLR2 as a result of downstream TLR4 signaling molecules and increased surface expression of specific antibodies against those receptors (Ibeagha-Awemu et al., 2008). The upregulation of TLR2 is likely due to some cross-talk among TLR2 and TLR4 receptors, as it is secondary to TLR4 activation and NF-κB production (Faure et al., 2001; Fan et al., 2003).

TLR4-dependent upregulation of TLR2 seems to depend on the presence of NF-κB sites on TLR2 (Ibeagha-Awemu et al., 2008). However, as several studies found no TLR2 involvement in LPS signaling (Heine et al., 1999; Takeuchi et al., 1999), the mammary epithelial cells may respond differently to the presence of some pathogenic compounds compared to other cell types (Ibeagha-Awemu et al., 2008). Goldammer et al. (2004) observed a coordinated upregulation of TLR2 as well as TLR4 in experimentally-induced *Staphylococcus aureus* mastitis

in dairy cows, although *Staphylococcus aureus* is generally expected only to upregulate TLR2. Mammary epithelial cells appear not only to possess the required immune repertoires to mount a robust defense against *Escherichia coli*, but also to adapt toward an effective response to different types of mastitis pathogens (Ibeagha-Awemu et al., 2008).

It is important to note that excessive production of pro-inflammatory cytokines following infection may lead to injury of mammary epithelial cells and can also induce serious systemic disorders such as chronic enterocolitis and atherosclerosis or even septic shock (Takeda and Akira, 2005). The study by Kobayashi et al. (2013) clearly demonstrated negative relationships between the concentration of inflammatory cytokines (i.e. IL-1β and IL-8) in mammary blood and milk production parameters. Impaired milk production in dairy cows experiencing SARA might be partly attributable to local inflammatory processes, which decrease the available nutrients or milk component precursors for milk component synthesis.

LPS that enter the mammary tissue might also activate neutrophils which, in turn, can produce large amounts of bactericidal molecules, including proteins, peptides and reactive oxygen species (ROS; Dong et al., 2011). Since ROS are unstable oxygen-containing molecules which respond to other molecules (ranging from proteins to lipids to DNA and RNA in cells), they promote oxidation which causes tissue damage (Abuelo et al., 2015; Zebeli et al., 2015). Production of ROS exceeding the antioxidative potential results in oxidative stress. This causes dysfunctional inflammation which can then cause metabolic stress, thereby increasing cows' susceptibility to health disorders (Sordillo and Aitken, 2009). It has been reported that the secretory tissue of the mammary gland is highly sensitive to LPS (Schmitz et al., 2004; Blum et al., 2000), due to high levels of oxygen free radicals and metabolites of lipid peroxidation in the mammary gland, especially in high-producing cows (Shi et al., 2016). SARA can thus be a risk factor for udder health.

Prolonged SARA episodes impair the antioxidant mechanism in the liver and mammary gland of lactating cows (Abaker et al., 2017; Memon et al., 2019). This mechanism involves an increased concentration of malondialdehyde (MDA) and mitogen-activated protein kinase (MAPK) pro-inflammatory genes. It also involves a decreased level of nuclear factor erythroid 2-related factor 2 (Nrf2) which is associated with protein expression and antioxidant genes in the mammary gland tissue of dairy cows experiencing SARA (Memon et al., 2019). The Nrf2 protein plays a key role in the expression of antioxidant defenses against ROS triggered by inflammation (Kansanen et al., 2013). A reduced expression of Nrf2 indicates suppression of antioxidant status which subsequently induces oxidative stress in mammary gland tissue.

The local immune response in the mammary gland caused by the translocation of LPS or other immunogenic compounds into the mammary

gland might also impair the immune defense mechanisms of the teat and even destroy its barrier function (Pareek et al., 2005; Dong et al., 2011). Cows experiencing SARA might also be more vulnerable to bacterial invasion and colonization in the mammary tissue compared to healthy cows (Sordillo and Streicher, 2002). After bacteria overcome the teat and streak canal barriers – as the first line of anatomical defenses – they might evade the mammary gland's cellular and humoral defense mechanisms (Sordillo and Streicher, 2002; Zhao and Lacasse, 2008). Besides the well-known shifts in the rumen microbiome due to SARA, high-grain feeding may also affect the composition of milk microbiota. A recent study revealed a higher proportion of several mastitis-causing pathogens, such as *Stenotrophomonas maltophilia*, *Streptococcus parauberis* and *Brevundimonas diminuta*, in dairy cows experiencing SARA (Zhang et al., 2015). Cows suffering from SARA showed a higher abundance of several psychrotrophic bacteria, such as *Brevundimonas*, *Sphingobacterium*, *Alcaligenes*, *Enterobacter* and *Lactobacillus* in milk compared to healthy cows. High-grain feeding may therefore enhance the risk of dairy cows suffering from gram-negative mastitis and also decrease raw milk quality and safety and limit the shelf life of processed milk (Zhang et al., 2015).

5 Conclusions

This review provides an update of the current knowledge about the effects of SARA, and the resulting translocation of endogenous LPS from the gastrointestinal tract into the circulatory system, on liver health, systemic metabolism, udder metabolism and health in dairy cows. The current body of evidence also suggests that endogenous LPS can invade the mammary gland after breaking the milk-blood barrier, thereby eliciting a local immune response resembling the damage of the blood-milk barrier caused by exogenous LPS. Endogenous LPS due to prolonged bouts of SARA might modify the mammary inflammation caused by exogenous LPS sources and may lower the protective capability of the udder in affected cows.

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7 Where to look for further information

A basic introduction to start this chapter is Zebeli and Metzler-Zebeli, 'Interplay between rumen digestive disorders and diet-induced inflammation in dairy cattle' (see Zebeli and Metzler-Zebeli, 2012 in the reference for full details).

Furthermore, there are current review/research projects designed to support the rumen health regarding subacute rumen acidosis incidence, including:

1 Invited review: Practical feeding management recommendations to mitigate the risk of subacute ruminal acidosis in dairy cattle (https://www.ncbi.nlm.nih.gov/pubmed/29153519).
2 Diagnosis and management of subacute ruminal acidosis in dairy herds (https://www.sciencedirect.com/science/article/abs/pii/S0749072017300579).
3 Supplementing phytogenic compounds or autolyzed yeast modulates ruminal biogenic amines and plasma metabolome in dry cows experiencing subacute ruminal acidosis (https://www.sciencedirect.com/science/article/pii/S0022030218306672).

8 References

Abaker, J. A., Xu, T. L., Jin, D., Chang, G. J., Zhang, K. and Shen, X. Z. 2017. Lipopolysaccharide derived from the digestive tract provokes oxidative stress in the liver of dairy cows fed a high-grain diet. *J. Dairy Sci.* 100(1), 666–78. doi:10.3168/jds.2016-10871.

Abuelo, A., Hernandez, J., Benedito, J. L. and Castilo, C. 2015. The importance of the oxidative status of dairy cattle in the periparturient period: revisiting antioxidant supplementation. *J. Anim. Physiol. Anim. Nutr.* 99(6), 1003–16. doi:10.1111/jpn.12273.

Aditya, S., Humer, E., Pourazad, P., Khiaosa-ard, R., Huber, J. and Zebeli, Q. 2017. Intramammary infusion of *Escherichia coli* lipopolysaccharide negatively affects feed intake, chewing, and clinical variables, but some effects are stronger in cows experiencing subacute rumen acidosis. *J. Dairy Sci.* 100, 1363–77.

Akira, S., Uematsu, S. and Takeuchi, O. 2006. Pathogen recognition and innate immunity. *Cell* 124(4), 783–801. doi:10.1016/j.cell.2006.02.015.

Ametaj, B. N., Bradford, B. J., Bobe, G., Nafikov, R. A., Lu, Y., Young, J. W. and Beitz, D. C. 2005. Strong relationships between mediators of the acute phase response and fatty liver in dairy cows. *Can. J. Anim. Sci.* 85(2), 165–75. doi:10.4141/A04-043.

Ametaj, B. N., Zebeli, Q. and Iqbal, S. 2010a. Nutrition, microbiota, and endotoxin-related diseases in dairy cows. *R. Bras. Zootec.* 39(suppl. spe), 433–44. doi:10.1590/S1516-35982010001300048.

Ametaj, B. N., Zebeli, Q., Saleem, F., Psychogios, N., Lewis, M. J., Dunn, S. M., Xia, J. and Wishart, D. S. 2010b. Metabolomics reveals unhealthy alterations in rumen metabolism with increased proportion of cereal grain in the diet of dairy cows. *Metabolomics* 6(4), 583–94. doi:10.1007/s11306-010-0227-6.

Aschenbach, J. R., Zebeli, Q., Patra, A. K., Greco, G., Amasheh, S. and Penner, G. B. 2019. Symposium review: the importance of the ruminal epithelial barrier for a healthy and productive cow. *J. Dairy Sci.* 102(2), 1866–82. doi:10.3168/jds.2018-15243.

Atkinson, O. 2013. A cross-sectional survey to investigate prevalence of and clinical indicators for Subacute Ruminal Acidosis (SARA) in lactating cows on UK dairy farms. Diploma in Cattle Health and Production. Dissertation. Royal College of Veterinary Surgeons, London.

Bannerman, D. D., Paape, M. J., Hare, W. R. and Sohn, E. J. 2003. Increased levels of LPS-binding protein in bovine blood and milk following bacterial lipopolysaccharide challenge. *J. Dairy Sci.* 86(10), 3128–37. doi:10.3168/jds.S0022-0302(03)73914-9.

Beeman, N., Webb, P. G. and Baumgartner, H. K. 2012. Occludin is required for apoptosis when claudin-claudin interactions are disrupted. *Cell Death Dis.* 3(2), e273. doi:10.1038/cddis.2012.14.

Berkes, J., Visvanathan, V. K., Savkovic, S. D. and Hecht, G. 2003. Intestinal epithelial responses to enteric pathogens: effects on the tight junction barrier, ion transport, and inflammation. *Gut* 52(3), 439–51. doi:10.1136/gut.52.3.439.

Beutler, B. and Rietschel, E. T. 2003. Innate immune sensing and itsroots: the story of endotoxin. *Nat. Rev. Immunol.* 3(2), 169–76. doi:10.1038/nri1004.

Blum, J. W., Dosogne, H., Hoeben, D., Vangroenweghe, F., Hammon, H. M., Bruckmaier, R. M. and Burvenich, C. 2000. Tumor necrosis factor-α and nitrite/nitrate responses during acute mastitis induced by *Escherichia coli* infection and endotoxin in dairy cows. *Domest. Anim. Endocrinol.* 19(4), 223–35. doi:10.1016/s0739-7240(00)00079-5.

Bramley, E., Lean, I. J., Fulkerson, W. J., Stevenson, M. A., Rabiee, A. R. and Costa, N. D. 2008. The definition of acidosis in dairy herds predominantly fed on pasture and concentrates. *J. Dairy Sci.* 91(1), 308–21. doi:10.3168/jds.2006-601.

Carlsson, A., Björck, L. and Persson, K. 1989. Lactoferrin and lysozyme in milk during acute mastitis and their inhibitory effect in Delvotest P. *J. Dairy Sci.* 72(12), 3166–75. doi:10.3168/jds.S0022-0302(89)79475-3.

Ceciliani, F., Ceron, J. J., Eckersall, P. D. and Sauerwein, H. 2012. Acute phase protein in ruminants. *J. Proteomics* 75(14), 4207–31. doi:10.1016/j.jprot.2012.04.004.

Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J. and Gusovsky, F. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J. Biol. Chem.* 274(16), 10689–92. doi:10.1074/jbc.274.16.10689.

da Silva Correia, J., Soldau, K., Christen, U., Tobias, P. S. and Ulevitch, R. J. 2001. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex transfer from CD14 to TLR4 and MD-2. *J. Biol. Chem.* 276(24), 21129–35. doi:10.1074/jbc.M009164200.

Dong, G., Liu, S., Wu, Y., Lei, C. Z., Zhou, J. and Zhang, S. 2011. Diet-induced bacterial immunogens in the gastrointestinal tract of dairy cows: impacts on immunity and metabolism. *Acta. Vet. Scand.* 53(1), 48. doi:10.1186/1751-0147-53-48.

Dong, G., Qiu, M., Ao, C. Z., Khas-Erdene, J., Khas-Erdene, Wang, X., Zhang, Z. and Yang, Y. 2014. Feeding a high-concentrate corn straw diet induced epigenetic alterations in the mammary tissue of dairy cows. *PLoS ONE* 9(9), e107659. doi:10.1371/journal.pone.0107659.

Eckel, E. F. and Ametaj, B. N. 2016. Invited review: role of bacterial endotoxins in the etiopathogenesis of periparturient diseases of transition dairy cows. *J. Dairy Sci.* 99(8), 5967–90. doi:10.3168/jds.2015-10727.

Emmanuel, D. G., Dunn, S. M. and Ametaj, B. N. 2008. Feeding high proportions of barley grain stimulates an inflammatory response in dairy cows. *J. Dairy Sci.* 91(2), 606–14. doi:10.3168/jds.2007-0256.

Enemark, J. M. D. 2008. The monitoring, prevention and treatment of sub-acute ruminal acidosis (SARA): a review. *Vet. J.* 176(1), 32–43. doi:10.1016/j.tvjl.2007.12.021.

Enemark, J. M. D. and Jørgensen, R. J. 2001. Subclinical rumen acidosis as a cause of reduced appetite in newly calved dairy cows in Denmark: results of a poll among Danish dairy practitioners. *Vet. Quart.* 23(4), 206–10. doi:10.1080/01652176.2001.9695115.

Erridge, C., Bennett-Guerrero, E. and Poxton, I. R. 2002. Structure and function of lipopolysaccharides. *Microbes Infect.* 4(8), 837–51. doi:10.1016/s1286-4579(02)01604-0.

Fan, J., Frey, R. S. and Malik, A. B. 2003. TLR4 signaling induces TLR2 expression in endothelial cells via neutrophil NADPH oxidase. *J. Clin. Invest.* 112(8), 1234–43. doi:10.1172/JCI18696.

Faure, E., Thomas, L., Xu, H., Medvedev, A., Equils, O. and Arditi, M. 2001. Bacterial lipopolysaccharide and IFN-gamma induce toll-like receptor 2 and toll-like receptor 4 expression in human endothelial cells: role of NF-kappa B activation. *J. Immunol.* 166(3), 2018–24. doi:10.4049/jimmunol.166.3.2018.

Gallay, P., Heumann, D., Le, D., Barras, C. and Glauser, M. P. 1994. Mode of action of anti-lipopolysaccharide-binding protein antibodies for prevention of endotoxemic shock in mice. *Proc. Natl. Acad. Sci. U.S.A.* 91(17), 7922–6. doi:10.1073/pnas.91.17.7922.

Garrett, E. F., Nordlund, K. V., Goodger, W. J. and Oetzel, G. R. 1997. A cross-sectional field study investigating the effect of periparturient dietary management on ruminal pH in early lactation dairy cows. *J. Dairy Sci.* 80(1), 169.

Goad, D. W, Goad, C. L. and Nagaraja, T. G. 1998. Ruminal microbial and fermentative changes associated with experimentally induced subacute acidosis in steers. *J. Anim. Sci.* 76, 234–41.

Goldammer, T., Zerbe, H., Molenaar, A., Schuberth, H. J., Brunner, R. M., Kata, S. R. and Seyfert, H. M. 2004. Mastitis increases mammary mRNA abundance of beta-defensin 5, toll-like-receptor 2 (TLR2), and TLR4 but not TLR9 in cattle. *Clin. Diagn. Lab. Immunol.* 11(1), 174–85. doi:10.1128/cdli.11.1.174-185.2004.

Gott, P. N., Hogan, J. S. and Weiss, W. P. 2015. Effects of various starch feeding regimens on responses of dairy cows to intramammary lipopolysaccharide infusion. *J. Dairy Sci.* 98(3),1786–96. doi:10.3168/jds.2014-8638.

Gozho, G. N., Plaizier, J. C., Krause, D. O., Kennedy, A. D. and Wittenberg, K. M. 2005. Subacute ruminal acidosis induces ruminal lipopolysaccharide endotoxin release and triggers an inflammatory response. *J. Dairy Sci.* 88(4), 1399–403. doi:10.3168/jds.S0022-0302(05)72807-1.

Gruffat, D., Durand, D., Graulet, B. and Bauchart, D. 1996. Regulation of VLDL synthesis and secretion in the liver. *Reprod. Nutr. Dev.* 36(4), 375–89. doi:10.1051/rnd:19960404.

Hailemariam, D., Mandal, R., Saleem, F., Dunn, S. M., Wishart, D. S. and Ametaj, B. N. 2014a. Identification of predictive biomarkers of disease state in transition dairy cows.*J. Dairy Sci.* 97(5), 2680–93. doi:10.3168/jds.2013-6803.

Hailemariam, D., Mandal, R., Saleem, F., Dunn, S. M., Wishart, D. S. and Ametaj, B. N.. 2014b. Metabolomics approach reveals altered plasma amino acid and sphingolipid profiles associated with pathological state in transition dairy cows. *Curr. Metab.* 2, 184–95.

Heine, H., Kirschning, C. J., Lien, E., Monks, B. G., Rothe, M. and Golenbock, D. T. 1999. Cutting edge: cells that carry a null allele for toll-like receptor 2 are capable of responding to endotoxin. *J. Immunol.* 162(12), 6971–5.

Humer, E., Khol-Parisini, A., Gruber, L., Gasteiner, J., Abdel-Raheem, ShM. and Zebeli, Q. 2015. Long-term reticuloruminal pH dynamics and markers of liver health in early-lactating cows of various parities fed diets differing in grain processing. *J. Dairy Sci.* 98(9), 6433–48. doi:10.3168/jds.2015-9522.

Humer, E., Petri, R. M., Aschenbach, J. R., Bradford, B. J., Penner, G. B., Tafaj, M., Südekum, K. H. and Zebeli, Q. 2018a. Invited review: practical feeding management recommendations to mitigate the risk of subacute ruminal acidosis in dairy cattle. *J. Dairy Sci.* 101(2), 872–88. doi:10.3168/jds.2017-13191.

Humer, E., Kröger, I., Neubauer, V., Schedle, K., Reisinger, N. and Zebeli, Q. 2018b. Supplementing phytogenic compounds or autolyzed yeast modulates ruminal biogenic amines and plasma metabolome in cows experiencing subacute rumen acidosis. *J. Dairy Sci.* 101(10), 9559–74. doi:10.3168/jds.2018-14744.

Humer, E., Aditya, S. and Zebeli, Q. 2018c. Innate immunity and metabolomic responses in dairy cows challenged intramammarily with lipopolysaccharide after subacute ruminal acidosis. *Animal* 12(12), 2551–60. doi:10.1017/S1751731118000411.

Ibeagha-Awemu, E. M., Lee, J. W., Ibeagha, A. E., Bannerman, D. D., Paape, M. J. and Zhao, X. 2008. Bacterial lipopolysaccharide induces increased expression of toll-like receptor (TLR) 4 and downstream TLR signaling molecules in bovine mammary epithelial cells. *Vet. Res.* 39(2), 11. doi:10.1051/vetres:2007047.

Ingman, W. V., Glynn, D. J. and Hutchinson, M. R. 2014. Inflammatory mediators in mastitis and lactation insufficiency. *J. Mammary. Gland. Biol. Neoplasia* 19(2), 161–7. doi:10.1007/s10911-014-9325-9.

Iqbal, S. 2013. Novel mucosal vaccines to improve immune and health status of periparturient dairy cows and increase their productive potentials. PhD Thesis. University of Alberta.

Jin, D., Chang, G., Zhang, K., Guo, J., Xu, T. and Shen, X. 2016. Rumen-derived lipopolysaccharide enhances the expression of lingual antimicrobial peptide in mammary glands of dairy cows fed a high-concentrate diet. *BMC. Vet. Res.* 12(1), 128. doi:10.1186/s12917-016-0755-z.

Jouany, J. P., Demeyer, D. I. and Grain, J. 1988. Effect of defaunating the rumen. *Anim. Feed Sci. Technol.* 21(2–4), 229–65. doi:10.1016/0377-8401(88)90105-8.

Kansanen, E., Kuosmanen, S. M., Leinonen, H. and Levonen, A. L. 2013. The Keap1-Nrf2 pathway: mechanisms of activation and dysregulation in cancer. *Redox Biol.* 1(1), 45–9. doi:10.1016/j.redox.2012.10.001.

Khafipour, E., Krause, D. O. and Plaizier, J. C. 2009. A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. *J. Dairy Sci.* 92(3), 1060–70. doi:10.3168/jds.2008-1389.

Khiaosa-Ard, R., Pourazad, P., Aditya, S., Humer, E. and Zebeli, Q. 2018. Factors related to variation in the susceptibility to subacute ruminal acidosis in early lactating Simmental cows fed the same grain-rich diet. *Anim. Feed Sci. Technol.* 238, 111–22. doi:10.1016/j.anifeedsci.2018.02.005.

Khovidhunkit, W., Kim, M. S., Memon, R. A., Shigenaga, J. K., Moser, A. H., Feingold, K. R. and Grunfeld, C. 2004. Effects of infection and inflammation on lipid and lipoprotein metabolism:mechanisms and consequences to the host. *J. Lipid Res.* 45(7), 1169–96. doi:10.1194/jlr.R300019-JLR200.

Kim, K. N., Ko, Y. J., Yang, H. M., Ham, Y. M., Roh, S. W., Jeon, Y. J., Ahn, G., Kang, M. C., Yoon, W. J., Kim, D. and Oda, T. 2013. Anti-inflammatory effect of essential oil and its constituents from fingered citron (*Citrus medica* L. var. *sarcodactylis*) through blocking JNK, ERK and NF-κB signaling pathways in LPS-activated RAW 264.7 cells. *Food Chem. Toxicol.* 57, 126–31. doi:10.1016/j.fct.2013.03.017.

Kitkas, G. C., Valergakis, G. E., Karatzias, H. and Panousis, N. 2013. Subacute ruminal acidosis: prevalence and risk factors in Greek dairy herds. *Iran. J. Vet. Res.* 14, 183–9.

Kleen, J. L. and Cannizzo, C. 2012. Incidence, prevalence and impact of SARA in dairy herds. *Anim. Feed. Sci. Technol.* 172(1–2), 4–8. doi:10.1016/j.anifeedsci.2011.12.003.

Kleen, J. L., Hooijer, G. A., Rehage, J. and Noordhuizen, J. P. T. M. 2003. Subacute ruminal acidosis (SARA): a review. *J. Vet. Med. A* 50(8), 406–14. doi:10.1046/j.1439-0442.2003.00569.x.

Kleen, J. L., Hooijer, G. A., Rehage, J. and Noordhuizen, J. P. T. M. 2004. Rumenocentesis (rumen puncture): a viable instrument in herd health diagnosis. *DTW. Dtsch. Tierarztl. Wochenschr.* 111(12), 458–62.

Kleen, J. L., Hooijer, G. A., Rehage, J. and Noordhuizen, J. P. T. M. 2009. Subacute ruminal acidosis in Dutch dairy herds. *Vet. Rec.* 164(22), 681–3. doi:10.1136/vr.164.22.681.

Kleen, J. L., Upgang, L. and Rehage, J. 2013. Prevalence and consequences of subacute ruminal acidosis in German dairy herds. *Acta Vet. Scand.* 55, 48. doi:10.1186/1751-0147-55-48.

Kobayashi, K., Oyama, S., Numata, A., Rahman, M. M. and Kumura, H. 2013. Lipopolysaccharide disrupts the milk-blood barrier by modulating claudins in mammary alveolar tight junctions. *PLoS ONE* 8(4), e62187. doi:10.1371/journal.pone.0062187.

Kröger, I., Humer, E., Neubauer, V., Reisinger, N. A., Aditya, S. and Zebeli, Q. 2017. Modulation of chewing behavior and reticular pH in nonlactating cows challenged with concentrate-rich diets supplemented with phytogenic compounds and autolyzed yeast. *J. Dairy Sci.* 100(12), 9702–14. doi:10.3168/jds.2017-12755.

Kröger, I., Humer, E., Neubauer, V., Reisinger, N. and Zebeli, Q. 2019. Feeding diets moderate in physically effective fibre alters eating and feed sorting patterns without improving ruminal pH, but impaired liver health in dairy cows. *Animals (Basel)* 9(4). doi:10.3390/ani9040128.

Kushibiki, S., Hodate, K., Shingu, H., Hayashi, T., Touno, E., Shinoda, M. and Yokomizo, Y. 2002. Alterations in lipid metabolism induced by recombinant bovine tumornecrosis factor-alpha administration to dairy heifers. *J. Anim. Sci.* 80(8), 2151–7. doi:10.2527/2002.8082151x.

Lacetera, N. 2016. Metabolic stress, heat shock proteins, and innate immune response. In: Amadori, M. (Ed.), *The Innate Immune Response to Noninfectious Stressors. Human and Animal Models*. Elsevier, Oxford, pp. 107–32.

Lechowski, R. 1997. The influence of metabolic acidosis in new-born calves on biochemical profile of the liver. *Comp. Haem. Int.* 7(3), 172–6. doi:10.1007/BF02652597.

Lee, J. W., Paape, M. J., Elsasser, T. H. and Zhao, X. 2003. Elevated milk soluble CD14 in bovine mammary glands challenged with *Escherichia coli* lipopolysaccharide. *J. Dairy Sci.* 86(7), 2382–9. doi:10.3168/jds.S0022-0302(03)73832-6.

Le Floćh, N., Melchior, D. and Obled, C. 2004. Modifications of protein and amino acid metabolism during inflammation and immune system activation. *Livest. Prod. Sci.* 87(1), 37–45. doi:10.1016/j.livprodsci.2003.09.005.

Li, S., Khafipour, E., Krause, D. O., Kroeker, A., Rodriguez-Lecompte, J. C., Gozho, G. N. and Plaizier, J. C. 2012. Effects of subacute ruminal acidosis challenges on fermentation and endotoxins in the rumen and hindgut in dairy cows. *J. Dairy Sci.* 95(1), 294–303. doi:10.3168/jds.2011-4447.

Liepa, G. U., Beitz, D. C. and Linder, J. R. 1978. Cholesterol synthesis in ruminating and nonruminating goats. *J. Nutr.* 108(3), 535–43. doi:10.1093/jn/108.3.535.

Marchesini, G., De Nardi, R., Gianesella, M., Stefani, A. L., Morgante, M., Barberio, A., Andrighetto, I. and Segato, S. 2013. Effect of induced ruminal acidosis on blood variables in heifers. *BMC Vet. Res.* 9, 98.doi:10.1186/1746-6148-9-98.

McGuire, K., Jones, M., Werling, D., Williams, J. L., Glass, E. J. and Jann, O. 2006. Radiation hybrid mapping of all 10 characterized bovine Toll-like receptors. *Anim. Genet.* 37(1), 47–50. doi:10.1111/j.1365-2052.2005.01364.x.

Memon, M. A., Wang, Y., Xu, T., Ma, N., Zhang, H., Roy, A. C., Aabdin, Z. U. and Shen, X. 2019. Lipopolysaccharide induces oxidative stress by triggering MAPK and Nrf2 signalling pathways in mammary glands of dairy cows fed a high-concentrate diet. *Microb. Pathog.* 128, 268–75.doi:10.1016/j.micpath.2019.01.005.

Morgante, M., Stelleta, C., Berzaghi, P., Gianasella, M. and Andrighetto, I. 2007. Subacute rumen acidosis in lactating cows: an investigation in intensive Italian dairy herds. *J. Anim. Physiol. Anim. Nutr.* 91(5–6), 226–34. doi:10.1111/j.1439-0396.2007.00696.x.

Morris, M. and Li, L. 2012. Molecular mechanisms and pathological consequences of endotoxin tolerance and priming. *Arch. Immunol. Ther. Exp. (Warsz.)* 60(1), 13–8. doi:10.1007/s00005-011-0155-9.

Nagaraja, T. G. and Lechtenberg, K. F. 2007. Acidosis in feedlot cattle. *Vet. Clin. North Am. Food Anim. Pract.*23, 333–50.

Nagaraja, T. G., Bartley, E. E., Fina, L. R. and Anthony, H. D. 1978. Relationship of rumen gram-negative bacteria and free endotoxin to lactic acidosis in cattle. *J. Anim. Sci.* 47(6), 1329–37. doi:10.2527/jas1978.4761329x.

Neubauer, V., Petri, R., Humer, E., Kröger, I., Mann, E., Reisinger, N., Wagner, M. and Zebeli, Q. 2018. High-grain diets supplemented with phytogenic compounds or autolyzed yeast modulate ruminal bacterial community and fermentation in dry cows. *J. Dairy Sci.* 101(3), 2335–49. doi:10.3168/jds.2017-13565.

Nilsson, A. and Duan, R. D. 2006. Absorption and lipoprotein transport of sphingomyelin. *J. Lipid Res.* 47(1), 154–71. doi:10.1194/jlr.M500357-JLR200.

Nocek, J. E. 1997. Bovine acidosis: implications on laminitis. *J. Dairy Sci.* 80(5), 1005–28. doi:10.3168/jds.S0022-0302(97)76026-0.

Nordlund, K. V. and Garrett, E. F. 1994. Rumenocentesis: a technique for collecting rumen fluid for the diagnosis of subacute rumen acidosis in dairy herds. *Bov. Pract.* 28, 109–12.

Nordlund, K. V., Garrett, E. F. and Oetzel, G. R. 1995. Herd-based rumenocentesis: a clinical approach to the diagnosis of subacute rumen acidosis. *Compend. Contin. Educ. Vet.* 17, S48–56.

O'Grady, L., Doherty, M. L. and Mulligan, F. J. 2008. Subacute ruminal acidosis (SARA) in grazing Irish dairy cows. *Vet. J.* 176(1), 44–9. doi:10.1016/j.tvjl.2007.12.017.

Pareek, R., Wellnitz, O., Van Dorp, R., Burton, J. and Kerr, D. 2005. Immunorelevant gene expression in LPS-challenged bovine mammary epithelial cells. *J. Appl. Genet.* 46(2), 171–7.

Plaizier, J. C., Krause, D. O., Gozho, G. N. and McBride, B. W. 2008. Subacute ruminal acidosis in dairy cows: the physiological causes, incidence and consequences. *Vet. J.* 176(1), 21–31. doi:10.1016/j.tvjl.2007.12.016.

Plaizier, J. C., Khafipour, E., Li, S., Gozho, G. N. and Krause, D. O. 2012. Subacute ruminal acidosis (SARA), endotoxins, and health consequences. *Anim. Feed. Sci. Technol.* 172(1–2), 9–21. doi:10.1016/j.anifeedsci.2011.12.004.

Plaizier, J. C., Danesh Mesgaran, M., Derakhshani, H., Golder, H., Khafipour, E., Kleen, J. L., Lean, I., Loor, J., Penner, G. and Zebeli, Q. 2018. Review: enhancing gastrointestinal health in dairy cows. *Animal* 12(s2), s399–418. doi:10.1017/S1751731118001921.

Pourazad, P., Khiaosa-Ard, R., Qumar, M., Wetzels, S. U., Klevenhusen, F., Metzler-Zebeli, B. U. and Zebeli, Q. 2016. Transient feeding of a concentrate-rich diet increases the severity of subacute ruminal acidosis in dairy cattle. *J. Anim. Sci.* 94(2), 726–38. doi:10.2527/jas.2015-9605.

Razak, M. A., Begum, P. S., Viswanath, B. and Rajagopal, S. 2017. Multifarious beneficial effect of nonessential amino acid, glycine: a review. *Ox. Med. Cell. Long.* doi:10.1155/2017/1716701.

Saleem, F., Ametaj, B. N., Bouatra, S., Mandal, R., Zebeli, Q., Dunn, S. M. and Wishart, D. S. 2012. A metabolomics approach to uncover the effects of grain diets on rumen health in dairy cows. *J.Dairy Sci.* 95(11), 6606–23. doi:10.3168/jds.2012-5403.

Schlingmann, B., Overgaard, C. E., Molina, S. A., Lynn, K. S., Mitchell, L. A., Dorsainvil White, S., Mattheyses, A. L., Guidot, D. M., Capaldo, C. T. and Koval, M. 2016. Regulation of claudin/zonula occludens-1 complexes by hetero-claudin interactions. *Nat. Commun.* 7, 12276. doi:10.1038/ncomms12276.

Schmitz, S., Pfaffl, M. W., Meyer, H. H. D. and Bruckmaier, R. M. 2004. Short-term changes of mRNA expression of various inflammatory factors and milk proteins in mammary tissue during LPS-induced mastitis. *Domest. Anim. Endocrinol.* 26(2), 111–26. doi:10.1016/j.domaniend.2003.09.003.

Schumann, R. R., Rietschel, E. T. and Loppnow, H. 1994. The role of CD14 and lipopolysaccharide-binding protein (LBP) in the activation of different cell types by endotoxin. *Med. Microbiol. Immunol.* 183(6), 279–97. doi:10.1007/bf00196679.

Shi, H., Guo, Y., Liu, Y., Shi, B., Guo, X., Jin, L. and Yan, S. 2016. The *in vitro* effect of lipopolysaccharide on proliferation, inflammatory factors and antioxidant enzyme activity in bovine mammary epithelial cells. *Anim. Nutr.* 2(2), 99–104. doi:10.1016/j.aninu.2016.03.005.

Sordillo, L. M. and Aitken, S. L. 2009. Impact of oxidative stress on the health and immune function of dairy cattle. *Vet. Immunol. Immunopathol.* 128(1–3), 104–9. doi:10.1016/j.vetimm.2008.10.305.

Sordillo, L. M. and Streicher, K. L. 2002. Mammary gland immunity and mastitis susceptibility. *J. Mammary Gland. Biol. Neoplasia* 7(2), 135–46. doi:10.1023/a:1020347818725.

Stone, W. C. 1999. The effect of subclinical rumen acidosis on milk components. In: *Proceeding of the Cornell Nutrition Conference of Feed Manufacturers*, Syracuse, NY. Cornell University, Ithaca, NY, pp. 40–6.

Stone, W. C. 2004. Nutritional approaches to minimize subacute ruminal acidosis and laminitis in dairy cattle. *J. Dairy Sci.* 87(E. Suppl.), E13–26. doi:10.3168/jds.S0022-0302(04)70057-0.

Strandberg, Y., Gray, C., Vuocolo, T., Donaldson, L., Broadway, M. and Tellam, R. 2005. Lipopolysaccharide and lipoteichoic acid induce different innate immune immune responses in bovine mammary epithelial cells. *Cytokine* 31(1), 72–86. doi:10.1016/j.cyto.2005.02.010.

Swanson, K., Gorodetsky, S., Good, L., Davis, S., Musgrave, D., Stelwagen, K., Farr, V. and Molenaar, A. 2004. Expression of a beta-defensin mRNA, lingual antimicrobial

peptide, in bovine mammary epithelial tissue is induced by mastitis. *Infect. Immun.* 72(12), 7311–4. doi:10.1128/IAI.72.12.7311-7314.2004.

Tajik, J., Nadalian, M. G., Raoofi, A., Mohammadi, G. R. and Bahonar, A. R. 2009. Prevalence of subacute ruminal acidosis in some dairy herds of Khorasan Razavi province, Northeast of Iran. *Iran. J. Vet. Res.* 10, 28–32.

Takeda, K. and Akira, S. 2005. Toll-like receptors in innate immunity. *Int. Immunol.* 17(1), 1–14. doi:10.1093/intimm/dxh186.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K. and Akira, S. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 11(4), 443–51. doi:10.1016/s1074-7613(00)80119-3.

Wall, S. K., Hernandez-Castellano, L. E., Ahmadpour, A., Bruckmaier, R. M. and Wellnitz, O. 2016. Differential glucocorticoid-induced closure of the blood-milk barrier during lipopolysaccharide- and lipoteichoicacid-induced mastitis in dairy cows. *J. Dairy Sci.* 99(9), 7544–53. doi:10.3168/jds.2016-11093.

Wellnitz, O., Arnold, E. T. and Bruckmaier, R. M. 2011. Lipopolysaccharide and lipoteichoic acid induce different immune responses in the bovine mammary gland. *J. Dairy Sci.* 94(11), 5405–12. doi:10.3168/jds.2010-3931.

Wu, T., Wang, C., Ding, L., Shen, Y., Cui, H., Wang, M. and Wang, H. 2016. Arginine relieves the inflammatory response and enhances the casein expression in bovine mammary epithelial cells induced by lipopolysaccharide. *Mediators Inflamm.* 2016, 9618795. doi:10.1155/2016/9618795.

Yang, W. Z. and Beauchemin, K. A. 2006. Increasing the physically effective fiber content of dairy cow diets may lower efficiency of feed use. *J. Dairy Sci.* 89(7), 2694–704. doi:10.3168/jds.S0022-0302(06)72345-1.

Zbinden, C., Stephan, R., Johler, S., Borel, N., Bünter, J., Bruckmaier, R. M. and Wellnitz, O. 2014. The inflammatory response of primary bovine mammary epithelial cells to Staphylococcus aureus strains is linked to the bacterial phenotype. *PLoS ONE* 9(1), e87374. doi:10.1371/journal.pone.0087374.

Zebeli, Q. and Ametaj, B. N. 2009. Relationships between rumen lipopolysaccharide and mediators of inflammatory response with milk fat production and efficiency in dairy cows. *J. Dairy Sci.* 92(8), 3800–9. doi:10.3168/jds.2009-2178.

Zebeli, Q. and Metzler-Zebeli, B. U. 2012. Interplay between rumen digestive disorders and diet-induced inflammation in dairy cattle. *Res. Vet. Sci.* 93(3), 1099–108. doi:10.1016/j.rvsc.2012.02.004.

Zebeli, Q., Dijkstra, J., Tafaj, M., Steingass, H., Ametaj, B. N. and Drochner, W. 2008. Modeling the adequacy of dietary fiber in dairy cows based on the responses of ruminal pH and milk fat production to composition of the diet. *J. Dairy Sci.* 91(5), 2046–66. doi:10.3168/jds.2007-0572.

Zebeli, Q., Mansmann, D., Steingass, H. and Ametaj, B. N. 2010. Balancing diets for physically effective fibre and ruminally degradable starch: a key to lower the risk of sub-acute rumen acidosis and improve productivity of dairy cattle. *Livest. Sci.* 127(1), 1–10. doi:10.1016/j.livsci.2009.09.003.

Zebeli, Q., Dunn, S. M. and Ametaj, B. N. 2011. Perturbations of plasma metabolites correlated with the rise of rumen endotoxin in dairy cows fed diets rich in easily degradable carbohydrates. *J. Dairy Sci.* 94(5), 2374–82. doi:10.3168/jds.2010-3860.

Zebeli, Q., Ghareeb, K., Humer, E., Metzler-Zebeli, B. U. and Besenfelder, U. 2015. Nutrition, rumen health and inflammation in the transition period and their role on

overall health and fertility in dairy cows. *Res. Vet. Sci.* 103, 126–36. doi:10.1016/j.rvsc.2015.09.020.

Zhang, R., Huo, W., Zhu, W. and Mao, S. 2015. Characterization of bacterial community of raw milk from dairy cows during subacute ruminal acidosis challenge by high-throughput sequencing. *J. Sci. Food Agric.* 95(5), 1072–9. doi:10.1002/jsfa.6800.

Zhang, K., Chang, G., Xu, T., Xu, L., Guo, J., Jin, D. and Shen, X. 2016. Lipopolysaccharide derived from the digestive tract activates inflammatory gene expression and inhibits casein synthesis in the mammary glands of lactating dairy cows. *Oncotarget* 7(9), 9652–65. doi:10.18632/oncotarget.7371.

Zhao, X. and Lacasse, P. 2008. Mammary tissue damage during bovine mastitis: causes and control. *J. Anim. Sci.* 86(13Suppl.), 57–65. doi:10.2527/jas.2007-0302.

Zhou, J., Dong, G., Ao, C., Zhang, S., Qiu, M., Wang, X., Wu, Y., Erdene, K., Jin, L., Lei, C. and Zhang, Z. 2014. Feeding a high-concentrate corn straw diet increased the release of endotoxin in the rumen and pro-inflammatory cytokines in the mammary gland of dairy cows. *BMC. Vet. Res.* 10, 172. doi:10.1186/s12917-014-0172-0.

Part 4

Nutritional strategies to optimise ruminal function

Chapter 18

Role of the rumen microbiome in pasture-fed ruminant production systems

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1 Introduction

Ruminant animals do not harbour the requisite digestive enzymes for the degradation of plant material, predominantly structural polysaccharides (Flint et al., 2012). Despite this they are adept at efficiently utilising the stored energy in an array of forages that is directly unavailable to the vast majority of mammals. In spite of this disadvantage, the ruminant animal plays a crucial role in feeding the global population, which is set to surpass 9 billion by the year 2050 (UN, 2017). Human reliance on a few livestock species such as cattle, buffalo, sheep and goats as a food source, has therefore developed as a consequence of the ability of ruminants to transform the inaccessible energy stored in plants into high-quality sources of protein and energy. Projections estimate global agriculture output will need to attain a near-50% increase by the year 2050 (FAO, 2017). Indeed, further dependence on the unique ability of ruminants to convert forage into high-quality sources of food will likely occur to meet the required increase in agricultural output and thus to meet global food demand.

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The capability of ruminant animals to gain their nutrient requirements from a forage-based diet arises from the symbiotic relationship that has developed over the past 50 million years between the microbial population in the rumen and the host (Sasson et al., 2017). Microbes residing in the rumen including bacteria, fungi, protozoa and archaea have developed unique methods to obtain nutrition. Some bacteria, fungi and protozoa are capable of directly degrading food sources ingested by the animal. Other microbes, primarily archaea and some bacteria, rely on the fermentation products of other organisms to supply substrate for utilisation. Indeed, the host animal is similar to microbes situated at lower trophic levels in the rumen ecosystem, as the host is highly dependent on the production of volatile fatty acids (VFAs) by members of the rumen microbiome. The production of VFAs in the rumen is estimated to supply around 63% of the energy requirements of ruminants (Bergman, 1990). The microbes of the rumen obtain benefits of this symbiotic relationship via the large supply of substrate from the feed ingested by the animal, along with the anaerobic conditions of the rumen, which are critical for many of these obligate anaerobes.

Within livestock production systems, feed is an extensive cost and therefore has major ramifications on farm profitability (Kenny et al., 2018). Under natural circumstances, forage-based diets predominate within ruminant production systems. Similarly, given its cost competitiveness in comparison to grain, grazed pasture has long been utilised as a key feed source for ruminant livestock. However, particularly in the Western world, grain is included in the diet in an effort to augment animal performance and/or as a supplement during periods of inadequate pasture. The feeding of grasses as well as other forages such as legumes not only has been recognised as beneficial to farm profitability but also limits, to a degree, competition between humans and livestock for scarce arable land resources.

Complementary to farm profitability is the identification of animals with improved capacity for the efficient use of feed for productive purposes. Feed efficiency is often classified as a multifactorial and complex trait, which can be described in a multitude of ways, including as a ratio of animal feed intake to weight gain or as a regression of the expected versus the actual intake of the animal (Kenny et al., 2018). The precise biological means which separate animals in terms of efficiency are not well understood. While diet has been accepted to induce considerable change to the composition and function of the rumen microbiome in numerous studies, there is increasing evidence that the rumen microbiome may vary in accordance with the feed efficiency status of the host animal (Guan et al., 2008; Carberry et al., 2012; Hernandez-Sanabria et al., 2012; Shabat et al., 2016; Ellison et al., 2017; McGovern et al., 2018; Delgado et al., 2019). Indeed, variation in the abundance of certain rumen microbes may influence the ruminant's ability to extract energy from feed (Cantalapiedra-Hijar et al., 2018).

Importantly, feed efficiency as a trait cannot be observed as independent of the environment in which the animal is situated, as the nutritional management that the animal is exposed to is likely to have a major influence on the expression of such traits (Kenny et al., 2018). Indeed, there is evidence of different diets being capable of altering the ruminant's level of efficiency, with an animal's degree of feed efficiency leading to a re-ranking across diets. Feed efficiency, particularly residual feed intake (RFI), is a trait which has a relatively high degree of repeatability across differing production phases (Kelly et al., 2010). However, while a moderately repeatable trait, the re-ranking of animals in terms of efficiency due to dietary change has been reported in previous studies (Durunna et al., 2012; Thompson, 2015) and would imply that the genetics of the animal could influence the capacity of the ruminal microflora to ferment nutrients from various feed types.

Increasing evidence suggests a probable correlation between an animal's feed efficiency status and their associated greenhouse gas (GHG) output production. A variety of studies have illustrated that more feed-efficient animals are capable of both reduced methane emissions (Table 4) and increased retention of dietary nitrogen (Sharma et al., 2018). This relationship is remarkably conceivable, considering the production of methane is estimated to result in a 6–12% loss of the animal's gross energy intake (GEI) in the methanogenesis process (Johnson and Johnson, 1995; Pacheco et al., 2014). Considering the mounting pressure on global livestock production to reduce the carbon footprint of the sector, the breeding of a more efficient animal both harnesses the ability to reduce emissions and increases farm profitability permanently.

Along with altering the composition of the rumen, dietary intervention can impact animal performance. For example, grazing high-quality pastures has provided evidence of reduced methane output (Jonker et al., 2018b). Similarly, the feeding of legumes has been associated with reductions in methane yield in some studies (Enriquez-Hidalgo et al., 2014a; Niderkorn et al., 2015). Indeed, grassland management also has a role to play in the performance not only of the sward but also of the animal, with decreases in methane emissions (Hart et al., 2009; Wims et al., 2010) and improvements to average daily gain (ADG; Boland et al., 2013) noted on higher-quality swards of lower pre-grazing herbage mass in cattle.

The aim of this chapter is to provide a better understanding (or greater insight) into the relationship between the rumen microbiome and feed efficiency within the context of pasture-based ruminant production systems. In addition, we will focus on the role of forages, mainly grasses and legumes, and their ability to alter the rumen microbiome and the resulting impact on animal performance. Firstly, we describe the main rumen characteristics associated with feed efficiency and methanogenesis and then proceed to discuss the potential impact of pasture composition and management on ruminal function

and animal performance. A strong emphasis will be placed on cattle production systems with further examples taken from other ruminant species and *in vitro* studies where appropriate.

2 Diet and rumen microbiome

As alluded to earlier, ruminants *per se* lack the necessary enzymes *per se* capable of degrading complex plant polysaccharides. Therefore, ruminants rely on a symbiotic relationship with the anaerobic microbial ecosystem which they harbour within their forestomach in order to ferment and subsequently extract necessary nutrients from forage material (Henderson et al., 2015). The unique co-evolution of the ruminant and its rumen microbiome allows for the conversion of human indigestible plant matter into high-quality dairy and meat products to the benefit of global society.

In particular, in countries where a temperate climate dominates year round, such as Ireland or New Zealand, forage is utilised as the main feed source for ruminants. However, regardless of whether they originate from forage or grain, plant carbohydrates are by far the most important nutrient source to the rumen microbial population, with a continued supply imperative to support microbial growth (Hungate, 1966). It is well established that different feed sources can alter the performance of livestock. Variability in the composition of the diet has long been identified as a leading factor regulating the composition and potential functionality of the ruminal microbiome (Hungate, 1966; Carberry et al., 2012, 2014a; Henderson et al., 2015; Ellison et al., 2017). Therefore, diet-associated differences in performance are most likely due to the impact diet has on the rumen microbiome, which subsequently dictates the efficiency with which nutrients are extracted from ingested feed (McCann et al., 2014a).

Many studies demonstrate variation in the response of the rumen microbial community to different diets. For example, the abundance of archaea and protozoa was significantly reduced in the rumen microbiome of cross-bred beef steers fed a diet in excess of 90% concentrates when compared to counterparts consuming a diet composed of near-equal quantities of concentrate and forage (Rooke et al., 2014). Similarly, alterations to the methanogen proportion of the microbiome have also been reported as the dietary concentrate level increases, with increases to the total methanogen population, *Methanosphaera stadtmanae, Methanobrevibacter ruminantium* and *Methanobrevibacter smithii* observed on a high concentrate compared to high forage diet (Carberry et al., 2014b). Continuing with a focus on the impact of grain, studies found that beef steers undergoing a gradual dietary alteration from a high forage to a high concentrate diet have corresponding increases in the bacterial population of *Megasphaera elsdenii, Streptococcus bovis, Selenomonas ruminantium and Prevotella bryantii* as the proportion of

concentrate in the diet increased with an equivalent decrease in the population of *Butyrivibrio fibrisolvens* and *Fibrobacter succinogenes* (Fernando et al., 2010).

Modern rumen microbiological research has a strong ecological focus, with various microbial groups being thought to occupy specific niches within the rumen ecosystem. Niche occupation by various rumen microbes contributes to explaining why variation is often observed in the rumen microbial population when animals are fed different diets. For example, the rumen bacteria *F. succinogens*, which specialises in degrading cellulose (Stewart et al., 1997), has been shown to produce extracellular cellulases (Forsberg et al., 1981) and as a result has been shown to have an increased ruminal abundance in cattle fed forage diets (Henderson et al., 2015).

As changes to the microbial population occur, fluctuations to the end products of rumen fermentation (such as VFAs, lactate, CO_2, H_2 and CH_4) are also expected due to the variation in the role of specific microbes in the rumen. The production of cellulases is reserved for a select cohort of cellulolytic rumen bacteria, fungi and protozoa, with the end products they produce being utilised by other rumen microbes incapable of degrading cellulose directly (Wolin et al., 1997). As such, depending on the trophic level of a microbe, fluctuations in the production of end products are likely to have varying implications. Co-cultures of *F. succinogens* with *S. ruminantium* have been demonstrated to support the growth of *S. ruminantium* on cellulose due to the supply of succinate by *F. succinogens* (Scheifinger and Wolin, 1973). While the direct impact of the dietary composition on primary degraders can be easily comprehended, the indirect and knock-on effects of rumen fermentation at additional trophic levels must not be forgotten.

3 Cellulose degradation in the rumen

Ruminants are capable of utilising fibrous feedstuffs that more simple stomached mammals, such as humans are unable to degrade (Morgavi et al., 2010). The structural polysaccharides, cellulose, hemicellulose and pectin are the predominant sources of energy for rumen microbes available in forages (Dehority, 1991). Degradation of the cell wall is completed by a combination of rumen bacteria and fungi with a lesser contribution considered from the protozoa (Chesson and Forsberg, 1997).

Different strategies have been developed by the various microbial groups to utilise cellulose, with some of the adaptations discussed later. Indeed, a variety of polysaccharides of plant origin are degraded by rumen microbes; however, the focus here will be on cellulose degradation with reference to the utilisation of other plant products made throughout the chapter. The degradation of cellulose is catalysed by a host of enzymes including glycohydrolases and endoglucanases. Enzymatic action results in plant polysaccharides being

converted to soluble oligosaccharides which can be absorbed by the cell for further processing (Arntzen et al., 2017). Enzymatic degradation of the plant cell wall is limited to the polysaccharide present on the surface of the ingested plant particle (Chesson and Forsberg, 1997). The mechanistic model of degradation of plant cell walls proposed by Chesson (1993) suggests that degradation of the cell wall does not occur in a discriminatory manner; rather, enzymatic action is targeted towards whichever polysaccharide is exposed at the surface of ingested plant matter. Figure 1 illustrates a simplistic version of the enzymatic degradation of plant particle matter with cellulose embedded in hemicellulose.

3.1 Bacterial degradation of cellulose

F. succinogenes, Ruminococcus flavefaciens and *Ruminococcus albus* are the dominant cellulose-degrading microbes (Weimer, 1996). While members of *Fibrobacter* and *Ruminococcus* persist as the main celluloytic bacteria, they seem to employ different methods for the degradation of lignocellulose compounds. Over half of the rumen cellulose glycohydrolases have been identified as originating from members of both genera along with over a third of hemicellulose glycohydrolases (Dai et al., 2015).

Both *R. flavefaciens* and *R. albus,* members of the order Clostridiales*,* are known to degrade plant biomass through the production of cellulosomes, multi-enzyme complexes consisting of dockerins, for the attachment of enzymes, and structural proteins called scaffoldin (Artzi et al., 2017). Cellulosome complexes promote the production of cellulases and hemicellulases synergistically for the efficient metabolism of plant cell-wall polysaccharides (Bayer et al., 2004; Han et al., 2004; Devendran et al., 2016). While deviations occur, a cellulosome complex allows the attachment of various enzymes to the outer surface of rumen cellulolytic bacteria promoting their ability to orderly degrade a multitude of complex plant polysaccharides. Differences in the complexity of the cellulosomes occur between *R. flavefaciens* and *R. albus,* with the former capable of synthesising a large number of dockerins and multiple scaffoldin allowing for increased enzymatic activity (Artzi et al., 2017; Seshadri et al., 2018).

The polysaccharide source and constituent subunits (cellulose, pectin, xylan, cellobiose, etc.) have been shown to impact gene expression and the enzyme profile of the cellulosome and to subsequently influence the composition of the complex (Han et al., 2004). For example, when cultured in media containing alternative carbon sources, gene expression for substrate-specific enzymes within the cellulsome of *Clostridium cellulovorans* was elevated in tandem with the specific substrates. Expression of *cbpA*, a cellulose-binding protein, was elevated in mediums containing cellulose, as was *xynA*, encoding for xylanase, when cultured on medium containing xylan. An extensive review of bacterial cellulosomes has been previously conducted (Artzi et al., 2017).

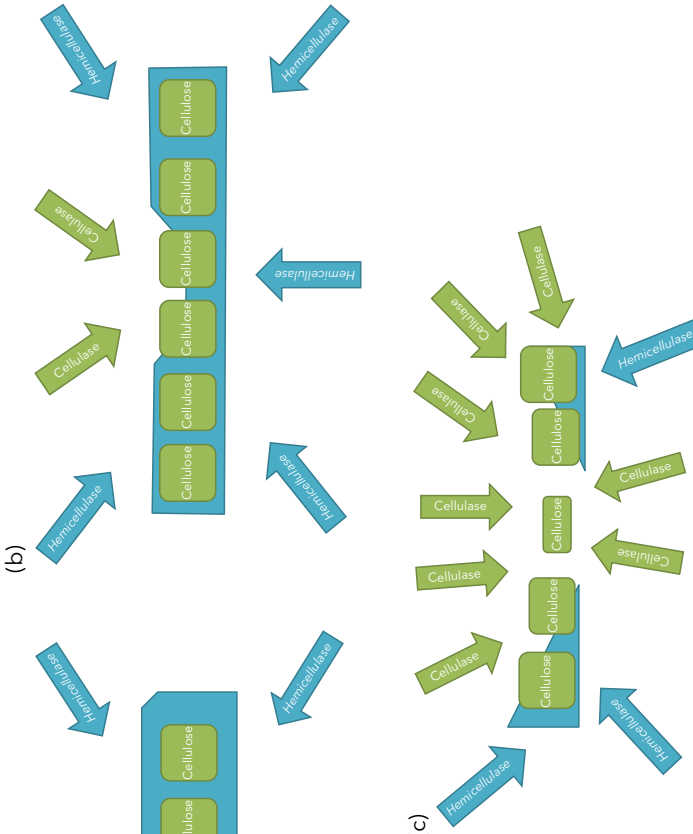

Figure 1 A schematic representation of the enzymatic degradation of plant matter, with cellulose embedded in hemicellulose, performed by rumen microbes based on the model proposed by Chesson (1993). Arrows indicate active enzymes. Colour of enzyme corresponds to targeted substrate; that is, blue arrow represents hemicellulase-targeting hemicellulose, while green arrows represent cellulase-targeting cellulose. (a) Hemicellulase-targeting hemicellulose component of the plant particle, (b) hemicellulose beginning to be degraded with exposure of cellulose and production of cellulase and (c) cellulase production increased as further exposure of cellulose occurs.

F. succinogenes requires close contact with its substrate for efficient degradation but does not produce a cellulosome for the degradation of cellulose, instead relying on a combination of outer-membrane proteins (Jun et al., 2007; Suen et al., 2011). Within the genome of *F. succinogenes S85*, ten genes known to be associated with slime moulds have been identified and are suggested to play a role in cellulose adhesion (Suen et al., 2011). In addition, outer-membrane pilin is suggested to encourage the adhesion of *F. succinogenes* to cellulose. Proteomic analysis conducted by Jun et al. (2007) compared the binding capabilities of *F. succinogenes* and adhesion mutant strains, Ad1 and Ad4. Their investigation found the production of a type IV pilin protein by *F. succinogenes* only, with the production of the protein lacking from both mutant strains. It was concluded that the production of pilin protein has a role to play in the attachment of *F. succinogenes* to cellulose, since the mutant types lacked both a strong adherence to crystalline cellulose and the capability to synthesise the pilin protein.

F. succinogenes has the ability to hydrolyse numerous plant polysaccharides; however, it is only able to utilise cellulose for growth and is therefore hypothesised to hydrolyse various complex carbohydrates to gain access to cellulose (Suen et al., 2011; Dai et al., 2015). Outer-membrane vesicles provide a protective transport vehicle for proteins to act in a concentrated manner on distant targets (Kulp and Kuehn, 2010) and have been suggested to be the predominant method of plant cell degradation within the studied *Fibrobacter* (Arntzen et al., 2017). Differential expression of genes with response to substrate has also been observed in *F. succinogenes* (Neumann et al., 2018).

Due to the different methods for degradation of cellulose, variation in the fermentation end products is expected. Succinate dominates as the end product of fermentation by *F. succinogenes* followed by acetate (Gokarn et al., 1997) while hydrogen (H_2) production is not associated with the species (Joblin et al., 2002). Contrastingly, the cellulolytic *Ruminococcus* are known H_2, acetate, formate and CO_2 producers (Zheng et al., 2014; Rooke et al., 2014). *R. flavefaciens* has also been responsible for succinate production but to a lesser degree compared to *F. succinogenes* (Gokarn et al., 1997) with further reductions in succinate, and increased acetate production, noted when co-cultured with methanogens (Latham and Wolin, 1977; Wolin et al., 1997).

Prevotella has been identified as one of the most common taxa within the microbiome (Stewart et al., 1997; Stevenson and Weimer, 2007; Henderson et al., 2015) and has been identified as having the capabilities to degrade hemicellulose (Rubino et al., 2017), starch, xylan and pectin (Matsui et al., 2000), as well as having a functional role in nitrogen utilisation (Kim et al., 2017). While similarities exist in the role members of the genus play within the rumen, there seems to be uniqueness at the species level. For example, *in vitro*

work conducted by Matsui et al. (2000) reported differences in the growth rate and enzyme production of *P. ruminicola, P. bryantii, P. albensis* and *P. brevis* in response to different growth media.

Prevotella, while having a role in the degradation of fibre, is probably not a key cellulolytic member, although it has been shown to grow on cellobiose (Matsui et al., 2000) but rather has a role to play in exposing cellulose to other rumen microbes (Huws et al., 2016; Rubino et al., 2017). *Prevotella* has been shown to play an important role in the primary colonisation of plant tissues, with the abundance of the bacteria increasing post-incubation of forage *in vitro* (Mayorga et al., 2016; Elliott et al., 2018). In addition, 14% and 13.5% of rumen oligosaccharide-degrading enzymes and hemicellulases are encoded by *Prevotella* (Dai et al., 2015).

Similarly, other members of the order Clostridiales are known to play a role in fibre degradation. Members of the genus *Clostridium* have been shown to produce cellulosomes while the genus *Eubacterium* has been shown to encode for cellulases and hemicellulases (Dai et al., 2015). Members of *Lachnospiraceae* including *Butyrivibrio* and *Pseudobutyrivibrio* have the ability to ferment hemicellulose and xylans (Krause et al., 2003) and may thus have a role in exposing cellulose also, yet some members have shown a greater abundance in the less-efficient rumen (Shabat et al., 2016).

Colonisation of ingested fibre in the rumen is initially conducted by organisms that target easily fermentable soluble carbohydrates (Brulc et al., 2009), with the main component of fibre degradation occurring during the secondary colonisation. The disappearance of plant dry matter (DM) has been shown to significantly increase after 4 h of incubation (2.8% 1–2 h vs. 31.7% 4–8 h) (Huws et al., 2016). Also, the proportion of *Lachnospiraceae* has shown a concurrent rise after 4 h in the rumen, with the proportion of *Pseudobutyrivibrio* and *Butyrivibrio* following a similar pattern (Mayorga et al., 2016; Huws et al., 2016). As such due to the near-tandem increase in the proportion of *Lachnospiraceae* and disappearance of plant matter, it is quite probable that members of this family are actively involved in fibre degradation and most likely exposure of cellulose for other organisms. Table 1 outlines the main fermentation end products from the dominant fibrolytic bacteria of the rumen.

3.2 Fungi and cellulose

Anaerobic fungi belong to the phylum *Neocallimastigomycota* (Gruninger et al., 2014) and are known to play a key role in the degradation of plant material due to their ability to synthesise a wide range of potent enzymes and physically rupture plant structures, via the rhizoid (Orpin, 1977; Choudhury et al., 2015; Huws et al., 2018). Eleven genera of rumen fungi have been described

Table 1 Fermentation end products of main fibrolytic bacteria

Genus	Species	Main fermentation end products	H_2	CO_2	Study
Fibrobacter	*succinogenes*	A, S	N	N	Gokarn et al. (1997), Joblin et al. (2002)
Ruminoccus	*flauefaciens*	A, F, S	Y	Y	Latham and Wolin (1977)
	albus	A, F	Y	Y	Miller and Wolin (1973), Zheng et al. (2014)
Butyrivibrio	*fibrisolvens*	B, F, L (A)	Y	Y*	Marounek and Dušková (1999), Emerson and Weimer (2017)
Pseudobutyrivibrio	*ruminis*	B, F, L (A)	_	_	Van Gylswyk et al. (1996)
Lachnospira	*multiparus*	A, F (L)	Y	Y	Dušková and Marounek (2001)
Prevotella	*rumincola*	A, F, P, S	M	Y*	Marounek and Dušková (1999), Emerson and Weimer (2017)
	albenis	A, F, P, S	Y	Y*	Emerson and Weimer (2017)
	revis	A, F, P, S	M	N*	Emerson and Weimer (2017)
	byranti	A, F, P, S	M	N*	Emerson and Weimer (2017)

[1] Order of end products *does not* correspond to that of which is produced most.
[2] Abbreviations: A = Acetate, B = Butyrate, F = Formate, L = Lactate, P = Propionate, S = Succinate, Y = Yes, N = No, M = Minor.
[3] Products in parentheses represent minor amounts.
* Calculation based on Emerson and Weimer (2017), whereby the production of CO_2 was estimated based on the stoichiometry of CO_2 production from the pathways of other known rumen end products of fermentation using the equation (CO_2 = acetate + 2(butyrate) − succinate).

including *Orpinomyces, Neocallimastix, Cyllamyces, Piromyces, Anaeromyces* and *Caecomyces* (Choudhury et al., 2015; Gruninger et al., 2014), with five new genera – *Buwchfawromyces* (Callaghan et al., 2015) and *Oontomyces* (from the camel forestomach; Dagar et al., 2015), *Pecoramyces* (Hanafy et al., 2017), *Liebetanzomycespolymorphus* (Joshi et al., 2018) and *Feramyces* (Hanafy et al., 2018) – revealed via molecular techniques. From this point onwards, reference to rumen fungi will be confined to anaerobic genera and species.

An appreciation of the key role of fungi in rumen fermentation is evidently clear from the work of Gordon and Phillips (1993), whereby the removal of fungi from the rumen of cross-bred wethers fed a straw-based diet was found to reduce feed intake by 40%. Some rumen fungi, particularly *Piromyces* and *Neocallimastix*, have been shown to have little preference and differences in performance with regard to substrate, but may have increased lignocelluloytic capabilities, even displaying increased growth rates of 20% on sources of crude lignocellulose (Solomon et al., 2016). However, recent work comparing the transcriptome of four common rumen fungi has provided evidence that fungal niches may exist within the rumen (Gruninger et al., 2018). The previous authors

identified nearly double the amount of transcripts of CAZymes encoding for pectinase in *Piromyces rhizinflata*, which could suggest a role for increased utilisation of pectin, along with differential proportions of transcripts amongst all the studied fungi. Over 8.5% of rumen cellulases have been shown to be encoded within the genus of *Neocallimastix* and *Piromyces* (Dai et al., 2015). Similar to cellulose-degrading bacteria, rumen fungi are also known to produce cellulosomes (Haitjema et al., 2017).

The fibrolytic nature of fungi has been the subject of much interest recently from the biotechnology industry (Ribeiro et al., 2016; Edwards et al., 2017), further emphasising the potency and efficient nature of fungal-derived cellulolytic enzymes. The predominant end products of rumen fungal fermentation from pure cultures are considered to be formate, acetate, lactate, ethanol, CO_2 and H_2, with trace or minor amounts of succinate and no production of propionate and butyrate (Borneman et al., 1989; Edwards et al., 2017).

3.3 Protozoa and cellulose degradation

Protozoa are postulated to play an active role in fibre digestion in the rumen and produce fermentation end products similar to bacterial ones, including acetate, butyrate and H_2 (Choudhury et al., 2015). Cellulase and hemicellulase production by rumen protozoa has been reported (Williams and Coleman, 1997). More recently, cDNA libraries from ciliate protozoa obtained from cannulated sheep displayed the production of xylanase and cellulases by members of the order Entodiniomorphida, along with pectate lyases and pectin degradation proteins (Ricard et al., 2006). In excess of 9% and 3% of rumen cellulases and hemicellulases were found to be encoded within the genera *Epidinium* and *Polyplastron* (Dai et al., 2015).

Rumen protozoa are known to enact predatory behaviour on bacteria and fungi (Williams and Coleman, 1997) and have been shown to reduce the degradation of cellulose *in vitro* when incubated with fungi (Morgavi et al., 1994). Coinciding with this is the well-established fact that the rumen microbiome is capable of functioning when protozoa are removed. Figure 2 illustrates the brief differences in the faunated and defaunated rumen and Figure 3 displays rumen ciliate protozoa under microscopic analysis.

Newbold et al. (2015) conducted a meta-analysis of 23 *in vivo* rumen defaunation studies and deduced that digestibility of organic matter (OM), neutral detergent fibre (NDF) and acid detergent fibre was reduced by 7%, 20% and 16%, respectively, following removal of protozoa from the rumen. Additionally, *in vitro* work with pure and mixed cultures of protozoa, bacteria and fungi obtained from a single lactating jersey cow and incubated on orchard grass hay, showed a significant reduction in the degradation capabilities of protozoa, compared with monocultures of fungi and bacteria appearing to be two times

more efficient (Lee et al., 2000). Results from the same study also indicated a reduction in degradation when protozoa were co-cultured with bacteria and fungi. A similar pattern of Bermuda grass degradation was observed for the production of endoglucanase on carboxymethyl cellulose assays.

An analysis of the contribution of protozoa to cellulose breakdown in the rumen is complicated and hampered due to the difficulties in maintaining protozoal communities in axenic cultures (Newbold et al., 2015). However, their removal from the rumen was estimated to reduce rumen fungi (92%), *R. albus* and *R. flavefaciens* (22%) (Newbold et al., 2015), and therefore may play a key role in the release of enzymes targeting plant tissues (Williams and Coleman, 1997).

Further, *omic*-based studies are required to better understand the role of ciliate protozoa within the functioning rumen; however, an increase in the number of sequenced rumen protozoan genomes is required. Recently, the first draft of a macronuclear genome sequence of a rumen protozoa, *Entodinium caudatum*, has been published (Park et al., 2018), which is likely to benefit our understanding of these difficult-to-grow microbes into the future.

Therefore, while there is mounting evidence that rumen protozoa are capable of the production of cellulase, their role as a key cellulolytic member of the rumen microbiome is likely to be revealed as more protozoan genomes are published. Equally beneficial would be the discovery of new methods to allow for the axenic culturing of these microbes.

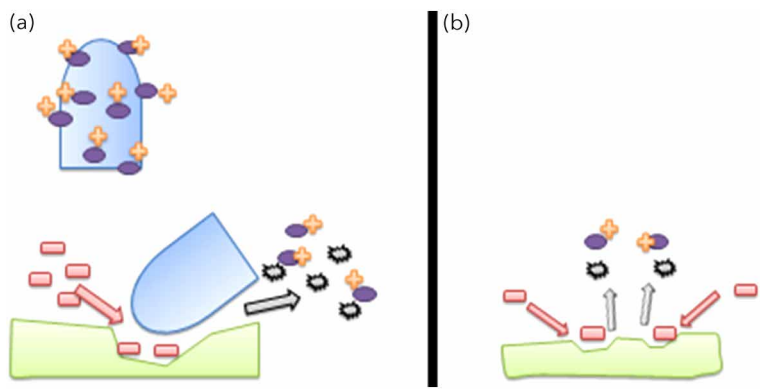

Figure 2 Illustration detailing the differences between the faunated and defaunated rumen. (a) Faunated rumen. Protoza (blue) degrading a feed particle (green). During the degradation process, bacteria (red) access digestible component of the feed particle with end products of fermentation (black) produced by both bacteria and protozoa being utilised by methanogens (purple) to produce methane (orange). Methanogens attach to protozoa utilising the excess hydrogen produced from the protozoal hydrogenosome. (b) Defaunated rumen. Degradation of ingested feed particle in the absence of protozoa results in a reduction in the products of fermentation and methane production.

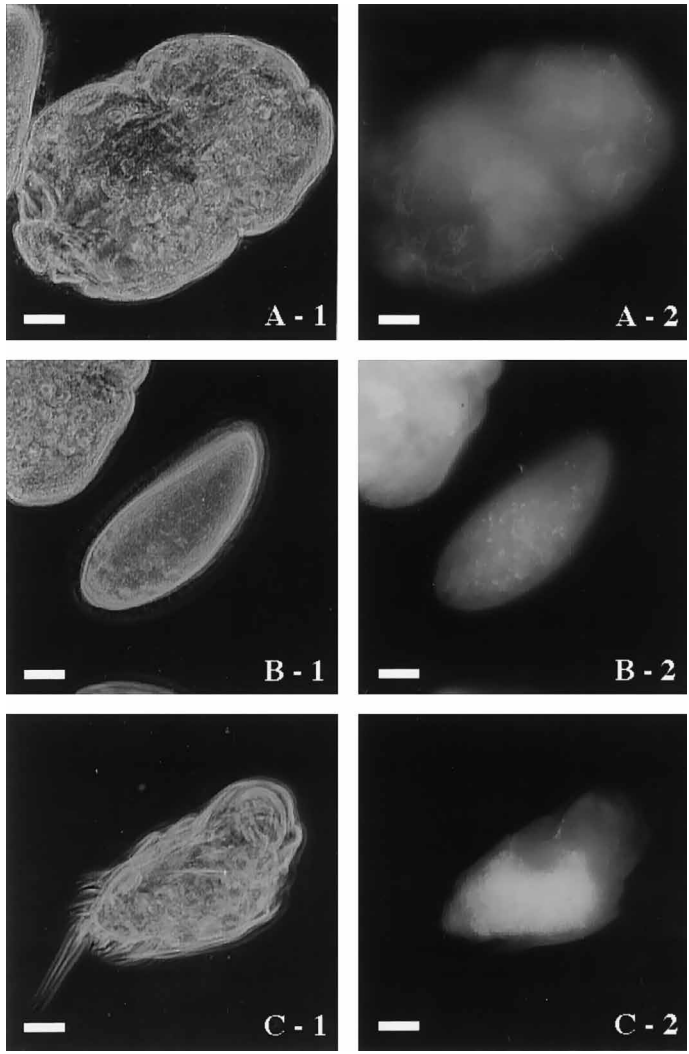

Figure 3 Rumen ciliate protozoa adapted from Tokura et al. (1999). 1: phase contrast microscopy, 2: fluorescence microscopy. A: *Polyplastron multivesiculatum*, B: *Isotricha prostoma*, C: *Ophryoscolex caudatus*. Size bar indicates 20 μm.

4 The rumen microbiome and feed efficiency

An improvement in the feed efficiency of ruminants is highly desired as a method of reducing feed costs. Feed efficiency as a trait can be described either as a ratio, such as the feed conversion ratio (FCR) or in the context of residuals from regression-based equations, such as residual feed intake (RFI) (Berry and Crowley, 2013). Traditionally, the FCR, ratio of feed intake to gain,

was the measure of efficiency, but RFI, the difference between actual feed intake and that expected to be required for maintenance and growth, has become a preferred choice of measurement (Kenny et al., 2018). From the meta-analysis of 39 studies across a variety of breeds from different countries, Berry and Crowley (2013) estimated the heritability of RFI and FCR to be 0.33 and 0.23 for growing animals. In the previous study, it should be noted that heritability estimates for RFI ranged from 0.07 to 0.62. Recently, heritability estimates of 0.40 and 0.20 for RFI and FCR were determined from a population of nearly 2,000 Australian Angus steers (Torres-Vázquez et al., 2018).

As stated in the previous sections, the rumen is the primary site for the digestion of feed and diet can be viewed as one of the main contributors to variation in the composition of the rumen microbiome. Therefore, feed efficiency as a trait cannot be observed independent of the environment in which the animal is managed (Kenny et al., 2018). As feed efficiency is considered a key economically important trait, this section will focus on summarising the literature in an effort to characterise members of the rumen microbiome associated with feed efficiency.

An ever-increasing body of metagenomic- and metatranscriptomic-based studies point towards an influence of host feed-efficiency status on the composition and putative functionality of the rumen microbiome. Indeed, the contribution of metagenomic-based studies is beginning to increase in this area. The focus of earlier studies has mainly been on the appearance of certain taxa with their function in the rumen considered based on correlations with traits, fermentation products or evidence from *in vitro* and other laboratory-based experiments. Therefore, as the number of both metatranscriptomic and metagenomic studies increase, so too will the understanding of how the rumen and its microbes function *in vivo*. In general, most studies have shown minor but significant variations in the rumen microbiome between animals differing in feed efficiency (Cantalapiedra-Hijar et al., 2018). However, as comprehended by the previous authors, variation in the population of rumen microbes with respect to feed efficiency could also be a consequence of changes in feed intake or rumen retention time associated with divergence in an animal's level of feed efficiency.

Apart from the role methanogens play in diverting dietary energy away from the host through the methanogenesis process, identifying specific rumen microbes as being consistently linked with host feed-efficiency status is a difficult task. Indeed, differences in the size of the methanogen population are not always associated with differences in host RFI status (Carberry et al., 2014a; Dini et al., 2019). However, it is more likely that differences in the species of methanogen, rather than the total number, are more likely to be associated with RFI. Total methanogen numbers did not differ between efficiency groups in steers; however, *Methanosphaera stadtmanae* and *Methanobrevibacter sp.*

strain AbM4 were 1.92 and 2.26 times more abundant in high RFI animals (Zhou et al., 2009). A subtle increase in operational taxonomic units (OTU) assigned to *Methanobrevibacter smithii* genotypes was observed in high RFI beef heifers (Carberry et al., 2014b) while two OTUs assigned as *Methanobrevibacter millerae YE315* (97%) and *Methanobrevibacter AbM4* (99%) were identified as having a negative association with RFI (McGovern et al., 2018). More recently, using a metagenome-based approach, an increased abundance of *Methanobrevibacter* was found in less feed-efficient dairy cows fed a mixed diet (Delgado et al., 2019). Changes to both the species and structure of the methanogen population deemed to be beneficial towards feed efficiency may result in a less-efficient methanogenesis process. In addition, variation in other rumen microbes which supply substrate to the methanogens or increases in alternative hydrogen sinks could also play a role (Delgado et al., 2019). The composition of the methanogen population, rather than the abundance of methanogens, appears to be associated with methane production (Tapio et al., 2017). Therefore, those species found to be negatively correlated with methane production could potentially be beneficial towards feed efficiency although further work is required. *Methanobrevibacter sp*. strain AbM4 was found to be present in lower abundance in lactating dairy cows with increased feed efficiency and reduced methane yield (Arndt et al., 2015) providing support for this hypothesis. A significantly enriched methanogenesis pathway and increased abundance of *Methanobrevibacter ruminantium* have been reported in the rumen microbiome of less-efficient dairy cows (Shabat et al., 2016), further linking specific methanogens with feed efficiency and potentially methane output.

Focus in the published literature has largely been on the relationship of host feed-efficiency status with ruminal prokaryotic microbes, although some studies, implementing a qPCR approach, have used general primers for protozoa and fungi, but have found little variation with regard to feed-efficiency status (Carberry et al., 2012). As such, a more targeted approach with the selection of specific primers capable of identifying individual differences in members of the rumen fungal and protozoal communities could allow for a more enhanced investigation into the association of eukaryotes with feed efficiency. However, a meta-analysis of rumen protozoal defaunation studies concluded that the removal of rumen protozoa was capable of improving FCR and increasing ADG (9%), improved the utilisation of energy for fattening (11%) and reduced heat production (5%; Newbold et al., 2015). Given the plausible indirect relationship between feed efficiency and methane production, reference to studies investigating the impact of fungi and protozoa on methane have been included in later sections.

As previously mentioned, *Prevotella* is often described as one of the most abundant genera in the rumen and has a variety of roles in the degradation of

various substrates. A further appreciation of the complex nature of this genus can be observed in its relationship with feed efficiency. For example, at the genus level in dairy cows, multiple OTUs assigned to the bacteria *Prevotella* were observed to have differential abundance in both high and low RFI animals (Jewell et al., 2015). Similarly, variation in the association of OTUs allocated as *Prevotella* with both positive and negative correlations with RFI has been reported in bulls (McGovern et al., 2018). Additional inconsistency can be observed across studies with an increase in the abundance of OTUs assigned to *Prevotella* detected in feed inefficient Brahman bulls grazing pasture (McCann et al., 2014b) while the opposite relationship was noted for Holstein dairy cows fed a mixed diet (Delgado et al., 2019). Furthermore, no clear evidence of a link between *Prevotella* and inefficient steers was observed by Myer et al. (2015).

Interestingly, diet would appear to have an effect on the association of different species of *Prevotella* with host feed efficiency. For instance, *P. ruminicola* was recognised as having a higher abundance in feed-efficient wethers on a concentrate-based diet with the opposite occurring on a forage-based diet (Ellison et al., 2017). Similar effects were noted on other species of *Prevotella* in the aforementioned study. As discussed earlier, different growth rates of species of *Prevotella* have been demonstrated when grown on different media *in vitro*. Therefore at the species level, both diet and host feed efficiency are likely to influence the presence of different *Prevotella*.

Based on the previously mentioned literature, an increased abundance of *Prevotella* would appear to be associated with a lower level of feed efficiency on a forage-based diet with a inverse relationship occurring on a concentrate-based diet. Future studies may look to confirm this relationship by comparing the rumen microbiome and feed efficiency on contrasting diets using a metagenomic-based approach.

It should be noted that, while an in-depth discussion into the analysis of sequencing data is not intended for this chapter, the identification of multiple OTUs assigned within a genus is an associated drawback of the grouping of sequences into OTUs based on a predefined similarity. The reader is referred elsewhere for information on the drawbacks of bioinformatics methods associated with grouping sequences into OTUs (Callahan et al., 2017).

The genus *Ruminococcus* is another group of microbes which has been found to have a varying relationship with RFI. *R. albus* was shown to have a 1.7-fold greater abundance in low RFI beef heifers when fed a high forage diet (Carberry et al., 2012). In the same study, irrespective of diet, the relative abundance of *R. albus* was also noted as having a marginal increase in low RFI animals ($P = 0.08$). Similarly, 16S rRNA sequencing of both liquid and solid rumen samples obtained from Simmental bulls, albeit on a high concentrate diet, has shown at the genus level, *Ruminococcus* to be negatively correlated with RFI and hence in association with a more efficient rumen (McGovern et al.,

2018). However, a higher abundance of *R. albus* has been shown to also be associated with high RFI wethers when consuming a high forage diet, with a concurrent greater abundance of *R. brommi* in low RFI animals (Ellison et al., 2017). Interestingly, in the same study, when fed a high concentrate diet, *R. albus* was found to be in a greater abundance in the low RFI wethers. Not all strains of *R. albus* are known to be capable of utilising cellulose to the same degree or at all (Morris and Cole, 1987), and this could explain the variation observed in the relationship of these taxa with RFI. Interestingly, *R. flavefaciens* has also been found in a greater abundance in less feed-efficient wethers irrespective of diet (Ellison et al., 2017). Conflictingly, the consistency of a significantly higher abundance of *R. flavefaciens* in less-efficient animals has not been maintained in other studies (Carberry et al., 2012; Shabat et al., 2016). As a result of the observed variation between species of *Prevotella* and *Ruminococcus* discussed previously, further *in vivo* investigations capable of detecting species and strain level differences will benefit the understanding of these groups of bacteria with regard to feed efficiency.

Other bacteria found to be related to an improved level of feed efficiency include *F.succinogenes* (Elolimy et al., 2018; McGovern et al., 2018), *Shuttleworthia* (Jewell et al., 2015), *M. elsdenii, Coprococcus catus* (Shabat et al., 2016) and *Eubacterium ruminantium* (Elolimy et al., 2018) with negative impacts on efficiency associated with *Psuedobutyivibrio ruminis* and *Succinivibrio dextrinsolven* (Shabat et al., 2016); however, concentrates formed a high proportion of the diet within all these studies. Members of *Succinivibrionaceae* are known producers of succinate, a substrate utilised for propionate production, and have been shown to have positive correlations with milk yield (Indugu et al., 2017). However, the synthesis of propionate from succinate would appear to be less efficient when compared to that which is formed via the acrylate pathway (Shabat et al., 2016). As a result, this may explain the negative association between members of *Succinivibrionaceae* and host feed efficiency. In addition, unclassified *Succinivibrionaceae* has also been reported as having a moderately negative association with ruminal papillae width in growing lambs (Yang et al., 2018).

Metagenomics, accompanied by metatranscriptomics, has the potential to provide the resolution required to better understand the relationship between the rumen microbiome with feed efficiency. However, it appears that diet has a strong covariate role when investigating the relationship between feed efficiency and the rumen microbiome. Previously conducted research by our own group and others has indicated an effect of diet on feed efficiency with changes in the association of certain microbes with host feed efficiency occurring across different diets (Carberry et al., 2012; Ellison et al., 2017). It has been proposed that a more efficient rumen has a less diverse cohort of microbes allowing for a more targeted approach towards energy extraction

from feed. Whole-genome sequencing of the rumen contents of lactating dairy cows divergent for RFI, consuming a 70:30 concentrate to forage diet, revealed a decrease in the diversity of microbial species and genes in the efficient group (Shabat et al., 2016); however, the authors suggest that this phenomenon may be diet dependant. Again, using a shotgun metagenomics approach, Patil et al. (2018) observed a numerically higher but non-significant ($P = 0.073$) difference in diversity in low RFI Targhee ewe lambs consuming a forage-based diet. In addition, when diversity was calculated to account for the metabolic networks between both high and low RFI groups, a greater diversity of metabolic networks and enzymes was found in the rumen microbiome of efficient animals.

Although differences between both studies could be species related, the impact diet has on feed efficiency should not be discounted, for a predominantly forage-based diet provides the rumen with a suite of more structurally complex polysaccharides than that of a concentrate food source. As livestock progress from forage to a concentrate-based diet, a reduction in metabolic network complexity is observed (Wolff et al., 2017). The variation described in the two previous studies (Shabat et al., 2016; Patil et al., 2018) further suggests that characteristics of the efficient rumen are most probably diet dependant. In summary, a more diverse microbiome may be favoured when consuming forage diets to facilitate enhanced degradation of complicated polysaccharides. In contrast, for a more digestible grain-based diet, a less diverse microbiome may allow for a more focused fermentation with resources better targeted; however, further investigations are warranted to investigate this theory with respect to feed efficiency.

4.1 Association of volatile fatty acids (VFAs) profile and feed efficiency

The relationship between feed efficiency and VFA production seems to also be influenced by diet with contrasting results observed for efficiency levels depending on whether forage or concentrate-based diets were fed. Indeed, precursors for propionate production are often favoured as they are considered competitive pathways to methane production (McAllister and Newbold, 2008) and therefore act to supply more energy to the animal. A higher concentration of VFAs has been recorded in the rumen of feed-efficient dairy cows fed a high concentrate diet, along with increases in propionate and butyrate concentration (Shabat et al., 2016). Low RFI finishing lambs consuming a 75% concentrate diet have been shown to have the corresponding increases in butyrate production compared to high RFI animals, but had lower propionate production with no difference in total VFA concentration (Liang et al., 2017). Contrasting results are displayed in trials when a high forage diet is offered, where no differences in

VFAs have been reported (Fitzsimons et al., 2013, 2014a,b; McDonnell et al., 2016); however, a reduction in propionate concentration was noted in low RFI grass silage fed heifers in the study of McDonnell et al. (2016), and although not a VFA, ammonia concentration was found to be reduced in low RFI pregnant beef cows (Fitzsimons et al., 2014a). Table 2 highlights the VFA profile of animals from a variety of studies investigating differences in RFI. It would appear from averaging the proportions of the main VFAs (acetate, propionate and butyrate) that no clear association with RFI is evident.

4.2 Association of digestibility and feed efficiency

In Table 2, focusing on studies which reported on ammonia (NH_3) concentration in the rumen and where forage was the predominant diet, on average low RFI animals had a 14.5% increased concentration of ammonia (mg/L). The relevance of this may be small, considering the low reporting of rumen NH_3 concentrations; however, it could be an indication of the increased nitrogen digestibility often associated with low RFI animals. Although comparisons to high concentrate diets are limited due to a low representation of data associated with ruminal concentration of ammonia, the divergence may not be as pronounced. High concentrate diets would appear to often be more energy dense and therefore would presumably allow for a more balanced fermentation with regard to protein and energy requirements in the rumen promoting increased utilisation of excess ammonia by rumen bacteria, as discussed in later sections.

