Belchiolina Beatriz Fonseca
Heriberto Fernandez
Daise Aparecida Rossi *Editors*

Campylobacter spp. and Related Organisms in Poultry

Pathogen-Host Interactions, Diagnosis and Epidemiology

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We dedicate this book

to our families and friends... because they are the raison for being of our existence and ongoing inspiration of life

to our students who have been ... they made us grow in the way of education

to our students will be ... they will allow us to move forward with them to meet the future

Foreword

This book fills a gap in the literature about *Campylobacter* spp. and related organisms in poultry, taking a modern approach to the relationship between *Campylobacter* and poultry, the poultry industry, and public health. Because this book on *Campylobacter* in poultry originated in Latin America, the data herein are specific to this geographic region.

The study and understanding of *Campylobacter*'s relationship to poultry are important because chickens are large reservoirs of these microorganisms. In addition, poultry are responsible for several enteric disorders in humans caused by *Campylobacter*.

This book is divided didactically into 11 chapters. The information is presented in a logical sequence to aid in the understanding of *Campylobacter* spp. in poultry. This is a modern presentation of a didactic work, aiming to provide technical knowledge to students and researchers.

The main themes of *Campylobacter* in poultry are covered in this book, including the presentation of the microorganism, isolation and identification, colonization of *Campylobacter* in poultry, and its effect on immune response. The traditional approach to *Campylobacter* being a commensal or a pathogen is also discussed, as well as the epidemiology of *Campylobacter* in farms, its control in commercial poultry production, and its ability to survive and multiply in poultry industry. Other important aspects of *Campylobacter* are also covered, such as antimicrobial resistance and incidence of other species of *Campylobacter* (non-jejuni/coli *Campylobacter*) and other related genera, such as *Arcobacter* and *Helicobacter*, in poultry.

All 23 experts who collaborated on this book have experience in their subjects of expertise, allowing the inclusion of their personal knowledge that has not been formally published elsewhere and thus further enhancing the work. Another great feature of this book is that it brings together experts from three continents, including the countries of Brazil, Chile, Costa Rica, the United States, South Africa, the United Kingdom and Germany.

The authors and editors—Profs. Belchiolina Beatriz Fonseca, Daise Aparecida Rossi, and Heriberto Fernandez—made use of their experience. With more than two dozen researchers working around the same theme, they gave us a great work. I am sure that this book will contribute significantly to the literature and be useful to all who need an update on *Campylobacter* in poultry.

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About the Authors

Dr. Belchiolina Beatriz Fonseca is Professor of Avian Disease and Poultry Production in Faculty of Veterinary Medicine at Universidade Federal de Uberlândia, Minas Gerais, Brasil. Before her academic work she has worked in the field as a Veterinary Medical with chickens and broiler breeders. She has started research about *Campylobacter* in poultry since 2005 when studied in Chile with Dr. Heriberto Fernandéz. Thenceforward, she did her research about *Campylobacter* with Dr. Daise Rossi in poultry. The main focus of the author is epidemiology and the relationship of commensal or disease between and campylobacter in poultry. She is member of the Latin American Network of Researchers in *Campylobacteraceae* (Relacampy).

Dr. Heriberto Fernández is Full Professor of Clinical Microbiology and former-director of the Institute of Clinical Microbiology, Faculty of Medicine, Universidad Austral de Chile.

During the initial years of his career he involved in microbiological diagnosis of zoonotic bacterial agents. Since 1980 their research lines turned towards *Campylobacter* and related microorganisms and their epidemiological relationships among humans, animals, the environment and food production. For the past 35 years he conducted many research projects in this field. As a professor he spent much of his time to teach about *Campylobacter*, not only to undergraduate and graduate students and professionals of his country, but also to others in many countries of Latin America. He was also invited as a speaker at many conferences and scientific meetings in almost all South American countries and some in Central America. He was trainer in

different courses in Argentina and Mexico of the WHO SalmSurvNet (now renamed GFN).

Dr. Fernández was invited to be member of the WHO Experts Committee on *Campylobacter*, the Chilean Agency for Food Safety and the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR), belonging to the latter until June 2014.

In 2014, he obtained a grant from the Ecuadorian Secretariat for Higher Education, Science, Technology and Innovation to develop a Prometeo Project at the Technical University of Loja to build academic capacities on *Campylobacter* and develop research projects in this field. At this time he created the Latin American Network of Researchers in *Campylobacteraceae* (www.relacampy.cl).

Dr. Daise Aparecida Rossi is Professor of Microbiology, zoonotic disease and hygiene and vigilance in food at Faculty of Veterinary Medicine in the Universidade Federal de Uberlândia, Minas Gerais, Brasil. She has experience in veterinary microbiology, mainly with food microbiology and epidemiology, with expertise in foodborne zoonoses and food safety. She has done several important works on *Campylobacter* and *Salmonella* general food as chicken, milk, cheese, and other meat. Her research team also developed projects in Brazil about the prevalence of *Campylobacter* in animal products, their virulence, resistance to antimicrobials, and capacity of biofilm formation. She is member of the Latin American Network of Researchers in *Campylobacteraceae* (Relacampy) and SSAN-UNASUL (Program of the development of strategies of Socio-educational or Social Technology character on Sovereignty, Food and Nutritional Security).

Chapter 1
About *Campylobacter* spp.

Patrícia Giovana Hoepers, Gustavo Medina, Daise Aparecida Rossi and Heriberto Fernandez

Abstract Members of the *Campylobacter* genus are Gram-negative, microaerophilic, small bacilli with polar flagellation. Bacterial movement has a typical corkscrew characteristic. There are many species associated with different diseases. This chapter describes the taxonomy, genetic structure, and general characteristics of the *Campylobacter* genus focusing on thermophilic species and/or those that colonize the intestinal tract of birds.

Keywords Taxonomy · Genetic structure · Thermophilic *Campylobacter*

1.1 Introduction

The genus *Campylobacter* was initially classified as *Vibrio* spp. due to their spiral morphology. The genus *Campylobacter* was first proposed by Sebald and Véron (1963) and included just two species, *Campylobacter fetus* and *C. bubulus*. However, most of the scientific community continued to refer to this bacteria as *Vibrio fetus* and *Vibrio bubulus* until further serological, biochemical, and DNA base composition analysis were made and *Campylobacter* was established as a distinct, recognizable genus (On and Harrington 2001).

The increased interest in *Campylobacter* took place after the study of Butzler et al. (1973) that indicated their high prevalence in human diarrhea. The understanding in growth characteristics and isolations methods resulted in 12 new species

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or subspecies were discovered in a range of different diseases and habitats from 1974 to 1988 (Vandamme and Groossens 1992). Since then, the number of *Campylobacter* species discovered has increased, at present the genus consists of 26 species, two provisional species, and nine subspecies (Kaakoush et al. 2015).

Campylobacter spp. are normal intestinal inhabitants of a wide variety of animals and avian species but frequently pathogens of humans. The incidence and prevalence of campylobacteriosis have increased in both developed and developing countries over the past 10 years. *Campylobacter jejuni* is the one of the most widespread infectious diseases agent of the last century (Kaakoush et al. 2015). Humans usually are infected through food or waterborne routes. In humans *C. jejuni* colonizes the intestinal epithelium and often causes a mild watery diarrhea to a severe, bloody diarrheal illness. The U.S. Food-Borne Diseases Active Surveillance Network (1996–2012) reports an annual incidence of 14.3 per 100,000 populations for *Campylobacter* infection (Gilliss et al. 2013). The estimated annual costs of campylobacteriosis are $1.7 billion in the United States (Batz et al. 2012). In the European Union states members, the incidence of *Campylobacter* infections range from 29.9 to 13,500 per 1,000,000 population in 2009 (Havelaar et al. 2013).

In addition to gastrointestinal infection, *Campylobacter* species also cause a range of clinical manifestations that occurs after an episode of enteritis, or a post-infectious immune disorder. These manifestations include Guillain-Barré syndrome, Miller Fisher syndrome, brain abscesses and meningitis, bacteremia, sepsis, endocarditis and myocarditis, reactive arthritis, and clinical manifestations that result in complications in the reproductive tract (Kaakoush et al. 2015). The Guillian-Barré syndrome, an acute demyelinating disease of the peripheral nervous system is the most important post-infectious complication of *C. jejuni* (Allos 1997).

Poultry products are considered the most significant source of human campylobacteriosis, with up to 80 % of fresh broiler meat contaminated at the retail sale (EFSA 2014). This contamination probably occurs during the evisceration stage of the slaughter process, when gut contents containing high concentration of *Campylobacter* spp. cell per gram in colonized birds contaminated carcasses on the production line (Nagel et al. 2013).

1.2 *Campylobacter* spp. Taxonomy

Campylobacter genus belongs to the Proteobacteria phylum that consists of over 200 genera and represents the largest and most diverse group of organisms and contains the majority of Gram-negative species. This phylum is divided in subdivisions: Alpha-, Beta-, Gamma-, Delta-, and Epsilonproteobacteria (Gupta 2000). *Campylobacter* spp. and *Helicobacter* spp. are the most commonly studied genera within the Epsilonproteobacteria group, both belonging to the order Campylobacterales (Gilbreath et al. 2011).

The genus *Helicobacter* spp. are Gram-negative, spiral-shaped microaerophiles, that can be separated in two main categories, gastric and nongastric *Helicobacter*

spp. *Helicobacter pylori* is a gastric species and represents the major pathogen within the genus (Blaser 1998). *H. pylori* colonizes half of the global population (Dunn et al. 1997). No environmental reservoirs have been discovered for *H. pylori*, the transmission is thought to be primarily at young age via person-to-person contact (Fiedorek et al. 1991; Ferguson et al. 1993; Kabir 2004). Outcomes of chronic infection range from asymptomatic and mild gastritis to more severe diseases such as peptic ulcer disease and gastric cancer (Blaser et al. 1995; Blaser 1998; Ernst and Gold 2000).

Formerly known as aerotolerant *Campylobacter*, genus *Arcobacter* was included in the family *Campylobacteraceae* in 1991 (Vandamme and De Ley 1991). Currently 21 species are recognized in the genus (Lastovica et al. 2014; Whiteduck-Léveillée et al. 2015). Species *A. butzleri*, *A. cryaerophilus*, *A. skirrowii,* and *A. thereius* have been associated mainly with enteritis in humans and abortion, mastitis and gastrointestinal disorders in animals (Anderson et al. 1993; On et al. 2002; Wybo et al. 2004; Van den Abeele et al. 2014). *A. butzleri* has been isolated from products of animal origin, with high prevalence in poultry, followed by pork and beef (Rivas et al. 2004; Gude et al. 2005). It has been suggested that *Campylobacter* isolates overgrow *Arcobacter* spp., which are not routinely studied with the clinical methods of isolation in clinical laboratories (Vandenberg et al. 2004). Although *A. butzleri* was the fourth most common *Campylobacter*-like organism isolated from human stools, there have been few human diarrhea cases reported (Prouzet-Mauleon et al. 2006; Collado and Figueiras 2011).

1.2.1 General Characteristics

Campylobacter spp. are Gram-negative, spiral, rod-shaped, or curved bacteria with a single polar flagellum, bipolar flagella, or no flagellum, depending on the species, are motile bacteria, and presents a typical movement of corkscrew. *Campylobacter* spp. are non-spore-forming, approximately 0.2–0.8 μm by 0.5–5 μm (Gilbreath et al. 2011). Refer to the electronmicrograph (Fig. 11.1) of this volume.

C. jejuni can also present filamentous form in broth cultures in a microaerobic atmosphere, this occurs on the entry into stationary phase (Griffiths 1993; Wright et al. 2009), elongated cells are also identified in scanning electron micrographs of *Campylobacter* biofilms (Brown et al. 2014). Ghaffar et al. (2015) showed that filamentous forms of *Campylobacter* showed more intracellular ATP content and enhanced survival in water at 4 and 37 °C when compared to spiral forms, suggesting that filaments are adapted to survive extra-intestinal environments and that filamentous morphology should be take into account in the methodology of isolation of *Campylobacter*.

Thermotolerant *Campylobacter* spp. frequently causes bacterial gastroenteritis in humans. This group contains species such as *C. jejuni*, *C. coli,* and *C. lari* that grows preferentially between 37 and 42 °C, but do not proliferate below 30 °C (Penner 1988). *C. jejuni* and *C. coli* are well known causes of diarrhea; patients

infected experience acute watery or bloody diarrhea, fever, weight loss, and cramps that last, on average, 6 days (Man 2011). *C. jejuni* is considered the most frequent cause of food-borne bacterial gastroenteritis in the world and is estimated to infect 1 % of Europe Union (EU) population each year. Predominant sources of infection are contaminated meat, mainly chicken, raw milk, and water (Allos 2001). In EU up to 80 % of retail poultry carcasses can be contaminated (EFSA 2010).

Clinical manifestation of *C. jejuni* infection range from mild watery to severe, inflammatory and bloody diarrhea accompanied with abdominal pain and fever (Allos 2001). Following an incubation period of approximately 24–72 h, symptoms develop, the most common manifestation is acute gastroenteritis, pain can be generalized or localized (Blakelock and Beasley 2003). In developing countries, infection is generally restricted to children, less than 5 years old, and mild clinical disease is the most common outcome (Oberhelman and Taylor 2000). In contrast, in developed countries, young adults (15–24 years) are also susceptible (Friedman et al. 2000). The immune system status of the host has been indicated as important factor in the disease pathogenesis since patients that acquired the disease abroad generally present clinical characteristics of the disease observed in their country of origin (Oberhelman and Taylor 2000).

Campylobacter spp. have a microaerophilic nature and requires a hydrogen-enriched atmosphere at 37 °C for its efficient cultivation in vitro. Optimal cultivation conditions for most *C. jejuni* isolates are provided in the Cape Town Protocol (Lastovica 2006). Several methods based on selective enrichment and selective agar media have been used for the isolation of *Campylobacter* species, mainly thermotolerant species, nevertheless the membrane filtration onto antibiotic-free Tryptose Blood agar plates has been proved to be efficient specially in isolation of antibiotic sensitive *Campylobacter* spp. (Lastovica 2006; Kinzelman et al. 2008; Jacob et al. 2011). Biochemical tests can be used to differentiate *Campylobacter* from related genera and identify species (Vandamme et al. 2005; Lastovica et al. 2014). Hippurate hydrolysis distinguishes *C. jejuni* from *C. coli*. *C. jejuni* has the ability to hydrolyze hippurate, whereas *C. coli* present a negative result (Lastovica 2006). On and Holmes (1991) present results on the use of 25 phenotypic tests for the differentiation of *Campylobacter, Helicobacter,* and *Arcobacter*. Molecular identification is also used mainly due the fastidious growth of *Campylobacter*, special requirements for optimal growth, the relatively and narrow spectrum of biochemical reactivity. The genes *hipO*, *cdtA,* and *pepT* specific for *C. jejuni*, *C. coli,* and *C. lari* were selected to differentiate these species by polymerase chain reaction (He et al. 2010; Vondrakova et al. 2014).

Although *C. jejuni* does not grow at laboratory in lower temperatures, respiration and ATP generation at temperatures as 4 °C and maintenance of metabolic activities at low temperatures for an extended time period is observed (Hazeleger et al. 1998). The ability to survive in low temperatures explains why refrigerated carcasses of poultry contaminated in the slaughter process are a common source of *C. jejuni* infections (Bhaduri and Cottrell 2004).

C. jejuni is a chemoheterotrophic bacterium with restricted carbohydrate catabolism, it is incapable to use glucose and other carbohydrates as growth substrates

and thus *Campylobacter* is generally considered to be a non-saccharolytic bacterium. The inability to catabolize carbohydrates is due the absence of the glycolytic enzyme phosphofructokinase of the Embden-Meyerhof-Parnas (EMP) pathway and incomplete pentose phosphate (PPP) and Entner Doudoroff (ED) pathways (Parkhill et al. 2000; Velayudhan and Kelly 2002). Interestingly, the incapability to catabolize glucose distinguishes *C. jejuni* from its close relative *H. pylori* (Mendz et al. 1993). Despite this, studies have shown a novel L-fucose pathway in certain strains like *C. jejuni* NCTC 11168, this metabolic diversity can be explained by the occurrence of a 9 Kb genomic island found in this strain in the open reading frame *cj0480* to *cj0490*, which are absent in *C. jejuni* 81–176 (Hofreuter et al. 2006; Muraoka and Zhang 2011; Stahl et al. 2011). This gene region encodes for a putative fucose permease FucP. It was shown that FucP enhanced the growth of *C. jejuni* NCTC 11168 when cultivated in chemically defined media containing 25 or 50 mM fucose as an additional carbon and energy source. Besides, fucose transport in *C. jejuni* in vivo provided competitive advantage during colonization of the piglet infection mode (Muraoka and Zhang 2011; Stahl et al. 2011).

Although the genome of *C. jejuni* harbors the enzymes required for gluconeogenesis synthesis of glucose from phosphoenolpyruvate (PEP) (Parkhill et al. 2000) it has not been experimentally proved yet (Hofreuter 2014). *C. jejuni* catabolizes organic acids like lactate, pyruvate, acetate, and intermediates of tricarboxylic acid (TCA) cycle, these are substrates that are used by this pathogen to feed its intermediary metabolism and cope with its necessities for carbohydrate, lipid, and protein biosynthesis (Elharrif and Megraud 1986; Westfall et al. 1986).

The utilization of amino acids is fundamental in fueling the central metabolism of *C. jejuni*, and protects bacteria from osmotic and oxidative stress (Booth and Higgins 1990). However, few glucogenic amino acids are degraded by this pathogen and support its proliferation. Chemotaxis is the ability of bacterial cells to detect temporal changes in the chemical concentration of their surrounding environment and associated with flagella-mediated motility play an important role in the intestinal colonization and invasion of epithelial intestinal cells of avian and mammalian hosts (Szymanski et al. 1995; Josenhans and Suerbaum 2002). Lysine, glucosamine, succinic acid, arginine, and thiamine have been described as chemorepellents (Rahman et al. 2014). Aspartate, glutamate, proline, and serine are growth-promoting amino acids for the majority of *C. jejuni* (Leach et al. 1997; Guccione et al. 2008; Hofreuter et al. 2008).

Growth-promoting amino acids utilization in liquid cultures occurs in sequential phases accordingly with its role in the pathogen's metabolism. Aspartate and serine are first catabolized and facilitate the rapid growth of *C. jejuni* followed by glutamate. Proline seems to be a less-preferred growth substrate of *C. jejuni* because its consumption from the culture medium occurred less rapidly in comparison to the depletion of aspartate, serine, and glutamate (Leach et al. 1997). Accordingly, L-aspartate, L-glutamate, and L-serine but not L-proline are effective chemoattractants for *C. jejuni* (Hugdahl et al. 1988; Vegge et al. 2009). Aspartate is an important carbon and energy source for *C. jejuni* as it directly feeds the TCA cycle by the aspartate ammonia lyse AspA catalyzing the deamination to fumarate

(Guccione et al. 2008). This AspA-catalyzed reaction of aspartate to fumarate also plays a role in the response of *C. jejuni* 81–176 to high pressure and its recovery from cell injury (Bieche et al. 2012). Besides, fumarate can be converted to oxaloacetate, which can be used as substrate for the gluconeogenesis and synthesis of essential carbohydrates (Sellars et al. 2002). Aspartate is also the precursor for the biosynthesis of several proteinogenic amino acids (lysine, methionine, threonine, isoleucine) as well as β-alanine (Hofreuter 2014).

The transport protein SdaC is responsible for the import of serine, the *sdaC* gene is organized in an operon with *sdaA* encoding for a serine dehydratase that catalyzes the deamination of serine to pyruvate (Velayudhan et al. 2004). Studies indicated that serine utilization is a variable catabolic characteristic of *C. jejuni* because not all tested isolates were able to grow with this amino acid as a unique carbon source. No molecular differences were observed to explain this fact as *C. jejuni* strains unable to utilize serine have no mutations in the *sdaA* and *sdaC* genes though the serine dehydratase activity was fairly reduced (Hofreuter et al. 2008).

The Peb ABC transporter system encoded by the *peb* locus is responsible for the intake of glutamate by *C. jejuni* (Pei and Blaser 1993). The depletion of uptake of glutamate in mutations of the permease PaqP and the ATPase PaqQ of the Paq (pathogenesis-associated glutamine) ABC transporter system implies that they also play a role in the glutamate acquisition (Lin et al. 2009). In contrast with other members of *Campylobacteraceae*, *C. jejuni* lacks glutamate which implies that glutamate is not converted directly to 2-oxoglutarate through deamination. Glutamate can be either converted to glutamine by the type I glutamine synthetase GlnA or is substrate of the aspartate: glutamate transaminase AspB catalyzing the generation of aspartate and 2-oxoglutarate from oxaloacetate and glutamate (Guccione et al. 2008). The inactivation of *aspB* implies severe growth problems for *C.jejuni* indicating the important role that the AspB plays in the *C. jejuni* metabolism (Novik et al. 2010).

Hofreuter et al. (2008), in a directed mutagenesis approach, showed that the growth of *C. jejuni* 81–176 with proline is mediated by the PutP transporter and the enzyme PutA. Although PutP is common in Gram-positive and Gram-negative and highly conserved in *C. jejuni* and shows about 80 % amino acid identity to respective transporter proteins of *C. coli*, *C. lari*, *C. upsaliensis,* and *C. fetus*, whereas no homologs are present in other *Campylobacter* species. Moreover, the proline symporter protein ($PutP_{Cj}$) of *C. jejuni* shows 75 % identity to the PutP transporter ($PutP_{Hp}$) of *H. pylori* (Hofreuter et al. 2012). The PutA enzyme of *C. jejuni* uses FAD and NADH as cofactors; it catalyzes the oxidation of the imported proline to glutamate (Hofreuter 2014).

Several putative peptidases and proteases are encoded by the *C. jejuni* genome, some named ClpP, HtrA, CJJ81176_1086, CJJ81176_1228, Cj0511, or Pgp1, have been associated with the virulence of *C. jejuni* (Brondsted et al. 2005; Novik et al. 2010; Boehm et al. 2012; Karlyshev et al. 2014). Although the role of peptidases in the catabolism and nutrient acquisition of *C. jejuni* has not been characterized in detail so far, the great importance of amino acid catabolism for the proliferation of

C. jejuni suggests that peptides may also be important growth-promoting substrates for this pathogen (Adibi and Mercer 1973).

In a study that compared the carbon source of utilization between strains of *C. jejuni* and *C. coli* differences in the metabolism of propionic acid were observed. While all *C. jejuni* tested failed to use it, *C. coli* strains were able to use this carbon source. This ability was linked with the presence of propanoate CoA ligase and 2-methyl-synthase genes in *C. coli* and absence in *C. jejuni*. The authors suggest that the presence or absence of these genes is a reliable marker for the identification of *C. coli* or *C. jejuni* (Wagley et al. 2014).

1.2.2 Genetic Structure

Campylobacter spp. have small genomes (1.6–2.0 megabases), sequencing the genome of *C. jejuni* has revealed the presence of hypervariable sequences that consists of homopolymeric tracts, they were found in genes encoding the biosynthesis or modification of surface structures such as the capsule, lipooligosaccharides (LOS) or flagellum (Parkhill et al. 2000; Young et al. 2007). Mechanisms such as phase variation, gene duplication and deletion, frameshifts and point mutations are responsible for the variations in these structures (Linton et al. 2000; Gilbert et al. 2002; Guerry et al. 2002; Karlyshev et al. 2002, 2005). The capacity of *C. jejuni* to take up DNA from the environment leads to the recombination between strains, which allows the generation of more genetic variation. The horizontal transfer of both plasmid and chromosomal DNA occurs both in vitro and during chick colonization, indicating that natural transformation could have an important role in genome diversity and in the spread of new factors like antibiotic resistance, even in the absence of selective pressure (Wilson et al. 2003; Avrain et al. 2004).

Differences in virulence of individual *C. jejuni* isolates are possibly correlated with dissimilarities in motility and surface structures involved in the direct interaction with the host. These structures include lipooligosaccharide, the capsule, flagella, and the glycosylation pattern of the flagellin (Wilson et al. 2010).

Lipooligosaccharide (LOS) plays an important role in the host immune avoidance of *C. jejuni* and is high variable. Several structures of LOS resemble human neural gangliosides and this can explain the autoimmune disorder caused by the pathogen, including the Guillain-Barré syndrome, a paralytic neuropathy that occurs in after 1 in every 1000 cases of campylobacteriosis, and Miller Fisher syndrome, a variant of Guillain-Barré syndrome. The current hypothesis is that a susceptible human host generates autoantibodies that target both the bacterial ganglioside-like lipooligosaccharide (LOS) structures and human peripheral nerve gangliosides, which triggers axonal degeneration and demyelination of the peripheral nerves (Nachamkin et al. 1998). Mutations in various genes that are involved in LOS biosynthesis imply in changes in resistance, adherence to and invasion to INT 407 cells (Fry et al. 2000).

The *C. jejuni* capsule is important for serum resistance, the adherence, and invasion of epithelial cells and chick colonization (Karlyshev et al. 2000; Szymanski et al. 2003). Structures of the capsules of several *C. jejuni* have been determined, and extensive variation has been observed. The capsule structure of *C. jejuni* strain RM1221 has been determined and surprisingly includes 6-deoxy-D-manno-heptose and D-xylose (Gilbert et al. 2007), which are two sugars that are not often detected in bacterial polysaccharides. McNally et al. (2005, 2006) found *C. jejuni* strains that display teichoic acid-like capsular polysaccharide and also strains that presented hyaluronic acid-like capsules. An in vitro study with hyper-invasive strains of *C. jejuni* showed that this phenotype displays mosaicism in the capsular polysaccharide region and a highly variable capsule region of genetic imports from *C. jejuni* subsp. *doylei* and *C. lari* (Baig et al. 2015).

The flagellum consists of two highly similar flagellin subunits, FlaA, and FlaB (Guerry et al. 1991), and is heavily glycosylated, which is decisive in flagella structure and function in *Campylobacter*. The whole flagellar apparatus involves the coordinated assembly of 40–100 proteins (Chen et al. 2011), is modified by covalent *O*-linked attachment of modified pseudaminic or legionaminic acid sugars (Thibault et al. 2001; McNally et al. 2007) and glycosylation has been shown to be essential for flagella assembly (Goon et al. 2003; Asakura et al. 2013). Expression of flagella genes is tightly regulated, with genes involved in the secretion apparatus subject to expression from σ^{54}-dependent promoters, and the major flagellin and several effectors requiring σ^{28} (Nuijten et al. 1990; Carrillo et al. 2004; Wosten et al. 2010). The two-component FlgSR system (Hendrixson and DiRita 2003; Wosten et al. 2004) and the FlgM anti-sigma factor (Wosten et al. 2010) are also involved in regulating transcription of flagellar genes. Flagella may be further regulated via phase variation as a result of polymeric A/T tracts within the *flgR* gene (Hendrixson 2006).