The rumen of individuals identified as having an increased feed efficiency has been suggested to have an enhanced ability to extract and absorb nutrients from ingested feed. Particularly in relation to nitrogen, it has been shown previously that more efficient animals have an increased metabolism of nitrogen which is deemed to compensate for a reduced DMI (Rius et al., 2012). The rumen papillae epithelium of higher feed-efficient bulls and steers has also been found to be thicker, suggesting greater metabolic activity (Lam et al., 2018).

The rumen metatranscriptomic profile of feed-efficient beef cattle is proposed to display increased activity in pathways associated with digestion, cell proliferation and survivability (Guan et al., 2017). Additional metatranscriptome studies of the rumen epithelium have shown that the clustering of efficient steers separates from that of non-efficient animals. For example, an increased expression of genes suggested to be associated with paracellular permeability for increased nutrient absorption was noted in feed-efficient cross-bred steers (Kong et al., 2016). Complimentary to this, a tendency for increased expression of solute carrier genes such as *SLC16A3* was observed in more efficient steers and heifers (Elolimy et al., 2018). Finally, Patil et al. (2018) discovered a tendency for the metabolic profile of the rumen microbiome of feed-efficient

Table 2 VFA profile of high (H) and low (L) residual feed intake (RFI) animals from various studies employing a variety of diets

Animal	Sex	Main diet component	pH	RFI	Total VFA (mM/L)	Acetate%	Propionate%	Butyrate%	A:P	Valerate%	NH_3 (mg/L)
Dairy[1]	F	Concentrate	–	H	79.3	41.94	24.53	22.72	–	3.97	–
Beef	M	Concentrate	5.72	H	95.1	51.9	30.5	12.5	1.97	5.1	43.6
Sheep	M	Concentrate	6.65	H	131.02	53.5	30.51[a]	13.44[a]	2.04[b]	1.6	–
Beef	M	Concentrate	–	H	55.35	56.4	32.6	6.1[a*]	1.73[a*]	1.2	–
Beef	M	Concentrate	–	H	58.55	54.48	31.45	9.51	1.87	1	(0.096mM)
Beef[2]	M	Concentrate	–	H	86.025	52.285	33.98	8.695	1.695	1.735	(0.15mM)
Beef[3]	M	TMR	–	H	73.8	49.7	32.4	10.2		3.12	–
Beef[2]	F	G/GS/TMR	6.8	H	119	66.2	20	10.8	3.42	1.3	–
Dairy	F	Grass	6.09	H	145.2	61.5	21.7	11.5	2.9	2.1	(12.9mM)[a]
Beef	F	Grass	6.57	H	87.6	66.9	24.1	10.6	3.27	2.1	110.6
Beef	F	Grass Silage	6.88	H	71.3	61.3	24.1	10.9	2.57	3.9	105.5
Beef	F	Grass Silage	6.85	H	80	62.3	14.1	20.4	4.6	3.2	38.2
Beef[3]	F	Grass Silage	6.77	H	54.3	68	16.6	10.3	4.1	4.9	20.7[a]
Beef[4]	F	Grass Silage	6.84	H	85.2	68.3	18.9[a]	10.4	3.66[c]	2.4	73.5
Sheep	M	Alfalfa pellets	–	H	–	65.2	17.3	14.1	–	1.5	–
		Average	6.57		87.88	59.85	25.33	11.39	2.74	2.51	65.35

[1] Concentration of VFAs converted from total reported as mM. A:P also calculated from mM.
[2] Concentration of VFAs reported as an average across diets.
[3] Recordings on farm.
[4] Pregnant animals.
[a,b] Means with a differing subscript infer significant differences, as per the original paper, between H and L groups.
[a*,b*] VFAs converted to % based on mM data reported. Differing subscripts with star indicates significant differences reported in mM.

Lactate (mg/L)	RFI	pH	Total VFA (mM/L)	Acetate%	Propionate%	Butyrate%	A:P	Valerate%	NH_3 (mg/L)	Lactate (mg/L)	Study
–	L	–	88.14	40.4	25.32	25	–	4.25	–	–	Shabat et al. (2016)
121.4	L	5.76	91.3	54	27.5	13.9	2.27	4.6	52.2	127.2	Fitzsimons et al. (2014b)
–	L	6.33	142.04	49.23	39.89[b]	8.23[b]	1.32[b]	1.82	–	–	Liang et al. (2017)
–	L	–	96.74	54.4	25.9	15[b*]	2.11[b*]	1.7	–	–	Guan et al. (2008)
–	L	–	64.17	54.92	33.41	7.26	1.69	1.04	(0.11mM)	–	Hernandez-Sanabria et al. (2010)
–	L	–	81.205	53.665	33.615	8.255	1.745	1.505	(0.14mM)	–	Hernandez-Sanabria et al. (2012)
–	L	–	74.9	49.1	33.1	10	–	3.33	–	–	Lam et al. (2018)
–	L	6.9	116	66.7	19.6	10.5	3.46	1.3	–	–	McDonnell et al. (2016)
–	L	6.11	142	62	21.41	11.7	2.9	1.8	(15.5mM)[b]		Rius et al. (2012)
40.6	L	6.42	93.4	65.7	24.6	11.3	3.14	2.1	134.8	61	Lawrence et al. (2013)
33.1	L	6.94	68.7	60.9	24.6	10.5	2.45	3.6	118.6	40.5	Lawrence et al. (2013)
29.4	L	6.81	75.2	62.5	18.2	16.1	3.5	3.2	53.7	16.6	Fitzsimons et al. (2013b)
24	L	6.98	54	67.4	16.5	10.4	4.1	5.5	10.2[b]	18.9	Fitzsimons et al. (2014a)
20.2	L	6.82	79.9	67.1	20.2[b]	10.3	3.33[d]	2.7	90.5	24.0	Lawrence et al. (2011)
–	L	–	–	66.2	18.1	12.5	–	1.5	–	–	Ellison et al. (2017)
44.78		6.56	90.74	59.56	25.88	11.14	2.61	2.55	76.67	48.03	

ewe lambs to synthesise more metabolites that are potentially transferred to the host, subsequently resulting in more established links between the microbe and host metabolic networks. Therefore, the more efficient rumen microbiome appears to encompass a metabolic network better aligned with that of host, therefore allowing for enhanced utilisation of beneficial end products of ruminal fermentation (Table 2).

5 The rumen microbiome and methane production

Methane-producing microbes belong to the domain Archaea, which produce methane as a metabolic end product (Deppenmeier and Müller, 2008) (Table 3). The rumen provides a unique environment characterised by a relatively rapid passage rate, and a readily available supply of CO_2 and H_2 resulting in a community of archaea distinct to other anoxic systems (Patra et al., 2017). Methanogens found in the liquid fraction of the rumen and attached to feed particles or rumen protozoa are considered to contribute most to methanogenesis (Morgavi et al., 2010).

As the sole producers of methane, a plausible hypothesis would consider an increased abundance of methanogens within the rumen to be associated with a higher output of methane emissions by the animal. However, it would seem that the composition rather than size of methanogen community in the rumen is more closely associated with methane production (Tapio et al., 2017). In cattle, no differences were noted in the overall relative abundance of archaea between high and low methane-emitting dairy cows (Danielsson et al., 2017) while weak correlations of archaea abundance and methane yield (CH_4 g/kg DMI) were reported in steers (Wallace et al., 2014). Similarly, with sheep, no difference in the relative abundance of archaea was noted in high and low methane-emitting (Kittelmann et al., 2014) or methane-yielding (Shi et al., 2014) groups. In contrast, high-methane-emitting cross-bred steers were found to have double the relative abundance of total methanogens compared to low

Table 3 The three main methanogenesis pathways in the rumen with estimates of free energies from reactions

Pathway	Formula	$\Delta G^{\circ\prime}$a (kJ/mol CH_4)
Hydrogenotrophic methanogenesis[1]	$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	−135 to −130.4
Methylotrophic methanogenesis[2]	$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	−113 to −112.5
Acetoclastic methanogenesis	$CH_3COOH \rightarrow CH_4 + CO_2$	−36 to −33

Table adapted and modified from Liu and Whitman (2008) and Ferry (2012). The reader is directed towards these publications for a more in-depth discussion on the biochemistry of methanogenesis.
[1] Formate is also capable of being used however not included separately due to its conversion to CO_2 prior to being utilised.
[2] Most thermodynamically favourable methyl-based reaction.

methane-emitting animals when metagenomic techniques were conducted (Auffret et al., 2017).

With respect to the composition of the methanogen rumen population, Danielsson et al. (2017) reported differences in the abundance of specific members of the archaeal communities of high and low methane-emitting dairy cattle. It was reported that an increased relative abundance of *M. gottcshalkii* was associated with high methane-emitting cows and a higher abundance of *M. ruminantium* found in the rumen of low methane-emitting animals. A similar association between an increased abundance of *M. gottcshalkii* in rumen samples obtained from high methane-emitting groups was also reported in sheep (Shi et al., 2014). The variation in the abundance of *M. ruminantium* and *M. gottcshalkii* could be as a result of differences in the expression of the different forms of methyl coenzyme M reductase (*mcr*) (Tapio et al., 2017). The *Methanobrevibacter* clade can be segmented into two subgroups, the SGMT (*M. smithii, M. millerae, M. thaueri* and *M. gottschalkii*) and RO clade (*M. ruminantium* and *M. olleyae*), with the SGMT clade capable of synthesising both *mcrI* and *mcrII* and the RO subgroup possessing only *mcrI* (Leahy et al., 2010; Tapio et al., 2017).

Expression of both *mcrI* and *mcrII* is regulated by H_2 availability in the rumen, with *mcrI* and *mcrII* expression occurring in the presence of low and high concentrations of ruminal H_2 (Reeve et al., 1997). Therefore, the association of a higher abundance of *M. gottschalkii* in high methane-emitting cattle and sheep could be indicative of an increased concentration of H_2 in these animals. Accordingly, a greater presence of SGMT methanogens in the rumen could be evidence of increased abundance of rumen microbes that synthesis substrates for methanogenesis (Danielsson et al., 2017). Similarly, as *mcrI* is produced in a low H_2 environment, a greater presence of RO methanogens may be suggestive of a rumen microbiome harbouring a lower abundance of H_2-producing-microbes and/or greater abundance of H_2-utilising microbes and thus associated with less methane production. Evidence of the relationship between SGMT and RO with high and low H_2-producing bacteria is evident from the work of Kittelmann et al. (2013), whereby a positive correlation between the abundance *M. gottschalkii* with *Ruminococcaceae* ($R = 0.90$) and *M. ruminantium* with *Fibrobacteraceae* ($R = 0.72$) was observed. Interestingly, in the same study, a negative correlation between the abundance of *M. gottschalkii* and *M. ruminantium* ($R = -0.51$) was noted, which the authors suggested to be as a result of competition for methanogenesis substrate. Therefore, strategies which increase the abundance of the RO clade in the rumen may be beneficial in terms of lowering methane output.

Similar to the methanogen composition in the rumen, the abundance of specific bacteria, particularly those that produce H_2 are associated with methane production (Tapio et al., 2017). Rumen bacteria vary greatly in

substrate specificity as well as different groups of bacteria being associated with H_2 production and utilisation (Stewart et al., 1997). Kittelmann et al. (2014) identified in sheep three different ruminotypes associated with methane emissions. Ruminotypes Q and S were correlated with low methane-emitting sheep and harboured higher abundances of bacterial communities associated with propionate production and a combination of lactate and succinate respectively. However, in contrast to this, ruminotype H found in high methane-emitting animals was characterised by a higher abundance of H_2 producers. Importantly, propionate is an alternative electron acceptor to H_2 and is associated with lower production of methane (Janssen, 2010). Succinate and lactate are also precursors of propionate production (Wolin et al., 1997). In general they are associated with low or no H_2 production (Kittelmann et al., 2014), thus leading to less H_2 being available for methanogenesis. Wallace et al. (2015) reported a four-fold reduction in the abundance of the succinate-producing bacteria family *Succinivibrionaceae* in high compared to low methane-emitting steers. Similar higher abundances of propionate-producing bacterial groups in low compared to high methane-emitting animals have been noted elsewhere (Kamke et al., 2016; Edwards et al., 2017). Lactate-producing bacteria such as *Sharpea* and *Kandleria* have been shown to maintain similar levels of lactate, formate and acetate production in pure and co-cultures with methanogens *in vitro* (Kumar et al., 2018). As such, it is hypothesised that the occurrence of these bacteria in the rumen acts to lower the availability of H_2, therefore leading to a decrease in the availability of the substrate for methanogenesis (Kittelmann et al., 2014; Kamke et al., 2016).

Inevitably, protozoa have a role in methanogenesis as key H_2 producers (Morgavi et al., 2012), with a strong positive linear relationship between $\log_{10}$ protozoal numbers and methane yield being reported in the meta-analysis of rumen protozoa experiments reported by Guyader et al. (2014). Meta-analysis studies have estimated protozoal removal to reduce methane emissions by 11% but have shown insignificant decreases in methanogen numbers (Newbold et al., 2015). The contrast in reports could be explained by the discovery that the adhesion-like protein (Mru_1499) in *M. ruminantium* M1, which is capable of binding to the surface of a wide range of rumen protozoa and also to H_2-producing *Butyrivibrio proteoclasticus*, suggesting this methanogen may be capable of continued methanogenesis after defaunation (Ng et al., 2016).

The refaunation work conducted by Belanche et al. (2015) showed increased methane emissions in sheep as they progressed from being protozoa free to monofaunated and fully faunated with the rumen fluid of control animals. Unlike with bacterial populations, identifying specific protozoa associated with methane is not as widely reported. Belanche et al. (2015) showed a greater diversity and a 14.4 times greater total abundance of protozoa in their fully faunated animals but did not report statistical differences between the groups

in terms of methane emissions or concentration of methanogens. Furthermore, Kittelmann et al. (2014) did not report any distinctive clustering of different ciliate protozoal communities in low and high methane-emitting sheep when conducting amplicon sequencing analysis.

Similar to protozoa, reports of a relationship between specific rumen fungal populations and methane has not been reported upon in great detail. Studies in sheep have failed to define clear differences in the clustering of fungal communities of high and low methane-emitting animals (Kittelmann et al., 2014) while studies in dairy cattle have failed to define clear correlations between the fungal populations present in rumen samples and methane emissions (Cunha et al., 2017). In the study of Cunha et al. (2017), some 73.19% of the fungal samples were identified as unclassified, and therefore poor identification could be a contributory factor towards the lack of reported correlations while it was also acknowledged that sampling method could impact identification, as anaerobic fungi are more commonly found attached to feed particles. Work in anaerobic digesters inoculated with fungi originating from the rumen of fistulated cattle, showed a positive correlation between fungi numbers and methane emissions (Aydin et al., 2017), perhaps indicating the concentration of the fungal population as a whole to be associated with methane emissions.

6 Methane production and residual feed intake

An ever-increasing body of research is providing evidence of a relationship between feed efficiency and methane production. An increasing trend for more efficient animals to produce less methane has been observed in a variety of studies (Nkrumah et al., 2006; Hegarty et al., 2007; Fitzsimons et al., 2013; Alemu et al., 2017; Sharma et al., 2018). Such a relationship seems inherently plausible considering the production of methane in the rumen is estimated to avert as much as 12% of the GEI of the animal towards methanogenesis (Johnson and Johnson, 1995) and therefore away from animal production.

The consistency of this trend has been challenged by the results from studies however, whereby animals identified as being more efficient were found to have an increase in methane yield (McDonnell et al., 2016; Flay et al., 2019). It has been hypothesised that the increased digestive ability of animals selected as being more efficient results in an increase in the availability of substrates for methanogensis (Flay et al., 2019). In effect, the rumen of a more efficient animal could have an increased capacity to ferment and therefore release more energy from ingested feed. Thus, the reduction in DMI associated with low RFI animals, coupled with a more efficient fermentation, most likely indicates their ability to maintain adequate energy from less feed. However, the reduction in DMI and increased capacity to degrade more indigestible

components of feed, may also lead to an increased retention time in the rumen, which is subsequently associated with increased methane production (Goopy et al., 2014). In sheep, as feed intake increases, a significant linear increase and reduction in rumen passage rate and methane yield has been observed (Hammond et al., 2014). In addition, a concurrent linear reduction in total mean tract and mean rumen retention time was observed as intake increased, although there was some deviation with respect to the measurement technique utilised.

Recently, a smaller rumen and a longer small intestine have been reported in low RFI lambs (Zhang et al., 2017). In the same study, low RFI lambs had an 18% reduction in DMI compared to their high RFI counterparts. The reduced DMI often associated with low RFI animals could therefore be as a result of reduced capacity associated with a smaller rumen and the observed increases in digestibility could be due to increased absorption ability of the small intestine. Although further studies are needed, reductions in methane yield and emissions may appear to have a positive association with high feed efficiency on high digestible diets, where rumen retention time is most likely reduced, and DMI less restricted.

The different associations between RFI and methane yield observed in studies could be as a result of the diet offered or sex as postulated by Flay et al. (2019). Table 4 showcases the results of a variety of studies which have reported on the association of feed efficiency (mainly RFI) and methane production. It would appear that in general more efficient animals are associated with a reduction in daily emissions and methane yield regardless of the diet fed. However, when forage is fed as the predominant diet, the same relationship does not seem to be upheld, and (although only reported in a small number of studies) it would appear that low RFI animals had a 6.5% decrease in daily emissions but a 7.5% increase in methane yield.

If the diet offered to animals during the RFI measurement period is different from that consumed during the methane measurement period, the associated reduction in methane output with low RFI animals may not be maintained. For example, the selection of progeny for RFI based on the estimated breeding value (EBV) of their parents, whose RFI was calculated on a high grain diet, did not appear to translate into a reduction in methane production when low RFI offspring consumed a pasture-based diet (Velazco et al., 2017). Therefore, while feed and management practices capable of reducing daily methane emissions are likely to have positive influences on feed efficiency and animal performance the impact of the proportion of forage in the diet on methane yield and the influence this has on feed efficiency require further investigation.

Indeed, it could be hypothesised that the appearance of certain taxa within the rumen could have different effects on feed efficiency and methane

Table 4 Methane emissions per day (MP) and methane yield (MY) from studies feeding different diets investigating the effects of residual feed intake (RFI) on methane emissions

Species	Main diet component	High RFI	DMI	ADG	MP	MY	Low RFI	DMI	ADG	MP	MY	Significant	Study	Notes
Beef[1] GF	Barley silage	0.292	7.9	1.03	222.2[a]	28.5	−0.25	7.4	1.14	202.5[b]	27.7	MP	Alemu et al. (2017)	
Beef[2] RC	Barley silage	0.292	6.3	1.03	164.5	26.5	−0.25	6	1.14	156.3	26.5	ND	Alemu et al. (2017)	
Beef	Barley concentrate	–	14.13	1.229	190.2[a]	14.7	–	8.38	1.126	142.3[b]	16.3	MP	Hegarty et al. (2007)	
Dairy	Alfalfa	–	12.4	–	256	20.7[b]	–	11.3	–	253	22.7[a]	MY	Flay et al. (2019)	
Beef[3]	High quality grass	0.68	14.0	–	227.24	–	−0.69	13.1	–	181	–	NA	Jones et al. (2011)	g/day were calculated as an estimate from g per LW as reported
Beef[3]	Low quality grass	0.68	10.7	–	125.06	–	−0.69	10.2	–	132.6	–	NA	Jones et al. (2011)	also done on EBV DMI also calculated as being per 500kgBW
Beef	Grass silage	0.54	7.96	0.6	297[a]	36	−0.49	6.95	0.59	260[b]	38	MP	Fitzsimons et al. (2013)	NH_3 much higher
Beef	Grass silage/ Pasture/TMR	0.66	7.54	1.52	146	20.2	−0.74	7.18	1.55	156	22.4	ND	McDonnell et al. (2016)	

(*Continued*)

Table 4 (Continued)

Species	Main diet component	High RFI	DMI	ADG	MP	MY	Low RFI	DMI	ADG	MP	MY	Significant	Study	Notes
Beef	TMR	0.83	10.6	0.8	265[a]	28.1[a]	−0.78	9.33	0.83	194[b]	20.3[b]	MP, MY	Dini et al. (2019)	
Nellore	TMR	0.362	8.12	0.547	163[a]	25.7	−0.506	7.09	0.467	144[b]	24.4	MP	Mercadante et al. (2015)	
Buffalo	TMR	0.05	9.2	0.74	222.2[a]	24.8	−0.05	9.5	0.76	163.4[b]	17.2	MP	Sharma et al. (2017)	
Nellore	TMR	0.787	7.86	1.12	107	11.4	−0.683	6.31	1.11	101	11.9		Oliveira et al. (2018)	
Dairy[4]	Low concentrate	0.44	20.9	–	635	30.7	−0.62	18.6	–	595	32.4	ND	Olijhoek et al. (2018)	
Dairy[4]	Low concentrate	0.98	15	–	499	32.6	−0.74	14.9	–	491	32.5	ND	Olijhoek et al. (2018)	
Dairy[4]	High concentrate	0.44	23.7	–	493	28.2	−0.62	21.6	–	467	27.9	ND	Olijhoek et al. (2018)	
Dairy[4]	High concentrate	0.98	17.8	–	504	21.4	−0.74	17	–	527	24.5	ND	Olijhoek et al. (2018)	

RFI = Residual feed intake (kg DMI/day).
MP = Methane production (g/day), MY=Methane yield (CH_4 g/ kg DMI).
ND = No difference.
[1] Methane emissions estimated using GreenFeed System.
[2] Methane emissions estimated using Respiration Chambers.
[3] Daily methane emissions reported as corrected to 500 kg live weight in study. Values for daily emissions reported here are conversions of original values to that of the mean live weight per group. RFI also estimated on EBV of parents.
[4] Emissions reported as litres per day.

production, particularly when a forage diet is consumed. For example, the celluloytic *Ruminococcus* are known producers of H_2. When co-cultured with methanogens, *R. albus* has been shown to increase acetate and H_2 production, which therefore has the effect of increasing the supply of more H_2 for methanogenesis (Wolin et al., 1997). However, *R. albus* has already been shown to play an active role in fibre degradation and has been shown to be both associated with low and high RFI animals. Therefore, while consuming a diet high in fibre, the presence of *Ruminococcus* is most likely favourable as it is beneficial towards fibre degradation and presumably increasing the supply of energy to the animal. However, the increased fermentation may also result in more substrate availability for methane emission. In addition increases in abundance of the *Succinivibrionaceae* has been associated with reduced methane production due to succinate being a precursor for propionate production, yet members of the family have been shown to be negatively correlated with feed efficiency. Further investigation is warranted to delve into the actions of these potentially antagonistic bacterial species to gain a better understanding of their role and how it relates to methane production and RFI, with an increased focus on forage-based systems.

7 The impact of forage plants on animal performance

As discussed previously, copious amounts of plant matter across the globe are unable to be utilised as a food source by humans. As such, global society relies on the ruminant and their symbiotic relationship with their rumen microbiome to convert indigestible plant matter, into high-quality dairy and meat products for human consumption. Forage traditionally dominates as the main food source for ruminant livestock globally, particularly in countries where a temperate climate dominates year round, such as Ireland or New Zealand. As predominantly grass-based systems are utilised for livestock production, incorporating the maximum proportion of grass in the diet is often the objective of the aforementioned countries. There is a tendency for the inclusion of high quantities of grain in beef finishing and high-yielding dairy systems; however, grass has long been established as the cheapest feed source for ruminant production much to the benefit of farm profitability. However, compared to grain, sources of forage tend to be less energy dense and therefore grains are often used to enhance animal performance.

Variation across and within the nutritional component of plant species exists. A study was conducted exploring differences in the quantity of protein, carbohydrates, fibre, lignin and minerals of some 136 forage plants across 30 different countries (Lee, 2018). On average, grasses contained more fibre than legumes (NDF; 59% vs. 42%) with the opposite being observed with respect to protein (15% vs. 21%). However, a large variation in the nutritional component

within species was found. For example, NDF, dry matter digestibility and crude protein (CP) content of *Lolium perenne* (perennial ryegrass) ranged from 34–62%, 56–86% and 6–34%, respectively (Lee, 2018).

Briefly, there are primarily two types of polysaccharides contained within the plant: those associated with storage, such as starch, and those labelled structural, which make up most of the plant cell wall (Chesson and Forsberg, 1997). The majority of plant cell walls can be split into primary and secondary, with the primary cell wall mainly associated with the young plant and secondary wall with that of a more mature stage in the plant's life (Fig 4).

The growing cell wall consists primarily of cellulose microfibrils (Cosgrove, 2005) which are embedded in a complex matrix of hemicellulose, mainly consisting of xylans (xyloglucan and arabinoxylan) and pectin polysaccharides, which help to provide strength to the cell wall (Harholt et al., 2010; Zamil and Geitmann, 2017). The cellulose component of the primary cell wall is estimated to range from 15–40% (Cosgrove and Jarvis, 2012). Due to the high cellulose and lower xylan content, degradation of most of the primary cell wall is estimated to take about 8–12 h, with the remaining material mainly consisting of the more slowly degraded xylans (Chesson and Forsberg, 1997). Plant cell walls are attached together via the pectin-rich middle lamella (Zamil and Geitmann, 2017). Finally, lignin is primarily associated with the secondary cell wall and maturation of the plant. The secondary cell wall is primarily composed of similar cellulose microfibrils bound by hemicelluloses with pectin being replaced by lignin (Cosgrove and Jarvis, 2012; Zamil and Geitmann, 2017).

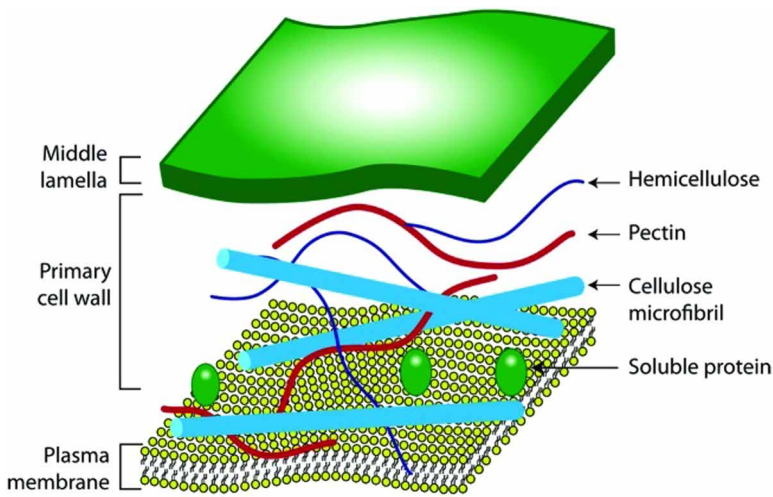

Figure 4 Illustration of the plant cell wall. Source: adapted from Flint et al. (2012).

7.1 Grassland management and animal performance

Differences in the nutritional values between forage species allow for the selection of different forage crops to the benefit of animal performance. Indeed, as observed by Lee (2018), there is a wide range of nutritive values within similar species of forage crop. While this chapter is not intended to provide an in-depth knowledge of pasture management, the importance of grassland management for optimising the efficiency of animal production must be considered.

The concentration of fibre within a plant negatively influences digestibility within the rumen due to its slower and sometimes indigestible nature (Buxton and Redfearn, 1997). It has been speculated that the rate of fibre digestion in the rumen is not limited by the presence of fibrolytic microbes but rather limited by the availability of cellulose for degradation (Weimer, 1996). In essence, it is the access of the rumen microbes to the fermentable proportion of the plant cell, rather than their absence in the rumen, which alters fermentation. Lignin, as a structural plant compound, is deposited in the plant cell wall as the plant grows, providing rigidity and protection of cell-wall polysaccharides from microbial degradation (Vanholme et al., 2010). While the deposition of lignin is beneficial to the plant's structural capabilities, the compound negatively impacts the digestibility of forages (Jung and Allen, 1995) and animal performance (Duble et al., 1971) due to its resistance to fungal and bacterial degradation (Raffrenato et al., 2017). Although lignin degradation is minimal if at all possible in the rumen, it would appear that microbes are capable of degrading the non-lignin proportion of lignin carbohydrate complexes as proposed in the model described by Chesson (1993). Therefore, both the proportion of indigestible lignin and the positioning of the component in the cell wall, including if it is presented at the surface of the ingested feed particle, are capable of limiting degradation.

Common management of the grazing sward targets animal consumption of the grass plant during the growth phase when herbage quality and digestibility is optimum and secondary cell walls are less defined. However, it is vital that a balance between quantity and quality of the pasture is achieved for pasture systems (Curran et al., 2010) and therefore the plant must be allowed grow to ensure an optimum supply of herbage. Assuming good grassland management, low herbage swards (due to the plant being in a stage of regrowth) have been shown to produce less stem and greater digestibility (Holmes et al., 1992). Increases in sward CP and organic matter digestibility and decreases in NDF are reported when swards are grazed at lower herbage mass (Curran et al., 2010; Wims et al., 2010; Boland et al., 2013). Coinciding with this an increase and decrease in the proportion of leaf and stem, respectively, is associated with swards with a lower herbage

mass and pre-grazing height (Wims et al., 2010). Increased milk production, ADG and reductions in methane emissions have been reported in both dairy and beef animals grazing higher-quality swards (Hart et al., 2009; Wims et al., 2010; Curran et al., 2010; Boland et al., 2013; Muñoz et al., 2016). It is highly likely that the benefits to animal performance obtained as a result of improved sward quality are associated with beneficial changes to the rumen microbiome.

Currently, the impact of sward quality with respect to the rumen microbiome with the use of next-generation sequencing (NGS) technologies has not been evaluated in great detail. The impact of sward digestibility on the rumen microbiome has been investigated previously; however, only the quantity, and not the type, of bacteria and fungi were investigated, with neither influenced by sward digestibility (Hart et al., 2009). Studies using more advanced microbial ecology techniques studying variation in sward quality and digestibility as a result of grassland management practices are likely to discover changes to the rumen microbiome due to differences in animals performance reported relative to sward quality.

7.2 Legumes and animal performance

When compared to grasses, legumes tend to have a reduced proportion of fibre and increased CP and are often included in the grazing sward due to their unique ability to fix atmospheric nitrogen into a usable means for plants. Indeed, they are also suggested to be more rapidly digestible, often resulting in an increase in DMI (Table 5) associated with their occurrence in the feed.

It has been shown previously that the pectin within the cell wall of red clover and lucerne is more rapidly degraded by rumen microbes in comparison to other plant structural components (Chesson and Monro, 1982). In addition, when compared to grasses, the degradation of clover would appear to occur more rapidly when inoculated with cultures of the pectin utilising bacteria, *L. multiparus* (Cheng et al., 1979). Due to the higher pectin content in clover, increased degradation of clover compared to grass in the presence of *L. multiparus* is expected. When inoculated with rumen fluid, the breakdown of the legume cell walls occurs differently to that of the grass cell wall, with cellular disorganisation, rather than cellular indentation being implemented (Cheng et al., 1980). Therefore, both the increased degradation of legumes and concurrent disruption of the legume plant cell wall may result in the increased digestibility associated with the feeding of legumes. The cellular segregation associated with the feeding of legumes may result in a reduction in the particle size of ingested feed. Subsequently, this would allow for an increased passage through the rumen and could explain why legumes are often associated with rapid digestibility and increased DMI (Table 5).

Table 5 Animal performance on various legumes in comparison to grasses (mainly Perennial ryegrass)

Forage	Species	DMI	MP	MY	ADG	Milk Yield	Study
White Clover v Grass							
	Sheep	–	–	–	↑	–	Grace et al. (2018)
	Sheep	↑	↑	ND	–	–	Niderkorn et al. (2017)
	Sheep[1]	ND	ND	ND	–	–	Hammond et al. (2011)
	Dairy	↑	–	–	–	↑	Dewhurst et al. (2003)
	Dairy	–	ND	↓	–	ND	Enriquez-Hidalgo et al. (2014a)
	Dairy	↑	↑	↓	–	↑	Lee et al. (2004a)
Red Clover v Grass							
	Sheep	↑	ND	↓	–	–	Niderkorn et al. (2015)
	Sheep	↑	–	–	↑	–	Fraser et al. (2004)
	Dairy	↑	–	–	–	↑	Dewhurst et al. (2003)
Lucerne/Alfalfa v Grass							
	Beef[2]	↓	ND	↑	–	–	Chaves et al. (2006)
	Beef[3]	ND	ND	ND	–	–	Chaves et al. (2006)
	Dairy	↑	–	↑	–	↑	Dewhurst et al. (2003)
	Sheep	↑	–	–	↑	–	Fraser et al. (2004)
	Goat	ND	ND	ND	–	–	Puchala et al. (2012)

DMI = Dry matter intake, ADG = Average daily gain, MP = Methane production per day, MY = Methane yield.
ND = No difference.
[1] Mean of three studies used for analysis. Studies investigated different feeding levels.
[2] Intakes estimated using alkane method.
[3] Intakes estimated using Cornell Net Carbohydrate and Protein System model.
[2,3]Mean of three sites used.
ND = no difference.
– = not reported.

Across 35 studies, the inclusion of white clover (WC) *(Trifolium repens)* in the grazing sward has been shown to increase both milk yield and milk solids yield by 1.4 kg and 0.12 kg, respectively (Dineen et al., 2018). Perennial ryegrass (PRG) and WC mixed swards have also been shown to increase life time ADG, reduce days to slaughter, increase carcass weight and reduce parasitic burden in lambs in comparison to PRG-only swards (Grace et al., 2018).

Grazing of mixed swards containing WC has resulted in a reduction in daily methane yield in dairy cows (Enriquez-Hidalgo et al., 2014a), with indoor feeding trials of PRG with increased levels of WC showing a similar effect (Lee et al., 2004a). However, the corresponding benefits of WC inclusion in a PRG-dominant diet have not been observed in sheep (Niderkorn et al., 2017). Unfortunately, rumen microbial profiling has only been reported for the grazing

dairy cows (Smith et al., 2019); however, VFA analysis was reported both in the sheep trial conducted by Niderkorn et al. (2017), with the VFA analysis of fistulated cattle grazing, and in the dairy cows with methane measurement in Enriquez-Hidalgo et al. (2014a,b). Both Enriquez-Hidalgo et al.'s (2014b) and Niderkorn et al.'s (2017) results showed similar trends of slight reductions in butyrate and increased ammonia concentration when WC was included in the diet.

The proportion of CP was significantly increased with a corresponding increase to WC content in the diet (Niderkorn et al., 2017), with a slight numeric increase noted in the studies of Enriquez-Hidalgo et al. (2014a,b). All three studies also showed a corresponding decrease in NDF with WC inclusion in the diet.

The reason for the reduction in methane yield associated with the feeding of WC is not fully understood. It has been suggested that the increase ($P<0.07$) in DMI associated with WC may be the reason (Enriquez-Hidalgo et al., 2014a), as an elevated DMI is indicative of a reduced rumen residency time, with both previously associated with reductions in methane yield (Hammond et al., 2014; Goopy et al., 2014). However, in both studies, dairy cows and sheep consuming a diet containing WC tended to have a higher DMI compared to animals on the PRG-only diet, and therefore a faster rumen passage may not be a consistent explanation for the mitigation effects of WC.

Apart from the use of different species and the associated impacts this may have had, the major difference in the experimental methods between Niderkorn et al. (2017) and Enriquez-Hidalgo et al. (2014a,b) is due to the latter animals grazing WC as part of a mixed pasture system, with animals in the former receiving a monocultures of WC and PRG in a mixture. As a result, the methane abatement potential of WC may only be obtainable in a mixed grazing pasture system. Changes to the morphology of the WC have been noted in mixed swards (Guy et al., 2018). Therefore, some as of yet unidentified interspecies interaction between WC and PRG when sown together, which would not occur in the monocultures used in Niderkorn et al. (2017), may have resulted in the observed differences between the studies.

In addition, the fertiliser regime differed between both studies. Swards grazed by the dairy cows received the same fertiliser regime of 260 kg of N/ha (60 kg/ha urea and 200 kg/ha calcium ammonium nitrate) while the PRG swards in the sheep study received a total of 100 kg/ha (type not specified) with no fertiliser applied to the monocultures of WC. The application of fertiliser to PRG/WC mixed swards has been shown to reduce nitrogen fixation (Harris and Clark, 1996; Ledgard et al., 2001). In conjunction with this, a negative correlation has been shown to exist between WC nitrogen fixation and nitrate uptake from the soil (Griffith et al., 2000). Such phenotypic alterations due to fertiliser application

may also have some as of yet unknown benefit in terms of methane reduction. Rumen microbial analysis, of the dairy cows from Enriquez-Hidalgo et al. (2014a), conducted by Smith et al. (2019) reported a reduction in the relative abundance of archaea by 21% in conjunction with a modest negative effect on *Methanobrevibacter* in the PRG/WC group. An increase in milk fat was also noted in the same group, which could indicate a higher production of acetate in the rumen PRG/WC animals (Urrutia and Harvatine, 2017), while fistulated cows co-grazing with these animals had a higher ammonia concentration in the rumen compared to their PRG counterparts (Enriquez-Hidalgo et al., 2014b). The aforementioned rumen characteristics associated with the feeding of PRG/WC swards are similar to those which are observed due to the inclusion of nitrate in the diet. *In vitro* work has shown the ability of nitrate to reduce methanogen abundance along with decreases in methane and butyrate production while increasing the proportion of acetate (Zhou et al., 2012; Liu et al., 2017). Nitrate has been suggested to act as a more competitive sink for H_2 than for CO_2 (Zhou et al., 2012), therefore directing H_2 away from methanogenesis. In addition, a reduction in methanogens was noted when nitrate was supplemented to steers (Zhao et al., 2018) while ruminal acetate and ammonia production also increased with nitrate supplementation in lactating dairy cows, although no significant rise in milk fat was reported (Farra and Satter, 1971). Although only a hypothesis, future studies combining rumen microbial analysis and methane estimations will investigate the inorganic nitrogen content of PRG/WC and PRG swards under the same fertiliser regime, to test if differences which could explain the reduction in methane yield exist.

Not many studies investigating the impact of WC on the rumen microbial profile *in vivo* have been conducted to date. Recently, the rumen microbiome of four lactating Holstein Friesian cows, strip grazing pure swards of PRG and WC was investigated in a cross over design (Bowen et al., 2018). While the primary focus of that study was on differences in the composition of samples taken from either the liquid or the solid phase of the rumen, further examination of the mean abundance of microbes discovered alterations in the microbial community composition with respect to diet. Data from our own lab investigating the rumen microbiome of the dairy cows used in the study of Enriquez-Hidalgo et al. (2014a) has also found significant differences in the rumen microbiome (Smith et al., 2019), which could explain the reductions in methane yield observed in the study as previously mentioned.

Increased concentrations of ammonia in the rumen microbiome along with decreased partitioning of nitrogen in the faeces was found in lactating dairy cows with a negative RFI (Rius et al., 2012). Although increases in urinary nitrogen were not detected in the study, the authors conceded an increased ammonia concentration in the rumen is likely indicative of increased ruminal

proteolysis and subsequently increased urinary nitrogen losses. As discussed, while increased concentrations of ammonia have been associated with reductions in methane production *in vitro,* the positive impact this has *in vivo* on environmental benefits could potentially be negated by increased urinary nitrogen output resulting in heightened nitrous oxide production. Interestingly reduced nitrogen output has also been observed in buffalo heifers with a lower RFI (Sharma et al., 2018) as well as reduced concentrations of ammonia in the rumen of low RFI pregnant heifers (Fitzsimons et al., 2014a). Recent work in Romeny wethers consuming three varieties of PRG estimated rumen concentrations of ammonia to be strongly correlated with daily urinary nitrogen in grams and nitrogen partitioned in the urine (0.72 and 0.64) while negatively correlated with both daily retained nitrogen in grams and nitrogen retained relative to intake (−0.54 and −0.61) (Jonker et al., 2018a). Therefore, while efforts to increase the concentration of ammonia in the rumen may benefit methane mitigation strategies, negative impacts on RFI and increased nitrogen losses may be an associated consequence.

Red clover (RC; *Trifolium pratense*) has also shown promise as a plant with mitigation potential. Similar to the evidence presented on WC, *in vitro* experiments have determined reductions in methane emission associated with RC compared to grasses (Navarro-Villa et al., 2011; Belanche et al., 2013) with a reduction in methane yield found *in vivo* using Texel sheep (Niderkorn et al., 2015). Similar to WC, the inclusion of RC has also been shown to increase milk yield and solids. Interestingly, when compared with each other, swards of mixed grass species, with either RC or WC included, showed no difference in milk yield, although WC was found to slightly increase milk protein (Steinshamn and Thuen, 2008). Milk production was also shown to increase in dairy cows when fed silages that contained mixtures of RC or WC with grass; however, it was also noted that the efficiency of dietary N conversion to milk N was decreased with the inclusion of the clovers (Dewhurst et al., 2003). Reduced days to slaughter (38 days vs. 66 days), increased live weight gain (305 g/day vs. 184 g/day) and a higher kill out percentage (48% vs. 46%) has been observed in lambs finished on RC compared to PRG (Fraser et al., 2004).

RC inclusion in the diet has been shown to alter the rumen microbiome when compared to grass. Increases in the DNA concentrations of *B. proteoclasticus* and *R. albus* with decreases to *R. flavefaciens* and *F. succinogenes* were found in the rumen of crossbred steers fed RC silage compared to grass silage (Huws et al., 2010). Similarly, increased abundances of *Butyrivibrio* were associated with RC compared to PRG *in vitro* (Elliott et al., 2018). Reductions in the aforementioned population of ruminococci and *F. succinogenes*, along with a reduced population of *B. fibrisolvens, P. ruminicola*, *S. ruminantium*, fungi and methanogens, were discovered *in vitro* (Belanche et al., 2013). Interestingly, it would seem from the literature that the impact of RC on protozoa are

inconsistent with some data showing no effects on total protozoa (Belanche et al., 2013) and other studies finding variation in the impact amongst different types of protozoa (Niderkorn et al., 2015). Amplicon sequence data has reported a decrease in *Butyrivibrio* in steers fed RC silage in a 70:30 ration with concentrates and with oilseed and vaccenic acid compared to those fed on grass hay with the same additives (Petri et al., 2014). In addition, *Fibrobacter* was noted as being in higher abundance in the RC animals. A decrease in the abundance of *Psuedobutyrivibrio* has been shown to be associated with *in vitro* fermentation of RC when compared to PRG (Elliott et al., 2018). Reductions in the ruminal abundance of *Psuedobutyrivibrio* could be deemed a positive impact of RC inclusion in the diet, as the genus has previously been discussed as having a negative impact on RFI, and the abundance of the taxa has been shown to be reduced in low methane *in vitro* studies (Mi et al., 2017).

Legumes, particularly WC, are known to have a higher concentration of pectin compared to grasses (Thomson, 1984), and members of the genus *Butyrivibrio* are known degraders of pectin. As such, the associated increases of *Butyrivibrio* on RC could simply be as a result of increased substrate for the fermentation by this species. Interestingly, the changes to the VFA profiles in the WC studies and changes associated with reductions in methane could be due to pectin-associated fermentation alterations. Both *B. fibrisolvens* and *P. ruminicola* have exhibited reductions in the proportion of butyrate produced *in vitro* with corresponding increases in acetate production when incubated on a source of pectin compared to glucose (Marounek and Dušková, 1999). Changes in the end products of such highly abundant microbes could explain the corresponding changes to the VFA profiles associated with the feeding of legumes. In addition, significantly reduced amounts of H_2 and formate were reported for *B. fibrisolvens* and could result in less substrate for methanogens. No difference in the proportion of *Butyrivibrio* but a tendency for increased *Lachnospiraceae* and a significant rise in the proportion of *Lachnospira* were noted in the samples of animal grazing pure WC (Bowen et al., 2018). Similarly, *Lachnospira multiparus* was shown to have increased acetate and reduced formate and H_2 when incubated on medium of pectin compared to glucose *in vitro* (Dušková and Marounek, 2001) which could also have an impact on reduced methane production. This interesting relationship between feeding of clover to ruminants and its impact on both methane production and the rumen microbiome warrants further investigation.

The feeding of RC to animals is often associated with greater nitrogen efficiency due to the presence of enzyme polyphenol oxidase (PPO) protecting plant proteins from degradation in the rumen (Huws et al., 2018). The enzyme PPO becomes activated on exposure to oxygen, converting phenols into quinones which act to crosslink with cellular components such as proteins, amines and amides, yielding reductions in proteolysis (Lee et al., 2004b). Although RC is

postulated as having benefits towards reduced protein degradation in the rumen due to PPO, the activation of the enzyme must occur outside of the rumen during mastication or silage production (Lee, 2014). Reductions in *R. flavefaciens* population have been noted previously when animals were fed a high-fibre, low-protein diet, along with a tendency for reduced *F. succinogenes* and fungi (Belanche et al., 2012). Reductions in the above microbes due to the presence of RC within the diet therefore suggest evidence of PPO having a limiting factor on the supply of nitrogen to the effected microbes.

Nitrogen retention has been shown to be reduced when RC and WC are consumed as the sole forage, although improvements in nitrogen retention in milk have been noted for both RC and WC when fed with grass or concentrates (Dewhurst et al., 2003). Similarly, RC at an inclusion rate of 25% was shown to increase nitrogen retention with respect to nitrogen intake in grams, compared to grass only swards, from 0.218 to 0.273 (Niderkorn et al., 2015). The beneficial nature of PPO is only realised when the diet is balanced with an efficient supply of fermentable energy to allow microbial protein production; otherwise, nitrogen content is considerably higher than demand of the animal and that of which can be turned into microbial protein (Lee, 2014). Ultimately, when nitrogen intake is above the requirements of the animal, the positive effects of PPO are negated.

Interestingly, both WC and RC inclusion in the diet has been shown to increase DMI in the majority of the studies mentioned (Dewhurst et al., 2003; Fraser et al., 2004; Niderkorn et al., 2015, 2017). Increased DMI has also known to be associated with passage rate and retention time in the rumen, which has shown to be beneficial towards a reduction in methane emission (Pinares-Patiño et al., 2003; Goopy et al., 2014). Therefore, the beneficial effects of both clovers with regard to milk production, growth rate and mitigation could be as a result of increased DMI-reducing rumen retention time. However, as previously discussed, the increase in DMI associated with clover feeding and the benefit this has on methane production is somewhat questionable.

Lucerne has also shown benefits with regard to animal performance. The feeding of lucerne has led to increased body weights of ewe lambs and their offspring during lactation (Corner-Thomas et al., 2014). Similar benefits have also been noted with finishing lambs grazing lucerne or PRG pastures, with those consuming lucerne having increased live weight gain and reduced days to slaughter (Fraser et al., 2004). Supplementation of milk replacer and starter with lucerne has also been shown to increase the abundance of *Ruminococcus* after weaning (Yang et al., 2018) and significantly increased papillae length (Yang et al., 2015). Potentially, the benefits of increased body weight in lambs could be due to positive impacts on the rumen papillae development or equally a reduction in ciliate protozoa, which has been associated with the feeding of lucerne to goats (Puchala et al., 2012). Contrastingly, a linear increase in total

protozoa numbers was noted with increases in the proportion lucerne fed silage as part of 60:40 TMR in favour of forage, to dairy cows (Hassanat et al., 2014). Lucerne contains the secondary plant compound saponin, which is capable of interacting with cholesterol in the membrane of rumen protozoa resulting in cellular fracture (Wina et al., 2005). In addition, the inclusion of lucerne hay compared to Chinese ryegrass (*Leymus chinensis*) resulted in an increase in the abundance of *Selenomonas* and members of *Prevotella;* however, no significant difference in the quantity or profile of VFAs was reported (Zhang et al., 2014).

In terms of a plant with mitigation potential, the benefits of lucerne seem inconclusive. Table 5 highlights the impact the aforementioned legumes have had on animal performance. The majority of studies comparing lucerne with grasses have shown an increase in DMI. Unlike the benefits of increased DMI on methane production associated with some WC and RC studies, evidence of similar results with lucerne does not appear to be as pronounced and most likely warrants further investigation. Similarly, the impact of lucerne on nitrogen efficiency compared to grasses does not seem to be optimum. Dewhurst et al. (2003) reported pure lucrene silages to have the lowest nitrogen efficiency compared to pure clover and clover mixed with grass silages and was also predicted to lead to excess nitrogen excretion using the Cornell Net Carbohydrate and Protein System model applied to *in vitro* measurements (Chaves et al., 2006).

Fundamentally, the true mitigation advantage of legume inclusion in the diet may be realised through a reduction in methane intensity. From the previously discussed sections, there is evidence of legumes increasing milk production parameters and carcass weights. As such, legumes may provide an opportunity for a cost-effective way to increase animal production without the subsequent increase in methane emissions. Indeed, although the feeding of legumes is reported to decrease nitrogen retention, such a negative impact may be offset by the evidence of comparable PRG sward performance between those receiving applications of synthetic nitrogen fertiliser and pastures containing WC (Enriquez-Hidalgo et al., 2018). Therefore, WC inclusion in the grazing sward may help to sustainably increase animal production and potentially decrease the amount of GHG output per kg of livestock product produced.

The reader should proceed with caution in evaluating the findings from some studies whereby pure cultures of legumes were implemented. Under normal production systems, grazing pure swards of some legumes for long periods of time would not be normal practice; rather, legumes would be included in mixes with grasses. Therefore, results from studies with pure swards should be cautiously accepted and there may be a need to form future studies under more practical conditions like those at farm level.

7.3 Grasses and animal performance

PRG is one of the most common species sown in pasture-based systems due to the superior growth yields and persistence in the grazing sward associated with the grass. There is potential for the identification of grass varieties with mitigation potential as a practical means to reduced emissions associated with livestock (Jonker et al., 2018b). Indeed, in New Zealand, a genetically modified high lipid ryegrass has been bred and may offer mitigation potential; however, no data has been published yet (Eckard and Clark, 2018). Different varieties of PRG have been shown to have varying results with regard to reducing methane emissions.

Recent work on Romeny wethers grazing a conventional diploid, high sugar diploid and a tetraploid variety was conducted investigating the effects of each variety on methane emissions and nitrogen partitioning (Jonker et al., 2018a,b). Both daily methane emissions and methane yield were reduced in wethers consuming the high sugar and tetraploid varieties in comparison to that of the conventional diploid variety, with reductions greatest for those consuming the tetraploid.

However, within the same group of sheep with a similar nitrogen intake, there was increased daily urinary nitrogen from animals on the high sugar and tetraploid varieties as well as an increase in nitrogen partitioning in urine in the tetraploid variety. A reduction in the apparent tract digestibility of OM on a dry matter basis (DOMD) was noted as having played a role in the decreased nitrogen retention in sheep grazing the tetraploid variety. This again highlights the importance of energy availability for efficient nitrogen utilisation in the rumen. Similarly, the reduction in methane emissions (both daily and yield) could have been, as a result of a decrease in the DOMD, accompanied by a reduced DMI, leading to less substrate availability for methanogenesis. Animal performance across all varieties, when ewes and lambs of 3 and 6 months of age, showed season had an effect on varieties such that ADG was highest on the high sugar variety in spring and highest on the tetraploid variety in autumn; however, live weight gains per hectare was similar across all varieties (Cosgrove et al., 2015).

Although overall methane output from sheep grazing the high sugar variety was higher than that of the tetraploid (16.2 g/day vs. 14.6 g/day; 19.4 g/kg DMI vs. 18.4 g/kg DMI), the numerically lower nitrogen partitioning in urine compared to the tetraploid variety could highlight a benefit of this high sugar grass to mitigate when compared to conventional diploids, as it reduced methane emissions and had an intermediate impact on nitrogen partitioning. In lactating dairy cows the feeding of dried high sugar variety AberMagic has been shown to reduce and increase nitrogen partitioning, as a proportion of nitrogen intake, in urine and faeces (Staerfl et al., 2012). Although the same

study noted a reduction in milk yield, no difference in methane emissions and substantially higher CP in the low sugar variety were found, which may have explained differences to animal performance.

An extensive review of high sugar grasses has been conducted (Edwards et al., 2007). Within the review, high sugar grasses were shown to decrease partitioning of nitrogen in urine in 3 out of 7 studies, increase milk yield in 3 out of 14 studies, increased feed intake in beef studies and lamb live weight gain was found to increase in 2 out of 5 studies. Therefore, as eluded to by the authors, further studies into the role of high sugar forages in animal production are required, coupled with the observed variation shown in the response to methane emissions.

Very little data exists regarding the impact of fresh high sugar grasses on the rumen microbiome. However, a few studies have examined high sugar hay with low-quality hay at different levels of concentrate inclusions. Although not directly comparable, such studies may provide evidence of the potential impacts of high sugar grasses on the rumen. In non-lactating dairy cows, the rumen microbiome was found to be significantly altered by high sugar hay, with further changes noted with increased concentrations of the higher-quality hay (Klevenhusen et al., 2017). Regardless of concentration supplementation, both rumen liquid and solid samples from low-quality hay clustered together indicating the type of hay was capable of changing the structure of the rumen. At the family level in the liquid fraction, the abundance of *Succinivibrionaceae* was found to decrease in linearly coinciding with increases in the high sugar hay. At the genus level, *Ruminobacter* also followed a similar pattern. Interestingly in the solid fraction, the abundance of *Fibrobacter* was found to be reduced when low and high sugar hay was fed at the same concentrate inclusion rate, with *Fibrobacter* gradually increasing as concentrate level decreased. The impact on *Fibrobacter* was hypothesised to be as a result of higher fibre content in the low-quality hay. Finally, it was noted that a linear decrease in propionate and butyrate and increase in acetate and isobutyrate as high sugar hay concentration in the diet increased. Unfortunately, milk yield was not recorded on these animals, nor were parameters associated with nitrogen retention. Rumen ammonia concentration in both the liquid and solid fraction appeared to be higher on both dietary extremes most likely as a result of a higher CP in the high sugar hay.

With relation to high sugar pastures, it would appear that animal performance is very much likely to be impacted by the nutrient composition of the grass, with CP level seemingly variable amongst the high sugar grasses. Evidence from the review by Edwards et al. (2007) would suggest that increasing the water-soluble carbohydrates (WSC):CP within high sugar grasses has potential to increase nitrogen retention. It is assumed that an increase in the fermentable energy results in an increased utilisation of ammonia by

microbes for growth. High sugar grasses have been analysed under a variety of modelling conditions (Ellis et al., 2011). It was conceived that nitrogen efficiency increases were subject to multiple conditions; however, increasing the content of WSC at the expenses of CP and increasing the WSC:CP was associated with reductions in the amount of nitrogen excreted in urine relative to nitrogen intake. A strong negative correlation (−0.90) between the WSC:CP of the grass and excretion of nitrogen in urine was also noted. It was also predicted that increases in the WSC at the expense of CP would reduce milk yield (−1.5 kg/day); however, a gain of (+2.4 kg/day) was predicted by increasing the WSC content at the expense of NDF. Carbohydrates are essential for microbial growth and therefore should a steady supply be lacking, the uptake of ammonia being utilised for growth by rumen microbes is below optimum (Hristov and Jouany, 2005). The feeding of high sugar grasses under more holistic conditions including conducting investigations of the rumen microbiome is warranted for a better understanding of the relationship between high sugar grasses and feed efficiency, milk yield and environmental outputs.

8 Conclusion

The ability of ruminant animals to produce high-quality meat and milk from human indigestible plant matter has and will underpin unprecedented growth in the global population for future decades. However, the environmental impact associated with the increased number of livestock required to meet such global demand for animal-based products must be addressed.

A variety of forages are fed to domesticated ruminants with differing impacts on animal performance. Equally, the economic benefits associated with pasture-based systems, due to the cost-effective nature of grazed forage, have resulted in the high usage of such systems in ruminant production. The nutrient profile of the diet offered to the animal varies dependant on the species of forage offered which likely impacts both the composition and functionality of the rumen ecosystem. Equally, pasture management influences the anatomy of the plant which can alter the nutrient composition of the offered forage with subsequent consequences to animal performance, with further investigations required to determine their effects on the rumen microbiome. In general, targeting grazing of the plant when it is in a stage of regrowth is practised due to the plant being more digestible and therefore more beneficial to the animal. Coinciding with this, the ingestion of more digestible plant matter is likely to reduce methanogenesis as a result of a decreased retention time in the rumen.

Both strategic dietary management and the influence of animal breeding on the rumen microbiome offer potentially feasible options to improve the sustainability of global livestock production (Tapio et al., 2017). The breeding

of ruminant animals with a more efficient rumen microbial ecosystem, offers potentially permanent gains to GHG mitigation efforts. However, in the short to medium term, dietary intervention, particularly in early life (Yanez-Ruiz et al., 2010), offers an alternative means to alter the efficiency of the ruminal fermentation process.

Feed-efficiency measurements for cattle are traditionally conducted in a feedlot setting where grain makes up the majority of the diet offered to the animal. As a result, grain has been the main dietary component in the majority of studies that have investigated the relationship between feed efficiency, the rumen microbiome and methane emissions. Notwithstanding this, however, there is an urgent need to improve our understanding of the impact of a high forage diet on the rumen given that fresh and/or ensiled pasture makes up a substantial proportion of the annual feed budget for ruminant livestock with its consequent impact on both the composition and functionality of the rumen microbiome.

The role of specific prokaryotic rumen microbes in determining the feed-efficiency status of the host as well as on the impact of the latter on its environment has become better understood with the recent technological advances in NGS available to researchers. Nevertheless, due to reduced representation in the available microbial databases, the identification and function of rumen fungi and protozoa in the rumen is less understood. Rumen eukaryotes, particularly anaerobic fungi, possess potent capabilities to degrade forage. Therefore advancing our knowledge of their role, both *in vitro* and *in vivo*, is required.

Finally, there is a need for a more holistic approach to be implemented when considering practices with mitigation potential. Certain methods may have antagonistic consequences to methane and nitrogen output in ruminants and as a result need to be considered when validating the overall mitigation potential of management strategies.

9 Where to look for further information

Future research in this area is focussed on enhancing the ability of the rumen microbiome to digest forage-based diets to improve feed efficiency and ultimately reduce feed costs. This will be enabled by increased knowledge of the constituents and the function of the rumen microbiome through culturing, deeper sequencing and improved query databases. The application of CAZymes and the extraction in the form of meat and milk, which could be produced industrially at large scale and added to feed, is another future goal. As discussed in this chapter, major international efforts are ongoing to reduce the carbon footprint of agriculture and specifically to reduce methane emissions from ruminants across all feeding systems. As such, nutritional feedings strategies

including feed additive supplementation to abate methane emissions are being tested internationally. Breeding strategies are being developed to breed beef and dairy cattle with improved feed efficiency and reduced methane emissions. This work is ongoing at Teagasc, Ireland, INRA, France, AgResearch, New Zealand, University of Alberta and Agriculture, AgriFood Canada, the USDA, USA and other institutions across the world. Research across these institutions are linked and supported by international agencies such as the Livestock Research Group of Global Research Alliance for Climate Change.

10 References

Alemu, A. W., Vyas, D., Manafiazar, G., Basarab, J. A. and Beauchemin, K. A. 2017. Enteric methane emissions from low- and high-residual feed intake beef heifers measured using GreenFeed and respiration chamber techniques. *J. Anim. Sci.* 95(8), 3727–37. doi:10.2527/jas.2017.1501.

Arndt, C., Powell, J. M., Aguerre, M. J., Crump, P. M. and Wattiaux, M. A. 2015. Feed conversion efficiency in dairy cows: repeatability, variation in digestion and metabolism of energy and nitrogen, and ruminal methanogens. *J. Dairy. Sci.* 98(6), 3938–50. doi:10.3168/jds.2014-8449.

Arntzen, M. Ø, Varnai, A., Mackie, R. I., Eijsink, V. G. H. and Pope, P. B. 2017. Outer membrane vesicles from *Fibrobacter succinogenes* S85 contain an array of carbohydrate-active enzymes with versatile polysaccharide-degrading capacity. *Environ. Microbiol.* 19(7), 2701–14. doi:10.1111/1462-2920.13770.

Artzi, L., Bayer, E. A. and Morais, S. 2017. Cellulosomes: bacterial nanomachines for dismantling plant polysaccharides. *Nat. Rev. Microbiol.* 15(2), 83–95. doi:10.1038/nrmicro.2016.164.

Auffret, M. D., Stewart, R., Dewhurst, R. J., Duthie, C. A., Rooke, J. A., Wallace, R. J., Freeman, T. C., Snelling, T. J., Watson, M. and Roehe, R. 2017. Identification, comparison, and validation of robust rumen microbial biomarkers for methane emissions using diverse *Bos taurus* breeds and basal diets. *Front. Microbiol.* 8, 2642. doi:10.3389/fmicb.2017.02642.

Aydin, S., Yildirim, E., Ince, O. and Ince, B. 2017. Rumen anaerobic fungi create new opportunities for enhanced methane production from microalgae biomass. *Algal Res.* 23, 150–60. doi:10.1016/j.algal.2016.12.016.

Bayer, E. A., Belaich, J. P., Shoham, Y. and Lamed, R. 2004. The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. *Annu. Rev. Microbiol.* 58, 521–54. doi:10.1146/annurev.micro.57.030502.091022.

Belanche, A., Doreau, M., Edwards, J. E., Moorby, J. M., Pinloche, E. and Newbold, C. J. 2012. Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. *J. Nutr.* 142(9), 1684–92. doi:10.3945/jn.112.159574.

Belanche, A., Lee, M. R. F., Moorby, J. M. and Newbold, C. J. 2013. Comparison of ryegrass and red clover on the fermentation pattern, microbial community and efficiency of diet utilisation in the rumen simulation technique (Rusitec). *Anim. Prod. Sci.* 53(10), 1052–64. doi:10.1071/AN12183.

Belanche, A., De La Fuente, G. and Newbold, C. J. 2015. Effect of progressive inoculation of fauna-free sheep with holotrich protozoa and total-fauna on rumen fermentation, microbial diversity and methane emissions. *FEMS Microbiol. Ecol.* 91(3), fiu026. doi:10.1093/femsec/fiu026.

Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* 70(2), 567–90. doi:10.1152/physrev.1990.70.2.567.

Berry, D. P. and Crowley, J. J. 2013. CELL BIOLOGY SYMPOSIUM: genetics of feed efficiency in dairy and beef cattle. *J. Anim. Sci.* 91(4), 1594–613. doi:10.2527/jas.2012-5862.

Boland, T. M., Quinlan, C., Pierce, K. M., Lynch, M. B., Kenny, D. A., Kelly, A. K. and Purcell, P. J. 2013. The effect of pasture pregrazing herbage mass on methane emissions, ruminal fermentation, and average daily gain of grazing beef heifers. *J. Anim. Sci.* 91(8), 3867–74. doi:10.2527/jas.2013-5900.

Borneman, W. S., Akin, D. E. and Ljungdahl, L. G. 1989. Fermentation products and plant cell wall-degrading enzymes produced by monocentric and polycentric anaerobic ruminal fungi. *Appl. Environ. Microbiol.* 55(5), 1066–73.

Bowen, J. M., McCabe, M. S., Lister, S. J., Cormican, P. and Dewhurst, R. J. 2018. Evaluation of microbial communities associated with the liquid and solid phases of the rumen of cattle offered a diet of perennial ryegrass or white clover. *Front. Microbiol.* 9, 2389. doi:10.3389/fmicb.2018.02389.

Brulc, J. M., Antonopoulos, D. A., Miller, M. E., Wilson, M. K., Yannarell, A. C., Dinsdale, E. A., Edwards, R. E., Frank, E. D., Emerson, J. B., Wacklin, P., Coutinho, P. M., Henrissat, B., Nelson, K. E. and White, B. A. 2009. Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. *PNAS* 106(6), 1948–53. doi:10.1073/pnas.0806191105.

Buxton, D. R. and Redfearn, D. D. 1997. Plant limitations to fiber digestion and utilization. *J. Nutr.* 127(5 Suppl.), 814S–8S.

Callaghan, T. M., Podmirseg, S. M., Hohlweck, D., Edwards, J. E., Puniya, A. K., Dagar, S. S. and Griffith, G. W. 2015. *Buwchfawromyces eastonii* gen. nov., sp. nov.: a new anaerobic fungus (Neocallimastigomycota) isolated from buffalo faeces. *MycoKeys* 9, 11–28. doi:10.3897/mycokeys.9.9032.

Callahan, B. J., McMurdie, P. J. and Holmes, S. P. 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J.* 11(12), 2639–43. doi:10.1038/ismej.2017.119.

Cantalapiedra-Hijar, G., Abo-Ismail, M., Carstens, G. E., Guan, L. L., Hegarty, R., Kenny, D. A., McGee, M., Plastow, G., Relling, A. and Ortigues-Marty, I. 2018. Review: biological determinants of between-animal variation in feed efficiency of growing beef cattle. *Animal* 12(S2), s321–35. doi:10.1017/S1751731118001489.

Carberry, C. A., Kenny, D. A., Han, S., McCabe, M. S. and Waters, S. M. 2012. Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. *Appl. Environ. Microbiol.* 78(14), 4949–58. doi:10.1128/AEM.07759-11.

Carberry, C. A., Kenny, D. A., Kelly, A. K. and Waters, S. M. 2014a. Quantitative analysis of ruminal methanogenic microbial populations in beef cattle divergent in phenotypic residual feed intake (RFI) offered contrasting diets. *J. Anim. Sci. Biotechnol.* 5(1), 41. doi:10.1186/2049-1891-5-41.

Carberry, C. A., Waters, S. M., Kenny, D. A. and Creevey, C. J. 2014b. Rumen methanogenic genotypes differ in abundance according to host residual feed intake phenotype and diet type. *Appl. Environ. Microbiol.* 80(2), 586–94. doi:10.1128/AEM.03131-13.

Chaves, A. V., Thompson, L. C., Iwaasa, A. D., Scott, S. L., Olson, M. E., Benchaar, C., Veira, D. M. and McAllister, T. A. 2006. Effect of pasture type (alfalfa vs. grass) on methane and carbon dioxide production by yearling beef heifers. *Can. J. Anim. Sci.* 86(3), 409–18. doi:10.4141/A05-081.

Cheng, K. J., Dinsdale, D. and Stewart, C. S. 1979. Maceration of clover and grass leaves by *Lachnospira multiparus*. *Appl. Environ. Microbiol.* 38(4), 723–9.

Cheng, K. J., Fay, J. P., Howarth, R. E. and Costerton, J. W. 1980. Sequence of events in the digestion of fresh legume leaves by rumen bacteria. *Appl. Environ. Microbiol.* 40(3), 613–25.

Chesson, A. 1993. Mechanistic models of forage cell wall degradation. In: Jung, H. G., Buxton, D. R., Hatfield, R. D. and Ralph, J. (Eds), *Forage Cell Wall Structure and Digestibility*. ASA-CSSA-SSSA, Madison, WI, pp. 347–76.

Chesson, A. and Forsberg, C. 1997. Polysaccharide degradation by rumen microorganisms. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem*. Blackie Academic and Professional, London, pp. 329–81.

Chesson, A. and Monro, J. A. 1982. Legume pectic substances and their degradation in the ovine rumen. *J. Sci. Food Agric.* 33(9), 852–9. doi:10.1002/jsfa.2740330907.

Choudhury, P. K., Salem, A. Z. M., Jena, R., Kumar, S., Singh, R. and Puniya, A. K. 2015. Rumen microbiology: an overview. In: Puniya, A., Singh, R. and Kamra, D. (Eds), *Rumen Microbiology: From Evolution to Revolution*. Springer, New Delhi.

Corner-Thomas, R. A., Kemp, P. D., Morris, S. T. and Kenyon, P. R. 2014. Grazing alternative herbages in lactation increases the liveweight of both ewe lambs and their progeny at weaning. *Anim. Prod. Sci.* 54(10), 1741–6. doi:10.1071/AN14229.

Cosgrove, D. J. 2005. Growth of the plant cell wall. *Nat. Rev. Mol. Cell. Biol.* 6(11), 850–61. doi:10.1038/nrm1746.

Cosgrove, D. J. and Jarvis, M. C. 2012. Comparative structure and biomechanics of plant primary and secondary cell walls. *Front. Plant Sci.* 3(204). doi:10.3389/fpls.2012.00204.

Cosgrove, G., Taylor, P. and Jonker, A. 2015. Sheep performance on perennial ryegrass cultivars differing in concentration of water-soluble carbohydrate. *J. N. Z. Grassl. Assoc.* 77, 123–30.

Cunha, C. S., Veloso, C. M., Marcondes, M. I., Mantovani, H. C., Tomich, T. R., Pereira, L. G. R., Ferreira, M. F. L., Dill-McFarland, K. A. and Suen, G. 2017. Assessing the impact of rumen microbial communities on methane emissions and production traits in Holstein cows in a tropical climate. *Syst. Appl. Microbiol.* 40(8), 492–9. doi:10.1016/j.syapm.2017.07.008.

Curran, J., Delaby, L., Kennedy, E., Murphy, J. P., Boland, T. M. and O'Donovan, M. 2010. Sward characteristics, grass dry matter intake and milk production performance are affected by pre-grazing herbage mass and pasture allowance. *Livest. Sci.* 127(2–3), 144–54. doi:10.1016/j.livsci.2009.09.004.

Dagar, S. S., Kumar, S., Griffith, G. W., Edwards, J. E., Callaghan, T. M., Singh, R., Nagpal, A. K. and Puniya, A. K. 2015. A new anaerobic fungus (*Oontomyces anksri* gen. nov., sp. nov.) from the digestive tract of the Indian camel (*Camelus dromedarius*). *Fungal Biol.* 119(8), 731–7. doi:10.1016/j.funbio.2015.04.005.

Dai, X., Tian, Y., Li, J., Luo, Y., Liu, D., Zheng, H., Wang, J., Dong, Z., Hu, S. and Huang, L. 2015. Metatranscriptomic analyses of plant cell wall polysaccharide degradation

by microorganisms in the cow rumen. *Appl. Environ. Microbiol.* 81(4), 1375–86. doi:10.1128/AEM.03682-14.

Danielsson, R., Dicksved, J., Sun, L., Gonda, H., Muller, B., Schnurer, A. and Bertilsson, J. 2017. Methane production in dairy cows correlates with rumen methanogenic and bacterial community structure. *Front. Microbiol.* 8, 226. doi:10.3389/fmicb.2017.00226.

Dehority, B. A. 1991. Effects of microbial synergism on fibre digestion in the rumen. *Proc. Nutr. Soc.* 50(2), 149–59. doi:10.1079/pns19910026.

Delgado, B., Bach, A., Guasch, I., González, C., Elcoso, G., Pryce, J. E. and Gonzalez-Recio, O. 2019. Whole rumen metagenome sequencing allows classifying and predicting feed efficiency and intake levels in cattle. *Sci. Rep.* 9(1), 11. doi:10.1038/s41598-018-36673-w.

Deppenmeier, U. and Müller, V. 2008. Life close to the thermodynamic limit: how methanogenic archaea conserve energy. *Results Probl. Cell Differ.* 45, 123–52.

Devendran, S., Abdel-Hamid, A. M., Evans, A. F., Iakiviak, M., Kwon, I. H., Mackie, R. I. and Cann, I. 2016. Multiple cellobiohydrolases and cellobiose phosphorylases cooperate in the ruminal bacterium *Ruminococcus albus* 8 to degrade cellooligosaccharides. *Sci. Rep.* 6, 35342. doi:10.1038/srep35342.

Dewhurst, R. J., Fisher, W. J., Tweed, J. K. S. and Wilkins, R. J. 2003. Comparison of grass and legume silages for milk production. 1. Production responses with different levels of concentrate. *J. Dairy Sci.* 86(8), 2598–611. doi:10.3168/jds.S0022-0302(03)73855-7.

Dineen, M., Delaby, L., Gilliland, T. and McCarthy, B. 2018. Meta-analysis of the effect of white clover inclusion in perennial ryegrass swards on milk production. *J. Dairy Sci.* 101(2), 1804–16. doi:10.3168/jds.2017-12586.

Dini, Y., Cajarville, C., Gere, J. I., Fernandez, S., Fraga, M., Pravia, M. I., Navajas, E. A. and Ciganda, V. S. 2019. Association between residual feed intake and enteric methane emissions in Hereford steers. *Transl. Anim. Sci.* 3(1), 239–46. doi:10.1093/tas/txy111.

Duble, R. L., Lancaster, J. A. and Holt, E. C. 1971. Forage characteristics limiting animal performance on warm-season perennial grasses. *Agron. J.* 63(5), 795–8. doi:10.2134/agronj1971.00021962006300050042x.

Durunna, O. N., Colazo, M. G., Ambrose, D. J., McCartney, D., Baron, V. S. and Basarab, J. A. 2012. Evidence of residual feed intake reranking in crossbred replacement heifers. *J. Anim. Sci.* 90(3), 734–41. doi:10.2527/jas.2011-4264.

Dušková, D. and Marounek, M. 2001. Fermentation of pectin and glucose, and activity of pectin-degrading enzymes in the rumen bacterium *Lachnospira multiparus*. *Lett. Appl. Microbiol.* 33(2), 159–63. doi:10.1046/j.1472-765x.2001.00970.x.

Eckard, R. J. and Clark, H. 2018. Potential solutions to the major greenhouse-gas issues facing Australasian dairy farming. *Anim. Prod. Sci.* doi:10.1071/AN18574.

Edwards, G. R., Parsons, A., Rasmussen, S. and Bryant, R. H. 2007. High sugar ryegrasses for livestock systems in New Zealand. *Proc. N. Z. Grassland Assoc.* 66, 161–71.

Edwards, J. E., Forster, R. J., Callaghan, T. M., Dollhofer, V., Dagar, S. S., Cheng, Y., Chang, J., Kittelmann, S., Fliegerova, K., Puniya, A. K., Henske, J. K., Gilmore, S. P., O'Malley, M. A., Griffith, G. W. and Smidt, H. 2017. PCR and omics based techniques to study the diversity, ecology and biology of anaerobic fungi: insights, challenges and opportunities. *Front. Microbiol.* 8, 1657. doi:10.3389/fmicb.2017.01657.

Elliott, C. L., Edwards, J. E., Wilkinson, T. J., Allison, G. G., McCaffrey, K., Scott, M. B., Rees-Stevens, P., Kingston-Smith, A. H. and Huws, S. A. 2018. Using 'omic approaches to compare temporal bacterial colonization of *Lolium perenne*, *Lotus corniculatus*,

and *Trifolium pratense* in the rumen. *Front. Microbiol.* 9, 2184. doi:10.3389/fmicb.2018.02184.

Ellis, J. L., Dijkstra, J., Bannink, A., Parsons, A. J., Rasmussen, S., Edwards, G. R., Kebreab, E. and France, J. 2011. The effect of high-sugar grass on predicted nitrogen excretion and milk yield simulated using a dynamic model. *J. Dairy Sci.* 94(6), 3105–18. doi:10.3168/jds.2010-4059.

Ellison, M. J., Conant, G. C., Lamberson, W. R., Cockrum, R. R., Austin, K. J., Rule, D. C. and Cammack, K. M. 2017. Diet and feed efficiency status affect rumen microbial profiles of sheep. *Small Rumin. Res.* 156, 12–9. doi:10.1016/j.smallrumres.2017.08.009.

Elolimy, A. A., Abdelmegeid, M. K., McCann, J. C., Shike, D. W. and Loor, J. J. 2018. Residual feed intake in beef cattle and its association with carcass traits, ruminal solid-fraction bacteria, and epithelium gene expression. *J. Anim. Sci. Biotechnol.* 9, 67. doi:10.1186/s40104-018-0283-8.

Emerson, E. L. and Weimer, P. J. 2017. Fermentation of model hemicelluloses by *Prevotella* strains and *Butyrivibrio fibrisolvens* in pure culture and in ruminal enrichment cultures. *Appl. Microbiol. Biotechnol.* 101(10), 4269–78. doi:10.1007/s00253-017-8150-7.

Enriquez-Hidalgo, D., Gilliland, T., Deighton, M. H., O'Donovan, M. and Hennessy, D. 2014a. Milk production and enteric methane emissions by dairy cows grazing fertilized perennial ryegrass pasture with or without inclusion of white clover. *J. Dairy Sci.* 97(3), 1400–12. doi:10.3168/jds.2013-7034.

Enriquez-Hidalgo, D., Hennessy, D., Gilliland, T., Egan, M., Mee, J. F. and Lewis, E. 2014b. Effect of rotationally grazing perennial ryegrass white clover or perennial ryegrass only swards on dairy cow feeding behaviour, rumen characteristics and sward depletion patterns. *Livest. Sci.* 169, 48–62. doi:10.1016/j.livsci.2014.09.002.

Enriquez-Hidalgo, D., Gilliland, T. J., Egan, M. and Hennessy, D. 2018. Production and quality benefits of white clover inclusion into ryegrass swards at different nitrogen fertilizer rates. *J. Agric. Sci.* 156(3), 378–86. doi:10.1017/S0021859618000370.

FAO. 2017. *The Future of Food and Agriculture – Trends and Challenges*. FAO, Rome.

Farra, P. A. and Satter, L. D. 1971. Manipulation of the ruminal fermentation. III. Effect of nitrate on ruminal volatile fatty acid production and milk composition. *J. Dairy Sci.* 54(7), 1018–24. doi:10.3168/jds.S0022-0302(71)85965-9.

Fernando, S. C., Purvis, H. T., Najar, F. Z., Sukharnikov, L. O., Krehbiel, C. R., Nagaraja, T. G., Roe, B. A. and Desilva, U. 2010. Rumen microbial population dynamics during adaptation to a high-grain diet. *Appl. Environ. Microbiol.* 76(22), 7482–90. doi:10.1128/AEM.00388-10.

Ferry, J. G. (Ed.). 2012. *Methanogenesis: Ecology, Physiology, Biochemistry & Genetics*. Springer Science and Business Media.

Fitzsimons, C., Kenny, D. A., Deighton, M. H., Fahey, A. G. and McGee, M. 2013. Methane emissions, body composition, and rumen fermentation traits of beef heifers differing in residual feed intake. *J. Anim. Sci.* 91(12), 5789–800. doi:10.2527/jas.2013-6956.

Fitzsimons, C., Kenny, D. A., Fahey, A. G. and McGee, M. 2014a. Feeding behavior, ruminal fermentation, and performance of pregnant beef cows differing in phenotypic residual feed intake offered grass silage. *J. Anim. Sci.* 92(5), 2170–81. doi:10.2527/jas.2013-7438.

Fitzsimons, C., Kenny, D. A. and McGee, M. 2014b. Visceral organ weights, digestion and carcass characteristics of beef bulls differing in residual feed intake offered a high concentrate diet. *Animal* 8(6), 949–59. doi:10.1017/S1751731114000652.

Flay, H. E., Kuhn-Sherlock, B., Macdonald, K. A., Camara, M., Lopez-Villalobos, N., Donaghy, D. J. and Roche, J. R. 2019. Hot topic: selecting cattle for low residual feed intake did not affect daily methane production but increased methane yield. *J. Dairy Sci.* 102(3), 2708–13. doi:10.3168/jds.2018-15234.

Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P. and Forano, E. 2012. Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* 3(4), 289–306. doi:10.4161/gmic.19897.

Forsberg, C. W., Beveridge, T. J. and Hellstrom, A. 1981. Cellulase and xylanase release from *Bacteroides succinogenes* and its importance in the rumen environment. *Appl. Environ. Microbiol.* 42(5), 886–96.

Fraser, M. D., Speijers, M. H. M., Theobald, V. J., Fychan, R. and Jones, R. 2004. Production performance and meat quality of grazing lambs finished on red clover, lucerne or perennial ryegrass swards. *Grass Forage Sci.* 59(4), 345–56. doi:10.1111/j.1365-2494.2004.00436.x.

Gokarn, R. R., Eiteman, M. A., Martin, S. A. and Eriksson, K. E. 1997. Production of succinate from glucose, cellobiose, and various cellulosic materials by the ruminal anaerobic bacteria *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*. *Appl. Biochem. Biotechnol.* 68(1–2), 69–80. doi:10.1007/bf02785981.

Goopy, J. P., Donaldson, A., Hegarty, R., Vercoe, P. E., Haynes, F., Barnett, M. and Oddy, V. H. 2014. Low-methane yield sheep have smaller rumens and shorter rumen retention time. *Br. J. Nutr.* 111(4), 578–85. doi:10.1017/S0007114513002936.

Gordon, G. L. R. and Phillips, M. W. 1993. Removal of anaerobic fungi from the rumen of sheep by chemical treatment and the effect on feed consumption and *in vivo* fibre digestion. *Lett. Appl. Microbiol.* 17(5), 220–3. doi:10.1111/j.1472-765X.1993.tb01451.x.

Grace, C., Lynch, M. B., Sheridan, H., Lott, S., Fritch, R. and Boland, T. M. 2018. Grazing multispecies swards improves ewe and lamb performance. *Animal* 17, 1–9. doi:10.1017/S1751731118003245.

Griffith, G. S., Cresswell, A., Jones, S. and Allen, D. K. 2000. The nitrogen handling characteristics of white clover (*Trifolium repens* L.) cultivars and a perennial ryegrass (*Lolium perenne* L.) cultivar. *J. Exp. Bot.* 51(352), 1879–92. doi:10.1093/jexbot/51.352.1879.

Gruninger, R. J., Puniya, A. K., Callaghan, T. M., Edwards, J. E., Youssef, N., Dagar, S. S., Fliegerova, K., Griffith, G. W., Forster, R., Tsang, A., McAllister, T. and Elshahed, M. S. 2014. Anaerobic fungi (phylum *Neocallimastigomycota*): advances in understanding their taxonomy, life cycle, ecology, role and biotechnological potential. *FEMS Microbiol. Ecol.* 90(1), 1–17. doi:10.1111/1574-6941.12383.

Gruninger, R. J., Nguyen, T. T. M., Reid, I. D., Yanke, J. L., Wang, P., Abbott, D. W., Tsang, A. and McAllister, T. 2018. Application of transcriptomics to compare the carbohydrate active enzymes that are expressed by diverse genera of anaerobic fungi to degrade plant cell wall carbohydrates. *Front. Microbiol.* 9, 1581–. doi:10.3389/fmicb.2018.01581.

Guan, L. L., Nkrumah, J. D., Basarab, J. A. and Moore, S. S. 2008. Linkage of microbial ecology to phenotype: correlation of rumen microbial ecology to cattle's feed efficiency. *FEMS Microbiol. Lett.* 288(1), 85–91. doi:10.1111/j.1574-6968.2008.01343.x.

Guan, L., Li, F., Bulumulla, A. and Zhou, M. 2017. The role of rumen microbiome on feed efficiency of grazing cattle. *28th Annual Florida Ruminant Nutrition Symposium*, 770, 137.

Guy, C., Hennessy, D., Gilliland, T. J., Coughlan, F. and McCarthy, B. 2018. Growth, morphology and biological nitrogen fixation potential of perennial ryegrass-white clover swards throughout the grazing season. *J. Agric. Sci.* 156(2), 188–99. doi:10.1017/S0021859618000199.

Guyader, J., Eugene, M., Noziere, P., Morgavi, D. P., Doreau, M. and Martin, C. 2014. Influence of rumen protozoa on methane emission in ruminants: a meta-analysis approach. *Animal* 8(11), 1816–25. doi:10.1017/S1751731114001852.

Haitjema, C. H., Gilmore, S. P., Henske, J. K., Solomon, K. V., De Groot, R., Kuo, A., Mondo, S. J., Salamov, A. A., Labutti, K., Zhao, Z., Chiniquy, J., Barry, K., Brewer, H. M., Purvine, S. O., Wright, A. T., Hainaut, M., Boxma, B., Van Alen, T., Hackstein, J. H. P., Henrissat, B., Baker, S. E., Grigoriev, I. V. and O'Malley, M. A. 2017. A parts list for fungal cellulosomes revealed by comparative genomics. *Nat. Microbiol.* 2(8), 17087. doi:10.1038/nmicrobiol.2017.87.

Hammond, K. J., Hoskin, S. O., Burke, J. L., Waghorn, G. C., Koolaard, J. P. and Muetzel, S. 2011. Effects of feeding fresh white clover (*Trifolium repens*) or perennial ryegrass (*Lolium perenne*) on enteric methane emissions from sheep. *Anim. Feed Sci. Tech.* 166–167, 398–404. doi:10.1016/j.anifeedsci.2011.04.028.

Hammond, K. J., Pacheco, D., Burke, J. L., Koolaard, J. P., Muetzel, S. and Waghorn, G. C. 2014. The effects of fresh forages and feed intake level on digesta kinetics and enteric methane emissions from sheep. *Anim. Feed Sci. Tech.* 193, 32–43. doi:10.1016/j.anifeedsci.2014.04.005.

Han, S. O., Cho, H. Y., Yukawa, H., Inui, M. and Doi, R. H. 2004. Regulation of expression of cellulosomes and noncellulosomal (hemi)cellulolytic enzymes in *Clostridium cellulovorans* during growth on different carbon sources. *J. Bacteriol.* 186(13), 4218–27. doi:10.1128/JB.186.13.4218-4227.2004.

Hanafy, R. A., Elshahed, M. S., Liggenstoffer, A. S., Griffith, G. W. and Youssef, N. H. 2017. *Pecoramyces ruminantium*, gen. nov., sp. nov., an anaerobic gut fungus from the feces of cattle and sheep. *Mycologia* 109(2), 231–43. doi:10.1080/00275514.2017.1317190.

Hanafy, R. A., Elshahed, M. S. and Youssef, N. H. 2018. *Feramyces austinii*, gen. nov, sp. nov., an anaerobic gut fungus from rumen and fecal samples of wild Barbary sheep and fallow deer. *Mycologia* 110(3), 513–25. doi:10.1080/00275514.2018.1466610.

Harholt, J., Suttangkakul, A. and Vibe Scheller, H. 2010. Biosynthesis of pectin. *Plant Physiol.* 153(2), 384–95. doi:10.1104/pp.110.156588.