C. jejuni flagellum-mediated motility is a prerequisite in both human disease and successful colonization in animals (Wassenaar et al. 1993). Studies have reported the association between the presence of intact flagellum and the ability of *C. jejuni* to adhere and invade cells (Grant et al. 1993; Nachamkin et al. 1993). Besides conferring the ability to swim toward intestinal epithelial cells for *Campylobacter*, which is a critical step for subsequent cell invasion (Lee et al. 1986; Szymanski et al. 1995), the flagellar Type III secretion system is utilized to secrete non-flagellar effector proteins, which are implied in virulence (Poly et al. 2007; Barrero-Tobon and Hendrixson 2012; Neal-McKinney and Konkel 2012). Moreover, motility is essential for chemotaxis, and various chemotaxis-defective mutants are attenuated in animal models of disease or show reduced immunopathology (Takata et al. 1992; Yao et al. 1994; Bereswill et al. 2011).

C. jejuni harbors both *N* and *O*-linked glycosylation systems (Szymanski et al. 1999; Wacker et al. 2002). The targets of the *O*-linked glycosylation system are flagellin (Guerry et al. 2006) and recently reported major outer membrane proteins. As these components are highly immunogenic glycosylated, major outer membrane proteins may play an underestimated role in the auto immune diseases (Mahdavi et al. 2014). The predominant *O*-glycans attached to the *Campylobacter* flagellum

are derivatives of pseudaminic acid or legionaminic acid, which are C9 sugars related to sialic acid (Nothaft and Szymanki 2010).

1.2.3 Campylobacter *spp. in Poultry*

Many species of domestic poultry such as chickens, turkeys, ducks, geese, and wild birds are frequently infected with thermophilic *Campylobacter*, primarily *C. jejuni* and *C. coli* (Sahin et al. 2015). Birds are naturally infected via the fecal-oral route; the organisms colonize primarily large blind ceca and colon and to a lesser extent the small intestines. Reports of *Campylobacter* isolation from liver, spleen, and blood suggests that *Campylobacter* may invade intestinal epithelial cells and become systemic (Sanyal et al. 1984; Knudsen et al. 2006). The horizontal transmission occurs rapidly. The fecal shedding of the bacterium and the coprophagic behavior of the chicken allows the overall prevalence within a flock near to 100 % at the slaughter age (Berndtson et al. 1996; Cardinale et al. 2004; Barrios et al. 2006). Sources of flock infection include old litter, untreated drinking water, domestic pets, other farm animals, wildlife species, flies insects, farm equipment and transport vehicles and farm workers, vertical transmission lacks of evidence (Sahin et al. 2015).

Reports of *Campylobacter*-positive poultry flocks vary widely from 2 to 100 %. The variations of prevalence are seen between regions, seasons, productions system, and flock age. In Iceland, the prevalence was approximately 15 %, whereas in Dutch, France, Spain, and US was respectively 63, 71.9, 62.9, and 63.6 % (Stern et al. 2005; Berghaus et al. 2013; Allain et al. 2014; Torralbo et al. 2014; Sandberg et al. 2015). Prevalence of *Campylobacter* shows a peak in warm months (Allain et al. 2014; Sandberg et al. 2015).

Young birds, less than 2–3 weeks of age are rarely infected with *Campylobacter*, regardless the avian species and the production types (FSA 2008; Allen et al. 2011; El-Adawy et al. 2012; Hermans et al. 2012). Maternal antibodies play an important role in the absence of detection of *Campylobacter* in young birds; studies have shown that they are widely present and in addition with the strain of the bacterium are determinants for the colonization of young chickens by *Campylobacter* (Sahin et al. 2003; Konkel et al. 2007; Hermans et al. 2012; Chaloner et al. 2014). Organic and free-range flocks are more likely to have high prevalence of *Campylobacter* than in conventional production systems (Engvall 2004; Allen et al. 2011).

In poultry, mainly in broiler chickens, *C. jejuni* is the predominant species colonizing the flocks, followed by *C. coli* and rarely other species. Otherwise *C. coli* is reported as the predominant species in turkey flocks. In a study in turkey farms in the US, Kashoma et al. (2014) found *Campylobacter* spp. prevalence of 55.9 %; *C. coli* represented the majority of the isolates with 72.1 % and *C. jejuni* only 5.3 %. *C. coli* also has been reported as the dominant species in colonizing organic and free-range chicken (El-Shibiny et al. 2005; Colles et al. 2008).

Although *Campylobacter* had been considered a harmless component of the commensal intestinal microbiota of chickens, reports suggest that *Campylobacter* is capable of inducing damage to the intestinal epithelia by compromising intracellular tight-junctions, modulating the barrier functions, and stimulating poorly-regulated host inflammatory responses (Rees et al. 2008; Awad et al. 2014; Humphrey et al. 2014), Humphrey et al. (2014) showed that the pathogen can lead to intestinal inflammation and diarrhea in fast growing breeds of broiler. Moreover, a link has been suggested between the presence of *Campylobacter* in poultry flocks and increased incidence of leg pathologies such as pododermatitis and hock burn (Bull et al. 2008; Williams et al. 2013), such pathologies are probably due humid litter caused by diarrheal feces. More studies are necessary for the understanding of *Campylobacter* impact in the poultry health and thus in the production costs.

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Chapter 2
Isolation and Identification of *Campylobacter* spp. in Poultry

Omar A. Oyarzabal and Heriberto Fernández

Abstract Poultry products, especially chicken meat, continue to be important sources of campylobacteriosis in humans. This chapter reviews the current methods used for the isolation and identification of *Campylobacter* spp. from chicken products. Emphasis is placed on the enrichment protocols, plate media, and most used, practical confirmation methods. The incorporation of molecular techniques and some of the methodologies used in some Latin American countries to detect *Campylobacter* spp. from poultry are summarized. Finally, some perspectives in future trends are provided.

Keywords Isolation · Identification · Culture media · Rapid methods · Molecular methods · Poultry · Food samples

2.1 Introduction

Campylobacteriosis is the generic name for the disease produced by bacteria belonging to the genus *Campylobacter*. Within the genus *Campylobacter* there are several bacterial species that can produce disease in humans and domestic animals (Man 2011), but *Campylobacter jejuni* and *Campylobacter coli* are the most important species from the public health stand point and are responsible for almost 98 % of all the confirmed human cases of campylobacteriosis (Gilliss et al. 2013).

The epidemiology of campylobacteriosis is complex and there are still several factors that are not well understood, even in developed countries. There are several

Authors do not endorse any particular assay or manufactures of assays discussed in the chapter.

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risk factors that have not been fully elucidated but that may prove to be important in the interpretation of the geographical variations (Ailes et al. 2012) and even within ethnic groups (Quinlan 2013) in the appearance of this disease. The incidence of campylobacteriosis varies according to countries. For instance, the reported incidence in the USA is 14 cases every 100,000 people, while the incidence in Australia is more than 100 cases every 100,000 people and excluding New South Wales (Anonymous 2014). In South America, there is also a variation in the prevalence of this disease by country, and although there are no consistent figures, this disease continues to have a major impact in public health (Fernández 2011).

The actual reported number of cases represents only confirmed cases and therefore there are many more cases that go underreported annually. Yet, with this incomplete reporting system campylobacteriosis represent one of the most important bacterial diseases transmitted by foods worldwide.

The foods most commonly implicated in cases of campylobacteriosis are meats, especially poultry meat and giblets, raw milk, and raw oysters. This chapter will focus on the methods most commonly used for the isolation of *Campylobacter* spp. from poultry meat, with emphasis on the methods suggested by food regulatory agencies from developed countries. The section about identification will focus on the protocols that are most commonly used in food microbiology laboratories and with special emphasis on the methods based on the identification using polymerase chain reaction (PCR) protocols.

2.2 Isolation

2.2.1 Isolation from Fecal Material of Live Chickens

Campylobacteriosis is largely considered as a foodborne disease. Poultry meat, primarily chicken meat, is an important source of transmission of *Campylobacter*. Some estimates suggest that up to 80 % of all cases of human campylobacteriosis are attributed to the transmission by chicken meat worldwide (Bahrndorff et al. 2013). Poultry meat gets contaminated during processing, at the slaughter house, at different stages. Defeathering and evisceration are important steps at which contamination with feathers/skin and intestinal content from the birds will occur. The subsequent full elimination of the bacteria from the meat is not completely achievable throughout the rest of the processing steps, and *Campylobacter* can survive (López et al. 2003) through storage and contaminate the kitchen of end users at home.

The isolation of *Campylobacter* from feces in commercial poultry farms is important for epidemiological studies of this agent. There is an extensive scientific literature on the methods for isolation of *Campylobacter* from fecal material and the best approach is the use of direct plating of feces on selective agar plates and the subsequent incubation of the plates at 42 °C under microaerobic conditions and for up

to 48 h. In South America, a modified protocol includes a pre-enrichment step of 24 h and a subsequence transfer to selective plates for up to 48 h. This protocol has been used to isolate *Campylobacter* from backyard hens and chickens in Southern Chile, with results showing a prevalence of 23–77 % of *Campylobacter* (Fernández 1992; Fernández et al. 1993; Fernández and Torres 2000). The pre-enrichment step increases the isolation rate by 20 % (Fernández 1992). With the same protocol, the prevalence of *Campylobacter* in poultry in a low-income community in Buenos Aires, Argentina was 40 % (López et al. 2003), whereas in Southern Brazil the prevalence of *Campylobacter* in 26 small, family farms with mixed flocks for meat and eggs production was 26 % (Gomes et al. 2006). In a study comparing direct plating versus pre-enrichment in 22 broiler flocks aged 3–5 weeks in Brazil, Kuana et al. (2008) found no statistical differences between the pre-enrichment and direct plating methods. However, the total rate of positive flocks detected by the pre-enrichment method amounted to 99.0 % (95/96), compared to 97.9 % (94/96) in direct plating.

2.2.2 Isolation from Poultry Products

2.2.2.1 Enrichment of Food Samples

The isolation of *Campylobacter* from foods is based on the enrichment of the samples in selective broths, the transfer of the enriched sample to selective agar plates and the identification of presumptive colonies grown on agar plates. This isolation protocol relies heavily on the use of selective agents and a high incubation temperature (42 °C) to reduce the competition from other microorganisms, mainly bacteria and yeasts, in the samples. It is important to keep in mind that high temperatures should be used only when suspecting the presence of thermotolerant species of *Campylobacter*, which are *C. jejuni*, *C. coli*, *C. lari,* and some strains of *C. upsaliensis* (Gharst et al. 2013).

If other, non-thermotolerant *Campylobacter* species are known or suspected in the samples, it is recommended that the isolation procedure be performed with incubation temperatures of 37 °C. However, the most important species in foods are *C. jejuni* and *C. coli* and most isolation protocols can be performed at incubation temperatures of 42 °C, especially in poultry samples. Some enrichment protocols suggest an initial temperature of 37 °C for the first 3–4 h of enrichment to help potentially injured *Campylobacter* cells to recover, but there are no scientific works or any important studies that justify the use of this initial temperature, or that suggest that a significantly larger proportion of samples will become positive if this variation in the protocol is included. The Cape Town Protocol (Lastovica 2006) utilizes an initial isolation temperature of 37 °C, presumptive colonies are reincubated at both 37 and 42 °C, allowing isolation of thermophilic and non-thermophilic *Campylobacter* spp. from chicken meat.

Traditionally, enrichment broths have been incubated under atmosphere containing a reduced oxygen level, usually atmospheres that are called "microaerobic" and are comprised of 5 % O_2, 10 % CO_2, and 85 % N_2. However, atmospheres with low oxygen levels are naturally generated in enrichment broths and therefore static incubation is enough to provide the adequate environment for *Campylobacter* cells to grow and multiply (Zhou et al. 2011).

Until recently, it was thought that the enrichment broths contained many different nutrients from which *Campylobacter* cells could grow. Yet, we now know that the presence of selective agents is more important than the nutrient composition of the broth for the successful isolation of *Campylobacter* spp. from food samples. For instance, buffered peptone water is sufficient for the isolation of *Campylobacter* from poultry meat (Oyarzabal et al. 2007, 2013). Among the selective agents for enrichment broths and plate media, cefoperazone (sodium salt) is the antibiotic most effective against competing bacteria present in the foods. Several enrichment broths incorporate, besides cefoperazone, vancomycin to control the growth of Gram-positive bacteria, trimethoprim, and amphotericin B as an antifungal agent. For many years, we have been suggesting the use of only cefoperazone, as a broad spectrum antibiotic, and amphotericin B. We use approximately 33 mg of cefoperazone and 4–10 mg of amphotericin B per liter of medium. It is difficult to predict how contaminated the food sample is, but this combination of antibiotics appears to be a good compromise for the isolation of *Campylobacter* spp. from poultry meat. An alternative to control high background flora is the addition of vancomycin at concentrations of 20 mg per liter, but we prefer to use filter membranes for the transfer of enriched samples to plate media and reduce the use of antibiotics (Speegle et al. 2009; Gharst et al. 2013). Some antibiotics used in antibiotic selective plates will suppress the growth of *Campylobacter* spp. (Lastovica, unpublished).

Due to an increase in the appearance of *Escherichia coli* strains expressing extended-spectrum beta-lactamase, some reports suggest the addition of tazobactam through the isolation procedure. This compound is more chemically stable than clavulanic acid or sulbactam; thus, tazobactam is more suitable for restoring the selectivity of CCDA (charcoal-cefoperazone-deoxycholate agar) and other media for the isolation of *Campylobacter* (Smith et al. 2015).

The traditional time for enrichment of samples is 48 h and the attempts to reduce the time to 24 h resulted in a larger proportion of samples identified as false negative (Liu et al. 2009; Oyarzabal et al. 2007). Yet, the transfer of enriched samples at 24 h will help identify the samples with higher number of naturally occurring *Campylobacter*. Using this methodology, Simaluiza et al. (2015) reported a prevalence of 62.7 % of *Campylobacter* positive samples in chicken livers for human consumption in Southern Ecuador.

The examination of enrichment broth at 24 h with PCR methods have not resulted in reliable identification. At 48 h, the use of PCR may have some benefits and some commercial systems, such as the BAX® (Dupont, Qualicon, Wilmington,

DE, USA) and iQ-Check™ (Bio-Rad Laboratories, Hercules, CA, USA) have been validated for detection at 48 h and for the use with poultry carcass rinse collected in chicken processing plants.

2.2.2.2 Growth on Selective Plates

In general, agar plates for isolation are based on the addition of either blood or charcoal. The original intention when adding blood or charcoal was to provide some substances that would reduce, or quench, oxygen in the medium. But nowadays these substances are usually added for differentiation purposes. A newer group of plate media are some chromogenic agars, but laboratories must buy premade media and therefore the cost of isolation increases substantially. In most countries, the plate most commonly used is CCDA (Bolton and Robertson 1982; Bolton and Coates 1983). This medium is one of the most economic alternatives for use in food microbiology laboratories and although identifying colonies may take some time, *Campylobacter* colonies have unique characteristics that make them be easily identifiable by trained personnel. Therefore, CCDA is a good differential plate for isolation purposes. The incubation time for plates is 48 h, although colonies can be identified at 36 h of incubation at 42 °C and under microaerobic conditions.

Other types of plates are those with the addition of blood. These plates have similar isolation efficiency as CCDA plates for isolation of *Campylobacter* from poultry products (Oyarzabal et al. 2005; Potturi-Venkata et al. 2007). In general, blood plates are supplemented with the some antibiotics incorporated in CCDA. In general, a personnel working in food microbiology laboratories like blood plates more because it is easier for them to learn how to identify presumptive *Campylobacter* colonies. The beta hemolysis from the growth of *Campylobacter* is a good selective way to identify presumptive *Campylobacter* colonies. However, this beta hemolysis is not unique to *Campylobacter* colonies and our experience indicates that charcoal-based plates are more reliable in the identification of presumptive *Campylobacter* colonies than blood-based plates. In addition, *Campylobacter* colonies tend to grow deeper than just the surface in blood plates due to the breakage of the agar surface during the streaking process. Figures 2.1 and 2.2 show the typical *Campylobacter* colonies in blood agar and CCDA plates respectively.

The antibiotics used in plate media are the same and at the same concentrations of those antibiotics used in enrichment media. In some cases, vancomycin could be added if the sample is suspected to have a large contamination with background microflora. The cefoperazone/amphotericin B has worked well for the authors in the isolation of *Campylobacter* from poultry products (Williams and Oyarzabal 2012). Yet, a simple modification during the transfer of enrichment media to agar plates can make a large impact in the reduction of antibiotics used in the enrichment step. This modification includes the use of filter membranes with pores of 0.45 or 0.65 μm. Several different variations of these filter membranes have been used for more than 50 years in the isolation of *Campylobacter* in veterinary (Plumer et al. 1962) and clinical samples, and in some cases the membranes were used on agar plates without

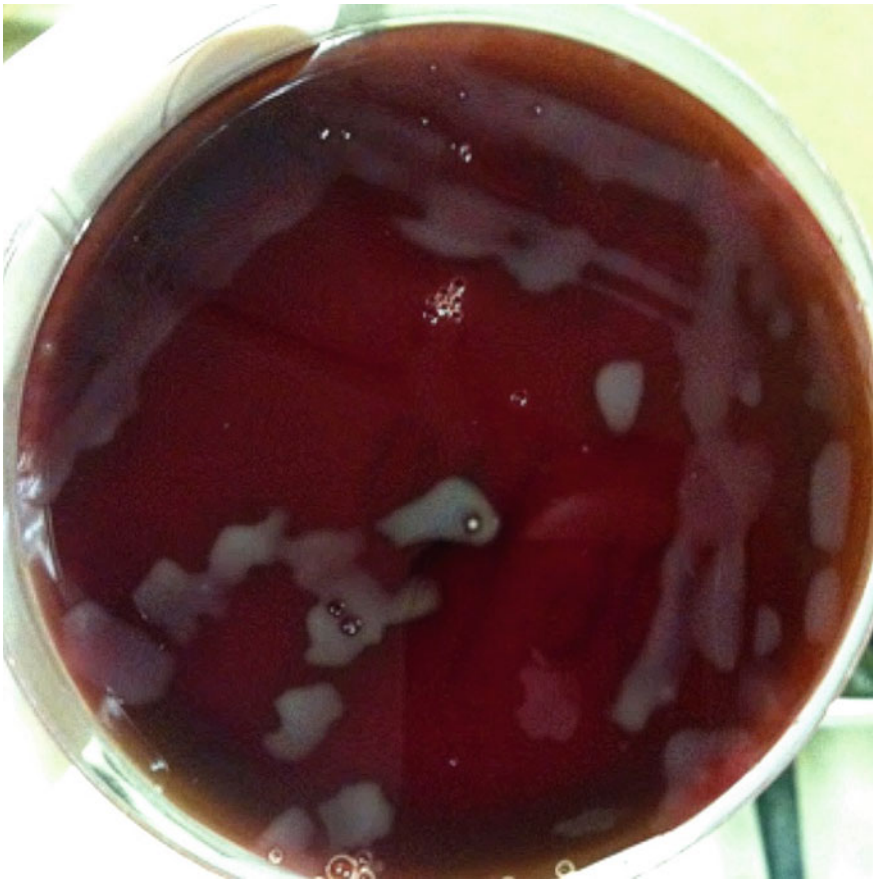

Fig. 2.1 Typical *Campylobacter* colonies in blood agar

any selective agents (Steele and McDermott 1978; Lastovica 2006). However, the use of these filters to isolate *Campylobacter* spp. from food samples did not start until the 1990s (Baggerman and Koster 1992). In our laboratories, we started the use of filter membranes in 2008 with very good results (Speegle et al. 2009). Some recent publications have also highlighted the practicality and usefulness of these filters membranes to isolate *Campylobacter* (Bi 2013).

These filters allow for food particles and large cells to be retained on the surface while the smaller, mobile *Campylobacter* cells pass through. We place one filter on top of an agar plate (charcoal- or blood-based), deposit approximately 100 μl of the enriched broth on top of the filter, and wait approximately 15–20 min before removing the filter with disinfected tweezers. Filter membranes with pores of 0.65 μm are adequate to isolate *Campylobacter* spp. and we prefer the use of selective media with at least 33 mg/L of cefoperazone to inhibit the growth of contaminating bacteria that can still pass through these filters (Speegle et al. 2009).

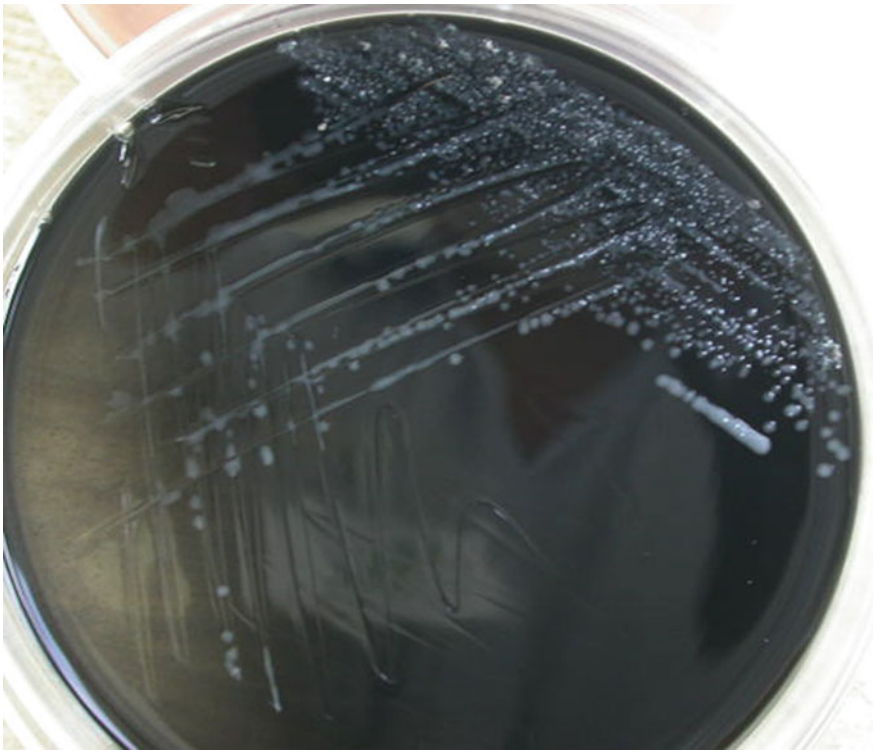

Fig. 2.2 *Campylobacter* colonies on CCDA plates

In general, these filter membranes help improve the efficacy of isolation of plate media irrespective of the formulation of the media (Chon et al. 2012). Figures 2.3 and 2.4 show the filtration procedure and the obtained colonies after incubation.

There are few chromogenic agar plates that are already commercially available in the USA, Europe, and Latin American countries. Some of these plates have been validated for isolation of *Campylobacter* spp., primarily from meat, poultry meat, carcass rinse, and environment samples (Table 2.1), and all current chromogenic agars have been found to be equally sensitive to traditional plates for identification of *Campylobacter* spp. from food samples (Ahmed et al. 2012; Seliwiorstow et al. 2014; Teramura et al. 2015). The first chromogenic agar that appeared in the market was CampyFood ID agar (bioMerieux, Marcy l'Etoile, France), a plate that has performed similarly to CCDA for the isolation of *Campylobacter* spp. from naturally contaminated poultry samples. Yet, there may be other bacterial species growing on the plate and therefore this is not completely differential (Habib et al. 2008; Habib et al. 2011). In a study performed in Chile, CampyFood ID agar had a higher isolation rate than mCCDA in chicken meat, with a percentage of positive samples of 83 % for CampyFood Agar and 67 % for mCCDA (Fernández-Riquelme 2011). Figure 2.5 shows *Campylobacter* colonies in CampyFood ID agar (red colonies).

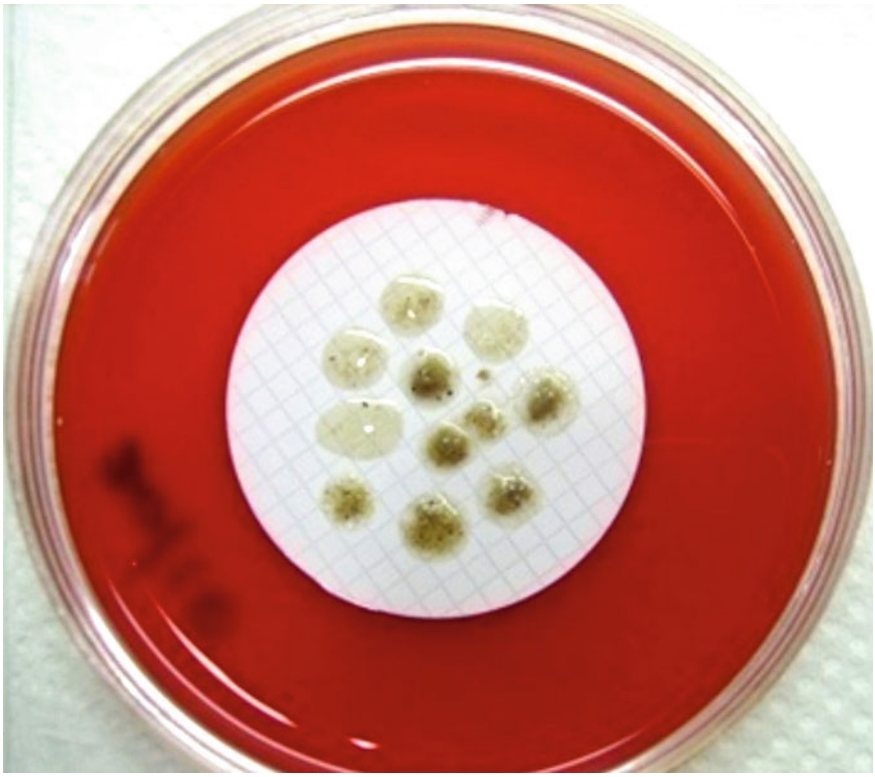

Fig. 2.3 Filtration procedure before incubation with filter membranes with pores of 0.65 μm in selective media

All of the media, broth and plates, used for isolation of *Campylobacter* have been modifications of media developed more than 30 years ago when generating microaerobic conditions in microbiology laboratories was more challenging that in current times. All of these media had the addition of substances that bind to oxygen to help produce a microaerobic environment that allows for *Campylobacter* to grow and to protect the cells from hydrogen peroxide. For instance, the addition of blood and charcoal to media was done with the intention of reducing the oxygen level in the media throughout the isolation process. Other substances that were commonly added were sodium metabisulfite, sodium pyruvate (which is supposed to also be a source of energy) and ferrous sulfate. However, the addition of blood or charcoal provides for the needed oxygen quenching substances and the added "differential" properties to the media to easily visualize the colonies on the plates. We have found that Brucella agar and even tryptic soy agar not supplemented with charcoal or blood are equally efficient for the isolation of *Campylobacter* from enriched samples. However, without a differential substance most colonies look similar and it is very difficult to identify presumptive *Campylobacter* colonies.