Harris, S. L. and Clark, D. A. 1996. Effect of high rates of nitrogen fertiliser on white clover growth, morphology, and nitrogen fixation activity in grazed dairy pasture in northern New Zealand. *N. Z. J. Agr. Res.* 39(1), 149–58. doi:10.1080/00288233.1996.9513173.

Hart, K. J., Martin, P. G., Foley, P. A., Kenny, D. A. and Boland, T. M. 2009. Effect of sward dry matter digestibility on methane production, ruminal fermentation, and microbial populations of zero-grazed beef cattle. *J. Anim. Sci.* 87(10), 3342–50. doi:10.2527/jas.2009-1786.

Hassanat, F., Gervais, R., Massé, D. I., Petit, H. V. and Benchaar, C. 2014. Methane production, nutrient digestion, ruminal fermentation, N balance, and milk production of cows fed timothy silage- or alfalfa silage-based diets. *J. Dairy Sci.* 97(10), 6463–74. doi:10.3168/jds.2014-8069.

Hegarty, R. S., Goopy, J. P., Herd, R. M. and McCorkell, B. 2007. Cattle selected for lower residual feed intake have reduced daily methane production. *J. Anim. Sci.* 85(6), 1479–86. doi:10.2527/jas.2006-236.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Global Rumen Census Collaborators and Janssen, P. H. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5, 14567. doi:10.1038/srep14567.

Hernandez-Sanabria, E., Guan, L. L., Goonewardene, L. A., Li, M., Mujibi, D. F., Stothard, P., Moore, S. S. and Leon-Quintero, M. C. 2010. Correlation of particular bacterial PCR-denaturing gradient gel electrophoresis patterns with bovine ruminal fermentation parameters and feed efficiency traits. *Appl. Environ. Microbiol.* 76(19), 6338–50. doi:10.1128/AEM.01052-10.

Hernandez-Sanabria, E., Goonewardene, L. A., Wang, Z., Durunna, O. N., Moore, S. S. and Guan, L. L. 2012. Impact of feed efficiency and diet on adaptive variations in the bacterial community in the rumen fluid of cattle. *Appl. Environ. Microbiol.* 78(4), 1203–14. doi:10.1128/AEM.05114-11.

Holmes, C. W., Hoogendoorn, C. J., Ryan, M. P. and Chu, A. C. P. 1992. Some effects of herbage composition, as influenced by previous grazing management, on milk production by cows grazing on ryegrass/white clover pastures. 1. Milk production in early spring: effects of different regrowth intervals during the preceding winter period. *Grass Sci.* 47(4), 309–15. doi:10.1111/j.1365-2494.1992.tb02276.x.

Hristov, A. and Jouany, J. 2005. Factors affecting the efficiency of nitrogen utilization in the rumen. In: Hristov, A. N. and Pfeffer, E. (Eds), *Nitrogen and Phosphorus Nutrition of Cattle: Reducing the Environmental Impact of Cattle Operations*. CAB International, Wallingford, UK, pp. 117–66.

Hungate, R. E. 1966. Variations in the rumen. In: *The Rumen and Its Microbes*. Academic Press, New York and London, pp. 376–418.

Huws, S. A., Lee, M. R., Muetzel, S. M., Scott, M. B., Wallace, R. J. and Scollan, N. D. 2010. Forage type and fish oil cause shifts in rumen bacterial diversity. *FEMS Microbiol. Ecol.* 73(2), 396–407. doi:10.1111/j.1574-6941.2010.00892.x.

Huws, S. A., Edwards, J. E., Creevey, C. J., Rees Stevens, P., Lin, W., Girdwood, S. E., Pachebat, J. A. and Kingston-Smith, A. H. 2016. Temporal dynamics of the metabolically active rumen bacteria colonizing fresh perennial ryegrass. *FEMS Microbiol. Ecol.* 92(1), fiv137. doi:10.1093/femsec/fiv137.

Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., Muñoz-Tamayo, R., Forano, E., Waters, S. M., Hess, M., Tapio, I., Smidt, H., Krizsan, S. J., Yáñez-Ruiz, D. R., Belanche, A., Guan, L., Gruninger, R. J., McAllister, T. A., Newbold, C. J., Roehe, R., Dewhurst, R. J., Snelling, T. J., Watson, M., Suen, G., Hart, E. H., Kingston-Smith, A. H., Scollan, N. D., Do Prado, R. M., Pilau, E. J., Mantovani, H. C., Attwood, G. T., Edwards, J. E., McEwan, N. R., Morrisson, S., Mayorga, O. L., Elliott, C. and Morgavi, D. P. 2018. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Front. Microbiol.* 9, 2161. doi:10.3389/fmicb.2018.02161.

Indugu, N., Vecchiarelli, B., Baker, L. D., Ferguson, J. D., Vanamala, J. K. P. and Pitta, D. W. 2017. Comparison of rumen bacterial communities in dairy herds of different production. *BMC Microbiol.* 17(1), 190. doi:10.1186/s12866-017-1098-z.

Janssen, P. H. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Anim. Feed Sci. Tech.* 160(1–2), 1–22. doi:10.1016/j.anifeedsci.2010.07.002.

Jewell, K. A., McCormick, C. A., Odt, C. L., Weimer, P. J. and Suen, G. 2015. Ruminal bacterial community composition in dairy cows is dynamic over the course of two

lactations and correlates with feed efficiency. *Appl. Environ. Microbiol.* 81(14), 4697–710. doi:10.1128/AEM.00720-15.

Joblin, K. N., Matsui, H., Naylor, G. E. and Ushida, K. 2002. Degradation of fresh ryegrass by methanogenic co-cultures of ruminal fungi grown in the presence or absence of *Fibrobacter succinogenes*. *Curr. Microbiol.* 45(1), 46–53. doi:10.1007/s00284-001-0078-5.

Johnson, K. A. and Johnson, D. E. 1995. Methane emissions from cattle. *J. Anim. Sci.* 73(8), 2483–92. doi:10.2527/1995.7382483x.

Jones, F. M., Phillips, F. A., Naylor, T. and Mercer, N. B. 2011. Methane emissions from grazing Angus beef cows selected for divergent residual feed intake. *Anim. Feed Sci. Tech.* 166–167, 302–7. doi:10.1016/j.anifeedsci.2011.04.020.

Jonker, A., Cheng, L., Edwards, G. R., Molano, G., Taylor, P. S., Sandoval, E. and Cosgrove, G. P. 2018a. Nitrogen partitioning differs in sheep offered a conventional diploid, a high sugar diploid or a tetraploid perennial ryegrass cultivar at two feed allowances. *Anim. Feed Sci. Tech.* 245, 32–40. doi:10.1016/j.anifeedsci.2018.09.004.

Jonker, A., Molano, G., Sandoval, E., Taylor, P. S., Antwi, C., Olinga, S. and Cosgrove, G. P. 2018b. Methane emissions differ between sheep offered a conventional diploid, a high-sugar diploid or a tetraploid perennial ryegrass cultivar at two allowances at three times of the year. *Anim. Prod. Sci.* 58(6), 1043–8. doi:10.1071/AN15597.

Joshi, A., Lanjekar, V. B., Dhakephalkar, P. K., Callaghan, T. M., Griffith, G. W. and Dagar, S. S. 2018. *Liebetanzomyces polymorphus* gen. et sp. nov., a new anaerobic fungus (*Neocallimastigomycota*) isolated from the rumen of a goat. *MycoKeys* 40(40), 89–110. doi:10.3897/mycokeys.40.28337.

Jun, H. S., Qi, M., Gong, J., Egbosimba, E. E. and Forsberg, C. W. 2007. Outer membrane proteins of *Fibrobacter succinogenes* with potential roles in adhesion to cellulose and in cellulose digestion. *J. Bacteriol.* 189(19), 6806–15. doi:10.1128/JB.00560-07.

Jung, H. G. and Allen, M. S. 1995. Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. *J. Anim. Sci.* 73(9), 2774–90. doi:10.2527/1995.7392774x.

Kamke, J., Kittelmann, S., Soni, P., Li, Y., Tavendale, M., Ganesh, S., Janssen, P. H., Shi, W., Froula, J., Rubin, E. M. and Attwood, G. T. 2016. Rumen metagenome and metatranscriptome analyses of low methane yield sheep reveals a Sharpea-enriched microbiome characterised by lactic acid formation and utilisation. *Microbiome* 4(1), 56. doi:10.1186/s40168-016-0201-2.

Kelly, A. K., McGee, M., Crews Jr., D. H., Sweeney, T., Boland, T. M. and Kenny, D. A. 2010. Repeatability of feed efficiency, carcass ultrasound, feeding behavior, and blood metabolic variables in finishing heifers divergently selected for residual feed intake. *J. Anim. Sci.* 88(10), 3214–25. doi:10.2527/jas.2009-2700.

Kenny, D. A., Fitzsimons, C., Waters, S. M. and McGee, M. 2018. Invited review: improving feed efficiency of beef cattle – the current state of the art and future challenges. *Animal* 12(9), 1815–26. doi:10.1017/S1751731118000976.

Kim, J. N., Méndez-García, C., Geier, R. R., Iakiviak, M., Chang, J., Cann, I. and Mackie, R. I. 2017. Metabolic networks for nitrogen utilization in *Prevotella ruminicola* 23. *Sci. Rep.* 7(1), 7851. doi:10.1038/s41598-017-08463-3.

Kittelmann, S., Seedorf, H., Walters, W. A., Clemente, J. C., Knight, R., Gordon, J. I. and Janssen, P. H. 2013. Simultaneous amplicon sequencing to explore co-occurrence

patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLoS ONE* 8(2), e47879. doi:10.1371/journal.pone.0047879.

Kittelmann, S., Pinares-Patiño, C. S., Seedorf, H., Kirk, M. R., Ganesh, S., McEwan, J. C. and Janssen, P. H. 2014. Two different bacterial community types are linked with the low-methane emission trait in sheep. *PLoS ONE* 9(7), e103171. doi:10.1371/journal.pone.0103171.

Klevenhusen, F., Petri, R. M., Kleefisch, M. T., Khiaosa-Ard, R., Metzler-Zebeli, B. U. and Zebeli, Q. 2017. Changes in fibre-adherent and fluid-associated microbial communities and fermentation profiles in the rumen of cattle fed diets differing in hay quality and concentrate amount. *FEMS Microbiol. Ecol.* 93(9), fix100. doi:10.1093/femsec/fix100.

Kong, R. S. G., Liang, G., Chen, Y., Stothard, P. and Guan, le L. 2016. Transcriptome profiling of the rumen epithelium of beef cattle differing in residual feed intake. *BMC Genomics* 17, 592. doi:10.1186/s12864-016-2935-4.

Krause, D. O., Denman, S. E., Mackie, R. I., Morrison, M., Rae, A. L., Attwood, G. T. and McSweeney, C. S. 2003. Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiol. Rev.* 27(5), 663–93. doi:10.1016/S0168-6445(03)00072-X.

Kulp, A. and Kuehn, M. J. 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu. Rev. Microbiol.* 64, 163–84. doi:10.1146/annurev.micro.091208.073413.

Kumar, S., Treloar, B. P., Teh, K. H., McKenzie, C. M., Henderson, G., Attwood, G. T., Waters, S. M., Patchett, M. L. and Janssen, P. H. 2018. Sharpea and Kandleria are lactic acid producing rumen bacteria that do not change their fermentation products when co-cultured with a methanogen. *Anaerobe* 54, 31–8. doi:10.1016/j.anaerobe.2018.07.008.

Lam, S., Munro, J. C., Zhou, M., Guan, L. L., Schenkel, F. S., Steele, M. A., Miller, S. P. and Montanholi, Y. R. 2018. Associations of rumen parameters with feed efficiency and sampling routine in beef cattle. *Animal* 12(7), 1442–50. doi:10.1017/S1751731117002750.

Latham, M. J. and Wolin, M. J. 1977. Fermentation of cellulose by *Ruminococcus flavefaciens* in the presence and absence of *Methanobacterium ruminantium*. *Appl. Environ. Microbiol.* 34(3), 297–301.

Lawrence, P., Kenny, D. A., Earley, B., Crews, D. H. and McGee, M. 2011. Grass silage intake, rumen and blood variables, ultrasonic and body measurements, feeding behavior and activity in pregnant beef heifers differing in phenotypic residual feed intake. *Journal of Animal Science* 89, 3248–61.

Lawrence, P., Kenny, D. A., Earley, B. and McGee, M. 2013. Intake of conserved and grazed grass and performance traits in beef suckler cows differing in phenotypic residual feed intake. *Livestock Science* 152, 154–66.

Leahy, S. C., Kelly, W. J., Altermann, E., Ronimus, R. S., Yeoman, C. J., Pacheco, D. M., Li, D., Kong, Z., McTavish, S., Sang, C., Lambie, S. C., Janssen, P. H., Dey, D. and Attwood, G. T. 2010. The genome sequence of the rumen methanogen *Methanobrevibacter ruminantium* reveals new possibilities for controlling ruminant methane emissions. *PLoS ONE* 5(1), e8926. doi:10.1371/journal.pone.0008926.

Ledgard, S. F., Sprosen, M. S., Penno, J. W. and Rajendram, G. S. 2001. Nitrogen fixation by white clover in pastures grazed by dairy cows: temporal variation and effects of nitrogen fertilization. *Plant Soil* 229(2), 177–87. doi:10.1023/A:1004833804002.

Lee, M. R. F. 2014. Forage polyphenol oxidase and ruminant livestock nutrition. *Front. Plant Sci.* 5, 694. doi:10.3389/fpls.2014.00694.

Lee, M. A. 2018. A global comparison of the nutritive values of forage plants grown in contrasting environments. *J. Plant Res.* 131(4), 641–54. doi:10.1007/s10265-018-1024-y.

Lee, S. S., Ha, J. K. and Cheng, K. 2000. Relative contributions of bacteria, protozoa, and fungi to in vitro degradation of orchard grass cell walls and their interactions. *Appl. Environ. Microbiol.* 66(9), 3807–13. doi:10.1128/aem.66.9.3807-3813.2000.

Lee, J., Woodward, S., Waghorn, G. and Clark, D. 2004a. Methane emissions by dairy cows fed increasing proportions of white clover (*Trifolium repens*) in pasture. *Proc. N. Z. Grassland Assess.* 66, 151–5.

Lee, M. R., Winters, A. L., Scollan, N. D., Dewhurst, R. J., Theodorou, M. K. and Minchin, F. R. 2004b. Plant-mediated lipolysis and proteolysis in red clover with different polyphenol oxidase activities. *J. Sci. Food Agric.* 84(13), 1639–45. doi:10.1002/jsfa.1854.

Liang, Y. S., Li, G. Z., Li, X. Y., Lü, J. Y., Li, F. D., Tang, D. F., Li, F., Deng, Y., Zhang, H., Wang, Z. L. and Weng, X. X. 2017. Growth performance, rumen fermentation, bacteria composition, and gene expressions involved in intracellular pH regulation of rumen epithelium in finishing Hu lambs differing in residual feed intake phenotype. *J. Anim. Sci.* 95(4), 1727–38. doi:10.2527/jas.2016.1134.

Liu, Y. and Whitman, W. B. 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Ann. N. Y. Acad. Sci.* 1125, 171–89. doi:10.1196/annals.1419.019.

Liu, L., Xu, X., Cao, Y., Cai, C., Cui, H. and Yao, J. 2017. Nitrate decreases methane production also by increasing methane oxidation through stimulating NC10 population in ruminal culture. *AMB Express* 7(1), 76. doi:10.1186/s13568-017-0377-2.

Marounek, M. and Dušková, D. 1999. Metabolism of pectin in rumen bacteria *Butyrivibrio fibrisolvens* and *Prevotella ruminicola*. *Lett. Appl. Microbiol.* 29(6), 429–33. doi:10.1046/j.1472-765X.1999.00671.x.

Matsui, H., Ogata, K., Tajima, K., Nakamura, M., Nagamine, T., Aminov, R. I. and Benno, Y. 2000. Phenotypic characterization of polysaccharidases produced by four Prevotella type strains. *Curr. Microbiol.* 41(1), 45–9. doi:10.1007/s002840010089.

Mayorga, O. L., Kingston-Smith, A. H., Kim, E. J., Allison, G. G., Wilkinson, T. J., Hegarty, M. J., Theodorou, M. K., Newbold, C. J. and Huws, S. A. 2016. Temporal metagenomic and metabolomic characterization of fresh perennial ryegrass degradation by rumen bacteria. *Front. Microbiol.* 7, 1854. doi:10.3389/fmicb.2016.01854.

McAllister, T. A. and Newbold, C. J. 2008. Redirecting rumen fermentation to reduce methanogenesis. *Aust. J. Exp. Agric.* 48(2), 7–13. doi:10.1071/EA07218.

McCann, J. C., Wickersham, T. A. and Loor, J. J. 2014a. High-throughput methods redefine the rumen microbiome and its relationship with nutrition and metabolism. *Bioinform. Biol. Insights* 8, 109–25. doi:10.4137/BBI.S15389.

McCann, J. C., Wiley, L. M., Forbes, T. D., Rouquette Jr., F. M. and Tedeschi, L. O. 2014b. Relationship between the rumen microbiome and residual feed intake-efficiency of Brahman bulls stocked on bermudagrass pastures. *PLoS ONE* 9(3), e91864. doi:10.1371/journal.pone.0091864.

McDonnell, R. P., Hart, K. J., Boland, T. M., Kelly, A. K., McGee, M. and Kenny, D. A. 2016. Effect of divergence in phenotypic residual feed intake on methane emissions, ruminal fermentation, and apparent whole-tract digestibility of beef heifers across three contrasting diets. *J. Anim. Sci.* 94(3), 1179–93. doi:10.2527/jas.2015-0080.

McGovern, E., Kenny, D. A., McCabe, M. S., Fitzsimons, C., McGee, M., Kelly, A. K. and Waters, S. M. 2018. 16S rRNA sequencing reveals relationship between potent cellulolytic genera and feed efficiency in the rumen of bulls. *Front. Microbiol.* 9, 1842–. doi:10.3389/fmicb.2018.01842.

Mercadante, M. E. Z., Caliman, A. P. D. M., Canesin, R. C., Bonilha, S. F. M., Berndt, A., Frighetto, R. T. S., Magnani, E. and Branco, R. H. 2015. Relationship between residual feed intake and enteric methane emission in Nellore cattle. *R. Bras. Zootec.* 44(7), 255–62. doi:10.1590/S1806-92902015000700004.

Mi, J., Zhou, J., Huang, X. and Long, R. 2017. Lower methane emissions from yak compared with cattle in *Rusitec fermenters*. *PLoS ONE* 12(1), e0170044. doi:10.1371/journal.pone.0170044.

Miller, T. L. and Wolin, M. J. 1973. Formation of hydrogen and formate by *Ruminococcus albus*. *J. Bacteriol.* 116(2), 836–46.

Morgavi, D. P., Sakurada, M., Mizokami, M., Tomita, Y. and Onodera, R. 1994. Effects of ruminal protozoa on cellulose degradation and the growth of an anaerobic ruminal fungus, *Piromyces* sp. strain OTS1, *in vitro*. *Appl. Environ. Microbiol.* 60(10), 3718–23.

Morgavi, D. P., Forano, E., Martin, C. and Newbold, C. J. 2010. Microbial ecosystem and methanogenesis in ruminants. *Animal* 4(7), 1024–36. doi:10.1017/S1751731110000546.

Morgavi, D. P., Martin, C., Jouany, J. P. and Ranilla, M. J. 2012. Rumen protozoa and methanogenesis: not a simple cause-effect relationship. *Br. J. Nutr.* 107(3), 388–97. doi:10.1017/S0007114511002935.

Morris, E. J. and Cole, O. J. 1987. Relationship between cellulolytic activity and adhesion to cellulose in *Ruminococcus albus*. *Microbiology* 133(4), 1023–32. doi:10.1099/00221287-133-4-1023.

Muñoz, C., Letelier, P. A., Ungerfeld, E. M., Morales, J. M., Hube, S. and Pérez-Prieto, L. A. 2016. Effects of pregrazing herbage mass in late spring on enteric methane emissions, dry matter intake, and milk production of dairy cows. *J. Dairy Sci.* 99(10), 7945–55. doi:10.3168/jds.2016-10919.

Myer, P. R., Smith, T. P. L., Wells, J. E., Kuehn, L. A. and Freetly, H. C. 2015. Rumen microbiome from steers differing in feed efficiency. *PLoS ONE* 10(6), e0129174. doi:10.1371/journal.pone.0129174.

Navarro-Villa, A., O'Brien, M., Lopez, S., Boland, T. M. and O'Kiely, P. 2011. *In vitro* rumen methane output of red clover and perennial ryegrass assayed using the gas production technique (GPT). *Anim. Feed Sci. Tech.* 168(3-4), 152–64. doi:10.1016/j.anifeedsci.2011.04.091.

Neumann, A. P., Weimer, P. J. and Suen, G. 2018. A global analysis of gene expression in *Fibrobacter succinogenes* S85 grown on cellulose and soluble sugars at different growth rates. *Biotechnol. Biofuels* 11, 295. doi:10.1186/s13068-018-1290-x.

Newbold, C. J., De La Fuente, G., Belanche, A., Ramos-Morales, E. and McEwan, N. R. 2015. The role of ciliate protozoa in the rumen. *Front. Microbiol.* 6, 1313. doi:10.3389/fmicb.2015.01313.

Ng, F., Kittelmann, S., Patchett, M. L., Attwood, G. T., Janssen, P. H., Rakonjac, J. and Gagic, D. 2016. An adhesin from hydrogen-utilizing rumen methanogen *Methanobrevibacter ruminantium* M1 binds a broad range of hydrogen-producing microorganisms. *Environ. Microbiol.* 18(9), 3010–21. doi:10.1111/1462-2920.13155.

Niderkorn, V., Martin, C., Rochette, Y., Julien, S. and Baumont, R. 2015. Associative effects between orchardgrass and red clover silages on voluntary intake and digestion in

sheep: evidence of a synergy on digestible dry matter intake. *J. Anim. Sci.* 93(10), 4967–76. doi:10.2527/jas.2015-9178.

Niderkorn, V., Martin, C., Le Morvan, A., Rochette, Y., Awad, M. and Baumont, R. 2017. Associative effects between fresh perennial ryegrass and white clover on dynamics of intake and digestion in sheep. *Grass For. Sci.* 72(4), 691–9. doi:10.1111/gfs.12270.

Nkrumah, J. D., Okine, E. K., Mathison, G. W., Schmid, K., Li, C., Basarab, J. A., Price, M. A., Wang, Z. and Moore, S. S. 2006. Relationships of feedlot feed efficiency, performance, and feeding behavior with metabolic rate, methane production, and energy partitioning in beef cattle. *J. Anim. Sci.* 84(1), 145–53. doi:10.2527/2006.841145x.

Olijhoek, D. W., Løvendahl, P., Lassen, J., Hellwing, A. L. F., Höglund, J. K., Weisbjerg, M. R., Noel, S. J., McLean, F., Højberg, O. and Lund, P. 2018. Methane production, rumen fermentation, and diet digestibility of Holstein and Jersey dairy cows being divergent in residual feed intake and fed at 2 forage-to-concentrate ratios. *J. Dairy Sci.* 101(11), 9926–40. doi:10.3168/jds.2017-14278.

Oliveira, L. F., Ruggieri, A. C., Branco, R. H., Cota, O. L., Canesin, R. C., Costa, H. J. U. and Mercadante, M. E. Z. 2018. Feed efficiency and enteric methane production of Nellore cattle in the feedlot and on pasture. *Anim. Prod. Sci.* 58(5), 886–93. doi:10.1071/AN16303.

Orpin, C. G. 1977. *Invasion of plant tissue in the rumen by the flagellate Neocallimastix frontalis*. *J. Gen. Microbiol.* 98(2), 423–30. doi:10.1099/00221287-98-2-423.

Pacheco, D., Waghorn, G. and Janssen, P. H. 2014. Decreasing methane emissions from ruminants grazing forages: a fit with productive and financial realities? *Anim. Prod. Sci.* 54(9), 1141–54. doi:10.1071/AN14437.

Park, T., Wijeratne, S., Meulia, T., Firkins, J. and Yu, Z. 2018. Draft macronuclear genome sequence of the ruminal ciliate *Entodinium caudatum*. *Microbiol. Resour. Announc.* 7(1), e00826-18. doi:10.1128/MRA.00826-18.

Patil, R. D., Ellison, M. J., Wolff, S. M., Shearer, C., Wright, A. M., Cockrum, R. R., Austin, K. J., Lamberson, W. R., Cammack, K. M. and Conant, G. C. 2018. Poor feed efficiency in sheep is associated with several structural abnormalities in the community metabolic network of their ruminal microbes. *J. Anim. Sci.* 96(6), 2113–24. doi:10.1093/jas/sky096.

Patra, A., Park, T., Kim, M. and Yu, Z. 2017. Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. *J. Anim. Sci. Biotech.* 8, 13. doi:10.1186/s40104-017-0145-9.

Petri, R. M., Mapiye, C., Dugan, M. E. R. and McAllister, T. A. 2014. Subcutaneous adipose fatty acid profiles and related rumen bacterial populations of steers fed red clover or grass hay diets containing flax or sunflower-seed. *PLoS ONE* 9(8), e104167. doi:10.1371/journal.pone.0104167.

Pinares-Patiño, C., Ulyatt, M. J., Lassey, K. R., Barry, T. N. and Holmes, C. W. 2003. Rumen function and digestion parameters associated with differences between sheep in methane emissions when fed chaffed lucerne hay. *J. Agr. Sci.* 140(2), 205–14.

Puchala, R., Animut, G., Patra, A. K., Detweiler, G. D., Wells, J. E., Varel, V. H., Sahlu, T. and Goetsch, A. L. 2012. Effects of different fresh-cut forages and their hays on feed intake, digestibility, heat production, and ruminal methane emission by Boer x Spanish goats. *J. Anim. Sci.* 90(8), 2754–62. doi:10.2527/jas.2011-4879.

Raffrenato, E., Fievisohn, R., Cotanch, K. W., Grant, R. J., Chase, L. E. and Van Amburgh, M. E. 2017. Effect of lignin linkages with other plant cell wall components on *in vitro* and

in vivo neutral detergent fiber digestibility and rate of digestion of grass forages. *J. Dairy Sci.* 100(10), 8119–31. doi:10.3168/jds.2016-12364.

Reeve, J. N., Nolling, J., Morgan, R. M. and Smith, D. R. 1997. Methanogenesis: genes, genomes, and who's on first? *J. Bacteriol.* 179(19), 5975–86. doi:10.1128/jb.179.19.5975-5986.1997.

Ribeiro, G. O., Gruninger, R. J., Badhan, A. and McAllister, T. A. 2016. Mining the rumen for fibrolytic feed enzymes. *Anim. Front.* 6(2), 20–6. doi:10.2527/af.2016-0019.

Ricard, G., McEwan, N. R., Dutilh, B. E., Jouany, J. P., Macheboeuf, D., Mitsumori, M., McIntosh, F. M., Michalowski, T., Nagamine, T., Nelson, N., Newbold, C. J., Nsabimana, E., Takenaka, A., Thomas, N. A., Ushida, K., Hackstein, J. H. P. and Huynen, M. A. 2006. Horizontal gene transfer from Bacteria to rumen Ciliates indicates adaptation to their anaerobic, carbohydrates-rich environment. *BMC Genomics* 7, 22. doi:10.1186/1471-2164-7-22.

Rius, A. G., Kittelmann, S., Macdonald, K. A., Waghorn, G. C., Janssen, P. H. and Sikkema, E. 2012. Nitrogen metabolism and rumen microbial enumeration in lactating cows with divergent residual feed intake fed high-digestibility pasture. *J. Dairy Sci.* 95(9), 5024–34. doi:10.3168/jds.2012-5392.

Rooke, J. A., Wallace, R. J., Duthie, C. A., McKain, N., De Souza, S. M., Hyslop, J. J., Ross, D. W., Waterhouse, T. and Roehe, R. 2014. Hydrogen and methane emissions from beef cattle and their rumen microbial community vary with diet, time after feeding and genotype. *Br. J. Nutr.* 112(3), 398–407. doi:10.1017/S0007114514000932.

Rubino, F., Carberry, C., Waters, S. M, Kenny, D., McCabe, M. S. and Creevey, C. J. 2017. Divergent functional isoforms drive niche specialisation for nutrient acquisition and use in rumen microbiome. *ISME J.* 11(4), 932–44. doi:10.1038/ismej.2016.172.

Sasson, G., Kruger Ben-Shabat, S., Seroussi, E., Doron-Faigenboim, A., Shterzer, N., Yaacoby, S., Berg Miller, M. E., White, B. A., Halperin, E. and Mizrahi, I. 2017. Heritable bovine rumen bacteria are phylogenetically related and correlated with the cow's capacity to harvest energy from its feed. *mBio* 8(4), 12. doi:10.1128/mBio.00703-17.

Scheifinger, C. C. and Wolin, M. J. 1973. Propionate formation from cellulose and soluble sugars by combined cultures of *Bacteroides succinogenes* and *Selenomonas ruminantium*. *Appl. Microbiol.* 26(5), 789–95.

Seshadri, R., Leahy, S. C., Attwood, G. T., Teh, K. H., Lambie, S. C., Cookson, A. L., Eloe-Fadrosh, E. A., Pavlopoulos, G. A., Hadjithomas, M., Varghese, N. J., Paez-Espino, D., Hungate1000 Project collaborators, Perry, R., Henderson, G., Creevey, C. J., Terrapon, N., Lapebie, P., Drula, E., Lombard, V., Rubin, E., Kyrpides, N. C., Henrissat, B., Woyke, T., Ivanova, N. N. and Kelly, W. J. 2018. Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nat. Biotech.* 36, 359–67.

Shabat, S. K., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., White, B. A., Shterzer, N. and Mizrahi, I. 2016. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J.* 10(12), 2958–72. doi:10.1038/ismej.2016.62.

Sharma, V. K., Kundu, S. S., Datt, C., Prusty, S., Kumar, M. and Sontakke, U. B. 2018. Buffalo heifers selected for lower residual feed intake have lower feed intake, better dietary nitrogen utilisation and reduced enteric methane production. *J. Anim. Physiol. Anim. Nutr.* 102(2), e607–14. doi:10.1111/jpn.12802.

Shi, W. B., Moon, C. D., Leahy, S. C., Kang, D. W., Froula, J., Kittelmann, S., Fan, C., Deutsch, S., Gagic, D., Seedorf, H., Kelly, W. J., Atua, R., Sang, C., Soni, P., Li, D., Pinares-Patino,

C. S., McEwan, J. C., Janssen, P. H., Chen, F., Visel, A., Wang, Z., Attwood, G. T. and Rubin, E. M. 2014. Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. *Genome Res.* 24(9), 1517–25. doi:10.1101/gr.168245.113.

Smith, P., Enriquez-Hidalgo, D., Hennessy, D., McCabe, M., Kenny, A., Kelly, A. K. and Waters, S. 2019. Archaea and members of the family, Lachnospiraceae, are altered in the rumen microbiome of cattle divergent in methane yield grazing perennial ryegrass swards with and without white clover. *Proc. Br. Soc. Anim. Sci.* 86.

Solomon, K. V., Haitjema, C. H., Henske, J. K., Gilmore, S. P., Borges-Rivera, D., Lipzen, A., Brewer, H. M., Purvine, S. O., Wright, A. T., Theodorou, M. K., Grigoriev, I. V., Regev, A., Thompson, D. A. and O'malley, M. A. 2016. Early-branching gut fungi possess a large, comprehensive array of biomass-degrading enzymes. *Science* 351(6278), 1192–5. doi:10.1126/science.aad1431.

Staerfl, S. M., Amelchanka, S. L., Kälber, T., Soliva, C. R., Kreuzer, M. and Zeitz, J. O. 2012. Effect of feeding dried high-sugar ryegrass ('AberMagic') on methane and urinary nitrogen emissions of primiparous cows. *Livest. Sci.* 150(1–3), 293–301. doi:10.1016/j.livsci.2012.09.019.

Steinshamn, H. and Thuen, E. 2008. White or red clover-grass silage in organic dairy milk production: grassland productivity and milk production responses with different levels of concentrate. *Livest. Sci.* 119(1–3), 202–15. doi:10.1016/j.livsci.2008.04.004.

Stevenson, D. M. and Weimer, P. J. 2007. Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl. Microbiol. Biotechnol.* 75(1), 165–74. doi:10.1007/s00253-006-0802-y.

Stewart, C., Flint, H. and Bryant, M. 1997. The rumen bacteria. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem*. Springer Netherlands, pp. 10–72.

Suen, G., Weimer, P. J., Stevenson, D. M., Aylward, F. O., Boyum, J., Deneke, J., Drinkwater, C., Ivanova, N. N., Mikhailova, N., Chertkov, O., Goodwin, L. A., Currie, C. R., Mead, D. and Brumm, P. J. 2011. The complete genome sequence of *Fibrobacter succinogenes* S85 reveals a cellulolytic and metabolic specialist. *PLoS ONE* 6(4), e18814. doi:10.1371/journal.pone.0018814.

Tapio, I., Snelling, T. J., Strozzi, F. and Wallace, R. J. 2017. The ruminal microbiome associated with methane emissions from ruminant livestock. *J. Anim. Sci. Biotech.* 8, 7. doi:10.1186/s40104-017-0141-0.

Thompson, S. C. 2015. The effect of diet type on residual feed intake and the use of infrared thermography as a method to predict efficiency in beef bulls. MSc Thesis. University of Manitoba.

Thomson, D. 1984. The nutritive value of white clover. *British Grassland Society Occasional Symposium* 16, 78–92.

Tokura, M., Tajima, K. and Ushida, K. 1999. Isolation of *Methanobrevibacter* sp. as a ciliate-associated ruminal methanogen. *J. Gen. Appl. Microbiol.* 45, 43–7.

Torres-Vázquez, J. A., Van Der Werf, J. H. J. and Clark, S. A. 2018. Genetic and phenotypic associations of feed efficiency with growth and carcass traits in Australian Angus cattle. *J. Anim. Sci.* 96(11), 4521–31. doi:10.1093/jas/sky325.

United Nations, Department of Economic and Social Affairs, Population Division 2017. World population prospects: the 2017 revision, key findings and advance tables. Working Paper No. ESA/P/WP/248.

Urrutia, N. L. and Harvatine, K. J. 2017. Acetate dose-dependently stimulates milk fat synthesis in lactating dairy cows. *J. Nutr.* 147(5), 763–9. doi:10.3945/jn.116.245001.

Van Glyswyk, N. O., Hippe, H. and Rainey, F. A. 1996. *Pseudobutyrivibrio ruminis* gen. nov., sp. nov., a butyrate-producing bacterium from the rumen that closely resembles *Butyrivibrio fibrisolvens* in phenotype. *Int. J. Syst. Bacteriol.* 46(2), 559–63. doi:10.1099/00207713-46-2-559.

Vanholme, R., Demedts, B., Morreel, K., Ralph, J. and Boerjan, W. 2010. Lignin biosynthesis and structure. *Plant Physiol.* 153(3), 895–905. doi:10.1104/pp.110.155119.

Velazco, J. I., Herd, R. M., Cottle, D. J. and Hegarty, R. S. 2017. Daily methane emissions and emission intensity of grazing beef cattle genetically divergent for residual feed intake. *Anim. Prod. Sci.* 57(4), 627–35. doi:10.1071/AN15111.

Wallace, R. J., Rooke, J. A., Duthie, C. A., Hyslop, J. J., Ross, D. W., McKain, N., De Souza, S. M., Snelling, T. J., Waterhouse, A. and Roehe, R. 2014. Archaeal abundance in post-mortem ruminal digesta may help predict methane emissions from beef cattle. *Sci. Rep.* 4, 5892. doi:10.1038/srep05892.

Wallace, R. J., Rooke, J. A., McKain, N., Duthie, C. A., Hyslop, J. J., Ross, D. W., Waterhouse, A., Watson, M. and Roehe, R. 2015. The rumen microbial metagenome associated with high methane production in cattle. *BMC Genomics* 16, 839. doi:10.1186/s12864-015-2032-0.

Weimer, P. J. 1996. Why don't ruminal bacteria digest cellulose faster? *J. Dairy Sci.* 79(8), 1496–502. doi:10.3168/jds.S0022-0302(96)76509-8.

Williams, A. G. and Coleman, G. S. 1997. The rumen protozoa. In: Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem*, Springer, pp. 73–139.

Wims, C. M., Deighton, M. H., Lewis, E., O'Loughlin, B., Delaby, L., Boland, T. M. and O'Donovan, M. 2010. Effect of pregrazing herbage mass on methane production, dry matter intake, and milk production of grazing dairy cows during the mid-season period. *J. Dairy Sci.* 93(10), 4976–85. doi:10.3168/jds.2010-3245.

Wina, E., Muetzel, S. and Becker, K. 2005. The impact of saponins or saponin-containing plant materials on ruminant production – a review. *J. Agric. Food Chem.* 53(21), 8093–105. doi:10.1021/jf048053d.

Wolff, S. M., Ellison, M. J., Hao, Y., Cockrum, R. R., Austin, K. J., Baraboo, M., Burch, K., Lee, H. J., Maurer, T., Patil, R., Ravelo, A., Taxis, T. M., Truong, H., Lamberson, W. R., Cammack, K. M. and Conant, G. C. 2017. Diet shifts provoke complex and variable changes in the metabolic networks of the ruminal microbiome. *Microbiome* 5(1), 60. doi:10.1186/s40168-017-0274-6.

Wolin, M. J., Miller, T. L. and Stewart, C. S. 1997. Microbe-microbe interactions. Hobson, P. N. and Stewart, C. S. (Eds), *The Rumen Microbial Ecosystem*. Springer, pp. 467–91.

Yanez-Ruiz, D. R., Macias, B., Pinloche, E. and Newbold, C. J. 2010. The persistence of bacterial and methanogenic archaeal communities residing in the rumen of young lambs. *FEMS Microbiol. Ecol.* 72(2), 272–8. doi:10.1111/j.1574-6941.2010.00852.x.

Yang, B., He, B., Wang, S. S., Liu, J. X. and Wang, J. K. 2015. Early supplementation of starter pellets with alfalfa improves the performance of pre- and postweaning Hu lambs. *J. Anim. Sci.* 93(10), 4984–94. doi:10.2527/jas.2015-9266.

Yang, B., Le, J., Wu, P., Liu, J., Guan, L. L. and Wang, J. 2018. Alfalfa intervention alters rumen microbial community development in Hu lambs during early life. *Front. Microbiol.* 9, 574–. doi:10.3389/fmicb.2018.00574.

Zamil, M. S. and Geitmann, A. 2017. The middle lamella—more than a glue. *Phys. Biol.* 14(1), 015004. doi:10.1088/1478-3975/aa5ba5.

Zhang, R., Zhu, W., Zhu, W., Liu, J. and Mao, S. 2014. Effect of dietary forage sources on rumen microbiota, rumen fermentation and biogenic amines in dairy cows. *J. Sci. Food Agric.* 94(9), 1886–95. doi:10.1002/jsfa.6508.

Zhang, X., Wang, W., Mo, F., La, Y., Li, C. and Li, F. 2017. Association of residual feed intake with growth and slaughtering performance, blood metabolism, and body composition in growing lambs. *Sci. Rep.* 7(1), 12681. doi:10.1038/s41598-017-13042-7.

Zhao, L., Meng, Q., Li, Y., Wu, H., Huo, Y., Zhang, X. and Zhou, Z. 2018. Nitrate decreases ruminal methane production with slight changes to ruminal methanogen composition of nitrate-adapted steers. *BMC Microbiol.* 18(1), 21. doi:10.1186/s12866-018-1164-1.

Zheng, Y., Kahnt, J., Kwon, I. H., Mackie, R. I. and Thauer, R. K. 2014. Hydrogen formation and its regulation in *Ruminococcus albus*: involvement of an electron-bifurcating [FeFe]-hydrogenase, of a non-electron-bifurcating [FeFe]-hydrogenase, and of a putative hydrogen-sensing [FeFe]-hydrogenase. *J. Bacteriol.* 196(22), 3840–52. doi:10.1128/JB.02070-14.

Zhou, M., Hernandez-Sanabria, E. and Guan, L. L. 2009. Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. *Appl. Environ. Microbiol.* 75(20), 6524–33. doi:10.1128/AEM.02815-08.

Zhou, Z., Yu, Z. and Meng, Q. 2012. Effects of nitrate on methane production, fermentation, and microbial populations in in vitro ruminal cultures. *Bioresour. Technol.* 103(1), 173–9. doi:10.1016/j.biortech.2011.10.013.

Chapter 19

Optimising ruminal function: the role of silage and concentrate in dairy cow nutrition to improve feed efficiency and reduce methane and nitrogen emissions

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1 Introduction

Ruminant farm animals contribute significantly to global greenhouse gas (GHG) emissions including enteric methane (CH_4) and nitrous oxide (N_2O). There has therefore been a lot of research in recent years in finding nutritional means to reduce GHG emissions especially from the dairy production sector. There is a considerable diversity of potential dietary mitigation options under development. These include feeds, feed management and rumen modifiers (i.e. feeding specific substances that directly or indirectly inhibit methanogenesis) or using biological control directed at reducing methanogens (Knapp et al., 2014). Some of the feed additives such as CH_4 inhibitor 3-nitrooxypropanol (3-NOP) are very promising not only in intensive dairy cow diets (Dijkstra et al., 2018; Van Gastelen et al., 2019), but also in all-forage cattle diets (Martinez-Fernandez et al., 2018). These applications are not yet available in practice. However, nutritional mitigation strategies based on the altering forage and concentrate components of the diet are easily available and often more adaptable and applicable into practice than the available rumen modifiers. It has

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been estimated that the potential of feeding and nutrition (including improved forage quality, feeding grain and dietary lipids) to lower GHG emissions usually range from low to medium (10–30%) (Hristov et al., 2013; Knapp et al., 2014). However, Hristov et al. (2013) concluded that improving the forage quality and the overall efficiency of dietary nutrient use is an effective way of decreasing CH_4 emission intensity in terms of CH_4 per unit of animal product.

The effectiveness of dietary strategies to mitigate GHG emissions (be they forage or concentrate-based strategies) depends largely on their effects on rumen volatile fatty acid (VFA) fermentation. Any change in dietary composition in favour of propionate production reduces CH_4 owing to reducing equivalents, while diets favouring acetate and butyrate formation generate H_2 for methanogenesis and thus increase CH_4 production as reviewed by Knapp et al. (2014). Other fermentation processes, such as rumen protein degradation and assimilation into microbial protein as well as biohydrogenation of fatty acids taking place in the rumen, contribute to the balance of H_2. The former results in either a net consumption or net production of H_2, while the latter results in net consumption of H_2.

As forages are the main component of dairy cow diets, local production of high-quality forage is of utmost importance to dairy farmers in maintaining and ensuring profitable milk production. The choice of a GHG-mitigating nutritional strategy appropriate at farm level depends a lot on the geographical location of the farm, which largely determines the climatic conditions and the forage plant species available for silage making. For instance, in hot regions silage crops are influenced by high temperatures negatively affecting crop yield and nutritive value while, in cold regions, a short and cool growing season may limit the use of crops such as maize sensitive to cool temperature as reviewed by Bernardes et al. (2018). This is often the case in northern areas such as in Northern Europe, where grass species rather than the use of maize predominate in silage production. According to Bernardes et al. (2018) climatic conditions affect all stages of silage production with temperature being the most limiting factor. Silage production is dependent both on controllable factors, for example, plant species and stage of maturity, harvesting and ensiling methods and the use of additives, and uncontrollable climate-related factors. This makes the process vulnerable to considerable annual variation in silage fermentation quality and nutritive value.

Concentrate-based strategies include increasing the proportion of concentrate in the diet and/or altering the type of carbohydrate (e.g. fibre vs. starch) or type of lipid (e.g. fatty acid profile) supplementation in the concentrate. Even though concentrate components such as grains and oilseeds in the diet are not as sensitive to annual changes in nutritive value relative to forages, their contribution to dairy cow feed ration depends a lot on dry matter (DM) intake of forages and digestibility of dietary components, for example, fibre in the

diet. According to Hristov et al. (2013) inclusion of concentrate feeds in the diet likely decreases CH_4 emission intensity particularly when inclusion is above 40% of dietary DM and rumen function is not impaired. On the other hand, decreasing forage to concentrate ratio in dairy cow diets contradicts moves to reduce the use of human-edible components such as grains in dairy cow feed rations. A sustainable dairy cow feeding strategy should make the most of the unique ability of ruminant animals to convert human-inedible biomass to high-quality animal-derived protein foods, that is, milk and meat.

In this chapter the authors focus on reviewing recent literature on dairy cow physiological or milk production studies conducted using silage and/or concentrate-supplemented diets, including measurements on CH_4 production. The chapter looks at their dietary effects on production parameters, feed efficiency (FE), nitrogen-use efficiency (NUE) and CH_4 emission intensity in dairy cows in temperate zone conditions with emphasis on Europe and North America. The authors assess the potential of plant species, stage of maturity of silage crops, as well as the factors such as dietary forage to concentrate ratio and concentrate composition, to reduce the environmental footprint of dairy cow production without compromising animal performance.

2 Role of silage: grass, forage legumes and maize

2.1 Silage plant species

Climatic conditions within the temperate area vary considerably and thus there is a large variation in forage species available for silage making. Major plant species include grasses, forage legumes and maize. In northern areas, short and cool growing seasons with the rigours of a cold winter limit the choice of perennial grasses and legumes for silage making (Bernardes et al., 2018). The most widely used perennial forage species include timothy (*Phelum pratense*), perennial ryegrass (*Lolium perenne* L.) and various fescue species such as meadow fescue (*Festuca pratensis*) and tall fescue (*Festuca arundinacae* L.), and legume species such as red clover (*Trifolium pratense*) and lucerne (*Medicago sativa* L.) (Wilkinson and Rinne, 2018; Bernardes et al., 2018). Though the production of lucerne is limited to southern parts of the area, advancing climate change may enable its production further north in future, for example, in Scandinavia (Järvenranta et al., 2016).

The maize plant (*Zea mays* L.) is a tropical crop in origin but, as a valuable forage crop, it is used wherever maize can grow, from temperate regions to the tropics. It is characterised by high quantities of low-cost starch per hectare coupled with a relatively high concentration of metabolizable energy, which makes this crop very attractive to farmers (Wilkinson and Rinne, 2018). Plant breeders have developed earlier-maturing maize varieties, which can be used for whole-crop silage making in northern conditions. Thus, the area of

maize cultivation for silage making is gradually expanding to the north, with advancing climate change possibly contributing to this shift as well. The use of biodegradable film in cultivation also enables earlier sowing and harvesting of maize in the marginal areas of Northern Europe such as in Scandinavian countries.

Cool-season forage grasses are characterised by high digestibility attributed to low temperature and long day length, which delay lignification of cell walls (Huhtanen et al., 2013; Bernardes et al., 2018). As the decline in the rate of digestibility due to advancing maturity is slower with legumes than grasses, mixing of these plant species for silage making is beneficial, extending the optimal harvesting period for these herbages (Kuoppala, 2010). However, concentration of non-structural carbohydrates (NSC) including water soluble carbohydrates (WSC) and starch of these forages is often low and varies a lot depending on the climatic conditions. It affects ensiling potential and feed value of grass and legume forages and can be manipulated with harvesting time and ensiling methods such as the use of additives (Vanhatalo and Jaakkola, 2016). There are also so-called high-sugar ryegrass cultivars rich in WSC available for silage making (Moorby et al., 2006).

2.2 Grass silages

Of the grass silage management factors altering forage, maturity stage at harvest has the greatest CH_4-mitigating potential while N fertilisation rate, use of additives or high-sugar ryegrass cultivars have no effect (Table 1). Feeding ryegrass-timothy (Warner et al., 2016, 2017), ryegrass (Brask et al., 2013a) or timothy (Pang et al., 2018) silage harvested from an early relative to late stage of maturity in high-forage dairy cow diets has significantly increased DM intake, energy-corrected milk yield (ECM) and FE, and decreased CH_4 emission intensity in terms of g CH_4 per kg ECM up to 20% (Warner et al., 2016, 2017). Moreover, reductions in CH_4 with improved silage quality were independent of the DM intake, and were smaller at late rather than early stages of lactation (Warner et al., 2017). They were not attributable to the acetate to propionate ratio, which was unchanged owing to the grass maturity (Brask et al., 2013a; Warner et al., 2016). However, these positive results were achieved at the expense of simultaneously decreasing NUE, which was remarkably high (−35%) when very early-cut leafy stage grass silage was used (Warner et al., 2017) reflecting the much higher crude protein (CP) content of the leafy stage than the late heading herbage. High N-fertilisation rate of grass forage (150 vs. 65 kg N/ha) was also in relation to reduced NUE owing to a 5%-unit higher CP content of the silage with high N-fertilisation level (Warner et al., 2016).

The positive DM intake and milk production responses of dairy cows to grass silage harvested at early stage of maturity are well established (e.g. Rinne,

Table 1 The effects of substituting basal forage with forage differing in plant maturity, ensiling method or plant species on dairy cow performance

				Change in % relative to control[1]					CH_4-emission intensity, g CH_4/kg ECM			
Basal forage[2]	Plant species/ Variety	Substituting forage[2]	F:C[3]	DMI	ECM	FE	NUE	Rumen C2 / C3	Control	Test	Change in %	Reference
Grass silage												
Late cut	Ryegrass[4]	Early cut, Primary growth	65:35	9	11	2	−7	−2	16.9	14.7	−13	Brask et al. (2013a)
Late cut	Timothy	Early cut, Primary growth	60:40	10	13	3	−17	NR	14.0	13.1	−7	Pang et al. (2018)
Late cut, Low N	Ryegrass:Timothy	Early cut, Sec. growth	80:20	6	32	25	−13	18	15.9	12.5	−21	Warner et al. (2016)
Late cut, High N		Early cut, Sec. growth		20	34	12	−8	−1	16.3	13.1	−20	
Low N		High N		−4	−2	2	−19	−4	15.2	14.9	−2	
Late cut[5]	Ryegrass:Timothy	Very early-cut (leafy) Primary growth[7]	80:20	9	12	6	−35	NR	14.0	11.2	−20	Warner et al. (2017)
Late cut[6]				4	11	8	−35	NR	12.9	10.2	−21	
No additive	Ryegrass:Timothy	Inoculated grass	75:25	3	2	−1	4	NR	16.0	16.0	0	Ellis et al. (2016)
Low sugar	Ryegrass	High sugar	100:0	−8	−15	−8	46	NR	16.5	17.2	4	Staerfl et al. (2012)
Legume silage												
Grass	Timothy	Lucerne	60:40	9	−2	−10	−15	−1	12.8	13.4	5	Hassanat et al. (2014)
Red clover:grass 30:70	NR:Timothy	Red clover:grass 70:30	60:40	−1	0	1	−10	NR	15.2	15.5	5	Gidlund et al. (2017)
Grass:sainfoin: maize 86:0:14	NR	Grass:sainfoin:maize 42:42:16	70:30	5	7	2	−5	NR	15.0	13.9	−7	Huyen et al. (2016)

(*Continued*)

Table 1 (*Continued*)

Basal forage[2]	Plant species/ Variety	Substituting forage[2]	F:C[3]	Change in % relative to control[1]					CH_4-emission intensity, g CH_4/kg ECM			Reference
				DMI	ECM	FE	NUE	Rumen C2 / C3	Control	Test	Change in %	
Maize silage												
Early-cut maize	LG30218	Late-cut maize	80:20	0	0	0	−3	13	12.8	11.9	−7	Hatew et al. (2016)
Conventional maize	NR	Brown midrib maize	65:35	6	8	1	5	−4	14.0	12.6	−10	Hassanat et al. (2017)
Barley	Cut at soft dough	Maize, two-thirds at milkline	60:40	20	15	−4	6	−13	15.0	14.9	−1	Benchaar et al. (2014)
Grass	NR	Maize	80:20	8	7	−1	18	6	16.6	15.0	−10	Van Gastelen et al. (2015)
Early-cut grass	Ryegrass[4]	Maize	65:35	−1	0	2	24	−22	14.7	13.8	−6	Brask et al. (2013a)
Late-cut grass				7	11	4	15	−23	16.9	13.8	−19	
Grass:maize 75:25	NR	Grass:maize 25:75	50:50	11	5	−5	−7	NR	15.0	14.3	−5	Reynolds et al. (2010)
Grass:maize 75:25[8]	Third cut ryegrass	Grass:maize 25:75	50:50	28	9	−15	−10	NR	16.3	14.2	−13	Hammond et al. (2016)
Same diets as above[9]				19	24	4	15	NR	16.9	16.2	−4	
Grass:maize 75:25	NR	Grass:maize 25:75	50:50	7	−2	−8	1	NR	12.9	12.0	7	Livingstone et al. (2015)
Grass	Ryegrass: cocksfoot:fescue	Maize at vitreous stage	45:55	0	−2	−2	6	−5	14.9	13.4	−10	Doreau et al. (2014)

Red clover	NR	Maize	60:40	0	2	2	7	−16	14.6	14.1	−4	Benchaar et al. (2015)
Lucerne	NR	Maize	60:40	5	−1	−6	14	−26	13.9	14.4	3	Hassanat et al. (2013)
Lucerne:maize 80:20	NR	Lucerne:maize 20:80	55:45	−1	0	1	15	−18	17.8	18.1	2	Arndt et al. (2015)

[1]Dry matter intake (DMI), Energy-corrected milk (ECM) calculated according to Sjaunja et al. (1991), Feed efficiency (FE) calculated as ECM / DM intake, Nitrogen-use efficiency (NUE) calculated as N output in milk/N intake, Ruminal acetate to propionate ratio (rumen C2/C3) calculated from their molar proportions in the rumen fluid, Not reported and not calculable (NR), [2] For forage mixtures the proportions of components (%) on a DM basis are given, [3]Forage to concentrate ratio on a DM basis (F:C), [4]Contained clover <10%, [5]Cows 96 days in milk; [6]Cows 218 days in milk, [7]Treatment consisting leafy stage grass contained 5% of chopped wheat straw, [8]Methane measured with GreenFeed, [9]Methane measured with respiratory chamber.

2000; Harrison et al., 2003) as is also the concomitant high N content of silages leading to losses of N from animals (e.g. Rinne et al., 1997). Extremely early harvest of grass forage such as that used by Warner et al. (2017) is thus not recommended. However, using silages made from early-cut primary growth grass improves FE, decreases CH_4 emission intensity and ensures good milk production level of high-producing dairy cows, though with compromises in NUE. Decreases in CH_4 owing to harvest at early maturity stage seem not to be related to rumen fermentation pattern, as effects of advancing maturity of ensiled grass on molar proportion of propionate have been small and inconsistent (Harrison et al., 2003; Warner et al., 2016). High nitrate content in early maturity grass silage (Warner et al., 2016) or, for example, increased microbial growth (Knapp et al., 2014) due to high energy content in early maturity grass silage may possibly have served as an alternative H_2 sink to propionate, thus explaining decreases in CH_4. High-sugar content ryegrass grass silage improved NUE in all-silage diet (Table 1; Staerfl et al., 2012) in agreement with beef cattle production studies (Merry et al., 2006), but reduced milk production parameters with minor effect on CH_4 emission intensity. According to Bertilsson et al. (2017), elevated WSC levels in high-sugar ryegrass silage were achieved at the expense of CP and fibre but their effects on dairy cow performance were minor. However, positive effects were attributed to a more favourable distribution of N in terms of more N to milk and faeces and less N to urine. Overall, the issue of improving low NUE of grass forage silages clearly warrants further research. Despite earlier positive production responses of silage inoculants to milk production (e.g. Muck et al., 2018), no such effect nor reduced CH_4 emission intensity owing to inoculated grass silage was seen in a study by Ellis et al. (2016) (Table 1).

2.3 Forage legume silages

Data on the effects of forage legume silages on CH_4 intensity in dairy production is scarce (Table 1). Replacing timothy with lucerne in dairy cow diets was not effective in reducing CH_4 emissions but led to increased DM intake, impaired FE and especially reduced NUE (Hassanat et al., 2014; Table 1). Inclusion of sainfoin (*Onobrychis viciifolia*) containing condensed tannins in grass-silage based silage increased DM intake and ECM yield but led only to a minor decrease in CH_4-emission intensity (Huyen et al., 2016; Table 1). Replacing a mixture of timothy and red clover silage of 70:30 with a mixture of red clover and timothy silage of 70:30 in dairy cows did not affect DM intake, ECM production, FE or CH_4-emission intensity but led to a reduction in NUE (Gidlund et al., 2017; Table 1). This is in agreement with findings of Van Dorland et al. (2007) showing that replacing part of ryegrass silage with red or white clover silage did not affect CH_4 emissions but slightly enhanced N losses to the environment.

Even so, according to a review of Phelan et al. (2015), forage legumes have generally resulted in lower CH_4 emissions per kg of milk or meat produced when compared to grasses. However, they emphasise that this occurs only when the forage legume has had higher feed intake and ruminal passage rates than grasses, or with legumes that contain condensed tannins.

Results from comparing forage legume and grass silage-based diets in general demonstrate the superior DM intake and milk production potential of forage legumes over grasses (Vanhatalo and Jaakkola, 2016). Moreover, forage legumes are often considered as an economically profitable alternative to grass and/or maize-based forages owing to their ability to provide biologically fixed nitrogen, which serves as an effective means to reduce dependence on synthetic N fertilisers and thus fossil energy (Vanhatalo and Jaakkola, 2016). The higher intake characteristics of legume than grass silages, despite lower digestibility, have been attributed to their lower fibre content, more rapid fermentation and particle breakdown in the rumen, and higher passage from the rumen (Kuoppala et al., 2009; Kuoppala, 2010; Dewhurst, 2013).

Contradictory results on CH_4 mitigation potential of forage legumes may be related to the varying silage fermentation quality and proportions of forage legume in the silages studied. It should be noted, that forage legumes are often grown in mixtures with grasses or other plants rather than as pure stands owing to higher annual herbage yield in mixtures (Phelan et al., 2015). Reduced NUE with forage legume-based diets rather than with grass silage diets stems from their inherently high CP concentrations, especially with lucerne. However, there are also differences between forage legume species such as red clover and lucerne in their N fractions, which may differently affect NUE as reviewed by Dewhurst (2013). Clearly, more research is needed on the potential of forage legumes to reduce CH_4 emission intensity and their effects on NUE.

2.4 Maize silages

According to Hatew et al. (2016) increasing maturity of whole-plant maize from very early (20% DM) to late stage (40% DM) at harvest effectively reduced CH_4 emission intensity on high-forage diet but did not affect DM intake, ECM yield, FE or NUE (Table 1). This was caused by the markedly increased starch content, decreased ruminal fractional rate of degradation of starch and decreased neutral detergent fibre (NDF) content with the advancing maturity of maize crop. However, despite higher starch intake, this was not attributable to decreased rumen pH and increased propionate as expected. Instead, the acetate to propionate ratio tended to increase with increasing maturity of maize. These results suggest that harvesting whole-plant maize at a higher maturity instead of the currently recommended practise (30–35% DM, for example, Khan et al., 2015) has the potential to reduce enteric CH_4 emissions.

Maize cultivars developed for potentially higher cell wall digestibility and intake properties (Jung et al., 2011) relative to conventional maize cultivar have been compared recently (Hassanat et al., 2017; Table 1). It was shown that replacing conventional maize silage (DM 40%, starch 269 g/kg DM) with more digestible brown midrib maize silage (BMCS; DM 34%, starch 283 g/kg DM) on high-forage diet not only increased DM intake and ECM yield, but also improved NUE and reduced CH_4 emission intensity. Again, reduction in CH_4 was not attributed to rumen fermentation pattern, which was unchanged between the treatments. Moreover, it was demonstrated that, by using BMCS, N excretion in manure reduced and potential N volatilisation was avoided by shifting N excretion from urine to faeces. Nevertheless, increased volatile solid content (i.e. degradable organic matter excretion) in the manure was observed giving rise to CH_4 emissions from manure storage. However, maize silage type (Falkone vs. LG30224) was of little importance in terms of dairy cow performance and CH_4 emissions despite the lower rumen NDF digestibility and higher starch content of Falkone in a diet where proportion of maize in the forage was 65% (De Boever et al., 2017).

Replacing barley silage (DM 32%, starch 139 g/kg DM) completely with maize silage (DM 31%, starch 322 g/kg DM) significantly increased DM intake, milk yield and NUE, but did not affect FE or CH_4 emission intensity (Benchaar et al., 2014; Table 1). However, increasing proportion of maize silage at the expense of barley silage in the diet reduced the CH_4 energy losses in association with lower ruminal acetate to propionate ratio. The improved NUE with increasing maize silage in the diet was due to decreased urinary losses suggesting low potential for N_2O and ammonia emissions from manure.

2.5 Maize silage versus grass and forage legume silages

Because of the inherently high energy value and low CP concentration of the maize crop, it has been of interest to study whether mixing or replacing grasses or legumes high in CP with maize silage in the diet leads to beneficial environmental effects in terms of increasing NUE and reducing CH_4 emission intensity. Replacing grass silage with maize silage (DM 32%, starch 322 g/kg DM) on restricted high-forage diet improved NUE and reduced CH_4 emission intensity but did not affect FE or milk production, except for increased milk protein yield (Van Gastelen et al., 2015; Table 1). Reduction in CH_4 was not associated with acetate to propionate ratio, which was unchanged between the treatments. Replacing early-cut or late-cut grass silages in the high-forage diet with maize silage (DM 31%, starch 150 g/kg DM) had no major effect on DM intake or milk yield, but NUE was improved particularly in relation to early-cut grass silage, and CH_4-emission intensity reduced particularly in relation to late-cut grass silage (Brask et al., 2013a; Table 1). In these dietary conditions

reductions in CH_4 were attributed to clearly decreased acetate to propionate ratio in the rumen and to lower ruminal fibre digestibility with maize silage diets.

Increasing the proportion of maize in the mixture of grass and maize silage from 25% to 75% on 50:50 forage to concentrate ratio diet increased DM intake and milk yield and decreased CH_4 emission intensity, while effects on FE and NUE were variable and more inconsistent (Reynolds et al., 2010; Hammond et al., 2016; Table 1). However, using a similar experimental setup Livingstone et al. (2015, Table 1) did not find differences between these silage treatments in any of the parameters mentioned above, owing to the exceptionally low-NDF content of the grass herbage. Using a low-forage diet, Doreau et al. (2014) (Table 1) did neither find any differences in these parameters, except for lower CH_4-emission intensity with maize silage diets. Even so, ruminal acetate to propionate ratio was unchanged between the treatments.

Red clover and lucerne silages have been replaced with maize silage on high-forage diets but with minor effects on DM intake, production parameters and CH_4-emission intensity, while NUE was clearly improved with maize silages (Hassanat et al., 2013; Benchaar et al., 2015; Arndt et al., 2015; Table 1). In these studies, maize silage maturity varied in the range of 36–38% DM and of 290–339 g/kg DM starch, while starch contents of legumes were less than 18 g/kg DM (Hassanat et al., 2013; Benchaar et al., 2015). Despite unaffected CH_4-emission intensity, rumen pH and ruminal acetate to propionate ratios were clearly decreased when red clover or lucerne was replaced with maize silage in the diet (Hassanat et al., 2013; Benchaar et al., 2015; Arndt et al., 2015). The positive changes for lower urinary and faecal N with maize silage at the expense of forage legume silages in these studies would likely result in lower ammonia and N_2O emissions. However, the reduced fibre digestion in the rumen with starch containing maize silage diets may lead to increased CH_4 emissions from manure storage (Hassanat et al., 2013).

It seems that replacing grass or legume forage silages with maize silage consistently leads to environmental benefits such as reducing CH_4-emission intensity on high-forage grass silage-based diets, and improved NUE, especially on forage legume silages high in CP without compromises in milk production. However, despite increased amounts of starch with increasing proportion of maize silage in the high-forage grass diet, reductions in CH_4-emission intensity are not necessarily related to changes in rumen fermentation pattern with reduced pH and decreased acetate to propionate ratio as expected. For instance, replacing grass silage with maize silage maintained high rumen pH and increased rumen butyrate in high-forage diet (Van Gastelen et al., 2015). Thus, the reduction in rumen pH enhancing the production of propionate (Dijkstra et al., 2011) does not necessarily occur in a high-forage diet. As maize starch is fairly resistant to rumen fermentation, it is susceptible to enzymatic digestion in the small intestine (Owens et al., 1986). Consequently, a possible

shift from acetate to butyrate in favour of less H_2 production in the rumen (e.g. Moss et al., 2000), and reduced H_2 production in the rumen owing to a partial shift of starch digestion to the lower tract may contribute to reduced CH_4 production with maize-containing diets. Nevertheless, a critical dietary concentration of starch is probably required to alter ruminal methanogenesis and decrease CH_4 production (Hassanat et al., 2013; Van Gastelen et al., 2015).

It is interesting that the clearly decreased acetate to propionate ratio with maize silage diets at the expense of forage legume diets (Hassanat et al., 2013; Benchaar et al., 2015; Arndt et al., 2015) did not lead to reduced CH_4-emission intensity. This may be related to the type of carbohydrate in forage NDF, which affects CH_4 emissions (Arndt et al., 2015). They found that the fermentation of maize NDF yielded substantially more CH_4 than fermentation of lucerne NDF. Thus, the greater CH_4 emission expected from greater amount of NDF in lucerne was counterbalanced by a decreasing emission per gram of lucerne NDF fermented. A similar difference in NDF fermentation between maize and lucerne was found in the study by Hassanat et al. (2013). Moreover, in the study of Brask et al. (2013a), less NDF was digested in the rumen with maize silage compared to grass silage diets despite a comparable NDF concentration in the diets. Further research on the effects of forage carbohydrate type on CH_4 emissions and rumen digestion kinetics is needed. The studies should ideally simultaneously examine rumen fermentation, digestion kinetics and microbiota together with GHG and milk production to give in-depth view on mechanism affecting the rumen function and GHG formation. The positive changes on N losses owing to increasing proportion of maize in the diet suggest that the dietary shift in forage source from forage legumes to maize silage results in lower NH_3 and N_2O emissions from manure (Hassanat et al., 2013; Arndt et al., 2015).

Nevertheless, it should be emphasised that conclusions about the potential of a feeding strategy to reduce GHG emissions also depend on whether a strategy is feasible on the farm rather just on the level of the individual animal (Van Middelaar et al., 2013). At an animal level increasing maize silage at the expense of grass and grass silage in dairy cow diets is a promising strategy with an immediate effect on GHG emissions. However, application of this strategy to average intensive Dutch farms would lead to problems with EU regulations when reducing grassland area. On the other hand, applying this strategy to intensive farming to reduce the area of grassland would lead to higher emissions owing to land use change, that is, ploughing grassland into maize land.

3 Role of concentrates: lipids, carbohydrates and protein

3.1 Lipids

Level of lipid supplementation. Lipid supplements are widely used to increase diet-energy density to meet the energy requirements of high-producing

dairy cows during early and mid-lactation and to improve energy utilization for milk production. However, dietary lipid content should not exceed 6–7% in DM (review by Beauchemin et al., 2008), otherwise a depression of DM intake, ruminal fibre digestibility and further milk production may occur, thus counteracting the advantages of increased diet-energy density (Bayat et al., 2017; Halmemies-Beauchet-Filleau et al., 2017). Lipids are also one of the most effective and practicable means to improve milk or meat fatty acid composition and mitigate GHG emissions of ruminants in industrialised countries, but their effectiveness depends on multiple factors. These include level of lipid supplementation, fatty acid profile of lipid supplement (e.g. chain length and level of unsaturation), a form in which the lipid is given (e.g. oil vs. full-fat seeds) and the type of basal diet (reviews by Eugène et al., 2008; Beauchemin et al., 2008; Shingfield et al., 2013; Table 2). To support long-term health of human consumers, the aim is to decrease the proportion of saturated fatty acids and to increase those of *cis*-monounsaturated fatty acids and omega-3 fatty acids, and improve the balance of omega-6 and omega-3 fatty acids in ruminant products (Shingfield et al., 2013).

Over a broad range of dietary conditions, Martin et al. (2010) reported a mean decrease in CH_4 emissions of 3.8% with each 1% addition of supplemental lipid in diet DM. One or more mechanisms may contribute to CH_4 mitigation potential of different lipids in the rumen. These include lower amount of organic matter (OM) fermented in the rumen (lipid decreases DM intake and/or replaces rumen fermentable ingredients in the ruminant diet), direct toxicity or inhibition of rumen cellulolytic bacteria, methanogens and/or attached protozoa, a shift in ruminal fermentation from acetate to propionate (that consume rather than produce H_2) and biohydrogenation of fatty acids in case of unsaturated lipid supplements (review by Martin et al., 2010).

Medium-chain saturated fatty acids. Medium-chain saturated fatty acid sources such as myristic acid (14:0) or coconut oil rich in lauric acid (12:0) and 14:0 have reduced ruminal CH_4-emission intensity up to 30% when fed at 3.3–5% in diet DM for lactating cows. The primary mechanism is probably through decrease in DM intake (Table 2) and fermentable OM content in the rumen (Bayat et al., 2018). Medium-chain saturated fatty acids may also exhibit toxic effects on methanogens (Beauchemin et al., 2008) or protozoa (Hristov et al., 2011b) and impair fibre digestion (Hollmann et al., 2012), but these effects are inconsistent between studies. Recently Bayat et al. (2018) reported only minor alterations in the diversity of specific microbial taxa and no effect on total quantities of bacteria, methanogenic archaea or ciliate protozoa or fibre digestion, despite a significant decrease in ruminal CH_4 production. Inclusion of medium-chain saturates in the diet in general improves FE and NUE in milk production, but concomitant sharp decrease in ECM yield (Table 2) together with relatively high price of these lipid supplements make applications in commercial dairy

Table 2 The effects of supplemental lipid on dairy cow performance

Lipid source	Form	Lipid dosage in diet DM	Basal forage component[2]	F:C[3]	Change in % relative to unsupplemented control diet[1]					CH_4-emission intensity, g CH_4 per kg ECM			Reference
					DMI	ECM	FE	NUE	Rumen C2 / C3	Control diet	Lipid diet	Change in %	
Medium-chain saturated fatty acids													
Myristic acid	oil	5%	Maize silage:grass haylage:hay 55:35:10	60:40	−7	−10	−4	−1	NR	28.4	20.4	−28	Odongo et al. (2007)
Myristic acid	methyl ester	5%	Grass silage	60:40	−31	−20	17	2	−6	22.7	18.8	−17	Bayat et al. (2018)
Coconut	oil	1.3%	Maize:lucerne:grass	50:50	−7	4	12	9	NR	13.9	12.9	−7	Hollmann et al. (2012)
		2.7%	silages 75:15:10		−22	−18	4	11	NR	13.9	14.4	3	
		3.3%			−29	−24	8	22	NR	13.9	9.9	−29	
Monounsaturated fatty acids													
Rapeseed	cake	2–3%	Grass:maize silages	50:50	3	11	8	8	−1	14.6	13.6	−7	Brask et al. (2013b)
	crushed		55:45		−2	−8	−6	4	−1	14.6	12.1	−17	
	oil				−14	4	21	6	7	14.6	12.0	−18	
Rapeseed	crushed	3%	Maize silage		−5	−10	−6	7	−2	13.7	14.0	3	Brask et al. (2013a)
			Early grass silage[4]	65:35	−2	−1	1	0	−1	15.6	13.9	−11	
			Late grass silage[4]		1	3	3	3	1	17.8	16.1	−10	
Rapeseed	crushed	2%	Maize:grass silages 75:25	50:50	2	4	1	4	NR	14.7	14.2	−3	Kliem et al. (2019)
Rapeseed	oil	5%	Grass silage	60:40	−12	3	17	11	3	22.7	17.5	−23	Bayat et al. (2018)

Polyunsaturated fatty acids													
Safflower	oil	5%	Grass silage	60:40	−6	2	9	6	0	22.7	17.5	−23	Bayat et al. (2018)
Soybean	oil	3.5%	Maize silage:lucerne haylage:hay 45:40:15	65:35	−3	0	4	NR	NR	18.3	18.2	0	Sauer et al. (1998)
Sunflower	oil	5%	Grass silage	65:35	−2	−2	0	−6	−5	18.9	14.5	−23	Bayat et al. (2017)
				35:65	−11	−16	−6	13	3	14.2	14.5	2	
Camelinaseed	oil	5%	Grass silage	50:50	−12	−16	−5	6	−5	15.4	13.0	−16	Bayat et al. (2015)
Linseed	whole seed	5%	Maize silage:grass hay	65:35	−2	−1	0	−6	NR	17.7	15.9	−10	Martin et al. (2008)
	extruded		90:10		−16	−16	−1	4	NR	17.7	13.1	−26	
	oil				−26	−26	0	12	NR	17.7	8.5	−52	
Linseed	extruded	1.8%	Maize silage:grass hay	60:40	−2	−15	−14	−2	−9	15.4	17.1	11	Ferlay et al. (2013)
		3.6%	90:10		−5	−16	−12	−3	−14	15.4	15.9	4	Martin et al. (2016)
		5.4%			−11	−5	7	12	−27	15.4	9.4	−39	
		1.8%	Grass hay	50:50	−9	−4	6	5	−6	19.8	17.4	−12	
		3.6%			−4	5	10	8	−6	19.8	15.3	−23	
		5.4%			−4	−3	1	12	−12	19.8	12.2	−39	
Linseed	extruded	2%	Maize:grass silages	50:50	0	2	2	4	NR	14.7	13.4	−9	Kliem et al. (2019)
Linseed and palm mix	Ca-salts of oil mix	2%	75:25	50:50	−2	3	6	1	NR	14.7	12.8	−13	
Linseed	oil	4%	Maize silage	60:40	−9	−14	−6	1	−22	14.1	12.1	−14	Benchaar et al (2015)
			Red clover silage	60:40	−2	2	4	5	−4	14.6	13.0	−11	
Linseed	oil	5%	Grass silage	60:40	−8	3	12	5	2	22.7	17.5	−23	Bayat et al. (2018)

(*Continued*)

Table 2 (*Continued*)

Lipid source	Form	Lipid dosage in diet DM	Basal forage component[2]	F:C[3]	Change in % relative to unsupplemented control diet[1]					CH_4-emission intensity, g CH_4 per kg ECM			Reference
					DMI	ECM	FE	NUE	Rumen C2 / C3	Control diet	Lipid diet	Change in %	
Fish	oil	0.8%	Maize silage:lucerne hay: grass hay	52:48[5]	4	8	4	−7	−1	13.5	12.6	−7	Pirondini et al. (2015)
		0.8%	55:25:20	52:48[6]	−2	−3	−1	1	0	12.4	13.2	6	
Algae	meal	0.3%	Lucerne hay	74:26	0	−6	−6	3	2	21.8	23.9	10	Moate et al. (2013)
rich in 22:6*n*-3		0.6%			−6	−14	−8	5	−1	21.8	25.7	18	
		1%			−11	−15	−5	0	4	21.8	24.1	11	
Algae	meal	0.3%[7]	Maize:grass silages	70:30	0	−12	−12	0	NR	9.5	11.1	16	Klop et al. (2016)
rich in 22:6n-3			70:30										

[1]Dry matter intake (DMI), Energy-corrected milk (ECM) calculated according to Sjaunja et al. (1991), Feed efficiency (FE) calculated as ECM/DM intake, Nitrogen-use efficiency (NUE) calculated as N output in milk/N intake, Ruminal acetate to propionate ratio (rumen C2/C3) calculated from their molar proportions in the rumen fluid, Not reported and not calculable (NR), [2]For forage mixtures the proportions of components (%) on a DM basis are given, [3]Forage to concentrate ratio on a DM basis (F:C), [4]Contains <10% in DM clover, [5]Concentrate low in starch, [6]Concentrate high in starch, [7]Supplemental DHA % in diet DM.

farms unlikely. Furthermore, dietary 12:0 and 14:0 supplementations increase their incorporation in bovine milk fat (Odongo et al., 2007; Hollmann et al., 2012; Bayat et al., 2018) that is a nutritionally undesirable change for human consumers.

Unsaturated fatty acids. Plant-unsaturated fatty acids such as oleic acid (18:1*n*-9) and essential fatty acids linoleic acid (18:2*n*-6) and α-linolenic acid (18:3*n*-3) are considered beneficial to human health. Thus, their use in dairy cow diets that typically results in increases of these omega-fatty acids in ruminant milk and meat together with a decrease in saturated fatty acids could be a viable way for CH_4 mitigation.

Trials with lactating dairy cows clearly indicate that the effects of lipids on animal performance and rumen methanogenesis are proportional to the level of supply and unsaturation (Table 2). As for saturated fatty acids, the primary mechanism is probably through a decrease in DM intake (Table 2). A dose-response trial by Martin et al. (2016), with three levels of 18:3*n*-3-rich linseed supplementation up to 5.4% of lipid in the diet DM, decreased ruminal CH_4 emission intensity up to 39%, with inconsistent effects at lower levels due to differences in the composition of the basal diets. Besides the lower level of DM intake, the decrease in enteric CH_4 production was attributed to a decreased ruminal acetate to propionate ratio and number of protozoa, whereas the number of ruminal methanogens and fibre digestibility remained unaltered. A modest lipid inclusion (1–2% in diet DM) does not suppress feed intake, but it alters milk fatty acid composition (Halmemies-Beauchet-Filleau et al., 2011). At a 5% lipid inclusion level in diet DM, rapeseed rich in 18:1*n*-9 and safflower seed, sunflower seed and camelina seed rich in 18:2*n*-6 have resulted in lower decreases in CH_4-emission intensity (up to 23%) compared to linseed rich in 18:3*n*-3 which has achieved at best 39–52% decreases (Table 2). In most studies presented in Table 2 the experimental periods are of 4–6 weeks, but recently Alstrup et al. (2015) demonstrated that plant lipids suppress ruminal methanognesis throughout the entire lactation. However, more studies comprising entire lactation are needed to confirm the persistency of lipids to mitigate ruminal CH_4 emissions.

It is generally thought that intact oilseeds give partial protection of oil against microbial metabolism, and/or limit the effects of oil on ruminal microbes and nutrient digestibility. However, Martin et al. (2008) reported no difference in OM and fibre digestibility between whole intact linseed, extruded linseed and linseed oil diets. Though pure oil is often more effective to mitigate CH_4 production in the rumen, processed oilseeds (e.g. crushed by milling, extruded, or as pressed cakes) are preferred because of less adverse effects on DM intake and generally lower price (Beauchemin et al., 2008; Table 2). In addition, administering high levels of dietary unsaturated fatty acids as a part of a total mixed ratio (TMR) results in a lower decrease in DM intake (Bayat et al., 2015) than incorporation into concentrates fed separately

to forage (Halmemies-Beauchet-Filleau et al., 2017). This is probably due to a more sudden release of unsaturated fatty acids in the rumen in a separate feed relative to TMR, as high amounts of free unsaturated fatty acids are toxic to cellulolytic bacteria (Maia et al., 2007).

The basal diet greatly affects the production and enteric CH_4 emission response to lipids. On predominantly forage diets (diets rich in fibre) based on 50% or more DM of grass silage, red clover silage or grass hay, up to 5% unsaturated lipid supplements in diet DM maintain ECM yield and improve FE. Furthermore, there is a dose-dependent and consistent decrease in ruminal CH_4 emissions (Table 2). In contrast, on starchy diets (diets based on maize silage or rich in concentrate starch), the ECM yield and FE are often compromised at low lipid inclusion levels (2–4% in diet DM) and the effects on ruminal CH_4 emissions are variable between studies (Table 2). At high lipid inclusion levels (4–5.5% in diet DM), ruminal CH_4-emission intensity is more consistently reduced, but the reduction is often accompanied by a decrease in ECM production (Table 2). Therefore, unsaturated lipid supplements to mitigate CH_4 emissions best suit diets rich in fibre, but their use in starchy diets is of limited interest due to the negative effects on ECM yield. This is probably linked to a much more detrimental effect of unsaturated lipid on rumen fibre digestion on diets high in starch relative to high-fibre diets, leading to a decrease in acetate formation in the rumen and further milk fat content (Benchaar et al., 2015; Bayat et al., 2017). In addition, PUFA supplementation in a starchy diet readily directs rumen biohydrogenation from *trans*-11 to *trans*-10 route, some *trans*-10 isomers being potential antilipogenic agents in the bovine mammary gland, thus causing milk fat depression (MFD; review by Shingfield et al., 2010; Ventto et al., 2017).

Feed N-use efficiency in milk protein synthesis is in general unaltered or slightly improved by unsaturated lipid supplements in the diet (Table 2). Milk protein content or yield may in some cases decrease due to lipid in the diet, as is also the case with feed and CP intake (Benchaar et al., 2015; Halmemies-Beauchet-Filleau et al., 2017; Bayat et al., 2018), which explains the unaffected or improved conversion efficiencies. The possible decrease in milk protein synthesis due to lipid supplementation has been attributed to negative effects on energy intake, limitation in glucose supply and microbial protein synthesis (review by Lock and Shingfield, 2004; Halmemies-Beauchet-Filleau et al., 2017).

Eicosapentaenoic acid (20:5*n*-3) and docosahexaenoic acid (22:6*n*-3) present in fish oil or specific algae products have had a strong CH_4-supressing effect when tested in vitro (Martin et al., 2010). However, when fed at low levels (up to 1% in diet DM) to lactating dairy cows, ECM yield and FE decreased without any improvement in ruminal CH_4 emissions. This suggests that lipid supplements rich in 20- and 22-carbon polyunsaturated fatty acids are not a useful tool to mitigate the GHG emissions of dairy cows in practice.

3.2 Carbohydrates

Level of concentrate supplementation. Decreasing forage to concentrate ratio (i.e. an increase in concentrate starch inclusion) in the dairy cow diet generally improves feed intake (Table 3) due to the reduced contribution of forage fibre with high bulk density (review of Allen, 2000). In addition, the greater proportion of concentrates in the diet may be associated with more extensive OM digestion in the rumen, reflecting the greater inherent digestibility of NSC in concentrates (starch and sugars) relative to structural forage carbohydrates (hemicellulose and cellulose; Bayat et al., 2017). Consistent with this concentrate level in the diet is an increase in the total tract digestibility of starch, but fibre digestibility is also often simultaneously impaired (Niu et al., 2016; Bayat et al., 2017), which may counteract the overall impact of starchy concentrate on OM digestibility. The changes in feed intake are reflected in ECM production leading to unaffected FE except for lipid-rich diets (Table 3). There is a trend for an increase in milk protein and a decrease in milk fat in response to concentrate supplementation (Aguerre et al., 2011; Niu et al., 2016; Bayat et al., 2017). The increase in milk protein may be driven by greater inclusion of cereals with high-metabolizable energy density in the diet. As the level of starchy concentrate in the diet increases, there is a concomitant decrease in forage intake and thus forage fibre. The ruminal fermentation of NDF results in lipogenic VFA in the rumen that may account for the decrease in milk fat on diets low in forage.

The low and high concentrate diets reported in Table 3 were all isonitrogenous (CP 15–18% in DM) except for Olijhoek et al. (2018), where the high-concentrate diet had a 20% higher CP content relative to a low-forage diet, leading to a reduced NUE. The general improvement in NUE in concentrate-rich diets (Table 3) is probably a result of a better balance of degradable protein and energy available for microbes in the rumen as indicated by lower rumen ammonia concentrations in high-concentrate diets (Bayat et al., 2017) and higher relative contribution of good-quality protein feeds in dietary protein.

The reduction of ruminal CH_4 production at increasing levels of concentrate in the diet is well established (Table 3; Martin et al., 2010). Cattle CH_4 emissions are relatively constant for diets containing up to 30–40% of concentrate in DM, decreasing rapidly to low levels for diets containing 80–90% concentrate common in some beef production systems (Martin et al., 2010). Replacing forage structural (fibrous) carbohydrates with NSC (starch and sugar) in concentrates drives changes in the rumen physico-chemical environment and microbial populations, favouring starch-fermenting microbes and propionate formation (Martin et al., 2010). However, even marked reductions in ruminal CH_4 formation are not always accompanied by a shift towards propionate in the rumen VFA (Aguerre et al., 2011). The forage in their study was a mixture of maize and grass silage, and rumen fermentation in cattle fed grass silage-based

Table 3 The effects of concentrate level and diet starch content on dairy cow performance

Main ingredients of control concentrate (CC)	CC in diet DM, %	Main ingredient difference of substituting concentrate (SC)	SC in diet DM, %	Starch in SC vs CC diets DM, %	Basal forage component	Change in % relative to control concentrate diet[1]					CH_4 emission intensity, g CH_4 per kg ECM			Reference
						DMI	ECM	FE	NUE	Rumen C2 / C3	CC diet	SC diet	Change in %	
Maize grain, soya bean meal and hulls	32	More maize	39	23 vs. 20	Maize-lucerne silage 50:50[2]	0	−1	0	3	−1	18.9	17.2	−9	Aguerre et al. (2011)
			46	26 vs. 20		3	3	0	3	−4	18.9	16.8	−11	
			53	29 vs. 20		4	3	−1	7	4	18.9	15.2	−20	
Maize grain, soya bean meal, dry distillers grains	47	More maize and soya bean	63	32 vs. 21	Lucerne hay	3	1	−2	10	NR	14.8	13.7	−8	Niu et al. (2016)
Barley, wheat, rapeseed meal	35	More cereals	65	32 vs. 14	Grass silage	23	15	−6	−4	−28	18.9	14.2	−25	Bayat et al. (2017)
Same as above plus plant oil		Same as above plus plant oil		29 vs. 11		11	−2	−12	15	−22	14.5	14.5	0	
Barley, rapeseed cake, soya bean meal	32	More barley	61	22 vs. 11	Grass-clover silage	15	10	−4	−15	−31	15.3	12.7	−17	Olijhoek et al. (2018)

[1]Dry matter intake (DMI), Energy-corrected milk (ECM) calculated according to Sjaunja et al. (1991), Feed efficiency (FE) calculated as ECM/DM intake, Nitrogen-use efficiency (NUE) calculated as N output in milk/N intake, Ruminal acetate to propionate ratio (rumen C2/C3) calculated from their molar proportions in the rumen fluid, [2]Silage mixture containing 50% of maize and 50% of lucerne silage on a DM basis, Not reported and not calculable (NR).

diets appears to be rather resistant to increased concentrate supplementation (Huhtanen et al., 2013). On the other hand, Bayat et al. (2017) reported a significant decrease in acetate to propionate ratio (−28%) and CH_4-emission intensity (−25%) in response to increased concentrate supplementation from 35% to 65% in DM in grass silage-based diets. However, when the diet contained supplemental lipids, there was no decrease in CH_4 despite a marked decrease in acetate to propionate ratio (−22%) that was similar to the decrease with an unsupplemented diet. It should be noted that VFA concentrations in the rumen fluid do not directly reflect VFA production, but the ruminal balance of production and absorption of VFA. This may contribute to the apparent inconsistencies between the VFA profile in the rumen fluid and ruminal CH_4 production in some cases as suggested by Aguerre et al. (2011).

In addition to the pattern of rumen fermentation, the reduction of rumen pH in high-concentrate diets may also contribute to decreased ruminal CH_4 production via the decrease of protozoal numbers (Martin et al., 2010) or direct inhibition of methanogenesis below pH 6 (Van Kessel and Russell, 1996) or both. Although inclusion of high levels of concentrate in the diet of dairy cows is an effective CH_4-mitigation strategy (Table 3), it has disadvantages associated with increased risk for sub-acute rumen acidosis (SARA) (see review by Krause and Oetzel, 2006), competition with human food sources, GHG emissions during grain production and high feed cost. In addition, fibrous forage rather than starchy concentrate is the predominant natural component in cattle diet they are adapted to utilise.

Carbohydrate source. Fibrous, human-inedible by-products from the food and bioenergy industries could be used to partly or totally replace starch-rich cereal grains in the diet of high-producing dairy cows, and could provide a cost-effective and sustainable feeding strategy promoting a circular economy. In addition, fibre-rich diets promote rumen and animal health as opposed to starch and other readily fermentable carbohydrates that are at high amounts known to greatly modify the rumen environment through a decrease in pH and, consequently, to predispose to SARA (Krause and Oetzel, 2006).

Feeding dairy cows a fibre-rich concentrate with pulp, soya bean hulls or cereal bran as a main carbohydrate ingredient has resulted in similar DM intakes, ECM production, FE and NUE compared to concentrates rich in cereal starch under variable isonitrogenous dietary conditions, in terms of forage to concentrate ratio and forage type (Table 4). It should, however, be noted that all these trials were made in mid- to late lactation with ECM production around 30 kg/d. Therefore, at higher milk production and nutrient demand levels in early and peak lactation, the effects on animal performance may be different. At early lactation (30 days in milk), Piccioli-Cappelli et al. (2014) reported no difference in DM intake or lactation performance between dairy cows fed diets with low or high readily fermentable carbohydrates (starch plus sugars

Table 4 The effects of concentrate carbohydrate and protein source and protein level on dairy cow performance

Control concentrate (CC) main ingredients	Difference in substituting concentrate (SC)	In SC vs. CC diet DM, %	Basal forage component[2]	F:C[3]	Change in % relative to control concentrate diet[1]					CH_4 emission intensity, g CH_4 per kg ECM			Reference
					DMI	ECM	FE	NUE	Rumen C2 / C3	CC diet	SC diet	Change in %	
Carbohydrate source		**Starch**											
Citrus and sugar beet pulps, soya bean hulls, palm kernel extract	Wheat, wheatfeed	15 vs. 10	GS:MS 70:30	70:30	3	0	−3	−4	NR	13.0	13.3	2	Hart et al. (2015)
		19 vs. 14	GS:MS 30:70		2	−2	−4	3	NR	12.6	12.1	−4	
Maize meal, soya bean hulls	Less maize meal, more soya bean hulls	28 vs. 24[4]	MS:LH:GH 55:25:20	50:50	0	3	3	1	0	13.5	12.6	−8	Pirondini et al. (2015)
		28 vs. 24[5]	MS:LH:GH 55:25:20		−6	−8	−2	10	1	12.4	13.2	5	
Sugar beet pulp, wheat bran, palm kernel cake	Oats, barley, wheat	15 vs. 3	Early GS	66:34	5	1	−4	−2	NR	13.1	13.0	1	Pang et al. (2018)
		15 vs. 3	Late GS		2	7	2	1	NR	14.0	13.9	1	
Beet pulp, soya bean hull, dried distillers maize grains, wheat bran	Wheat, maize grain, wheat middling, wheat starch	23 vs. 6[6]	GS:GH 85:15	50:50	−3	−3	0	9	−13	14.9	13.3	−11	Bougouin et al. (2018)
		23 vs. 6[7]	GS:GH 85:15		−4	−1	3	1	−14	15.3	11.9	−22	
Protein level		**Protein**											
No protein feed	Soya bean meal	17 vs. 15	GS	60:40	0	1	1	−9	NR	17.5	16.9	−3	Gidlund et al. (2015)
		19 vs. 15	GS		1	5	3	−18	NR	17.5	15.9	−9	
		21 vs. 15	GS		0	3	3	−25	NR	17.5	17.8	2	

	Rapeseed meal	17 vs. 15	GS		1	3	1	−8	NR	17.5	16.9	−3	
		18 vs. 15	GS		4	7	2	−14	NR	17.5	16.1	−8	
		20 vs. 15	GS		2	7	5	−19	NR	17.5	15.8	−10	
No protein feed	Rapeseed meal	16 vs. 15	GS:RCS 70:30	60:40	5	8	3	−1	NR	16.7	15.6	−7	Gidlund et al. (2017)
		17 vs. 15	GS:RCS 70:30		10	10	0	−14	NR	16.7	15.2	−9	
		19 vs. 15	GS:RCS 70:30		10	6	−4	−22	NR	16.7	14.9	−11	
		17 vs. 16	GS:RCS 30:70		4	3	−1	−4	NR	17.8	15.0	−16	
		19 vs. 16	GS:RCS 30:70		9	4	−5	−14	NR	17.8	16.4	−8	
		19 vs. 16	GS:RCS 30:70		7	7	0	−20	NR	17.8	16.3	−8	
Less soya bean protein	More soya bean protein	16 vs. 14	GS:MS 75:25	50:50	8	1	−6	−10	NR	16.7	15.6	−7	Reynolds et al. (2010)[8]
		18 vs. 14	GS:MS 75:25		0	6	6	−22	NR	16.7	15.2	−9	
Less soya bean protein, rapeseed meal	More soya bean protein, rapeseed meal	16 vs. 14	GS:MS 25:75		2	0	−1	−13	NR	17.8	15.0	−16	
		18 vs. 14	GS:MS 25:75		5	2	−3	−24	NR	17.8	16.4	−8	
Less soya bean meal	More soya bean meal	19 vs. 15[9]	LH	45:55	0	3	3	−18	NR	14.4	14.0	−3	Niu et al. (2016)
Protein source		**Protein**											
Soya bean meal	Rapeseed meal	19 vs. 19	GS	60:40	2	2	0	5	NR	16.9	16.3	−4	Gidlund et al. (2015)
Soya bean meal	Faba bean milled	16 vs. 16	LS:MS 65:35	55:45	0	−2	−2	−3	1	15.0	15.2	1	Cherif et al. (2018)
	Faba bean rolled	16 vs. 16	LS:MS 65:35		1	−2	−3	−3	9	15.0	15.4	3	

(*Continued*)

Table 4 (*Continued*)

Control concentrate (CC) main ingredients	Difference in substituting concentrate (SC)	In SC vs. CC diet DM, %	Basal forage component[2]	F:C[3]	Change in % relative to control concentrate diet[1]					CH_4 emission intensity, g CH_4 per kg ECM			Reference
					DMI	ECM	FE	NUE	Rumen C2 / C3	CC diet	SC diet	Change in %	
Soya bean meal: rapeseed meal 65:35[10]	Faba bean 16%	16 vs. 16	GS	60:40	0	5	5	5	NR	17.6	15.9	−9	Johnston et al. (2019)
	33%	16 vs. 16	GS		0	0	−1	2	NR	17.6	16.2	−8	
	47%	16 vs. 16	GS		−1	1	2	−8	NR	17.6	16.9	−4	
Rapeseed meal	Faba bean	19 vs. 19	GS	60:40	−2	−4	−3	4	NR	15.2	16.1	6	Ramin et al. (2017)
	Pea	18 vs. 19	GS		0	−6	−6	−6	NR	15.2	16.7	10	

[1]Dry matter intake (DMI), Energy-corrected milk (ECM) calculated according to Sjaunja et al. (1991), Feed efficiency (FE) calculated as ECM / DM intake, Nitrogen-use efficiency (NUE) calculated as N output in milk/N intake, Ruminal acetate to propionate ratio (rumen C2/C3) calculated from their molar proportions in the rumen fluid, Not reported and not calculable (NR), [2]Grass (G), Hay (H), Lucerne (L), Maize (M), Red clover (RC), Silage (S), for forage mixtures the proportions of components (%) on a DM basis are presented in the appearing order, [3]Forage to concentrate ratio in DM basis (F:C), [4]Without fish oil in the diet, [5]With fish oil in the diet, [6]Without bicarbonate in the diet, [7]With bicarbonate in the diet, [8]Personal communication of C.K. Reynolds for diet formulation and milk composition, [9]Mean of two F:C ratios 53:47 and 38:62, [10]Protein feed mixture containing 65% of soya bean meal and 35% of rapeseed meal on a DM basis.

18% versus 25 % in diet DM, milk yield level 37 kg/d). However, the alterations in the concentrations of energy metabolites and hormones in blood, together with body weight loss, indicated mobilization of body reserves in the low-starch diet. This was in contrast to the high-starch diet which resulted in positive energy balance and some body weight gain. It seems that a large proportion if not all cereal starch can be replaced by fibrous by-products of high digestibility such as sugar beet pulp and soya bean hulls up to milk production level of 30 kg/d without a significant decrease in dairy cow lactation performance. Furthermore, Cabezas-Garcia et al. (2017) demonstrated recently that it is possible to replace barley and late-cut grass silage with early-cut grass silage of high energy value without compromising ECM yield, NUE and CH_4-emission intensity. In their study, the proportion of concentrate in the diet DM incrementally decreased from 60% to 45% and the starch from 25% to 17%, whereas NDF gradually increased from 36% to 42%.

Though Benchaar et al. (2001) modelled that replacing fibrous concentrate with a starch alternative reduces CH_4 emissions, a critical dietary concentration of starch of 20 to 22% in DM is required to mitigate ruminal methanogenesis (Tables 3 and 4; Hassanat et al., 2013). In lipid-supplemented diets, however, even a dietary starch content of 28% in DM did not alter ruminal CH_4 formation (Pirondini et al., 2015, Table 4; Bayat et al., 2017, Table 3). The decrease in ruminal protozoa population and the shift of rumen fermentation towards propionate seem to be the main factors for reduced CH_4-emission intensity on high-starch concentrate diets (Pirondini et al., 2015; Bougouin et al., 2018). Though high-starch diets are promising in mitigating CH_4-emission intensity in dairy cattle, the level of cereal starch needed to obtain significant reduction is very high. This contradicts the traditional role of ruminants in their ability to convert fibrous biomass inedible to monogastrics to high-quality human food.

3.3 Protein

Level of protein supplementation. Conventional good-quality protein sources in rapeseed and soya bean meals typically increase DM intake of dairy cows (see meta-analysis of Huhtanen et al., 2011) though the effect has been negligible in some cases (Table 4). The increase in feed intake may at least in part be attributed to improved digestibility of dietary fibre and CP (Broderick, 2003; Jaakkola et al., 2009), though better amino acid balance of conventional protein feeds or increased nutrient demand due to higher milk production may also contribute (Gidlund et al., 2017). The improved ECM and milk protein yields in response to good-quality CP supplementation probably results from higher supply of essentials amino acids to mammary glands (Gidlund et al., 2017).

However, the increase in ECM production above dietary CP content of 14–15% is in general rather moderate and seems to level out or even decline

at high dietary CP concentrations above 18–20% (Table 4; Broderick, 2003). Interestingly, the production responses of rapeseed meal have been similar, irrespective of a wide range of forage CP concentration (Jaakkola et al., 2009; Gidlund et al., 2017 in Table 4). Replacing soya bean meal with dehydrated lucerne also resulted in lower (−7%) milk yield (Doreau et al., 2014). All this indicates the superiority of protein in rapeseed and soya bean to that in forage to enhance milk production.

Dietary CP concentration is the best predictor of NUE in dairy production (see meta-analysis by Huhtanen and Hristov, 2009). Indeed, the linear decrease of NUE in response to incremental supply of dietary CP is consistently reported (Table 4), with efficiency typically ranging between 40% and 20% (Dijkstra et al., 2011). With low-protein diets, faecal N excretion represents a larger proportion of N intake (up to 50%) than urine N (as low as 25%) but, as dietary protein increases, the contribution of faecal N decreases and the environmentally labile urinary N exponentially increases up to 60% of N intake (Dijkstra et al., 2011). In part this is related to the failure of rumen microbes to utilise the excess degradable protein since the primary route of removing excess ammonia from the rumen is conversion to urea in the liver, followed by excretion in urine (Castillo et al., 2000).

The most obvious reason why an increased dietary level of CP could in theory reduce ruminal CH_4 emissions is that the fermentation of protein produces less CH_4 than fermentation of carbohydrates (Bannink et al., 2006). The potential to decrease ruminal CH_4-emission intensity by increased protein supplementation has been small in practise; the low to medium inclusion of protein feed resulting in the smallest ruminal CH_4-emission intensity (at best around −15%) together with the biggest improvement in lactation performance (Table 4). The excess of dietary CP in dairy cow ratios is unwanted since protein feeds are expensive and the improvement in milk and CH_4 production diminish or even reverse at high CP levels (Table 4), leading to a more significant N load released into the environment via manure and urine.

Protein source. Inclusion of rapeseed meal in dairy ratios generally increases DM intake, ECM yield and milk protein yield more than soybean meal (review by Huhtanen et al., 2011; Table 4) and other protein sources (review by Martineau et al., 2013). Huhtanen et al. (2011) suggested that the greater milk production responses with rapeseed meal is due to increased or more balanced amino acid supply (histidine in particular), the greater energy demand for milk production also affecting DM intake. This results in slightly improved NUE in rapeseed-supplemented diets relative to soya bean (Table 4). Reports comparing the effects of rapeseed meal to soya bean on enteric CH_4 production are scarce. Gidlund et al. (2015; Table 4) reported a marginal decrease in CH_4-emission intensity using rapeseed relative to soya bean meal across a wide range of dietary CP levels.

Grain legume faba bean (*Vicia faba*) and pea (*Pisum sativum*) seeds are relatively rich in protein (23–30% of DM) and starch (45–50% of DM), making them an interesting home-grown protein and energy source for dairy cow feed in temperate areas. The protein of these alternative grain legumes is more rumen degradable and lower in methionine than that of rapeseed or soybean, which may limit the lactation performance of dairy cows (see review by Halmemies-Beauchet-Filleau et al., 2018). Replacing protein in soya bean meal partially or completely with faba beans or peas has, however, resulted in similar bovine lactation performance (Halmemies-Beauchet-Filleau et al., 2018, Table 4). In contrast, the milk production responses of alternative grain legumes are often inferior compared to the rapeseed meal (Halmemies-Beauchet-Filleau et al., 2018; Table 4). Puhakka et al. (2016) reported a decreased milk protein yield together with an increased milk urea concentration. The proportion of N excreted in urine suggested less efficient use of protein in faba beans than in rapeseed, leading to increased N emissions to environment. However, the NUE of alternative grain legumes seems rather similar to soya bean and rapeseed meal in most studies (Table 4). The inclusion of faba beans or peas in the dairy cow diet could increase starch intake and shift rumen fermentation towards propionate, thus mitigating ruminal CH_4 production. However, in recent studies (Ramin et al., 2017; Cherif et al., 2018; Johnston et al., 2019) the effect on CH_4-emission intensity has been negligible (Table 4).

4 Case study: Effects of milled rapeseed on milk production, milk fat composition and ruminal CH_4 emissions of dairy cows in practical farm conditions

Introduction. Besides mitigating ruminal CH_4 production, unsaturated lipids such as 18:1*n*-9-rich oil from rapeseeds have great potential to modify the lipid composition of ruminant meat and milk by decreasing the proportion of saturated fatty acids and increasing that of unsaturated fatty acids. This is significant as milk and dairy products contribute significantly to human 12:0, 14:0 and palmitic acid (16:0) consumption, with excessive intake of these saturated fatty acids increasing the risk of cardiovascular disease as well as lowered insulin sensitivity (see review by Shingfield et al., 2013). The form of lipid inclusion in the dairy cow diet affects lipid bioavailability and final product composition. Milling of rapeseeds has been found to be necessary to release lipids within seeds for efficient absorption (Kairenius et al., 2009). Milled rapeseeds in the diet resulted in a similar fatty acid profile in bovine milk as free rapeseed oil, with the exception of lower increase in *trans*-fatty acids. Milling whole oilseeds as needed also minimises the risk for oxidative deterioration of unsaturated lipid during feed storage relative to pure oil. The aim of the

study described here was therefore to examine the effects of milled rapeseed on milk fat composition and ruminal CH_4 emissions of dairy cows in practical farm conditions.

Materials and methods. The study was conducted at the University of Helsinki Viikki research farm in Finland (for details, see Halmemies-Beauchet-Filleau et al., 2019). The whole Finnish Ayrshire milk herd was fed a control diet for 3 weeks (period 1) followed by rapeseed lipid-rich diet for 4 weeks (period 2). After this, all cows were switched back to the control diet (3 weeks, period 3). Forage-rich dairy cow TMR based on high-quality grass silage (digestible OM 696 g/kg DM, 60% in TMR DM) were fed *ad libitum*. The pre-wilted grass silage (predominantly timothy and meadow fescue) was from the first cut and ensiled with formic acid-based additives in big bales. Concentrates in TMR (40% in TMR DM) comprised home-grown cereals, rapeseed feeds as protein supplement, molassed sugar beet pulp and vitamins and minerals. Rapeseed protein was isonitrogenously supplied either as a lipid extracted meal (control diet) or full-fat seeds milled daily during TMR preparation using an ordinary hammer mill (sieve pore size 6–8 mm) (test diet).

The amount of additional rapeseed lipids in the test diet was ca. 50 g/kg diet DM. Cereal in the control diet was barley and in the test diet oats. When visiting the milking robot (Lely Astronaut A3, Lely, Maassluis, the Netherlands), cows producing less than 30, between 30 and 40 and over 40 kg of milk per day at the beginning of the trial received 3, 4 or 5 kg of standard concentrate per day throughout the study. The milking robot was equipped with GreenFeed system (C-Lock Inc., Rapid City, SD, USA) that measures ruminal CH_4, carbon dioxide and H_2 emissions.

Results and discussion. Cows had no health concerns when fed the test diet, but DM intake was decreased by, on average, 4% relative to control diet (for details, see Halmemies-Beauchet-Filleau et al., 2019). This is not unexpected because lipid supplementation often suppresses DM intake at high inclusion rates (review of Huhtanen et al., 2008; Halmemies-Beauchet-Filleau et al., 2017). As ECM was unaffected by the test diet, the FE was marginally improved from 1.34 to 1.40 (ECM kg per DM intake kg) compared with control diet. Protein yield and milk urea content were also unaffected by the form of rapeseed protein in the diet. Though the test diet had no effect on milk fat yield, it altered milk fat composition (Table 5). The total saturated fatty acid content of milk fat from the test diet was 17% lower than from the control diet (Table 5). Furthermore, the 10- to 16-carbon-saturated fatty acids, regarded as the key blood cholesterol-increasing fatty acids in humans, were substantially lower in milk from the test than in the milk from the control diet. Indeed, increased supply of long-chain fatty acids is known to inhibit de novo synthesis of saturated fatty acids in the mammary gland (see review of Shingfield et al., 2010). The total monounsaturated fatty acids were 58% higher in milk fat from the test

Table 5 Fatty acid composition of tank milk

Fatty acid, g/100 g total fatty acids	Control diet	Test diet	Change in %
10:0	3.9	2.0	−49
12:0	4.6	2.2	−52
14:0	13	8.5	−35
16:0	31	21	−31
18:0	9.7	18	+82
18:1*n*-9	16	28	+70
18:2*n*-6	1.3	1.1	
18:3*n*-3	0.4	0.4	
Total saturated fatty acids	74	61	−17
Total monounsaturated fatty acids	23	36	+58
Total polyunsaturated fatty acids	2.6	2.3	
Total *trans*-fatty acids	3.6	5.0	

Source: adapted from Halmemies-Beauchet-Filleau et al. (2019).

diet than the control diet that principally originated from 18:1*n*-9. The effect of milled rapeseeds on polyunsaturated fatty acids in milk was marginal. Furthermore, milk fat and fat-rich dairy products with a high monounsaturated fatty acid content are less susceptible to oxidative deterioration (Lin et al., 1996) compared with milk fat enriched with polyunsaturated fatty acids (Havemose et al., 2006).

Ruminal CH_4, carbon dioxide and H_2 emissions were decreased by 18%, 5% and 36%, respectively, with the test diet relative to the control. Milled rapeseeds substantially decreased H_2 load and CH_4 formation in the rumen of dairy cows fed diets rich in highly digestible grass silage (Fig. 1). The small decrease in DM intake cannot account for all the diminution in the ruminal H_2 and CH_4 emissions observed in the test diet. It is likely that the rumen fermentation pattern shifted towards propionate that increases H_2 utilisation. Rapeseed or other unsaturated lipids in the dairy cow diet have been shown to decrease the ruminal acetate to propionate ratio in some (Hristov et al., 2011a; Table 2), but not in all studies (Table 2). Though ruminal biohydrogenation of unsaturated fatty acids is an alternative H_2 sink as well, its significance to overall ruminal methanogenesis is generally considered very low (Martin et al., 2010). The CH_4 emission intensity was 12.1 versus 15.1 g CH_4 per kg ECM for test and control diet, respectively. In an average dairy herd in Finland that produces 10 300 ECM per year per cow and has 45 dairy cows, a change from the control diet typical to the production system in the area to test diet would thus represent an annual decrease of 1 390 t in ruminal CH_4 emissions. This corresponds to the withdrawal of CH_4 production of the whole herd of about 2 months in a year.

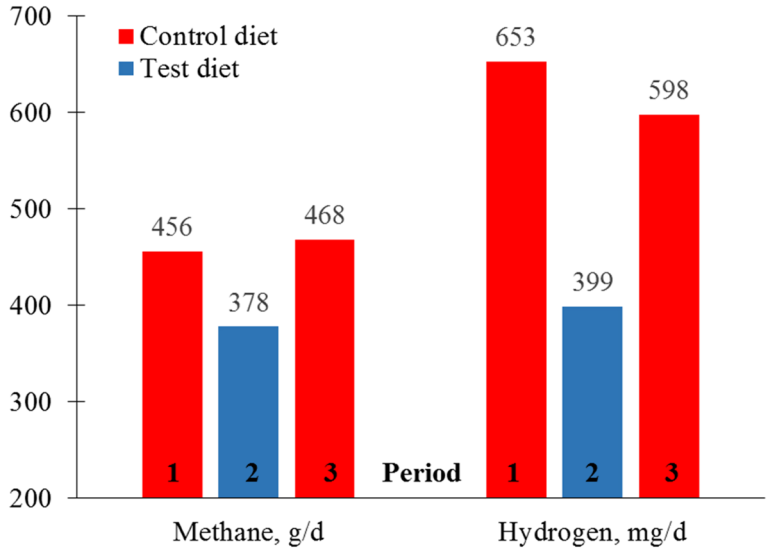

Figure 1 Ruminal gas emissions of dairy cows fed control diet in period 1, test diet in period 2 and again control diet in period 3. Source: adapted from Halmemies-Beauchet-Filleau et al. (2019).

Conclusions. Replacing rapeseed meal with milled rapeseeds (supplying 5% of lipid in diet DM) in a dairy cow diet based on highly digestible grass silage had no adverse effects on milk production, FE or animal health. Milled rapeseeds improved milk fat profile by decreasing the proportion of cholesterol-raising medium-chain saturated fatty acids and increasing that of 18:1*n*-9 in a whole herd level. In addition, milled rapeseeds fed at a commercially practical level substantially suppressed ruminal CH_4 production.

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5 Summary and future trends

Nutritional strategies available for mitigating GHG emissions from dairy cow production include various rumen modifiers (currently under development) and forage and/or concentrate-based dietary strategies currently available for practical use. Given the concern for reducing human-edible feed ingredients such as grains in animal feed, forage-based dietary strategies should be emphasised, especially with ruminant animals specialised in fibre digestion. In temperate areas, major plant species available for making silage include

grasses, forage legumes and maize but their availability in various areas depends on local climatic conditions.

Grass silages. Altering forage maturity at harvest has the greatest potential to reduce the environmental footprint of cool-season grass silages in dairy production. Harvesting grass herbage at an early rather than late maturity stage has led to increased DM intake, ECM, FE and reduced CH_4-emission intensity with dairy cows, though at the expense of reduced NUE. The trade-offs between reduced CH_4 emissions and reduced NUE are complex and clearly warrant further research. Other management factors such as N fertilization rate, use of additives in ensiling or high-sugar grass cultivars were of minor importance.

Forage legume silages. Limited data on the effects of forage legume silages on CH_4-emission intensity in comparison to grasses together with reduced NUE suggest minor potential for forage legumes to reduce the environmental footprint of dairy production. In contrast, the literature suggests lower CH_4-emission intensity for forage legumes than grasses, provided that higher DM intake potential and ruminal passage rates characteristic to forage legumes take place. Contrasting results may be attributable to the practice of growing and feeding forage legumes in mixtures with grasses or other plants as well as the large variation in silage nutritive and fermentation quality between years. Further research on the potential of forage legumes to reduce the environmental footprint of dairy production is needed to fully exploit their beneficial effects on forage production, feed DM intake and animal performance.

Maize silages. Starch containing maize silage is characterised by high metabolizable energy and low CP contents, which makes it a highly valuable forage crop and compatible to be mixed with grasses and legumes high in CP. The means available for reducing the environmental footprint of maize silage include advancing the maturity of maize crop at harvest to a late stage (40% DM) and using maize cultivars developed for higher cell wall digestibility and intake properties such as brown midrib maize. These methods may have the potential to reduce CH_4-emission intensity up to 10% on high-forage diets.

Replacing grass or legume forage silages with maize silage consistently leads to environmental benefits in terms of reducing CH_4-emission intensity on high-forage grass silage-based diets, and improving NUE especially on forage legume silages high in CP without compromises in milk production. Nevertheless, further research is needed to optimise the use of these forages in dairy production. There is a particular need for more research on the effects of forage legume N fractions on NUE and forage carbohydrate type on CH_4 emissions. There also need to be life cycle analyses comparing the environmental effects of using maize and perennial silage crops.

Lipids in concentrate. Unsaturated plant lipids at levels up to 5% in diet DM have the potential to mitigate ruminal CH_4 emissions in a dose-dependent manner by 20–40% in diets based on conserved grass or forage legumes without negative effects on animal performance in terms of ECM yield and FE. The effect of lipids seems to persist throughout the entire lactation, but more long-term studies are needed to confirm this. At high lipid inclusion rates, feeding lipid as a part of TMR is preferred to separate concentrate feeding. In contrast, on starchy diets (based on maize silage or rich in concentrate starch), lipid supplementation is of limited interest due to the negative effect on ECM yield. This is probably linked to more detrimental effect of unsaturated lipid on rumen fibre digestion when basal diet contains significant amounts of starch.

Carbohydrates in concentrate. Increasing the proportion of cereal starch in the dairy cow diet in general improves feed intake, ECM yield and NUE. A critical dietary concentration of starch of 20–22% in the diet DM is required to mitigate ruminal methanogenesis. Decreases of 20–25% have been reported when the starch content has reached 20–32% in the diet DM. However, high inclusion of readily fermentable carbohydrates from cereals predisposes cows to SARA and competes with human nutrition. Fibrous, human-inedible by-products of food and bioenergy industries provide a cost-effective and ethically sound feeding strategy that promotes a circular economy. Soya bean hulls, sugar beet pulp and cereal bran have partly or totally replaced starch-rich cereal grains in the diet of dairy cows without a decrease in animal performance or increase in ruminal CH_4. However, the production level of mid-lactation cows has not exceeded 30 kg/d in these studies, so more research is needed at higher levels of milk production and at early lactation to confirm these promising findings.

Protein in concentrate. Good-quality protein sources such as rapeseed and soya bean meals typically increase DM intake of dairy cows though the effects have been negligible in some cases. Low to medium inclusion of protein feed (dietary CP content of 15–18% depending on the CP of the basal forage) results in the smallest ruminal CH_4-emission intensity (at best around −15%) together with the biggest improvement in lactation performance. The excess of dietary CP in dairy cow ratios (CP above 18–20% in DM) is unwanted since protein feeds are expensive, and improvements in milk and CH_4 production diminish or even reverse at the highest CP levels, leading only to a more significant N load to environment via manure and urine. Interestingly, the protein in conventional dairy cow protein feeds using rapeseed and soya bean is superior to that in forage in enhancing milk production. Rapeseed protein is slightly superior to soya bean and faba bean and pea in terms of lactation performance but in terms of ruminal CH_4 emissions, the differences between these protein sources are negligible. Faba beans and peas are promising home-grown protein and energy sources for dairy cow feed in temperate areas due to their relatively

high CP and starch content. More research is needed to find ways to improve the CP utilization of forage and alternative grain legumes to improve their NUE in milk production.

6 Where to look for further information

The following review articles or meta-analysis provide a good overview of the subject:

- Dewhurst, R. J. 2013. Milk production from silage: comparison of grass, legume and maize silages and their mixtures. *Agric. Food Sci.* 22, 57–69.
- Huhtanen, P. and Hristov, A. N. 2009. A meta-analysis of the effects of dietary protein concentration and degradability on milk protein yield and milk N efficiency in dairy cows. *J. Dairy Sci.* 92, 3222–32.
- Hristov, A. N., Oh, J., Firkins, J. L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H. P. S., Adesogan, A. T., Yang, W., Lee, C., Gerber, P. J., Henderson, B. and Tricarico, J. M. 2013. Special topics – mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. *J. Anim. Sci.* 91, 5045–69.
- Ramin, M. and Huhtanen, P. 2013. Development of equations for predicting methane emissions from ruminants. *J. Dairy Sci.* 96, 2476–93.

7 References

Aguerre, M. J., Wattiaux, M. A., Powell, J. M., Broderick, G. A. and Arndt, C. 2011. Effect of forage-to-concentrate ratio in dairy cow diets on emission of methane, carbon dioxide, and ammonia, lactation performance, and manure excretion. *J. Dairy Sci.* 94(6), 3081–93. doi:10.3168/jds.2010-4011.

Allen, M. S. 2000. Effects of diet on short-term regulation of feed intake by lactating dairy cattle. *J. Dairy Sci.* 83(7), 1598–624. doi:10.3168/jds.S0022-0302(00)75030-2.

Alstrup, L., Hellwing, A. L. F., Lund, P. and Weisbjerg, M. R. 2015. Effect of fat supplementation and stage of lactation on methane production in dairy cows. *Anim. Feed Sci. Technol.* 207, 10–9. doi:10.1016/j.anifeedsci.2015.05.017.

Arndt, C., Powell, J. M., Aguerre, M. J. and Wattiaux, M. A. 2015. Performance, digestion, nitrogen balance, and emission of manure ammonia, enteric methane, and carbon dioxide in lactating cows fed diets with varying alfalfa silage-to-corn silage ratios. *J. Dairy Sci.* 98(1), 418–30. doi:10.3168/jds.2014-8298.

Bannink, A., Kogut, J., Dijkstra, J., France, J., Kebreab, E., Van Vuuren, A. M. and Tamminga, S. 2006. Estimation of the stoichiometry of volatile fatty acid production in the rumen of lactating cows. *J. Theor. Biol.* 238(1), 36–51. doi:10.1016/j.jtbi.2005.05.026.

Bayat, A. R., Kairenius, P., Stefánski, T., Leskinen, H., Comtet-Marre, S., Forano, E., Chaucheyras-Durand, F. and Shingfield, K. J. 2015. Effect of Camelina oil or live yeasts (*Saccharomyces cerevisiae*) on ruminal methane production, rumen fermentation,

and milk fatty acid composition in lactating cows fed grass silage diets. *J. Dairy Sci.* 98(5), 3166–81. doi:10.3168/jds.2014-7976.

Bayat, A. R., Ventto, L., Kairenius, P., Stefański, T., Leskinen, H., Tapio, I., Negussie, E., Vilkki, J. and Shingfield, K. J. 2017. Dietary forage to concentrate ratio and sunflower oil supplement alter rumen fermentation, ruminal methane emissions, and nutrient utilization in lactating cows. *Transl. Anim. Sci.* 1(3), 277–86. doi:10.2527/tas2017.0032.

Bayat, A. R., Tapio, I., Vilkki, J., Shingfield, K. J. and Leskinen, H. 2018. Plant oil supplements reduce methane emissions and improve milk fatty acid composition in dairy cows fed grass silage-based diets without affecting milk yield. *J. Dairy Sci.* 101(2), 1136–51. doi:10.3168/jds.2017-13545.

Beauchemin, K. A., Kreuzer, M., O'Mara, F. and McAllister, T. A. 2008. Nutritional management for enteric methane abatement: a review. *Aust. J. Exp. Agric.* 48(2), 21–7. doi:10.1071/EA07199.

Benchaar, C., Pomar, C. and Chiquette, J. 2001. Evaluation of dietary strategies to reduce methane production in ruminants: a modelling approach. *Can. J. Anim. Sci.* 81(4), 563–74. doi:10.4141/A00-119.

Benchaar, C., Hassanat, F., Gervais, R., Chouinard, P. Y., Petit, H. V. and Masse, D. I. 2014. Methane production, digestion, ruminal fermentation, nitrogen balance, and milk production of cows fed corn silage- or barley silage-based diets. *J. Dairy Sci.* 97(2), 961–74. doi:10.3168/jds.2013-7122.

Benchaar, C., Hassanat, F., Martineau, R. and Gervais, R. 2015. Linseed oil supplementation to dairy cows fed diets based on red clover silage or corn silage: effects on methane production, rumen fermentation, nutrient digestibility, N balance, and milk production. *J. Dairy Sci.* 98(11), 7993–8008. doi:10.3168/jds.2015-9398.

Bernardes, T. F., Daniel, J. L. P., Adesogan, A. T., McAllister, T. A., Drouin, P., Nussio, L. G., Huhtanen, P., Tremblay, G. F., Bélanger, G. and Cai, Y. 2018. Silage review: unique challenges of silages made in hot and cold regions. *J. Dairy Sci.* 101(5), 4001–19. doi:10.3168/jds.2017-13703.

Bertilsson, J., Åkerlind, M. and Eriksson, T. 2017. The effects of high-sugar ryegrass/red clover silage diets on intake, production, digestibility, and N utilization in dairy cows, as measured in vivo and predicted by the NorFor model. *J. Dairy Sci.* 100(10), 7990–8003. doi:10.3168/jds.2017-12874.

Bougouin, A., Ferlay, A., Doreau, M. and Martin, C. 2018. Effects of carbohydrate type or bicarbonate addition to grass silage-based diets on enteric methane emissions and milk fatty acid composition in dairy cows. *J. Dairy Sci.* 101(7), 6085–97. doi:10.3168/jds.2017-14041.

Brask, M., Lund, P., Hellwing, A. L. F., Poulsen, M. and Weisbjerg, M. R. 2013a. Enteric methane production, digestibility and rumen fermentation in dairy cows fed different forages with and without rapeseed fat supplementation. *Anim. Feed Sci. Technol.* 184(1–4), 67–79. doi:10.1016/j.anifeedsci.2013.06.006.

Brask, M., Lund, P., Weisbjerg, M. R., Hellwing, A. L. F., Poulsen, M., Larsen, M. K. and Hvelplund, T. 2013b. Methane production and digestion of different physical forms of rapeseed as fat supplements in dairy cows. *J. Dairy Sci.* 96(4), 2356–65. doi:10.3168/jds.2011-5239.

Broderick, G. A. 2003. Effects of varying dietary protein and energy levels on the production of lactating dairy cows. *J. Dairy Sci.* 86(4), 1370–81. doi:10.3168/jds.S0022-0302(03)73721-7.

Cabezas-Garcia, E. H., Krizsan, S. J., Shingfield, K. J. and Huhtanen, P. 2017. Effects of replacement of late-harvested grass silage and barley with early-harvested silage on milk production and methane emissions. *J. Dairy Sci.* 100(7), 5228–40. doi:10.3168/jds.2016-12444.

Castillo, A. R., Kebreab, E., Beever, D. E. and France, J. 2000. A review of efficiency of nitrogen utilisation in lactating dairy cows and its relationship with environmental pollution. *J. Anim. Feed Sci.* 9(1), 1–32. doi:10.22358/jafs/68025/2000.

Cherif, C., Hassanat, F., Claveau, S., Girard, J., Gervais, R. and Benchaar, C. 2018. Faba bean (*Vicia faba*) inclusion in dairy cow diets: effect on nutrient digestion, rumen fermentation, nitrogen utilization, methane production, and milk performance. *J. Dairy Sci.* 101(10), 8916–28. doi:10.3168/jds.2018-14890.

De Boever, J. L., Goossens, K., Peiren, N., Swanckaert, J., Ampe, B., Reheul, D., Brabander, D. L., De Campeneere, S. and Vandaele, L. 2017. The effect of maize silage type on the performances and methane emission of dairy cattle. *J. Anim. Physiol. Anim. Nutr.* 101(5), e246–56. doi:10.1111/jpn.12598.

Dewhurst, R. J. 2013. Milk production from silage: comparison of grass, legume and maize silages and their mixtures. *AFSci* 22(1), 57–69. doi:10.23986/afsci.6673.

Dijkstra, J., Oenema, O. and Bannink, A. 2011. Dietary strategies to reducing N excretion from cattle: implications for methane emissions. *Curr. Opin. Env. Sust.* 3(5), 414–22. doi:10.1016/j.cosust.2011.07.008.

Dijkstra, J., Bannik, A., France, J., Kebreab, E. and Van Gastelen, S. 2018. Short communication: antimethanogenic effects of 3-nitroxypropanol depend on supplementation dose, dietary fiber content, and cattle type. *J. Dairy Sci.* 101(10), 9041–7. doi:10.3168/jds.2018-14456.

Doreau, M., Ferlay, A., Rochette, Y. and Martin, C. 2014. Effects of dehydrated Lucerne and soya bean meal on milk production and composition, nutrient digestion, and methane and nitrogen losses in dairy cows receiving two different forages. *Animal* 8(3), 420–30. doi:10.1017/S1751731113002206.

Ellis, J. L., Hindrichsen, I. K., Klop, G., Kinley, R. D., Milora, N., Bannik, A. and Dijkstra, J. 2016. Effects of lactic acid bacteria silage inoculation on methane emission and productivity of Holstein Friesian dairy cattle. *J. Dairy Sci.* 99(9), 7159–74. doi:10.3168/jds.2015-10754.

Eugène, M., Massé, D., Chiquette, J. and Benchaar, C. 2008. Meta-analysis on the effects of lipid supplementation on methane production in lactating dairy cows. *Can. J. Anim. Sci.* 88(2), 331–7. doi:10.4141/CJAS07112.

Ferlay, A., Doreau, M., Martin, C. and Chilliard, Y. 2013. Effects of incremental amounts of extruded linseed on the milk fatty acid composition of dairy cows receiving hay or corn silage. *J. Dairy Sci.* 96(10), 6577–95. doi:10.3168/jds.2013-6562.

Gidlund, H., Hetta, M., Krizsan, S. J., Lemosquet, S. and Huhtanen, P. 2015. Effects of soybean meal or canola meal on milk production and methane emissions in lactating dairy cows fed grass silage-based diets. *J. Dairy Sci.* 98(11), 8093–106. doi:10.3168/jds.2015-9757.

Gidlund, H., Hetta, M. and Huhtanen, P. 2017. Milk production and methane emissions from dairy cows fed a low or high proportion of red clover silage and an incremental level of rapeseed expeller. *Liv. Sci.* 197, 73–81. doi:10.1016/j.livsci.2017.01.009.

Halmemies-Beauchet-Filleau, A., Kokkonen, T., Lampi, A. M., Toivonen, V., Shingfield, K. J. and Vanhatalo, A. 2011. Effect of plant oils and Camelina expeller on milk fatty acid

composition in lactating cows fed diets based on red clover silage. *J. Dairy Sci.* 94(9), 4413–30. doi:10.3168/jds.2010-3885.

Halmemies-Beauchet-Filleau, A., Shingfield, K. J., Simpura, I., Kokkonen, T., Jaakkola, S., Toivonen, V. and Vanhatalo, A. 2017. Effect of incremental amounts of Camelina oil on milk fatty acid composition in lactating cows fed diets based on a mixture of grass and red clover silage and concentrates containing *Camelina expeller*. *J. Dairy Sci.* 100(1), 305–24. doi:10.3168/jds.2016-11438.

Halmemies-Beauchet-Filleau, A., Rinne, M., Lamminen, M., Mapato, C., Ampapon, T., Wanapat, M. and Vanhatalo, A. 2018. Alternative and novel feeds for ruminants: nutritive value, product quality and environmental aspects. *Animal* 12(s2), s295–309. doi:10.1017/S1751731118002252.

Halmemies-Beauchet-Filleau, A., Jaakkola, S., Kokkonen, T., Turpeinen, A., Givens, D. I. and Vanhatalo, A. 2019. Rapeseed lipids to decrease saturated fatty acids in milk and ruminal methane emissions of dairy cows. In: *Proceedings of the 10th Nordic Feed Science Conference*, pp. 69–73.

Hammond, K. J., Jones, A. K., Humphries, D. J., Crompton, L. A. and Reynolds, C. K. 2016. Effects of diet forage source and neutral detergent fiber content on milk production of dairy cattle and methane emissions determined using GreenFeed and respiration chamber techniques. *J. Dairy Sci.* 99(10), 7904–17. doi:10.3168/jds.2015-10759.

Harrison, J., Huhtanen, P. and Collins, M. 2003. Perennial grasses. In: Buxton, D. R., Muck, R. E. and Harrison, J. H. (Eds), *Silage Science and Technology*. American Society of Agronomy, Crop Science Society of America and Soil Science of Society of America, Madison, WI, pp. 635–747.

Hart, K. J., Huntington, J. A., Wilkinson, R. G., Bartram, C. G. and Sinclair, L. A. 2015. The influence of grass silage-to-maize silage ratio and concentrate composition on methane emissions, performance and milk composition of dairy cows. *Animal* 9(6), 983–91. doi:10.1017/S1751731115000208.

Hassanat, F., Gervais, R., Julien, C., Massé, D. I., Lettat, A., Chouinard, P. Y., Petit, H. V. and Benchaar, C. 2013. Replacing alfalfa silage with corn silage in dairy cow diets: effects on enteric methane production, ruminal fermentation, digestion, N balance, and milk production. *J. Dairy Sci.* 96(7), 4553–67. doi:10.3168/jds.2012-6480.

Hassanat, F., Gervais, R., Massé, D. I., Petit, H. V. and Benchaar, C. 2014. Methane production, nutrient digestion, ruminal fermentation, N balance, and milk production of cows fed timothy silage-or alfalfa silage-based diets. *J. Dairy Sci.* 97(10), 6463–74. doi:10.3168/jds.2014-8069.

Hassanat, F., Gervais, R. and Benchaar, C. 2017. Methane production, ruminal fermentation characteristics, nutrient digestibility, nitrogen excretion, and milk production of dairy cows fed conventional or brown midrib corn silage. *J. Dairy Sci.* 100(4), 2625–36. doi:10.3168/jds.2016-11862.

Hatew, B., Bannink, A., Van Laar, H., De Jonge, L. H. and Dijkstra, J. 2016. Increasing harvest maturity of whole-plant corn silage reduces methane emission of lactating dairy cows. *J. Dairy Sci.* 99(1), 354–68. doi:10.3168/jds.2015-10047.

Havemose, M. S., Weisbjerg, M. R., Bredie, W. L. P., Poulsen, H. D. and Nielsen, J. H. 2006. Oxidative stability of milk influenced by fatty acids, antioxidants, and copper derived from feed. *J. Dairy Sci.* 89(6), 1970–80. doi:10.3168/jds.S0022-0302(06)72264-0.

Hollmann, M., Powers, W. J., Fogiel, A. C., Liesman, J. S., Bello, N. M. and Beede, D. K. 2012. Enteric methane emissions and lactational performance of Holstein cows fed

different concentrations of coconut oil. *J. Dairy Sci.* 95(5), 2602–15. doi:10.3168/jds.2011-4896.

Hristov, A. N., Domitrovich, C., Wachter, A., Cassidy, T., Lee, C., Shingfield, K. J., Kairenius, P., Davis, J. and Brown, J. 2011a. Effect of replacing solvent-extracted canola meal with high-oil traditional canola, high-oleic acid canola, or high-erucic acid rapeseed meals on rumen fermentation, digestibility, milk production, and milk fatty acid composition in lactating dairy cows. *J. Dairy Sci.* 94(8), 4057–74. doi:10.3168/jds.2011-4283.

Hristov, A. N., Lee, C., Cassidy, T., Long, M., Heyler, K., Corl, B. and Forster, R. 2011b. Effects of lauric and myristic acids on ruminal fermentation, production, and milk fatty acid composition in lactating dairy cows. *J. Dairy Sci.* 94(1), 382–95. doi:10.3168/jds.2010-3508.

Hristov, A. N., Oh, J., Firkins, J. L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H. P. S., Adesogan, A. T., Yang, W., Lee, C., Gerber, P. J., Henderson, B. and Tricarico, J. M. 2013. Special topics—mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. *J. Anim. Sci.* 91(11), 5045–69. doi:10.2527/jas.2013-6583.

Huhtanen, P. and Hristov, A. N. 2009. A meta-analysis of the effects of dietary protein concentration and degradability on milk protein yield and milk N efficiency in dairy cows. *J. Dairy Sci.* 92(7), 3222–32. doi:10.3168/jds.2008-1352.

Huhtanen, P., Rinne, M. and Nousiainen, J. 2008. Evaluation of concentrate factors affecting silage intake of dairy cows: a development of the relative total diet intake index. *Animal* 2(6), 942–53. doi:10.1017/S1751731108001924.

Huhtanen, P., Hetta, M. and Swensson, C. 2011. Evaluation of canola meal as a protein supplement for dairy cows: a review and a meta-analysis. *Can. J. Anim. Sci.* 91(4), 529–43. doi:10.4141/cjas2011-029.

Huhtanen, P., Jaakkola, S. and Nousiainen, J. 2013. An overview of silage research in Finland: from ensiling innovation to advances in dairy cow feeding. *AFSci* 22(1), 35–56. doi:10.23986/afsci.6632.

Huyen, N. T., Desrues, O., Alferink, S. J. J., Zandstra, T., Verstegen, M. W. A., Hendriks, W. H. and Pellikaan, W. F. 2016. Inclusion of sainfoin (*Onobrychis viciifolia*) silage in dairy cow rations affects nutrient digestibility, nitrogen utilization, energy balance, and methane emissions. *J. Dairy Sci.* 99(5), 3566–77. doi:10.3168/jds.2015-10583.

Jaakkola, S., Saarisalo, E. and Heikkilä, T. 2009. Formic acid treated whole crop barley and wheat silages in dairy cow diets: effects of crop maturity, proportion in the diet, and level and type of concentrate supplementation. *AFSci* 18(3–4), 234–56. doi:10.2137/145960609790059569.

Järvenranta, K., Kuoppala, K., Rinne, M. and Virkajärvi, P. 2016. Legumes in ruminant production systems in European cold climates – limitations and opportunities. Legume Perspectives Issue 12, April 2016, pp. 34–5.

Johnston, D. J., Theodoridou, K. and Ferris, C. P. 2019. The impact of field bean inclusion level in dairy cow diets on cow performance and nutrient utilisation. *Liv. Sci.* 220, 166–72. doi:10.1016/j.livsci.2018.12.015.

Jung, H. G., Mertens, D. R. and Phillips, R. L. 2011. Effect of reduced ferulate-mediated lignin/arabinoxylan cross-linking in corn silage on feed intake, digestibility, and milk production. *J. Dairy Sci.* 94(10), 5124–37. doi:10.3168/jds.2011-4495.

Kairenius, P., Toivonen, V., Ahvenjärvi, S., Vanhatalo, A., Givens, D. I. and Shingfield, K. J. 2009. Effects of rapeseed lipids in the diet on ruminal lipid metabolism and milk

fatty acid composition in cows fed grass silage based diets. In: Ruminant Physiology: *Digestion*, Metabolism and Effects of Nutrition on Reproduction and Welfare. *Proceedings of the XIth Interface Symposium Ruminant Physiology*. Wageningen Academic Publishing, the Netherlands, pp. 232–3.

Khan, N. A., Yu, P., Ali, M., Cone, J. W. and Hendriks, W. H. 2015. Nutritive value of maize silage in relation to dairy cow performance and milk quality. *J. Sci. Food Agric.* 95(2), 238–52. doi:10.1002/jsfa.6703.

Kliem, K. E., Humphries, D. J., Kirton, P., Givens, D. I. and Reynolds, C. K. 2019. Differential effects of oilseed supplements on methane production and milk fatty acid concentrations in dairy cows. *Animal* 13(2), 309–17. doi:10.1017/S1751731118001398.

Klop, G., Hatew, B., Bannink, A. and Dijkstra, J. 2016. Feeding nitrate and docosahexaenoic acid affects enteric methane production and milk fatty acid composition in lactating dairy cows. *J. Dairy Sci.* 99(2), 1161–72. doi:10.3168/jds.2015-10214.

Knapp, J. R., Laur, G. L., Vadas, P. A., Weiss, W. P. and Tricarico, J. M. 2014. Invited review: Enteric methane in dairy cattle production: quantifying the opportunities and impact of reducing emissions. *J. Dairy Sci.* 97(6), 3231–61. doi:10.3168/jds.2013-7234.

Krause, K. M. and Oetzel, G. R. 2006. Understanding and preventing subacute ruminal acidosis in dairy herds: a review. *Anim. Feed Sci. Technol.* 126(3–4), 215–36. doi:10.1016/j.anifeedsci.2005.08.004.

Kuoppala, K. 2010. Influence of harvesting strategy on nutrient supply and production of dairy cows consuming diets based on grass and red clover silage. Doctoral dissertation. MTT Science, no. 11, 49p.

Kuoppala, K., Ahvenjärvi, S., Rinne, M. and Vanhatalo, A. 2009. Effects of feeding grass or red clover silage cut at two maturity stages in dairy cows. 2. Dry matter intake and cell wall digestion kinetics. *J. Dairy Sci.* 92(11), 5634–44. doi:10.3168/jds.2009-2250.

Lin, M. P., Sims, C. A., Staples, C. R. and O'Keefe, S. F. 1996. Flavor quality and texture of modified fatty acid high monoene, low saturate butter. *Food Res. Int.* 29(3–4), 367–71. doi:10.1016/0963-9969(96)00017-8.

Livingstone, K. M., Humphries, D. J., Kirton, P., Kliem, K. E., Givens, D. I. and Reynolds, C. K. 2015. Effects of forage type and extruded linseed supplementation on methane production and milk fatty acid composition of lactating dairy cows. *J. Dairy Sci.* 98(6), 4000–11. doi:10.3168/jds.2014-8987.

Lock, A. L. and Shingfield, K. J. 2004. Optimizing milk composition. In: Kebreab, E., Mills, J. and Beever, D. E. (Eds), *Dairying -Using Science to Meet Consumers Needs*. Br. Soc. Anim. Sci. Publ. 29. Nottingham University Press, Loughborough, UK, pp. 107–88.

Maia, M. R., Chaudhary, L. C., Figueres, L. and Wallace, R. J. 2007. Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen. *Antonie Leeuwenhoek* 91(4), 303–14. doi:10.1007/s10482-006-9118-2.

Martin, C., Rouel, J., Jouany, J. P., Doreau, M. and Chilliard, Y. 2008. Methane output and diet digestibility in response to feeding dairy cows crude linseed, extruded linseed, or linseed oil. *J. Anim. Sci.* 86(10), 2642–50. doi:10.2527/jas.2007-0774.

Martin, C., Morgavi, D. P. and Doreau, M. 2010. Methane mitigation in ruminants: from microbe to the farm scale. *Animal* 4(3), 351–65. doi:10.1017/S1751731109990620.

Martin, C., Ferlay, A., Mosoni, P., Rochette, Y., Chilliard, Y. and Doreau, M. 2016. Increasing linseed supply in dairy cow diets based on hay or corn silage: effect on enteric

methane emission, rumen microbial fermentation, and digestion. *J. Dairy Sci.* 99(5), 3445–56. doi:10.3168/jds.2015-10110.

Martineau, R., Ouellet, D. R. and Lapierre, H. 2013. Feeding canola meal to dairy cows: a meta-analysis on lactational responses. *J. Dairy Sci.* 96(3), 1701–14. doi:10.3168/jds.2012-5740.

Martinez-Fernandez, G., Duval, S., Kinderman, M., Schirra, H. J., Denman, S. E. and McSweeney, C. S. 2018. 3-NOP vs. halogenated compound: methane production, ruminal fermentation and microbial community response in forage fed cattle. *Front. Microbiol.* 9, 1–13.

Merry, R. J., Lee, M. R. F., Davies, D. R., Dewhurst, R. J., Moorby, J. M., Scollan, N. D. and Theodorou, M. K. 2006. Effects of high-sugar ryegrass silage and mixtures with red clover silage on ruminant digestion. 1. In vitro and in vivo studies of nitrogen utilization. *J. Anim. Sci.* 84(11), 3049–60. doi:10.2527/jas.2005-735.

Moate, P. J., Williams, S. R. O., Hannah, M. C., Eckard, R. J., Auldist, M. J., Ribaux, B. E., Jacobs, J. L. and Wales, W. J. 2013. Effects of feeding algal meal high in docosahexaenoic acid on feed intake, milk production, and methane emissions in dairy cows. *J. Dairy Sci.* 96(5), 3177–88. doi:10.3168/jds.2012-6168.

Moss, A. R., Jouany, J.-P. and Newbold, J. 2000. Methane production by ruminants: its contribution to global warming. *Ann. Zootech.* 49(3), 231–53. doi:10.1051/animres:2000119.

Moorby, J. M., Evans, R. T., Scollan, N. D., MacRae, J. C. and Theodorou, M. K. 2006. Increased concentration of water-soluble carbohydrate in perennial ryegrass (*Lolium perenne* L.). Evaluation in dairy cows in early lactation. *Grass Sci.* 61(1), 52–9. doi:10.1111/j.1365-2494.2006.00507.x.

Muck, R. E., Nadeau, E. M. G., McAllister, T. A., Contreras-Govea, F. E., Santos, M. C. and Kung, L. 2018. Silage review: recent advances and future uses of silage additives. *J. Dairy Sci.* 101(5), 3980–4000. doi:10.3168/jds.2017-13839.

Niu, M., Appuhamy, J. A. D. R. N., Leytem, A. B., Dungan, R. S. and Kebreab, E. 2016. Effect of dietary crude protein and forage contents on enteric methane emissions and nitrogen excretion from dairy cows simultaneously. *Anim. Prod. Sci.* 56(3), 312–21. doi:10.1071/AN15498.

Odongo, N. E., Or-Rashid, M. M., Kebreab, E., France, J. and McBride, B. W. 2007. Effect of supplementing myristic acid in dairy cow rations on ruminal methanogenesis and fatty acid profile in milk. *J. Dairy Sci.* 90(4), 1851–8. doi:10.3168/jds.2006-541.

Olijhoek, D. W., Løvendahl, P., Lassen, J., Hellwing, A. L. F., Höglund, J. K., Weisbjerg, M. R., Noel, S. J., McLean, F., Hojberg, O. and Lund, P. 2018. Methane production, rumen fermentation, and diet digestibility of Holstein and Jersey dairy cows being divergent in residual feed intake and fed at 2 forage-to-concentrate ratios. *J. Dairy Sci.* 101(11), 9926–40. doi:10.3168/jds.2017-14278.

Owens, F., Zinn, R. A. and Kim, Y. K. 1986. Limits to starch digestion in the ruminant small intestine. *J. Anim. Sci.* 63, 1634–48.

Pang, D., Yan, T., Trevisi, E. and Krizsan, S. J. 2018. Effect of grain-or by-product-based concentrate fed with early-or late-harvested first-cut grass silage on dairy cow performance. *J. Dairy Sci.* 101(8), 7133–45. doi:10.3168/jds.2018-14449.

Phelan, P., Moloney, A. P., McGeough, E. J., Humphreys, J., Bertilsson, J., O'Riordan, E. G. and O'Kiely, P. 2015. Forage legumes for grazing and conserving in ruminant production systems. *Crit. Rev. Plant Sci.* 34(1–3), 281–326. doi:10.1080/07352689.2014.898455.

Piccioli-Cappelli, F., Loor, J. J., Seal, C. J., Minuti, A. and Trevisi, E. 2014. Effect of dietary starch level and high rumen-undegradable protein on endocrine-metabolic status, milk yield, and milk composition in dairy cows during early and late lactation. *J. Dairy Sci.* 97(12), 7788–803. doi:10.3168/jds.2014-8336.

Pirondini, M., Colombini, S., Mele, M., Malagutti, L., Rapetti, L., Galassi, G. and Crovetto, G. M. 2015. Effect of dietary starch concentration and fish oil supplementation on milk yield and composition, diet digestibility, and methane emissions in lactating dairy cows. *J. Dairy Sci.* 98(1), 357–72. doi:10.3168/jds.2014-8092.

Puhakka, L., Jaakkola, S., Simpura, I., Kokkonen, T. and Vanhatalo, A. 2016. Effects of replacing rapeseed meal with fava bean at 2 concentrate crude protein levels on feed intake, nutrient digestion, and milk production in cows fed grass silage–based diets. *J. Dairy Sci.* 99(10), 7993–8006. doi:10.3168/jds.2016-10925.

Ramin, M., Höjer, A. and Hetta, M. 2017. The effects of legume seeds on the lactation performance of dairy cows fed grass silage-based diets. *AFSci* 26(3), 129–37. doi:10.23986/afsci.64417.

Reynolds, C. K., Crompton, L. A., Mills, J. A. N., Humphries, D. J., Kirton, P., Relling, A. E., Misselbrook, T. H., Chadwick, D. R. and Givens, D. I. 2010. Effects of diet protein level and forage source on energy and nitrogen balance and methane and nitrogen excretion in lactating dairy cows. In: Crovetto, G. M. (Ed.), *Energy and Protein Metabolism and Nutrition. Proceedings of the 3rd EAAP International Symposium Energy Protein Metabolism and Nutrition*. Wageningen Academic Publishing, the Netherlands, pp. 463–4.

Rinne, M., Jaakkola, S. and Huhtanen, P. 1997. Grass maturity effects on cattle fed silage-based diets. 1. Organic matter digestion, rumen fermentation and nitrogen utilization. *Anim. Feed Sci. Technol.* 67(1), 1–17. doi:10.1016/S0377-8401(96)01141-8.

Rinne, M. 2000. Influence of the timing of the harvest of primary grass growth on herbage quality and subsequent digestion and performance in the ruminant animal. Academic dissertation. University of Helsinki, Department of Animal Science Publications no. 54. Helsinki University Press, Helsinki, 42p.

Sauer, F. D., Fellner, V., Kinsman, R., Kramer, J. K. G., Jackson, H. A., Lee, A. J. and Chen, S. 1998. Methane output and lactation response in Holstein cattle with monensin or unsaturated fat added to the diet. *J. Anim. Sci.* 76(3), 906–14. doi:10.2527/1998.763906x.

Shingfield, K. J., Bernard, L., Leroux, C. and Chilliard, Y. 2010. Role of trans fatty acids in the nutritional regulation of mammary lipogenesis in ruminants. *Animal* 4(7), 1140–66. doi:10.1017/S1751731110000510.

Shingfield, K. J., Bonnet, M. and Scollan, N. D. 2013. Recent developments in altering the fatty acid composition of ruminant-derived foods. *Animal* 7(Suppl. 1), 132–62. doi:10.1017/S1751731112001681.

Sjaunja, L. O., Bævre, L., Junkkarinen, L., Pedersen, J. and Setälä, J. 1991. A Nordic proposal for an energy corrected milk (ECM) formula. In: *Proceedings of the 27th Biennial Session of the International Committee for Animal Recording (ICAR)*, Paris, France. Wageningen Academic Publishing, the Netherlands, pp. 156–7.

Staerfl, S. M., Amelchanka, S. L., Kälber, T., Soliva, C. R., Kreuzer, M. and Zeitz, J. O. 2012. Effect of feeding dried high-sugar ryegrass ('AberMagic') on methane and urinary nitrogen emission of primiparous cows. *Liv. Sci.* 150, 203–301.

Van Dorland, H. A., Wettstein, H. -R., Leuenberger, H. and Kreuzer, M. 2007. Effect of supplementation of fresh and ensiled clovers to ryegrass on nitrogen loss

and methane emission of dairy cows. *Liv. Sci.* 111(1–2), 57–69. doi:10.1016/j.livsci.2006.11.015.

Van Gastelen, S., Antunes-Fernandes, E. C., Hettinga, K. A., Klop, G., Alferink, S. J. J., Hendriks, W. H. and Dijkstra, J. 2015. Enteric methane production, rumen volatile fatty acid concentrations, and milk fatty acid composition in lactating Holstein-Friesian cows fed grass silage-or corn silage-based diets. *J. Dairy Sci.* 98(3), 1915–27. doi:10.3168/jds.2014-8552.

Van Gastelen, S., Dijkstra, J. and Bannink, A. 2019. Are dietary strategies to mitigate enteric methane emission equally effective across dairy cattle, beef cattle, and sheep? *J. Dairy Sci.* 102(7), 6109–30. doi:10.3168/jds.2018-15785.

Vanhatalo, A. and Jaakkola, S. 2016. Intake and performance with temperate forage legume-based ruminant production systems. *Legume Perspect.* (12), 17–9.

Van Kessel, J. A. S. and Russell, J. B. 1996. The effect of pH on ruminal methanogenesis. *FEMS Microb. Ecol.* 20(4), 205–10. doi:10.1016/0168-6496(96)00030-X.

Van Middelaar, C. E., Berentsen, P. B. M., Dijkstra, J. and De Boer, I. J. M. 2013. Evaluation of a feeding strategy to reduce greenhouse gas emissions from dairy farming: the level of analysis matters. *Agric. Syst.* 121, 9–22. doi:10.1016/j.agsy.2013.05.009.

Ventto, L., Leskinen, H., Kairenius, P., Stefański, T., Bayat, A. R., Vilkki, J. and Shingfield, K. J. 2017. Diet-induced milk fat depression is associated with alterations in ruminal biohydrogenation pathways and formation of novel fatty acid intermediates in lactating cows. *Br. J. Nutr.* 117(3), 364–76. doi:10.1017/S0007114517000010.

Warner, D., Hatew, B., Podesta, S. C., Klop, G., Van Gastelen, S., Van Laar, H., Dijkstra, J. and Bannink, A. 2016. Effects of nitrogen fertilisation rate and maturity of grass silage on methane emission by lactating dairy cows. *Animal* 10(1), 34–43. doi:10.1017/S1751731115001640.

Warner, D., Bannink, A., Hatew, B., Van Laar, H. and Dijkstra, J. 2017. Effects of grass silage quality and level of feed intake on enteric methane production in lactating dairy cows. *J. Anim. Sci.* 95(8), 3687–700. doi:10.2527/jas.2017.1459.

Wilkinson, J. M. and Rinne, M. 2018. Highlights of progress in silage conservation and future perspectives. *Grass Sci.* 73(1), 40–52. doi:10.1111/gfs.12327.

Chapter 20

The use of feedlot/cereal grains in improving feed efficiency and reducing by-products such as methane in ruminants

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1 Introduction

Cattle are a marvel of evolutionary processes because they convert low-quality feedstuffs, such as forages, into high-quality protein sources for humans. The ability of the ruminant to convert sunlight into meat, milk, and fiber is extraordinary and is mediated through the symbiotic relationship between the host animal and its resident gastrointestinal microbial consortium

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(Hungate, 1966). The stepwise degradation of feeds by the members of this mixed microbial microorganism fermentation is crucial to production of volatile fatty acids (VFAs) upon which the animal depends for energy (Russell, 2002). However, the molar proportions of each VFA produced by the ruminal microbial ecosystem vary depending on which dietary ingredient or feedstuff is fermented by the resident microbial population, which can have profound impacts on animal production efficiency, carcass quality, and food safety (Russell and Hespell, 1981; Depenbusch et al., 2008; Verdu et al., 2015; Wilson et al., 2016). While the ruminant animal clearly evolved to degrade forages, the ability to ferment feeds is not limited to forages, but includes the ability to degrade cereal grains such as wheat, corn, barley, sorghum, and oats.

The environment of the rumen is highly reduced (Russell, 2002), meaning that reducing equivalent disposal (e.g. NADH NAD) can become limiting, imperiling the continuation of the fermentation of feedstuffs. Interspecies hydrogen transfer to regenerate NAD results in methane production (Iannotti et al., 1973; Thiele and Zeikus, 1988), and is a clear keystone to the symbiotic relationships that occur within the ruminal microbial population. However, the fundamental importance of interspecies hydrogen transfer to methane underlines the critical role of methane as a reducing equivalent sink to allow anaerobic degradation of feedstuffs to continue unless an alternative electron sink is provided. Despite the benefits the ruminal microbial population derives from methane production, it is both a significant loss of carbon and energy, representing from 2% to 8% of the GE to the animal (Dong et al., 2006) and in some situations methane can represent up to 12% of the DE (Johnson and Johnson, 1995), especially when cattle are fed forages. It should be noted that methane is a potent and notable greenhouse gas that is of increasing global importance.

The contribution of livestock to global climate change is caused by either the direct (e.g. enteric fermentation) or indirect (e.g. feedstuff production) emission of greenhouse gases (GHGs) (Steinfeld et al., 2006; Beauchemin et al., 2009; Haque, 2018). Carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) are the main GHGs produced by the livestock over the course of their production (Hungate, 1966). Among livestock, ruminants are the key contributors to the GHG, approximately 80% of the total emissions in the sector (Opio et al., 2012), while pigs and poultry only have approximately 9% and 8% contribution, respectively (Gerber et al., 2013). Ruminants consume plants that utilize CO_2 in photosynthesis; therefore, the CO_2 emitted by animals is not viewed as a net contributor to the climate change (Steinfeld et al., 2006). Consequently, CH_4 and N_2O are considered the primary GHGs caused by enteric fermentation and feed production in ruminant production systems, having massive global warming potentials (GWP) of 25 and 298 CO_2 equivalent, respectively (Forster et al., 2007; Eckard et al., 2010).

Since the mid-1940s, the US beef production industry has increasingly relied upon the use of grain feeding to produce a wholesome, high-quality protein source. By feeding starch-containing feeds such as corn, the end products of the microbial fermentation are altered, with significant impacts upon fermentation efficiency and host physiology. Starch fermentation yields a greater proportion of propionate, which leads to increased levels of intramuscular marbling in grain-fed cattle. Other chapters of this book will address the end products of the rumen microbial fermentation and inefficiencies inherent to the ruminal fermentation, but in this chapter, we will address the impacts of feeding grain to cattle to reduce deleterious fermentation by-products such as methane.

2 Types of cereal grains fed to cattle

Beef production in the United States is localized largely in the central one-third of the nation, primarily in the Great Plains. The proximity to the Grain Belt means that shipping grain to the cattle is logistically and economically feasible, therefore many of our beef cattle are grown in feedlots located in the central high plains. Cereal grains are a large part of the US agricultural economy with over 475 million metric tons produced in 2016, including wheat, corn, barley, sorghum, rice, and oats. While some cereal grain production is for human consumption, a large portion is used in the livestock feed industry because of the ability to meet high-energy demands associated with modern levels of production. Starch is often the primary nutrient used to promote increased levels of production in ruminant diets, and cereal grains are an effective source of starch (Theurer, 1986).

The kernel of cereal grains primarily consists of three main parts including the bran (or outer hull), the endosperm (primary location of starch), and the germ (or embryo). Although grain is a good source of starch, the cell wall (outer layer) is virtually indigestible in livestock and often requires further processing before feeding. Some common processing procedures include dry rolling, grinding, cracking, steam rolling, steam flaking, pelleting, and the addition of enzymes. Dry rolling, cracking, and grinding are often grouped together because each of these mechanical processes increases the surface area of the grain for digestion by breaking the seed coat and reducing particle size (Rowe et al., 1999). Steam rolling includes heating the grain through a steam cabinet to soften the kernels before rolling. Steam flaking involves processing the corn under steam pressure before rolling into a flake. This latter method partially gelatinizes the present starch as well as breaks the seed coat and endosperm, making the grain more susceptible to degradation by amylolytic bacteria (Rowe et al., 1999). Pelleting combines smaller particles together to make a large particle that allows control of site and rate of digestion by altering the density and size of the final particle created (Rowe et al., 1999). Among the list

of grains produced in the United States each year, the primary cereal grains used in livestock rations are barley, corn, and wheat.

2.1 Barley

Barley (*Hordeum vulgare L.)* ranks fourth in the production quantity after corn, rice, and wheat. In 2014, the production of barley totaled over 144 million tons with the top five producers being Russia, France, Germany, Australia, and Ukraine. Although total acres of barley harvested has steadily decreased in the last five years, more productive varieties and farming practices have allowed the total quantity to increase. Barley's nutritional value makes it an appropriate supplement to cattle, especially in systems where high levels of production are optimal. Starch generally makes up between 52% and 60% of the barley kernel while protein and energy are around 13% and 85%, respectively. Although cereal grains are typically low in calcium and high in phosphorus, barley provides a greater amount of calcium and ß-glucans when compared to corn, wheat, and sorghum. Inside the barley grain, multiple types of starch have been identified including waxy, normal, and high amylose (Zhu, 2017). Although the nutritional value of barley is beneficial for feeding cattle, the kernel is surrounded by a fibrous hull that is rather indigestible in the rumen, requiring some processing, such as dry rolling, to maximize utilization (Beauchemin et al., 1994). Some studies have shown that dry rolling barley can increase digestibility from whole barley by 32.7% (Toland, 1976).

2.2 Corn

Corn *(Zea Mays L.)* is the most common cereal grain used in livestock feed because of the nutritional value, productivity, and availability. Not only can corn grain be fed to cattle (processed or whole), but there are multiple forms and by-products available from corn production including distiller's grain (wet and dry), corn silage, and corn gluten (Firkins et al., 1985; Fron et al., 1996). The livestock feed industry benefits greatly from many corn by-products that are created during the production of ethanol such as stillage, condensed distiller's solubles, wet distiller's grain, and dry distiller's grains. Stillage is a liquid product that is created from mash distillation that can be used to replace water in cattle diets to decrease dry matter intake without compromising performance. Stillage can also be dehydrated to create condensed distillers solubles or syrup. The solid fraction created from ethanol production is known the distillers grain. Distiller's grain is a highly digestible alternative to whole corn and has a higher energy value because of the increased fat content. The mechanical processing of corn increases digestibility by 5–10% and increases nutrient availability by cracking the pericarp (smooth outer surface), that is basically indigestible in the

rumen, to allow microbes to access the starch inside the kernel. While whole corn generally consists of 70–72% starch, 10% protein, and 87% TDN, the crude protein content of corn by-products can range from 20% to 35% and TDN from 70% to 100% (Beauchemin et al., 1994).

2.3 Wheat

Wheat *(Triticum aestivum L.)* is an important part of the livestock feeding industry because of its versatility. While most cereal grains are planted in the spring and grow during the summer, wheat can be planted in the fall to provide a good source of grazing for livestock during the winter. Aside from grazing, wheat can be used to make hay, silage, or can be fed as a grain. The wheat seed is generally made up of 60% starch and has approximately 16% protein and 80% TDN or energy. The wheat kernel is composed of three parts: bran (outer), endosperm (85–86% starch), and the germ (embryo). The bran, or outer layer of the wheat kernel, can escape ruminal fermentation so further processing, such as steam flaking or dry rolling, is required (Kreikemeier et al., 1990). If whole wheat is fed, chewing time tends to increase as well as dry matter disappearance or digestibility tends to decrease. Much like other cereal grains, wheat can be used as an effective source of energy in rations but calcium supplementation may be required since wheat is low in calcium and high in phosphorus. Compared to other grains, wheat has shown, when further processed, to have the greatest dry matter disappearance in animal studies (Herrera-Saldana et al., 1990).

2.4 Other cereal grains

Oats *(Avena sativa)* rank sixth in global cereal grain production after wheat, corn, rice, barley, and sorghum (Stevens et al., 2004). Much like corn, oats have many different uses in the livestock feeding industry including grazing, fodder, silage, haylage, straw, and hay. Although there are other uses for oats, Stevens et al. (2004) reported that 74% of oats are used in livestock feed. Similar to barley, oats offer a valuable source of ß-glucans and mineral, while providing around 13% crude protein. Although oats have a softer kernel than other cereal grains, which creates difficulty in the milling process, the kernel is composed of 60% starch which makes it an effective source of energy in livestock feeds. Sorghum *(Sorghum bicolor)* is not used as extensively as other cereal grains (Etuk et al., 2012). Sorghum offers a higher percentage of starch than oats (74% vs. 60%) with slightly less protein (12.3% vs. 13%). While sorghum behaves in a similar fashion to corn when fed to livestock, when fed as a forage, grain, or silage, only 51% of the sorghum produced is used as livestock feed. Consideration of plant maturity when feeding sorghum is critical as young, green plants contain a compound

called dhurrin. Dhurrin is a cyanogenic glycoside that yields hydrogen cyanide (HCN) during hydrolysis which is lethal to animals (Etuk et al., 2012).

3 Cereal grain production

Cereal grain production is a vital part of the world economy with over 600 million ha of production in 2010. However, all cereal grains are not produced in every part of the world as there are several factors that determine where crops are produced including environmental, cultural, and economic (Awika, 2011). The main factor that determines crop production location is the local environmental conditions.

3.1 Corn

Corn is the most important of all cereal grains in terms of production with over 800 million metric tons produced in 2010 with 40% produced by the United States (Awika, 2011). Corn is relatively drought and frost susceptible, so it is grown in areas with plenty of rainfall or ability to irrigate and is planted in the spring which allows it to grow in the warm summer months. Corn is produced in a wide variety of states in the United States with the highest concentration being in the Midwest states or the 'corn belt'. The states that make up the 'corn belt' are primarily Indiana, Illinois, Iowa, Minnesota, Missouri, Nebraska, and Kansas with some surrounding states being included as well.

The United States Department of Agriculture (USDA) defines a value-added product as changing the physical state or form of a product to enhance its value. In this sense, cattle serve as a tremendous value-added product of corn. Cattle have evolved to form a symbiotic relationship with the microbes that inhabit their rumen and allow them to efficiently survive on feedstuffs that would normally be unavailable to the animal. The microbes inside the rumen can degrade the starch present in corn (and all cereal grains) to produce VFA that provide energy to the animal and promote growth at a conversion rate of 5–7 pounds of feed per pound of gain, roughly. Feed corn commonly grown is relatively unsuitable for human consumption, but feeding to cattle changes the form of that product into a highly digestible animal protein and adds significant value. For example, the average bushel of corn weighs 56 pounds and is currently worth $3.61 per bushel, which is roughly $0.065/lb. The current live cattle prices are $1.26/lb while a grain-fed, choice ribeye steak is worth $9.99/lb from a popular grocery store in the United States. With this information, it can be calculated that 1000 pounds of corn is worth $65, and 1000 pounds of live cattle are worth $1260, though this vastly simplified calculation ignores some important other costs (e.g. varying feed conversion efficiency, cost of maintaining feedlots, transport, opportunity cost, debt service, etc.) that are associated with producing cattle

to market weight and bringing the product to consumers' plates. Corn is an important part of cattle rations because of its ability to add significant amounts of energy in the production systems where high levels of performance are demanded. With the vast difference in price for corn and cattle, producers could certainly benefit from viewing cattle as a value-added product of corn.

3.2 Wheat

Wheat is arguably the most versatile cereal grain. Wheat can be grown in a wider range of environments as it is more resistant to drought and temperature variation so it can be grown in both winter and summer months (Awika, 2011). Because of its versatility across different climates and temperatures, wheat production is distributed across parts of the United States and Canada. The 'wheat belt' extends from central Texas to central Alberta including the states of Oklahoma, Kansas, Nebraska, Montana, and the Dakotas.

3.3 Barley

Barley is more temperature tolerant and can survive in colder climates than other cereal grains. The majority of barley production in the United States is in the north/northwestern states of Montana, North Dakota, Idaho, and Washington with an average of 205 million bushels produced between 2008 and 2012. In North America, Canada produces the majority of barley as they produce about 40% more than the United States.

3.4 Other cereal grains

Sorghum and oats are produced in much lower quantities in the United States when compared to corn, wheat, barley, and even rice (although rice is not generally used as a livestock feed). The majority of oat production in North America, much like barley, occurs in Canada. The highest concentration of oat production in the United States takes place in Iowa, Minnesota, South Dakota, North Dakota, and Wisconsin. Sorghum is rather tolerant to heat and drought when compared to other cereal grains which makes it a popular crop in African countries (Awika, 2011), and in the United States it is mainly grown in dry lands of the 'sorghum belt' that stretches from southern Texas to South Dakota including Oklahoma, New Mexico, Colorado, Kansas, and Nebraska.

4 Dietary factors affecting methane production by ruminants

Most of the enteric CH_4 is generated by ruminants via ruminal fermentation of carbohydrates, proteins, and, to some extent, lipids by the bacterial, protozoal, and fungal populations under anaerobic conditions, leading to the production

of VFAs, mainly acetate, propionate, and butyrate that are used by the animal as a source of energy. The production of gases (CO_2, H_2, and CH_4) is a by-product of fermentation, which is removed through eructation (Boadi et al., 2004; Kebreab et al., 2006; Martin et al., 2010). Fermentation is an oxidative process, during which reduced cofactors (NADH, NADPH, FADH) are re-oxidized (NAD^+, $NADP^+$, FAD^+) through dehydrogenation reactions generating H_2 in the rumen. Once produced, H_2 is utilized by methanogenic archaea, a microbial group distinct from Eubacteria, to reduce CO_2 or formate to CH_4 (McAllister and Newbold, 2008; Martin et al., 2010). However there are some ruminal methanogens such as Methanomassiliicoccaceae that generate methane by reducing methyl groups from other metabolites (e.g. methylamines) (Martinez-Fernandez et al., 2018). This route can be important to ruminal methane production because pectin contains a higher ratio of methylated compounds. Moreover, production of acetate liberates H_2, whereas propionate serves as a net H_2 sink. Consequently, diets that increase propionate and decrease acetate in the rumen are often associated with a reduction in ruminal CH_4 production, given that less H_2 is available to methanogens for reducing from CO_2 to CH_4 (Beauchemin et al., 2009).

The amount of emitted CH_4 by the ruminants depends on various factors including carbohydrate intake, type of the carbohydrate sources, ruminal pH, residence time in the rumen, rate of ruminal fermentation, and rate of methanogenesis. The daily emission of enteric CH_4 can be inhibited by reducing feed intake and/or fermentation rate in the rumen; however, animal growth performance should be considered. Fat supplemented diets lower the carbohydrate fermentation in the rumen due to the replacement of carbohydrate with lipids; however, fiber digestibility may be adversely affected, leading to decreased feed conversion efficiency in the ruminant (Martin et al., 2008; Beauchemin et al., 2009). Dry matter intake (DMI) is another major factor affecting methane production in ruminants, and a positive relationship of DMI and methane production is reported. However, methane production per unit of intake (g CH_4 /kg DMI) decreases with increasing DMI, suggesting a higher rumen turnover leading to a lower digestibility of the diet (Buddle et al., 2011).

Type of carbohydrate plays a critical role in CH_4 production as it can impact ruminal pH and subsequently alter the microbiota present. The digestibility of cellulose and hemicellulose is highly related to methane production compared to soluble carbohydrate (Hook et al., 2010). A positive relationship was reported between digestibility of hemicellulose and methane emission in non-lactating cows fed forage; however, a negative relationship was reported between digestibility of cellulose and methane emission (Holter and Young, 1992). Another study concluded that there seems to be a curvilinear relationship between methane production and proportion of concentrate in the ration, with methane losses of 6–7% of gross energy (GE) remaining constant at

30–40% concentrate levels in the ration and then reducing to 2–3% of GE with a concentrate level of 80–90% (Sauvant and Giger-Reverdin, 2007). On the other hand, in dairy cows increasing the level of concentrates to inhibit CH_4 emitted is not promising due to the fact that milk quality is adversely affected by the concentrate levels over 50% of the ration (Beauchemin et al., 2008).

It is critical to consider that elevated amounts of rapidly fermentable carbohydrates in a ration can lead to a higher passage rate from the rumen and lower ruminal retention time and pH, directing methanogenesis from slower degrading carbohydrates toward the hindgut and manure (Hindrichsen et al., 2006; Hook et al., 2010). Grinding forage feed prior to the consumption by the ruminants seems to reduce methane production, most likely by increasing the ruminal digestion and flow rate through the gastrointestinal tract (GIT); therefore, less time is available for ruminal methane production (Johnson and Johnson, 1995). Moreover, the ruminal fermentation of rapidly fermentable carbohydrates can improve the production of VFAs; however, if VFA production is higher than the absorption capacity from the rumen, the pH will drop, resulting in a risk of subacute ruminal acidosis (SARA) and imbalance of the rumen microbiota (Plaizier et al., 2008).

5 The role of starch and forage in methane formation

Starch is the primary nutrient used in cattle rations to add increased levels of digestible energy when performance at high levels is required (Theurer, 1986). There are two components of starch, amylose and amylopectin. Amylose is a linear molecule that only accounts for roughly 20–30% of starch and is made up of primarily α 1–4 linkages with very few α 1–6 branch points (Fig. 1).

Amylopectin, on the other hand, is a larger (although less dense) molecule that is described as 'fluffy' because of its considerable amount of α 1–6 linkages in combination with α 1–4 bonds as well. Generally, cereal grains

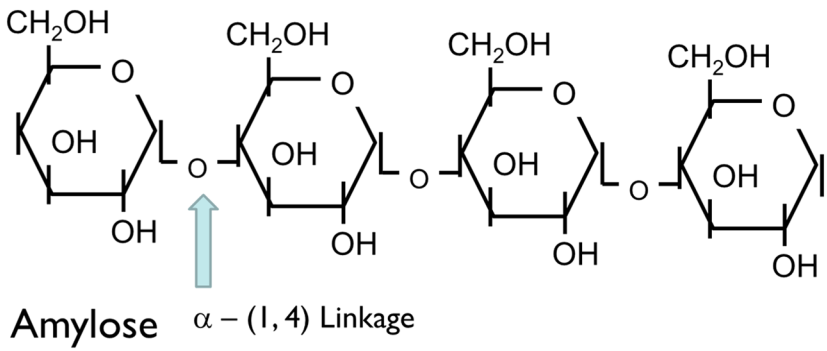

Figure 1 Structure of amylose (starch), characterized by α-(1, 4) linkages.

are composed of greater than 50% starch with varying sizes of starch granules depending on the ratio of amylose to amylopectin. The size of the starch granule is negatively correlated to the amount of amylose present. Amylose is broken down into sugars by enzymes such as amylases (α and ß) and is rapidly fermented to produce VFA (acetate and propionate) and lactate. Amylopectin is also broken down by enzymes such as amylase (α and ß), but because of the increased amount of α 1–6 branch points, both sugars and limit dextrins are produced (Fig. 2).

The sugars are rapidly fermented inside the rumen while limit dextrans require additional enzymes, such as limit dextranase, for complete degradation. Starch is fermented in the rumen by amylolytic bacteria (i.e. *Streptococcus bovis, Prevotella ruminicola, Ruminobacter* (formerly *Bacteroides*) *amylophilus, Selenomonas ruminantium, Succinomonas amylolytica*) to produce acetate, propionate, and lactate. As starch is fermented inside the rumen, propionate production increases and the acetate to propionate ratio decreases, which provides more available energy to the animal. While lactate can be detrimental to the animal, due to acidosis, balancing the ration with sufficient forage can prevent many of the negative effects.

Starch is a key source of glucogenic energy for high-yield dairy cows and a vital fuel for rumen microbes in the form of fermentable energy (Koenig et al., 2003). Once in the rumen, starch is mainly broken down by amylolytic bacteria and also by protozoa and fungi to some degree. The enzymes produced by rumen microorganisms are capable of hydrolyzing amylose and amylopectin glycosidic bonds, releasing various glucooligosaccharides.