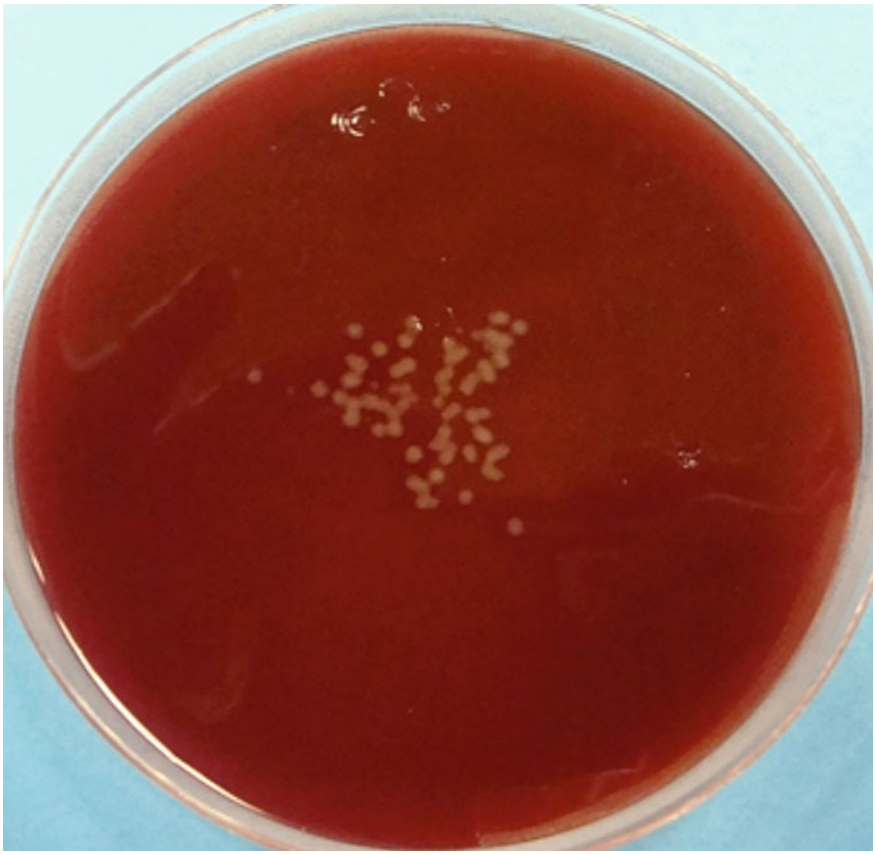

Fig. 2.4 Obtained colonies after filtration procedure and incubation over 48 h

Different substances have been added to plate media to generate microaerobic conditions on the surface of the plate. One of these substances, Oxyrase® (Oxyrase, Inc. Mansfield, Ohio), is an enzyme system that help produce anaerobic conditions in a wide variety of bacteriological broth media. However, as stated in previous section in this chapter, microaerobic conditions are naturally created in broth media and therefore the addition of any oxygen quenching substance is less important than the addition of selective agents that allow for the suppression of competing bacteria and for *Campylobacter* to multiply to detectable numbers. The addition of this enzyme system for plate media appears to be more appropriate, but the media have to be poured on special plates (OxyDish™), which makes the isolation procedure more expensive. We are not aware of any microbiology laboratory using Oxyrase® for the routine isolation of *Campylobacter* spp.

Table 2.1 Methods for detection of *Campylobacter* spp. from foods that have received validation by AOAC International[a]

Type of method	Method name	Manufacturer	Validated matrices
PCR-based	BAX® System Real-Time PCR Assay for *Campylobacter jejuni*, coli, and lari	DuPont Nutrition and Health Diagnostics	Feces on cloacae swabs (levels above 100 cfu/g). Ready-to-eat turkey product and chicken (25 g), or carcass rinses (30 mL)
	Campylobacter real-time PCR	Eurofins Genescan	Chicken raw meat, feces on cloacae swabs, disposal shoe covers with chicken feces
	iQ-Check™ *Campylobacter* real-time PCR	Bio-Rad Laboratories	Chicken carcass rinse (30 mL), turkey carcass sponge, raw ground chicken (25 g)
ELISA-based	VIDAS® *Campylobacter* (CAM)	BioMérieux	Meat products (25 g) and production environment samples. Fresh raw pork, raw chicken breast, processed chicken nuggets (25 g), chicken carcass rinse, turkey carcass sampled with sponge
Chromogenic agars	CampyFood Agar (CFA)	BioMérieux	Meat, poultry products 25 g, and production environment samples. Fresh raw pork, raw chicken breast, processed chicken nuggets (25 g), chicken carcass rinse, turkey carcass sampled with sponge
	CASA® (*Campylobacter* Selective Agar) for enumeration of *Campylobacter* spp	BioMérieux	Meat products, poultry products, and environmental samples
	RAPID' *Campylobacter*/ Agar	Bio-Rad Laboratories	Meat products, and meat product and production environment samples
	Brilliance™ CampyCount Agar	Oxoid Ltd, part of Thermo Fisher Scientific	Poultry products
Lateral flow	Singlepath® *Campylobacter*	Merck KGaA	Raw ground chicken, raw ground turkey (25 g), pasteurized milk
	Veriflow™ *Campylobacter*	Invisible Sentinel, Inc	Chicken carcass rinse

[a]These methods target *Campylobacter jejuni*, *C. coli*, *C. lari,* or *Campylobacter* spp. The table has been modified from the Validated Test Kit table available at the website of the U.S. Department of Agriculture

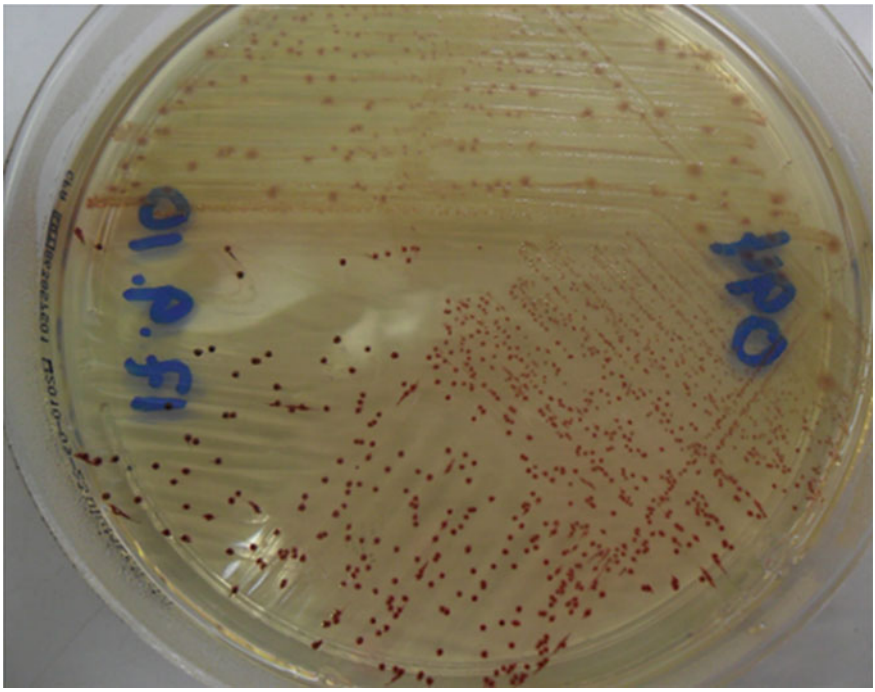

Fig. 2.5 *Campylobacter* colonies in CampyFood ID agar (*red* colonies)

2.3 Identification

It is important to remember that presumptive colonies on agar plates must be confirmed though a method that has been validated when reporting the results from samples that are under regulatory compliance, such as the performance standard for *Campylobacter* in whole chicken carcasses in processing plants inspected by the Food Safety and Inspection Service of the U.S. Department of Agriculture in the USA.

There are several available options for the identification of presumptive colonies on agar plates. Until approximately 20 years ago the use of few biochemical tests was still common for identification of strains to the genus and even species level. Over the years, the use of these biochemical tests for *Campylobacter* spp. has almost completely disappeared from food microbiology laboratories because of the lack of reliability of these techniques to identify strains to the species level. M'ikanatha et al. (2012) found considerable variation in laboratory protocols, detection methods, and isolation rates of *Campylobacter* in a study of 176 clinical laboratories. Some reference laboratories however, may still use some of these biochemical tests under strict protocols.

The current clinical and food microbiology laboratories use latex agglutination tests, ELISA and PCR tests to confirm colonies to the genus and sometimes to the

species level. The antibody-based techniques, such as the latex tests, lateral flow devices, and ELISA, can be used for the confirmation of isolates to the genus level but are not very robust for identification as the species level. For species identification, the polymerase chain reaction (PCR) technique has several advantages and has been employed for several years in the identification of *Campylobacter* spp. to the species level and in different laboratories. As it was mentioned earlier in this chapter, the species of importance are *C. jejuni* and *C. coli* and therefore a multiplex PCR with only two pair of primers can provide information to the species level for all common food isolates from chicken products.

2.3.1 Latex Agglutination Tests

These tests are based on polyclonal antibodies and have been in the market for more than 20 years. There have been several laboratories that have owned some of the antibodies that were developed in early 1990s. All these tests are based on the agglutination of *Campylobacter* cells in the presence of polyclonal antibodies that normally react with fluellin or other proteins present on the cell walls. The latex particles are covered by the antibodies (immunoglobulins) that usually react with *C. jejuni*, *C. coli,* and *C. lari*. The methodology for confirmation of isolates suggested in the Microbiology Laboratory Guidebook of the U.S. Department of Agriculture includes the use of a latex test and phase contrast microscopy, which is not commonly used in research laboratories (Anonymous 2013). Three commercially available latex agglutination tests are available and have been evaluated (Miller et al. 2008).

2.3.2 ELISA Tests

Most of the current ELISA tests in the market are for the confirmation of presumptive colonies isolated from clinical samples. Most of the food microbiology laboratories do not employ ELISA. One exception is the use of the VIDAS® *Campylobacter* (bioMerieux, Marcy l'Etoile, France), which is an immuno-based test that is almost completely automated and that has been validated for several food matrices and has been in used for several years (Liu et al. 2009; Reiter et al. 2010). In Chile, this system has been in use by some of the laboratories testing poultry products and was used in a study aimed at detecting the prevalence of *Campylobacter* spp. in chicken and turkey samples (Fernández-Riquelme 2011).

2.3.3 PCR Methods

There are several PCR assays for incorporation in food laboratories. Some of the PCR methods are commercially available and have a high level of automation. PCR

assays have several advantages that make them easy to incorporate in laboratories. One advantage is that samples can be treated with heat to stop the action of enzymes and still leave the DNA available for identification purposes. PCR assays are also very specific for identification of unique DNA fragment that allow for identification to the species level and even sometime at the infra-species level.

In the last 10 years, the cost of the DNA methods has decreased substantially and the protocols have been simplified considerably to allow for systems that are almost completely automated. There are some PCR assays that identify isolates only to the genus level and therefore the results are expressed as *Campylobacter* spp. As described, some of these assays are almost completely automated and their protocol include an enrichment step and the equipment necessary to perform the actual PCR, which is a real-time PCR assay in all the commercially available PCR systems. In this cases, the operator only have to load the sample and the reagents to the equipment to perform the assay. The enrichment of the sample is still an important step to allow for *Campylobacter* cells to multiply to detectable levels. Most PCR assays have a sensitivity of approximately 3 Log CFU/g or ml of samples. Therefore, an enrichment step is needed for the samples that carry low number of *Campylobacter* cells. The enrichment step also increases the chances of having live cells in the sample, which will increase the probability of confirming the presumptive result found from testing the enriched samples with the PCR assays. When performing PCR assays, the use of stringent protocols for handling the samples are important to minimize the probability of cross-contamination that can results in samples identified as positive by PCR but are not confirmed through the use of plate media. This type of problems occur more frequently with clinical sample, especially stool samples, where the sample may have had a large number of *Campylobacter* spp. but the handling of the samples (freezing/thawing, etc.) could result in the inactivation of the cells and lack of growth on plates but still a detection by PCR assays.

The use of multiplex PCR assays have allowed for the detection of more than one species of *Campylobacter* in the same poultry sample. In these cases, both *C. jejuni* and *C. coli* were detected in the samples after enrichment (Oyarzabal et al. 2007). This is not surprising due to the fact of the large number of *Campylobacter* cells colonizing live chickens. However, these findings do point out to the complexity of the epidemiology of *Campylobacter* in live chickens and the resulting contamination of food products. Several of the research multiplex PCR assays that have been used in our laboratories have been validated through a large number of samples tested in different studies and are relatively simple to incorporate (Linton et al. 1997; Cloak and Fratamico 2002; Oyarzabal et al. 2005, 2007; Persson and Olsen 2005; Zhou et al. 2011). Yet, like other molecular techniques, the incorporation of PCR requires the initial training of laboratory personnel and the investment in equipment for identification of the amplified products with methods other than the traditional gel electrophoresis protocols. To avoid staining gels with ethidium bromide, real-time PCR protocols are the best choice but they are more expensive than conventional PCR assays. In South American countries, PCR assays

have been incorporated in clinical and food microbiology laboratories, primarily in Chile, Brazil, and Costa Rica (Rivera et al. 2011; Silva et al. 2014; Zumbado-Gutiérrez et al. 2014).