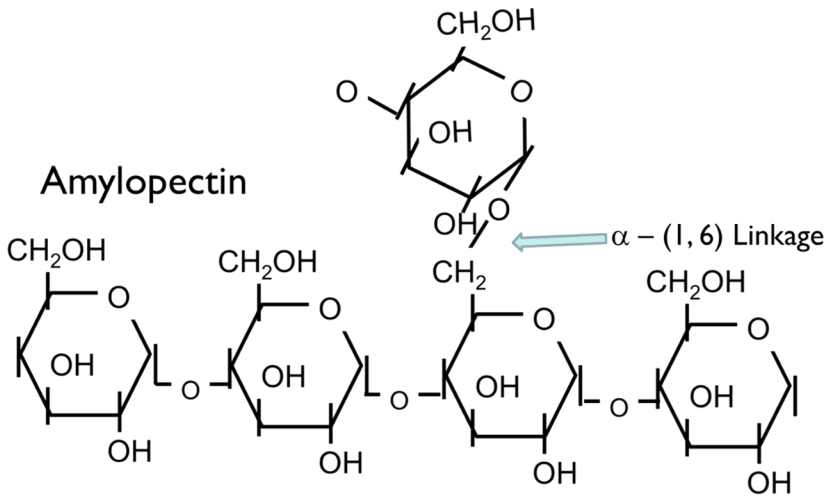

Figure 2 Structure of amylopectin, characterized by α-(1, 4) and α-(1, 6) linkages.

The post-ruminal process of starch breakdown is triggered by pancreatic α-amylase secretion, hydrolyzing amylose and amylopectin into dextrins and smaller glucooligosaccharides. The process is ended by the action of maltase and isomaltase secreted in the intestine, though it is inferred that starch digestion in the ruminant small intestine is limited because evolutionarily little starch reached the small intestine of ruminants (Huntington, 1997; Ortega-Cerrilla and Mendoza-Martínez, 2003; Gómez et al., 2016). The site of starch breakdown impacts the substrates absorbed in ruminants. Ruminal breakdown of starch decreases enteric CH_4 formation, leading to an alternative H_2 sink to methanogenesis, and produces VFAs for absorption and supplies energy to microbial protein synthesis; however, decreased starch digestibility is considered beneficial regarding the preventive effect against acidosis and increase in the supply of glucogenic substrates. On the other hand, starch breakdown in the ruminant small intestine leads to higher energetic efficiency compared to degradation via microbial fermentation due to a reduction in CH_4 production, fermentation heat losses, and greater efficiency of metabolizable energy utilization (Huhtanen and Sveinbjörnsson, 2006; Gómez et al., 2016). It is important to note that lower ruminal starch digestion is not correlated with an increase in its small intestinal digestion; however, it is linked to higher hindgut and lower total tract digestibility (Larsen et al., 2009).

Grain selection in the diet is also critical to the production levels of methane, as well as production efficiency of milk and meat, which offers potential to reduce methanogenesis via current technology and feeding systems (Martin et al., 2010; Moate et al., 2016). Changing the diet of Australian cattle from corn to wheat resulted in a significant decrease in methane yield (g CH_4/kg DMI) (Moate et al., 2018). Researchers have demonstrated that diets containing wheat rather than corn had lower methane yield (11.1 vs. 19.5 g CH_4/kg DMI, respectively) and methane efficiency (7.6 vs. 15.7 g CH_4/kg milk) (Moate et al., 2019). However, it was noted that some cattle were 'adaptive' to changing the diet over time in regard to milk and methane production metrics, but not all cattle responded the same way (Moate et al., 2018). Thus is apparent that the availability of starch in grains does play an important role in ruminal methanogenesis, but the mode of action of the reduction of methane production is unclear currently, and this offers a distinct opportunity for analysis of the changes in the microbiome and how this can impact methane production.

Forage quality has an impact on CH_4 formation in the rumen. High-quality forage (i.e. young plants) can decrease CH_4 production by changing the fermentation pathway since it has greater levels of readily fermentable carbohydrates and less neutral detergent fiber (NDF), resulting in a higher digestibility and passage rate (Beever et al., 1986). In contrast, mature forage leads to a higher CH_4 production yield (e.g. methane produced/kg DMI) due to a higher C:N ratio, resulting in a decrease in the digestibility (Milich, 1999),

which contrasts with the generally accepted relationship that increasing N available with mature forages can increase forage digestibility.

Different types of forage can also affect CH_4 emission because of the differences in their chemical composition. Cereal forages can have substantial levels of starch, favoring propionate production over acetate and can decrease ruminal CH_4 formation (Beauchemin et al., 2008). Legume forage has a lower CH_4 yield due to the presence of condensed tannins, a low-fiber content, a high DMI, and a fast passage rate (Beauchemin et al., 2008); however, results from other studies focusing on the effect of forage type on methane yield are inconsistent (Benchaar et al., 2001; Hammond et al., 2013). It has been stated that C4 grasses produce more CH_4 than the C3 plants (Archimède et al., 2011). Additionally, forage processing and preservation can impact CH_4 emission as well. For example, when forages are chopped or pelleted, they can decrease the CH_4 production per kg of DMI since smaller particles undergo less breakdown in the rumen (Boadi et al., 2004; Martin et al., 2010). Ensiled forages have a tendency to cause less CH_4 formation due to the fact that the digestible oligosaccharides in ensiled forages are fermented during the process (Boadi et al., 2004).

6 H_2 sinks in the rumen and methane production

Methanogenesis is vital for a desirable performance of the rumen because it prevents H_2 accumulation by serving as a reducing equivalent sink. The elimination of methane production would result in an inhibition of dehydrogenase activity involved in the oxidation of reduced cofactors (Wolin, 1975; McAllister and Newbold, 2008). The microbial fermentation of substrates produces various end products that are not equivalent in terms of H_2 output; therefore, accumulated H_2 inhibits microbes in the rumen to oxidize the cofactors having a role in electron transfer in the rumen, resulting in less energy from the fermentation process (Beauchemin et al., 2009; Martin et al., 2010). Acetate and butyrate production in the rumen leads to a release of H_2 and favors CH_4 production, while the propionate formation is a competitive pathway for H_2 utilization (Boadi et al., 2004). Efficiency is a complex production trait to define, but animals that were defined as efficient in gain did not differ in specific methane production or methanogenic bacterial populations from animals that were less efficient in gain (Freetly et al., 2015).

The rumen is an anaerobic fermentation chamber where microbial populations are in a symbiotic relationship, exchanging metabolites to improve each other's growth; therefore, this interaction is called 'cross-feeding' (Schultz and Breznak, 1979). Methane formation is considered as cross-feeding between H_2-producing microbial populations (e.g. fibrolytic fungi and bacteria) and H_2-utilizing methanogens, allowing removal of H_2 and improvement in

fiber fermentation (Kobayashi, 2010). Current strategies to reduce methane production need to consider alternative H_2 sinks to methanogenesis. There are several H_2-consuming pathways that occur in the rumen. Methanogenesis is the major one followed by propionate production (i.e. fumarate reduction). Other pathways such as nitrate- and nitrite-reduction, reductive acetogenesis, and biohydrogenation of unsaturated fatty acids have a minor role in H_2 consumption in the rumen (Kobayashi, 2010).

Monensin has gained an interest as a mitigation strategy for CH_4 production, because it has an inhibitory effect on protozoa and gram-positive bacteria, including ruminococci, streptococci, and lactobacilli that are supplying methanogens with substrate for methanogenesis (Russell and Strobel, 1989). The monensin selects for gram-negative microorganisms, leading to a shift toward propionate production in the rumen, therefore, it is hypothesized that monensin does not affect CH_4 production by limiting methanogens (Martin et al., 2010), but inhibits the growth of the bacteria and protozoa supplying a substrate for methanogenesis (Bergen and Bates, 1984; Russell and Strobel, 1989). This hypothesis is supported by the fact that when rumen fluid was administered with monensin in vitro, CH_4 production decreased until H_2 was supplied, at which time CH_4 formation resumed (Russell and Strobel, 1989).

Monensin is generally supplemented to the diet as a premix or is provided via a slow-release capsule (Beauchemin et al., 2008). Studies indicated that the effect of monensin on decreasing CH_4 production seems to be dose-dependent, with lower doses (10–15 ppm) leading to an effective milk response in dairy cows but no effect on CH_4, whereas higher doses (24–35 ppm) decreased CH_4 production (Eckard et al., 2010). However, there have been contradictory results from different studies in terms of the inhibitory effect of monensin in terms of sustainability. In a study, it has been reported that long-term administration of monensin to dairy cows continuously decreased methane by 7% for 6 months without adverse effect on milk yield (Odongo et al., 2007; Ellis et al., 2008). It has been reported that monensin (33 mg/kg) decreased CH_4 emissions in beef cattle by up to 30%, but levels returned to pre-treatment levels within 2 months (Guan et al., 2006).

Dicarboxylic acids such as fumarate and malate are precursors to produce propionate in the rumen and can act as an alternative H_2 sink and mitigate methanogenesis (Nisbet and Martin, 1993; Martin and Streeter, 1995; McAllister and Newbold, 2008). It has been reported by the studies that supplementation of fumaric acid or malic acid reduced CH_4 production (Callaway and Martin, 1996; McGinn et al., 2004; Foley et al., 2009; Wood et al., 2009). However, the authors stated that the effect of organic acid supplementation seems to be affected by diet. Greater reductions in methane production were obtained when diets were supplemented with high concentrate, most likely caused by a greater effect on the acetate to propionate (A:P) ratio in the rumen, in addition

to its ability to function as a H_2 sink. High levels of organic acid supplementation decrease DMI and ruminal pH, adversely affecting fiber fermentation in the rumen (Beauchemin et al., 2009). This can be overcome by encapsulation of organic acids with fat in order to slow their release in the rumen (Wallace et al., 2006; Martin et al., 2010), but to date it has been considered expensive as a mitigation strategy.

7 Using cereal grains to improve feed efficiency and reduce methane production

In general, ruminants lose from 2% to 12% of their ingested energy as methane (Ferrell, 1988; Harper et al., 1999). Thus, strategies that can reduce emissions of methane from cattle are beneficial not only to the environment but also to the animal, as they normally result in improved animal performance if the C and H_2 are captured in a form available to the animal as metabolizable energy. If H_2 is simply captured in another form or lost as H_2 gas then it will still be lost to the animal. Simply reducing the populations of methanogens can reduce methane production (at least temporarily), but this methane reduction alone does not affect animal performance (Patra and Saxena, 2009; Hook et al., 2010; Wright and Klieve, 2011). Thus it is imperative that the energy lost as methane must be redirected into forms that are usable by the animal, such as propionate, so that more metabolizable energy reaches the animal. As has been noted above, production of methane by cattle is affected by numerous factors such as the addition of ionophores and lipids to diets, the level of feed intake, and even by the type of diet fed to the cattle (Johnson and Johnson, 1995; Grainger and Beauchemin, 2011). In this section, the authors focus on the latter factor: the type of diet offered to cattle. More specifically, the authors focus on the use of cereal grains and how their increased proportion of inclusion in the diet (at the expense of roughage sources) can affect feed efficiency and production of methane.

Fermentation of rapidly degradable carbohydrates (e.g. starch) in the rumen promotes production of propionate, creating an alternative hydrogen sink to methanogenesis (Grainger and Beauchemin, 2011). In addition, this type of fermentation lowers ruminal pH, inhibits the growth of rumen methanogens and decreases rumen protozoal numbers. It has been suggested that ruminal protozoa serve to sequester starch as they engulf and subsequently ferment starch granules. As further evidence of the role that protozoa play in ruminal starch fermentation, more Entodinium were associated with corn than barley diets (Xia et al., 2015). A reduction in the protozoa population also contributes to methane mitigation as it limits the transfer of hydrogen from protozoa to methanogens (Grainger and Beauchemin, 2011).

A study conducted by Harper and collaborators measured methane emissions from cattle under grazing and feedlot conditions. Among their

findings, authors reported that when cattle were grazed on pasture, they wasted an equivalent of 7.7–8.4% of their GE intake as methane. However, when the same group of heifers was fed a highly digestible, high-grain diet, the amount of energy lost as methane decreased to only 1.9–2.2% of their GE intake. This difference of almost four times in magnitude demonstrates that cattle fed low-quality (high-fiber) diets produce more methane than cattle fed high-quality, high-grain diets (Harper et al., 1999). Similarly, in an effort to quantify the amount of methane produced at different ratios of forage to concentrate, Hales et al. (2014) fed diets varying from 2% to 14% alfalfa hay to beef steers. These authors detected a linear reduction in dry matter digestibility, digestible energy, and metabolizable energy as the level of forage increased (or as levels dry-rolled corn decreased) in the steers' diet. This resulted in lower energy retained as body weight gain. This lower retained energy was attributed to a couple of factors which included a higher loss of energy as methane, since steers had greater levels of forage in their diets. For instance, the percentage of energy intake lost as methane increased from 3.07% to 4.18% as the level of forage in the diet varied from 2% to 14%. The authors concluded that methane losses increased linearly as the level of forage increased in the diet, which coincided with less energy being retained by the animals.

Essentially, fermentation of cell wall components (cellulose and hemicellulose) results in higher acetate to propionate molar ratios, which leads to higher methane yields compared to the fermentation of soluble carbohydrates (Johnson and Johnson, 1995). Because the quantity of methane generated is related to the end products produced from carbohydrate fermentation in the rumen (Fahey and Berger, 1988), higher amounts of acetate at the expense of propionate results in higher methane production. As illustrated in Fig. 3, if

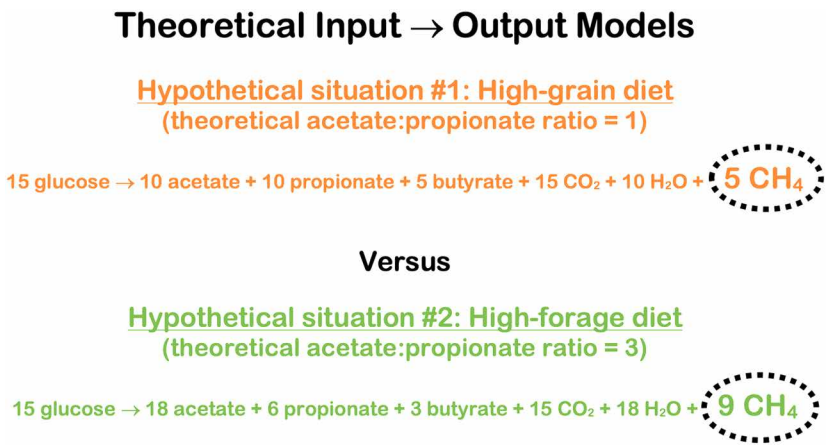

Figure 3 Theoretical models illustrating the ruminal fermentation of 15 molecules of glucose under two hypothetical situations: High-grain and high-forage diets.

a high-grain or a high-forage diet is fed to cattle, resulting in distinct acetate to propionate ratios, the same amount of glucose available for fermentation would result in very different quantities of methane being formed. In fact, for the two examples presented in Fig. 1 the amount of methane would be 1.8 times greater in the high-forage diet, compared to the high-grain diet. Thus, there is a positive relationship between acetate and methane, and a negative relationship between propionate and methane. According to Fahey and Berger (1988), these relationships stem from the fact that when the propionate is being formed, more C and H atoms present in glucose are accounted for. In contrast, more H atoms are released when glucose is converted to acetate, resulting in greater quantities of methane being formed.

About two-thirds of the methane produced in nature originate from acetate, more specifically, from the methyl group of acetate (Ferry, 1992; Wolfe, 1993). As previously stated, from the productivity stand point, methane is considered a waste of energy. Therefore, one of the reasons why animal performance is normally improved when more propionate is produced (instead of acetate) is because more C and H atoms become propionate instead of methane, increasing the metabolizable energy level of the diet (Fahey and Berger, 1988). In practical situations, the ratio of acetate to propionate normally varies from 0.9 to 4.0, and consequently, corresponding methane losses vary widely as well. However, as highlighted in this section, the literature clearly shows that feeding more grains to ruminants can substantially decrease their methane emissions. Not only can it lower methane emissions per kg of DM consumed, but it can also reduce total methane emissions per kg of final product (e.g. milk, meat) since more grains normally improve animal performance. Therefore, the development of strategies to mitigate emissions of methane by cattle is critical and achieving this goal while not reducing or even improving animal performance is highly desirable; therefore, increasing the levels of cereal grains fed to cattle 'kills two birds with one stone'.

8 Microbiology of cereal grain fermentation

The rumen microflora is a dense and diverse consortium of archaea, bacteria, fungi, and protozoa all competing for resources through the diet of their ruminant host. Uniquely, these microorganisms have given the ruminant animal an evolutionary niche in which the microbes are able to utilize complex carbohydrates, such as cellulose and hemicellulose (fibrolytic microbes), through certain metabolic pathways which yield fermented by-products that are absorbed and utilized by the animal. The ruminant host in return provides a warm (39°C), anaerobic, and nutrient-rich media for the microbes to thrive. Starch-fermenting microbes contain alpha-amylase and beta-amylase which allow them to degrade large polysaccharides of glucose in forms of amylose and amylopectin.

Generally speaking, most starch and forage fermentation results in short-chain fatty acid production (formate, acetate, propionate, butyrate, and branch-chain SCFA), adenosine triphosphate production for microbial cell energy, microbial crude protein synthesis, and heat + gas (methane and carbon dioxide) as an energetic loss to the animal and microbes.

Drs. Robert Hungate and Marvin Bryant are the forebearers of modern ruminal microbiology and microbial ecology (Krause et al., 2013), and were two of the first ruminant microbiologists to study and isolate individual ruminal microbes in pure culture to further understand the symbiotic relationship which exists between microbes and the ruminant animal (Hungate, 1944, 1947; Bryant, 1959). Lastly, a ruminant animal relies on the majority of fermentation end products from foregut fermentation in the rumen, but just like in a monogastric digestive system, there is a second site of fermentation in the lower digestive tract of a ruminant which accounts for 10–20% of the energy for the animal (Russell, 2002). In vitro studies have shown that specific methane production from cecal digesta is 6.8% of the rumen digesta (Freetly et al., 2015). Furthermore, no differences in methanogenic bacterial populations were observed between the two sites of digestion. However, for the purposes of this publication the predominant starch utilizing microbes and methane-producing archaea found in the rumen will be discussed in full.

The early work of Hungate and Bryant led to the general classifications of microbes into primary niche categories: fibrolytic, simple carbohydrate, obligate amino acid, and methane-producing fermenting microorganisms. Although it is easy to divide these ruminal microbes into categories, it is important to note that current technology has indicated that the microbial diversity may be larger than scientists have previously recognized. The microbial ecology of the rumen is very rich, and a variety of starch-fermenting bacteria and methane-producing archaea are known because they have all been grown and studied in pure culture experimentations. Table 1 below illustrates the niches and by-products of the starch-degrading and methane-producing microbes modified from Russell (2002).

As research has moved from culture-based methodologies to Next-Generation Sequencing and the Hungate 1000 project data, further information about changes in the microbial population have emerged (McAllister et al., 2015; Seshadri et al., 2018). Using starch as a selective agent, ruminal fluid cultures had increased *Prevotella* populations associated with starch feeding (Bandarupalli, 2017). However many of the bacterial species thought to be responsible for most of the starch fermentation in the rumen based on in vitro studies (e.g. *Streptococus bovis*, *Ruminobacter amylophilus*, *Succinomonas amylolytica*, *Butyrivibrio fibrisolvens*) were not detected at high populations in cattle-fed corn or barley diets (Xia et al., 2015). Cattle with frothy bloat from grain rations had increased populations of *Clostridium*, *Eubacterium*, and *Butyrivibrio*, while having reduced *Prevotella* populations (Pitta et al., 2016).

Table 1 Ruminal species and their niche(s) and by-products

Species	Primary niches	Products
Butyrivibrio fibrisolvens	cellulose, hemicellulose, starch, pectin, and sugar	butyrate, formate, lactate, and acetate
Ruminobacter amylophilus	starch	succinate, formate, and acetate
Selenomonas ruminantium	sugar, starch, and lactate	lactate, acetate, propionate, butyrate, and H_2
Prevotella ruminicola, P. albensis, P. bryantii, P. brevis	starch, hemicellulose, pectin, β-glucans, protein	succinate, acetate, formate, propionate
Succinomonas amylolytica	starch	succinate, acetate, propionate
Streptococcus bovis	starch, sugar	lactate, acetate, formate, ethanol
Methanobrevibacter ruminantium	H_2, carbon dioxide, formate	methane
Methanosphaera stadtmaniae[a]	H_2, methanol	methane

[a] Information is cited from Liu and Whitman (2008).
Source: modified from Russell (2002).

9 Bacteria and archaea involved in fermentation

9.1 Butyrivibrio fibrisolvens

Butyrivibrio sp. are versatile, gram-negative, rod-shaped microbes which have enzyme capabilities of degrading pentose and hexose sugars, along with starch and hemicellulose. Bryant first isolated the microbe and classified this genus appropriately based on its characteristic butyrate production: hexose, pyruvate, acetyl CoA, butyrate ($NADP^+$ regeneration), and acetate (ATP generation). Some *Butyrivibrio* sp. have butyrate kinase and will never generate lactate; however, the *Butyrivibrio* sp. that do not have butyrate kinase will revert carbon skeletons to lactate when acetate is absent.

9.2 Ruminobacter amylophilus

The ruminal bacteria *R. amylophilus* is a great model for starch degradation because it only uses α 1–4 polymers of glucose for energy, and it was the first reported ruminal bacterium demonstrated to exhibit starch-binding sites on the cell surface similar to the intestinal *Bacteroides* (Anderson, 1995). *R. amylophilus* is a gram-negative, anaerobic, and proteolytic (only with ammonia nitrogen) microbe that relies on polysaccharide degradation of maltose, maltodextrins, and starch.

9.3 Selenomonas ruminantium

Selenomonas ruminantium is a gram-negative and strictly anaerobic bacterium with a rod-shaped structure and unique flagella that allows the microbe to turn/spin on both axes. *S. ruminantium* can grow very rapidly in the presence of sugar, and ferments almost strictly in a homolactic manner (pyruvate to lactic acid). Although *Sel. ruminantium*'s niche is related to monosaccharides or disaccharides, in the absence of simple sugars *Sel. ruminantium* can utilize lactic acid and dextrins from other starch-fermenting microbes as its main source of ATP generation.

9.4 Prevotella sp.

Prevotella ruminicola was the first microbe to be reclassified from the *Bacteroides ruminicola* classification because of its sensitivity to bile salts and hexose monophosphate pathway (Russell, 2002). Around the mid-1990s, *P. ruminicola* was then further reclassified into a variety of species (*ruminicola*, *albensis*, *bryantii*, and *brevis*) based on the enzymatic capabilities and xylose utilization: carboxymethyl cellulase negative, produced deoxyribonuclease, and no xylose utilization; *Prevotella brevis*, carboxymethyl cellulase positive, produced deoxyribonuclease, and utilized xylose; *Prevotella bryantii*, and carboxymethyl cellulase positive and did not produce deoxyribonuclease; *Prevotella ruminicola* (Avgustin et al., 1997). *Prevotella sp.* are pleomorphic rod-shaped bacteria which generate a large amount of succinate, utilize multiple substrates for energy except for cellulose, and are in typically the most abundant genus within the rumen.

9.5 Succinomonas amylolytica

Succinomonas amylolytica is a strict anaerobe, rod to coccoid shaped cell that occupies a starch-degrading niche in the rumen (Bryant et al., 1957). *Suc. amylolytica* grows well in cultures rich in trypticase and yeast when rumen fluid is removed but does not grow at all with no bicarbonate as indicated. *Suc. amylolytica* produces little amounts of propionate compared to its major by-products: succinate and acetate.

9.6 Streptococcus bovis

Streptococcus bovis is a gram-positive, facultative anaerobe which grows the fastest in reduced environments in the absence of oxygen, and is ovoid in shape. *Str. bovis* is one of the fastest growing bacteria in the rumen with a published doubling time as short as 24 min (Russell and Robinson, 1984). However, there are unfortunate repercussions of this fast doubling time because *Str. bovis*'

homolactic fermentations produce excessive amounts of lactic acid when sugars and starches are readily available which results in decrease in the pH and an increase in the acidotic conditions for ruminant animals.

9.7 Archaea involved in fermentation

Methanobrevibacter ruminantium and *Methanosphaera stadtmanae*, both belong to the archaea family Methanobacteriaceae. Methane is a major by-product of ruminal fermentation and can produce as much as roughly 400 liters from mature cattle in a day. Archaea are widely distributed in cattle, and *Methanobrevibacter gottschalkii* and *Methanobrevibacter ruminantium* accounted for up to 74% of all archaea (Henderson et al., 2015). Five dominant methanogen groups comprised more than 89% of the archeal communities (Henderson et al., 2015).

Ruminant microbiologists and nutritionists have investigated alternatives to reduce methane production because as much as 10% of dietary GE is lost through methane production (Blaxter, 1962). However, methanogenesis is a natural process in the rumen and is needed to maintain low hydrogen-ion concentrations by methanogens which utilize H^+ and CO_2 and produce methane as a by-product. Methane-producing bacteria tend to have a stronger symbiotic relationship and effectiveness with fibrolytic microbes versus starch-degrading bacteria. It has long been understood that when cereal grains are added to a rumen diet, the propionate increases and methane production decreases (Van Kessel and Russell, 1996; Czerkawski, 1986). Frothy bloat is a disease encountered in many feedlot cattle as a result of gas being captured in the viscous ruminal fluid of cattle fed higher grain diets, and archeael populations (most notably *Methanobrevibacter*) were higher in bloated cattle compared to controls (Pitta et al., 2016) (Fig. 4).

10 Feed retention time

One of the factors that contributes most to degradation of feedstuffs and absorption of nutrients is the duration feedstuffs are retained in the rumen before passing through the reticulo-omasal orifice and into the rest of the GIT. The retention time of feedstuffs in the rumen varies depending on a variety of factors, one of which being the diet fed to the animal. Concentrate diets high in readily fermentable starches decrease the retention time, as they are broken down rapidly. Increasing the dilution rate in a RUSITEC (rumen simulation technique) fermenter with ovine rumen fluid inoculum showed an increase in DMI as well as VFA and ammonia-N production (Martínez et al., 2009).

When retention time of feedstuffs in the rumen decreases, the rate of methane production by the methanogens in the rumen also decreases because

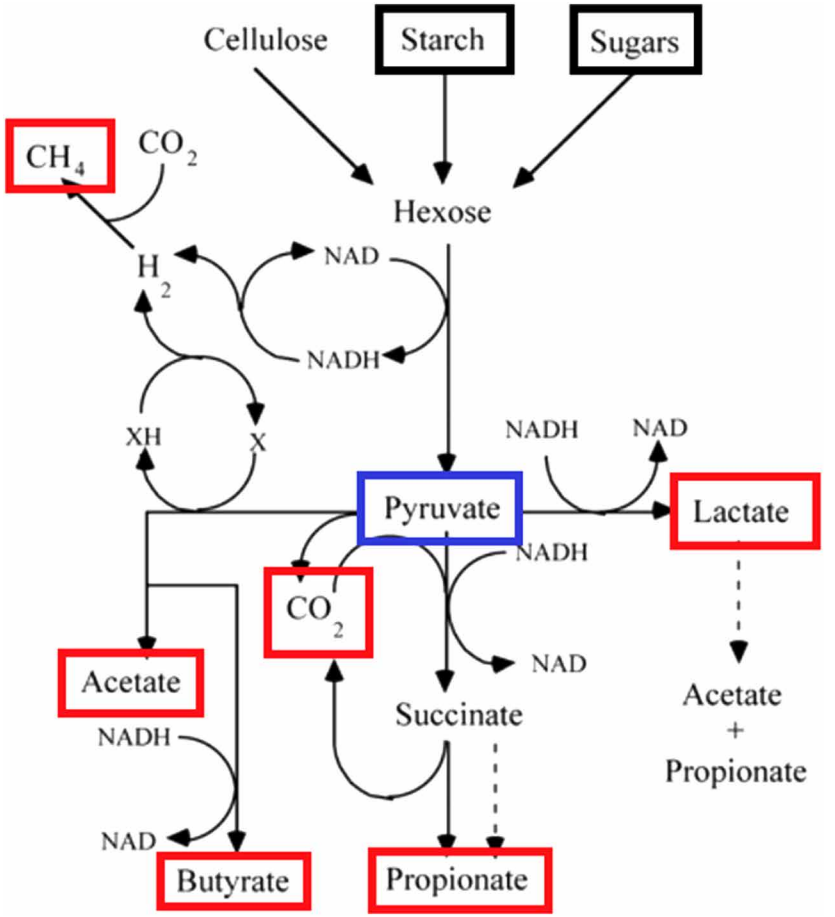

Figure 4 The black boxes indicate simple carbohydrate substrates in ruminant diets. The red boxes indicate end products of microbial fermentation. The blue box highlights pyruvate, which is the pivot point/intermediate for several microbial pathways. Source: modified from Russell and Rychlik (2001).

methanogens have a relatively slow growth rate and are unable to maintain adequate numbers to sustain greater methane production. An increase in the dilution rate of rumen fluid can inhibit the growth of methanogens, limiting their abundance in the rumen (Eun et al., 2004) and ultimately leading to a reduction in the methane they are able to produce. This effect can be seen by altering the diet or other factors. Okine et al. (1989) found that displacing free volume in the rumen with inert materials, methane production decreased 29% along with an increase in rumen fluid dilution rate of 43%. A quarter of the variation in methane production by a rumen could be explained by how

quickly the fluid is washed out of the rumen during and after feeding (Okine et al., 1989).

An animal can also be genetically selected for a particular level of methane production, and they have similar patterns between methane production and rumen retention time. Sheep producing less methane have a decrease in the retention times of both the solid and liquid dilution rates accounting for 59% and 70% of the variation in methane production, respectively (Goopy et al., 2014). Sheep that produced less methane also had a lower amount of particulate (g DM) in their rumen than sheep producing more methane (Goopy et al., 2014). A positive relationship exists between methane production and rumen liquid and particulate mean retention times. As noted numerous times above, methane production is correlated to ruminal retention time.

11 Acidosis and other negative feed effects

If too much grain is introduced into the diet too quickly, the pH of the rumen can drop rapidly, causing ruminal acidosis. Acidosis can be either acute or subacute (chronic). Acute acidosis occurs when the pH of the rumen drops abruptly after cattle consume a lot of readily fermentable starch. Subacute acidosis occurs when the pH of the rumen is consistently too low (pH between 5.1 and 5.6) which can happen in feedlot settings where cattle are pushed to their limits when it comes to concentrates in the diet (Brown et al., 2006). Subacute acidosis does not manifest itself into observable symptoms and the cattle may not even appear to be sick until death occurs (Owens et al., 1998). Not all animals are affected the same by adding grain to the diets. On the same diet, some cattle have shown severe adverse effects, and some have only shown mild effects such as diarrhea (Brown et al., 2006). Regardless of severity, treatment is needed because it can cause animals to either reduce their DMI or go 'off-feed' completely. In order to not suddenly and drastically disrupt the microbial populations within the ruminal environment, using a step-up ration can gradually allow the ruminal microbial population to adapt and reduce abundance of *Str. bovis* which can overgrow, rapidly produce lactic acid, and decrease the pH of the rumen (Wells et al., 1997). This helps the microbes in the rumen adapt to the new environment, so they are better suited for it.

A drop in ruminal pH cause risk for acidosis but itcan also damage the lining of the rumen epithelium causing keratinization or parakeratosis which is a hardening of the ruminal epithelial cells used for absorption. A diet with 60% grain showed an increase in the ruminal epithelial cell thickening in goats when compared to 0% and 30% grain diets. This damage ultimately leads to a decrease in absorption of nutrients and VFAs from the rumen because they cannot travel across the epithelial cells into the bloodstream to be absorbed

by the animal (Hinders and Owen, 1965). The lack of absorption causes a decrease in the energy availability to the animal and negatively impacts the overall production of the animal.

Not only does feeding high-grain diets decrease the pH and damage the lining of the rumen, there is also an increase in the prevalence liver abscesses. When the ruminal epithelium becomes damaged by low pH associated with feeding starch, pathogens have an opportunity to colonize the rumen. An example is the anaerobic bacteria *Fusobacterium necrophorum*, which is the main cause of liver abscesses (Nagaraja and Chengappa, 1998). After colonization, the bacteria can enter the blood stream through the damaged epithelium and eventually end up being filtered by the liver where it can cause abscesses (Nagaraja and Chengappa, 1998). This process can occur during all levels of production in both dairy and beef operations; however, it is most common in feedlots when the cattle are placed on a high-concentrate diet.

Liver abscesses are a major concern in the feedlot system with 12–32% of young feedlot cattle exhibiting significant liver damage at slaughter (Brink et al., 1990). This causes economic loss for packers because they cannot sell the liver and must also spend more time trimming the carcass. Not only do liver abscesses cause the liver to be condemned, but the animal's prior performance also gets affected. Cattle with liver abscesses have a lower feed intake and less gain (Brink et al., 1990). A preventive measure in the past, the antibiotic tylosin phosphate has been included in the diet to reduce ruminal *F. necrophorum*, prevent liver abscesses, and increase weight gain (Brown et al., 1975).

12 Summary

The use of starch-containing cereal grains as a feedstuff improves the efficiency because these energy-dense feedstuffs are more fermentable than forages and the fermentation of starch generally results in a shift in VFAs and less GHGs. The reduction of the reducing equivalent sink (CH_4) for disposal of accumulating NADH from catabolic reactions further ensures an increase in the production of propionate, a reduced VFA. Because propionate is a glucogenic VFA, it yields more ATP when metabolized by the ruminant animal and is also associated with higher levels of intramuscular marbling. Furthermore, because the grain shifts the fermentation profile away from acetate production, there is a concomitant reduction in CO_2 production by the ruminal carbohydrate fermentation, which ensures an improvement in sustainability of animal production. Thus, feeding ruminants cereal grains has profound impact on animal physiology as well as efficiency, and reduces the environmental footprint of ruminant animal production.

Feeding high-starch diets to cattle has many benefits to carcass quality and growth efficiency in addition to reducing methane production. However,

there are still negative consequences to grain feeding, including lowering of pH and inducing acidosis in cattle, as well as liver abscesses. High-starch rations have benefits to producers and environmental sustainability that are important to maintain, but the biochemical basis of these benefits must be more well-characterized. The impact of starch feeding on the composition of the microbial population of the rumen and lower GIT is profound, and is related to the efficiency as well as the sustainability of beef production. Until these benefits derived from starch feeding can be replicated via other methods, then feeding high-grain rations will be favored, when viewed from both the economic and environmental lenses.

13 Where to look for further information

The topic of grain feeding in cattle is inextricably enmeshed with current discussions of environmental impact and long-term production sustainability. However, this present chapter is focused primarily on the impact within the animal, and what effects these diets have on the microbial population. We must understand the succession of events that occur in the ruminal microbial population when cattle are fed high-starch rations. If we can fully understand the shifts in the microbiome and metabolome of the rumen, then we can begin to prepare specific interventions or remedies to ameliorate the negative impacts (such as acidosis, both acute and sub-acute) and reduce production of wasteful end products (e.g. methane and CO_2) of fermentation. Furthermore, we must understand the effect of other least-cost feed components on the microbial populations as well and their impacts on animal health, immunity, and food safety.

There are many organizations involved in research that are aimed at furthering our understanding of the impact of high-grain rations on the rumen ecosystem and physiology. In the United States, the Agricultural Research Service, most notably at the Meat Animal Research Center, along with the Rowett Research Institute in Aberdeen (now a part of University of Aberdeen) and the Roslin Institute, as well as INRA in France, and of course CSIRO in Australia. Many university researchers around the world have been involved in expanding our understanding, frankly too many to list here because of the quality scientists that would inevitably be inadvertently left out of the list. Research in this arena is highly active and ongoing, and new discoveries are made yearly Many of the most active researchers in this field attend the Congress on Gastrointestinal Function (https://www.congressgastrofunction.org/) as well as the companion INRA-Aberdeen Joint Symposium on Gastrointestinal Microbiology. One of the best new resources available to examine many of the issues described here is: Tedeschi and Nagaraja (2020).

14 References

Anderson, K. L. 1995. Biochemical analysis of starch degradation by *Ruminobacter amylophilus* 70. *Applied and Environmental Microbiology* 61(4), 1488–91. doi:10.1128/AEM.61.4.1488-1491.1995.

Archimède, H., Eugène, M., Marie Magdeleine, C., Boval, M., Martin, C., Morgavi, D. P., Lecomte, P. and Doreau, M. 2011. Comparison of methane production between C3 and C4 grasses and legumes. *Animal Feed Science and Technology* 166–167, 59–64. doi:10.1016/j.anifeedsci.2011.04.003.

Avgustin, G., Wallace, R. J. and Flint, H. J. 1997. Phenotypic diversity among ruminal isolates of *Prevotella ruminicola*: proposal of *Prevotella brevis* sp. nov., *Prevotella bryantii* sp. nov., and *Prevotella albensis* sp. nov. and redefinition of *Prevotella ruminicola*. *International Journal of Systematic Bacteriology* 47(2), 284–8. doi:10.1099/00207713-47-2-284.

Awika, J. M. 2011. Major cereal grains production and use around the world. *ACS Symposium Series* 1089(1), 1–13. doi:10.1021/bk-2011-1089.ch001.

Bandarupalli, V. 2017. Identification of novel rumen bacteria using starch as a selective nutrient in batch cultures. *Journal of Animal Science* 95(suppl_4), 305. doi:10.2527/asasann.2017.623.

Beauchemin, K. A., McAllister, T. A., Dong, Y., Farr, B. I. and Cheng, K. J. 1994. Effects of mastication on digestion of whole cereal grains by cattle. *Journal of Animal Science* 72(1), 236–46. doi:10.2527/1994.721236x.

Beauchemin, K. A., Kreuzer, M., O'mara, F. and McAllister, T. A. 2008. Nutritional management for enteric methane abatement: a review. *Australian Journal of Experimental Agriculture* 48(2), 21–7. doi:10.1071/EA07199.

Beauchemin, K. A., McAllister, T. A. and McGinn, S. M. 2009. Dietary mitigation of enteric methane from cattle. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* 4(35), 1–18. doi:10.1079/PAVSNNR20094035.

Beever, D. E., Dhanoa, M. S., Losada, H. R., Evans, R. T., Cammell, S. B. and France, J. 1986. The effect of forage species and stage of harvest on the processes of digestion occurring in the rumen of cattle. *The British Journal of Nutrition* 56(2), 439–54. doi:10.1079/bjn19860124.

Benchaar, C., Pomar, C. and Chiquette, J. 2001. Evaluation of dietary strategies to reduce methane production in ruminants: a modelling approach. *Canadian Journal of Animal Science* 81(4), 563–74. doi:10.4141/A00-119.

Bergen, W. G. and Bates, D. B. 1984. Ionophores: their effect on production efficiency and mode of action. *Journal of Animal Science* 58(6), 1465–83. doi:10.2527/jas1984.5861465x.

Blaxter, K. 1962. The energy metabolism of ruminants. In: Blaxter, K. (Ed.), *The Energy Metabolism of Ruminants*. Charles C. Thomas, Springfield, IL, pp. 197–200.

Boadi, D., Benchaar, C., Chiquette, J. and Massao, D. 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: update review. *Canadian Journal of Animal Science* 84(3), 319–35. doi:10.4141/A03-109.

Brink, D. R., Lowry, S. R., Stock, R. A. and Parrott, J. C. 1990. Severity of liver-abscesses and efficiency of feed-utilization of feedlot cattle. *Journal of Animal Science* 68(5), 1201–7. doi:10.2527/1990.6851201x.

Brown, H., Bing, R. F., Grueter, H. P., McAskill, J. W., Cooley, C. O. and Rathmacher, R. P. 1975. Tylosin and chlortetracycline for the prevention of liver abscesses, improved weight gains and feed efficiency in feedlot cattle. *Journal of Animal Science* 40(2), 207–13. doi:10.2527/jas1975.402207x.

Brown, M. S., Ponce, C. H. and Pulikanti, R. 2006. Adaptation of beef cattle to high-concentrate diets: performance and ruminal metabolism. *Journal of Animal Science* 84(Suppl.), E25–33. doi:10.2527/2006.8413_supple25x.

Bryant, M. P. 1959. Bacterial species of the rumen. *Bacteriological Reviews* 23(3), 125–53. doi:10.1128/MMBR.23.3.125-153.1959.

Bryant, M. P., Small, N., Bouma, C. and Chu, H. 1957. *Bacteriodes ruminicola* N. Sp. and *Succinimonas amylolytica* the new genus and species. *Journal of Bacteriology* 76, 15–23.

Buddle, B. M., Denis, M., Attwood, G. T., Altermann, E., Janssen, P. H., Ronimus, R. S., Pinares-Patiño, C. S., Muetzel, S. and Wedlock, D. N. 2011. Strategies to reduce methane emissions from farmed ruminants grazing on pasture. *The Veterinary Journal* 188(1), 11–7. doi:10.1016/j.tvjl.2010.02.019.

Callaway, T. R. and Martin, S. A. 1996. Effects of organic acid and monensin treatment on in vitro mixed ruminal microorganism fermentation of cracked corn. *Journal of Animal Science* 74(8), 1982–9. doi:10.2527/1996.7481982x.

Czerkawski, J. W. 1986. *An Introduction to Rumen Studies*. Elsevier Press, Oxford, UK.

Depenbusch, B. E., Nagaraja, T. G., Sargeant, J. M., Drouillard, J. S., Loe, E. R. and Corrigan, M. E. 2008. Influence of processed grains on fecal pH, starch concentration, and shedding of *Escherichia coli* O157 in feedlot cattle. *Journal of Animal Science* 86(3), 632–9. doi:10.2527/jas.2007-0057.

Dong, H., Mangino, J., McAllister, T. A., Hatfield, J. L., Johnson, D. E., Lassey, K. R., Lima, M. A. d. and Romanovskaya, A. 2006. Emissions from livestock and manure management. In: Eggleston, S., Buendia, L., Miwa, K., Ngara, T. and Tanabe, K. (Eds), *Guidelines for National Greenhouse Gas Inventories* (vol. 4). Institute for Global Environmental Strategies, Hayama, Japan, pp. 10.11–87.

Eckard, R. J., Grainger, C. and De Klein, C. A. M. 2010. Options for the abatement of methane and nitrous oxide from ruminant production: a review. *Livestock Science* 130(1–3), 47–56. doi:10.1016/j.livsci.2010.02.010.

Ellis, J. L., Dijkstra, J., Kebreab, E., Bannink, A., Odongo, N. E., McBride, B. W. and France, J. 2008. Aspects of rumen microbiology central to mechanistic modelling of methane production in cattle. *The Journal of Agricultural Science* 146(2), 213–33. doi:10.1017/S0021859608007752.

Etuk, E., Ifeduba, A., Okata, U., Chiaka, I., Okoli, I. C., Okeudo, N., Esonu, B., Udedibie, A. and Moreki, J. 2012. Nutrient composition and feeding value of sorghum for livestock and poultry: a review. *Journal of Animal Science Advances* 2(6), 510–24.

Eun, J. S., Fellner, V. and Gumpertz, M. L. 2004. Methane production by mixed ruminal cultures incubated in dual-flow fermentors. *Journal of Dairy Science* 87(1), 112–21. doi:10.3168/jds.S0022-0302(04)73148-3.

Fahey, G. C. and Berger, L. L. 1988. Carbohydrate nutrition of ruminants. In: Church, D. C. (Ed.), *The Ruminant Animal: Digestive Physiology and Nutrition*. Prentice Hall, Englewood Cliffs, NJ, pp. 269–97.

Ferrell, C. L. 1988. Energy metabolism. In: Church, D. C. (Ed.), *The Ruminant Animal: Digestive Physiology and Nutrition*. Prentice Hall, Englewood Cliffs, NJ.

Ferry, J. G. 1992. Methane from acetate. *Journal of Bacteriology* 174(17), 5489–95. doi:10.1128/jb.174.17.5489-5495.1992.

Firkins, J. L., Berger, L. L. and Fahey Jr., G. C. 1985. Evaluation of wet and dry distillers grains and wet and dry corn gluten feeds for ruminants. *Journal of Animal Science* 60(3), 847–60. doi:10.2527/jas1985.603847x.

Foley, P. A., Kenny, D. A., Callan, J. J., Boland, T. M. and O'Mara, F. P. 2009. Effect of DL-malic acid supplementation on feed intake, methane emission, and rumen fermentation in beef cattle. *Journal of Animal Science* 87(3), 1048–57. doi:10.2527/jas.2008-1026.

Forster, P., Ramaswamy, V., Artaxo, P., Berntsen, T., Betts, R., Fahey, D. W., Haywood, J., Lean, J., Lowe, D. C., Myhre, G., Nganga, J., Prinn, R., Raga, G., Schulz, M. and Van Dorland, R. 2007. Changes in atmospheric constituents and in radiative forcing. In: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K. B., Tignor, M. and Miller, H. L. (Eds), *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press, Cambridge, UK and New York, NY.

Freetly, H. C., Lindholm-Perry, A. K., Hales, K. E., Brown-Brandl, T. M., Kim, M., Myer, P. R. and Wells, J. E. 2015. Methane production and methanogen levels in steers that differ in residual gain. *Journal of Animal Science* 93(5), 2375–81. doi:10.2527/jas.2014-8721.

Fron, M., Madeira, H., Richards, C. and Morrison, M. 1996. The impact of feeding condensed distillers byproducts on rumen microbiology and metabolism. *Animal Feed Science and Technology* 61(1–4), 235–45. doi:10.1016/0377-8401(95)00943-4.

Gerber, P. J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A. and Tempio, G. 2013. *Tackling Climate Change through Livestock: a Global Assessment of Emissions and Mitigation Opportunities*. Food and Agriculture Organization of the United Nations (FAO), Rome.

Gómez, L. M., Posada, S. L. and Olivera, M. 2016. Starch in ruminant diets: a review. *Revista Colombiana de Ciencias Pecuarias* 29(2), 77–90. doi:10.17533/udea.rccp.v29n2a01.

Goopy, J. P., Donaldson, A., Hegarty, R., Vercoe, P. E., Haynes, F., Barnett, M. and Oddy, V. H. 2014. Low-methane yield sheep have smaller rumens and shorter rumen retention time. *The British Journal of Nutrition* 111(4), 578–85. doi:10.1017/S0007114513002936.

Grainger, C. and Beauchemin, K. A. 2011. Can enteric methane emissions from ruminants be lowered without lowering their production? *Animal Feed Science and Technology* 166–167, 308–20. doi:10.1016/j.anifeedsci.2011.04.021.

Guan, H., Wittenberg, K. M., Ominski, K. H. and Krause, D. O. 2006. Efficacy of ionophores in cattle diets for mitigation of enteric methane. *Journal of Animal Science* 84(7), 1896–906. doi:10.2527/jas.2005-652.

Hales, K. E., Brown-Brandl, T. M. and Freetly, H. C. 2014. Effects of decreased dietary roughage concentration on energy metabolism and nutrient balance in finishing beef cattle. *Journal of Animal Science* 92(1), 264–71. doi:10.2527/jas.2013-6994.

Hammond, K. J., Burke, J. L., Koolaard, J. P., Muetzel, S., Pinares-Patiño, C. S. and Waghorn, G. C. 2013. Effects of feed intake on enteric methane emissions from sheep fed fresh white clover (*Trifolium repens*) and perennial ryegrass (*Lolium perenne*) forages. *Animal Feed Science and Technology* 179(1–4), 121–32. doi:10.1016/j.anifeedsci.2012.11.004.

Haque, M. N. 2018. Dietary manipulation: a sustainable way to mitigate methane emissions from ruminants. *Journal of Animal Science and Technology* 60(1), 15. doi:10.1186/s40781-018-0175-7.

Harper, L. A., Denmead, O. T., Freney, J. R. and Byers, F. M. 1999. Direct measurements of methane emissions from grazing and feedlot cattle. *Journal of Animal Science* 77(6), 1392–401. doi:10.2527/1999.7761392x.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Janssen, P. H., Abecia, L., Angarita, E., Aravena, P., Arenas, G. N., Ariza, C., Attwood, G. T., Avila, J. M., Avila-Stagno, J., Bannink, A., Barahona, R., Batistotti, M., Bertelsen, M. F., Brown-Kav, A., Carvajal, A. M., Cersosimo, L., Chaves, A. V., Church, J., Clipson, N., Cobos-Peralta, M. A., Cookson, A. L., Cravero, S., Carballo, O. C., Crosley, K., Cruz, G., Cucchi, M. C., De La Barra, R., De Menezes, A. B., Detmann, E., Dieho, K., Dijkstra, J., Dos Reis, W. L. S., Dugan, M. E. R., Ebrahimi, S. H., Eythórsdóttir, E., Fon, F. N., Fraga, M., Franco, F., Friedeman, C., Fukuma, N., Gagić, D., Gangnat, I., Grilli, D. J., Guan, L. L., Miri, V. H., Hernandez-Sanabria, E., Gomez, A. X. I., Isah, O. A., Ishaq, S., Jami, E., Jelincic, J., Kantanen, J., Kelly, W. J., Kim, S. H., Klieve, A., Kobayashi, Y., Koike, S., Kopecny, J., Kristensen, T. N., Krizsan, S. J., LaChance, H., Lachman, M., Lamberson, W. R., Lambie, S., Lassen, J., Leahy, S. C., Lee, S. S., Leiber, F., Lewis, E., Lin, B., Lira, R., Lund, P., Macipe, E., Mamuad, L. L., Mantovani, H. C., Marcoppido, G. A., Márquez, C., Martin, C., Martinez, G., Martinez, M. E., Mayorga, O. L., McAllister, T. A., McSweeney, C., Mestre, L., Minnee, E., Mitsumori, M., Mizrahi, I., Molina, I., Muenger, A., Munoz, C., Murovec, B., Newbold, J., Nsereko, V., O'Donovan, M., Okunade, S., O'Neill, B., Ospina, S., Ouwerkerk, D., Parra, D., Pereira, L. G. R., Pinares-Patino, C., Pope, P. B., Poulsen, M., Rodehutscord, M., Rodriguez, T., Saito, K., Sales, F., Sauer, C., Shingfield, K., Shoji, N., Simunek, J., Stojanović-Radić, Z., Stres, B., Sun, X., Swartz, J., Tan, Z. L., Tapio, I., Taxis, T. M., Tomkins, N., Ungerfeld, E., Valizadeh, R., Van Adrichem, P., Van Hamme, J., Van Hoven, W., Waghorn, G., Wallace, R. J., Wang, M., Waters, S. M., Keogh, K., Witzig, M., Wright, A. D. G., Yamano, H., Yan, T., Yanez-Ruiz, D. R., Yeoman, C. J., Zambrano, R., Zeitz, J., Zhou, M., Zhou, H. W., Zou, C. X. and Zunino, P. 2015. Rumen microbial community composition varies with diet, host, but a core microbiome is found across a wide geographical range. *Scientific Reports* 5.

Herrera-Saldana, R. E., Huber, J. T. and Poore, M. H. 1990. Dry matter, crude protein, and starch degradability of five cereal grains. *Journal of Dairy Science* 73(9), 2386–93. doi:10.3168/jds.S0022-0302(90)78922-9.

Hinders, R. G. and Owen, F. G. 1965. Relation of ruminal parakeratosis development to volatile fatty acid absorption. *Journal of Dairy Science* 48(8), 1069–73. doi:10.3168/jds.S0022-0302(65)88393-X.

Hindrichsen, I. K., Wettstein, H. -R., Machmüller, A. and Kreuzer, M. 2006. Methane emission, nutrient degradation and nitrogen turnover in dairy cows and their slurry at different milk production scenarios with and without concentrate supplementation. *Agriculture, Ecosystems and Environment* 113(1–4), 150–61. doi:10.1016/j.agee.2005.09.004.

Holter, J. B. and Young, A. J. 1992. Methane prediction in dry and lactating Holstein cows. *Journal of Dairy Science* 75(8), 2165–75. doi:10.3168/jds.S0022-0302(92)77976-4.

Hook, S. E., Wright, A. D. and McBride, B. W. 2010. Methanogens: methane producers of the rumen and mitigation strategies. *Archaea* 2010, 945785. doi:10.1155/2010/945785.

Huhtanen, P. and Sveinbjörnsson, J. 2006. Evaluation of methods for estimating starch digestibility and digestion kinetics in ruminants. *Animal Feed Science and Technology* 130(1–2), 95–113. doi:10.1016/j.anifeedsci.2006.01.021.

Hungate, R. E. 1944. Studies on cellulose fermentation. I: The culture and physiology of an anaerobic cellulose-digesting bacterium. *Journal of Bacteriology* 48(5), 499–513. doi:10.1128/JB.48.5.499-513.1944.

Hungate, R. E. 1947. Studies on cellulose fermentation. III: The culture and isolation of cellulose-decomposing bacteria from the rumen of cattle. *Journal of Bacteriology* 53(5), 631–45. doi:10.1128/JB.53.5.631-645.1947.

Hungate, R. E. 1966. *The Rumen and Its Microbes*. Academic Press, New York, NY.

Huntington, G. B. 1997. Starch utilization by ruminants: from basics to the bunk. *Journal of Animal Science* 75(3), 852–67. doi:10.2527/1997.753852x.

Iannotti, E. L., Kafkewitz, D., Wolin, M. J. and Bryant, M. P. 1973. Glucose fermentation products of *Ruminococcus albus* grown in continuous culture with *Vibrio succinogenes*: changes caused by interspecies transfer of H_2. *Journal of Bacteriology* 114(3), 1231–40. doi:10.1128/JB.114.3.1231-1240.1973.

Johnson, K. A. and Johnson, D. E. 1995. Methane emissions from cattle. *Journal of Animal Science* 73(8), 2483–92. doi:10.2527/1995.7382483x.

Kebreab, E., Clark, K., Wagner-Riddle, C. and France, J. 2006. Methane and nitrous oxide emissions from Canadian animal agriculture: a review. *Canadian Journal of Animal Science* 86(2), 135–57. doi:10.4141/A05-010.

Kobayashi, Y. 2010. Abatement of methane production from ruminants: trends in the manipulation of rumen fermentation. *Asian-Australasian Journal of Animal Sciences* 23(3), 410–6. doi:10.5713/ajas.2010.r.01.

Koenig, K. M., Beauchemin, K. A. and Rode, L. M. 2003. Effect of grain processing and silage on microbial protein synthesis and nutrient digestibility in beef cattle fed barley-based diets. *Journal of Animal Science* 81(4), 1057–67. doi:10.2527/2003.8141057x.

Krause, D. O., Nagaraja, T. G., Wright, A. D. G. and Callaway, T. R. 2013. Board-invited review: rumen microbiology: leading the way in microbial ecology. *Journal of Animal Science* 91(1), 331–41. doi:10.2527/jas.2012-5567.

Kreikemeier, K. K., Harmon, D. L., Brandt Jr., R. T., Nagaraja, T. G. and Cochran, R. C. 1990. Steam-rolled wheat diets for finishing cattle: effects of dietary roughage and feed intake on finishing steer performance and ruminal metabolism. *Journal of Animal Science* 68(7), 2130–41. doi:10.2527/1990.6872130x.

Larsen, M., Lund, P., Weisbjerg, M. R. and Hvelplund, T. 2009. Digestion site of starch from cereals and legumes in lactating dairy cows. *Animal Feed Science and Technology* 153(3–4), 236–48. doi:10.1016/j.anifeedsci.2009.06.017.

Liu, Y. and Whitman, W. B. 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic Archaea. *Annals of the New York Academy of Sciences* 1125(1), 171–89. doi:10.1196/annals.1419.019.

Martin, S. A. and Streeter, M. N. 1995. Effect of malate on *in vitro* mixed ruminal microorganism fermentation. *Journal of Animal Science* 73(7), 2141–5. doi:10.2527/1995.7372141x.

Martin, C., Rouel, J., Jouany, J. P., Doreau, M. and Chilliard, Y. 2008. Methane output and diet digestibility in response to feeding dairy cows crude linseed, extruded linseed, or linseed oil. *Journal of Animal Science* 86(10), 2642–50. doi:10.2527/jas.2007-0774.

Martin, C., Morgavi, D. P. and Doreau, M. 2010. Methane mitigation in ruminants: from microbe to the farm scale. *Animal: an International Journal of Animal Bioscience* 4(3), 351–65. doi:10.1017/S1751731109990620.

Martínez, M. E., Ranilla, M. J., Ramos, S., Tejido, M. L. and Carro, M. D. 2009. Effects of dilution rate and retention time of concentrate on efficiency of microbial growth, methane production, and ruminal fermentation in *Rusitec fermenters*. *Journal of Dairy Science* 92(8), 3930–8. doi:10.3168/jds.2008-1975.

Martinez-Fernandez, G., Duval, S., Kindermann, M., Schirra, H. J., Denman, S. E. and McSweeney, C. S. 2018. 3-NOP vs. Halogenated compound: methane production, ruminal fermentation and microbial community response in forage fed cattle. *Frontiers in Microbiology* 9(1582), 1582. doi:10.3389/fmicb.2018.01582.

McAllister, T. A. and Newbold, C. J. 2008. Redirecting rumen fermentation to reduce methanogenesis. *Australian Journal of Experimental Agriculture* 48(2), 7–13. doi:10.1071/EA07218.

McAllister, T. A., Meale, S. J., Valle, E., Guan, L. L., Zhou, M., Kelly, W. J., Henderson, G., Attwood, G. T. and Janssen, P. H. 2015. RUMINANT NUTRITION SYMPOSIUM: use of genomics and transcriptomics to identify strategies to lower ruminal methanogenesis. *Journal of Animal Science* 93(4), 1431–49. doi:10.2527/jas.2014-8329.

McGinn, S. M., Beauchemin, K. A., Coates, T. and Colombatto, D. 2004. Methane emissions from beef cattle: effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. *Journal of Animal Science* 82(11), 3346–56. doi:10.2527/2004.8211 3346x.

Milich, L. 1999. The role of methane in global warming: where might mitigation strategies be focused? *Global Environmental Change* 9(3), 179–201. doi:10.1016/S0959-3780(98)00037-5.

Moate, P. J., Deighton, M. H., Williams, S. R. O., Pryce, J. E., Hayes, B. J., Jacobs, J. L., Eckard, R. J., Hannah, M. C. and Wales, W. J. 2016. Reducing the carbon footprint of Australian milk production by mitigation of enteric methane emissions. *Animal Production Science* 56(7), 1017–34. doi:10.1071/AN15222.

Moate, P. J., Jacobs, J. L., Hannah, M. C., Morris, G. L., Beauchemin, K. A., Alvarez Hess, P. S., Eckard, R. J., Liu, Z., Rochfort, S., Wales, W. J. and Williams, S. R. O. 2018. Adaptation responses in milk fat yield and methane emissions of dairy cows when wheat was included in their diet for 16 weeks. *Journal of Dairy Science* 101(8), 7117–32. doi:10.3168/jds.2017-14334.

Moate, P. J., Williams, S. R. O., Deighton, M. H., Hannah, M. C., Ribaux, B. E., Morris, G. L., Jacobs, J. L., Hill, J. and Wales, W. J. 2019. Effects of feeding wheat or corn and of rumen fistulation on milk production and methane emissions of dairy cows. *Animal Production Science* 59(5), 891–905. doi:10.1071/AN17433.

Nagaraja, T. G. and Chengappa, M. M. 1998. Liver abscesses in feedlot cattle: a review. *Journal of Animal Science* 76(1), 287–98. doi:10.2527/1998.761287x.

Nisbet, D. J. and Martin, S. A. 1993. Effects of fumarate, L-malate, and an *Aspergillus oryzae* fermentation extract on D-lactate utilization by the ruminal bacterium *Selenomonas ruminantium*. *Current Microbiology* 26, 136–.

Odongo, N. E., Bagg, R., Vessie, G., Dick, P., Or-Rashid, M. M., Hook, S. E., Gray, J. T., Kebreab, E., France, J. and McBride, B. W. 2007. Long-term effects of feeding monensin on methane production in lactating dairy cows. *Journal of Dairy Science* 90(4), 1781–8. doi:10.3168/jds.2006-708.

Okine, E. K., Mathison, G. W. and Hardin, R. T. 1989. Effects of changes in frequency of reticular contractions on fluid and particulate passage rates in cattle. *Journal of Animal Science* 67(12), 3388–96. doi:10.2527/jas1989.67123388x.

Opio, C., Gerber, P. and Mottet, A. 2012. *Greenhouse Gas Emission from Ruminant Supply Chains*. Food and Agriculture Organization/World Health Organization.

Ortega-Cerrilla, M. E. and Mendoza-Martínez, G. 2003. Starch digestion and glucose metabolism in the ruminant: a review. *Interciencia* 28, 380–6.

Owens, F. N., Secrist, D. S., Hill, W. J. and Gill, D. R. 1998. Acidosis in cattle: a review. *Journal of Animal Science* 76(1), 275–86. doi:10.2527/1998.761275x.

Patra, A. K. and Saxena, J. 2009. Dietary phytochemicals as rumen modifiers: a review of the effects on microbial populations. *Antonie van Leeuwenhoek* 96(4), 363–75. doi:10.1007/s10482-009-9364-1.

Pitta, D. W., Pinchak, W. E., Indugu, N., Vecchiarelli, B., Sinha, R. and Fulford, J. D. 2016. Metagenomic analysis of the rumen microbiome of steers with wheat-induced frothy bloat. *Frontiers in Microbiology* 7(689), 689. doi:10.3389/fmicb.2016.00689.

Plaizier, J. C., Krause, D. O., Gozho, G. N. and McBride, B. W. 2008. Subacute ruminal acidosis in dairy cows: the physiological causes, incidence and consequences. *The Veterinary Journal* 176(1), 21–31. doi:10.1016/j.tvjl.2007.12.016.

Rowe, J. B., Choct, M. and Pethick, D. W. 1999. Processing cereal grains for animal feeding. *Australian Journal of Agricultural Research* 50(5), 721–36. doi:10.1071/AR98163.

Russell, J. B. 2002. *Rumen Microbiology and Its Role in Ruminant Nutrition*. Cornell University Press, Ithaca, NY.

Russell, J. B. and Hespell, R. B. 1981. Microbial rumen fermentation. *Journal of Dairy Science* 64(6), 1153–69. doi:10.3168/jds.S0022-0302(81)82694-X.

Russell, J. B. and Robinson, P. H. 1984. Compositions and characteristics of strains of *Streptococcus bovis*. *Journal of Dairy Science* 67(7), 1525–31. doi:10.3168/jds.S0022-0302(84)81471-X.

Russell, J. B. and Rychlik, J. L. 2001. Factors that alter rumen microbial ecology. *Science* 292(5519), 1119–22. doi:10.1126/science.1058830.

Russell, J. B. and Strobel, H. J. 1989. Effect of ionophores on ruminal fermentation. *Applied and Environmental Microbiology* 55(1), 1–6. doi:10.1128/AEM.55.1.1-6.1989.

Sauvant, D. and Giger-Reverdin, S. 2007. Empirical modelling by meta-analysis of digestive interactions and CH_4 production in ruminants. In: *Energy and Protein Metabolism and Nutrition*. Publication – European Association for Animal Production (EAAP), 124. Presented at 2nd EAAP International Symposium on Energy and Protein Metabolism and Nutrition, Vichy, FRA, 09–13 September 2007. Wageningen Academic Publishers, Wageningen, the Netherlands, pp. 561–2. Available at: https://prodinra.inra.fr/record/13493.

Schultz, J. E. and Breznak, J. A. 1979. Cross-feeding of lactate between *Streptococcus lactis* and *Bacteroides* sp. isolated from termite hindguts. *Applied and Environmental Microbiology* 37(6), 1206–10. doi:10.1128/AEM.37.6.1206-1210.1979.

Seshadri, R., Leahy, S. C., Attwood, G. T., Teh, K. H., Lambie, S. C., Cookson, A. L., Eloe-Fadrosh, E. A., Pavlopoulos, G. A., Hadjithomas, M., Varghese, N. J., Paez-Espino, D., Hungate1000 Project Collaborators, Palevich, N., Janssen, P. H., Ronimus, R. S., Noel, S., Soni, P., Reilly, K., Atherly, T., Ziemer, C., Wright, A.-D., Ishaq, S., Cotta, M., Thompson, S., Crosley, K., McKain, N., Wallace, R. J., Flint, H. J., Martin, J. C., Forster, R. J., Gruninger, R. J., McAllister, T., Gilbert, R., Ouwerkerk, D., Klieve, A., Jassim, R. A., Denman, S., McSweeney, C., Rosewarne, C., Koike, S., Kobayashi,

Y., Mitsumori, M., Shinkai, T., Cravero, S., Cucchi, M. C., Perry, R., Henderson, G., Creevey, C. J., Terrapon, N., Lapebie, P., Drula, E., Lombard, V., Rubin, E., Kyrpides, N. C., Henrissat, B., Woyke, T., Ivanova, N. N. and Kelly, W. J. 2018. Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nature Biotechnology* 36, 359.

Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., Rosales, M., Rosales, M. and de Haan, C. 2006. *Livestock's Long Shadow: Environmental Issues and Options*. Food and Agriculture Organization, Rome, Italy.

Stevens, E., Armstrong, K., Bezar, H., Griffin, W. and Hampton, J. 2004. Fodder oats an overview. In: Suttie, J. M. and Reynolds, S. G. (Eds), *Fodder Oats: a World Overview*. Plant Production and Protection Series No. 33. Food and Agriculture Organization, Rome.

Tedeschi, L. O. and Nagaraja, T. G. 2020. *Phibro Rumen Health Compendium*. Texas A&M University Press, College Station, TX.

Theurer, C. B. 1986. Grain processing effects on starch utilization by ruminants. *Journal of Animal Science* 63(5), 1649–62. doi:10.2527/jas1986.6351649x.

Thiele, J. H. and Zeikus, J. G. 1988. Control of interspecies electron flow during anaerobic digestion: significance of formate transfer versus hydrogen transfer during syntrophic methanogenesis in flocs. *Applied and Environmental Microbiology* 54(1), 20–9. doi:10.1128/AEM.54.1.20-29.1988.

Toland, P. 1976. The digestibility of wheat, barley or oat grain fed either whole or rolled at restricted levels with hay to steers. *Australian Journal of Experimental Agriculture* 16(78), 71–5. doi:10.1071/EA9760071.

Van Kessel, J. S. and Russell, J. B. 1996. The effect of pH on ruminal methanogenesis. *FEMS Microbiology Ecology* 20(4), 205–10. doi:10.1016/0168-6496(96)00030-X.

Verdu, M., Bach, A. and Devant, M. 2015. Effect of concentrate feeder design on performance, eating and animal behavior, welfare, ruminal health, and carcass quality in Holstein bulls fed high-concentrate diets. *Journal of Animal Science* 93(6), 3018–33. doi:10.2527/jas.2014-8540.

Wallace, R. J., Wood, T. A., Rowe, A., Price, J., Yanez, D. R., Williams, S. P. and Newbold, C. J. 2006. Encapsulated fumaric acid as a means of decreasing ruminal methane emissions. *International Congress Series* 1293, 148–51. doi:10.1016/j.ics.2006.02.018.

Wells, J. E., Krause, D. O., Callaway, T. R. and Russell, J. B. 1997. A bacteriocin-mediated antagonism by ruminal lactobacilli against *Streptococcus bovis*. *FEMS Microbiology Ecology* 22(3), 237–43. doi:10.1111/j.1574-6941.1997.tb00376.x.

Wilson, B. K., Holland, B. P., Step, D. L., Jacob, M. E., Vanoverbeke, D. L., Richards, C. J., Nagaraja, T. G. and Krehbiel, C. R. 2016. Feeding wet distillers grains plus solubles with and without a direct-fed microbial to determine performance, carcass characteristics, and fecal shedding of *Escherichia coli* O157:H7 in feedlot heifers. *Journal of Animal Science* 94(1), 297–305. doi:10.2527/jas.2015-9601.

Wolfe, R. S. 1993. An historical overview of methanogenesis. In: Ferry, J. G. (Ed.), *Methanogenesis*. Chapman and Hall Publishers, New York, NY, pp. 1–32.

Wolin, M. J. 1975. Interactions between the bacterial species in the rumen. In: McDonald, W. and Warner, A. C. I. (Eds), *Digestion and Metabolism in the Ruminant*. Univ. New Eng. Pub. Unit, Armidale, Australia, pp. 134–48.

Wood, T. A., Wallace, R. J., Rowe, A., Price, J., Yáñez-Ruiz, D. R., Murray, P. and Newbold, C. J. 2009. Encapsulated fumaric acid as a feed ingredient to decrease ruminal methane

emissions. *Animal Feed Science and Technology* 152(1–2), 62–71. doi:10.1016/j.anifeedsci.2009.03.006.

Wright, A. G. and Klieve, A. V. 2011. Does the complexity of the rumen microbial ecology preclude methane mitigation? *Animal Feed Science and Technology* 166–167, 248–53. doi:10.1016/j.anifeedsci.2011.04.015.

Xia, Y., Kong, Y., Seviour, R., Yang, H. E., Forster, R., Vasanthan, T. and McAllister, T. 2015. In situ identification and quantification of starch-hydrolyzing bacteria attached to barley and corn grain in the rumen of cows fed barley-based diets. *FEMS Microbiology Ecology* 91(8), fiv077. doi:10.1093/femsec/fiv077.

Zhu, F. 2017. Barley starch: composition, structure, properties, and modifications. *Comprehensive Reviews in Food Science and Food Safety* 16(4), 558–79. doi:10.1111/1541-4337.12265.

Chapter 21

Plant secondary compounds: beneficial roles in sustainable ruminant nutrition and productivity

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1 Introduction

The intensification of ruminant feeding systems to meet requirements of genetically improved animals and increase efficiency in livestock production is in many cases causing disturbances in the rumen microbial ecosystem. Meeting energy and nutrient requirements of ruminants while avoiding digestive and systemic metabolic disturbances is critical to ensure that milk and meat are produced from healthy animals in an efficient and cost-effective manner. The current intensive management systems encourage dietary inclusion of large amounts of cereal grains or easily degradable by-products to support high yields (Zebeli et al., 2010). Although these feeding practices seem to increase production in a short term, sometimes they do not cope with digestive physiology (Zebeli and Ametaj, 2009). The most important consequence thereof is an impaired rumen ecosystem, which can lead to lactic acidosis, intestinal inflammation and/or diarrhoea. Antibiotics at sub-therapeutic levels are commonly used to enhance the efficiency of converting feeds to gain (e.g. milk and meat) and/or prevent metabolic disorders and health problems. However, the restriction and the ban (e.g. European Union) on the use of antibiotics in several countries have prompted scientists and the feed industry to search for alternative products. Plant secondary compounds, also known as phytogenics or phyotochemicals (Patra, 2010), offer interesting

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opportunities in this regard as many plants produce secondary metabolites that, when extracted and concentrated, may exert modulation of the activity of gut microorganisms. Also, current trends indicate that consumers are increasingly rejecting the use of synthetic substances in food production; therefore, plant-derived compounds with growth-promoting activity are gaining a presence in the livestock feed additive market (Dhama et al., 2015). Accordingly, research on the use of phytogenics in ruminant nutrition has increased over the last two decades.

Phytochemical feed additives are an extremely large group of compounds with great diversity in chemical structure and bioactivity (Kroon and Williamson, 1999). The active compounds in plants vary widely depending on intrinsic factors, such as the plant part used, the harvest season and the geographical origin, and extrinsic factors, such as the additive production procedure (Ganguly, 2013). The great diversity of secondary plant derivatives is the result of an evolutionary process through which plants have developed defences against attacks by microorganisms, insects and herbivorous animals (Cowan, 1999). These compounds are a valuable source of a variety of additives with different biological activities that have the potential to promote the yield of producing animals. The wide varieties of phytochemical feed additives that have been used experimentally in animal production are difficult to classify, in part, because there are no concise definitions (Valenzuela-Grijalva et al., 2017). Several authors have proposed various classifications based on different criteria, the main ones being: (i) botanical origin, (ii) chemical composition, (iii) processing method and (iv) mechanisms of action. However, some compounds may exert their effect through different mechanisms, thus complicating their classification. Based on the different biological activities of phytochemicals, four principal mechanisms have been proposed that support the physiological changes observed in the studied animals and explain the effects on performance (Valenzuela-Grijalva et al., 2017):

1 Improving feed characteristics and animal feed intake
2 Modulating microbial activity
3 Enhancing nutrient digestion and absorption
4 Influencing anabolic activity on target tissues

Although it is recognized that the effect of plant secondary compounds might be a combination of some of the above mechanisms, this chapter will focus mainly on the modulating action on rumen microbiota (2). Because of the large number of components present in plant secondary compounds, it is most likely that their antimicrobial activity is not due to a single mode of action but involves several targets in the cells (Acamovic and Brooker, 2005). There are four recognized modes of action that explain how phytochemical

feed additives exert their antimicrobial effect and the changes that occur in rumen microbiota: (1) inhibition of cell wall synthesis, (2) disruption of cell wall structure and function (e.g. altering the permeability of the cytoplasmic membrane), (3) inhibition of nucleic acid synthesis and (4) inhibition of protein synthesis or unique bacterial metabolic pathway. These actions lead to the collapse of core cellular activities and, consequently, result in bacterial death or compromised growth (Patra and Saxena, 2009a). The specific mode of action displayed by each of the groups considered in this chapter will be discussed separately. Three main groups of secondary compounds are considered based on their chemical composition and classification used in previous reviews (Calsamiglia et al., 2007; Benchaar et al., 2008; Patra and Saxena, 2009a) and these are: (i) essential oils, (ii) tannins and (iii) saponins (Fig. 1).

The above described mechanisms can exert both anti-nutrient/toxic and beneficial effects in the ruminant. There is extensive literature on overtly toxic compounds in forages and how the ruminant counters these compounds

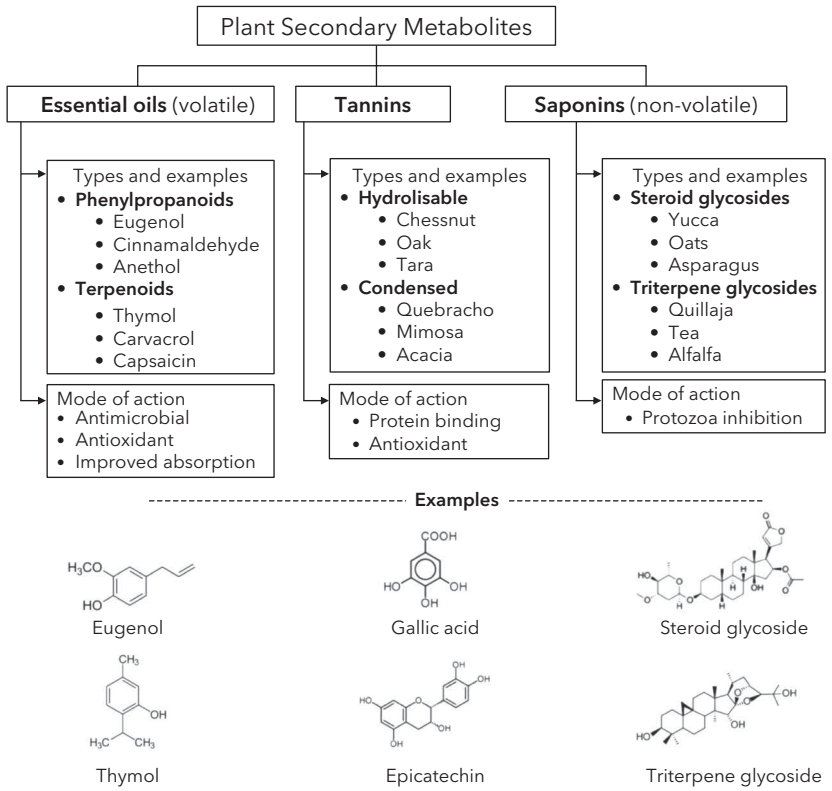

Figure 1 Classification and mode of action of plan secondary compounds.

(Estelle, 2010). However, this is beyond the scope of this chapter, which focuses on the positive impacts.

Understandably, most of the research conducted to screen plant secondary compounds to modulate rumen fermentation is laboratory based (i.e. *in vitro*) and has enabled the identification of a large number of compounds and their main constituents for their effects on rumen microbial fermentation (Patra and Saxena, 2011).

In vitro gas production (IVGP) systems have been used extensively for rapid screening of chemical substances, plant species, plant extracts and dietary ingredients on rumen fermentation. Such experimental approaches have allowed the mode of action of a range of chemicals (Busquet et al., 2005a; Bodas et al., 2008; García-González et al., 2008; Durmic et al., 2014) and dietary substrates (Patra and Yu, 2013; Hatew et al., 2015) to be investigated. Use of IVGP systems provides the possibility to evaluate a broad spectrum of chemical components alone or in different combinations over a wide range of concentrations (Busquet et al., 2005a; García-González et al., 2008). For example, Bodas et al. (2008) screened 450 plant extracts for their ability to inhibit methane production *in vitro* and found that 35 plant extracts decreased methane production by more than 15%, with 6 of these plant extracts having a methane inhibition greater than 25% without adverse effects on digestion or fermentation. However, *in vitro* approaches do not generate reliable information for agents that are effective over an extended period (Castro-Montoya et al., 2015), as it is discussed in the following sections. Furthermore, results from screening studies (Bodas et al., 2008; Durmic et al., 2014) are often inconclusive and may be conflicting due to variation in dosage, chemical structure of the test chemical or compound, diet, combination of treatments applied, adaptation of rumen microbes from donor animals or the form in which an agent is introduced into the system (Cardozo et al., 2004; Cardozo et al., 2005). Few direct comparisons (i.e. antimethanogenic compounds) between *in vitro* and *in vivo* approaches are available, and they have shown big discrepancies. As reviewed by Yáñez-Ruiz et al. (2016), a different outcome in the effectiveness of studies while using the same compounds and similar doses may be explained by a combination of factors: (i) compounds being tested are typically provided in 1-2 pulses via the rumen cannula that often coincide with feeding times, and as a consequence might not be rapidly and well mixed with rumen contents; (ii) differences in the degradation rate of the active compounds *in vitro* and *in vivo*; (iii) decrease in microbial density and changes in bacterial community structure of rumen contents during processing as inoculum for *in vitro* studies associated with the exposure of microorganisms to oxygen and the removal of solids during filtration (Soto et al., 2013); (iv) potential washout of some compounds from the rumen or absorption through the rumen wall and (v) adaptation of the rumen microbial

ecosystem to the tested compound *in vivo* that is not emulated by inoculated microbiota *in vitro*.

The impact of a wide range of plant secondary compounds on rumen fermentation has been studied *in vitro* and extensively reviewed (Frutos et al., 2004; Calsamiglia et al., 2007; Benchaar et al., 2008; Hart et al., 2008; Patra and Saxena, 2009a, 2011; Wang et al., 2012; Vasta et al., 2019). Cieslak et al. (2013) studied the mode of action of plant extracts pointing that essential oils, tannins and saponins differed in their mode of action within the rumen and concluded that whilst there was a significant body of work from *in vitro* studies, there was a need for more *in vivo* and production-based studies to truly describe the impact of the rumen microbial modulation by plant extracts on the overall feed efficiency (nitrogen and energy) and animal performance (i.e. milk and meat). This chapter presents the recent developments in the use of plant secondary compounds as feed additives in ruminant nutrition focusing on the results obtained through *in vivo* studies and in particular the interaction with the rumen function and microbiome. Antimicrobial properties, mechanisms of action, effects on ruminal protein metabolism, enteric methane production, animal performance, and associated challenges are discussed.

2 Essential oils (EO)

2.1 Composition

Essential oils (EO) (also referred as volatile or ether oils) are complex mixtures of volatile lipophilic secondary metabolites. They are typically extracted from plant material by boiling water and steam distillation, but other methods also include solvent extraction, supercritical CO_2 extraction and expression extraction (Benchaar and Greathead, 2011). Essential oils are plant specific and are responsible for a plant's characteristic flavour and fragrance (Greathead, 2003). They also play an important role in protecting the plant from abiotic and biotic stressors and acting as attractants to organisms that pollinate and disperse seeds (Wink and Schimmer, 2018). Essential oils can be extracted from all parts of a plant including leaves, roots, flowers, petals, seeds, fruits, stems and barks. The yield and the composition of essential oils can vary widely among plants depending on the plant species (Martinez et al., 2006), geographical location, climatic conditions, soils (Vokou et al., 1993), part of the plant (e.g. seeds vs. leaves; Delaquis et al., 2002), plant health (e.g. insect attacks) and the method of extraction (Anitescu et al., 1997).

Chemically, the major essential oils are typically composed of terpene or phenylpropene secondary metabolites (Fig. 1). Terpenes comprise structurally and functionally different types, are synthesised from isoprene (isopropenyl diphosphate) and may be classified based on the number of 5-C-base (C5) isoprene units that are part of their skeletons (Benchaar et al., 2007). If they

are linked to other elements (i.e. oxygen) they are known as terpenoids or isoprenoids (Benchaar et al., 2007). For example monoterpenes represent the most common molecules that comprise 90% of EO (i.e. linalool, carvacrol, thymol, limonene). On the other hand, the phenylpropanoids are aromatic compounds that are derived from phenylalanine and consist of C6 benzenic ring with a C3 propionic side chain (Hart et al., 2008). Examples of these compounds are cinnamaldehyde, eugenol and anethol. Also, some EO contain other compounds than terpene or phenylpropene; for example, garlic oils that comprise a large number of organosulphur compounds such as diallyl disulfide, diallyl sulfide or ajoenes.

2.2 Activity

Generally, EO are characterized by 2 or 3 main components in high concentrations where terpenes and terpenoids represent 20-70% of the components and explain the major biological effect (Okoh et al., 2010). It is important to note that the complexity of the properties depends upon whether these compounds have additive, antagonistic or synergistic effects (Burt, 2004). The previous works conducted to test the antimicrobial activity of different EO show variable antimicrobial potency against the same microbes (Burt, 2004; Pauli and Schilcher, 2010). There are many hypotheses to explain the EO antibacterial activities. An important characteristic of EO and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable (Knobloch et al., 1986; Sikkema et al., 1994). Leakage of ions and other cell contents can then occur (Carson et al., 2002; Ultee et al., 2002). Although a certain amount of leakage from bacterial cells may be tolerated without loss of viability, extensive loss of cell contents or the exit of critical molecules and ions will lead to microbial death (Denyer and Hugo, 1991). Components of EO also appear to act on cell proteins embedded in the cytoplasmatic membrane, and this is the case of ATPases (Knobloch et al., 1989). The possible mechanisms of such action could be the accumulation of lipophilic hydrocarbon molecules and disturbance of the lipid-protein interaction or direct interaction of the lipophilic compounds with hydrophobic parts of the protein (Sikkema et al., 1995). Due to their complex composition, it is probable that the antimicrobial activity of EO is due to an interaction with a large number of molecular targets, rather than a specific mode of action (Benchaar et al., 2007). Although greater sensitivity of gram-positive bacteria to EO has been reported due to their lack of protective outer membrane, some authors (Burt, 2004) observed increased potency against gram-negative bacteria that lost lipopolysaccharides in the outer membrane. For these reasons, the antibacterial properties of EO can be considered

non-specific and their effect on rumen microbial populations could be difficult to predict due to high variability in composition of EO and sensitivity of different microorganisms (Pauli and Schilcher, 2010). The difference in composition of the rumen microbiome among individual ruminant animals and limited understanding on the mode of antimicrobial activities of EO also contribute to the above challenge (Patra and Yu, 2015).

Also, fungi, protozoa and viruses can be affected by EO through the same biological mechanisms as described for bacteria (Jouany and Morgavi, 2007); however, less information is available on the impact on individual species or strains as exists for bacteria (Burt, 2004).

2.3 Effects of essential oils on rumen microbiome and function

The effect of EO on the major ruminal microbial populations has been studied primarily using cultivation-based methods before DNA-based molecular biology techniques took off. Initial works in the sixties (Nagy et al., 1964) demonstrated that adding EO significantly reduced rumen bacterial activity *in vitro*. Some bacteria, such as *Prevotella ruminicola*, *Clostridium sticklandii* and *Peptostreotococcus anaerobius*, appeared to be more sensitive to EO than other bacteria, such as *Streptococcus bovis* (McIntosh et al., 2003). Evans and Martin (2000) observed that 90 μg/ml of thymol inhibited the growth of *Selenomonas ruminantium* but not that of *S. bovis*, the latter of which required 180 μg/ml to be inhibited. Molecular biology-based techniques immediately showed their potential to better unveil the effect of EO on rumen microbial populations than cultivation-based methods. Essential oils appeared to be effective in reducing the abundance of methanogens, but they also affected the abundance of cellulolytic bacteria such as *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens* (Cobellis et al., 2016). Besides, protozoal population was decreased by most of the EO tested.

A few studies employed molecular techniques to determine the effects of EO on Hyper Ammonia-Producing (HAP) bacteria, which appeared to be sensitive to EO (Wallace et al., 2002; McIntosh et al., 2003). Patra and Yu (2012) confirmed the high sensitivity of *Prevotella ruminicola*, *Prevotella bryantii*, *Clostridium aminophilum*, *Clostridium sticklandii*, and *Butyrivibrio fibrisolvens* to most of the EO tested *in vitro*. Using microarray analysis, Patra and Yu (2015) also observed that the effect of EO on rumen bacterial community composition was dependent on the chemical nature of EO used. Compounds with a phenolic structure, such as oregano EO, demonstrated a stronger antibacterial activity than other EO in decreasing the abundance of the phylum Firmicutes, the class Clostridia, and the genus *Butyrivibrio* (Patra and Yu, 2015). However, oregano EO increased the abundances of some members of *Prevotella* probably due to its inhibitory effect on competing bacteria. Complex interactions occur

among EO, feed components and host, thus, correlation of the results from rumen fermentation characteristics (e.g. VFA, CH_4 and NH_3 production), feed degradability and microbial abundance could provide more information about rumen microbiome dynamics and function and allow for development of effective mitigation technologies. Further to studying the impact of using EO on CH_4 emissions, some have also quantified the impact on methanogens numbers with positive (Khorrami et al., 2015) and negative responses (Tomkins et al., 2015). However, the proven lack of relationship between methanogen numbers and CH_4 production (Morgavi et al., 2010) makes the quantification of this microbial group not very informative. In addition to studying the impact of using EO on the abundance of a limited number of species, a more holistic assessment of the whole microbiome would theoretically provide more insight. Schären et al. (2017) studied in transition cows the impact of monensin and a blend of EO on rumen fermentation, milk production and rumen bacterial population using Illumina sequencing. Interestingly, while monensin caused both an increase and a decrease of several bacterial groups, EO did not promote any change. It is worth pointing that in this study the addition of EO did not cause major changes in rumen fermentation and promoted no benefits in terms of DMI or milk yield. The results from Schären et al. (2017) also confirm the hypothesis that varying effects depending on cell-wall constitution and thickness might apply for monensin sensitivity rather than a clear-cut difference between gram-negative and gram-positive bacteria, which could be probably applied for EO.