2.4 Perspectives and Future Trends

There is a large body of research on the isolation and identification of *Campylobacter* spp. in poultry carcass rinses, with mainly samples collected in processing plants. As the methodology of testing for *Campylobacter* moves toward detection in retail samples, or in other segments in the farm-fork continuum, there will be a need to validate some of these methods for the new sample types or even for new matrices.

The use of chromogenic agars will increase as they provide a simpler system for detection, especially for small laboratories where there are limited resources or do not handle large volume of samples. But the trend in food microbiology laboratories is that of consolidation, with fewer laboratories with more automated equipment that can provide a very competitive price for testing and can deal with a very large number of samples.

One trend that is difficult to predict is the increase in small food processors across different regions. Some of these processors are small and have many limitations. If this trend continues, there will be a need for regional laboratories to capture the sampling coming from these small processing plants as they start to be scrutinized by regulatory agencies.

PCR assays and other versions of molecular techniques based on DNA detection will continue to expand and be incorporated in clinical and microbiology laboratories. Automation of the protocols, including sample handling and preparation, will increase and may even result in systems that can be adapted for the testing of small number of samples without significantly increase the cost. Different versions of microfluidic arrays have been generated in the past 10 years and some versions may find applicability in food microbiology laboratories. Most of these systems offer high sensitivity and reduced time for detection.

Sample validation and protocol standardization across different countries continues to be an area of expansion and challenges. And as we continue recreating food systems that provide local foods and are more segmented, there will be more challenges to the incorporation of testing methods that prioritize public health.

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Chapter 3
Colonization of *Campylobacter jejuni* in Poultry

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Abstract *Campylobacter jejuni* produces several virulence factors to colonize the poultry gastrointestinal tract. In commercial broiler chickens, this colonization appears to be predominantly commensal, with *C. jejuni* found in large numbers in the intestinal mucosa. The consumption of contaminated chicken meat is the major source of human campylobacteriosis, which makes the understanding of the mechanisms of colonization important in the search for alternatives for the treatment and prevention of such zoonosis. In the past few years, the research on the colonization mechanism of *C. jejuni* in chickens has significantly advanced. This chapter summarizes our increasing knowledge about the main virulence factors involved in the colonization of poultry.

Keywords Chicken · Colonization · Campylobacteriosis · Virulence factors

3.1 Introduction

Campylobacter jejuni is one of the most prevalent etiologic agents of food-borne human gastroenteritis in the developed countries (EFSA 2012). The high frequency of infection, severity of post-infection syndromes, and the increase of antimicrobial resistant strains made this pathogen as of major importance for public health (Svensson et al. 2015). The commercial broiler chickens are frequently highly colonized with *Campylobacter* and the consumption of contaminated chicken meat

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is the most incriminated source for the transmission of human campylobacteriosis, responsible for up to 80 % of cases (Hermans et al. 2011; EFSA 2013).

Despite this high prevalence in chickens, in the large majority of cases, these animals remaining asymptomatic (Young et al. 2007) and therefore, the bacteria is considered a commensal (Korolik et al. 1998; Stas 1999; Young et al. 2007; EFSA 2010; Hermans et al. 2012). Nevertheless, *C. jejuni* can invade the chicken's intestinal mucosa (Van Deun et al. 2008), and it can be isolated from other organs of the poultry's body (Cox et al. 2006; Whyte et al. 2006; Luber and Bartelt 2007; Medeiros et al. 2008; Jennings et al. 2011), including the blood circulation (Richardson et al. 2011).

The first association between *Campylobacter* and avian disease was the avian vibrionic hepatitis (AVH) (Tudor 1954; Lukas 1955; Hofstad et al. 1958; Moore 1958; Sevolan et al. 1958; Whenham et al. 1961). After these findings, AVH had little attention and the evidences were not sufficient to support an association between *C. jejuni* and the classic syndrome of liver disease (Soerjadi et al. 1982). Finally, after some years, Crawshaw and Young (2003) and Jennings et al. (2011) have reported the association between *C. jejuni* and AVH.

Some studies have been shown that chickens infected by *Campylobacter* spp. present negative consequences on growth and weight performances (Dhillon et al. 2006; Gharib-Naseri et al. 2012; Awad et al. 2014, 2015b). The colonization of the intestine has been associated with jejunal histomorphological changes (Lamb-Rosteski et al. 2008; Humphrey et al. 2014; Awad et al. 2015a), higher intestinal permeability (Awad et al. 2015a), altered intestinal electrolyte transport (Awad et al. 2015a), altered mRNA expression of intestinal nutrient transporters (Awad et al. 2014) with decrease in intestinal nutrient absorption (Awad et al. 2015b), and intracellular Ca^{2+} signaling interference (Awad et al. 2015b). The nutrient absorption impairment can lead to an accumulation of nutrients in the intestinal lumen and, for consequence, favors *C. jejuni* colonization (Guccione et al. 2008; Hofreuter et al. 2008; Awad et al. 2015b). Additionally, *C. jejuni* can lead to diarrhea in fast-growing broilers due to a strong inflammatory response to the infection (Humphrey et al. 2014). Finally, the inoculation of *C. jejuni* in chicken embryos results in high embryonic mortality (Lam et al. 1992; Fonseca et al. 2011).

Regardless the consequences of the presence of bacteria, colonization is part of the complex interactions between host and pathogen. The colonization is, therefore, a multifactorial process which depends on the genetic characteristics and virulence of the strains, as well as host characteristics.

Campylobacter jejuni pan genome contains 2427 gene families with estimated 1295 gene families found in its core genome (Friis et al. 2010). There is diversity of gene distribution in field strains associated with chicken colonization (Thibodeau et al. 2013) and isolates of *C. jejuni* differ in their gut colonizing ability (Ringoir and Korolik 2003; Hanel et al. 2009; Chaloner et al. 2014). While some strains fail to colonize the intestine of chickens or are rapidly cleared after colonization (transient colonizer), others are responsible for an efficient and sustained colonization of the poultry intestinal tract (Korolik et al. 1998; Ringoir and Korolik 2003; Hanel et al. 2009; Hermans et al. 2011).

The number of *C. jejuni* sufficient to colonize poultry is only 2 cfu in 1-day-old chicks and 200–1000 cfu in 14-day-old birds (Knudsen et al. 2006). Even under low amount of inoculum (less than 100 cfu) (Hendrixson and DiRita 2004), the number of *C. jejuni* in intestinal content of asymptomatic birds can reach 10^8 cfu g^{-1} (Dhillon et al. 2006) to 10^{10} cfu g^{-1} (Young et al. 2007; Larson et al. 2008). After ingestion, *C. jejuni* reaches the cecum and multiplies, stabilizing at 24 h after entrance (Coward et al. 2008). Probably due to the presence of maternally-derived antibodies, the young chicks became colonized only at an age of two to four weeks (Hermans et al. 2011).

In the past few years, the research on the colonization mechanism of *C. jejuni* in chickens has significantly advanced. This chapter summarizes our increasing knowledge about the main virulence factors involved in the colonization of poultry.

3.2 *C. jejuni* Virulence Factors for Colonization

The ability to adapt to hostile environments and to the host immune response seems to be a key factor to *C. jejuni*'s effective colonization of the gut. In order to colonize the intestinal tract of poultry, after fecal-oral transmission, microorganisms migrate toward intestinal environment driven by chemoattractant substances (chemotaxis). To reach the gastrointestinal tract, *C. jejuni* must tolerate many adverse environmental conditions such as: pH variation, oxygen limitation of the cecum, oxidative stress, increased osmotic pressure and the presence of digestive fluids, including bile salts (Louis and O'Byrne 2010). There is also the presence of some proteins with antimicrobial activities such as beta-defensin gallinacin-6 in the proximal digestive tract (van Dijk et al. 2007). To survive the stresses in the host environment, *C. jejuni* has several adaptive mechanisms (Bolton 2015).

Table 3.1 and Fig. 3.1 show a summary of *C. jejuni* colonization factors and the colonization process discussed in this chapter.

3.2.1 Chemotaxis

C. jejuni is attracted by bile, the amino acids aspartate, cysteine, serine and glutamate, and the salts of the organic acids citrate, fumarate, a-ketoglutarate, malate, pyruvate, and succinate (Hermans et al. 2011). In addition, the mucin component (the major constituent of the mucus glycoprotein), specifically L-fucose also act as chemoattractant. In vitro, L-asparagine, formate, and D-lactate are detected by the transmembrane methyl-accepting chemotaxis proteins (MCPs) of *C. jejuni* and are also involved in chemotaxis (Vegge et al. 2009).

The changes in the local concentration of a stimulus can be sensed directly in classical chemotaxis, by metabolism-independent fashion, often by MCPs also known as Transducer like proteins (Tlp). MCPs are signaling proteins, sensitive to

Table 3.1 *Campylobacter* virulence factors for colonization of the intestinal tract

Function	Encoding gene(s)	Virulence factor(s)
Chemotaxis	*cj0019c* or *docB* or *tlp10*	Methyl-accepting chemotaxis proteins (MCPs) also called transducer-like proteins (Tlp)
	cj0262c or *docC* or *tlp4*	
	tlp8	
	tlp6	
	cetA and *cetB*	*Campylobacter* energy taxis system proteins CetA (Tlp9) and CetB (Aer2)
	cheA, *cheB* and *cheR*	Chemotaxis proteins; Che A, B, R
	cheY	CheY, response regulator controlling flagellar rotation
	luxS	AI-2 biosynthesis enzyme
Motility	*flaA*	FlaA, the major flagellin protein
	flaB	FlaB, the major flagellin protein
	fliA	σ^{28} promoter regulates *flaA* gene expression
	rpoN	σ^{54} promoter regulates *flaB* gene expression
	flgS-flgR	Flagellar signal transduction system
	flgK	Possible flagellar hook associated protein
	maf5	Motility accessory factor 5 (flagellar biosynthesis)
	motAB	Flagellar motor components
Oxygen tension and oxidative stress defense	*dcuA* or *cj0088*	Anaerobic C4-dicarboxylate transporter
	dcuB or *cj0671*	
	dctA or *cj1192*	
	aspA or *cj0087*	Aspartate-ammonia lyase
	katA	Catalase (convert hydrogen peroxide to water and oxygen)
	sodB	Superoxide dismutase, antioxidant protein
	tpx	Thiol peroxidase
	bcp	Bacterioferritin comigratory protein
	dps	Bacterioferritin family proteins
	ahpC	Alkyl hydroperoxide reductase or alkylhydroperoxidase
	msrA/B	Methionine sulphoxide reductases
	cj1386	Ankyrin-containing protein involved in heme trafficking to catalase
	rrc or *cj0012c*	Rbo/Rbr like protein

(continued)

Table 3.1 (continued)

Function	Encoding gene(s)	Virulence factor(s)
	cj0358	Putative cytochrome c peroxidase
	fur	Ferric uptake regulator
	perR	Peroxide-sensing regulon
	cj1000	Putative transcriptional regulator (LysR family)
Factors which indirectly contribute to oxidative stress defense	*chaN*, *exbB1*, and *tonB2*	Cation transport/binding proteins
	acnB	Energy metabolism
	flhB, *flgD*, *flgH*, *flgI*, *flgK*, *flgL*, and *pseB*	Surface structures
	cj1623 and *flgP*	Membranes, lipoproteins, and porins
	cj0062c, *cj0260c*, *cj0344*, *cj1388*, and *cj1159c*	Hypothetical proteins
	pstC	Putative phosphate transport system permease protein
	flgR	σ^{54}—associated transcriptional activator
	cj0947c	Putative carbon-nitrogen hydrolase
Bile resistance	*cmeABC*	*Campylobacter* multidrug efflux pump
	cmeR	Transcriptional repressor of *cmeABC*
	cj0561c	Putative periplasmic protein
	cbrR (cj0643)	*Campylobacter* bile resistance orphan response regulator
Nutrient and micronutrient acquisition systems	*cfrA*and *cfrB*	Outer membrane ferric enterobactin FeEnt receptors
	feoB	Protein transport soluble ferrous
	chuA	Hemin uptake outer membrane protein
	cj0178	Putative transferring-bound iron utilization outer membrane receptor
	cj0143 or *znuA*	Putative zinc ABC transport system
Protein glycosylation	*plg*	N-linked glycosylation
	cj1496c	Glycoprotein with unknown function
Secretion system	*flhA*, *flhB*, *fliQ*, *fliP*, *fliO*and *fliR*	Components of the flagellar T3SS
	ciaB	73-kDa protein involved in adhesion
	cdtA, *cdtB* and *cdtC*	Cytolethal distending toxin subunits
	tssJ, *tssL* and *tssM*	Membrane-associated proteins (T6SS)
	tssB, *tssC*, *tssD* or *hcp*, *tssE* and *tssI* or *vgrG*	Proteins with function related to tailed bacteriophage components (T6SS)
	tssA, *tssF*, *tssG*, *tssH* (*tagH*) and *tssK*	Proteins with unknown function (T6SS)

(continued)

Table 3.1 (continued)

Function	Encoding gene(s)	Virulence factor(s)
Adhesion and invasion	*cadF*	Outer membrane protein
	pldA	Phospholipase A
	capA	*Campylobacter* adhesion protein A
	cj1279c or *flpA*	Fibronectin-like protein A
	peB1	Periplasmic binding protein
	jlpA	42-kDa lipoprotein involved in adhesion to Hep-2 cells
	virB11	Type IV secretion system
	peb3	Transport protein
	peb4	Chaperone involved in exporting proteins to the outer memberane