Numerous studies have been conducted *in vitro* (mostly using batch culture and some using fermenters) to test the ability of EO to modulate rumen microbial populations and to improve the efficiency of nutrient utilization (Calsamiglia et al., 2007). A fair proportion of them have shown positive effects on VFA production, protein metabolism, fibre digestion and CH_4 production. As stated previously, results obtained *in vivo* are less abundant and sometimes only report effects on either rumen fermentation or in productivity but less frequent for both. Given the large number of published works covering *in vitro* studies and for the reasons provided above, only studies conducted *in vivo* and mostly in the last 10 years (2010-2019) reporting effects on rumen function and productivity are considered in this work (Table 1).

Typically the inhibitory action of EO on some rumen microorganisms has been described as inhibition of deamination and methanogenesis, resulting in lower NH_3 N, methane and acetate, as well as higher propionate and butyrate concentrations (Busquet et al., 2005b; Cardozo et al., 2006). Moreover, the inhibition of protein degradation in the rumen can potentially increase the intestinal supply of amino acids to the animal host (Wallace, 2004). However, very rarely all these effects occur simultaneously (Hart et al., 2008). The reduction of NH_3-N production in the rumen has been associated with the inhibition of HAP bacteria (Russell et al., 1991). This group of bacteria

Table 1 Summary of recent publications describing the effect (ns, non-significant; +, increase; −, decrease) of essential oils on rumen fermentation and animal performance based on *in vivo* studies

	Study details				Rumen fermentation											Animal productivity				
Reference	Animal	Main compounds	g/kg DM	Exposure	DMI	pH	NH3	VFA	Ac	Pr	Bu	CH_4	Bac	Ptz	Meth	MY	Fat	Pr	ADG	FE
Blanch et al. (2016)	Dairy cattle	Cinnemaldehyde, garlic	200–400 mg/d	4 weeks	ns	ns	ns	ns	ns	+	ns	−				ns	−	−		
Braun et al. (2019)	Dairy cattle	BEO BIX12	1.2 g/ animal/d	20 d												+				+
Canaes et al. (2017)	Dairy goats	Citral oil	0.02–0.24 ml/kg BW	14 d	ns	ns	ns	ns	ns	ns	ns					ns	ns	ns		
Castro-Montoya et al. (2015)	Dairy catle	BEO Agolin ®	0.2 g/d	8 weeks								−				ns	ns	ns		
Chaves et al. (2011)	Lambs	Cinnmaldehyde	100–400 mg/kg BW	18 weeks	ns	ns	ns	ns	ns	ns	ns								ns	ns
Cobellis et al. (2016)	Sheep	Rosemary oil	1.75v %	21 d	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns					
Elcoso et al. (2019)	Dairy cattle	BEO Agiolin ®	1 g/d	8 weeks	ns	ns	ns	ns	+	−	ns	−				+	+	ns		
de Jesus et al. (2013)	Dairy cows	Cashew nut, castor oils	500mg/ kg BW	21 d	ns	ns	ns	ns	ns	+	ns					+	+	+		
Flores et al. (2013)	Dairy cattle	BEO XTrcat ®	200–600 mg/d	6 weeks		ns	ns	ns	ns	ns	ns					ns	ns	ns		
Giannenas et al. (2011)	Dairy sheep	BEO Crina ®	50–150 mg/kg DM	5 months		ns	ns	ns	ns	+	ns		+	+		+	ns	ns		

(*Continued*)

Table 1 (*Continued*)

	Study details				Rumen fermentation											Animal productivity				
Reference	Animal	Main compounds	g/kg DM	Exposure	DMI	pH	NH3	VFA	Ac	Pr	Bu	CH_4	Bac	Ptz	Meth	MY	Fat	Pr	ADG	FE
Ghizzi et al. (2018)	Dairy cows	Cardanol, cardol, ricinoleic	500mg/kg DM	5 weeks	ns	ns	ns	ns	ns	ns	ns					ns	ns	ns		
Hart et al. (2019)	Dairy cattle	BEO Agiolin ®	1 g/d	22 weeks	+	ns						−				+	ns	ns		
Kholifa et al. (2017)	Dairy goats	Rosemary	10g/d	12 weeks	ns	ns	ns	+	ns	+	ns					+	+	+		+
Kholifa et al. (2017)	Dairy goats	Lemon grass	10g/d	12 weeks	ns	ns	−	+	ns	+	ns					+	+	+		+
Khorrami et al. (2015)	Beef cattle	Thyme	0.5 g/d	21 d	ns	ns	ns	ns	ns	+	ns			−	−					
Khorrami et al. (2015)	Beef cattle	Cinnamon	0.5 g/d	21 d	ns	ns	ns	ns	ns	ns	ns			−	−					
Klop et al. (2017)	Dairy cattle	BEO Agolin ®	0.17 g/kg DM	2 weeks	ns	ns	ns	+	ns	ns	ns	−				ns	ns			
Lin et al. (2013)	Sheep	BEO	0.5–1 g/d	21 d	+	ns	ns	−	ns	ns	ns		ns	−	ns					
Ornaghi et al. (2017)	Beef cattle	Clove, cinnamon	3.5–7 g/d	187 d	+														+	+
Santosa et al. (2010)	Dairy cattle	BEO Agolin ®	1 g/d	21 d												ns	+			
Schären et al. (2017)	Dairy cattle	BEO Crina ®	1 g/d	3 weeks									ns	ns		ns	ns	ns		
Silva et al. (2018)	Dairy cattle	BEO Crina ®	44 mg/kg DM	21 d	ns	ns	ns	ns	ns	ns	ns					ns	ns	ns		

Silva et al. (2019)	Beef cattle	Cashew nut, castor oils	30 mg/kg DM	120 d	+															
Soltan et al. (2018)	Sheep	BEO GRASP ®	200–400/ kg DM	15 d	ns	ns	ns	ns	ns	+	ns	−		−						
Souza et al. (2019)	Beef cattle	BEO	2–4 g/ animal,/d	73 d															+	+
Spanghero et al. (2009)	Dairy cattle	BEO														ns	ns	ns	ns	
Tager and Krause (2011)	Dairy cattle	Cinnamaldehyde, eugenol	0.5–10 g/d	21 d	ns	ns	ns	ns	ns	ns	ns					ns				
Tager and Krause (2011)	Dairy cattle	capsicum	0.25 g/d	21 d	ns	ns	ns	ns	ns	ns	ns					ns				
Tekippe et al. (2011)	Dairy cattle	Oregano leaf	500 g/d	21 d																
Tekippe et al. (2013)	Dairy cattle	BEO XTract ®	525 mg/d	28 d	ns	ns	ns	ns	ns	ns	ns					ns	ns	ns		
Tomkins et al. (2015)	Beef cattle	BEO Crina ®	1–2 g/d	40 d	ns	ns	ns	ns	ns	ns	ns	ns			ns					
Wall et al. (2014)	Dairy cattle	BEO XTract	200–600 mg/d	42 d	+															
Yanga et al. (2010)	Beef cattle	Eugenol	400–600 mg/kg DM	21 d	ns	ns	ns	ns	ns	ns	ns			ns		+	ns	+	ns	

Abbreviations: BEO, blend of essential oils; DMI, DM intake; VFA, volatile fatty acids; C2, acetate; C3, propionate; C4, butyrate; Ptz, protozoa; Bac, bacteria; Meth, methanogens; MY, milk yield; Fat, milk fat %; Pr, milk protein content; ADG, average daily gain; FE, feed efficiency.

represents only about 1% of the rumen bacterial population (Russell, 1991), but they are characterized with high deamination ability and low capability to utilize carbohydrates (Eschenlauer et al., 2002). The HAP bacteria possess a very high deamination activity, which is influenced by the types and physical forms of the diets. All the known HAP bacteria are gram positive and highly sensitive to ionophores such as monensin (Eschenlauer et al., 2002). To date about 14 different bacterial species of HAP have been identified, but the highest deamination activity is attributed to *Clostridium sticklandii*, *Clostridium aminophilum* and *Prevotella ruminicola* (Szumacher-Strabel and Cieślak, 2012). Also the addition of some EO has been associated with the alteration of the pattern of bacterial colonization of starch-rich substrates. Inhibition of protein and starch degradation and HAP bacteria can have positive effects when animals are fed rations with high content in protein (Patra and Saxena, 2009a).

With regards to CH_4 production, three possible approaches to reduce the production by ruminants are considered: to directly inhibit methanogenesis, to reduce hydrogen production and to provide alternative pathways for hydrogen use (alternative sinks for disposal of hydrogen) in the rumen (Patra, 2011). The effects of EO have been associated with the first two, either by decreasing feed degradation (i.e. colonization) or by directly inhibiting protozoa and/or methanogens (Patra et al., 2017). As stated earlier, most studies seeking CH_4 reduction by using EO have been conducted *in vitro* and only a few studies have investigated their effects *in vivo* (Table 1). In most cases, *in vivo* studies showed conflicting results because of variation in dose and type of EO used. Due to variation in chemical composition of different EO, diet and feeding regime, it is also difficult to determine the dose of EO required to achieve effective EO concentration in the rumen (Bodas et al., 2012). Wang et al. (2009) showed that feeding a mixture of EO derived from oregano (0.25 g/d) to sheep for 15 days lowered CH_4, while Beauchemin and McGinn (2006) and Tomkins et al. (2015) found no decrease in CH_4 production from beef cattle that were fed a commercial blend of essential oils (BEO) (1 and 2 g/d; Crina® ruminants; Akzo Surface Chemistry Ltd., Hertfordshire, UK). More recently, two studies using a commercial BEO (Agolin®) have shown promising results in dairy cattle (Elcoso et al., 2019; Hart et al., 2019). From the evaluation of these studies it becomes clear that further assessment is needed to systematically evaluate EO using *in vivo* assays.

Despite growing interest in improving rumen functions through the use of EO, the mode of action and activities of EO remain poorly understood. Cobellis et al. (2016) recently reviewed the few groups or species of rumen microbes that have been examined with respect to their response to EO and highlighted that in most cases the studies were conducted *in vitro* and in those using *in vivo* approaches mostly quantified protozoa cells through microscope counting.

2.4 Effects of essential oils on animal performance

One of the proposed positive effects of EO on ruminants in production stage (lactating or growing) is an increase on feed intake. However, this is not always the case. While some *in vivo* studies using sheep or cows showed that EO supplementation did not affect feed intake (Yang et al., 2007; Wang et al., 2009), others reported decreases of feed intake by beef cattle and dairy cattle when fed cinnamaldehyde and eugenol (Cardozo et al., 2006; Calsamiglia et al., 2007). Out of the 24 *in vivo* studies analysed in this document (Table 1), only 5 reported an increase in feed intake and they were not necessarily the same that showed positive effects on production (either milk or meat). However, in most studies reporting feed efficiency, it was significantly improved, which consisted of an increased production without changing feed intake. A recent meta-analysis compiling experiments with long-term exposure to a commercial BEO (Agolin) showed an increase in milk yield (3.8%) and no changes in feed intake (Belanche et al., 2019). However, the mechanisms behind such an increase in performance are still to be elucidated. Previous works have shown enhanced digestion and absorption of metabolites in animals fed different EO (Franz and Novak, 2009), although this is clearer in monogastric animals (Muthusamy and Sankar, 2015). In ruminants, one of the most plausible mechanisms involve the modulation of the rumen microbial fermentation (Benchaar et al., 2012), but possibly not detectable by only measuring VFA and NH_3 concentrations. Other mechanisms cannot be ruled out though. The Chinese culture began to use EO in their traditional medicinal therapies 5000 years ago due to their wide variety of effects on health, including positive effects on cardiovascular diseases, some tumours, inflammatory processes and, in general, diseases in which there is a uncontrolled proliferation of free radicals (Calsamiglia et al., 2007). These properties depend on their ability to scavenge free radicals, inhibit peroxidation of membrane lipids, chelate metals and stimulate the activity of antioxidant enzymes (Lee et al., 2003). Also, improvement in nutrients absorption has been suggested as another factor (Oh et al., 1968). Braun et al. (2019) reported that the beneficial effects of EO on milk yield in dairy cows is linked to enhanced cation absorption through stimulation of cation-transporting proteins expressed in the rumen epithelium, resulting in an increased uptake of cations like calcium and ammonium. More studies are needed to verify whether these properties, in addition to enhanced digestion, are responsible for the increase in productivity observed in some studies.

The impact of adding EO on productivity has shown variable results. The first limitation is the low number of *in vivo* studies conducted and the sample size (number of animals per group) used, as often differences are numerical but do not reach statistical significance. On the other hand, EO are widely

used in commercial livestock farming worldwide but scientific reports from on-farm studies are scarce due to the difficulty of controlling and measuring key variables (i.e. individual animal feed intake) or the lack of resources and knowledge of enterprises to publish their results. In this chapter, 4 out of the 21 dairy studies that reported effects on milk yield, 8 found positive responses, with average 2-4% improvement. As for growing trials, 2 out of 5 reported positive effects. In many cases the improvement in milk yield or ADG was not a result of greater VFA concentration as described previously. One of the factors to consider in the different *in vivo* studies is the duration of the treatments with EO, which ranged from 14 days to 5 months (Table 1). A typical *in vivo* study involves a cross-over or Latin-square design in which all animals receive all treatments (control and EO at different levels) in subsequent periods. However, this design uses short periods of treatments and in most cases results in no beneficial effects. However, it is possible that an extended period of time is needed to discern treatment differences and that there might be carry-over effects in trials that use a short period crossover design. Recently Belanche et al. (2019) described that the effect of a commercial BEO (Agolin) required a minimum time of 4 weeks of exposure to reach an increase in fat and protein corrected milk production and feed utilization as well as a decrease in CH_4 production. These findings may explain the highly inconsistent results observed in the literature in relation to the use of different blends of EO and milk yield in short-term trials (Benchaar et al., 2008) an may be related to the unspecified mode of action and target species (Pauli and Schilcher, 2010) in addition to the complexity and redundancy of the rumen microbial ecosystem (Weimer, 2015).

3 Tannins

3.1 Composition

Tannins, together with flavonoids, are polyphenols which refer to a class of plant secondary metabolites with a phenolic moiety, bearing at least one hydroxyl substituent. From a chemical point of view it is difficult to define tannins since the term comprises a diverse type of oligomers and polymers (Harborne and Baxter, 1999; Schofield et al., 2001). Despite their heterogeneity, all of them have a high molecular weight and, to some extent, the capacity to form reversible and irreversible complexes with proteins, polysaccharides, alkaloids, nucleic acids and minerals, etc (Schofield et al., 2001; Frutos et al., 2004; Mueller-Harvey, 2006).

Tannins have traditionally been classified into two main groups: the condensed (CT) and the hydrolysable tannins (HT). Condensed tannins, or proanthocyanidins, are non-branched polymers of up to 50 flavonoids units

(flavan-3-ol, flavan-3,4-diol), and usually have a higher molecular weight than the HT (1000-20 000 Da compared to 500-3000 Da) (Mueller-Harvey, 2006). Hydrolysable tannins are nonflavonoid compounds which are classified as gallotannins and ellagitannins. In the first case, the core of HT is a glucose esterified to gallic acid, whereas in the second case glucose is esterified to both ellagic acid and gallic acid. As a result, these tannins are hydrolysable with acid, alkali, hot water and by enzymatic action.

Quebracho (*Schinopsis lorentzii*) and chestnut (*Castanea sativa*) have been the two most studied sources of CT and HT, respectively (Patra and Saxena, 2011). Tannins are widely distributed throughout the plant kingdom, especially among trees, shrubs and herbaceous leguminous plants (Rhoades, 1985; Perevolotsky, 1994). Moreover, the range of species over which tannin compounds are found has grown as detection techniques have developed. In general, tannins are more abundant in new leaves and flowers (which are more likely to be eaten by herbivores) (Rhoades, 1985; Terrill et al., 1992; Van Soest, 1994). As reported in many previous reviews environmental and seasonal factors as well as of phenological development influence the amount of tannins in the plant (Frutos et al., 2004). In general, high temperatures, water stress, extreme light intensities and poor soil quality increase the tannin content of plants (Rhoades, 1985; Van Soest, 1994). During their growth period, when plants produce a lot of biomass, few resources are available for synthesis of phenolic compounds. However, during flowering, when growth is reduced, excess carbon may be available for tannins synthesis (Iason et al., 1993).

The occurrence of tannins in ruminants' diets is very common, especially for grazing animals. In this sense, Fraisse et al. (2007) identified 43 botanical species with a total of 170 different phenolic compounds in the same pasture and estimated that a grazing cow could consume up to 500 g of those compounds per day. Many legume forages present in swards contain tannins, for example, sulla (*Hedysarum coronarium*), birdsfoot (*Lotus corniculatus*), big trefoil (*Lotus pedunculatus*), vetch (*Vicia sativa*), sainfoin (*Onobrychis coronarium*) and sericea (*Lespedeza cuneate*). In many regions, considering the competition between human being and livestock farming for the use of land and water, herbivores graze wild bushes and pastures rich in tannins such as safflower (*Carthamus tinctorius*), chicory (*Cichorium intybus)* or tropical plants (*Calliandra calothyrsus*, *Clitoria fairchildiana*, *Desmodium ovalifolium*, *Flemingia macrophylla*, *Leucaena leucocephala*, *Leucaena macrophylla* and *Leucaena pallida*). While in dry areas of the Mediterranean, sheep and goats browse tree leaves rich in tannins such as acacia (*Acacia cyanophylla*), oak (*Quercus pyrenaica*), argan tree (*Argania spinosa*) or carob (*Ceratonia siliqua*). Moreover, in recent years some agro-industry by-products containing considerable amounts of tannins have also been considered as feed additives for ruminants.

3.2 Activity

The reduction of ruminal protein degradation may be the most significant and well-known effect of tannins (Frutos et al., 2004). Tannins have a great affinity for proteins, and the neutral rumen pH favour the formation of tannin-protein complexes. In general, this reduction in protein degradation is associated with lower production of NH_3 N and greater non-NH_3 N flow to the duodenum (Frutos et al., 2004). Tannins from different plant species display a range of physical and chemical properties (Mangan, 1988), and therefore, have very diverse biological activities (Zucker, 1983). The high affinity of tannins for proteins is due to the high number of phenolic groups, which provide many possibilities for bonding to occur with the carbonyl groups of peptides (Leinmüller et al., 1991). The formation of such complexes is specific, both in terms of the tannin and protein involved, and the degree of affinity between the participating molecules residing in the chemical characteristics of each (Zucker, 1983; Mangan, 1988; Hagerman and Butler, 1991). The main factors that facilitate the formation of complexes include their relatively high molecular weight and their structural flexibility (Hagerman and Butler, 1991; Mueller-Harvey, 2006). The proteins that show the most affinity for tannins are relatively large and hydrophobic, with an open and flexible structure and are generally rich in proline (Mueller-Harvey, 2006). Tannins also form complexes with carbohydrates particularly hemicellulose, cellulose, starch and pectins. There is increasing evidence indicating that fibre degradation in the rumen can be drastically reduced in animals consuming tannins (McSweeney et al., 2001b).

The mechanisms by which tannins reduce ruminal degradation of dietary components are not well known but seem to rely on at least three modes of action: (i) substrate privation, (ii) enzyme inhibition and (iii) anti-microbial activity. With respect to substrate privation, it has been hypothesized that tannins prevent – or at least interfere with – the attachment of rumen microbes to feed particles (Frutos et al., 2004), which affect plant degradation (Chiquette et al., 1988; McAllister et al., 1994). Moreover, the formation of complexes with proteins and carbohydrates makes these nutrients inaccessible to microbes (Frutos et al., 2004). Tannins are also chelating agents, and this could reduce the availability of certain metallic ions necessary for the microbial metabolism. With respect to the enzyme inhibition, tannins can react with microbial (both bacterial and fungal) enzymes, inhibiting their activity (McSweeney et al., 2001b). Finally, tannins can have direct anti-microbial effect, e.g. by altering the permeability of their membrane (Frutos et al., 2004) which ultimately can shape the rumen microbiome. Chelating of metal ions by polyphenols could result in iron deprivation, a decrease in activity of metal enzymes and inhibition of the oxidative phosphorylation necessary for the microbial growth. Thus, metal chelators are known to have antimicrobial activities and cause indirect

but significant ecological changes (Smith et al., 2005). As a result of these antimicrobial properties, the degree of tolerance to tannins seems to widely differ between the different microbial groups in the rumen (e.g. bacteria, methanogens, protozoa and fungi). All these modes of action can differ depending on tannin source, and the differences between HT and CT in this respect are notorious.

Data on the effects of dietary tannins (both CT and HT) on ruminant nutrition and performance have been extensively reviewed in the past, by considering either the addition of purified substances or the natural tannins present in forages and concentrate feed ingredients (Makkar, 2003; Min et al., 2003). Therefore, here we focus on the most recent *in vivo* findings as illustrated in Table 2.

3.3 Effects of tannins on rumen function

Tannins have been shown to possess both detrimental and favourable effects, depending on the diet composition, the animal species, the tannin source and their level of inclusion in the diet (Table 2). There is a large body of studies indicating that high doses of tannins (both CT and HT) significantly reduced the voluntary feed intake and ultimately animal performance, if compared with low or medium doses (Frutos et al., 2004; Jayanegara et al., 2012). This is probably due to the reduction of palatability of the diet caused by the astringent activity of tannins. A recent meta-analysis (Min and Solaiman, 2018) found a negative linear correlation between CT content in the diet and DM intake in sheep, however, this response was quadratic in goats suggesting that low doses of tannins can stimulate feed intake in goats. Although concentrations can differ across studies and tannin sources, most recent studies have used tannins at concentrations between 5 and 60 mg/kg DM in ruminant diets.

In a meta-analysis based on *in vitro* and *in vivo* studies, Jayanegara et al. (2012) reported a linear decrease of total VFA concentration in relation to tannin supplementation, as well as a tendency to increase acetate molar proportion *in vitro*. However, the same work (Jayanegara et al., 2012) reported no significant effects of tannins on the total VFA and individual molar proportions based on *in vivo* data. Recent *in vivo* studies agree with these later observations suggesting that tannin supplementation can increase (5 studies), decrease (7 studies) but in most studies (28) had no effect on the rumen VFA concentration. Moreover, CT supplementation led in some cases (9 studies) to greater molar proportion of propionate. The reasons for this subtle shift in the rumen fermentation are still unknown but could rely on the formation of tannin-protein and tannin-fibre complexes. These complexes could have deleterious effects on fibrolytic bacteria given their high dependency on protein and fibre as substrate to produce acetate and butyrate as the main fermentation products (Belanche et al., 2012a).

Table 2 Summary of recent publications describing the effect (ns, non-significant; +, increase; –, decrease) of tannins on rumen fermentation and animal performance based on *in vivo* studies

	Study details					Rumen			
Reference	Animal	F:C	Plant	Form	g/kg DM	DMI	pH	NH3	VFA
Cieslak et al. (2012)	Dairy cow	60/40	Vaccinium vitis	CT−E	2		−−−	−−−	ns
Dschaak et al. (2011)	Dairy cow	59/41	Schinopsis sp.	CT−E	22	−−	ns	−	−
Dschaak et al. (2011)	Dairy cow	41/59	Schinopsis sp.	CT−E	22	−−	ns	−	−
Aguerre et al. (2016)	Dairy cow	51/49	Schinopsis sp.	CT−E	4 to 18	−−	ns	++	ns
Focant et al. (2019)	Dairy cow	64/36	Humulus lupulus	CT−E	2.9	ns			
Focant et al. (2019)	Dairy cow	64/36	Quercus sp.	HT−E	8.7	ns			
Focant et al. (2019)	Dairy cow	64/36	Humulus lupulus	HT−E	11	ns			
Benchaar et al. (2008)	Dairy cow	40/60	Schinopsis sp.	CT−E	6.4	ns	ns	ns	ns
Broderick et al. (2004)	Dairy cow	50/50	Lotus corniculatus	CT−P	6 to 17	+++			
Broderick et al. (2004)	Dairy cow	48/52	Lotus corniculatus	CT−P	5 to 15	+	ns	−	ns
Junior et al. (2017)	Cow	50/50	Acacia mearnsii	CT−E	2	ns	ns	ns	ns
Grainger et al. (2009)	Cow	88/12	Acacia mearnsii	CT−E	8 to 16	−			
Piñeiro-Vázquez et al. (2018)	Heifer	96/4	Schinopsis balansae	CT−E	7 to 29	ns	ns	ns	ns
Aboagye et al. (2019)	Heifer	95/5	Rhus chinensis	HT−E	15	ns	ns	ns	+
Aboagye et al. (2019)	Heifer	95/5	Taninic acid	CT−E	15	ns	ns	−	ns
Aboagye et al. (2019)	Heifer	95/5	Castanea sativa	HT−E	15	ns	ns	ns	ns
Dickhoefer et al. (2016)	Heifer	50/50	Schinopsis lorentzii	CT−E	1 to 10		−−−	ns	+++
Koenig and Beauchemin (2018)	Steer	9/91	Acacia mearnsii	CT−E	13	ns	ns	−	−−−
Doce et al. (2013)	Steer	100/0	Quercus pyrenaica	CT−L	76 to 112			−	ns
Koenig et al. (2018)	Steer	9.0/91	Acacia mearnsii	CT−E	13	ns	−	−	ns
Aboagye et al. (2018), Aboagye et al. (2018)	Steer	95/5	Castanea sativa	HT−E	2.5	ns	ns	−−	ns
Aboagye et al. (2018)	Steer	95/5	Castanea sativa	HT−E	15	ns	ns	−−	ns
Aboagye et al. (2018)	Steer	95/5	Various	HT+CT	2.5	ns	ns	−−	ns
Aboagye et al. (2018)	Steer	95/5	Various	HT+CT	15	ns	ns	−−	ns
Krueger et al. (2010)	Steer	9.0/91	Castanea sativa	HT−E	15	ns	ns/−	ns	ns
Krueger et al. (2010)	Steer	9.0/91	Acacia mearnsii	CT−E	15	ns	ns	ns	ns
Bhatta (2012)	Goats	65/35	Acacia mearnsii	CT+HT−E	3 to 6	ns	−	−	−
Gunun et al. (2016)	Goats	60/40	A thwaitesianum	CT−E	0.8 to 2	ns	ns	ns	−
Animut et al. (2008a)	Goats	100/0	Lespedeza striata	CT−P	50 to 150	ns	+	ns	ns
Animut et al. (2008b)	Goats	100/0	Schinopsis+Castanea	CT+HT−P	5	ns	ns	ns	ns
Abarghuei et al. (2011)	Sheep	67/33	Quercus libani	HT−L	3.8		ns	ns	
Dentinho et al. (2014)	Sheep	60/40	Cistus ladanifer	CT−E	0.1 to 2		ns	−	
Lima et al. (2019)	Sheep	60/40	Mimosa tenuiflora	CT−E	3	ns	−	ns	
Piñeiro-Vázquez et al. (2017)	Sheep	100/0	Various	CT−L	40 to 66	ns			ns
Rira et al. (2015)	Sheep	100/0	Various	CT−L	17 to 40	+++	−	ns	+++
Theodoridou et al. (2010)	Sheep	100/0	Onobrychis viciifolia	CT−P	3 to 13	ns		−−	ns

fermentation							Animal productivity													
C2	C3	C4	CH_4	Bac	Ptz	Meth	MY	Fat	Pr	MUN	BUN	OMd	NDFd	CPd	MN	UNE	FNE	Nr	ADG	FE
−	+	−	−−	ns	−−−		+	ns	ns			ns	ns							
++	++	+					ns	ns	ns	−−		ns	ns	ns				ns		++
ns	ns	ns					ns	ns	ns	−−		ns	ns	ns				ns		+
ns	ns	ns					ns	ns	+	ns	++					−	++	ns		++
			ns				ns	ns	ns			ns		ns		−	ns	ns		
			ns				ns	ns	ns			ns		−−		−−	+	ns		
			ns				++	ns	ns	ns		ns		ns		−−	ns	++		
ns	ns	ns			ns		ns	ns	ns	ns		ns	ns	ns						
							ns	ns	ns	−−		−−	−−	−−		−−	++	++		−−
ns	ns	ns					ns	ns	ns	−−		ns	ns	−−		−−	++	ns		ns
ns	ns	ns	ns		ns							ns								
			−−				−−	−	ns	ns						−−−	+++	ns		
ns	++	ns	−−									−	−	ns						
ns	ns	ns	−		ns						−−−	ns	ns	ns		+	ns	ns		
ns	ns	ns	ns		ns						−−−	ns	ns	−−−		ns	+++	ns		
ns	ns	ns	ns		ns						−−−	ns	ns	−−−		ns	+++	ns		
−−−	+	++													−−					
ns	−−	++									−−−	−−−	−−		−−−	−−	+++			
ns	+	ns										ns								
ns	ns	ns									−−−								ns	ns
ns	ns	−−			ns						ns					ns			ns	ns
ns	ns	ns	ns		ns						ns					ns			ns	ns
ns	ns	ns			ns						ns					−−			ns	ns
ns	ns	++	−		ns						ns					−−			ns	ns
ns	ns	ns	ns/+																ns	ns
ns	ns	ns/+	ns																ns	ns
+	ns	−	−									−	−	−		−	+	−		−
ns	+	ns	−	ns	ns						ns	ns	ns	ns	ns	−−	ns	++		
ns	−	ns	−−	ns	−−						ns	−−	−−	−−			++	++		
ns	ns	ns	ns	ns	ns						ns	ns	ns	−		ns		ns		
					−							−	−	−	−−		+			
ns	ns	ns										ns	ns	ns	−	ns	ns	ns		
			−		−−							ns	ns	ns						
ns	ns	ns	ns									ns	+	ns						
−−	++	ns		ns	ns	−						−								
ns	ns	ns			ns							ns		−−−		−	+++	ns		

(*Continued*)

Table 2 (*Continued*)

	Study details					Rumen			
Reference	Animal	F:C	Plant	Form	g/kg DM	DMI	pH	NH3	VFA
Malik et al. (2017)	Sheep	60/40	Ficus benghalensis	CT−L	11	ns		ns	ns
Malik et al. (2017)	Sheep	60/40	A. heterophyllus	CT−L	7.2	ns		ns	ns
Malik et al. (2017)	Sheep	60/40	Azadirachta indica	CT−L	7.4	−		ns	−
Liu et al. (2011)	Sheep	52/48	Castanea sativa	HT−E	1 to 3	ns	ns	−	ns
Vasta et al. (2010)	Sheep	30/70	Schinopsis lorentzii	CT−E	64				
Utsumi et al. (2013)	Sheep	61/39	Schinopsis sp.	CT−E	75	−	−		++
Utsumi et al. (2013)	Sheep	61/39	Schinopsis sp.	CT−E	75	−−−	+		ns
Śliwiński et al. (2002)	Lamb	55/45	Castanea sativa	HT−E	1 to 2	ns	ns	ns	ns
Salami et al. (2018)	Lamb	15/85	Castanea sativa	HT−E	40		−	ns	+
Salami et al. (2018)	Lamb	15/85	Caesalpinia spinosa	HT−E	40		ns	ns	ns
Salami et al. (2018)	Lamb	15/85	Acacia negra	CT−E	40		ns	ns	−
Salami et al. (2018)	Lamb	15/85	Uncaria gambir	CT−E	40		ns	ns	−
Jayanegara et al. (2012)	Various		Various	HT+CT	up to 177	+	ns	−−	ns
Min and Solaiman (2018)	Various		Various	HT+CT	up to 100	−		−	

Abbreviations: F:C, forage to concentrate ratio; DMI, DM intake; VFA, volatile fatty acids; C2, acetate; C3, propionate; C4, butyrate; Ptz, protozoa; Bac, bacteria; Meth, methanogens; MY, milk yield; Fat, milk fat %; Pr, milk protein content; OMd, OM digestibility; NDFd, NDF digestibility; CPd, CP

Tannins have been described to have an anti-microbial effect on some rumen microbes, by altering the permeability of membranes (Frutos et al., 2004). However, this anti-microbial effect is highly dependent on the dose and the nature of tannin as well as the microbial species. CT have a direct inhibitory effect on hemicellulases, endoglucanases, and proteolytic enzymes of several rumen microbes such as *Fibrobacter succinogenes, Butyrivibrio fibrisolvens, Ruminobacter amylophilus* and *Streptococcus bovis* (Vasta et al., 2019). Conversely, *Prevotella ruminicola* is able to counteract the negative effect of tannins by producing protective extracellular material (Jones et al., 1994). Moreover, several rumen bacteria and anaerobic fungi are able to completely degrade gallic acid (the main component of HT) to acetate and butyrate (Bhat et al., 1998). However, recent publications have not found a clear correlation between the content of HT in the diet and the accumulation of these fermentation products in the rumen (Table 2). A substantial decline in the rumen concentration of Firmicutes was observed when goats received chestnut and quebracho tannins, whereas phylum Bacteroidetes (generally gram-negative) increased in the rumen, and vice versa responses were described in the lower gut, possibly as a result of the dissociation of tannin-protein complexes in the lower gut (Min and Solaiman, 2018). Using two different sheep breeds (Texel and Blackbelly) supplemented with CT (17 to 40 mg/kg DM) from three different plant species

fermentation								Animal productivity													
C2	C3	C4	CH_4	Bac	Ptz	Meth	MY	Fat	Pr	MUN	BUN	OMd	NDFd	CPd	MN	UNE	FNE	Nr	ADG	FE
ns	ns	ns	−−		−−															
ns	ns	ns	−−		−−															
ns	ns	ns	−−−		−−															
ns	ns	ns	−		−	−													ns	ns
				ns	−−										+++					
ns	+	ns																		
−−−	+	ns																		
ns	ns	ns	ns	−	ns							ns	ns	ns		ns	ns	ns		
ns	ns	ns		ns	ns	ns														
ns	ns	ns		ns	−	ns														
ns	ns	+		ns	ns	−														
ns	ns	ns		ns	−	−														
ns	ns	ns	−−	ns	ns							−−	−−−	−−−				ns		
			−−−			−						−		−					ns	

digestibility; MN, microbial N flow; UNE, urinary N excretion; FNE, faecal N excretion; Nr, N retention; ADG, average daily gain; FE, feed efficiency.

(*Leucaena leucocephala*, *Gliricidia sepium* and *Manihot esculenta*), a decline in the rumen concentration of *R. flavefaciens* and methanogens was found; however, no differences were noted on the concentration of total bacteria, *R. albus, F. succcinogenes*, nor in the bacterial and methanogens diversity (Rira et al., 2015). McSweeney et al. (2001b) reported that condensed tannins from *Calliandra calothyrsus* decreased the numbers of cellulolytic bacteria, including populations of *F. succinogenes* and *Ruminococcus* spp, by contrast, protozoa and fungi and the microbial group containing *Bacteroides-Porphyromonas-Prevotella* bacteria appeared to be less affected. Another study (Vasta et al., 2010) showed that quebracho-supplemented sheep (64mg/kg DM) had higher rumen concentration of *B. fibrisolvens*. Moreover, a recent study showed that lambs supplemented with 4 different source of HT (chestnut and tara) and CT (mimosa and gambier) at 40 mg/kg DM had similar rumen concentration of the main bacterial and methanogen species but noted a substantial shift in the overall bacterial community structure when chestnut and mimosa, but not when tara and gambier were used (Salami et al., 2018). This discrepancy between different sources of tannin seems to support the hypothesis that the anti-microbial activity of tannins towards rumen microbes correlates with their molecular weight. Another work using molecular fingerprinting technique (Smith and Mackie, 2004) reported that the proportion of tannin-resistant

bacteria significantly increased in the rat gastrointestinal tract after exposure to condensed tannins. Sequencing analysis of DGGE bands and characterization of tannin-resistant isolates indicated that tannins selected for *Enterobacteriaceae* and *Bacteroides* species. Overall, these studies have revealed that tanning-containing diet or its extracts have a direct influence on the composition and diversity of the rumen and small and large intestinal microbiome (Min and Solaiman, 2018). However, these findings suggest that the effect of tannins on the rumen bacterial community is a multi-factorial effect which requires further studies using new generation of molecular techniques to fully elucidate their mode of action.

In vitro studies have suggested that tannins may have anti-microbial activity against anaerobic fungi (Patra and Saxena, 2009b). Liu et al. (2011) noted lower levels of anaerobic fungi in the rumen of sheep supplemented with chestnut HT; however, a more extensive study using various sources of HT and CT showed no impact on the fungal concentration in the rumen (Salami et al., 2018). Thus, fibre-degrading ability of rumen fungi may be less sensitive to the inhibitory effects of CT compared to cellulolytic bacteria (McSweeney et al., 2001b).

Various recent studies have shown a decrease in rumen methanogenesis in animals supplemented with tannins (Table 2). Min and Solaiman (2018) reported a negative correlation between the CT content in the diet and CH_4 emissions. This inhibition seems to rely on the anti-microbial effect against methanogens and on an indirect decrease in H_2 production as a result of decreased fibre digestion and protozoal population in the rumen (Patra et al., 2017). Various studies have shown lower methanogens concentrations in the rumen of sheep supplemented with tannins (Liu et al., 2011; Rira et al., 2015; Min and Solaiman, 2018) without affecting the overall methanogens community structure (Salami et al., 2018). Only *Methanobrevibacter* spp has been reported to linearly decrease with increasing CT content in the diet (Min and Solaiman, 2018). Studies in structure-activity relationships have shown that types and molecular weights of tannins are important in determining their potential in lowering CH_4 production and abundance of rumen methanogens, with high-molecular-weight CT being more potent (Patra et al., 2017). Min et al. (2003) reported that the optimal level of CT in the diet to decrease rumen methanogenesis and optimize sheep production was between 22 and 40 mg/kg DM. Although HT also can affect methanogens, these are usually more toxic to the animal and therefore tend to be used at lower inclusion rates than CT (Patra and Saxena, 2011). Nevertheless, the role of tannins in reducing CH_4 emissions is variable, sometimes confounded with the nutritional characteristics of the plant containing tannins, and little scientific evidence has been published on the actual mode of action. This calls for more work in this research area.

Effects of tannins on rumen protozoa have traditionally been considered as conflicting. A small proportion of studies reported an increase in protozoal

numbers when animals are supplemented with tannins, while similar proportion of studies showed a decrease or no effect of tannins (Patra and Saxena, 2009a). This discrepancy across studies seems to indicate that tannins present in all types of plants are not equally effective on protozoa. Focussing on the most recent publications (Table 2) it can be observed that this anti-protozoal effect seems to be diet dependent. Indeed, most of the studies in which tannins led to a significant decline in the protozoal population were conducted with animals fed a substantial proportion of concentrate feed (between 33 and 82%); on the contrary, most studies using forage-based diets showed no significant effects. It is well established that moderate amounts of concentrate feeds increase rumen protozoa levels (Belanche et al., 2011), an aspect that could magnify the anti-protozoal activity of tannins. Previous studies have stated that this anti-protozoal activity may differ across protozoal species, in particular, holotrichs seem to be more susceptible to tannins than the most abundant group, the entodiniomorphs (Makkar, 2003; Patra and Saxena, 2009b). However, three recent studies have shown that sheep consuming oak leaves (Abarghuei et al., 2011), various plant leaves rich in CT (Rira et al., 2015) and fresh sainfoin (Theodoridou et al., 2010) had lower ruminal proportion of entodiniomorphs than their un-supplemented counterparts leading up to the complete removal of certain species (i.e. *Diplodinium* and *Eudiplodiniium*). These findings suggest that tannins also have an anti-protozoal activity against, an aspect that indirectly could favour the rumen N efficiency given the larger bacterial N breakdown capacity described for entodiniomorphs in comparison to holotrichs (Belanche et al., 2012a). Despite these new findings, further studies are needed to truly understand the role of the tannins as rumen microbial modulators.

3.4 Effects of tannins on animal performance

Numerous articles exist on the ability of tannins to reduce the digestibility of the diet. Tannins mainly exert this effect on proteins based on their ability to form hydrogen bonds that are stable between pH 3.5 and 8 (approximately). These complexes are stable at rumen pH but dissociate when the pH is below 3.5, such as the abomasum (pH 2.5-3), or greater than 8 such as in the duodenum (pH 8) (Frutos et al., 2004). As a result, tannin supplementation modifies the location of the protein degradation shifting from the rumen towards the intestine. This decrease in the rumen feed degradation is often not fully compensated with the increased intestinal digestibility, possibly due to the persistence in the intestine of tannin-protein complexes which failed to dissociate in the abomasum, to the formation of new complexes with digestive enzymes and feed proteins, or to changes in the intestinal absorption due to the interaction of tannin with mucosal protein that normally is digested in

the gut, but once complexed with CT becomes indigestible. As a result, one of the clearest pieces of evidence showing that tannins reduce the total tract digestibility of the feed is the higher faecal N excretion (FNE) with increased tannin content. Most recent studies agree that this increase in FNE is linked to a decrease in urinary N excretion in animals supplemented with tannins, an aspect that could minimize NH_3 volatilization and/or underwater pollution and ultimately the negative consequences for the environment (Gill et al., 2010). Moreover ruminants can benefit from dietary tannins supplementation when increases in the duodenal protein flow exceed the reduction in the absorption of amino acids from the intestine.

In terms of productivity, since tannin consumption can affect feed intake and its digestive utilization, there are likely to be consequences on the animal productivity. In general, high tannin intakes (above 50 g/kg DM) have a clear negative effect on productivity as a result of lower voluntary feed intake and feed digestibility and alteration of the animal physiology (higher salivation rate, mucosal perturbations, etc.) (Serrano et al., 2009). Early studies observed a reduction in the live-weight gain in lambs fed high levels of CT (>76 g/kg DM); however, some authors indicated that continued ingestion of tannins might lead to a partial adaptation with the disappearance – or at least the attenuation – of their harmful effects (Barry and Manley, 1984). The intake of under 50 g/kg DM of tannins improved the digestive utilization of feed by ruminants, mainly because of a reduction in ruminal protein degradation and greater amino acid absorption in the small intestine (Min and Solaiman, 2018). Recent studies have shown increased average daily gains of 8% (Sharifi and Chaji, 2019) and 35% (Pathak et al., 2017) in lambs supplemented with various sources of CT at levels between 10 and 30 g/kg DM. With respect to milk yield, most recent studies showed no significant effect on either milk yield or milk composition despite many of them showing a consistent decrease in milk urea N and plasma urea N (Tiemann et al., 2008; Aboagye et al., 2019). Low levels of urea N in conjunction with increased productivity indicates enhanced N efficiency by the animal. This in several studies has been reported as greater milk yield (+2.3% to +5%) in cows fed *Vaccinium vitis* (Cieslak et al., 2012) and oak CT (Focant et al., 2019) or higher feed efficiency (+2% to +7%) in those consuming quebracho tannins (Dschaak et al., 2011; Aguerre et al., 2016). The ability of plant extracts including tannins to modify the fatty acid composition of ruminant-derived products (i.e. milk and meat) is currently receiving great attention. Recent studies have shown that tannins can inhibit some bacteria (i.e. *Butiryvibrio proteoclasticus*) which participate in rumen fatty acid biohydrogenation which covert vaccenic acid to stearic acid (Vasta et al., 2010). Therefore, it has been hypothesized that tannins could be a useful tool to improve product quality by increasing levels of unsaturated fatty acids.

4 Saponins

4.1 Composition

Saponins are naturally occurring secondary compounds produced mainly by plants, but also by lower marine animals and some bacteria (Yoshiki et al., 1998). The name is derived from the ability to form stable foam in aqueous solutions similar to soaps. As described by Hart et al. (2008), saponins are high-molecular-weight glycosides in which sugars are linked to a hydrophobic aglycone (sapogenin) which may be triterpenoid or steroidal in nature. The aglycone may contain one or more unsaturated C–C bonds. The saponins that have one sugar molecule attached at the C3 position are monodesmoside saponins, and those that have an additional sugar moiety at the C26 or C28 position are bidesmoside saponins. The diversity and complexity of saponin structures arises from the variability of the aglycone structure, the nature of the side chains and the position of attachment of these moieties on the aglycone. It has been shown that the type of linkage and the sugar composition of the saponins are directly related to their biological activity (Teferedegne, 2000; Ramos-Morales et al., 2017).

Saponins occur in different parts of the plants such as the roots, tuber, bark, leaves, seed and fruit. Triterpenoid saponins are found principally in cultivated crops (mostly dicotyledons), while steroidal saponins occur in monocotyledons. However, some plants contain both triterpenoid and steroidal. Young leaves contain more saponins than mature leaves. Thus, they are normally found in tissues that are most vulnerable to fungal or bacterial attack or insect predation (Morrissey and Osbourn, 1999; Francis et al., 2002).

Although saponins can be found in a multitude of plant species, the present section only focuses on those of relevance in ruminant production (Table 3). Usually the saponin-rich plants themselves are not used as animal feed; rather, the saponins are extracted from specific plant parts and used as feed additives. Triterpenoid saponins have been detected in many legumes such as quillaja, alfalfa, peas, beans and soyabeans and also in chestnut, tea, quinoa, alliums, spinach, sugar beet, sunflower or ginseng. Steroid saponins are found in yucca, oats, asparagus, alliums, capsicum peppers, aubergine, tomato seeds or yam. In terms of importance, Yucca and Quillaja are the most common sources of saponins and their extracts are commercially available not only as feed additives for ruminants but also as foaming agents in beverages and emulsifying agents in cosmetics. *Yucca schidigera* is a desert plant native from North America, while *Quillaja saponaria* (soapbark tree) originated from Chile. Yucca extracts contain 4.4% of steroid saponins, while *Q. saponaria* extracts contains 10% of total saponins, having both extracts over 20 different active saponins isoforms (Wina et al., 2005). Moreover, in the last decades relative new commercial sources of saponins with potential use in animal production

Table 3 Summary of recent publications describing the effect (ns, non-significant; +, increase; -, decrease) of saponins on rumen fermentation and animal performance based on *in vivo* studies

	Study details				Rumen fermentation											Animal productivity										
Reference	Animal	F:C	Plant	g/kg DM	DMI	pH	NH3	VFA	C2	C3	C4	CH_4	Bac	Ptz	Meth	MY	Fat	Prot	OMd	NDFd	CPd	MN	UNE	FNE	Nr	ADG	FE
Valdez et al. (1986)	Dairy cows	45/55	*Yucca shidigera*	0.077			ns	+	ns	ns	ns		ns	−		ns	ns	ns	ns							+	
Holtshausen et al. (2009)	Dairy cows	51/49	*Yucca schidigera*	1	ns	ns	−	ns	ns	ns	ns	ns		ns		ns	ns	ns	ns	ns	ns					ns	−−
Holtshausen et al. (2009)	Dairy cows	51/49	*Quillaja saponaria*	1	ns	ns	ns	ns	ns	ns	ns	ns		ns		ns	ns	ns	ns	ns	ns					ns	ns
Holtshausen et al. (2009)	Dairy cows	51/49	*Yucca schidigera*	2.7	ns	ns	ns	ns	ns	ns	ns			ns		ns	ns	ns	ns	ns	ns						
Anantasook et al. (2015)	Dairy cows	50/50	*Samanea saman*	5.3	ns	ns	−	ns	−	++	ns	−−		−	−	+	ns	+									
Poungchompu et al. (2009)	Cows	Various	Various	6.3	ns	ns	ns	++	−−−	+++	ns	−−−	ns	−−−	ns				−	ns	ns						
Guyader et al. (2015)/Popova et al. (2019)	Cows	50/50	*Camellia sinensis*	5	−	ns	ns	+	ns	ns	ns	ns	ns	ns	ns				ns	ns	ns	ns	ns	ns	ns		
Molina-Botero et al. (2019)	Heifers	80/20	Various	3.3	ns				ns	ns	ns	ns	ns	ns	ns				ns	ns	ns					ns	
Molina-Botero et al. (2019)	Heifers	85/15	Various	6.6	ns				ns	ns	ns	ns	ns	ns	ns				ns	ns	ns					ns	
Molina-Botero et al. (2019)	Heifers	90/10	Various	10	ns				ns	ns	ns	ns	ns	ns	ns				ns	−	ns					ns	
Ramirez-Restrepo et al. (2016a)	Steers	15/85	*Camellia sinensis*	1.4 to 2.2	ns	ns		ns	ns	ns	+	ns	ns	+	ns												
Wang et al. (2019)	Steers	35/65	*Camellia oleifera*	9g/d		ns		+	−	ns	ns																
Ramirez-Restrepo (2016b)	Steers	15/85	*Cammelia sinensis*	0.8 to 3.0	ns	+		−	+	−	−	ns															

Malik et al. (2009)	Buffalo	60/40	*Medicago sativa*	6	+	ns	ns	ns	– +	ns	ns	– +											
Santoso et al. (2007)	Goats	70/30	*B. petersianum*	0.3 to 0.5	ns	++	–– –		– ++	–		-				–	–	––	++	––	++	++	
Zhou et al. (2012)	Goats	50/50	*Ilex kudingcha*	0.4 to 0.8	ns	ns	ns	ns	ns ns	ns						ns	ns	ns					
Klita et al. (1996)	Sheep	100/0	*Medicago sativa*	10 to 40	ns	ns		ns	ns ns	ns	ns	–––				-	-	-					
Yuan et al. (2007)	Sheep	60/40	*Camellia sp.*	5		–		ns	ns ns	ns	–––												
Canul-Solis et al. (2014)	Sheep	94/6	*Yucca shidigera*	1.8 to 7.4	ns	ns		ns	ns ns	ns	ns					ns	ns	ns					
Wang et al. (2009)	Sheep	75/25	*Yucca shidigera*	0.1	ns	ns	–––	+++	ns +	ns	––					ns	ns	ns					
Zhou et al. (2011)	Sheep	60/40	*Camellia sinensis*	3		–	++	ns	ns ns	ns	––	–	ns						++				
Wina et al. (2006)	Sheep	65/35	*Sapindus rarak*	12 to 18			––	ns	ns +	–––		–––				ns	ns	ns	ns	ns	ns		
Śliwiński et al. (2002)	Lambs	55/45	*Yuca schidigera*	0.002 to 0.03	ns	ns	ns	ns	ns ns	ns	ns	ns				ns	ns	ns		ns	ns	ns	
Mao et al. (2010)	Lambs	60/40	*Camellia sinensis*	4	ns	++	ns	+	ns ns	ns	–––	–	ns						++			ns	

Abbreviations: F:C, forage to concentrate ratio; DMI, DM intake; VFA, volatile fatty acids; C2, acetate; C3, propionate; C4, butyrate; Ptz, protozoa; Bac, bacteria; Meth, methanogens; MY, milk yield; Fat, milk fat %; Pr, milk protein content; OMd, OM digestibility; NDFd, NDF digestibility; CPd, CP digestibility; MN, microbial N flow; UNE, urinary N excretion; FNE, faecal N excretion; Nr, N retention; ADG, average daily gain; FE, feed efficiency.

have been isolated from tea seeds (*Camellia sinensis*), alfafa (*Medicago sativa*) and *Sapindus* fruits (e.g. *S. saponaria* and *S. rarak*) (Patra and Saxena, 2009a).

4.2 Activity

Ciliate protozoa are inhabitants but not essential denizens in the rumen ecosystem, and are primarily responsible for the substantial turnover of bacterial protein due to predation of rumen bacteria (Belanche et al., 2012a). Consequently, the N economy in ruminants might be improved by the elimination of protozoa (defaunation) from the rumen despite having negative effects on fibre degradation (Newbold et al., 2015). Thus it is generally agreed that removing or decreasing rumen protozoal levels results in increased ruminant productivity, particularly on low-protein diets. Moreover, since protozoa are key microbes in H_2 production in the rumen, defaunation has been suggested as effective methane mitigation strategy (Belanche et al., 2015), with an average reduction of 11% (Newbold et al., 2015). However, the practicality of removing protozoa from the rumen in commercial farming is still a challenge. Saponins kill or damage protozoa by forming complexes with sterols in the protozoal membrane surface. The membranes become impaired and eventually disintegrate (Wallace et al., 2002). Consequently, inhibition of protozoa has long been a target for research. Rumen protozoal species seems to differ in their sensitivity to saponins due to differences in the sterol composition of their cellular membranes leading to the suggestion that feeding saponins might lead to partial defaunation (Patra and Saxena, 2009b). *In vitro* studies have demonstrated that Yucca saponins (1%) inhibited the motion of ciliate protozoa (the cilia of entodiniomorphs and the contraction of holotrich) and decreased their predatory activity of rumen bacteria (Wallace et al., 1994). Several review publications have described in detail the consistent negative effects of various sources of saponins on the protozoal concentration and activity *in vitro* (Wina et al., 2005; Patra and Saxena, 2009b). However, the effects of these extracts under *in vivo* conditions seem to be more variable.

It has been observed that the inhibition of protozoal concentration plateaued at a certain dose specific for saponins type (−53% of protozoa for Yaccca, −29% for Quillaja and −40% for *Biophytum petersianum*), beyond which there was no further inhibition (Pen et al., 2006; Santoso et al., 2007). Therefore current research is focused on combining saponins of different types in order to study the potential synergistic anti-protozoal activities. Additionally, the composition of the diet has a strong influence on the protozoal community, therefore, it would be expected that the effects of saponins on ruminal protozoa could be diet dependent. Several authors have demonstrated this hypothesis suggesting that the decrease in the rumen protozoal levels was of a greater magnitude when saponins were added to high-forage diets rather than to

high-concentrate diets (Hess et al., 2003; Poungchompu et al., 2009). Similarly, saponins' effects also seem to differ across ruminant species, and a recent study has demonstrated that unlike other animal species, cattle do not have high tolerance to high doses of tea saponins (Ramírez-Restrepo et al., 2016a).

4.3 Effects of saponins on rumen function

Anti-protozoal activity of saponins is the most consistent effect in the rumen ecosystem, and most studies suggest that all protozoal species have a similar sensitivity to saponins since no clear diferences in the protozoal species distribution (i.e. holotrich and entodiniomorphids) have been observed between animals supplemented with saponins and their un-supplemented counterparts (Śliwiński et al., 2002; Holtshausen et al., 2009; Popova et al., 2019).

The effect of saponins on the rumen bacterial community has not been explored in detail and results are contradictory. For example, rumen concentration of *F. succinogenes* has been reported to increase when cattle are supplemented with *Samanea saman* plant material (Anantasook et al., 2015) and tea saponins at 2 mg/kg DM (Ramírez-Restrepo et al., 2016a), while the opposite was observed in sheep supplemented with tea saponins (3 mg/kg DM) (Zhou et al., 2011). This discrepancy on the effects of saponins on the rumen bacterial community also extends to *R. albus* and *R. flavefaciens* across *in vivo* studies (Ramírez-Restrepo et al., 2016b; Wang et al., 2019). These studies clearly suggest species-dependent effects of saponins on rumen bacteria, which might offer a selective manipulation of rumen metabolism. For example, it has been speculated that supplementation of high-concentrate diets with saponins may reduce the incidence of rumen acidosis by inhibiting the growth of lactic acid-producing bacteria (i.e. *Streptococcus bovis*) (Russell and Rychlik, 2001). Using next-generation sequencing to study the rumen microbiome, Belanche et al. (2016) described the mode of action of ivy fruit saponins on the rumen simulation technique (Rusitec). This study concluded that ivy fruit saponins have a minor impact on the bacterial community leading to no differences in the microbial fermentation. Instead, ivy saponins modified the structure of the methanogen community and decreased its diversity. This specific antimicrobial effect of ivy saponins against methanogens was considered its main anti-methanogenic mechanism which led to a substantial decrease in methane production (-40%).

Recent *in vivo* publications (Table 3) are in line with this observation suggesting that addition of saponins in the diets might decrease methane production, which is likely due to a decrease in protozoal numbers and/or methanogenic archaea. For example supplementation with yucca saponins at low doses (0.1 g/kg DM) has promoted a 15% methane reduction in sheep

(Wang et al., 2009). Similarly, tea saponins have shown consistent decreases in methane production in sheep (−9%) and lambs (−27%) (Yuan et al., 2007; Mao et al., 2010) with a reduction in the protozoal numbers. As a result of this anti-protozoal effect, the inhibition in methane production originated by tea saponins was nearly double (−11% vs −5.7%) in faunated than in defaunated lambs (Zhou et al., 2011). The direct inhibitory activities of some saponins on rumen archaea are thus dependent on the type of saponins, their concentration and the type of diet. For example, saponins of *S. rarak* fruits decreased methanogens concentrations at high doses (4 mg/ml) while lower doses have no effects (Wina et al., 2005). Similarly, addition of *S. sesban* showed a more pronounced decrease in methane production in concentrate than in forage-based diets, despite having no correlation with the observed decline in methanogens (Goel et al., 2008). Guo et al. (2008) using an *in vitro* model showed that tea saponin reduced methane production and the expression levels of methyl coenzyme-M reductase subunit A (mcrA), and this effect was mediated via reduction of protozoa cells and the methanogens associated to them. Therefore, the effect of saponins on rumen ciliate protozoa and methanogens are highly variable among studies. Patra et al. (2017) suggested that saponins probably have little direct effect on methanogens but exert an indirect effect by lowering the abundance of protozoa-associated methanogens. It has been demonstrated that protozoa-associated methanogens are very active and can contribute up to 37% of total methane production (Finlay et al., 1994).

Although many studies show that saponins depress rumen protozoa, some noted that the antiprotozoal effect is only transient and this represents the main drawback of using them as feed additive. Protozoa '*per se*' seem not to become resistant to saponins (Newbold et al., 1997) but rumen bacteria can adapt to the presence of saponins by developing the ability to deglycosylate saponins making them inactive (Patra and Saxena, 2009b). Several strategies have been proposed to overcome this limitation such as (i) the intermittent supplementation with saponins to prevent the bacterial adaptation (Newbold et al., 1997); (ii) the combination of different types of saponins (Molina-Botero et al., 2019) and (iii) the use of chemically modified saponins to make them more resistant to degradation in the rumen (Ramos-Morales et al., 2017). However, more research is needed to elucidate the effectiveness of these strategies *in vivo* to overcome the challenge of rumen adaptation to saponins.

4.4 Effects of sapponins on animal performance

The antiprotozoal effect of saponins would be expected to have mixed effects on ruminant productivity depending on the diets and saponins involved. Although defaunation is known to decrease fibre degradation in the rumen, it is generally agreed that has positive effects on ruminant

productivity (Newbold et al., 2015). These positive effects are particularly relevant when animals are fed low-protein diets given the negative impact of rumen protozoa on the N metabolism (Firkins et al., 2007). Therefore saponins supplementation is considered useful for animals with high protein requirements, when fed low true protein diets, but no limited in energy (Eugène et al., 2004). Most recent publications have shown no effects of saponins on animal performances (i.e. milk yield and liveweight gain). The lack of consistency on the effect of saponins on high yielding cows might be due in some studies to the administration of low levels of saponins, while in other studies, nutrient requirements are probably met from the diet without saponins supplementation, therefore saponins have very little margin to improve animal productivity. Despite that constraint, a small number of publications have reported an increase in milk yield (+10%) when dairy cows were supplemented with *Samanea saman* saponins extract (Anantasook et al., 2015) or in liveweight gain (+9%) when goats were fed tea saponins (Jadhav et al., 2017). Although it is difficult to explain the exact mechanism of action of saponins (or their metabolites) that enhanced animal productivity, some findings showed a relative consistent increase in microbial protein synthesis. The supplementation of goats with *Biophytum petersianum* saponins have been reported to increase up to 37% the microbial N flow (Santoso et al., 2007). Similar increments in microbial N flow (up 29%) have been observed in faunated sheep (Mao et al., 2010; Jadhav et al., 2017) but only 8.6% in defaunated sheep (Zhou et al., 2011). These observations seem to suggest that positive effect on the N metabolism is mostly mediated by the anti-protozoal effect of saponins.

5 Future trends and conclusion

Plants extracts include a wide range of compounds with potential application in ruminants feeding. In particular essential oils, tannins and saponins have been proven to display mechanisms that modify the rumen fermentation and improve animal production. This mostly includes more efficient use of dietary protein and energy and decreased methane emissions, which sometimes result in increased milk yield or liveweight gain. However, despite extensive research conducted in the last decades, the use of plant extracts as feed additives in ruminant livestock is still limited by several factors. In addition to those related to highly variable composition and dosage used, the lack of robust *in vivo* studies that confirm the outcomes observed *in vitro* is one of the main barriers. Transferring the dosage used *in vitro* to *in vivo* conditions is challenging, but without such studies the implementation of a supplementary successful strategy at farm level is not possible. Another important factor to consider is the duration of the *in vivo* tests. While many treatments (i.e. saponins) failed to have

persistent effects, others (i.e. essential oils) need a longer time of exposure to achieve a sustained improvement in productivity.

Future research needs to strength the collaboration between animal feed companies, including those developing and providing additives with plant extracts, and scientists to make use of existing on-farm data and to better understand the effect of plant extracts on animal health and performance.

6 References

Abarghuei, M., Rouzbehan, Y. and Alipour, D. 2011. Effect of oak (*Quercus libani* Oliv.) leave tannin on ruminal fermentation of sheep. *Journal of Agricultural Science and Technology* 13, 1021–32.

Aboagye, I. A., Oba, M., Castillo, A. R., Koenig, K. M., Iwaasa, A. D. and Beauchemin, K. A. 2018. Effects of hydrolyzable tannin with or without condensed tannin on methane emissions, nitrogen use, and performance of beef cattle fed a high-forage diet. *Journal of Animal Science* 96(12), 5276–86. doi:10.1093/jas/sky352.

Aboagye, I. A., Oba, M., Koenig, K. M., Zhao, G. Y. and Beauchemin, K. A. 2019. Use of gallic acid and hydrolyzable tannins to reduce methane emission and nitrogen excretion in beef cattle fed a diet containing alfalfa silage. *Journal of Animal Science* 97(5), 2230–44. doi:10.1093/jas/skz101.

Acamovic, T. and Brooker, J. D. 2005. Biochemistry of plant secondary metabolites and their effects in animals. *Proceedings of the Nutrition Society* 64(3), 403–12. doi:10.1079/pns2005449.

Aguerre, M. J., Capozzolo, M. C., Lencioni, P., Cabral, C. and Wattiaux, M. A. 2016. Effect of quebracho-chestnut tannin extracts at 2 dietary crude protein levels on performance, rumen fermentation, and nitrogen partitioning in dairy cows. *Journal of Dairy Science* 99(6), 4476–86. doi:10.3168/jds.2015-10745.

Anantasook, N., Wanapat, M., Cherdthong, A. and Gunun, P. 2015. Effect of tannins and saponins in *Samanea saman* on rumen environment, milk yield and milk composition in lactating dairy cows. *Journal of Animal Physiology and Animal Nutrition* 99(2), 335–44. doi:10.1111/jpn.12198.

Animut, G., Puchala, R., Goetsch, A. L., Patra, A. K., Sahlu, T., Varel, V. H. and Wells, J. 2008a. Methane emission by goats consuming diets with different levels of condensed tannins from lespedeza. *Animal Feed Science and Technology* 144(3–4), 212–27. doi:10.1016/j.anifeedsci.2007.10.014.

Animut, G., Puchala, R., Goetsch, A. L., Patra, A. K., Sahlu, T., Varel, V. H. and Wells, J. 2008b. Methane emission by goats consuming different sources of condensed tannins. *Animal Feed Science and Technology* 144(3–4), 228–41. doi:10.1016/j.anifeedsci.2007.10.015.

Anitescu, G., Doneanu, C. and Radulescu, V. 1997. Isolation of coriander oil: comparison between steam distillation and supercritical CO_2 extraction. *Flavour and Fragrance Journal* 12(3), 173–6. doi:10.1002/(SICI)1099-1026(199705)12:3<173::AID-FFJ630>3.0.CO;2-1.

Barry, T. N. and Manley, T. R. 1984. The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep: 2. Quantitative digestion of carbohydrates and proteins. *British Journal of Nutrition* 51(3), 493–504. doi:10.1079/bjn19840055.

Beauchemin, K. A. and McGinn, S. M. 2006. Methane emissions from beef cattle: effects of fumaric acid, essential oil, and canola oil. *Journal of Animal Science* 84(6), 1489–96. doi:10.2527/2006.8461489x.

Belanche, A., Abecia, L., Holtrop, G., Guada, J. A., Castrillo, C., De la Fuente, G. and Balcells, J. 2011. Study of the effect of presence or absence of protozoa on rumen fermentation and microbial protein contribution to the chyme. *Journal of Animal Science* 89(12), 4163–74 doi:10.2527/jas.2010-3703.

Belanche, A., De la Fuente, G., Moorby, J. M. and Newbold, C. J. 2012a. Bacterial protein degradation by different rumen protozoal groups. *Journal of Animal Science* 90(12), 4495–504. doi:10.2527/jas.2012-5118.

Belanche, A., Doreau, M., Edwards, J. E., Moorby, J. M., Pinloche, E. and Newbold, C. J. 2012b. Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. *Journal of Nutrition* 142(9), 1684–92. doi:10.3945/jn.112.159574.

Belanche, A., de la Fuente, G. and Newbold, C. J. 2015. Effect of progressive inoculation of fauna-free sheep with holotrich protozoa and total-fauna on rumen fermentation, microbial diversity and methane emissions. *FEMS Microbiology Ecology* 91(3), 1–10. doi:10.1093/femsec/fiu026.

Belanche, A., Pinloche, E., Preskett, D. and Newbold, C. J. 2016. Effects and mode of action of chitosan and ivy fruit saponins on the microbiome, fermentation and methanogenesis in the rumen simulation technique. *FEMS Microbiology Ecology* 92(1), 1–13. doi:10.1093/femsec/fiv160.

Belanche, A., Newbold, C., Morgavi, D., Bach, A., Zweifel, B. and Yañez-Ruiz, D. 2019. A meta-analysis describing the effects of a commercial essential oils blend on performance, digestion and methane emissions in dairy cows. *Animals* (in press).

Benchaar, C. and Greathead, H. 2011. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. *Animal Feed Science and Technology* 166–167, 338–55. doi:10.1016/j.anifeedsci.2011.04.024.

Benchaar, C., Petit, H. V., Berthiaume, R., Ouellet, D. R., Chiquette, J. and Chouinard, P. Y. 2007. Effects of essential oils on digestion, ruminal fermentation, rumen microbial populations, milk production, and milk composition in dairy cows fed alfalfa silage or corn silage. *Journal of Dairy Science* 90(2), 886–97. doi:10.3168/jds.S0022-0302(07)71572-2.

Benchaar, C., Calsamiglia, S., Chaves, A. V., Fraser, G. R., Colombatto, D., McAllister, T. A. and Beauchemin, K. A. 2008. A review of plant-derived essential oils in ruminant nutrition and production. *Animal Feed Science and Technology* 145(1–4), 209–28. doi:10.1016/j.anifeedsci.2007.04.014.

Benchaar, C., Lettat, A., Hassanat, F., Yang, W. Z., Forster, R. J., Petit, H. V. and Chouinard, P. Y. 2012. Eugenol for dairy cows fed low or high concentrate diets: effects on digestion, ruminal fermentation characteristics, rumen microbial populations and milk fatty acid profile. *Animal Feed Science and Technology* 178(3–4), 139–50. doi:10.1016/j.anifeedsci.2012.10.005.

Bhat, T. K., Singh, B. and Sharma, O. P. 1998. Microbial degradation of tannins–a current perspective. *Biodegradation* 9(5), 343–57. doi:10.1023/a:1008397506963.

Bhatta, R., Enishi, O., Yabumoto, Y., Nonaka, I., Takusari, N., Higuchi, K. and Takenaka, A. M. Kurihara, M. 2012. Methane reduction and energy partitioning in goats fed two concentrations of tannin from Mimosa spp. J. Agric. Sci, 84, 409–415..

Blanch, M., Carro, M. D., Ranilla, M. J., Viso, A., Vazquez-Anon, M. and Bach, A. 2016. Influence of a mixture of cinnamaldehyde and garlic oil on rumen fermentation, feeding behaviour and performance of lactating dairy cows. *Animal Feed Science and Technology* 219, 313–23. doi:10.1016/j.anifeedsci.2016.07.002.

Bodas, R., López, S., Fernandez, M., García-González, R., Rodríguez, A. B., Wallace, R. J. and González, J. S. 2008. *In vitro* screening of the potential of numerous plant species as antimethanogenic feed additives for ruminants. *Animal Feed Science and Technology* 145(1–4), 245–58. doi:10.1016/j.anifeedsci.2007.04.015.

Bodas, R., Prieto, N., García-González, R., Andrés, S., Giráldez, F. J. and López, S. 2012. Manipulation of rumen fermentation and methane production with plant secondary metabolites. *Animal Feed Science and Technology* 176(1–4), 78–93. doi:10.1016/j.anifeedsci.2012.07.010.

Braun, H. S., Schrapers, K. T., Mahlkow-Nerge, K., Stumpff, F. and Rosendahl, J. 2019. Dietary supplementation of essential oils in dairy cows: evidence for stimulatory effects on nutrient absorption. *Animal: an International Journal of Animal Bioscience* 13(3), 518–23. doi:10.1017/S1751731118001696.

Broderick, G. A., Udén, P., Murphy, M. L. and Lapins, A. 2004. Sources of variation in rates of *in vitro* ruminal protein degradation. *Journal of Dairy Science* 87(5), 1345–59. doi:10.3168/jds.S0022-0302(04)73284-1.

Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology* 94(3), 223–53. doi:10.1016/j.ijfoodmicro.2004.03.022.

Busquet, M., Calsamiglia, S., Ferret, A. and Kamel, C. 2005a. Screening for the effects of natural plant extracts and secondary plant metabolites on rumen microbial fermentation in continuous culture. *Animal Feed Science and Technology* 123, 597–613.

Busquet, M., Calsamiglia, S., Ferret, A., Cardozo, P. W. and Kamel, C. 2005b. Effects of cinnamaldehyde and garlic oil on rumen microbial fermentation in a dual flow continuous culture. *Journal of Dairy Science* 88(7), 2508–16. doi:10.3168/jds.S0022-0302(05)72928-3.

Calsamiglia, S., Busquet, M., Cardozo, P. W., Castillejos, L. and Ferret, A. 2007. Invited review: essential oils as modifiers of rumen microbial fermentation. *Journal of Dairy Science* 90(6), 2580–95. doi:10.3168/jds.2006-644.

Canaes, T. S., Zanferari, F., Maganhe, B. L., Takiya, C. S., Silva, T. H., Del Valle, T. A. and Rennó, F. P. 2017. Increasing dietary levels of citral oil on nutrient total tract digestibility, ruminal fermentation, and milk composition in Saanen goats. *Animal Feed Science and Technology* 229, 47–56. doi:10.1016/j.anifeedsci.2017.05.002.

Canul-Solis, J. R., Piñeiro-Vázquez, A. T., Briceño-Poot, E. G., Chay-Canul, A. J., Alayón-Gamboa, J. A., Ayala-Burgos, A. J., Aguilar-Pérez, C. F., Solorio-Sánchez, F. J., Castelán-Ortega, O. A. and Ku-Vera, J. C. 2014. Effect of supplementation with saponins from *Yucca schidigera* on ruminal methane production by Pelibuey sheep fed *Pennisetum purpureum* grass. *Animal Production Science* 54(10), 1834–7. doi:10.1071/AN14296.

Cardozo, P. W., Calsamiglia, S., Ferret, A. and Kamel, C. 2004. Effects of natural plant extracts on ruminal protein degradation and fermentation profiles in continuous culture. *Journal of Animal Science* 82(11), 3230–6. doi:10.2527/2004.82113230x.

Cardozo, P. W., Calsamiglia, S., Ferret, A. and Kamel, C. 2005. Screening for the effects of natural plant extracts at different pH on *in vitro* rumen microbial fermentation of a

high-concentrate diet for beef cattle. *Journal of Animal Science* 83(11), 2572–9. doi :10.2527/2005.83112572x.

Cardozo, P. W., Calsamiglia, S., Ferret, A. and Kamel, C. 2006. Effects of alfalfa extract, anise, capsicum, and a mixture of cinnamaldehyde and eugenol on ruminal fermentation and protein degradation in beef heifers fed a high-concentrate diet. *Journal of Animal Science* 84(10), 2801–8. doi:10.2527/jas.2005-593.

Carson, C. F., Mee, B. J. and Riley, T. V. 2002. Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. *Antimicrobial Agents and Chemotherapy* 46(6), 1914–20. doi:10.1128/aac.46.6.1914-1920. 2002.

Castro-Montoya, J., Peiren, N., Cone, J. W., Zweifel, B., Fievez, V. and De Campeneere, S. 2015. *In vivo* and *in vitro* effects of a blend of essential oils on rumen methane mitigation. *Livestock Science* 180, 134–42. doi:10.1016/j.livsci.2015.08.010.

Chaves, A. V., Dugan, M. E. R., Stanford, K., Gibson, L. L., Bystrom, J. M., McAllister, T. A., Van Herk, F. and Benchaar, C. 2011. A dose-response of cinnamaldehyde supplementation on intake, ruminal fermentation, blood metabolites, growth performance, and carcass characteristics of growing lambs. *Livestock Science* 141(2–3), 213–20. doi:10.1016/j.livsci.2011.06.006.

Chiquette, J., Cheng, K.-J., Costerton, J. W. and Milligan, L. P. 1988. Effect of tannins on the digestibility of two isosynthetic strains of birdsfoot trefoil (*Lotus corniculatus* L.) using *in vitro* and *in sacco* techniques. *Canadian Journal of Animal Science* 68(3), 751–60. doi:10.4141/cjas88-084.

Cieslak, A., Zmora, P., Pers-Kamczyc, E. and Szumacher-Strabel, M. 2012. Effects of tannins source (*Vaccinium vitis idaea* L.) on rumen microbial fermentation *in vivo*. *Animal Feed Science and Technology* 176(1–4), 102–6. doi:10.1016/j.anifeedsci.2012.07.012.

Cieslak, A., Szumacher-Strabel, M., Stochmal, A. and Oleszek, W. 2013. Plant components with specific activities against rumen methanogens. *Animal: an International Journal of Animal Bioscience* 7(Suppl. 2), 253–65. doi:10.1017/S1751731113000852.

Cobellis, G., Trabalza-Marinucci, M. and Yu, Z. 2016. Critical evaluation of essential oils as rumen modifiers in ruminant nutrition: a review. *Science of the Total Environment* 545–546, 556–68. doi:10.1016/j.scitotenv.2015.12.103.

Cowan, M. M. 1999. Plant products as antimicrobial agents. *Clinical Microbiology Reviews* 12(4), 564–82. doi:10.1128/CMR.12.4.564.

de Jesus, F., Del Valle, E. T. A., Calomeni, G. D., Silva, T. H., Takiya, C. S., Vendramini, T. H. A., Paiva, P. G., Silva, G. G., Netto, A. S. and Rennó, F. P. 2013. Influence of a blend of functional oils or monensin onnutrient intake and digestibility, ruminal fermentation andmilk production of dairy cows. *Animal Feed Science and Technology* 219, 59–67.

Delaquis, P. J., Stanich, K., Girard, B. and Mazza, G. 2002. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *International Journal of Food Microbiology* 74(1–2), 101–9. doi:10.1016/s0168-1605(01)00734-6.

Dentinho, M. T. P., Belo, A. T. and Bessa, R. J. B. 2014. Digestion, ruminal fermentation and microbial nitrogen supply in sheep fed soybean meal treated with *Cistus ladanifer* L. tannins. *Small Ruminant Research* 119(1–3), 57–64. doi:10.1016/j.smallrumres.2014.02.012.

Denyer, S. P. and Hugo, W. B. 1991. Biocide-induced damage to the bacterial cytoplasmic membrane. In: Denyer, S. P. and Hugo, W. B. (Eds), *Mechanisms of Action of Chemical Biocides*. Blackwell Scientific Publications, Oxford, UK, pp. 171–88.

Dhama, K., Latheef, S. K., Mani, S., Samad, H. A., Karthik, K., Tiwari, R., Khan, R. U., Alagawany, M., Farag, M. R., Alam, G. M., Laudadio, V. and Tufarelli, V. 2015. Multiple beneficial applications and modes of action of herbs in poultry health and production: a review. *International Journal of Pharmacology* 11(3), 152–76. doi:10.3923/ijp.2015.152.176.

Dickhoefer, U., Ahnert, S. and Susenbeth, A. 2016. Effects of quebracho tannin extract on rumen fermentation and yield and composition of microbial mass in heifers. *Journal of Animal Science* 94(4), 1561–75. doi:10.2527/jas.2015-0061.

Doce, R. R., Belenguer, A., Toral, P. G., Hervás, G. and Frutos, P. 2013. Effect of the administration of young leaves of *Quercus pyrenaica* on rumen fermentation in relation to oak tannin toxicosis in cattle. *Journal of Animal Physiology and Animal Nutrition* 97(1), 48–57. doi:10.1111/j.1439-0396.2011.01241.x.

Dschaak, C. M., Williams, C. M., Holt, M. S., Eun, J. S., Young, A. J. and Min, B. R. 2011. Effects of supplementing condensed tannin extract on intake, digestion, ruminal fermentation, and milk production of lactating dairy cows. *Journal of Dairy Science* 94(5), 2508–19. doi:10.3168/jds.2010-3818.

Durmic, Z., Moate, P. J., Eckard, R., Revell, D. K., Williams, R. and Vercoe, P. E. 2014. *In vitro* screening of selected feed additives, plant essential oils and plant extracts for rumen methane mitigation. *Journal of the Science of Food and Agriculture* 94(6), 1191–6. doi:10.1002/jsfa.6396.

Elcoso, G., Zweifel, B. and Bach, A. 2019. Effects of a blend of essential oils on milk yield and feed efficiency of lactating dairy cows. *Applied Animal Science* 35(3), 304–11. doi:10.15232/aas.2018-01825.

Eschenlauer, S. C. P., McKain, N., Walker, N. D., McEwan, N. R., Newbold, C. J. and Wallace, R. J. 2002. Ammonia production by ruminal microorganisms and enumeration, isolation, and characterization of bacteria capable of growth on peptides and amino acids from the sheep rumen. *Applied and Environmental Microbiology* 68(10), 4925–31. doi:10.1128/aem.68.10.4925-4931.2002.

Estelle, R. E. 2010. Coping with shrub secondary metabolites by ruminants. *Small Ruminant Research* 94(1–3), 1–9. doi:10.1016/j.smallrumres.2010.09.012.

Eugène, M., Archimède, H. and Sauvant, D. 2004. Quantitative meta-analysis on the effects of defaunation of the rumen on growth, intake and digestion in ruminants. *Livestock Production Science* 85(1), 81–97. doi:10.1016/S0301-6226(03)00117-9.

Evans, J. D. and Martin, S. A. 2000. Effects of thymol on ruminal microorganisms. *Current Microbiology* 41(5), 336–40. doi:10.1007/s002840010145.

Finlay, B. J., Esteban, G., Clarke, K. J., Williams, A. G., Embley, T. M. and Hirt, R. P. 1994. Some rumen ciliates have endosymbiotic methanogens. *FEMS Microbiology Letters* 117(2), 157–61. doi:10.1111/j.1574-6968.1994.tb06758.x.

Firkins, J. L., Yu, Z. and Morrison, M. 2007. Ruminal nitrogen metabolism: perspectives for integration of microbiology and nutrition for dairy. *Journal of Dairy Science* 90(Suppl. 1), E1–16. doi:10.3168/jds.2006-518.

Flores, A. J., Garciarena, A. D., Vieyra, J. M. H., Beauchemin, K. A. and Colombatto, D. (2013). Effects of specific essential oil compounds on the ruminal environment, milk production and milk composition of lactating dairy cows at pasture. Animal feed science and technology, 186(1-2), 20–26.

Focant, M., Froidmont, E., Archambeau, Q., Dang Van, Q. C. and Larondelle, Y. 2019. The effect of oak tannin (*Quercus robur*) and hops (*Humulus lupulus*) on dietary nitrogen efficiency, methane emission, and milk fatty acid composition of dairy cows fed a low-protein diet including linseed. *Journal of Dairy Science* 102(2), 1144–59. doi:10.3168/jds.2018-15479.

Fraisse, D., Carnat, A., Viala, D., Pradel, P., Besle, J. M., Coulon, J. B., Felgines, C. and Lamaison, J. L. 2007. Polyphenolic composition of a permanent pasture: variations related to the period of harvesting. *Journal of the Science of Food and Agriculture* 87(13), 2427–35. doi:10.1002/jsfa.2918.

Francis, G., Kerem, Z., Makkar, H. P. and Becker, K. 2002. The biological action of saponins in animal systems: a review. *British Journal of Nutrition* 88(6), 587–605. doi:10.1079/BJN2002725.

Franz, C. and Novak, J. 2009. Sources of essential oils. In: Başer, K. H. C. and Buchbauer, G. (Eds), *Handbook of Essential Oils: Science, Technology, and Applications*. CRC Press, Boca Raton, FL, pp. 48–90.

Frutos, P., Hervas, G., Giraldez, F. J. and Mantecon, A. R. 2004. Review. Tannins and ruminant nutrition. *Spanish Journal of Agricultural Research* 2(2), 191–202. doi:10.5424/sjar/2004022-73.

Ganguly, S. 2013. Herbal and plant derived natural products as growth promoting nutritional supplements for poultry birds: a review. *Journal of Pharmaceutical and Scientific Innovation* 2(3), 12–3. doi:10.7897/2277-4572.02323.

García-González, R., López, S., Fernandez, M., Bodas, R. and González, J. S. 2008. Screening the activity of plants and spices for decreasing ruminal methane production in vitro. *Animal Feed Science and Technology* 147(1–3), 36–52. doi:10.1016/j.anifeedsci.2007.09.008.

Ghizzi, L. G., Del Valle, T. A., Takiya, C. S., da Silva, G. G., Zilio, E. M. C., Grigoletto, N. T. S., Martello, L. S. and Rennó, F. P. 2018. Effects of functional oils on ruminal fermentation, rectal temperature, and performance of dairy cows under high temperature humidity index environment. *Animal Feed Science and Technology* 246, 158–66. doi:10.1016/j.anifeedsci.2018.10.009.

Giannenas,I., Skoufos, J., Giannakopoulos, C., Wiemann, M., Gortzi, O., Lalas, S.and Kyriazakis, I. 2011. Effects of essential oils on milk production, milk composition, and rumen microbiota in Chios dairy ewes. *Journal of Dairy Science* 94(11), 5569–77. doi:10.3168/jds.2010-4096.

Gill, M., Smith, P. and Wilkinson, J. M. 2010. Mitigating climate change: the role of domestic livestock. *Animal: an International Journal of Animal Bioscience* 4(3), 323–33. doi:10.1017/S1751731109004662.

Goel, G., Makkar, H. P. S. and Becker, K. 2008. Effects of *Sesbania sesban* and *Carduus pycnocephalus* leaves and Fenugreek (*Trigonella foenum-graecum* L.) seeds and their extracts on partitioning of nutrients from roughage-and concentrate-based feeds to methane. *Animal Feed Science and Technology* 147(1–3), 72–89. doi:10.1016/j.anifeedsci.2007.09.010.

Grainger, C., Clarke, T., Auldist, M. J., Beauchemin, K. A., McGinn, S. M., Waghorn, G. C. and Eckard, R. J. 2009. Potential use of *Acacia mearnsii* condensed tannins to reduce methane emissions and nitrogen excretion from grazing dairy cows. *Canadian Journal of Animal Science* 89(2), 241–51. doi:10.4141/CJAS08110.

Greathead, H. 2003. Plants and plant extracts for improving animal productivity. *Proceedings of the Nutrition Society* 62(2), 279–90. doi:10.1079/pns2002197.

Gunun, P., Wanapat, M., Gunun, N., Cherdthong, A., Sirilaophaisan, S. and Kaewwongsa, W. 2016. Effects of condensed tannins in mao (*Antidesma thwaitesianum* Muell. Arg.) seed meal on rumen fermentation characteristics and nitrogen utilization in goats. *Asian-Australasian Journal of Animal Sciences* 29(8), 1111–9. doi:10.5713/ajas.15.0552.

Guo, Y. Q., Liu, J. X., Lu, Y., Zhu, W. Y., Denman, S. E. and McSweeney, C. S. (2008). Effect of tea saponin on methanogenesis, microbial community structure and expression of mcrA gene, in cultures of rumen micro-organisms. Letters in Applied Microbiology, 47(5), 421–426..

Guyader, J., Eugène, M., Doreau, M., Morgavi, D. P., Gérard, C., Loncke, C. and Martin, C. 2015. Nitrate but not tea saponin feed additives decreased enteric methane emissions in nonlactating cows. *Journal of Animal Science* 93(11), 5367–77. doi:10.2527/jas.2015-9367.

Hagerman, A. and Butler, L. G. 1991. Tannins and lignins. In: Rosenthal, G. A. and Berenbaum, M. R. (Eds), Herbivores: Their Interactions with Secondary Plant Metabolites (vol. 1), pp. 355–88.