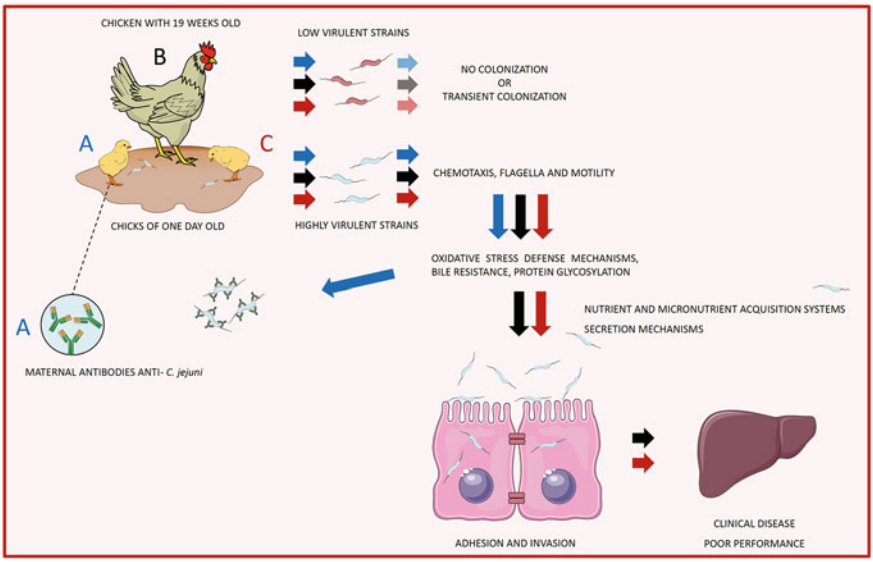

Fig. 3.1 *C. jejuni* **colonization of the poultry intestinal tract**. After ingestion, low virulent strains fail to colonize the intestine of chickens or are rapidly cleared after colonization. Highly virulent strains are responsible for an efficient and sustained colonization of the poultry intestinal tract. Probably due to the presence of maternally-derived antibodies, microorganisms are destroyed in some young chicks (*A*, *blue arrows*) or it becomes colonized only at an age of two to four weeks. Chickens (*B*, *black arrows*) and unprotected chicks (*C*, *red arrows*) are susceptible to *C. jejuni* colonization/infection. After entrance, *C. jejuni* reaches the cecum, evades the immune system, and multiplies due to its virulence factors. In the large majority of cases, the animals remain asymptomatic although there may be intestinal histomorphological and functional changes, for example due to the bacterial nutrient and micronutrient acquisition system that led to accumulation of nutrients in the intestinal lumen favoring bacterial colonization. In some cases, *C. jejuni* can adhere and invade the chicken's intestinal mucosa producing and secreting virulence factors and, as a consequence, causing disease

environmental components and responsible for translating signals changing motility, either towards the chemoattractants or against the direction to chemorepellents (Young 2008; Chandrashekhar et al. 2015).

C. jejuni has a high number of Tlps and due to this is characterized by a complex lifestyle and a markable ability to interact with the host and other bacteria (Lacal et al. 2010). In *C. jejuni*, Tlps are classified into three groups based on predicted domain structure and homology to chemoreceptors of other bacteria: Group A (Tlp1, 2, 3, 4, 7 and 10), Group B [Tlp9 (CetA)], and Group C Tlps (Tlp5, 6, and 8) (Marchant et al. 2002; Vegge et al. 2009; Chandrashekhar et al. 2015). The expression of Tlp genes can vary based on growth conditions, isolation source, and strains (Day et al 2012).

The mutation of *cj0019c* (DocB or Tlp10) and *cj0262c* (DocC or Tlp4) genes severely affected chick colonization capacity of *C. jejuni* (Hendrixson and DiRita 2004; Hartley-Tassell et al. 2010; Li et al. 2014; Chandrashekhar et al. 2015). Moreover, Tlp1 (aspartate receptor) was described as an important factor for chick colonization since a *tlp1*-isogenic mutant showed reduced colonization ability (Hendrixson and DiRita 2004; Hartley-Tassell et al. 2010). Additionally, these chemoreceptors (*tlp1*, *tlp4,* and *tlp10*) have been identified as being important for invasion of *C. jejuni* in mammalian and chicken embryo intestinal cells (Ziprin et al. 2001; Vegge et al. 2009). Colonization potential is reduced in mutantes Δ*tlp6* and Δ*tlp8* colonizing duodenum and jejunum of chickens (Chandrashekhar et al. 2015) while Δ*tlp9* mutant is still able to colonize all the examined segments of the intestine (Chandrashekhar et al. 2015). Despite the advances, the overall understanding of the importance of this class of proteins for chicken colonization should still be studied.

The CheR (methyltransferase) and CheB (methylesterase) enzymes are both involved in a methylation-dependent chemotaxis pathway being responsible for methylation of MCPs and thus, for its regulation. A Δ*cheBR* mutant had reduced ability to colonize chick cecum (Kanungpean et al. 2011).

The survival of *C. jejuni*, their migration towards favorable conditions and consequent colonization is due to chemotaxis. The *cet*A and *cet*B genes appear to be involved in chemotaxis by energy taxis. *C. jejuni* CetA and CetB proteins can alter flagellar rotation and direct the microorganism towards new environments with higher energy-producing (Hendrixson et al. 2001). CetA assist in energy taxis along with the signal sensing protein CetB (Marchant et al. 2002; Vegge et al. 2009).

C. jejuni uses phosphorylation and dephosphorylation cascade systems to control flagellar motility in response to environmental chemical changes (Miller et al. 2009). In cell culture or mouse model, the major pathway of chemotaxis signal transduction is the histidine kinase regulatory system and its regulatory protein is CheY (Hendrixson and DiRita 2004). When CheA (encoded by *cj0284c*) binds MCP chemoreceptor, there is a phosphorylation which transfers a phosphate group to CheY. Phosphorylated CheY interacts with the flagellar motor resulting in the redirection of their orientation (Miller et al. 2009; Hartley-Tassell et al. 2010). CheA is one of the fundamental components also for chemotaxis in poultry (Hu et al. 2014).

Autoinducer 2 (AI-2), encoded by the *luxS*, is a self-inducing quorum sensing molecule that controls phenotype of pathogenic bacteria regulating colonization and virulence (Schauder et al. 2001; Quiñones et al. 2009). Autoinducer 2 (AI-2) regulates functions such as motility, autoagglutination, biofilm formation, sensitivity to hydrogen peroxide, and the transcription of cytolethal distending toxin genes (*cdtABC*—the role of Cdt is discussed in more detail in Sect. 3.2.7 of this chapter) (Jeon et al. 2003, 2005; Reeser et al. 2007; He et al. 2008; Plummer 2012). Mutant strains that have lost *luxS* show decreased chemotaxis towards amino acids asparagine, aspartate, glutamate, or glutamine (Quiñones et al. 2009).

3.2.2 *Flagella and Motility*

Campylobacter is characterized by a rapid, darting motility that is mediated by polar flagella and these structures have long been recognized as crucial to pathogenesis of these bacteria. This organelle is not only involved in cell motility and chemotaxis, but also assumes a role in adherence and invasion of host cells, protein secretion, autoagglutination, and biofilm formation (Wassenaar et al. 1993; Guerry 2007).

The *Campylobacter* flagella are composed of a major flagellin, called FlaA, and a minor flagellin, called FlaB, encoded by two homologous genes, *flaA* and *flaB*, respectively (Nuijten et al. 1990; Guerry et al. 1991). The *flaA* gene is regulated by[1] σ^{28}, the classical flagellin promoter, whereas *flaB* is regulated by a σ^{54}-dependent promoter, which are responsible for flagellar biosynthesis and regulation of a large number of genes involved in motility, protein secretion, and invasion (Nuijten et al. 1990; Guerry et al. 1991; Kinsella et al. 1997; Hendrixson and DiRita 2003; Fernando et al. 2007; Bolton 2015). The flaA promoter is upregulated by chemotactic effector such as aspartate, glutamate, citrate, fumarate as well as bovine bile, deoxycholate, L-fucose, high osmolality and pH (Hermans et al. 2011). Moreover, *rpoN* (σ^{54}) and *fliA* (σ^{28}) and also *flgS/flgR* and *flgK* genes, assume an important role in the process of adhesion and colonization of the avian intestinal tract (Hendrixson and Dirita 2004; Wosten et al. 2004; Fernando et al. 2007; Bolton 2015).

One of the first findings, reported more than twenty years ago, indicates that intact and motile flagella are important colonization factors for *C. jejuni* in chickens (Nachamkin et al. 1993). Experiments with mutants have shown that FlaA but not FlaB is essential for colonization of chickens, although probably both are needed for full motility (Jones et al. 2004; Wassenaar et al. 1993; Neal-McKinney et al. 2010).

[1]A sigma factor (σ) is a bacterial transcription initiation factor that enables specific binding of RNA polymerase to gene promoters. These proteins are distinguished by their characteristic molecular weights (e.g. σ^{28} refers to the sigma factor with a molecular weight of 28 kDa).

The motility accessory factor 5 (*maf5*) is another important gene for the formation of flagellum and mutants of *maf5* showed a decrease in the capacity of adherence to the avian intestinal tract (Jones et al. 2004). The *motAB* genes from flagellar motor, which enables the rotation of the flagellum, are also essential for colonization of poultry, in addition to being involved in the defense against oxidative stress (Mertins et al. 2013).

Although motility is an important factor for the bacterial colonization (Wassenaar et al. 1993), immobile mutants may promote colonization in a short time with lesser bacterial load in the cecum (Hendrixson and DiRita 2004). This shows that there are many factors involved in the colonization of *C. jejuni* in poultry beyond motility.

3.2.3 Oxygen Tension and Oxidative Stress Defense

The ability to adapt to hostile environments and to the host immune response seems to be a key factor to *C. jejuni*'s effective colonization of the gut. In order to colonize the intestinal tract of poultry, microorganisms must tolerate many adverse environmental conditions in the gastrointestinal tract, such as pH variation, oxygen limitation of the cecum, oxidative stress, increased osmotic pressure, and the presence of digestive fluids, including bile salts (Louis and O'Byrne 2010). There is also the presence of some proteins with antimicrobial activities such as beta-defensin gallinacin-6 in the proximal digestive tract (van Dijk et al. 2007). To survive the stresses in the host environment, *C. jejuni* has several adaptive mechanisms (Bolton 2015).

The intestinal environmental condition ranges from a relatively aerobic environment in the duodenum to a progressively anaerobic one in the ileum and cecum. To support the low oxygen demand in the cecum, *C. jejuni* uses other substances as fumarate and succinate as final electron acceptors. In low levels of oxygen there is an increase of expression of genes *dcuA* (*cj0088*), *dcuB* (*cj0671*), *dctA* (*cj1192*) whose function as an anaerobic C4-dicarboxylate transporter and *aspA* (*cj0087*) gene with function aspartate-ammonia lyase. This seems to be involved with a regulatory system not yet characterized, allowing *C. jejuni* to survive in an oxygen lacking environment (Woodall et al. 2005).

C. jejuni is a microaerophilic microorganism, which optical cultivation conditions are 5 % oxygen, 10 % carbon dioxide, and 85 % nitrogen (Bolton and Coates 1983). The presence of oxygen can create superoxide and hydrogen peroxide, which are partially reduced species that can oxidize biomolecules whose oxygen itself reacts poorly. Several different sources of reactive oxygen species (ROS) are produced during the colonization, including those from incomplete reduction of oxygen by *C. jejuni* metabolism, from the immune response and from the gut microbiota. *C. jejuni* contains different ROS detoxification pathways to survive oxidative stress and colonize its host. Detoxification enzymes as KatA (catalase), SodB (superoxide dismutase), Tpx (thiol peroxidase), Bcp (thiol peroxidase), Dps

(bacterioferritin), AhpC (alkylhydroperoxidase), MsrA/B, and Cj1386 (an ankyrin-containing protein involved in heme trafficking to catalase) have been identified and characterized (Pesci et al. 1994; Baillon et al. 1999; Ishikawa et al. 2003; Atack et al. 2008; Atack and Kelly 2008; Flint et al. 2012).

It has recently been shown that several other proteins are apparently directly involved in protective mechanisms against oxidative stress or indirectly involved contributing to metabolic rearrangements, which affect the endogenous production of ROS (Flint et al. 2014). The genes involved in the direct detoxification of oxidants include the well-characterized *katA*, *ahpC*, *sodB* and the newly reported *rrc* (*cj0012c*) and *cj0358* genes. Unlike the former genes, the last two not significantly affected the ability of *C. jejuni* to colonize the cecum of chicks (Palyada et al. 2009; Flint et al. 2014). The genes of cation transport/binding proteins (*chaN*, *exbB1*, and *tonB2*), energy metabolism (*acnB*), surface structures (*flhB*, *flgD*, *flgH*, *flgI*, *flgK*, *flgL*, and *pseB*), membranes, lipoproteins, and porins (*cj1623* and *flgP*), some hypothetical proteins (*cj0062c*, *cj0260c*, *cj0344*, *cj1388*, and *cj1159c*), besides *pstC* (Putative phosphate transport system permease protein), *flgR* (σ^{54}—associated transcriptional activator), and *cj0947c* (Putative carbon-nitrogen hydrolase) are also important factors indirectly contributing to antioxidant defense (Flint et al. 2014). The mutation of some of these genes was sufficient for significantly reducing the ability of strains to colonize chick ceca, revealing the significant role that oxidative stress defenses play during colonization (Flint et al. 2014).