Harborne, J. B. and Baxter, H. 1999. *The Handbook of Natural Flavonoids* (vols. 1 and 2). John Wiley and Sons, Hoboken, NJ.

Hart, K. J., Yanez-Ruiz, D. R., Duval, S. M., McEwan, N. R. and Newbold, C. J. 2008. Plant extracts to manipulate rumen fermentation. *Animal Feed Science and Technology* 147(1–3), 8–35. doi:10.1016/j.anifeedsci.2007.09.007.

Hart, K. J., Jones, H. G., Waddams, K. E., Worgan, H. J., Zweifel, B. and Newbold, C. J. 2019. An essential oil blend decreases methane emissions and increases milk yield in dairy cows. *Open Journal of Animal Sciences* 09(3), 259–67. doi:10.4236/ojas.2019.93022.

Hatew, B., Cone, J. W., Pellikaan, W. F., Podesta, S. C., Bannink, A., Hendriks, W. H. and Dijkstra, J. 2015. Relationship between *in vitro* and *in vivo* methane production measured simultaneously with different dietary starch sources and starch levels in dairy cattle. *Animal Feed Science and Technology* 202, 20–31. doi:10.1016/j.anifeedsci.2015.01.012.

Hess, H. D., Kreuzer, M., Díaz, T. E., Lascano, C. E., Carulla, J. E., Soliva, C. R. and Machmüller, A. 2003. Saponin rich tropical fruits affect fermentation and methanogenesis in faunated and defaunated rumen fluid. *Animal Feed Science and Technology* 109(1–4), 79–94. doi:10.1016/S0377-8401(03)00212-8.

Holtshausen, L., Chaves, A. V., Beauchemin, K. A., McGinn, S. M., McAllister, T. A., Odongo, N. E., Cheeke, P. R. and Benchaar, C. 2009. Feeding saponin-containing *Yucca schidigera* and *Quillaja saponaria* to decrease enteric methane production in dairy cows. *Journal of Dairy Science* 92(6), 2809–21. doi:10.3168/jds.2008-1843.

Iason, G. R., Hartley, S. E. and Duncan, A. J. 1993. Chemical composition of *Calluna vulgaris* (Ericaceae): do responses to fertilizer vary with phenological stage? *Biochemical Systematics and Ecology* 21(3), 315–21. doi:10.1016/0305-1978(93)90023-K.

Jadhav, R. V., Kannan, A., Bhar, R., Sharma, O. P., Bhat, T. K., Gulati, A., Rajkumar, K., Sharma, R., Mal, G., Singh, B. and Sharma, V. K. 2017. Effect of tea (*Camellia sinensis*) seed saponin supplementation on growth performance, nutrient utilization, microbial protein synthesis and hemato-biochemical attributes of gaddi goats. *Animal Nutrition and Feed Technology* 17(2), 255–68. doi:10.5958/0974-181X.2017.00025.7.

Jayanegara, A., Leiber, F. and Kreuzer, M. 2012. Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from *in vivo* and *in vitro*

experiments. *Journal of Animal Physiology and Animal Nutrition* 96(3), 365–75. doi:10.1111/j.1439-0396.2011.01172.x.

Jones, G. A., McAllister, T. A., Muir, A. D. and Cheng, K. J. 1994. Effects of saifoin (*Onobrychis viciifolia* Scop.) condensed tannins on growth and proteolysis by 4 strains of ruminal bacteria. *Applied and Environmental Microbiology* 60(4), 1374–8. doi:10.1128/AEM.60.4.1374-1378.1994.

Jouany, J. P. and Morgavi, D. P. 2007. Use of 'natural' products as alternatives to antibiotic feed additives in ruminant production. *Animal: an International Journal of Animal Bioscience* 1(10), 1443–66. doi:10.1017/S1751731107000742.

Junior, F. P., Cassiano, E. C. O., Martins, M. F., Romero, L. A., Zapata, D. C. V., Pinedo, L. A., Marino, C. T. and Rodrigues, P. H. M. 2017. Effect of tannins-rich extract from *Acacia mearnsii* or monensin as feed additives on ruminal fermentation efficiency in cattle. *Livestock Science* 203, 21–9. doi:10.1016/j.livsci.2017.06.009.

Kholifa, A. E., Matloupa, O. H., Morsy, T. A., Abdo, M. M., Abu Elella, A. A., Anele, U. Y. and Swanson, K. C. 2017. Rosemary and lemongrass herbs as phytogenic feed additives to improve efficient feed utilization, manipulate rumen fermentation and elevate milk production of Damascus goats. *Livestock Science* 204, 39–46. doi:10.1016/j.livsci.2017.08.001.

Khorrami, B., Vakili, A. R., Mesgaran, M. D. and Klevenhusen, F. 2015. Thyme and cinnamon essential oils: potential alternatives for monensin as a rumen modifier in beef production systems. *Animal Feed Science and Technology* 200, 8–16. doi:10.1016/j.anifeedsci.2014.11.009.

Klita, P. T., Mathison, G. W., Fenton, T. W. and Hardin, R. T. 1996. Effects of alfalfa root saponins on digestive function in sheep. *Journal of Animal Science* 74(5), 1144–56. doi:10.2527/1996.7451144x.

Klop, G., Dijkstra, J., Dieho, K., Hendriks, W. H. and Bannink, A. 2017. Enteric methane production in lactating dairy cows with continuous feeding of essential oils or rotational feeding of essential oils and lauric acid. *Journal of Dairy Science* 100(5), 3563–75. doi:10.3168/jds.2016-12033.

Knobloch, K., Weis, N. and Weigand, H. 1986. Mechanism of antimicrobial activity of essential oils. *Planta Medica* 52(6), 556–. doi:10.1055/s-2007-969370.

Knobloch, K., Pauli, A., Iberl, B., Weigand, H. and Weis, N. 1989. Antibacterial and antifungal properties of essential oil components. *Journal of Essential Oil Research* 1(3), 119–28. doi:10.1080/10412905.1989.9697767.

Koenig, K. M. and Beauchemin, K. A. 2018. Effect of feeding condensed tannins in high protein finishing diets containing corn distillers grains on ruminal fermentation, nutrient digestibility, and route of nitrogen excretion in beef cattle. *Journal of Animal Science* 96(10), 4398–413. doi:10.1093/jas/sky273.

Koenig, K. M., Beauchemin, K. A. and McGinn, S. M. 2018. Feeding condensed tannins to mitigate ammonia emissions from beef feedlot cattle fed high-protein finishing diets containing distillers grains. *Journal of Animal Science* 96(10), 4414–30. doi:10.1093/jas/sky274.

Kroon, P. A. and Williamson, G. 1999. Hydroxycinnamates in plants and food: current and future perspectives. *Journal of the Science of Food and Agriculture* 79(3), 355–61. doi:10.1002/(SICI)1097-0010(19990301)79:3<355::AID-JSFA255>3.0.CO;2-G.

Krueger, W. K., Gutierrez-Banuelos, H., Carstens, G. E., Min, B. R., Pinchak, W. E., Gomez, R. R., Anderson, R. C., Krueger, N. A. and Forbes, T. D. A. 2010. Effects of dietary tannin source on performance, feed efficiency, ruminal fermentation, and carcass

and non-carcass traits in steers fed a high-grain diet. *Animal Feed Science and Technology* 159(1–2), 1–9. doi:10.1016/j.anifeedsci.2010.05.003.

Lee, S. E., Hwang, H. J., Ha, J. S., Jeong, H. S. and Kim, J. H. 2003. Screening of medicinal plant extracts for antioxidant activity. *Life Sciences* 73(2), 167–79. doi:10.1016/s0024-3205(03)00259-5.

Leinmüller, E., Steingass, H. and Menke, K. 1991. Tannins in ruminant feedstuffs. In: Bittner, A. (Ed.), *Animal Research and Development* (vol. 33). Institut fur WissenschaftlicheZusammenarbeit, Germany, pp. 9–62.

Lima, P. R., Apdini, T., Freire, A. S., Santana, A. S., Moura, L. M. L., Nascimento, J. C. S., Rodrigues, R. T. S., Dijkstra, J., Garcez Neto, A. F., Queiroz, M. A. Á. and Menezes, D. R. 2019. Dietary supplementation with tannin and soybean oil on intake, digestibility, feeding behavior, ruminal protozoa and methane emission in sheep. *Animal Feed Science and Technology* 249, 10–7. doi:10.1016/j.anifeedsci.2019.01.017.

Liu, H., Vaddella, V. and Zhou, D. 2011. Effects of chestnut tannins and coconut oil on growth performance, methane emission, ruminal fermentation, and microbial populations in sheep. *Journal of Dairy Science* 94(12), 6069–77. doi:10.3168/jds.2011-4508.

Makkar, H. P. S. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. *Small Ruminant Research* 49(3), 241–56. doi:10.1016/S0921-4488(03)00142-1.

Malik, P., Singhal, K. and Deshpande, S. 2009. Effect of saponin rich lucerne fodder supplementation on rumen fermentation, bacterial and protozoal population in buffalo bulls. *Indian Journal of Animal Sciences* 79, 912–6.

Malik, P. K., Kolte, A. P., Baruah, L., Saravanan, M., Bakshi, B. and Bhatta, R. 2017. Enteric methane mitigation in sheep through leaves of selected tanniniferous tropical tree species. *Livestock Science* 200, 29–34. doi:10.1016/j.livsci.2017.04.001.

Mangan, J. L. 1988. Nutritional effects of tannins in animal feeds. *Nutrition Research Reviews* 1(1), 209–31. doi:10.1079/NRR19880015.

Mao, H.-L., Wang, J.-K., Zhou, Y.-Y. and Liu, J.-X. 2010. Effects of addition of tea saponins and soybean oil on methane production, fermentation and microbial population in the rumen of growing lambs. *Livestock Science* 129(1–3), 56–62. doi:10.1016/j.livsci.2009.12.011.

Martinez, S., Madrid, J., Hernandez, F., Megias, M. D., Sotomayor, J. A. and Jordan, M. J. 2006. Effect of thyme essential oils (*Thymus hyemalis* and *Thymus zygis*) and monensin on *in vitro* ruminal degradation and volatile fatty acid production. *Journal of Agricultural and Food Chemistry* 54(18), 6598–602. doi:10.1021/jf060985p.

McAllister, T. A., Bae, H. D., Jones, G. A. and Cheng, K. J. 1994. Microbial attachment and feed digestion in the rumen. *Journal of Animal Science* 72(11), 3004–18. doi:10.2527/1994.72113004x.

McIntosh, F. M., Williams, P., Losa, R., Wallace, R. J., Beever, D. A. and Newbold, C. J. 2003. Effects of essential oils on ruminal microorganisms and their protein metabolism. *Applied and Environmental Microbiology* 69(8), 5011–4. doi:10.1128/aem.69.8.5011-5014.2003.

McSweeney, C. S., Palmer, B., Bunch, R. and Krause, D. O. 2001a. Effect of the tropical forage calliandra on microbial protein synthesis and ecology in the rumen. *Journal of Applied Microbiology* 90(1), 78–88. doi:10.1046/j.1365-2672.2001.01220.x.

McSweeney, C. S., Palmer, B., McNeill, D. M. and Krause, D. O. 2001b. Microbial interactions with tannins: nutritional consequences for ruminants. *Animal Feed Science and Technology* 91(1–2), 83–93. doi:10.1016/S0377-8401(01)00232-2.

Min, B. R. and Solaiman, S. 2018. Comparative aspects of plant tannins on digestive physiology, nutrition and microbial community changes in sheep and goats: a review. *Journal of Animal Physiology and Animal Nutrition* 102(5), 1181–93. doi:10.1111/jpn.12938.

Min, B. R., Barry, T. N., Attwood, G. T. and McNabb, W. C. 2003. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. *Animal Feed Science and Technology* 106(1–4), 3–19. doi:10.1016/S0377-8401(03)00041-5.

Molina-Botero, I. C., Arroyave-Jaramillo, J., Valencia-Salazar, S., Barahona-Rosales, R., Aguilar-Pérez, C. F., Burgos, A. A., Arango, J. and Ku-Vera, J. C. 2019. Effects of tannins and saponins contained in foliage of Gliricidia sepium and pods of Enterolobium cyclocarpum on fermentation, methane emissions and rumen microbial population in crossbred heifers. *Animal Feed Science and Technology* 251, 1–11. doi:10.1016/j.anifeedsci.2019.01.011.

Morgavi, D. P., Forano, E., Martin, C. and Newbold, C. J. 2010. Microbial ecosystem and methanogenesis in ruminants. *Animal: an International Journal of Animal Bioscience* 4(7), 1024–36. doi:10.1017/S1751731110000546.

Morrissey, J. P. and Osbourn, A. E. 1999. Fungal resistance to plant antibiotics as a mechanism of pathogenesis. *Microbiology and Molecular Biology Reviews: MMBR* 63(3), 708–24. doi:10.1128/MMBR.63.3.708-724.1999.

Mueller-Harvey, I. 2006. Unravelling the conundrum of tannins in animal nutrition and health. *Journal of the Science of Food and Agriculture* 86(13), 2010–37. doi:10.1002/jsfa.2577.

Muthusamy, N. and Sankar, V. 2015. Phytogenic compounds used as a feed additives in poultry production. *International Journal of Science Environment and Technology* 4, 167–71.

Nagy, J. G., Steinhoff, H. W. and Ward, G. M. 1964. Effects of essential oils of sagebrush on deer rumen microbial function. *Journal of Wildlife Management* 28(4), 785–90. doi:10.2307/3798795.

Newbold, C. J., El Hassan, S. M., Wang, J., Ortega, M. E. and Wallace, R. J. 1997. Influence of foliage from African multipurpose trees on activity of rumen protozoa and bacteria. *British Journal of Nutrition* 78(2), 237–49. doi:10.1079/bjn19970143.

Newbold, C. J., de la Fuente, G., Belanche, A., Ramos-Morales, E. and McEwan, N. R. 2015. The role of ciliate protozoa in the rumen. *Frontiers in Microbiology* 6, 1313. doi:10.3389/fmicb.2015.01313.

Oh, H. K., Jones, M. B. and Longhurst, W. M. 1968. Comparison of rumen microbial inhibition resulting from various essential oils isolated from relatively unpalatable plant species. *Applied Environmental Microbiology* 16(1), 39–44. doi:10.1128/AEM.16.1.39-44.1968.

Okoh, O. O., Sadimenko, A. P. and Afolayan, A. J. 2010. Comparative evaluation of the antibacterial activities of the essential oils of *Rosmarinus officinalis* L. obtained by hydrodistillation and solvent free microwave extraction methods. *Food Chemistry* 120(1), 308–12. doi:10.1016/j.foodchem.2009.09.084.

Ornaghi, M. G., Passetti, R. A. C., Torrecilhas, J. A., Mottin, C., Vital, A. C. P., Guerrero, A., Sañudo, C., del Mar Campo, M. and Prado, I. N. 2017. Essential oils in the diet of young bulls: effect on animal performance, digestibility, temperament, feeding behaviour and carcass characteristics. *Animal Feed Science and Technology* 234, 274–83. doi:10.1016/j.anifeedsci.2017.10.008.

Pathak, A. K., Dutta, N., Pattanaik, A. K., Chaturvedi, V. B. and Sharma, K. 2017. Effect of condensed tannins from *Ficus infectoria* and *Psidium guajava* leaf meal mixture on nutrient metabolism, methane emission and performance of lambs. *Asian-Australasian Journal of Animal Sciences* 30(12), 1702–10. doi:10.5713/ajas.17.0060.

Patra, A. K. 2010. Meta-analyses of effects of phytochemicals on digestibility and rumen fermentation characteristics associated with methanogenesis. *Journal of the Science of Food and Agriculture* 90(15), 2700–8. doi:10.1002/jsfa.4143.

Patra, A. K. 2011. Effects of essential oils on rumen fermentation, microbial ecology and ruminant production. *Asian Journal of Animal and Veterinary Advances* 6(5), 416–28. doi:10.3923/ajava.2011.416.428.

Patra, A. K. and Saxena, J. 2009a. Dietary phytochemicals as rumen modifiers: a review of the effects on microbial populations. *Antonie Van Leeuwenhoek* 96(4), 363–75. doi:10.1007/s10482-009-9364-1.

Patra, A. K. and Saxena, J. 2009b. The effect and mode of action of saponins on the microbial populations and fermentation in the rumen and ruminant production. *Nutrition Research Reviews* 22(2), 204–19. doi:10.1017/S0954422409990163.

Patra, A. K. and Saxena, J. 2011. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. *Journal of the Science of Food and Agriculture* 91(1), 24–37. doi:10.1002/jsfa.4152.

Patra, A. K. and Yu, Z. 2012. Effects of essential oils on methane production and fermentation by, and abundance and diversity of, rumen microbial populations. *Applied and Environmental Microbiology* 78(12), 4271–80. doi:10.1128/AEM.00309-12.

Patra, A. K. and Yu, Z. 2013. Effects of coconut and fish oils on ruminal methanogenesis, fermentation, and abundance and diversity of microbial populations *in vitro*. *Journal of Dairy Science* 96(3), 1782–92. doi:10.3168/jds.2012-6159.

Patra, A. K. and Yu, Z. 2015. Essential oils affect populations of some rumen bacteria *in vitro* as revealed by microarray (RumenBactArray) analysis. *Frontiers in Microbiology* 6, 297. doi:10.3389/fmicb.2015.00297.

Patra, A., Park, T., Kim, M. and Yu, Z. 2017. Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. *Journal of Animal Science and Biotechnology* 8, 13. doi:10.1186/s40104-017-0145-9.

Pauli, A. and Schilcher, H. 2010. In vitro antimicrobial activities of essential oils monographed in the European pharmacopoeia 6th edition. In: Başer, K. H. C. and Buchbauer, G. (Eds), *Handbook of Essential Oils: Science, Technology, and Applications*. CRC Press, Boca Raton, FL, pp. 343–548.

Pen, B., Sar, C., Mwenya, B., Kuwaki, K., Morikawa, R. and Takahashi, J. 2006. Effects of *Yucca schidigera* and *Quillaja saponaria* extracts on *in vitro* ruminal fermentation and methane emission. *Animal Feed Science and Technology* 129(3–4), 175–86. doi:10.1016/j.anifeedsci.2006.01.002.

Perevolotsky, A. 1994. Tannins in Mediterranean woodland species: lack of response to browsing and thinning. *Oikos* 71(2), 333–40. doi:10.2307/3546282.

Piñeiro-Vázquez, A. T., Canul-Solis, J. R., Casanova-Lugo, F., Chay-Canul, A. J., Ayala-Burgos, A. J., Solorio-Sánchez, F. J., Aguilar-Pérez, C. F. and Ku-Vera, J. C. 2017. Emisión de metano en ovinos alimentados con *Pennisetum purpureum* y árboles que contienen taninos condensados. *Revista mexicana de ciencias pecuarias* 8(2), 111–9. doi:10.22319/rmcp.v8i2.4401.

Piñeiro-Vázquez, A. T., Canul-Solis, J. R., Jiménez-Ferrer, G. O., Alayón-Gamboa, J. A., Chay-Canul, A. J., Ayala-Burgos, A. J., Aguilar-Pérez, C. F. and Ku-Vera, J. C. 2018. Effect of condensed tannins from Leucaena leucocephala on rumen fermentation, methane production and population of rumen protozoa in heifers fed low-quality forage. *Asian-Australasian Journal of Animal Sciences* 31(11), 1738–46. doi:10.5713/ajas.17.0192.

Popova, M., Guyader, J., Silberberg, M., Seradj, A. R., Saro, C., Bernard, A., Gérard, C., Martin, C. and Morgavi, D. P. 2019. Changes in the rumen microbiota of cows in response to dietary supplementation with nitrate, linseed, and saponin alone or in combination. *Applied and Environmental Microbiology* 85(4), e02657-02618. doi:10.1128/AEM.02657-18.

Poungchompu, O., Wanapat, M., Wachirapakorn, C., Wanapat, S. and Cherdthong, A. 2009. Manipulation of ruminal fermentation and methane production by dietary saponins and tannins from mangosteen peel and soapberry fruit. *Archives of Animal Nutrition* 63(5), 389–400. doi:10.1080/17450390903020406.

Ramírez-Restrepo, C. A., O'Neill, C. J., López-Villalobos, N., Padmanabha, J., Wang, J. K. and McSweeney, C. 2016a. Effects of tea seed saponin supplementation on physiological changes associated with blood methane concentration in tropical Brahman cattle. *Animal Production Science* 56(3), 457–65. doi:10.1071/AN15582.

Ramírez-Restrepo, C. A., Tan, C., O'Neill, C. J., López-Villalobos, N., Padmanabha, J., Wang, J. and McSweeney, C. S. 2016b. Methane production, fermentation characteristics, and microbial profiles in the rumen of tropical cattle fed tea seed saponin supplementation. *Animal Feed Science and Technology* 216, 58–67. doi:10.1016/j.anifeedsci.2016.03.005.

Ramos-Morales, E., De La Fuente, G., Duval, S., Wehrli, C., Bouillon, M., Lahmann, M., Preskett, D., Braganca, R. and Newbold, C. J. 2017. Antiprotozoal effect of saponins in the rumen can be enhanced by chemical modifications in their structure. *Frontiers in Microbiology* 8, 399. doi:10.3389/fmicb.2017.00399.

Rhoades, D. F. 1985. Offensive-defensive interactions between herbivores and plants: their relevance in herbivore population dynamics and ecological theory. *American Naturalist* 125(2), 205–38. doi:10.1086/284338.

Rira, M., Morgavi, D. P., Archimède, H., Marie-Magdeleine, C., Popova, M., Bousseboua, H. and Doreau, M. 2015. Potential of tannin-rich plants for modulating ruminal microbes and ruminal fermentation in sheep. *Journal of Animal Science* 93(1), 334–47. doi:10.2527/jas.2014-7961.

Russell, J. B. 1991. Intracellular pH of acid-tolerant ruminal bacteria. *Applied and Environmental Microbiology* 57(11), 3383–4. doi:10.1128/AEM.57.11.3383-3384.1991.

Russell, J. B. and Rychlik, J. L. 2001. Factors that alter rumen microbial ecology. *Science* 292(5519), 1119–22. doi:10.1126/science.1058830.

Russell, J. B., Onodera, R. and Hino, T. 1991. Ruminal protein fermentation: new perspectives on previous contradictions. In: Tsuda, T., Sasaki, Y. and Kawashima, R. (Eds), *Physiological Aspects of Digestion and Metabolism in Ruminants*. Academic Press Limited, London, UK, pp. 691–7.

Salami, S. A., Valenti, B., Bella, M., O'Grady, M. N., Luciano, G., Kerry, J. P., Jones, E., Priolo, A. and Newbold, C. J. 2018. Characterisation of the ruminal fermentation and microbiome in lambs supplemented with hydrolysable and condensed tannins. *FEMS Microbiology Ecology* 94(5), fiy061. doi:10.1093/femsec/fiy061.

Santoso, B., Kilmaskossu, A. and Sambodo, P. 2007. Effects of saponin from *Biophytum petersianum* Klotzsch on ruminal fermentation, microbial protein synthesis and nitrogen utilization in goats. *Animal Feed Science and Technology* 137(1–2), 58–68. doi:10.1016/j.anifeedsci.2006.10.005.

Santosa, M. B., Robinson, P. H., Williams, P. and Losa, R. 2010. Effects of addition of an essential oil complex to the diet of lactating dairy cows on whole tract digestion of nutrients and productive performance. *Animal Feed Science and Technology* 157(1–2), 64–71. doi:10.1016/j.anifeedsci.2010.02.001.

Schären, M., Drong, C., Kiri, K., Riede, S., Gardener, M., Meyer, U., Hummel, J., Urich, T., Breves, G. and Dänicke, S. 2017. Differential effects of monensin and a blend of essential oils on rumen microbiota composition of transition dairy cows. *Journal of Dairy Science* 100(4), 2765–83. doi:10.3168/jds.2016-11994.

Schofield, P., Mbugua, D. M. and Pell, A. N. 2001. Analysis of condensed tannins: a review. *Animal Feed Science and Technology* 91(1–2), 21–40. doi:10.1016/S0377-8401(01)00228-0.

Serrano, J., Puupponen-Pimia, R., Dauer, A., Aura, A. M. and Saura-Calixto, F. 2009. Tannins: current knowledge of food sources, intake, bioavailability and biological effects. *Molecular Nutrition and Food Research* 53(Suppl. 2), S310–29. doi:10.1002/mnfr.200900039.

Sharifi, A. and Chaji, M. 2019. Effects of processed recycled poultry bedding with tannins extracted from pomegranate peel on the nutrient digestibility and growth performance of lambs. *South African Journal of Animal Science* 49(2), 290–300. doi:10.4314/sajas.v49i2.9.

Sikkema, J., de Bont, J. A. and Poolman, B. 1994. Interactions of cyclic hydrocarbons with biological membranes. *Journal of Biological Chemistry* 269(11), 8022–8.

Sikkema, J., de Bont, J. A. and Poolman, B. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiological Reviews* 59(2), 201–22. doi:10.1128/MMBR.59.2.201-222.1995.

Silva,G. G., Takiya, C. S., Del Valle, T. A., de Jesus, E. F., Grigoletto, N. T. S., Nakadonari, B., Cortinhas, C. S., Acedo, T. S. and Rennó, F. P. 2018. Nutrient digestibility, ruminal fermentation, and milk yield in dairy cows fed a blend of essential oils and amylase. *Journal of Dairy Science* 101(11), 9815–26. doi:10.3168/jds.2018-14789.

Silva, R. B. D., Pereira, M. N., Araujo, R. C. D., Silva, W. D. R. and Pereira, R. A. N. (2020). A blend of essential oils improved feed efficiency and affected ruminal and systemic variables of dairy cows. Translational Animal Science, 4(1), 183..

Śliwiński, B. J., Kreuzer, M., Wettstein, H. R. and Machmüller, A. 2002. Rumen fermentation and nitrogen balance of lambs fed diets containing plant extracts rich in tannins and saponins, and associated emissions of nitrogen and methane. *Archives of Animal Nutrition* 56(6), 379–92. doi:10.1080/00039420215633.

Smith, A. H. and Mackie, R. I. 2004. Effect of condensed tannins on bacterial diversity and metabolic activity in the rat gastrointestinal tract. *Applied and Environmental Microbiology* 70(2), 1104–15. doi:10.1128/aem.70.2.1104-1115.2004.

Smith, A. H., Zoetendal, E. and Mackie, R. I. 2005. Bacterial mechanisms to overcome inhibitory effects of dietary tannins. *Microbial Ecology* 50(2), 197–205. doi:10.1007/s00248-004-0180-x.

Soltan,Y. A., Natel, A. S., Araujo, R. C., Morsy, A. S. and Abdalla, A. L. 2018. Progressive adaptation of sheep to a microencapsulated blend of essential oils: ruminal

fermentation, methane emission, nutrient digestibility, and microbial protein synthesis. *Animal Feed Science and Technology* 237, 8–18. doi:10.1016/j.anifeedsci.2018.01.004.

Soto, E. C., Molina-Alcaide, E., Khelil, H. and Yáñez-Ruiz, D. R. 2013. Ruminal microbiota developing in different *in vitro* simulation systems inoculated with goats' rumen liquor. *Animal Feed Science and Technology* 185(1–2), 9–18. doi:10.1016/j.anifeedsci.2013.06.003.

Souza, K. Ad, Monteschio, JdO., Mottin, C., Ramos, T. R., Pinto, L. AdM., Eiras, C. E., Guerrero, A. and Prado, I. Nd. 2019. Effects of diet supplementation with clove and rosemary essential oils and protected oils (eugenol, thymol and vanillin) on animal performance, carcass characteristics, digestibility, and ingestive behavior activities for Nellore heifers finished in feedlot. *Livestock Science* 220, 190–5. doi:10.1016/j.livsci.2018.12.026.

Spanghero, M., Robinson, P. H., Zanfi, C. and Fabbro, E. 2009. Effect of increasing doses of a microencapsulated blend of essential oils on performance of lactating primiparous dairy cows. *Animal Feed Science and Technology* 153(1–2), 153–7. doi:10.1016/j.anifeedsci.2009.06.004.

Szumacher-Strabel, M. and Cieślak, A. 2012. Dietary possibilities to mitigate rumen methane and ammonia production. In: Liu, G. (Ed.), *Greenhouse Gases-Capturing, Utilization and Reduction*. IntechOpen. University of North Dakota, United States of America, pp. 199–218.

Tager, L. R. and Krause, K. M. 2011. Effects of essential oils on rumen fermentation, milk production, and feeding behavior in lactating dairy cows. *Journal of Dairy Science* 94(5), 2455–64. doi:10.3168/jds.2010-3505.

Teferedegne, B. 2000. New perspectives on the use of tropical plants to improve ruminant nutrition. *Proceedings of the Nutrition Society* 59(2), 209–14. doi:10.1017/s0029665100000239.

Tekippe, J. A., Hristov, A. N., Heyler, K. S., Cassidy, T. W., Zheljazkov, V. D., Ferreira, J. F. S., Karnati, S. K. and Varga, G. A. 2011. Rumen fermentation and production effects of *Origanum vulgare* L. leaves in lactating dairy cows. *Journal of Dairy Science* 94(10), 5065–79. doi:10.3168/jds.2010-4095.

Tekippe, J. A., Tacoma, R., Hristov, A. N., Lee, C., Oh, J., Heyler, K. S., Cassidy, T. W., Varga, G. A. and Bravo, D. 2013. Effect of essential oils on ruminal fermentation and lactation performance of dairy cows. *Journal of Dairy Science* 96(12), 7892–903. doi:10.3168/jds.2013-7128.

Terrill, T. H., Douglas, G. B., Foote, A. G., Purchas, R. W., Wilson, G. F. and Barry, T. N. 1992. Effect of condensed tannins upon body growth, wool growth and rumen metabolism in sheep grazing Sulla (*Hedysarum coronarium*) and perennial pasture. *Journal of Agricultural Science* 119(2), 265–73. doi:10.1017/S0021859600014192.

Theodoridou, K., Aufrère, J., Andueza, D., Pourrat, J., Le Morvan, A., Stringano, E., Mueller-Harvey, I. and Baumont, R. 2010. Effects of condensed tannins in fresh sainfoin (*Onobrychis viciifolia*) on *in vivo* and in situ digestion in sheep. *Animal Feed Science and Technology* 160(1–2), 23–38. doi:10.1016/j.anifeedsci.2010.06.007.

Tiemann, T. T., Lascano, C. E., Wettstein, H. R., Mayer, A. C., Kreuzer, M. and Hess, H. D. 2008. Effect of the tropical tannin-rich shrub legumes *Calliandra calothyrsus* and *Flemingia macrophylla* on methane emission and nitrogen and energy balance in growing lambs. *Animal: an International Journal of Animal Bioscience* 2(5), 790–9. doi:10.1017/S1751731108001791.

Tomkins, N. W., Denman, S. E., Pilajun, R., Wanapat, M., McSweeney, C. S. and Elliott, R. 2015. Manipulating rumen fermentation and methanogenesis using an essential oil and monensin in beef cattle fed a tropical grass hay. *Animal Feed Science and Technology* 200, 25–34. doi:10.1016/j.anifeedsci.2014.11.013.

Ultee, A., Bennik, M. H. and Moezelaar, R. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Applied and Environmental Microbiology* 68(4), 1561–8. doi:10.1128/aem.68.4.1561-1568.2002.

Utsumi, S. A., Cibils, A. F., Estell, R. E., Soto-Navarro, S. A., Chen, L. and Hallford, D. M. 2013. Effects of adding protein, condensed tannins, and polyethylene glycol to diets of sheep and goats fed one-seed juniper and low quality roughage. *Small Ruminant Research* 112(1–3), 56–68. doi:10.1016/j.smallrumres.2012.09.006.

Valdez, F. R., Bush, L. J., Goetsch, A. L. and Owens, F. N. 1986. Effect of steroidal sapogenins on ruminal fermentation and on production of lactating dairy cows. *Journal of Dairy Science* 69(6), 1568–75. doi:10.3168/jds.S0022-0302(86)80573-2.

Valenzuela-Grijalva, N. V., Pinelli-Saavedra, A., Muhlia-Almazan, A., Domínguez-Díaz, D. and González-Ríos, H. 2017. Dietary inclusion effects of phytochemicals as growth promoters in animal production. *Journal of Animal Science and Technology* 59, 8. doi:10.1186/s40781-017-0133-9.

Van Soest, J. P. 1994. *Nutritional Ecology of the Ruminant*. Comstock Publishing Associates, Cornell University Press, Ithaca, NY and London, UK.

Vasta, V., Yanez-Ruiz, D. R., Mele, M., Serra, A., Luciano, G., Lanza, M., Biondi, L. and Priolo, A. 2010. Bacterial and protozoal communities and fatty acid profile in the rumen of sheep fed a diet containing added tannins. *Applied and Environmental Microbiology* 76(8), 2549–55. doi:10.1128/AEM.02583-09.

Vasta, V., Daghio, M., Cappucci, A., Buccioni, A., Serra, A., Viti, C. and Mele, M. 2019. Invited review: plant polyphenols and rumen microbiota responsible for fatty acid biohydrogenation, fiber digestion, and methane emission: experimental evidence and methodological approaches. *Journal of Dairy Science* 102(5), 3781–804. doi:10.3168/jds.2018-14985.

Vokou, D., Kokkini, S. and Bessiere, J.-M. 1993. Geographic variation of Greek oregano (*Origanum vulgare* ssp. hirtum) essential oils. *Biochemical Systematics and Ecology* 21(2), 287–95. doi:10.1016/0305-1978(93)90047-U.

Wall, E. H., Doane, P. H., Donkin, S. S. and Bravo, D. 2014. The effects of supplementation with a blend of cinnamaldehyde and eugenol on feed intake and milk production of dairy cows. *Journal of Dairy Science* 97(9), 5709–17. doi:10.3168/jds.2014-7896.

Wallace, R. J. 2004. Antimicrobial properties of plant secondary metabolites. *Proceedings of the Nutrition Society* 63(4), 621–9. doi:10.1079/pns2004393.

Wallace, R. J., Arthaud, L. and Newbold, C. J. 1994. Influence of *Yucca shidigera* extract on ruminal ammonia concentrations and ruminal microorganisms. *Applied and Environmental Microbiology* 60(6), 1762–7. doi:10.1128/AEM.60.6.1762-1767.1994.

Wallace, R. J., McEwan, N. R., McIntosh, F. M., Teferedegne, B. and Newbold, C. J. 2002. Natural products as manipulators of rumen fermentation. *Asian-Australasian Journal of Animal Sciences* 15(10), 1458–68. doi:10.5713/ajas.2002.1458.

Wang, C. J., Wang, S. P. and Zhou, H. 2009. Influences of flavomycin, ropadiar, and saponin on nutrient digestibility, rumen fermentation, and methane emission from

sheep. *Animal Feed Science and Technology* 148(2–4), 157–66. doi:10.1016/j.anifeedsci.2008.03.008.

Wang, J. K., Ye, J. A. and Liu, J. X. 2012. Effects of tea saponins on rumen microbiota, rumen fermentation, methane production and growth performance—a review. *Tropical Animal Health and Production* 44(4), 697–706. doi:10.1007/s11250-011-9960-8.

Wang, B., Ma, M. P., Diao, Q. Y. and Tu, Y. 2019. Saponin-induced shifts in the rumen microbiome and metabolome of Young cattle. *Frontiers in Microbiology* 10, 356. doi:10.3389/fmicb.2019.00356.

Weimer, P. J. 2015. Redundancy, resilience, and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations. *Frontiers in Microbiology* 6, 296. doi:10.3389/fmicb.2015.00296.

Wina, E., Muetzel, S. and Becker, K. 2005. The impact of saponins or saponin-containing plant materials on ruminant production - a review. *Journal of Agricultural and Food Chemistry* 53(21), 8093–105. doi:10.1021/jf048053d.

Wina, E., Muetzel, S. and Becker, K. 2006. Effects of daily and interval feeding of *Sapindus rarak* saponins on protozoa, rumen fermentation parameters and digestibility in sheep. *Asian-Australasian Journal of Animal Sciences* 19(11), 1580–7. doi:10.5713/ajas.2006.1580.

Wink, M. and Schimmer, O. 2018. Modes of action of defensive secondary metabolites. In: Wink, M. and Schimmer, O. (Eds), *Annual Plant Reviews Book Series, Volume 3: Functions of Plant Secondary Metabolities and their Exploitation in Biotechnology*. John Wiley and Sons, Hoboken, NJ, pp. 18–137.

Yáñez-Ruiz, D. R., Bannink, A., Dijkstra, J., Kebreab, E., Morgavi, D. P., O'Kiely, P., Reynolds, C. K., Schwarm, A., Shingfield, K. J., Yu, Z. and Hristov, A. N. 2016. Design, implementation and interpretation of in vitro batch culture experiments to assess enteric methane mitigation in ruminants—a review. *Animal Feed Science and Technology* 216, 1–18. doi:10.1016/j.anifeedsci.2016.03.016.

Yang, W. Z., Benchaar, C., Ametaj, B. N., Chaves, A. V., He, M. L. and McAllister, T. A. 2007. Effects of garlic and juniper berry essential oils on ruminal fermentation and on the site and extent of digestion in lactating Cows1. *Journal of Dairy Science* 90(12), 5671–81. doi:10.3168/jds.2007-0369.

Yanga, W. Z., Benchaar, C., Ametaj, B. N. and Beauchemin, K. A. 2010. Dose response to eugenol supplementation in growing beef cattle: ruminal fermentation and intestinal digestion. *Animal Feed Science and Technology* 158(1–2), 57–64. doi:10.1016/j.anifeedsci.2010.03.019.

Yoshiki, Y., Kudou, S. and Okubo, K. 1998 Relationship between chemical structures and biological activities of triterpenoid saponins from soybean. *Bioscience, Biotechnology, and Biochemistry* 62(12), 2291–9. doi:10.1271/bbb.62.2291.

Yuan, Z., Zhang, C., Zhou, L., Zou, C., Guo, Y., Li, W., Liu, J. and Wu, Y. 2007. Inhibition of methanogenesis by tea saponin and tea saponin plus disodium fumarate in sheep. *Journal of Animal and Feed Sciences* 16(Suppl. 2), 560–5. doi:10.22358/jafs/74607/2007.

Zebeli, Q. and Ametaj, B. N. 2009. Relationships between rumen lipopolysaccharide and mediators of inflammatory response with milk fat production and efficiency in dairy cows. *Journal of Dairy Science* 92(8), 3800–9. doi:10.3168/jds.2009-2178.

Zebeli, Q., Mansmann, D., Steingass, H. and Ametaj, B. N. 2010. Balancing diets for physically effective fibre and ruminally degradable starch: a key to lower the risk of

sub-acute rumen acidosis and improve productivity of dairy cattle. *Livestock Science* 127(1), 1–10. doi:10.1016/j.livsci.2009.09.003.

Zhou, Y. Y., Mao, H. L., Jiang, F., Wang, J. K., Liu, J. X. and McSweeney, C. S. 2011. Inhibition of rumen methanogenesis by tea saponins with reference to fermentation pattern and microbial communities in Hu sheep. *Animal Feed Science and Technology* 166–167, 93–100. doi:10.1016/j.anifeedsci.2011.04.007.

Zhou, C. S., Xiao, W. J., Tan, Z. L., Salem, A. Z. M., Geng, M. M., Tang, S. X., Wang, M., Han, X. F. and Kang, J. H. 2012. Effects of dietary supplementation of tea saponins (Ilex kudingcha CJ Tseng) on ruminal fermentation, digestibility and plasma antioxidant parameters in goats. *Animal Feed Science and Technology* 176(1–4), 163–9. doi:10.1016/j.anifeedsci.2012.07.019.

Zucker, W. V. 1983. Tannins: does structure determine function? An ecological perspective. *American Naturalist* 121(3), 335–65. doi:10.1086/284065.

Chapter 22

The use of probiotics as supplements for ruminants

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1 Introduction

Good rumen function is one of the most important characteristics in cattle as it impacts their health and productivity and, thus, helps to ensure sufficient food production for a growing human population. In its life, a ruminant faces several periods of stress, from gestation and calving to the demands of high production of milk or meat. Current intensive farming practices are known to challenge digestive microbiota and put the animal at risk of developing metabolic disorders. Much research has been devoted recently for investigating the impact of some of these life cycle stages on rumen function and health. This research has shown that the rumen microbiota and the rumen epithelial wall play a key role to maintain optimal function during these stages.

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This chapter begins by reviewing critical periods in the ruminant lifecycle as targets for probiotics. It then looks at definitions of probiotics, delivery mechanisms and regulation. The rest of the chapter summarizes and assesses the range of research on the benefits and modes of action of probiotics, starting with their potential in young ruminants. It then considers the role of probiotics in adult ruminants in the following areas: feed efficiency, methane production, pathogen control and supporting the immune system.

2 Critical periods in the ruminant lifecycle as targets for probiotics

Calving is an extremely stressful event for the neonate ruminant, especially from the microbiological point of view. Colonization of the gastrointestinal tract (GIT) starts during and after the birth through direct contact with the mother and farm environment (Yeoman et al., 2018). Members of typical ruminal populations, such as methanogens, fibrolytic bacteria or Proteobacteria, have been detected in the rumen of calves less than 20 minutes after birth (Guzman et al., 2015) and are metabolically active (Guzman et al., 2016). Neonate calf physiology (i.e. esophageal groove closure reflex) prevents milk from entering the rumen compartment during the pre-weaning phase. However, the immaturity of the groove and consumption of small amounts of solids contribute to rumen microbial inoculation and a slow and immature development of rumen microbiota and physiology before weaning (Meale et al., 2017). Colonization of the rumen starts with aerobic and facultative anaerobic taxa which are gradually replaced by strict anaerobic microorganisms (Rey et al., 2014). Impairment in rumen development will affect nutrient digestion and impact the further growth and performance of the animal (Steele et al., 2016).

Digestive issues are one of the most common reasons for disease-related deaths among dairy calves and heifers. An average mortality rate as high as 56.4% has been observed in the pre-weaning period for heifers in the North American dairy industry (USDA, 2014a). Most calf diarrhea problems occur within the first 3 weeks of life (McGuirk, 2008). In a 2-year study in Estonia, metabolic and digestive disorders were identified as the main reason for on-farm mortality in calves under 1 month of age (43%), between 1–5 months (29.5%) and between 6–19 months of age (32.3%) (Mõtus et al., 2018). Poor production linked to digestive disorders is the identified cause of removing pre-weaned heifers in 21.1% of cases in the United States (USDA, 2014a).

At birth, calf immunity depends on colostrum and milk consumption which allows transfer of immunoglobulins (Ig) and neutrophils, as well as macrophages known to secrete immune-related components such as cytokines or antimicrobial peptides and proteins (Stelwagen et al., 2009). IgG is the main immunoglobulin in colostrum and its concentration is used to characterize

colostrum quality (Johnsen et al., 2019). Low quality or limited consumption of colostrum increases mortality and decreases calf growth (McGuirk and Collins, 2004), while feeding high-quality colostrum soon after birth increases calf weaning weight and BW gain (Priestley et al., 2013). These results highlight the link between diet and immune function. Colostral immunity is recognized as an essential part of GIT disease management in dairy calves (McGuirk, 2008). The proportion of *E. coli* attached to small intestinal tissue was significantly higher for calves deprived of colostrum when compared to colostrum-fed animals, whereas the proportion of *Bifidobacterium* was lower (Malmuthuge et al., 2015). In newborn lambs, the gut microbiome was speculated to provide critical signals involved in the establishment of a functional immune response, for example, in maintaining the development and function of lymphoid follicules in ileal Peyer patches (Reynolds and Morris, 1984).

Weaning usually occurs around 6–8 weeks of age for dairy calves and represents one of the most dramatic changes in rumen development, leading to important modifications in intestinal mass, immunity and metabolism (Baldwin et al., 2004). It is recognized that a badly managed weaning stage will often be associated with distress, depressed growth and diarrhea (Roth et al., 2009). At weaning, milk is abruptly or gradually removed from the calf diet and replaced by solid feed entering the rumen compartment. At that time, overall microbial activity increases, as shown by the growth of several members of rumen microbiota, the increase in ruminal enzyme production and resulting fermentative end-products (Jiao et al., 2015). Weaning leads to a strong and rapid physiological evolution of the rumen (e.g. in papillae development, volume expansion, increased rumen wall thickness) (reviewed in Meale et al., 2017). A decrease in microbial diversity has been observed immediately after weaning, probably due to drastic dietary change from the transition to a feed-based diet, extensive fermentation and fluctuating pH in the rumen compartment (Meale et al., 2016). Rumen development in calves is also affected by the physical form and chemical composition of starter diets (Khan et al., 2016).

The mucosal epithelium is considered to be a barrier between the GIT and the host. Epithelial cells play a key role in recognizing the rumen microbiome, pathogens and chemicals present in digesta, thus influencing development of the mucosal immune system (Malmuthuge et al., 2012). Toll-Like Receptors (TLRs) found on mucosal epithelium all along the GIT are down-regulated during continuous exposure to microbial ligands coming from rumen microbiota in order to limit a chronic and unnecessary inflammatory response. Gene expression of epithelial expressed molecules such as β-defensin (antimicrobial peptide) and PGLYRP1 (peptidoglycan recognition protein) are repressed prior to weaning but increase afterwards. Malmuthuge et al. (2012) suggested that TLRs drive primary innate immunity up to weaning but that

their role decreases in favor of other innate immune mechanisms as the animal ages. Bush et al. (2019) conducted a comprehensive transcriptomic study of the whole GIT of ruminants from birth to adulthood and observed a strong immune transcriptomic signature in rumen evolving with animal age.

Research focusing on the developing rumen has demonstrated that it may be possible to regulate microbial community development by controlling feeding management early in life, with subsequent effects later in the animal's life (Abecia et al., 2013, 2014; Yáñez-Ruiz et al., 2015). Alteration of the diet of goat kids to reduce methane emissions was shown to modify bacterial and archaeal rumen populations up to 4 months after treatment; there was also a maternal effect since modifications of bacterial groups and metabolite profiles were also associated with kids from treated mothers (Abecia et al., 2018). These results also highlight the potential long-term effect of maternal imprinting on rumen microbiota. A meta-analysis by Soberon and Van Amburgh (2013) observed that heifers fed milk ab-libitum during the pre-weaning period showed a greater milk yield in the first lactation than calves with a restricted milk diet during pre-weaning. Feeding a higher level of nutrients during the pre-weaning period will affect cell functions involved in the morphological and physiological development of the mammary gland and increase milk yield (Hare et al., 2019). These data suggest that dietary interventions in early life, although not specifically targeting the rumen, can have an impact on later productivity of the animal.

During their life ruminants will need to be transported from their place of birth and mixed with other animals in places such as another farm, feedlots or market auctions. In 2013, 28.6% of US dairy farms introduced new animals into their herd (USDA, 2014b). This period is associated with several stresses such as handling and transportation, feed or water privation, mixing with other animals or high temperature variations. It is well known that stress negatively affects the immune system (Blecha et al., 1984) which could be very detrimental at a time when the animal is more likely to be exposed to infectious agents as a result of commingling with other animals. Stress will impair cattle performance and health with a decreased feed intake being commonly observed (Hutcheson and Cole, 1986; Silanikove, 2000). Ashenafi et al. (2018) reviewed the effect of stresses associated with transportation over long distances and noted alteration in metabolism, immune competence and behavior, failures in reproduction and increases in morbidity and mortality.

Beef cattle during the fattening period or dairy cows during the lactating period are commonly fed high-grain diets in order to meet energy requirements. The animals are supplied with large amounts of readily fermentable starch or sugars, known to alter rumen microbial communities and functions (Petri et al., 2013a,b, 2018). As rumen pH falls, lactate producers may outnumber lactate utilizers leading to changes in the structure of rumen microbiota (Russell and

Wilson, 1996). The abundance of fibrolytic species *Ruminococcus* sp. and *Fibrobacter succinogenes* was shown to decrease in rumen of cows switching from a forage to high-grain diet. In contrast, lactate utilizers *Selenomonas ruminantium* and *Megasphaera elsdenii* increased at that time (Petri et al., 2013b). *Lactobacillus* and *Streptococcus* were observed to appear in the rumen of clinically acidotic cows, probably reflecting the tolerance of these species to low pH and their ability to proliferate on an excess of fermentable carbohydrate (Petri et al., 2013a). Low rumen pH for a prolonged period can negatively affect feed intake, microbial metabolism and nutrient degradation, and leads to physiological disorders such as acidosis, inflammation, laminitis, diarrhea and milk fat depression (Kleen et al., 2003; Villot et al., 2018).

This microbial dysbiosis may trigger the release of potential harmful molecules which impact animal health. Acidosis has been associated with release of lipopolysaccharide (LPS) from Gram-negative bacteria into the rumen and the hindgut (Khafipour et al., 2009; Khiaosa-Ard and Zebeli, 2018). LPS translocation has been observed from the rumen into the interior circulation which can trigger an inflammatory response with an increase in acute phase protein concentration in peripheral blood (reviewed in Plaizier et al., 2012). Histamine (and other biogenic amines) are produced by some rumen bacteria under low pH conditions (Silberberg et al., 2013; Wang et al., 2013), leading to an increase in rumen epithelial cell inflammatory response (Sun et al., 2017). Finally, a more global systemic inflammation response may be triggered by an activation of the immune system in the bloodstream or tissues, when a significant amount of nutrients and energy is diverted from maintenance of general body homeostasis and production, which ultimately results in poor animal performance with a significant economic impact (Zebeli et al., 2015). Higher expression of TLR2 and TLR4 was identified in the rumen papillaes of acidosis-resistant vs. sensitive steers indicating greater host innate immune response. The authors suggested that an increased expression of TLRs may protect the rumen epithelium from subacute ruminal acidosis (SARA) damage by stimulating the barrier function of the rumen in resistant steers (Chen et al., 2012). It has been shown that leukocytes present in saliva can migrate back and forth from the rumen cavity, starting a cross-talk with the lymphoid tissues in the oral cavity and cytokines or other mediators released by forestomach walls. In a field trial on 128 cows, it has been shown that concentrations of total Ig and IgM in rumen fluid were regulated by rumen pH levels, as well as by volatile fatty acids (VFA) concentrations. These findings suggest control by the innate immune system over metabolic activities in bovine forestomachs (Trevisi et al., 2018).

Epimural microbiota is attached to the ruminal epithelium and is in close contact with epithelial cells involved in inflammatory response modulation. Epimural microbial diversity is distinct from microbiota observed either in liquid or solid phases in the rumen (Sadet et al., 2007). Diversity of the rumen epimural

population is strongly affected with a shift from *Firmicutes* to *Proteobacteria* as concentrates are replaced by hay, although the expression of genes targeting intracellular pattern recognition receptor (TLR), barrier function, pH regulation, and nutrient uptake of rumen epithelium remain stable (Petri et al., 2018). Chen et al. (2011) observed the differences in epimural microbiota under high-hay or high-grain diet with *Treponema* sp., *Ruminobacter* sp., and *Lachnospiraceae* observed only with the high-grain diet. These results contradicted previous published data and the authors suggested that the host had a role in regulating microbial diversity and density and in the response to rumen environmental changes.

At the start of the lactation period, dairy cows are often unable to reach their energy requirements and need to mobilize body reserves, despite the alteration in diet from high-fiber to high-energy content. Rumen function should thus be maintained at its best to get the best reproductive performance and milk production. At that time, the rumen undergoes drastic changes impacting rumen microbiota, fermentation profile and epithelial permeability (Bach et al., 2018; Minuti et al., 2015). Zhu et al. (2018) observed changes in bacterial and archaeal communities which could be linked to short-chain fatty acids profiles over the transition period in primiparous dairy cows. The relative abundance of *Bacteroidetes* decreased and *Proteobacteria* increased after calving. Significant shifts in methanogenic community composition over the transition period were observed mainly for *Methanosphaera* and *Methanomassiliicoccus*, although the pattern varied across genera.

Rumen microbial composition appears to be linked to milk production. The ratio of *Firmicutes* to *Bacteroidetes* in rumen has been negatively correlated to feed efficiency (FE) and changes in milk fat yield in Holstein cattle (Jami et al., 2014). A study to determine the associations between the rumen microbiota and feed efficiency was conducted in a Holstein cattle using whole metagenome sequencing. A larger relative abundance of *Bacteroidetes* and *Prevotella* was observed in more efficient cows together with a lower relative abundance of *Firmicutes* and some members of the archaeal population (Delgado et al., 2019).

The transition period (from 3 weeks before to 3 weeks after calving) is linked with drastic effects on cow health and has been associated with a reduction of immune competence, a negative energy balance, hypocalcemia, an over-systemic inflammatory response (even in the absence of signs of microbial infection) and a state of oxidative stress (Trevisi and Minuti, 2018). The expression of genes involved in immune response modulation in rumen epithelium was observed to vary widely from two weeks before calving to 3 weeks post-partum (Bach et al., 2018). The inflammatory response is considered to be directly correlated with the increase in the release of ruminal endotoxins due to high-concentrate diets during lactation (Abaker et al., 2017). Ingvartsen et al. (2003) reviewed the diseases associated with high-yielding cows and

found that imbalanced immune status and diets lead to metabolic disorders and reproduction issues, mastitis and increased risk of ketosis and lameness.

Pitta et al. (2018) reviewed recent studies on microbes and dietary interactions to enhance the productivity of dairy cows. They have identified a number of strategies to manipulate rumen microbial processes (Fig. 1). The potential action of probiotics can be considered in different areas such as fiber digestion, protein supply and microbial growth, bio-hydrogenation and methane production. The ruminant is influenced by its microbiota and immune system but also by dietary interventions and environmental stresses. The manipulation of gastrointestinal microbiota in maintaining animal gut health, through understanding diversity, stability, metabolites and crosstalk with the epithelium and the underlying immune system, still needs further research (Gaggìa et al., 2010).

3 Definitions, delivery mechanisms and regulation

3.1 Definitions

The term probiotic comes from the Latin '*pro*' ('for') and the Greek '*bios*' ('life') and was firstly suggested in the 1960s in contrast to antibiotic ('against life') to define substances produced by protozoa that are able to support the growth of other microorganisms (Morelli and Capurso, 2012). In the late 1980s, Dr R. Fuller proposed a definition for probiotic as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal

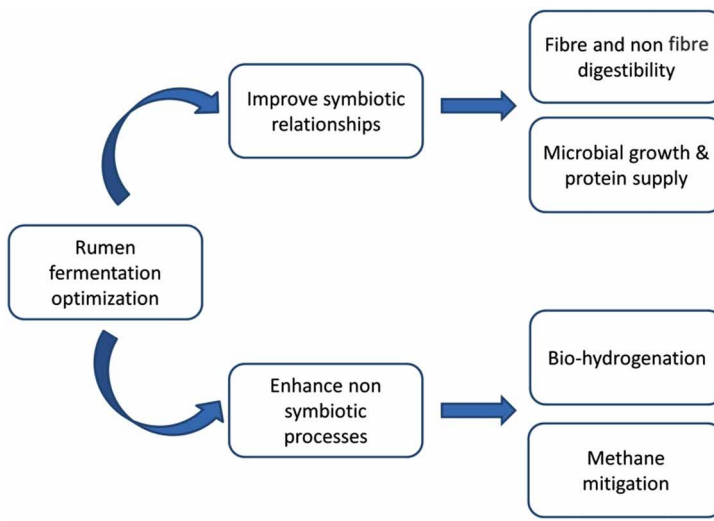

Figure 1 Strategies to manipulate rumen microbial processes to enhance rumen functions. Source: adapted from Pitta et al. (2018).

balance'. At that time, probiotics were only focused on livestock animals. With the expansion of use in humans, the Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) proposed a slightly different definition in 2001: 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'. This definition was reviewed in 2013 by a panel of scientific experts, the International Scientific Association for Probiotics and Prebiotics, who agreed that the FAO/WHO definition for probiotics was still relevant. In this chapter, we will only consider live microorganisms and not dead or inactivated microorganisms, such as yeast culture or microbial fractions, which are not probiotics as defined above.

We need to underline that, in the case of ruminants, the term 'probiotic' is in fact not commonly used. Indeed the strict definition is not totally appropriate for several reasons:

- the main digestive target is the rumen and not the intestine.
- the focus is mainly nutritional effects rather than health benefits.
- live microorganisms are being generally incorporated in feed so have been considered as feed supplements.
- in European Union (EU) and other countries, these microorganisms fall into the feed additive category (zootechnical additives) (EC Regulation 1831/2003).

In most countries, commercialized products containing live microorganisms are considered as feed additives (FA) or direct-fed microbials (DFM) and these terms will be used throughout this chapter rather than 'probiotic'.

3.2 Delivery mechanisms

In ruminants, microbial FA are delivered through the diet, either with a mineral/vitamin premix, in milk powder or included in pelleted concentrate. As the concentration of active cells per gram of pure additive is high, inclusion in feed is generally at a low percentage which ensures a precise amount of additive per animal. It is important to assess the compatibility of microbial FA with some minerals (i.e. copper) which may exhibit some toxicity toward the live cells. It is also important to ensure that the pelleting process, which involves conditions such as high temperature, high pressure and humidity, will not damage microbial cells. Sullivan and Bradford (2011) have raised this concern and compared different commercial active dry yeasts (ADY) containing highly concentrated live yeast cells fed to dairy cattle. Tested products failed to consistently meet product claims and viability was significantly diminished during storage at 40°C for 3 weeks. Loss in viability at elevated temperatures can be reduced when

ADY products are diluted with a premix containing vitamin trace minerals due to the antioxidant role of vitamins A and E and micro minerals such as selenium. FA producers need to ensure the right formulations to address these problems. In the case of inclusion into pelleted concentrates, some companies have proposed technologies such as micro-encapsulated beads to protect microbial FA against harsh feed manufacturing conditions and ensure optimal stability of live microorganisms up to the point of consumption. In case of combination with other feed supplements, such as essential oils, plant extracts or antibiotics, it is important to ensure that the live microorganisms will not be negatively affected by these compounds which are known to exert antimicrobial effects. A particular situation is feedlot cattle supplementation. In large-scale fattening operations, it is possible to feed the animals with liquid supplements containing DFM, primarily to control pathogens in the gut (discussed later in this chapter). In this case, DFM (bacteria) are delivered in refrigerated containers and fed daily to the herd, avoiding problems about stability in feed.

In many species including humans, the concept of probiotics is based on a daily distribution of live microorganisms as these microorganisms do not need to colonize the digestive tract to be active. In ruminants, microbial FA are also administered daily through feed. It has been shown that a repeated distribution of a live yeast product ensured a stable concentration of cells in the rumen of lambs (Durand-Chaucheyras et al., 1998). However, when supplementation was stopped, a decrease in live cell concentration was measured after 24h and a total loss from the rumen was observed after a few days, with some recovery from the feces of animals up to several days after the last incorporation of the yeast product in the diet.

Some yeasts, such as *Saccharomyces boulardii*, are recognized for their ability to survive in the gut and to reach the lower gut compartment alive, justifying its current application in pre-weaned ruminants in North America to promote intestinal health. Probiotic bacteria are mainly targeted at lower gut health. However, there is limited information about their potential survival in this part of the gut. This is, in part, due to the lack of detection methods allowing a specific detection/quantification of live/viable probiotic bacteria within the commensal bacterial population in the gut, since the latter often contains the same species as those used in the probiotic (Fomenky et al., 2017). However it is clear that the digestive environment of the ruminant provides hostile conditions for survival of probiotics. Research is needed to develop innovative formulations which could help deliver active microorganisms in the lower gut. Microencapsulation is an emerging technology used to protect probiotics against adverse environmental conditions. Different microencapsulation techniques can be used with materials such as alginate, chitosan, carrageenan, gums, gelatin, whey protein, starch and the use of compression coating (Riaz and Masud, 2013). It is important to find an optimal balance between capsule

Table 1 Yeast and bacteria species used as probiotics in ruminants

Yeasts	Bacteria
Saccharomyces cerevisiae	*Lactobacillus acidophilus/crispatus*
Saccharomyces boulardii	*Lactobacillus rhamnosus*
	Enterococcus faecium
	Bacillus subtilis
	Bacillus licheniformis
	Propionibacterium freudenreichii
	Propionibacterium acidipropionici
	Megasphaera elsdenii

characteristics, protection of the probiotic, added cost and ease of use. Table 1 summarizes microbial species, commercially available as probiotics in ruminants. Their effects and modes of action on the targeted species are described later in this chapter.

3.3 Regulation

In the EU, Regulation EC 1831/2003 on feed additives covers microbial FA applied to animal nutrition, including ruminants. The regulation applies to all FA and premixtures, but not to processing aids or to veterinary medicinal products as defined by Directive 2001/82/EC. Only authorized additives may be placed on the market and used. Authorizations are granted for use in feed intended for specific animal species or categories and for specific conditions of use. Microbial FA are classified into the category of zootechnical additives, either as digestibility enhancers or gut flora stabilizers. Authorizations are valid for 10 years throughout the European Economic Area and they are renewable for 10-year periods. The authorization procedure is a long and strict process (Fig. 2) which has led to a very small number of microbial additives actually authorized in EU for ruminants (dairy cattle, beef cattle, small ruminants and growing ruminants).

Other countries possess their own regulation process which can also be complex and demanding. In the United States, the designation 'Generally Recognized As Safe' (GRAS) is authorized by the Food and Drug Administration (FDA) when a chemical or substance added to food is considered safe by experts, and is thus exempted from the usual Federal Food, Drug, and Cosmetic Act (FFDCA) food additive tolerance requirements. Marketed probiotics are considered GRAS. In other countries where there is no specific regulation, microbial FA are generally considered as safe but no specific demonstration of efficacy is required.

There is a growing concern about antimicrobial resistance (AMR) that might be developed by gut bacteria when exposed repeatedly to antibiotics. In this context, probiotic bacteria carrying AMR genes could be a problem for

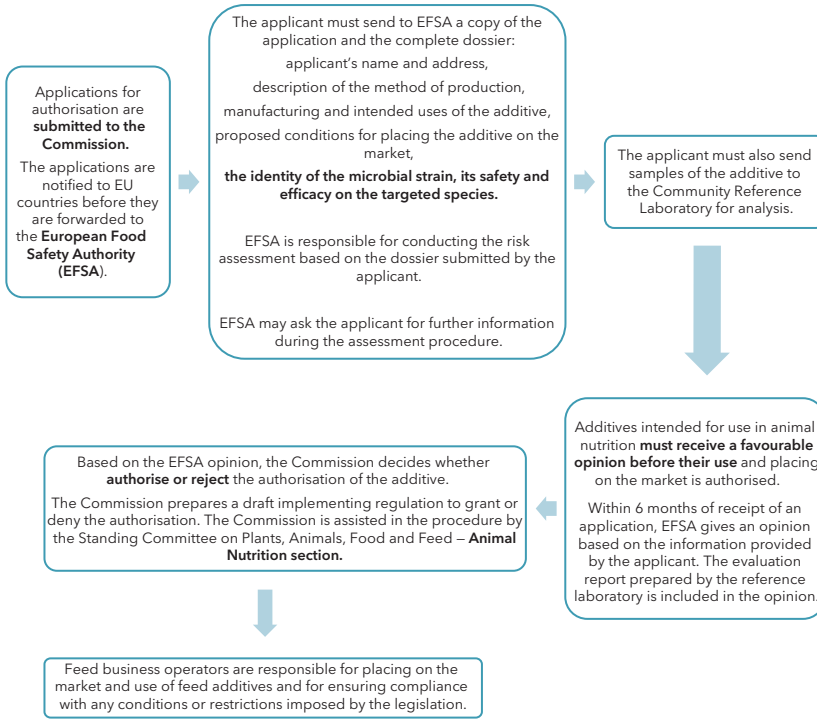

Figure 2 Current procedure for a microbial feed additive authorization for animal nutrition in EU (from Regulation (EC) N° 1831/2003 on additives for use in animal nutrition).

animal hosts. Amachawadi et al. (2018) have looked at commercial probiotics containing *Enterococcus faecium* for cattle and swine, in particular their sensitivity or resistance to antimicrobials and presence of virulence genes. They found that none of them harbored virulence genes. However, some exhibited AMR to medically important antimicrobials and were multi-resistant to chloramphenicol, erythromycin, penicillin, kanamycin, lincomycin and tetracycline, which suggests they might be a source of AMR in the animal gut. It is important to improve characterization of microbial strains used in ruminant feed, particularly by obtaining genomic information using whole genome sequencing (WGS), to ensure the complete safety of these microbials.

4 Benefits and modes of action of probiotics: young ruminants

As previously discussed, pre-weaning is the most critical period in the ruminant life cycle with the highest incidence of mortality and morbidity. Optimization of the pre-weaning phase is crucial to ensure successful herd management.

Microbial FA represent a promising opportunity for the dairy industry, particularly in the context of reducing antibiotic usage.

Currently available published trials do not report a consistent beneficial effect of microbial FA on performance (Alugongo et al., 2017; Fomenky et al., 2017; Galvão et al., 2005; He et al., 2017). This is probably due to variations in the design of studies, type of diet, FA strains and dosage, duration and mode of administration (in milk, in starter feed, or both, and combined or not with other additives), but also large individual variations observed among calves from the same herd. When microbial FA is given through starter feed, the low consumption of this solid feed generally observed during the first two weeks after birth prevents the calf from sufficient inoculation with live microbial cells, thus impacting efficacy of the supplement. For this reason, the distribution of microbial FA through milk has attracted as it allows a better control of the dose administered. When distributed in milk, the live probiotic reaches the intestine and can exert beneficial effects on gut health and the calf immune system.

As it has been noted, microbial inoculation of the rumen starts immediately at birth (Yeoman et al., 2018), and rapid changes occur in the composition of the ruminal bacterial community during the first days of life (Jami et al., 2013; Rey et al., 2012). In an experiment with lambs reared with their dams, the daily distribution of a live yeast additive (*Saccharomyces cerevisiae* CNCM I-1077) accelerated microbial establishment of functional communities in the rumen in very young lambs (Chaucheyras-Durand and Fonty, 2002). In gnotobiotically reared-lambs maintained in sterile isolators, the additive also stimulated the establishment of fibrolytic bacteria (Chaucheyras-Durand and Fonty, 2001). The same strain was found to improve microbial colonization in the maturing rumen of newborn artificially fed lambs, in particular ciliate protozoa, fungi and the fibrolytic bacterial species *Fibrobacter succinogenes* (Chaucheyras-Durand et al., unpublished data), with a potentially more specialized ecosystem toward efficient fiber degradation, as assessed by a dedicated DNA microarray (Comtet-Marre et al., 2018). This suggested a possible positive impact on fiber digestive efficiency (Chaucheyras-Durand, unpublished data). This yeast additive (*S. cerevisiae* CNCM I-1077) also had a beneficial effect in a calf performance trial up to 3 weeks post weaning, with growth and final weight both significantly increased, together with a better weight gain to feed ratio, probably because of improved microbial rumen maturation (Terré et al., 2015). Possible modes of action in the rumen may include an improved anaerobic environment, as redox potential of rumen contents of young lambs was shown to be lower with yeast supplementation, due to the capacity of live yeast cells to scavenge oxygen (Chaucheyras-Durand and Fonty, 2002). It may be also possible that yeast FA also supplies key nutrients, B vitamins or cofactors, able to promote growth and feed particle colonization by fibrolytic microbial populations (Chaucheyras-Durand et al., 2016).

It is noticeable that beneficial effects may depend on the initial health status of the young animal. A very good health status combined with optimal farm cleanliness and good husbandry, are among factors which may help to explain why no benefit of a microbial FA could be observed in the absence of any source of challenge or disease causing agents to the animals (Alugongo et al., 2017). In growth-retarded calves, the supplementation of *Bacillus* probiotics had a beneficial effect on body weight, feed intake, feed conversion and growth factors levels in serum. The volume of bacteria involved in production of energy and short chain fatty acids (such as *Proteobacteria, Rhodospirillaceae*, *Campylobacterales* and *Butyricimonas*) were increased, whereas undesirable mycoplasmas were decreased, compared to the non-supplemented group (Du et al., 2018). Administration of *Bacillus amyloliquefaciens* H57 to dairy calves was shown to increase weight gain although no significant differences were observed in rumen community structure. The low abundance of the *B. amyloliquefaciens* H57 in the rumen suggests that the probiotic was not directly responsible for weight gain but rather influenced animal behavior (feed consumption) or altered rumen community functions (Schofield et al., 2018).

Galvão et al. (2005) studied calves with low serum IgG concentrations due to lack of colostrum. Calves fed with milk replacer containing a *Saccharomyces boulardii* strain CNCM I-1079 had significantly less diarrhea and associated veterinary costs. *E. coli* is one of the main pathogens responsible for calf diarrhea and DFM are commonly used to alleviate diarrhea even in absence of the pathogen being identified. In a meta-analysis, Signorini et al. (2012) concluded that administration of a lactic acid bacteria (LAB) probiotic to pre-weaning dairy calves exerted a protective effect and reduced the incidence of diarrhea. Other studies have also shown prevention of diarrhea in dairy calves fed with *E. coli* Nissle 1917 (von Buenau et al., 2005) or with a mix of microorganisms (Mokhber-Dezfouli et al., 2007). A multispecies DFM bolus administrated to Holstein dairy calves suffering from diarrhea reduced duration of diarrhea (Renaud et al., 2019). A *Bacillus*-based DFM was evaluated in combination with an electrolyte given orally as a therapy against diarrhea in pre-weaned calves. Compared with the electrolyte alone, the associated additives did reduce *Clostridium perfringens* fecal shedding, decreased diarrhea severity and reduced treatment costs (Wehnes et al., 2009).

Recent findings have highlighted the potential positive role of the *S. boulardii* strain CNCM I-1079, distributed in milk replacer and in starter feed, on gut development in young dairy calves (Fomenky et al., 2017). Altered colon morphology and increased neutral mucin production were observed, suggesting earlier gut maturation in calves receiving the supplement. Acute phase proteins such as haptoglobin and C-reactive protein were also increased in the serum of probiotic-fed calves at weaning. Phagocytic activity

of polymorphonuclear neutrophils isolated from plasma was also stimulated. These data suggest a possible role of *S. boulardii* CNCM I-1079 in enhancing the innate immune and inflammatory response of calves during the stressful weaning period.

In the same study, two probiotics (*S. boulardii* CNCM I-1079 and *L. acidophilus BT-1386*) were found to have a significant impact on gut bacteria community structure in the pre-weaning period, particularly in the ileum (Fomenky et al., 2018). Both probiotics significantly reduced the relative abundance of potential pathogenic bacteria *Streptococcus* and *Tyzzerella* and increased that of the beneficial fiber-degrading bacteria *Fibrobacter*. The live yeast supplementation seemed to have greater effects on gut microbiota than the bacterial DFM. DFM may impact bile acid secretion, known to affect fat digestion and absorption, as well as protein and energy metabolism and gut microbiota regulation. As shown in pigs, activation of bile acid-regulated pathways strengthens intestinal protection against bacterial infection and associated secretion of fluids and electrolytes, reduces inflammation in the colon and increases levels of the growth hormone FGF19 (Ipharraguerre et al., 2018). Different types of mechanisms of action for *S. boulardii* have been reported, though most have only been demonstrated *in vitro* or with laboratory animal experiments (Pothoulakis, 2009; McFarland, 2010; Stier and Bischoff, 2016). *S. boulardii* has been classified as having three modes of action: luminal, trophic and mucosal-anti-inflammatory signaling effects. Within the intestinal lumen, *S. boulardii* may interfere with pathogenic adhesion through yeast cell wall manno-oligosaccharides-bacterial fimbriae interaction. They could neutralize toxins, preserve cellular physiology, interact with intestinal microbiota and induce changes in the short chain fatty acid profile.

5 Benefits and modes of action of probiotics: feed efficiency in adult ruminants

Improving feed efficiency (FE) is important for sustainable livestock production. Recent metagenomics studies have shown associations between rumen microbiota composition/functions and FE (Mizrahi and Jami, 2018). There are particular associations between several plant-cell wall degrading bacteria taxa, their fiber-degrading function and FE (Delgado et al., 2019). Using a 16S rDNA sequencing approach, McGovern et al. (2018) showed a negative correlation between *Fibrobacter succinogenes* abundance and residual feed intake (RFI), suggesting this cellulolytic and hemicellulolytic bacteria contributes to FE by providing a substrate to the host and to other microbial populations. Improving fiber digestibility in the rumen is therefore a target for probiotics. It may be achieved by:

- reducing the indigestible fiber fraction, and
- increasing the rate of fiber digestion, for example, by maintaining a ruminal environment able to promote the population of fiber-digesting bacteria.

In forage indigestible fiber (iNDF) is generally related to lignin content and, in particular, structural carbohydrates (cellulose and hemicellulose) 'trapped' within lignin. Lignin is not fully digested in the animal gastrointestinal tract as its biochemical degradation process involves oxidative pathways. However, the release of lignin-bounded carbohydrates would potentially increase the feed value of forage. McSweeney et al. (1994) observed that up to 33.6% of sorghum lignin degradation could be associated with the activity of the ruminal anaerobic fungus *Neocallimastix patriciarum*. The authors suggested that the degraded lignin fraction was a lignin carbohydrate complex solubilized through dissolution of xylan from the matrix rather than through lignin depolymerization.

Microbial FA could improve fiber degradation in the rumen in different ways. An indirect route is through pH stabilization effects (discussed in the further section). Another route is modification of the ruminal environment through strengthening anaerobic conditions and oxygen-scavenging properties (Chaucheyras-Durand et al., 2008; Chaucheyras-Durand and Fonty, 2002; Jouany, 2006; Jouany and Morgavi, 2007; Marden et al., 2008). These conditions promote fiber-degrading microbiota and their effect on plant-cell wall polysaccharides. Specific nutritional requirements for vitamins, peptides, amino acids, ammonia, organic acids or branched-chain fatty acids have been described for bacteria and fungi, and it is likely that microbial FA could supply these components to fibrolytic microorganisms.

Research has identified the potential of live yeast FA to enhance growth and activity of fiber-degrading rumen microorganisms (Chaucheyras-Durand et al., 2008). Mechanisms include an increase in fungal zoospore germination and cellulose degradation, promotion of growth and/or activities of fibrolytic bacterial strains of *Fibrobacter succinogenes, Ruminococcus albus*, *Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens*. Several studies report increases in abundance of *F. succinogenes* (AlZahal et al., 2017; Pinloche et al., 2013; Uyeno et al., 2017), *Ruminococcus* (Mosoni et al., 2007; Pinloche et al., 2013; Silberberg et al., 2013; Sousa et al., 2018) or rumen fungi (Ding et al., 2014) using DNA-based techniques (qPCR, DNA sequencing). Jiang et al. (2017) compared the effect of live and heat-killed yeast FA on rumen microbiota using Illumina MiSeq and qPCR. The supplementation of live yeast increased the relative abundance of *Ruminococcus* and *F. succinogenes*. The impact of a live yeast strain (*S. cerevisiae* CNCM I-1077) was confirmed in promoting colonization of fibrous substrates by cellulolytic bacteria (*F. succinogenes, R. flavefaciens, B. fibrisolvens*) and fungi. It was

observed that the degree of stimulation was dependent upon the nature of the substrate, and on the microbial species targeted (Chaucheyras-Durand et al., 2016). Feedstuffs with the highest levels of lignin and thereby with less easily accessible digestible carbohydrates were better degraded during yeast supplementation, suggesting an impact on the microbial breakdown of lignin-polysaccharide linkages, on which rumen fungi could be mostly active.

Live yeast has been found to promote the abundance of *Butyrivibrio fibrisolvens* on fibrous substrates. This bacterial species is known to possess ferulic and p-coumaric acid esterases which hydrolyze ester linkages between phenolic acids and xylan chains within the hemicellulose fraction, thereby exposing more polysaccharides to microbial enzymatic attack (McSweeney et al., 1998). Guedes et al. (2008) reported that the same live yeast strain increased fiber (NDF-neutral detergent fiber) degradation of corn silage samples. The yeast FA increased NDF degradation of the low-digestible corn silages more strongly than that of the high-digestible corn silage. These results suggest that live yeast could help to reduce indigestible NDF by promoting the action of bacteria and fungi involved in the hydrolysis of lignin-polysaccharide bonds.

Live yeast additives indirectly promote fiber degradation by stabilizing rumen pH in the case of ruminal acidosis. Using 18S and ITS sequencing, Ishaq et al. (2017) have shown that diet-induced subacute ruminal acidosis (SARA) modified the diversity of rumen fungi and protozoa and selected against fiber-degrading species. Cows supplemented with the live yeast product had a stabilized rumen pH and greater microbial diversity shifts which prevented a reduction in protozoa. The live yeast (*S. cerevisiae* Y1242) also increased the abundance of some dominant anaerobic OTU belonging to *F. succinogenes* and the abundance of genes encoding for specific microbial fibrolytic enzymes (AlZahal et al., 2017). It has been consistently reported that live yeast supplementation improves rumen fiber digestion *in vivo* (Chaucheyras-Durand et al., 2016; Dias et al., 2018; Ding et al., 2014; Ferraretto et al., 2012; Guedes et al., 2008; Sousa et al., 2018);

Ruminal acidosis is still commonly found in dairy and beef cattle, where a high amount of readily fermentable carbohydrates associated with low fiber in the diet may negatively impact rumen function due to high acid production and reduced buffering capacity (Villot et al., 2018). Although acidosis is not just a 'rumen disorder' but affects the total digestive tract (Plaizier et al., 2018), intervention strategies have focused on the rumen microbiota balance and rumen pH stabilization (Humer et al., 2018). There have been a number of studies of yeast FA in SARA prevention (Chaucheyras-Durand et al., 2016; Ipharraguerre et al., 2018; Jiang et al., 2017; Jouany, 2006; Jouany and Morgavi, 2007; Mizrahi and Jami, 2018). Rumen sensors (Villot et al., 2018) have been used to measure the beneficial effect of live yeast FA on ruminal pH.

Yeast FA promote shifts in microbial populations involved in release of fermentation acids (in particular lactate, which is a stronger acid than VFAs), and/or those implicated in lactic acid removal, leading to an optimized balance between lactate producers and lactate utilizers. Stimulation of growth and metabolism of lactate-utilizing bacteria, such as *Megasphaera elsdenii* or *Selenomonas ruminantium,* have been observed both *in vitro* in the presence of different live yeasts (Chaucheyras-Durand et al., 2008), and *in vivo* (Pinloche et al., 2013). The supply of growth factors, peptides, amino acids or vitamins has been proposed as a mechanism of action (Fonty and Chaucheyras-Durand, 2006). The inhibition of growth of *Streptococcus bovis*, one of the main bacterial species involved in lactate production, has been measured *in vitro* (Fonty and Chaucheyras-Durand, 2006). The impact of yeast FA on ruminal lactate concentration has been confirmed in *in vivo* studies (Chaucheyras-Durand et al., 2008; Kumprechtová et al., 2019; Reis et al., 2018).

Yeast FA can also alleviate butyric orientated acidosis (Brossard et al., 2004; Lettat et al., 2010). Brossard et al. (2006) reported the pH stabilizing effect of one strain of *S. cerevisiae* in sheep with butyric ruminal acidosis. This strain promoted ciliate Entodiniomorphid protozoa, which are known to engulf starch granules very rapidly and thus compete effectively with amylolytic bacteria (Owens et al., 1998). The effect of live yeast on ciliate protozoa has been reported in other studies (Chaucheyras-Durand and Fonty, 2002; Silberberg et al., 2013). The fact that ciliates digest starch at a slower rate than by amylolytic bacteria, and their main end-products of fermentation are VFAs rather than lactate, might explain why they have a stabilizing effect in the rumen by delaying fermentation. The promotion of ciliate protozoa, despite their association with methane production, could increase fiber digestibility as a recent rumen metatranscriptomic study showed that their contribution to fibrolysis appeared to be greater than previously thought (Comtet-Marre et al., 2017).

Better fiber digestion and stabilized rumen pH have been seen to benefit animal rumen health and its function by improvement of FE. De Ondarza et al. (2010) has investigated the effect of live yeast (*Saccharomyces cerevisiae* CNCM I-1077) on dairy cows by gathering performance data from 14 trials, clearly demonstrating that FE was improved. When targeting the cows fed diet above 30% NDF (high fiber diet, low SARA risk), FE was higher than the overall mean. The live yeast-treated animals produced an extra 40 g of milk per kg dry matter intake (DMI). In the case of a higher risk of SARA (low fiber diet, >25% starch), FE was even greater with an extra 80 g of milk per kg DMI. Other research has shown that when cows were fed with this live yeast product, eating behavior was modified with shorter intervals between meals (Bach et al., 2007; DeVries and Chevaux, 2014), indicating improvement in diet digestibility since intake was not affected. Improvement of rumen pH supports higher activity of fiber-degrading populations and thus explains the higher meal frequency.

A recent study using an endoscope to collect ruminal biopsies has evaluated the effect of a live yeast (*S. cerevisiae* CNCM I-1077) during the calving period (Bach et al., 2018). Lactate-producing *Streptococcus* and *Lactobacillus* genera (Derakhshani et al., 2016) and saccharolytic members of the Proteobacteria phylum (Zhu et al., 2017) increase post-partum, increasing the risk of SARA which affects the epithelium of the gastrointestinal tract (Steele et al., 2011). Results showed that supplementation of live yeast FA before calving increased expression of genes regulating inflammation and the epithelial barrier in the rumen (such as tight junction coding genes).

Few studies have looked at the potential of probiotics to alleviate inflammation or to control the immune system during acidosis. In heifers fed with high challenging diets (starch/fructose), a combination of the ionophore monensin and a live yeast FA (*S. cerevisiae* I-1077) was significantly reduced histamine concentration in rumen fluid (Golder et al., 2014). In other trials looking at the effect of microbial FA on rumen or plasma concentrations of inflammatory molecules, Garcia Diaz et al. (2018) have shown a decrease in plasmatic LPS concentrations as well as Serum Amyloid A (SAA), an acute phase protein, after a supplementation of steers by live yeasts (*S. cerevisiae* NCYC 996), but with no effect on ruminal or duodenal LPS concentrations. Silberberg et al. (2013) measured a significant decrease in plasma SAA levels with the use of live yeast FA, again with no effect on ruminal LPS concentrations. This may be because the large intestine rather than the rumen is the most probable site for LPS translocation in ruminants, because the monolayer intestinal epithelium is more prone to damage by acidity compared to the reticulo-rumen epithelium (Khiaosa-Ard and Zebeli, 2018).

Figure 3 summarizes the expected benefits of live yeast FA supplementation to ruminants at risk of subacute rumen acidosis (SARA).

When cattle are subjected to high temperature and humidity conditions (heat stress), the relative ruminal proportions of the *Clostridium*

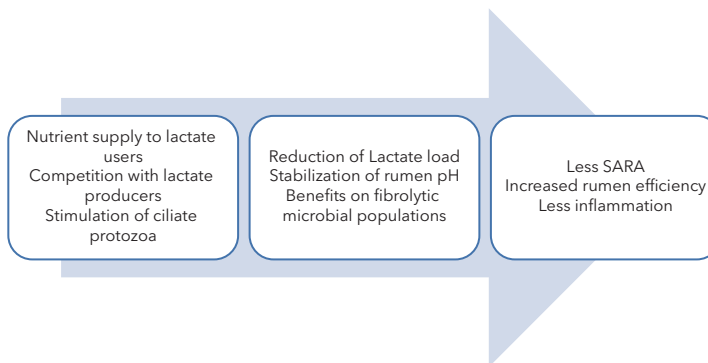

Figure 3 The benefits of live yeast FA in ruminants.

coccoides-*Eubacterium rectale* group and *Streptococcus* increase, while the genus *Fibrobacter* decrease, leading to microbial imbalance (Uyeno et al., 2010). Under these conditions, microbial FA could help to stabilize the rumen ecosystem and alleviate the negative impact of heat stress on cattle performance (Salvati et al., 2015).

Megasphaera elsdenii is as an ecologically important species within the rumen ecosystem as it removes lactate and thereby prevents acidosis. A recent metagenomic study, focusing on the rumen microbiome in low or high efficient cows, highlighted that genes that were the most enriched in the efficient cows' microbiomes were affiliated to *Megasphaera elsdenii*. Genes of the acrylate metabolic pathway, involved in the conversion of lactate into propionate were also enriched in the most efficient animals (Shabat et al., 2016). *M. elsdenii* has been considered as a FA to boost *M. elsdenii* concentrations in the rumen and speed up removal of lactate from the rumen (Arik et al., 2019; Muya et al., 2015; Yohe et al., 2018; Zebeli et al., 2012). However, it has not always been possible to measure beneficial effects (Yohe et al., 2018; Zebeli et al., 2012). In addition, preparation and delivery is challenging.

Other lactate-utilizing bacteria from the *Propionibacterium* genus have also been evaluated to alleviate the severity of SARA in high-grain fed cattle (Azad et al., 2017; Philippeau et al., 2017). It has been suggested associating them with *Lactobacilli* in order to promote lactic acid production, which would stimulate lactate-utilizing *Propionibacterium* (Lettat et al., 2012). A study using 16S rRNA gene sequencing reported beneficial effects of one *P. acidipropionici* strain P169 on the relative abundance of sequences affiliated to key lactate utilizers (*Veillonellaceae* and *Megasphaera),* and cellulolytic members of the bacterial families *Ruminococcaceae, Lachnospiraceae, Clostridiaceae, Christensenellaceae* which were enriched in the rumen microbiota of supplemented high-grain fed steers (Azad et al., 2017). Microbial metabolites were also affected (higher molar proportions of branched-chain fatty acids and increased concentration of ammonia) indicating an improved state of fibrolytic and proteolytic activity.

6 Benefits and modes of action of probiotics: methane production

The use of microbial FA is one possible option to decrease CH_4 emission from ruminants. Potential mechanisms of action include (Jeyanathan et al., 2014):

- direct inhibition of methanogenesis,
- promotion of alternative pathways which already exist in the rumen such as homoacetogenesis,
- fumarate reduction,
- propionate production through the acrylate pathway,
- nitrate/nitrite reduction,

- capnophily (CO_2 fixation), and
- anaerobic oxidation of methane (methanotrophy).

Only a few of these routes have been explored, probably because of a lack of understanding of these complex microbial metabolisms, and of their capacity to compete with methanogenesis. The homoacetogenic pathway has been found to promote functional ruminal activity in lambs deprived of methanogens but that acetogens were much less efficient in capturing H_2 than methanogens (Fonty et al., 2007). There are also problems of survival for candidate microbes since most of these alternative routes require strict anaerobic conditions. Finally, there are potential side-effects on fermentation efficiency or toxicity for the animal, for example, in the case of nitrite accumulation from nitrate supplementation which could cause methemoglobinaemia.

There have been several studies on the effect of *Saccharomyces cerevisiae* on rumen methanogenesis to promote alternative non-methanogenic pathways. The promotion of acetogenic bacteria has been shown at least *in vitro* (Chaucheyras et al., 1995; Nollet et al., 1997). Using gnotobiotic animal models, Chaucheyras-Durand et al. (2010b) showed that the composition of the cellulolytic community (hydrogen producers vs. non-hydrogen producers) may have an impact on H_2 accumulation and subsequent methane production in the rumen ecosystem. The promotion of fibrolytic organisms which do not produce any hydrogen, such as *Fibrobacter succinogenes*, may help limit methane emissions in the rumen. However, studies about yeast FA so far have failed to demonstrate a mitigating effect (Bayat et al., 2015; Chung et al., 2011). The increase in FE reported in the presence of live yeast FA (see earlier) should have an indirect effect on methane excretion, as it would decrease the amount of output/kg of milk/meat produced (Jeyanathan et al., 2014). A few bacteria have been tested for their anti-methanogenic potential. Results in sheep showed some efficacy with one *L. pentosus* strain (a 13% decrease in methane emission after 2 weeks which lasted throughout the 4 week-treatment) (Jeyanathan et al., 2016). A mix of three strains of *Propionibacterium* was also evaluated in beef cattle that fed a corn-finishing diet (Vyas et al., 2014) but showed no effect on enteric methane production; the authors suggested that the high starch content of the diet-induced high levels of propionate, and that these conditions may have reduced the *Propionibacterium* efficacy on methane mitigation.

7 Benefits and modes of action of probiotics: pathogen control

The use of antibiotics in livestock production has been linked to the development of resistant bacterial populations and the persistence of antibiotic residues in animal food products (Langford et al., 2003; Ramatla et al., 2017;

Seymour et al., 1988). Microbial FA supplementation has been considered as an alternative way to decrease pathogen loads in cattle as well as reduce the risk of transmission of zoonotic pathogens to humans. Salmonellosis was the second most important human zoonosis observed in the EU in 2017 (EFSA and ECDC, 2018), and *Salmonella* food-borne outbreaks have been associated with the consumption of beef products (EFSA and ECDC, 2018). *Salmonella* have been shown to be asymptomatically carried by cattle (Feye et al., 2016) although they are also capable of causing clinical diseases in animals. Diarrhea and enteric diseases caused by *Salmonella enterica* and *Escherichia coli* are a major cause of economic loss for cattle producers (USDA, 2014a; Cho and Yoon, 2014). Probiotics may help to eradicate pathogens through competitive exclusion, production of antimicrobial compounds and stimulation of the host immune defenses which will decrease pathogen colonization in the animal and the risk of infection.

Lactobacillus amylovorus C94 and *L. salivarius* C86 strains have demonstrated promising potential *in vitro* against *Salmonella enterica* isolated from cattle (Adetoye et al., 2018). DFM have been shown to have a positive effect in preventing *Salmonella* infection in steers treated daily with a mixture of *L. acidophilus* LA51 and *P. freudenreichii* PF24 (Tabe et al., 2008). Administration of the same DFM to beef cattle led to a significantly reduced prevalence of *Salmonella* at slaughter and a significant reduction of pathogen concentration in peripheral lymph nodes (PLN) associated with contamination of ground beef (Vipham et al., 2015). Soto et al. (2015) tested the effect of an inoculum composed of *Lactobacillus casei* DSPV318T, *L. salivarius* DSPV315T and *Pediococcus acidilactici* DSPV006T on young calves, and observed that daily administration of this inoculum coupled with lactose caused a decrease in the neutrophils/lymphocytes ratio, indicating an increase in immune response during the acute phase of infection (Soto et al., 2016). This product was previously observed to decrease severity of diarrhea in *Salmonella*-infected calves (Soto et al., 2015).

Shiga-toxin producing *E. coli* (STEC) (such as *E. coli* O157:H7) are of major concern due to their impact on human health (Caprioli et al., 2005; Chauret, 2011; EFSA and ECDC, 2018). DFM represent an efficient strategy to reduce fecal shedding of *E. coli* O157:H7 in beef cattle (Brashears and Chaves, 2017). *In vitro* it was shown that *Lactobacillus acidophilus* BT-1386 had a dose-dependent inhibitory effect on *E. coli* O157:H7 in feces (Chaucheyras-Durand et al., 2006). This strain and a live yeast strain of *Saccharomyces cerevisiae* CNCM I-1077 were able to significantly reduce pathogenic *E. coli* load in the rumen of sheep (Chaucheyras-Durand et al., 2010a). Suppression of *E. coli* O157:H7 was achieved in the rumen with *L. reuteri* LB1-7 coupled with glycerol. Reduced pathogen growth in rectal content was achieved following prior exposure to the same mixture in rumen fluid (Bertin et al., 2017). Those

in vitro results highlight the potential of DFM to decrease pathogen load in the digestive system.

A recent meta-analysis has shown that LAB (particularly a DFM combination of *Lactobacillus acidophilus* NP51 and *Propionibacterium freudenreichii* NP24) reduces the prevalence of *E. coli* O157:H7 fecal shedding (Wisener et al., 2015). In 2013, The Beef Industry Food Safety Council (BIFSCO) in the United States has recognized the efficiency of several bacterial strains in reducing *E. coli* O157:H7 in beef cattle and include them as part of Production Best Practice (Beef Industry Food Safety Council subcommittee on pre-harvest, 2013). Administration of a mixture of competitive-exclusion commensal *E. coli* to experimentally infected calves showed a significant reduction of fecal shedding for O157:H7 but also non-O157 serotypes of *E. coli* (Tkalcic et al., 2003). Using the same probiotic mixture, another study found treated calves shed significantly less non-O157 *E. coli* but no effect was observed on *E. coli* O157 (Zhao et al., 2003). In experimentally infected lambs, daily administration of *S. faecium* or a mixture of *S. faecium*, *L. acidophilus*, *L. casei*, *L. fermentum* and *L. plantarum* led to significant reductions in *E. coli* O157:H7 in feces (Lema et al., 2001). A probiotic mixture containing *Lactobacillus acidophilus* LC10, *Lactobacillus helveticus* LC3, *Lactobacillus bulgaricus* LC182, *Lactobacillus lactis*, *Streptococcus thermophilus* LC201 and *Enterococcus faecium* LAT E-253 was shown to significantly reduce the fecal shedding of pathogenic *E. coli* in sheep (Rigobelo et al., 2015). However, the limitation of STEC carriage is dependent upon many parameters which are difficult to control (such as diet, environmental factors, stress level, intermittent and seasonal shedding, occurrence of several serotypes within the herd, and so on (Bertin et al., 2011, 2013; Chaucheyras-Durand et al., 2010a, 2006; Dunière et al., 2011; Fremaux et al., 2006). Identification and implementation of the most efficient strategies should be based on a good understanding of STEC ecology and physiology in bovine GIT (Segura et al., 2018).

Probiotics can also be administered to cattle to treat non-digestive disorders. One of the most detrimental diseases in dairy industry is mastitis as it leads to decrease in production and higher treatment costs (Hogeveen and Østerås, 2005). Probiotics such as LAB can potentially reduce mastitis through colonizing the udder and building a beneficial biofilm that prevents pathogen colonization. Three strains of *Lactobacillus brevis* 1595 and 1597 and *Lactobacillus plantarum* 1610 have shown good colonization capacities in competing with pathogens in mammary gland colonization. They also exhibit anti-inflammatory properties, with lower IL-8 secretion by *E. coli*-stimulated bovine mammary epithelial cells (bMEC) (Bouchard et al., 2015). *Lactobacillus rhamnosus* ATCC7469 and *L. plantarum* 2/37 have been shown to form biofilms to displace *Staphylococcus* (Wallis et al., 2018, 2019). A bacteriocin-producing *Lactococcus lactis* DPC3174 was shown to be as effective as a conventional antibiotic to treat cow mastitis

(Klostermann et al., 2008). Two LAB strains (*Lactobacillus perolens* CRL 1724 and nisin Z producer *Lactococcus lactis* sub. *Lactis* CRL1655) were shown to modulate the host udder immune system and stimulate local and systemic defence lines *in vivo* in dairy cow at the dry-off stage (Pellegrino et al., 2017). In their review of the recent literature on the use of probiotics to treat bovine mastitis, Rainard and Foucras (2018) point that most trials with LAB are *in vitro* rather than *in vivo*. However, they confirm intra-mammary administration as the best approach for mastitis control with probiotics.

Probiotics can also be used in order to enhance reproduction performance. Bovine reproductive diseases can lead to lower milk production as well as impaired reproductive performance (Bellows et al., 2002). Acute metritis is an inflammation of the uterus after calving due to bacterial infection, with *E. coli* being the predominant pathogen initiating the disease (Kassé et al., 2016). Vaginal treatment with LAB probiotic was observed to reduce metritis prevalence up to 58% compared to control animals (Genís et al., 2018). Live yeast dietary supplementation has been shown to improve reproductive performance of dairy cows during heat stress through the alteration of hormones and ovarian follicular dynamics (Nasiri et al., 2018).

Bovine respiratory disease (BRD) results in significant losses for cattle producers (Fulton et al., 2002; Griffin et al., 2010). BRD is multifactorial but one of the predominant bacterial pathogens identified is *Mannheimia haemolytica*. Probiotic strains belonging to *Lactobacillus*, *Lactococcus* and *Paenibacillus* genera have been shown to adhere to bovine respiratory cells and inhibit *M. haemolytica* through competition and displacement *in vitro* (Amat et al., 2017). Intragastric administration of *Enterococcus faecalis* CECT7121 to mice was shown to induce an increase in humoral immune response against *Pasteurella multocida* and *M. haemolytica*. Probiotic-treated mice showed higher interferon-γ production indicating a higher cellular immune response (Díaz et al., 2018). The authors suggested that this probiotic could be used as a possible adjuvant in a vaccine strategy to enhance ruminant immune response. In beef heifers submitted to a bacterial and viral challenge (*M. haemolytica* and bovine herpesvirus-1), the supplementation of *Saccharomyces boulardii* CNCM I-1079 altered the leukogram (with a significant increase in neutrophil %, and an increase in monocyte % on the day following *Mannheimia* challenge), indicating an increase in the innate immune response (Kayser et al., 2019).

8 Benefits and modes of action of probiotics: effects on the immune system

As seen earlier in this chapter, there is significant interest in probiotic intervention to enhance immune function in neonate and pre-weaned ruminants. Research demonstrates that gut microbiota in early life has a key role in the shaping of

immune-competence later in life (Gensollen et al., 2016). In adult ruminants, probiotics may play a role in modulating the production of pro- vs. anti-inflammatory signals in immune function (Raabis et al., 2019). However, the way probiotics participate in host-microbial interaction and modulate immune function remains largely unknown (Ma et al., 2018). Modulation of the immune response associated with supplementation of the diet by probiotics may occur not only through the innate and adaptive immune system, but also through regulation of intestinal epithelium permeability, mucus secretion and competition within the microbial ecosystem where probiotics can secrete antimicrobial compounds (La Fata et al., 2018). This makes the picture very complex (Fig. 4).

Bach et al. (2018) assessed the effect of a live yeast FA (*S. cerevisiae* I-1077) on the expression of genes encoding for proteins involved in the immune response in cows around calving. Cows responded rapidly to the live yeast, with measurable changes in expression of genes involved in the immune response after 7 days of supplementation, including the expression of the anti-inflammatory IL-10 genes in the rumen and of the β-defensin gene in the colon. β-glucans or manno-oligosaccharides (e.g. *Saccharomyces cerevisiae*) have also been considered as immunostimulants or pathogen binders (Ganner and Schatzmayr, 2012; Goodridge et al., 2009; Li et al., 2016, 2018; Yuan et al., 2015). Other species of yeast such as *Debaryomyces hansenii* are also of growing interest as the cell wall β-glucans seem to induce a marked immune response in other animal species (Angulo et al., 2018, 2019). There has been great interest in improving the understanding of the role of yeast cell wall components on immune system modulation and host response using techniques such as atomic force microscopy (Schiavone et al., 2015, 2017).

There is a significant body of literature on the effect of selected bacteria on intestinal epithelial cell (IEC) response, mostly from human cell lines or in rodent models (La Fata et al., 2018). Villena et al. (2018) have suggested the use of bovine IEC to study the impact of immunobiotics, such as *Bifidobacteria* or *Lactobacillus*, on anti-viral immunity. They have reported beneficial effects of immunobiotics through activation of interferon regulatory factor-3 (IRF-3), increased production of anti-viral factors and regulation of inflammation mediators. The impact of bacterial probiotics (*Lactobacillus casei* BL23, *Lactococcus lactis* V7) has been studied on the innate immune response of bovine mammary cells stimulated by *Staphylococcus aureus* (Assis et al., 2015; Souza et al., 2018). Like yeasts bacteria cell wall composition and organization are likely to be involved in triggering host response (Lebeer et al., 2018).

There is a growing interest in the use of neurochemical-producing probiotics in the treatment of health and disease through the microbiota-gut-brain axis (Lyte, 2011). This is significant for ruminants since there is increasing

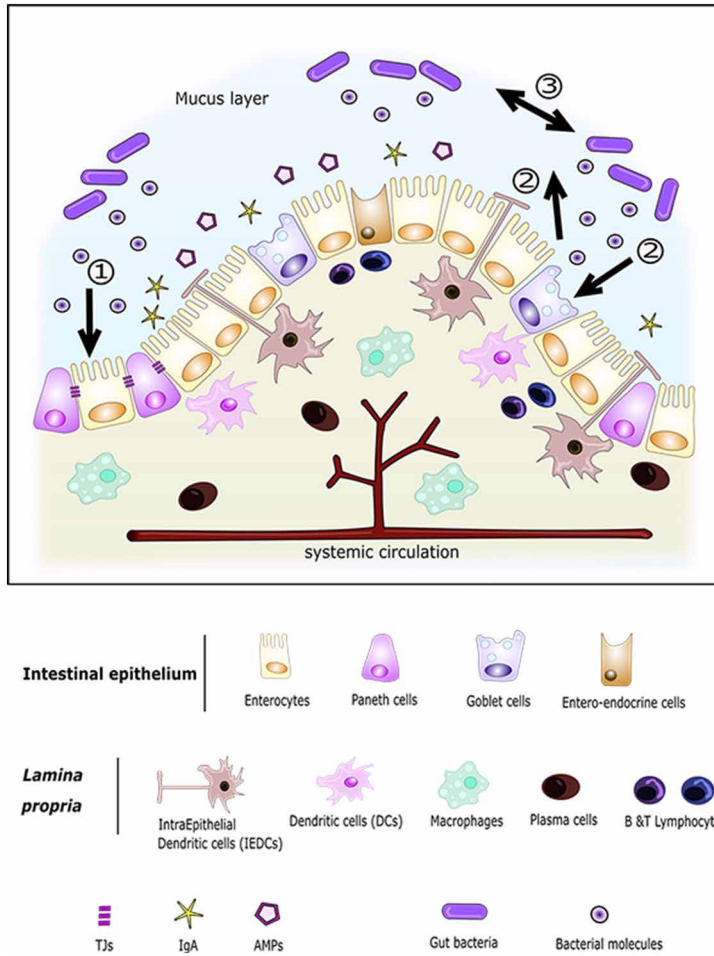

Figure 4 The intestinal barrier and the main actors involved in microbiota (gut bacteria or probiotics and their metabolites) – intestinal epithelium cross-talk. ① represents modulation of tight junction (TJ) proteins, ② shows the modulation of mucus secretion from goblet cells, ③ shows possible interactions between different members of gut microbiota (antimicrobial properties for example). Source: adapted from La Fata et al. (2018).

evidence to show that stress increases susceptibility to enteric infections (Freestone and Lyte, 2010). Recent findings report production of dopamine by *Enterococcus faecium* strains acting in the gastrointestinal tract (Villageliú and Lyte, 2018). Other microbial species used as probiotics are also able to produce a range of molecules involved in gut-brain axis communication (Lyte, 2011) (Table 2).

Table 2 Neurochemicals isolated by potential probiotic genera

Microbial genus	Neurochemical
Bifidobacterium, Lactobacillus	Gamma amino butyric acid (GABA)
Lactobacillus	Acetylcholine
Bacillus, Saccharomyces	Norepinephrine (Noradrenaline)
Enterococcus, Bacillus	Dopamine
Enterococcus	Serotonine

Source: adapted from Lyte (2011) and Villageliú and Lyte (2018).

9 Conclusions and future trends

As the research discussed in this chapter suggests, microbial FA use in livestock can improve profitability while maintaining animal welfare and farm sustainability. The evolution of commercially available probiotics will depend on regulatory constraints. In the EU, for example, the focus is on approving a single, well-defined microbial strain for one target species. It would help if regulators were able to accept combinations of microbial actives or even uncharacterized microbial consortia if they had proven benefits. Innovative techniques, such as microbial transplants (ruminal or fecal), are currently not allowed, nor are genetically modified probiotics authorized.

More research is needed to better understand the mechanisms by which microbial FA interact with gut microbiota and the host animal, in order to better select safe, robust, environmentally friendly and efficient additives which can be used easily by farmers. Multi-omic approaches will aid in deciphering metabolic pathways utilized by probiotics which can explain their benefits in the host animal. Once these pathways are characterized, it will be easier to use this understanding for more accurate and appropriate screening and selection of the best candidate for a given expected benefit. Most ruminant research has been focused on improving rumen function for FE or methane mitigation. However, there needs to be more research on how probiotics modulate host-microbial interactions and host immunity in order to optimize probiotic intervention strategies to improve animal gut health and productivity.

Other potential areas of research include maternal imprinting (on GIT microbiota establishment and immunity development in offspring) through microbial FA, although mechanisms of action remain largely unknown and require further research. Another exciting field is microbial endocrinology, a discipline at the interface of microbiology, endocrinology and neurophysiology. This can help understand better how stress affects ruminants health. To quote Freestone: 'happy, less stressed ruminants may be better-nourished animals and safer sources of meat' (Freestone and Lyte, 2010).

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11 Where to look for further information section

Further readings to get deeper information on the topics which were covered in this chapter:

- Chaucheyras-Durand, F. and Durand, H. 2010. Probiotics in animal nutrition and health. *Benef Microbes.* 1(1), 3–9. doi:10.3920/BM2008.1002 (Review).
- McCann, J. C., Elolimy, A. A. and Loor, J. J. 2017. Rumen microbiome, probiotics, and fermentation additives. *Vet. Clin. North Am. Food. Anim. Pract.* 33(3), 539–53. doi:10.1016/j.cvfa.2017.06.009.
- McSweeney, C. and Mackie, R. 2012. Micro-organisms and ruminant digestion: state of knowledge, trends and future prospects. Commission on Genetic Resources for Food and Agriculture. Background study paper NO. 61.
- Probiotics in animal nutrition. FAO report 2016. Available at: http://www.fao.org/3/a-i5933e.pdf.

Key societies, professional organisations or other websites worth visiting to keep up to date with trends:

- www.lallemandanimalnutrition.com.
- www.ruminantdigestivesystem.com.
- www6.ara.inrae.fr/medis.

Key journals or conferences (e.g. key conferences held regularly every year or few years):

- Beneficial Microbes Conference (https://www.bastiaanse-communication.com/BMC2018/) and associated journal.
- Congress on Gastro-intestinal Function (https://www.congressgastrofunction.org/).
- International Probiotic Conference (https://www.probiotic-conference.net/).
- Joint INRA-Rowett Symposium on gut microbiota (https://colloque.inrae.fr/inra-rowett-2016).

12 References

Abaker, J. A., Xu, T. L., Jin, D., Chang, G. J., Zhang, K. and Shen, X. Z. 2017. Lipopolysaccharide derived from the digestive tract provokes oxidative stress in the liver of dairy cows fed a high-grain diet. *J. Dairy Sci.* 100(1), 666–78. doi:10.3168/jds.2016-10871.

Abecia, L., Martín-García, A. I., Martínez, G., Newbold, C. J. and Yáñez-Ruiz, D. R. 2013. Nutritional intervention in early life to manipulate rumen microbial colonization and methane output by kid goats postweaning. *J. Anim. Sci.* 91(10), 4832–40. doi:10.2527/jas.2012-6142.

Abecia, L., Martínez-Fernandez, G., Waddams, K., Martín-García, A. I., Pinloche, E., Creevey, C. J., Denman, S. E., Newbold, C. J. and Yáñez-Ruiz, D. R. 2014. An antimethanogenic nutritional intervention in early life of ruminants modifies ruminal colonization by Archaea. *Archaea* 2014, 841463. doi:10.1155/2014/841463.

Abecia, L., Martínez-Fernandez, G., Waddams, K., Martín-García, A. I., Pinloche, E., Creevey, C. J., Denman, S. E., Newbold, C. J. and Yáñez-Ruiz, D. R. 2018. Analysis of the rumen microbiome and metabolome to study the effect of an antimethanogenic treatment applied in early life of kid goats. *Front. Microbiol.* 9, 2227. doi:10.3389/fmicb.2018.02227.

Adetoye, A., Pinloche, E., Adeniyi, B. A. and Ayeni, F. A. 2018. Characterization and anti-salmonella activities of lactic acid bacteria isolated from cattle faeces. *BMC Microbiol.* 18(1), 96. doi:10.1186/s12866-018-1248-y.

Alugongo, G. M., Xiao, J., Wu, Z., Li, S., Wang, Y. and Cao, Z. 2017. Review: utilization of yeast of Saccharomyces cerevisiae origin in artificially raised calves. *J. Anim. Sci. Biotechnol.* 8, 34. doi:10.1186/s40104-017-0165-5.

AlZahal, O., Li, F., Guan, L. L., Walker, N. D. and McBride, B. W. 2017. Factors influencing ruminal bacterial community diversity and composition and microbial fibrolytic enzyme abundance in lactating dairy cows with a focus on the role of active dry yeast. *J. Dairy Sci.* 100(6), 4377–93. doi:10.3168/jds.2016-11473.

Amachawadi, R. G., Giok, F., Shi, X., Soto, J., Narayanan, S. K., Tokach, M. D., Apley, M. D. and Nagaraja, T. G. 2018. Antimicrobial resistance of *Enterococcus faecium* strains isolated from commercial probiotic products used in cattle and swine. *J. Anim. Sci.* 96(3), 912–20. doi:10.1093/jas/sky056.

Amat, S., Subramanian, S., Timsit, E. and Alexander, T. W. 2017. Probiotic bacteria inhibit the bovine respiratory pathogen *Mannheimia haemolytica* serotype 1 *in vitro*. *Lett. Appl. Microbiol.* 64(5), 343–9. doi:10.1111/lam.12723.

Angulo, M., Reyes-Becerril, M., Tovar-Ramírez, D., Ascencio, F. and Angulo, C. 2018. *Debaryomyces hansenii* CBS 8339 β-glucan enhances immune responses and down-stream gene signaling pathways in goat peripheral blood leukocytes. *Dev. Comp. Immunol.* 88, 173–82. doi:10.1016/j.dci.2018.07.017.

Angulo, M., Reyes-Becerril, M., Cepeda-Palacios, R., Tovar-Ramírez, D., Esteban, M. Á. and Angulo, C. 2019. Probiotic effects of marine *Debaryomyces hansenii* CBS 8339 on innate immune and antioxidant parameters in newborn goats. *Appl. Microbiol. Biotechnol.* 103(5), 2339–52 doi:10.1007/s00253-019-09621-5.

Arik, H. D., Gulsen, N., Hayirli, A. and Alatas, M. S. 2019. Efficacy of Megasphaera elsdenii inoculation in subacute ruminal acidosis in cattle. *J. Anim. Physiol. Anim. Nutr.* 103(2), 416–26 doi:10.1111/jpn.13034.

Ashenafi, D., Yidersal, E., Hussen, E., Solomon, T. and Desiye, M. 2018. The effect of long distance transportation stress on cattle: a review. *Biomed. J. Sci. Tech. Res.* 3. doi:10.26717/BJSTR.2018.03.000908.

Assis, B. S., Germon, P., Silva, A. M., Even, S., Nicoli, J. R. and Le Loir, Y. 2015. *Lactococcus lactis* V7 inhibits the cell invasion of bovine mammary epithelial cells by *Escherichia coli* and *Staphylococcus aureus*. *Benef Microbes* 6(6), 879–86. doi:10.3920/BM2015.0019.

Azad, E., Narvaez, N., Derakhshani, H., Allazeh, A. Y., Wang, Y., McAllister, T. A. and Khafipour, E. 2017. Effect of *Propionibacterium acidipropionici* P169 on the rumen and faecal microbiota of beef cattle fed a maize-based finishing diet. *Benef. Microbes* 8(5), 785–99. doi:10.3920/BM2016.0145.

Bach, A., Iglesias, C. and Devant, M. 2007. Daily rumen pH pattern of loose-housed dairy cattle as affected by feeding pattern and live yeast supplementation. *Anim. Feed Sci. Technol.* 136(1–2), 146–53. doi:10.1016/j.anifeedsci.2006.09.011.

Bach, A., Guasch, I., Elcoso, G., Chaucheyras-Durand, F., Castex, M., Fàbregas, F., Garcia-Fruitos, E. and Aris, A. 2018. Changes in gene expression in the rumen and colon epithelia during the dry period through lactation of dairy cows and effects of live yeast supplementation. *J. Dairy Sci.* 101(3), 2631–40. doi:10.3168/jds.2017-13212.

Baldwin, R. L., McLeod, K. R., Klotz, J. L. and Heitmann, R. N. 2004. Rumen development, intestinal growth and hepatic metabolism in the pre- and postweaning ruminant. *J. Dairy Sci.* 87, E55–65. doi:10.3168/jds.S0022-0302(04)70061-2.

Bayat, A. R., Kairenius, P., Stefański, T., Leskinen, H., Comtet-Marre, S., Forano, E., Chaucheyras-Durand, F. and Shingfield, K. J. 2015. Effect of Camelina oil or live yeasts (*Saccharomyces cerevisiae*) on ruminal methane production, rumen fermentation, and milk fatty acid composition in lactating cows fed grass silage diets. *J. Dairy Sci.* 98(5), 3166–81. doi:10.3168/jds.2014-7976.

Beef Industry Food Safety Council subcommittee on pre-harvest. 2013. Production Best Practices (PBP) to aid in the control of foodborne pathogens in groups of cattle. BIFSCO, USA.

Bellows, D. S., Ott, S. L. and Bellows, R. A. 2002. Review: Cost of reproductive diseases and conditions in cattle. *Prof. Anim. Sci.* 18(1), 26–32. doi:10.15232/S1080-7446(15)31480-7.

Bertin, Y., Girardeau, J. P., Chaucheyras-Durand, F., Lyan, B., Pujos-Guillot, E., Harel, J. and Martin, C. 2011. Enterohaemorrhagic *Escherichia coli* gains a competitive advantage by using ethanolamine as a nitrogen source in the bovine intestinal content. *Environ. Microbiol.* 13(2), 365–77. doi:10.1111/j.1462-2920.2010.02334.x.

Bertin, Y., Chaucheyras-Durand, F., Robbe-Masselot, C., Durand, A., de la Foye, A., Harel, J., Cohen, P. S., Conway, T., Forano, E. and Martin, C. 2013. Carbohydrate utilization by enterohaemorrhagic *Escherichia coli* O157:H7 in bovine intestinal content. *Environ. Microbiol.* 15(2), 610–22. doi:10.1111/1462-2920.12019.

Bertin, Y., Habouzit, C., Dunière, L., Laurier, M., Durand, A., Duchez, D., Segura, A., Thévenot-Sergentet, D., Baruzzi, F., Chaucheyras-Durand, F. and Forano, E. 2017. *Lactobacillus reuteri* suppresses *E. coli* O157:H7 in bovine ruminal fluid: toward a pre-slaughter strategy to improve food safety? *PLoS ONE* 12(11), e0187229. doi:10.1371/journal.pone.0187229.

Blecha, F., Boyles, S. L. and Riley, J. G. 1984. Shipping suppresses lymphocyte blastogenic responses in Angus and Brahman × Angus feeder calves. *J. Anim. Sci.* 59(3), 576–83. doi:10.2527/jas1984.593576x.

Bouchard, D. S., Seridan, B., Saraoui, T., Rault, L., Germon, P., Gonzalez-Moreno, C., Nader-Macias, F. M., Baud, D., François, P., Chuat, V., Chain, F., Langella, P., Nicoli, J., Le Loir, Y. and Even, S. 2015. Lactic acid bacteria isolated from bovine mammary microbiota: potential allies against bovine mastitis. *PLoS ONE* 10(12), e0144831. doi:10.1371/journal.pone.0144831.

Brashears, M. M. and Chaves, B. D. 2017. The diversity of beef safety: a global reason to strengthen our current systems. *Meat Sci.* 132, 59–71. doi:10.1016/j.meatsci.2017.03.015.

Brossard, L., Martin, C., Chaucheyras-Durand, F. and Michalet-Doreau, B. 2004. Protozoa involved in butyric rather than lactic fermentative pattern during latent acidosis in sheep. *Reprod. Nutr. Dev.* 44(3), 195–206. doi:10.1051/rnd:2004023.

Brossard, L., Chaucheyras-Durand, F., Michalet-Doreau, B. and Martin, C. 2006. Dose effect of live yeasts on rumen microbial communities and fermentations during butyric latent acidosis in sheep: new type of interaction. *Anim. Sci.* 82(6), 829–36. doi:10.1017/ASC200693.

Bush, S. J., McCulloch, M. E. B., Muriuki, C., Salavati, M., Davis, G. M., Farquhar, I. L., Lisowski, Z. M., Archibald, A. L., Hume, D. A. and Clark, E. L. 2019. Comprehensive transcriptional profiling of the gastrointestinal tract of ruminants from birth to adulthood reveals strong developmental stage specific gene expression. *G3 Bethesda MD* 9(2), 359–73. doi:10.1534/g3.118.200810.

Caprioli, A., Morabito, S., Brugère, H. and Oswald, E. 2005. Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. *Vet. Res.* 36(3), 289–311. doi:10.1051/vetres:2005002.

Chaucheyras, F., Fonty, G., Bertin, G. and Gouet, P. 1995. *In vitro* H2 utilization by a ruminal acetogenic bacterium cultivated alone or in association with an archaea methanogen is stimulated by a probiotic strain of Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* 61(9), 3466–7.

Chaucheyras-Durand, F. and Fonty, G. 2001. Establishment of cellulolytic bacteria and development of fermentative activities in the rumen of gnotobiotically-reared lambs receiving the microbial additive *Saccharomyces cerevisiae* CNCM I-1077. *Reprod. Nutr. Dev.* 41(1), 57–68. doi:10.1051/rnd:2001112.

Chaucheyras-Durand, F. and Fonty, G. 2002. Influence of a probiotic yeast (*Saccharomyces cerevisiae* CNCM I-1077) on microbial colonization and fermentations in the rumen of newborn lambs. *Microb. Ecol. Health Dis.* 14(1), 30–6. doi:10.1080/089106002760002739.

Chaucheyras-Durand, F., Madic, J., Doudin, F. and Martin, C. 2006. Biotic and abiotic factors influencing *in vitro* growth of *Escherichia coli* O157:H7 in ruminant digestive contents. *Appl. Environ. Microbiol.* 72(6), 4136–42. doi:10.1128/AEM.02600-05.

Chaucheyras-Durand, F., Walker, N. D. and Bach, A. 2008. Effects of active dry yeasts on the rumen microbial ecosystem: past, present and future. *Anim. Feed Sci. Technol.* 145(1–4), 5–26. doi:10.1016/j.anifeedsci.2007.04.019.

Chaucheyras-Durand, F., Faqir, F., Ameilbonne, A., Rozand, C. and Martin, C. 2010a. Fates of acid-resistant and non-acid-resistant Shiga toxin-producing *Escherichia coli* strains in ruminant digestive contents in the absence and presence of probiotics. *Appl. Environ. Microbiol.* 76(3), 640–7. doi:10.1128/AEM.02054-09.

Chaucheyras-Durand, F., Masséglia, S., Fonty, G. and Forano, E. 2010b. Influence of the composition of the cellulolytic flora on the development of hydrogenotrophic microorganisms, hydrogen utilization, and methane production in the rumens of

gnotobiotically reared lambs. *Appl. Environ. Microbiol.* 76(24), 7931–7. doi:10.1128/AEM.01784-10.

Chaucheyras-Durand, F., Ameilbonne, A., Bichat, A., Mosoni, P., Ossa, F. and Forano, E. 2016. Live yeasts enhance fibre degradation in the cow rumen through an increase in plant substrate colonization by fibrolytic bacteria and fungi. *J. Appl. Microbiol.* 120(3), 560–70. doi:10.1111/jam.13005.

Chauret, C. 2011. Survival and control of *Escherichia coli* O157:H7 in foods, beverages, soil and water. *Virulence* 2(6), 593–601. doi:10.4161/viru.2.6.18423.

Chen, Y., Penner, G. B., Li, M., Oba, M. and Guan, L. L. 2011. Changes in bacterial diversity associated with epithelial tissue in the beef cow rumen during the transition to a high-grain diet. *Appl. Environ. Microbiol.* 77(16), 5770–81. doi:10.1128/AEM.00375-11.

Chen, Y., Oba, M. and Guan, L. L. 2012. Variation of bacterial communities and expression of toll-like receptor genes in the rumen of steers differing in susceptibility to subacute ruminal acidosis. *Vet. Microbiol.* 159(3–4), 451–9. doi:10.1016/j.vetmic.2012.04.032.

Cho, Y. I. and Yoon, K. J. 2014. An overview of calf diarrhea - infectious etiology, diagnosis, and intervention. *J. Vet. Sci.* 15(1), 1–17. doi:10.4142/jvs.2014.15.1.1.

Chung, Y. H., Walker, N. D., McGinn, S. M. and Beauchemin, K. A. 2011. Differing effects of 2 active dried yeast (*Saccharomyces cerevisiae*) strains on ruminal acidosis and methane production in nonlactating dairy cows. *J. Dairy Sci.* 94(5), 2431–9. doi:10.3168/jds.2010-3277.

Comtet-Marre, S., Parisot, N., Lepercq, P., Chaucheyras-Durand, F., Mosoni, P., Peyretaillade, E., Bayat, A. R., Shingfield, K. J., Peyret, P. and Forano, E. 2017. Metatranscriptomics reveals the active bacterial and eukaryotic fibrolytic communities in the rumen of dairy cow fed a mixed diet. *Front. Microbiol.* 8, 67. doi:10.3389/fmicb.2017.00067.

Comtet-Marre, S., Chaucheyras-Durand, F., Bouzid, O., Mosoni, P., Bayat, A. R., Peyret, P. and Forano, E. 2018. FibroChip, a functional DNA microarray to monitor cellulolytic and hemicellulolytic activities of rumen microbiota. *Front. Microbiol.* 9, 215. doi:10.3389/fmicb.2018.00215.

Delgado, B., Bach, A., Guasch, I., González, C., Elcoso, G., Pryce, J. E. and Gonzalez-Recio, O. 2019. Whole rumen metagenome sequencing allows classifying and predicting feed efficiency and intake levels in cattle. *Sci. Rep.* 9(1), 11. doi:10.1038/s41598-018-36673-w.

de Ondarza, M. B., Sniffen, C. J., Dussert, L., Chevaux, E., Sullivan, J. and Walker, N. 2010. Case Study: multiple-study analysis of the effect of live yeast on milk yield, milk component content and yield, and feed efficiency. *Prof. Anim. Sci.* 26(6), 661–6. doi:10.15232/S1080-7446(15)30664-1.

Derakhshani, H., Tun, H. M., Cardoso, F. C., Plaizier, J. C., Khafipour, E. and Loor, J. J. 2016. Linking peripartal dynamics of ruminal microbiota to dietary changes and production parameters. *Front. Microbiol.* 7, 2143. doi:10.3389/fmicb.2016.02143.

DeVries, T. J. and Chevaux, E. 2014. Modification of the feeding behavior of dairy cows through live yeast supplementation. *J. Dairy Sci.* 97(10), 6499–510. doi:10.3168/jds.2014-8226.

Dias, A. L. G., Freitas, J. A., Micai, B., Azevedo, R. A., Greco, L. F. and Santos, J. E. P. 2018. Effect of supplemental yeast culture and dietary starch content on rumen fermentation and digestion in dairy cows. *J. Dairy Sci.* 101(1), 201–21. doi:10.3168/jds.2017-13241.

Díaz, A. M., Almozni, B., Molina, M. A., Sparo, M. D., Manghi, M. A., Canellada, A. M. and Castro, M. S. 2018. Potentiation of the humoral immune response elicited by a commercial vaccine against bovine respiratory disease by *Enterococcus faecalis* CECT7121. *Benef Microbes* 9(4), 553–62. doi:10.3920/BM2017.0081.

Ding, G., Chang, Y., Zhao, L., Zhou, Z., Ren, L. and Meng, Q. 2014. Effect of *Saccharomyces cerevisiae* on alfalfa nutrient degradation characteristics and rumen microbial populations of steers fed diets with different concentrate-to-forage ratios. *J. Anim. Sci. Biotechnol.* 5(1), 24. doi:10.1186/2049-1891-5-24.

Du, R., Jiao, S., Dai, Y., An, J., Lv, J., Yan, X., Wang, J. and Han, B. 2018. Probiotic *Bacillus amyloliquefaciens* C-1 improves growth performance, stimulates GH/IGF-1, and regulates the gut microbiota of growth-retarded beef calves. *Front. Microbiol.* 9, 2006. doi:10.3389/fmicb.2018.02006.

Dunière, L., Gleizal, A., Chaucheyras-Durand, F., Chevallier, I. and Thévenot-Sergentet, D. 2011. Fate of *Escherichia coli* O26 in corn silage experimentally contaminated at ensiling, at silo opening, or after aerobic exposure, and protective effect of various bacterial inoculants. *Appl. Environ. Microbiol.* 77(24), 8696–704. doi:10.1128/AEM.06320-11.

Durand-Chaucheyras, F., Fonty, G., Bertin, G., Théveniot, M. and Gouet, P. 1998. Fate of Levucell SC I-1077 yeast additive during digestive transit in lambs. *Reprod. Nutr. Dev.* 38(3), 275–80. doi:10.1051/rnd:19980307.

EFSA, ECDC. 2018. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017 [WWW Document]. European Food Safety Authority. Available at: https://www.efsa.europa.eu/fr/efsajournal/pub/5500 (accessed on 03 June 2019).

Ferraretto, L. F., Shaver, R. D. and Bertics, S. J. 2012. Effect of dietary supplementation with live-cell yeast at two dosages on lactation performance, ruminal fermentation, and total-tract nutrient digestibility in dairy cows. *J. Dairy Sci.* 95(7), 4017–28. doi:10.3168/jds.2011-5190.

Feye, K. M., Anderson, K. L., Scott, M. F., Henry, D. L. and Dorton, K. L. 2016. Abrogation of *Salmonella* and *E. coli* O157:H7 in feedlot cattle fed a proprietary *Saccharomyces cerevisiae* fermentation prototype. *J. Vet. Sci. Technol.* 7(4) doi:10.4172/2157-7579.1000350.

Fomenky, B. E., Chiquette, J., Bissonnette, N., Talbot, G., Chouinard, P. Y. and Ibeagha-Awemu, E. M. 2017. Impact of *Saccharomyces cerevisiae* boulardii CNCMI-1079 and *Lactobacillus acidophilus* BT1386 on total lactobacilli population in the gastrointestinal tract and colon histomorphology of Holstein dairy calves. *Anim. Feed Sci. Technol.* 234, 151–61. doi:10.1016/j.anifeedsci.2017.08.019.

Fomenky, B. E., Do, D. N., Talbot, G., Chiquette, J., Bissonnette, N., Chouinard, Y. P., Lessard, M. and Ibeagha-Awemu, E. M. 2018. Direct-fed microbial supplementation influences the bacteria community composition of the gastrointestinal tract of pre- and post-weaned calves. *Sci. Rep.* 8(1), 14147. doi:10.1038/s41598-018-32375-5.

Fonty, G. and Chaucheyras-Durand, F. 2006. Effects and modes of action of live yeasts in the rumen. *Biol. (Bratisl.)* 61(6), 741–50. doi:10.2478/s11756-006-0151-4.

Fonty, G., Joblin, K., Chavarot, M., Roux, R., Naylor, G. and Michallon, F. 2007. Establishment and development of ruminal hydrogenotrophs in methanogen-free lambs. *Appl. Environ. Microbiol.* 73(20), 6391–403. doi:10.1128/AEM.00181-07.

Freestone, P. and Lyte, M. 2010. Stress and microbial endocrinology: prospects for ruminant nutrition. *Animal* 4(7), 1248–57. doi:10.1017/S1751731110000674.

Fremaux, B., Raynaud, S., Beutin, L. and Rozand, C. V. 2006. Dissemination and persistence of Shiga toxin-producing *Escherichia coli* (STEC) strains on French dairy farms. *Vet. Microbiol.* 117(2–4), 180–91. doi:10.1016/j.vetmic.2006.04.030.

Fulton, R. W., Cook, B. J., Step, D. L., Confer, A. W., Saliki, J. T., Payton, M. E., Burge, L. J., Welsh, R. D. and Blood, K. S. 2002. Evaluation of health status of calves and the impact on feedlot performance: assessment of a retained ownership program for postweaning calves. *Can. J. Vet. Res.* 66(3), 173–80.

Gaggìa, F., Mattarelli, P. and Biavati, B. 2010. Probiotics and prebiotics in animal feeding for safe food production. *Int. J. Food Microbiol.* 141(Suppl. 1), S15–28. doi:10.1016/j.ijfoodmicro.2010.02.031.

Galvão, K. N., Santos, J. E. P., Coscioni, A., Villaseñor, M., Sischo, W. M. and Berge, A. C. 2005. Effect of feeding live yeast products to calves with failure of passive transfer on performance and patterns of antibiotic resistance in fecal *Escherichia coli*. *Reprod. Nutr. Dev.* 45(4), 427–40. doi:10.1051/rnd:2005040.

Ganner, A. and Schatzmayr, G. 2012. Capability of yeast derivatives to adhere enteropathogenic bacteria and to modulate cells of the innate immune system. *Appl. Microbiol. Biotechnol.* 95(2), 289–97. doi:10.1007/s00253-012-4140-y.

Garcia Diaz, T., Ferriani Branco, A., Jacovaci, F. A., Cabreira Jobim, C., Bolson, D. C. and Pratti Daniel, J. L. 2018. Inclusion of live yeast and mannan-oligosaccharides in high grain-based diets for sheep: ruminal parameters, inflammatory response and rumen morphology. *PLoS ONE* 13(2), e0193313. doi:10.1371/journal.pone.0193313.

Genís, S., Cerri, R. L. A., Bach, À., Silper, B. F., Baylão, M., Denis-Robichaud, J. and Arís, A. 2018. Pre-calving intravaginal administration of lactic acid bacteria reduces metritis prevalence and regulates blood neutrophil gene expression after calving in dairy cattle. *Front. Vet. Sci.* 5, 135. doi:10.3389/fvets.2018.00135.

Gensollen, T., Iyer, S. S., Kasper, D. L. and Blumberg, R. S. 2016. How colonization by microbiota in early life shapes the immune system. *Science* 352(6285), 539–44. doi:10.1126/science.aad9378.

Golder, H. M., Celi, P., Rabiee, A. R. and Lean, I. J. 2014. Effects of feed additives on rumen and blood profiles during a starch and fructose challenge. *J. Dairy Sci.* 97(2), 985–1004. doi:10.3168/jds.2013-7166.

Goodridge, H. S., Wolf, A. J. and Underhill, D. M. 2009. Beta-glucan recognition by the innate immune system. *Immunol. Rev.* 230(1), 38–50. doi:10.1111/j.1600-065X.2009.00793.x.

Griffin, D., Chengappa, M. M., Kuszak, J. and McVey, D. S. 2010. Bacterial pathogens of the bovine respiratory disease complex. *Vet. Clin. North Am. Food Anim. Pract.* 26(2), 381–94. doi:10.1016/j.cvfa.2010.04.004.

Guedes, C. M., Gonçalves, D., Rodrigues, M. A. M. and Dias-da-Silva, A. 2008. Effects of a Saccharomyces cerevisiae yeast on ruminal fermentation and fibre degradation of maize silages in cows. *Anim. Feed Sci. Technol.* 145(1–4), 27–40. doi:10.1016/j.anifeedsci.2007.06.037.

Guzman, C. E., Bereza-Malcolm, L. T., De Groef, B. and Franks, A. E. 2015. Presence of selected methanogens, Fibrolytic bacteria, and proteobacteria in the gastrointestinal tract of neonatal dairy calves from birth to 72 hours. *PLoS ONE* 10(7), e0133048. doi:10.1371/journal.pone.0133048.

Guzman, C. E., Bereza-Malcolm, L. T., De Groef, B. and Franks, A. E. 2016. Uptake of milk with and without solid feed during the monogastric phase: effect on fibrolytic and methanogenic microorganisms in the gastrointestinal tract of calves. *Anim. Sci. J.* 87(3), 378–88. doi:10.1111/asj.12429.

Hare, K. S., Leal, L. N., Romao, J. M., Hooiveld, G. J., Soberon, F., Berends, H., Van Amburgh, M. E., Martín-Tereso, J. and Steele, M. A. 2019. Preweaning nutrient supply alters mammary gland transcriptome expression relating to morphology, lipid accumulation, DNA synthesis, and RNA expression in Holstein heifer calves. *J. Dairy Sci.* 102(3), 2618–30. doi:10.3168/jds.2018-15699.

He, Z. X., Ferlisi, B., Eckert, E., Brown, H. E., Aguilar, A. and Steele, M. A. 2017. Supplementing a yeast probiotic to pre-weaning Holstein calves: feed intake, growth and fecal biomarkers of gut health. *Anim. Feed Sci. Technol.* 226, 81–7. doi:10.1016/j.anifeedsci.2017.02.010.

Hogeveen, H. and Østerås, O. 2005. Mastitis management in an economic framework. In: *Mastitis in Dairy Production*. Wageningen Academic Publishers, Maastrich, Netherlands. doi:10.3920/978-90-8686-550-5.

Humer, E., Petri, R. M., Aschenbach, J. R., Bradford, B. J., Penner, G. B., Tafaj, M., Südekum, K. H. and Zebeli, Q. 2018. Invited review: practical feeding management recommendations to mitigate the risk of subacute ruminal acidosis in dairy cattle. *J. Dairy Sci.* 101(2), 872–88. doi:10.3168/jds.2017-13191.

Hutcheson, D. P. and Cole, N. A. 1986. Management of transit-stress syndrome in cattle: nutritional and environmental effects. *J. Anim. Sci.* 62(2), 555–60. doi:10.2527/jas1986.622555x.

Ingvartsen, K. L., Dewhurst, R. J. and Friggens, N. C. 2003. On the relationship between lactational performance and health: is it yield or metabolic imbalance that cause production diseases in dairy cattle? A position paper. *Livest. Prod. Sci.* 83(2–3), 277–308. doi:10.1016/S0301-6226(03)00110-6.

Ipharraguerre, I. R., Pastor, J. J., Gavaldà-Navarro, A., Villarroya, F. and Mereu, A. 2018. Antimicrobial promotion of pig growth is associated with tissue-specific remodeling of bile acid signature and signaling. *Sci. Rep.* 8(1), 13671. doi:10.1038/s41598-018-32107-9.

Ishaq, S. L., AlZahal, O., Walker, N. and McBride, B. 2017. An investigation into rumen fungal and protozoal diversity in three rumen fractions, during high-fiber or grain-induced sub-acute ruminal acidosis conditions, with or without active dry yeast supplementation. *Front. Microbiol.* 8, 1943. doi:10.3389/fmicb.2017.01943.

Jami, E., Israel, A., Kotser, A. and Mizrahi, I. 2013. Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J.* 7(6), 1069–79. doi:10.1038/ismej.2013.2.

Jami, E., White, B. A. and Mizrahi, I. 2014. Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. *PLoS ONE* 9(1), e85423. doi:10.1371/journal.pone.0085423.

Jeyanathan, J., Martin, C. and Morgavi, D. P. 2014. The use of direct-fed microbials for mitigation of ruminant methane emissions: a review. *Animal* 8(2), 250–61. doi:10.1017/S1751731113002085.

Jeyanathan, J., Martin, C. and Morgavi, D. P. 2016. Screening of bacterial direct-fed microbials for their antimethanogenic potential *in vitro* and assessment of their effect on ruminal fermentation and microbial profiles in sheep. *J. Anim. Sci.* 94(2), 739–50. doi:10.2527/jas.2015-9682.

Jiang, Y., Ogunade, I. M., Qi, S., Hackmann, T. J., Staples, C. R. and Adesogan, A. T. 2017. Effects of the dose and viability of Saccharomyces cerevisiae. 1. Diversity of ruminal microbes as analyzed by Illumina MiSeq sequencing and quantitative PCR. *J. Dairy Sci.* 100(1), 325–42. doi:10.3168/jds.2016-11263.

Jiao, J., Li, X., Beauchemin, K. A., Tan, Z., Tang, S. and Zhou, C. 2015. Rumen development process in goats as affected by supplemental feeding v. grazing: age-related anatomic development, functional achievement and microbial colonisation. *Br. J. Nutr.* 113(6), 888–900. doi:10.1017/S0007114514004413.

Johnsen, J. F., Viljugrein, H., Bøe, K. E., Gulliksen, S. M., Beaver, A., Grøndahl, A. M., Sivertsen, T. and Mejdell, C. M. 2019. A cross-sectional study of suckling calves' passive immunity and associations with management routines to ensure colostrum intake on organic dairy farms. *Acta Vet. Scand.* 61(1), 7. doi:10.1186/s13028-019-0442-8.

Jouany, J. P. 2006. Optimizing rumen functions in the close-up transition period and early lactation to drive dry matter intake and energy balance in cows. *Anim. Reprod. Sci.* 96(3–4), 250–64. doi:10.1016/j.anireprosci.2006.08.005.

Jouany, J. P. and Morgavi, D. P. 2007. Use of 'natural' products as alternatives to antibiotic feed additives in ruminant production. *Animal* 1(10), 1443–66. doi:10.1017/S1751731107000742.

Kassé, F. N., Fairbrother, J. M. and Dubuc, J. 2016. Relationship between *Escherichia coli* virulence factors and postpartum metritis in dairy cows. *J. Dairy Sci.* 99(6), 4656–67. doi:10.3168/jds.2015-10094.

Kayser, W. C., Carstens, G. E., Washburn, K. E., Welsh, T. H., Lawhon, S. D., Reddy, S. M., Pinchak, W. E., Chevaux, E. and Skidmore, A. L. 2019. Effects of combined viral-bacterial challenge with or without supplementation of *Saccharomyces cerevisiae* boulardii strain CNCM I-1079 on immune upregulation and DMI in beef heifers. *J. Anim. Sci.* 97(3), 1171–84 doi:10.1093/jas/sky483.

Khafipour, E., Krause, D. O. and Plaizier, J. C. 2009. A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. *J. Dairy Sci.* 92(3), 1060–70. doi:10.3168/jds.2008-1389.

Khan, M. A., Bach, A., Weary, D. M. and von Keyserlingk, M. A. G. 2016. Invited review: transitioning from milk to solid feed in dairy heifers. *J. Dairy Sci.* 99(2), 885–902. doi:10.3168/jds.2015-9975.

Khiaosa-Ard, R. and Zebeli, Q. 2018. Diet-induced inflammation: from gut to metabolic organs and the consequences for the health and longevity of ruminants. *Res. Vet. Sci.* 120, 17–27. doi:10.1016/j.rvsc.2018.08.005.

Kleen, J. L., Hooijer, G. A., Rehage, J. and Noordhuizen, J. P. T. M. 2003. Subacute ruminal acidosis (SARA): a review. *J. Vet. Med. A* 50(8), 406–14. doi:10.1046/j.1439-0442.2003.00569.x.

Klostermann, K., Crispie, F., Flynn, J., Ross, R. P., Hill, C. and Meaney, W. 2008. Intramammary infusion of a live culture of *Lactococcus lactis* for treatment of bovine mastitis: comparison with antibiotic treatment in field trials. *J. Dairy Res.* 75(3), 365–73. doi:10.1017/S0022029908003373.

Kumprechtová, D., Illek, J., Julien, C., Homolka, P., Jančík, F. and Auclair, E. 2019. Effect of live yeast (*Saccharomyces cerevisiae*) supplementation on rumen fermentation and metabolic profile of dairy cows in early lactation. *J. Anim. Physiol. Anim. Nutr.* 103(2), 447–55. doi:10.1111/jpn.13048.

La Fata, G., Weber, P. and Mohajeri, M. H. 2018. Probiotics and the gut immune system: indirect regulation. *Probiotics Antimicrob. Proteins* 10(1), 11–21. doi:10.1007/s12602-017-9322-6.

Langford, F. M., Weary, D. M. and Fisher, L. 2003. Antibiotic resistance in gut bacteria from dairy calves: a dose response to the level of antibiotics fed in milk. *J. Dairy Sci.* 86(12), 3963–6. doi:10.3168/jds.S0022-0302(03)74006-5.

Lebeer, S., Bron, P. A., Marco, M. L., Van Pijkeren, J.-P., O'Connell Motherway, M., Hill, C., Pot, B., Roos, S. and Klaenhammer, T. 2018. Identification of probiotic effector molecules: present state and future perspectives. *Curr. Opin. Biotechnol.* 49, 217–23. doi:10.1016/j.copbio.2017.10.007.

Lema, M., Williams, L. and Rao, D. R. 2001. Reduction of fecal shedding of enterohemorrhagic *Escherichia coli* O157:H7 in lambs by feeding microbial feed supplement. *Small Rumin. Res.* 39(1), 31–9. doi:10.1016/s0921-4488(00)00168-1.

Lettat, A., Nozière, P., Silberberg, M., Morgavi, D. P., Berger, C. and Martin, C. 2010. Experimental feed induction of ruminal lactic, propionic, or butyric acidosis in sheep. *J. Anim. Sci.* 88(9), 3041–46. doi:10.2527/jas.2010-2926.

Lettat, A., Nozière, P., Silberberg, M., Morgavi, D. P., Berger, C. and Martin, C. 2012. Rumen microbial and fermentation characteristics are affected differently by bacterial probiotic supplementation during induced lactic and subacute acidosis in sheep. *BMC Microbiol.* 12, 142. doi:10.1186/1471-2180-12-142.

Li, Z., You, Q., Ossa, F., Mead, P., Quinton, M. and Karrow, N. A. 2016. Assessment of yeast *Saccharomyces cerevisiae* component binding to *Mycobacterium avium* subspecies paratuberculosis using bovine epithelial cells. *BMC Vet. Res.* 12, 42. doi:10.1186/s12917-016-0665-0.

Li, Z., Kang, H., You, Q., Ossa, F., Mead, P., Quinton, M. and Karrow, N. A. 2018. *In vitro* bioassessment of the immunomodulatory activity of *Saccharomyces cerevisiae* components using bovine macrophages and *Mycobacterium avium* ssp. paratuberculosis. *J. Dairy Sci.* 101(7), 6271–86. doi:10.3168/jds.2017-13805.

Lyte, M. 2011. Probiotics function mechanistically as delivery vehicles for neuroactive compounds: microbial endocrinology in the design and use of probiotics. *Bioessays* 33(8), 574–81. doi:10.1002/bies.201100024.

Ma, T., Suzuki, Y. and Guan, L. L. 2018. Dissect the mode of action of probiotics in affecting host-microbial interactions and immunity in food producing animals. *Vet. Immunol. Immunopathol.* 205, 35–48. doi:10.1016/j.vetimm.2018.10.004.

Malmuthuge, N., Li, M., Fries, P., Griebel, P. J. and Guan, L. L. 2012. Regional and age dependent changes in gene expression of toll-like receptors and key antimicrobial defence molecules throughout the gastrointestinal tract of dairy calves. *Vet. Immunol. Immunopathol.* 146(1), 18–26. doi:10.1016/j.vetimm.2012.01.010.

Malmuthuge, N., Chen, Y., Liang, G., Goonewardene, L. A. and Guan, L. 2015. Heat-treated colostrum feeding promotes beneficial bacteria colonization in the small intestine of neonatal calves. *J. Dairy Sci.* 98(11), 8044–53. doi:10.3168/jds.2015-9607.

Marden, J. P., Julien, C., Monteils, V., Auclair, E., Moncoulon, R. and Bayourthe, C. 2008. How does live yeast differ from sodium bicarbonate to stabilize ruminal pH in high-yielding dairy cows? *J. Dairy Sci.* 91(9), 3528–35. doi:10.3168/jds.2007-0889.

McFarland, L. V. 2010. Systematic review and meta-analysis of *Saccharomyces boulardii* in adult patients. *World J. Gastroenterol.* 16(18), 2202–22. doi:10.3748/wjg.v16.i18.2202.

McGovern, E., Kenny, D. A., McCabe, M. S., Fitzsimons, C., McGee, M., Kelly, A. K. and Waters, S. M. 2018. 16S rRNA sequencing reveals relationship Between potent cellulolytic genera and feed efficiency in the rumen of bulls. *Front. Microbiol.* 9, 1842. doi:10.3389/fmicb.2018.01842.

McGuirk, S. M. 2008. Disease management of dairy calves and heifers. *Vet. Clin. North Am. Food Anim. Pract.* 24, 139–53. doi:10.1016/j.cvfa.2007.10.003.

McGuirk, S. M. and Collins, M. 2004. Managing the production, storage, and delivery of colostrum. *Vet. Clin. North Am. Food Anim. Pract.* 20, 593–603. doi:10.1016/j.cvfa.2004.06.005.

McSweeney, C. S., Dulieu, A., Katayama, Y. and Lowry, J. B. 1994. Solubilization of lignin by the ruminal anaerobic fungus *Neocallimastix patriciarum*. *Appl. Environ. Microbiol.* 60(8), 2985–89.

McSweeney, C. S., Dulieu, A. and Bunch, R. 1998. *Butyrivibrio* spp. and other xylanolytic microorganisms from the rumen have *Cinnamoyl esterase* activity. *Anaerobe* 4(1), 57–65. doi:10.1006/anae.1997.0130.

Meale, S. J., Li, S., Azevedo, P., Derakhshani, H., Plaizier, J. C., Khafipour, E. and Steele, M. A. 2016. Development of ruminal and fecal microbiomes are affected by weaning but not weaning strategy in dairy calves. *Front. Microbiol.* 7, 582. doi:10.3389/fmicb.2016.00582.

Meale, S. J., Chaucheyras-Durand, F., Berends, H., Guan, L. L. and Steele, M. A. 2017. From pre- to postweaning: transformation of the young calf's gastrointestinal tract. *J. Dairy Sci.* 100(7), 5984–95. doi:10.3168/jds.2016-12474.

Minuti, A., Palladino, A., Khan, M. J., Alqarni, S., Agrawal, A., Piccioli-Capelli, F., Hidalgo, F., Cardoso, F. C., Trevisi, E. and Loor, J. J. 2015. Abundance of ruminal bacteria, epithelial gene expression, and systemic biomarkers of metabolism and inflammation are altered during the peripartal period in dairy cows. *J. Dairy Sci.* 98(12), 8940–51. doi:10.3168/jds.2015-9722.

Mizrahi, I. and Jami, E. 2018. Review: The compositional variation of the rumen microbiome and its effect on host performance and methane emission. *Animal* 12(s2), s220–32. doi:10.1017/S1751731118001957.

Mokhber-Dezfouli, M. R., Tajik, P., Bolourchi, M. and Mahmoudzadeh, H. 2007. Effects of probiotics supplementation in daily milk intake of newborn calves on body weight gain, body height, diarrhea occurrence and health condition. *Pak. J. Biol. Sci.* 10(18), 3136–40. doi:10.3923/pjbs.2007.3136.3140.

Morelli, L. and Capurso, L. 2012. FAO/WHO guidelines on probiotics: 10 years later. *J. Clin. Gastroenterol.* 46(Suppl.), S1–2. doi:10.1097/MCG.0b013e318269fdd5.

Mosoni, P., Chaucheyras-Durand, F., Béra-Maillet, C. and Forano, E. 2007. Quantification by real-time PCR of cellulolytic bacteria in the rumen of sheep after supplementation of a forage diet with readily fermentable carbohydrates: effect of a yeast additive. *J. Appl. Microbiol.* 103(6), 2676–85. doi:10.1111/j.1365-2672.2007.03517.x.

Mõtus, K., Viltrop, A. and Emanuelson, U. 2018. Reasons and risk factors for beef calf and youngstock on-farm mortality in extensive cow-calf herds. *Animal* 12(9), 1958–66. doi:10.1017/S1751731117003548.

Muya, M. C., Nherera, F. V., Miller, K. A., Aperce, C. C., Moshidi, P. M. and Erasmus, L. J. 2015. Effect of *Megasphaera elsdenii* NCIMB 41125 dosing on rumen development, volatile fatty acid production and blood β-hydroxybutyrate in neonatal dairy calves. *J. Anim. Physiol. Anim. Nutr.* 99(5), 913–8. doi:10.1111/jpn.12306.

Nasiri, A. H., Towhidi, A., Shakeri, M., Zhandi, M., Dehghan-Banadaky, M. and Colazo, M. G. 2018. Effects of live yeast dietary supplementation on hormonal profile, ovarian follicular dynamics, and reproductive performance in dairy cows exposed to high ambient temperature. *Theriogenology* 122, 41–6. doi:10.1016/j.theriogenology.2018.08.013.

Nollet, L., Demeyer, D. and Verstraete, W. 1997. Effect of 2-bromoethanesulfonic acid and Peptostreptococcus productus ATCC 35244 addition on stimulation of reductive

acetogenesis in the ruminal ecosystem by selective inhibition of methanogenesis. *Appl. Environ. Microbiol.* 63(1), 194–200.

Owens, F. N., Secrist, D. S., Hill, W. J. and Gill, D. R. 1998. Acidosis in cattle: a review. *J. Anim. Sci.* 76(1), 275–86. doi:10.2527/1998.761275x.

Pellegrino, M., Berardo, N., Giraudo, J., Nader-Macías, M. E. F. and Bogni, C. 2017. *Bovine mastitis* prevention: humoral and cellular response of dairy cows inoculated with lactic acid bacteria at the dry-off period. *Benef. Microbes* 8(4), 589–96. doi:10.3920/BM2016.0194.

Petri, R. M., Schwaiger, T., Penner, G. B., Beauchemin, K. A., Forster, R. J., McKinnon, J. J. and McAllister, T. A. 2013a. Changes in the rumen epimural bacterial diversity of beef cattle as affected by diet and induced ruminal acidosis. *Appl. Env. Microbiol.* 79, 3744–55. doi:10.1128/AEM.03983-12.

Petri, R. M., Schwaiger, T., Penner, G. B., Beauchemin, K. A., Forster, R. J., McKinnon, J. J. and McAllister, T. A. 2013b. Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge. *PLoS ONE* 8(12), e83424. doi:10.1371/journal.pone.0083424.

Petri, R. M., Kleefisch, M. T., Metzler-Zebeli, B. U., Zebeli, Q. and Klevenhusen, F. 2018. Changes in the rumen epithelial microbiota of cattle and host gene expression in response to alterations in dietary carbohydrate composition. *Appl. Environ. Microbiol.* 84(12). doi:10.1128/AEM.00384-18.

Philippeau, C., Lettat, A., Martin, C., Silberberg, M., Morgavi, D. P., Ferlay, A., Berger, C. and Nozière, P. 2017. Effects of bacterial direct-fed microbials on ruminal characteristics, methane emission, and milk fatty acid composition in cows fed high- or low-starch diets. *J. Dairy Sci.* 100(4), 2637–50. doi:10.3168/jds.2016-11663.

Pinloche, E., McEwan, N., Marden, J. P., Bayourthe, C., Auclair, E. and Newbold, C. J. 2013. The effects of a probiotic yeast on the bacterial diversity and population structure in the rumen of cattle. *PLoS ONE* 8(7), e67824. doi:10.1371/journal.pone.0067824.

Pitta, D. W., Indugu, N., Baker, L., Vecchiarelli, B. and Attwood, G. 2018. Symposium review: understanding diet-microbe interactions to enhance productivity of dairy cows. *J. Dairy Sci.* 101(8), 7661–79. doi:10.3168/jds.2017-13858.

Plaizier, J. C., Khafipour, E., Li, S., Gozho, G. N. and Krause, D. O. 2012. Subacute ruminal acidosis (SARA), endotoxins and health consequences. *Anim. Feed Sci. Technol.* 172, 9–21. doi:10.1016/j.anifeedsci.2011.12.004.

Plaizier, J. C., Danesh Mesgaran, M., Derakhshani, H., Golder, H., Khafipour, E., Kleen, J. L., Lean, I., Loor, J., Penner, G. and Zebeli, Q. 2018. Review: enhancing gastrointestinal health in dairy cows. *Animal* 12(s2), s399–418. doi:10.1017/S1751731118001921.

Pothoulakis, C. 2009. Review article: anti-inflammatory mechanisms of action of Saccharomyces boulardii. *Aliment. Pharmacol. Ther.* 30(8), 826–33, Article: Anti-Inflammatory. doi:10.1111/j.1365-2036.2009.04102.x.

Priestley, D., Bittar, J. H., Ibarbia, L., Risco, C. A. and Galvão, K. N. 2013. Effect of feeding maternal colostrum or plasma-derived or colostrum-derived colostrum replacer on passive transfer of immunity, health, and performance of preweaning heifer calves. *J. Dairy Sci.* 96(5), 3247–56. doi:10.3168/jds.2012-6339.

Raabis, S., Li, W. and Cersosimo, L. 2019. Effects and immune responses of probiotic treatment in ruminants. *Vet. Immunol. Immunopathol.* 208, 58–66. doi:10.1016/j.vetimm.2018.12.006.

Rainard, P. and Foucras, G. 2018. A critical appraisal of probiotics for mastitis control. *Front. Vet. Sci.* 5, 251. doi:10.3389/fvets.2018.00251.

Ramatla, T., Ngoma, L., Adetunji, M. and Mwanza, M. 2017. Evaluation of antibiotic residues in raw meat using different analytical methods. *Antibiot. Basel Switz* 6(4). doi:10.3390/antibiotics6040034.

Reis, L. F., Sousa, R. S., Oliveira, F. L. C., Rodrigues, F. A. M. L., Araújo, C. A. S. C., Meira-Júnior, E. B. S., Barrêto-Júnior, R. A., Mori, C. S., Minervino, A. H. H. and Ortolani, E. L. 2018. Comparative assessment of probiotics and monensin in the prophylaxis of acute ruminal lactic acidosis in sheep. *BMC Vet. Res.* 14(1), 9. doi:10.1186/s12917-017-1264-4.

Renaud, D. L., Kelton, D. F., Weese, J. S., Noble, C. and Duffield, T. F. 2019. Evaluation of a multispecies probiotic as a supportive treatment for diarrhea in dairy calves: a randomized clinical trial. *J. Dairy Sci.* 102(5), 4498–505 doi:10.3168/jds.2018-15793.

Rey, M., Enjalbert, F. and Monteils, V. 2012. Establishment of ruminal enzyme activities and fermentation capacity in dairy calves from birth through weaning. *J. Dairy Sci.* 95(3), 1500–12. doi:10.3168/jds.2011-4902.

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O. and Monteils, V. 2014. Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. *J. Appl. Microbiol.* 116(2), 245–57. doi:10.1111/jam.12405.

Reynolds, J. D. and Morris, B. 1984. The effect of antigen on the development of Peyer's patches in sheep. *Eur. J. Immunol.* 14(1), 1–6. doi:10.1002/eji.1830140102.

Riaz, Q. U. A. and Masud, T. 2013. Recent trends and applications of encapsulating materials for probiotic stability. *Crit. Rev. Food Sci. Nutr.* 53(3), 231–44. doi:10.1080/10408398.2010.524953.

Rigobelo, E. E. C., Karapetkov, N., Maestá, S. A., Avila, F. A. and McIntosh, D. 2015. Use of probiotics to reduce faecal shedding of Shiga toxin-producing *Escherichia coli* in sheep. *Benef. Microbes* 6(1), 53–60. doi:10.3920/BM2013.0094.

Roth, B. A., Keil, N. M., Gygax, L. and Hillmann, E. 2009. Influence of weaning method on health status and rumen development in dairy calves. *J. Dairy Sci.* 92(2), 645–56. doi:10.3168/jds.2008-1153.

Russell, J. B. and Wilson, D. B. 1996. Why are ruminal cellulolytic bacteria unable to digest cellulose at low pH? *J. Dairy Sci.* 79(8), 1503–9. doi:10.3168/jds.S0022-0302(96)76510-4.

Sadet, S., Martin, C., Meunier, B. and Morgavi, D. P. 2007. PCR-DGGE analysis reveals a distinct diversity in the bacterial population attached to the rumen epithelium. *Animal* 1(7), 939–44. doi:10.1017/S1751731107000304.

Salvati, G. G. S., Morais Junior, N. N., Melo, A. C. S., Vilela, R. R., Cardoso, F. F., Aronovich, M., Pereira, R. A. and Pereira, M. N. 2015. Response of lactating cows to live yeast supplementation during summer. *J. Dairy Sci.* 98(6), 4062–73. doi:10.3168/jds.2014-9215.

Schiavone, M., Sieczkowski, N., Castex, M., Dague, E. and Marie François, J. 2015. Effects of the strain background and autolysis process on the composition and biophysical properties of the cell wall from two different industrial yeasts. *FEMS Yeast Res.* 15(2). doi:10.1093/femsyr/fou012.

Schiavone, M., Déjean, S., Sieczkowski, N., Castex, M., Dague, E. and François, J. M. 2017. Integration of biochemical, biophysical and transcriptomics data for investigating the structural and nanomechanical properties of the yeast cell wall. *Front. Microbiol.* 8, 1806. doi:10.3389/fmicb.2017.01806.

Schofield, B. J., Lachner, N., Le, O. T., McNeill, D. M., Dart, P., Ouwerkerk, D., Hugenholtz, P. and Klieve, A. V. 2018. Beneficial changes in rumen bacterial community profile in

sheep and dairy calves as a result of feeding the probiotic *Bacillus amyloliquefaciens* H57. *J. Appl. Microbiol.* 124(3), 855–66. doi:10.1111/jam.13688.

Segura, A., Bertoni, M., Auffret, P., Klopp, C., Bouchez, O., Genthon, C., Durand, A., Bertin, Y. and Forano, E. 2018. Transcriptomic analysis reveals specific metabolic pathways of enterohemorrhagic Escherichia coli O157:H7 in bovine digestive contents. *BMC Genomics* 19(1), 766. doi:10.1186/s12864-018-5167-y.

Seymour, E. H., Jones, G. M. and McGilliard, M. L. 1988. Persistence of residues in milk following antibiotic treatment of dairy cattle. *J. Dairy Sci.* 71(8), 2292–6. doi:10.3168/jds.S0022-0302(88)79806-9.

Shabat, S. K. B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., White, B. A., Shterzer, N. and Mizrahi, I. 2016. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J.* 10(12), 2958–72. doi:10.1038/ismej.2016.62.

Signorini, M. L., Soto, L. P., Zbrun, M. V., Sequeira, G. J., Rosmini, M. R. and Frizzo, L. S. 2012. Impact of probiotic administration on the health and fecal microbiota of young calves: A meta-analysis of randomized controlled trials of lactic acid bacteria. *Res. Vet. Sci.* 93(1), 250–8. doi:10.1016/j.rvsc.2011.05.001.

Silanikove, N. 2000. Effects of heat stress on the welfare of extensively managed domestic ruminants. *Livest. Prod. Sci.* 67(1–2), 1–18. doi:10.1016/S0301-6226(00)00162-7.

Silberberg, M., Chaucheyras-Durand, F., Commun, L., Mialon, M. M., Monteils, V., Mosoni, P., Morgavi, D. P. and Martin, C. 2013. Repeated acidosis challenges and live yeast supplementation shape rumen microbiota and fermentations and modulate inflammatory status in sheep. *Animal* 7(12), 1910–20. doi:10.1017/S1751731113001705.

Soberon, F. and Van Amburgh, M. E. 2013. Lactation Biology Symposium: the effect of nutrient intake from milk or milk replacer of preweaned dairy calves on lactation milk yield as adults: a meta-analysis of current data. *J. Anim. Sci.* 91(2), 706–12. doi:10.2527/jas.2012-5834.

Soto, L. P., Frizzo, L. S., Signorini, M. L., Zbrun, M. V., Lavari, L., Bertozzi, E., Sequeira, G. and Rosmini, M. 2015. Faecal culturable microbiota, growth and clinical parameters of calves supplemented with lactic acid bacteria and lactose prior and during experimental infection with *Salmonella* Dublin DSPV 595T. *Arch. Med. Vet.* 47(2), 237–44. doi:10.4067/S0301-732X2015000200017.

Soto, L. P., Astesana, D. M., Zbrun, M. V., Blajman, J. E., Salvetti, N. R., Berisvil, A. P., Rosmini, M. R., Signorini, M. L. and Frizzo, L. S. 2016. Probiotic effect on calves infected with *Salmonella* Dublin: haematological parameters and serum biochemical profile. *Benef. Microbes* 7(1), 23–33. doi:10.3920/BM2014.0176.

Sousa, D. O., Oliveira, C. A., Velasquez, A. V., Souza, J. M., Chevaux, E., Mari, L. J. and Silva, L. F. P. 2018. Live yeast supplementation improves rumen fibre degradation in cattle grazing tropical pastures throughout the year. *Anim. Feed Sci. Technol.* 236, 149–58. doi:10.1016/j.anifeedsci.2017.12.015.

Souza, R. F. S., Rault, L., Seyffert, N., Azevedo, V., Le Loir, Y. and Even, S. 2018. *Lactobacillus casei* BL23 modulates the innate immune response in *Staphylococcus aureus*-stimulated bovine mammary epithelial cells. *Benef. Microbes* 9(6), 985–95. doi:10.3920/BM2018.0010.

Steele, M. A., Kroom, J., Kahler, M., AlZahal, O., Hook, S. E., Plaizier, K. and McBride, B. W. 2011. Bovine rumen epithelium undergoes rapid structural adaptations during grain-induced subacute ruminal acidosis. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 300(6), R1515–23. doi:10.1152/ajpregu.00120.2010.

Steele, M. A., Penner, G. B., Chaucheyras-Durand, F. and Guan, L. L. 2016. Development and physiology of the rumen and the lower gut: targets for improving gut health. *J. Dairy Sci.* 99(6), 4955–66. doi:10.3168/jds.2015-10351.

Stelwagen, K., Carpenter, E., Haigh, B., Hodgkinson, A. and Wheeler, T. T. 2009. Immune components of bovine colostrum and milk. *J. Anim. Sci.* 87(13 Suppl.), 3–9. doi:10.2527/jas.2008-1377.

Stier, H. and Bischoff, S. C. 2016. Influence of *Saccharomyces boulardii* CNCM I-745 on the gut-associated immune system. *Clin. Expl. Gastroenterol.* 9, 269–79. doi:10.2147/CEG.S111003.

Sullivan, M. L. and Bradford, B. J. 2011. Viable cell yield from active dry yeast products and effects of storage temperature and diluent on yeast cell viability. *J. Dairy Sci.* 94(1), 526–31. doi:10.3168/jds.2010-3553.

Sun, X., Yuan, X., Chen, L., Wang, T., Wang, Z., Sun, G., Li, X., Li, X. and Liu, G. 2017. Histamine induces bovine rumen epithelial cell inflammatory response via NF-κB pathway. *Cell. Physiol. Biochem.* 42(3), 1109–19. doi:10.1159/000478765.

Tabe, E. S., Oloya, J., Doetkott, D. K., Bauer, M. L., Gibbs, P. S. and Khaitsa, M. L. 2008. Comparative effect of direct-fed microbials on fecal shedding of *Escherichia coli* O157:H7 and *Salmonella* in naturally infected feedlot cattle. *J. Food Prot.* 71(3), 539–44. doi:10.4315/0362-028X-71.3.539.

Terré, M., Maynou, G., Bach, A. and Gauthier, M. 2015. Effect of *Saccharomyces cerevisiae* CNCM I-1077 supplementation on performance and rumen microbiota of dairy calves. *Prof. Anim. Sci.* 31(2), 153–8. doi:10.15232/pas.2014-01384.

Tkalcic, S., Zhao, T., Harmon, B. G., Doyle, M. P., Brown, C. A. and Zhao, P. 2003. Fecal shedding of enterohemorrhagic *Escherichia coli* in weaned calves following treatment with probiotic *Escherichia coli*. *J. Food Prot.* 66(7), 1184–9. doi:10.4315/0362-028x-66.7.1184.

Trevisi, E. and Minuti, A. 2018. Assessment of the innate immune response in the periparturient cow. *Res. Vet. Sci.* 116, 47–54. doi:10.1016/j.rvsc.2017.12.001.

Trevisi, E., Riva, F., Filipe, J. F. S., Massara, M., Minuti, A., Bani, P. and Amadori, M. 2018. Innate immune responses to metabolic stress can be detected in rumen fluids. *Res. Vet. Sci.* 117, 65–73. doi:10.1016/j.rvsc.2017.11.008.

USDA. 2014a. Dairy 2014: health and management practices on U.S dairy operations.

USDA 2014b. Dairy 2014: dairy cattle management practices in the United States.

Uyeno, Y., Sekiguchi, Y., Tajima, K., Takenaka, A., Kurihara, M. and Kamagata, Y. 2010. An rRNA-based analysis for evaluating the effect of heat stress on the rumen microbial composition of Holstein heifers. *Anaerobe* 16(1), 27–33. doi:10.1016/j.anaerobe.2009.04.006.

Uyeno, Y., Akiyama, K., Hasunuma, T., Yamamoto, H., Yokokawa, H., Yamaguchi, T., Kawashima, K., Itoh, M., Kushibiki, S. and Hirako, M. 2017. Effects of supplementing an active dry yeast product on rumen microbial community composition and on subsequent rumen fermentation of lactating cows in the mid-to-late lactation period. *Anim. Sci. J.* 88(1), 119–24. doi:10.1111/asj.12612.

Villageliú, D. and Lyte, M. 2018. Dopamine production in *Enterococcus faecium*: a microbial endocrinology-based mechanism for the selection of probiotics based on neurochemical-producing potential. *PLoS ONE* 13(11), e0207038. doi:10.1371/journal.pone.0207038.

Villena, J., Aso, H., Rutten, V. P. M. G., Takahashi, H., van Eden, W. and Kitazawa, H. 2018. Immunobiotics for the bovine host: their interaction with intestinal epithelial

cells and their effect on antiviral immunity. *Front. Immunol.* 9, 326. doi:10.3389/fimmu.2018.00326.

Villot, C., Meunier, B., Bodin, J., Martin, C. and Silberberg, M. 2018. Relative reticulo-rumen pH indicators for subacute ruminal acidosis detection in dairy cows. *Animal* 12(3), 481–90. doi:10.1017/S1751731117001677.

Vipham, J. L., Loneragan, G. H., Guillen, L. M., Brooks, J. C., Johnson, B. J., Pond, A., Pond, N. and Brashears, M. M. 2015. Reduced burden of *Salmonella enterica* in bovine subiliac lymph nodes associated with administration of a direct-fed microbial. *Zoonoses Public Health* 62(8), 599–608. doi:10.1111/zph.12187.

von Buenau, R., Jaekel, L., Schubotz, E., Schwarz, S., Stroff, T. and Krueger, M. 2005. *Escherichia coli* strain Nissle 1917: significant reduction of neonatal calf diarrhea. *J. Dairy Sci.* 88(1), 317–23. doi:10.3168/jds.S0022-0302(05)72690-4.

Vyas, D., McGeough, E. J., Mohammed, R., McGinn, S. M., McAllister, T. A. and Beauchemin, K. A. 2014. Effects of Propionibacterium strains on ruminal fermentation, nutrient digestibility and methane emissions in beef cattle fed a corn grain finishing diet. *Animal* 8(11), 1807–15. doi:10.1017/S1751731114001657.

Wallis, J. K., Krömker, V. and Paduch, J. H. 2018. Biofilm formation and adhesion to bovine udder epithelium of potentially probiotic lactic acid bacteria. *AIMS Microbiol.* 4(2), 209–24. doi:10.3934/microbiol.2018.2.209.

Wallis, J. K., Krömker, V. and Paduch, J. H. 2019. Biofilm challenge: lactic acid bacteria isolated from bovine udders versus staphylococci. *Foods* 8(2). doi:10.3390/foods8020079.

Wang, D. S., Zhang, R. Y., Zhu, W. Y. and Mao, S. Y. 2013. Effects of subacute ruminal acidosis challenges on fermentation and biogenic amines in the rumen of dairy cows. *Livest. Sci.* 155(2–3), 262–72. doi:10.1016/j.livsci.2013.05.026.

Wehnes, C. A., Novak, K. N., Patskevich, V., Shields, D. R., Coalson, J. A., Smith, A. H., Davis, M. E. and Rehberger, T. G. 2009. Benefits of supplementation of an electrolyte scour treatment with a Bacillus-based direct fed microbial for calves. *Probiotics Antimicrob. Proteins* 1(1), 36–44. doi:10.1007/s12602-008-9004-5.

Wisener, L. V., Sargeant, J. M., O'Connor, A. M., Faires, M. C. and Glass-Kaastra, S. K. 2015. The use of direct-fed microbials to reduce shedding of *Escherichia coli* O157 in beef cattle: a systematic review and meta-analysis. *Zoonoses Public Health* 62(2), 75–89. doi:10.1111/zph.12112.

Yáñez-Ruiz, D. R., Abecia, L. and Newbold, C. J. 2015. Manipulating rumen microbiome and fermentation through interventions during early life: a review. *Front. Microbiol.* 6, 1133. doi:10.3389/fmicb.2015.01133.

Yeoman, C. J., Ishaq, S. L., Bichi, E., Olivo, S. K., Lowe, J. and Aldridge, B. M. 2018. Biogeographical differences in the influence of maternal microbial sources on the early successional development of the bovine neonatal gastrointestinal tract. *Sci. Rep.* 8(1), 3197. doi:10.1038/s41598-018-21440-8.

Yohe, T. T., Enger, B. D., Wang, L., Tucker, H. L. M., Ceh, C. A., Parsons, C. L. M., Yu, Z. and Daniels, K. M. 2018. Short communication: does early-life administration of a *Megasphaera elsdenii* probiotic affect long-term establishment of the organism in the rumen and alter rumen metabolism in the dairy calf? *J. Dairy Sci.* 101(2), 1747–51. doi:10.3168/jds.2017-12551.

Yuan, K., Mendonça, L. G. D., Hulbert, L. E., Mamedova, L. K., Muckey, M. B., Shen, Y., Elrod, C. C. and Bradford, B. J. 2015. Yeast product supplementation modulated humoral and mucosal immunity and uterine inflammatory signals in transition dairy cows. *J. Dairy Sci.* 98(5), 3236–46. doi:10.3168/jds.2014-8469.

Zebeli, Q., Terrill, S. J., Mazzolari, A., Dunn, S. M., Yang, W. Z. and Ametaj, B. N. 2012. Intraruminal administration of *Megasphaera elsdenii* modulated rumen fermentation profile in mid-lactation dairy cows. *J. Dairy Res.* 79(1), 16–25. doi:10.1017/S0022029911000707.

Zebeli, Q., Ghareeb, K., Humer, E., Metzler-Zebeli, B. U. and Besenfelder, U. 2015. Nutrition, rumen health and inflammation in the transition period and their role on overall health and fertility in dairy cows. *Res. Vet. Sci.* 103, 126–36. doi:10.1016/j.rvsc.2015.09.020.

Zhao, T., Tkalcic, S., Doyle, M. P., Harmon, B. G., Brown, C. A. and Zhao, P. 2003. Pathogenicity of enterohemorrhagic *Escherichia coli* in neonatal calves and evaluation of fecal shedding by treatment with probiotic *Escherichia coli*. *J. Food Prot.* 66(6), 924–30. doi:10.4315/0362-028x-66.6.924.

Zhu, Z., Noel, S. J., Difford, G. F., Al-Soud, W. A., Brejnrod, A., Sørensen, S. J., Lassen, J., Løvendahl, P. and Højberg, O. 2017. Community structure of the metabolically active rumen bacterial and archaeal communities of dairy cows over the transition period. *PLoS ONE* 12(11), e0187858. doi:10.1371/journal.pone.0187858.

Zhu, Z., Kristensen, L., Difford, G. F., Poulsen, M., Noel, S. J., Abu Al-Soud, W., Sørensen, S. J., Lassen, J., Løvendahl, P. and Højberg, O. 2018. Changes in rumen bacterial and archaeal communities over the transition period in primiparous Holstein dairy cows. *J. Dairy Sci.* 101(11), 9847–62. doi:10.3168/jds.2017-14366.

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