Campylobacter spp. has evolved several adaptative mechanisms in order to survive to ROS and oxygen tension. However, the regulation genes involved in these stress response are poorly understood. Most genes involved in oxidative stress response in *C. jejuni* are controlled by the regulators PerR and Fur (Palyada et al 2009; Butcher et al. 2010). Additionally, the *cj1000* gene, which encodes putative transcriptional regulator (LysR family), participates in the adaptation of bacteria to a low-oxygen environment; it is involved in energy metabolism and oxidative stress defenses; therefore, it seems to be important to colonization of 1-day old chicks (Dufour et al. 2013).

3.2.4 Bile Resistance

Campylobacter jejuni also needs to resist bile salts for successful colonization. The bile acids, such as cholates and deoxycholates (DOCs), are a type of detergent, which kills bacteria through disruption the lipid bilayer of cell membrane and unfolding and aggregation of proteins in the bacterial cytoplasm (Cremers et al. 2014; Gunn 2000). There are several mechanisms that affect the resistance of bacteria to bile, including modulation of the synthesis of lipooligosaccharide (LOS) and porins, active expulsion of bile by efflux pumps (e.g., *Campylobacter* multidrug efflux—CME, encoded by the operon *cmeABC*), the presence or mutation of regulatory genes (e.g., *cmeR*, *cbrR,* and *cj0561c*) which may induce or repress genes associated with bile resistance, and production of bile acid hydrolase

(Lin et al. 2005; Raphael et al. 2005; Woodall et al. 2005; Lin and Martines 2006; Guo et al. 2008; Merritt and Donaldson 2009; Dzieciol et al. 2011; Iwata et al. 2013).

3.2.5 Nutrient and Micronutrient Acquisition Systems

Bacterial colonization of the host gastrointestinal tract depends in part on the ability of the microorganism to acquire essential growth nutrients. *C. jejuni* influences the expression of nutrient transporter genes of chicken intestinal cells. In the presence of *C. jejuni* there is a decrease in gene expression carriers sodium/glucose co-transporter (SGLT-1), peptide transporter (PepT-1), glucose transporter (GLUT-2), cationic amino acid transporter (CAT-2) and excitatory amino acid transporter (EAAT-3), and L-type amino acid transporter (y(+)LAT-2) (Awad et al. 2014, 2015a). Indeed, recently it has been demonstrated that the glucose uptake is reduced due to *C. jejuni* infection, which coincided with a decrease in body weight gain of the infected birds. Additionally, a reduction in the amount of the amino acids (serine, proline, valine, leucine, phenylalanine, arginine, histidine, and lysine) in ileal digesta was observed (Awad et al. 2015b). It is possible that accumulation of nutrients in the intestinal lumen may favor *C. jejuni* replication and colonization. (Awad et al. 2014, 2015a).

C. jejuni needs iron and zinc ion for colonization, but these micronutrients are extremely limited within the host. Thereby, *C. jejuni* uses a wide variety of strategies to obtain these nutrients (Zeng et al. 2009, 2013; Xu et al. 2010; Naikare et al. 2013). Siderophores are high-affinity iron chelating compounds elaborated by bacteria to scavenge iron from the host (Neilands 1995). These small molecules are secreted into the extracellular environment, bind iron ions, and are then imported by highly specific outer membrane proteins (OMPs) of Gram-negative bacteria (Naikare et al. 2013). Enterobactin, salmochelin, and bacillibactin are examples of these chelating molecules (Raymond et al. 2003; Dertz et al. 2006; Muller et al. 2009). Although *C. jejuni* is unable to synthesize siderophores, it is able to utilize exogenous siderophores as an iron source and, therefore, presents several genes involved iron acquisition process (Palyada et al. 2009; Naikare et al. 2013). The OMP proteins, CfrA and CfrB, are responsible for capturing a wide variety of siderophores (Palyada et al. 2004; Xu et al. 2010; Naikare et al. 2013) totally dependent on the TonB-ExbB-ExbD energy transduction system, which provides the energy required to transport these ligands across the outer membrane of the bacterium (Naikare et al. 2013). *C. jejuni* also presents the FeoB protein considered the major route for the transport of ferrous iron across the cytoplasmic membrane (Naikare et al. 2006). Additionally, the *chuA* gene encodes ChuA protein, that uptake hemoglobin or hemin as an iron source and *cj0178* gene encodes Cj0178, a putative transferrin-bound iron utilization outer membrane receptor, which might be required for *C. jejuni* to colonize chicks (Palyada et al. 2004; Woodall et al. 2005). There are few studies on genes and proteins involved in the acquisition of zinc to

colonization in poultry, but Davis et al. (2009) found that a mutant lacking *cj0143* (*znuA*)—the periplasmic component of a putative zinc ATP-binding cassette (ABC) transport system—is growth deficient in zinc-limiting media, as well as in the chick gastrointestinal tract, decreasing colonization potential in chickens.

Finally, calcium is another important nutrient, particularly for the invasion process. *C. jejuni* induces the mobilization of intracellular Ca^{2+} which has effects on cellular functions and enables the cell invasion by microvillar cytoskeleton rearrangement (Hu et al. 2005; Awad et al 2015b).

3.2.6 Protein Glycosylation

Protein glycosylation is an enzyme-catalyzed covalent attachment of glycans in amino acid side chains of proteins via the N- or O-linkage (Lu et al 2015). *C. jejuni* modifies its flagellar proteins and major outer membrane proteins (MOMP) with O-linked glycans (Guerry et al. 2006; Mahdavi et al. 2014; Zebian et al. 2015), besides to modify numerous periplasmic and membrane proteins with N-linked glycans (Guerry et al. 2006). The glycosylation of MOMP is required for the optimal colonization of chickens by *C. jejuni* (Mahdavi et al. 2014). Protein N-glycosylation influences the pathogenesis of *C. jejuni* (Karlyshev et al. 2004) since mutations in this pathway produce a reduction at chicken colonization (Hendrixson and DiRita 2004). In chicken, protein N-linked glycosylation of *C. jejuni* surface proteins protects bacterial proteins from cleavage due to gut proteases (Alemka et al. 2013; Lu et al. 2015).

The *pglH* is a part of *plg* (protein glycosylation pathway) gene cluster and it is involved in protein glycosylation promoting the colonization process (Karlyshev et al. 2004). In addition to *pglH* gene, *pglE*, *pglF* (Hendrixson and DiRita 2004), and *cj1496c* genes (Kakuda and DiRita 2006), which are part of a multigene locus encoding a general protein glycosylation system, seems to be involved with colonization in chicks (Hendrixson and DiRita 2004).

3.2.7 Secretion System

The *C. jejuni* secretion mechanisms in chicken are a subject still little explored. Besides motility, flagellar apparatus has been likened to the type III secretion systems (T3SS), in which effectors molecules are injected directly into host cells (Konkel et al. 1999; Samuelson and Konkel 2013). A series of work have been published reporting the extracellular release of *C. jejuni* proteins through the flagellum, which represents a T3SS by definition (Desvaux et al. 2006). The first identified secreted factor was the *Campylobacter* invasion antigen (CiaB), a 73-kDa protein (Konkel et al. 1999, 2004). The CiaB protein is involved in exportation of other Cia proteins and in bacterial invasion of host cells (Konkel et al. 1999), and it

was reported that the invasion might be an important colonization determinant of *C. jejuni* in chicks because mutations in *ciaB* severely impair cecal colonization (Ziprin et al. 2001)

Cytolethal distending toxin (Cdt), encoded by three adjacent genes *cdtA*, *cdtB,* and *cdtC*, is another protein secreted by *C. jejuni*. Cdt is involved in the arrest of the cell cycle at the G2/M phase (cell cycle control), causing progressive cellular distension and inducing host cell apoptosis of human and chicken epithelial cells (Abuoun et al. 2005; Fonseca et al. 2015). The *cdtB* gene encodes the component responsible for the activity and toxicity of Cdt, while the *cdtA* and *cdtC* genes encode proteins responsible for binding and internalization into the host cell (Abuoun et al. 2005). Additionally, Cdt is involved in the pathogenesis of inflammatory diarrhea in humans (Whitehouse et al. 1998), but does not solely determine severity of infection and clinical outcome (Mortensen et al. 2011). The *cdt* genes are frequent in *C. jejuni* isolates from laying hens (Dipineto et al. 2011), as well as from broiler carcasses and vegetables at the points of sale (de Carvalho et al. 2014) and seem to be important for colonization since mutants that lack *cdt* cannot colonize chicks (Biswas et al. 2006).

Recently, the novel type VI secretion system (T6SS) has been recognized (Jani and Cotter 2010). The T6SS is composed of 13 conserved genes: *tssJ*, *tssL,* and *tssM* encoding membrane-associated proteins; genes *tssB*, *tssC*, *tssD* (*hcp*), *tssE,* and *tssI* (*vgrG*) encoding proteins with function related to tailed bacteriophage components; and genes *tssA*, *tssF*, *tssG*, *tssH* (*tagH*), and *tssK* encoding proteins with unknown function (Silverman et al. 2012). In vitro studies with human cells and murine macrophages and in vivo experiments with mice have shown that the T6SS of *C. jejuni* strains participates in host cell adhesion, invasion, adaptation to a specific bile salt, deoxycholic acid, which together contribute to the ability of *C. jejuni* to establish colonization (Lertpiriyapong et al. 2012).

3.2.8 Adhesion and Invasion

Bacterial adherence and entrance into epithelial cells are critical steps for colonization and disease development. In the adhesion process are involved the intact flagella, adhesins, and surface-exposed proteins (Guerry 2007; Bolton 2015). The genes *cadF*, *pldA*, *capA*, *cj1279c* (*flpA*), *peB1*, *jlpA*, *virB11*, *peb3,* and *peb4* have significant role in colonization of broiler chicks (Ziprin et al. 2001; Ashgar et al. 2007; Hiett et al. 2008; Flanagan et al. 2009; Bolton 2015).

The *Campylobacter* adhesion to fibronectin (CadF) outer membrane protein is a 37-kDa protein which binds to the extracellular matrix (ECM) protein fibronectin, a glycoprotein of the extracellular matrix of the intestinal tract (Konkel et al. 1997). It was determined that this adhesin is crucial for full binding capacity of *C. jejuni* to chicken epithelial cells (Flanagan et al. 2009). Other studies conducted through the generation of mutants showed that phospholipase A, encoded by *pldA*, assume a crucial role in successful colonization of chicken cecum. This fact can be due to the

localization of this protein in the outer membrane, what might be involved in the maintenance of the functional integrity of surface exposed adhesins in some strains (Ziprin et al. 1999, 2001; Dekker 2000). However, the biological function of *pldA* is not still known and, in contrast to the highly prevalent *cadF* gene, many *C. jejuni* isolates lack the *pldA* gene (Rizal et al. 2010).

The *Campylobacter* adhesion protein A, encoded by *capA* gene, is a protein that was first identified as an autotransporter lipoprotein. The first studies showed that a mutation of *capA* results in reduced ability to adhere to chicken intestinal cells, reduced invasion capacity in human epithelial cells, and abolished colonization in chicks (Ashgar et al. 2007). However, news studies revealed that mutation of *capA* did not produce a significant decrease in the colonization capacity (Flanagan et al. 2009). It is important to mention that since this gene is absent in many *C. jejuni* poultry isolates, the genuine contribution of *capA* to successful chick colonization remains unclear (Ashgar et al. 2007; Flanagan et al. 2009).

On the other hand, a new adhesin called fibronectin-like protein A (FlpA), a potential fibronectin binding protein, has been identified as a factor that assume a crucial role in adherence to chicken epithelial cells and in colonization of chickens by *C. jejuni* (Flanagan et al. 2009). Currently, it has been proposed that CadF and FlpA act together to target fibronectin for bacterial binding and subsequent invasion by *C. jejuni* (Eucker and Konkel 2012).

Other genes and their products which may have a function in adhesion include *jlpA*, which encodes a 42-kDa lipoprotein, involved in adhesion to Hep-2 cells; *C. jejuni* virulence plasmid (pVir) genes as *virB11*, producing a component of a type IV secretion system; *peb1A* which encodes the 21-kDa protein PEB1, a periplasmic binding protein; *peb3*, responsible for a transport protein involved in the utilization of 3-phosphoglycerate and *peb4* gene, encoding a chaperone, playing a key role in exporting proteins such as CadF to the outer membrane. The mutation of these genes significantly reduces adherence and invasion of strains (Bolton 2015).

Regarding the *C. jejuni* invasion process, most studies have been made in mammalian epithelial cells, however, is known that invasion is a relevant colonization determinant of *C. jejuni* in chicks since mutations in *ciaB*, components of the flagellar T3SS, as well as in the MCP genes, both important for colonization (see above), impair in vitro invasion assays (Ziprin et al. 2001; Hermans et al. 2011; Bolton 2015).

Until now it is not clear which mechanism of invasion, "zipper" or "trigger," is used by *C. jejuni*. Various researchers indicated that *C. jejuni* induces membrane ruffling in a contact-dependent manner followed by host cell entry, first with its flagellar tip followed by the opposite flagellar end (Krause-Gruszczynska et al. 2007, 2011; Boehm et al. 2012), surprisingly sharing some features of both "zipper" and "trigger" mechanisms. It may be that *C. jejuni* has developed during evolution a strategy which shares features of both of these mechanisms, but future studies are necessary to clear up how the cell invasion occurs and what are the forces triggered by the host cell that mediate engulfment, uptake, and also membrane closure behind the bacteria entrance.

Chapter 6 addresses the invasion in more detail, discussing the types of invasion routes, intracellular traffic, virulence factors, and other aspects of colonization of poultry cells.

3.3 Conclusions

In summary, the broiler chicken gut is often colonized by *C. jejuni* and although in some cases there is a disease, in most of them, the established host-pathogen interaction is an asymptomatic colonization. *C. jejuni* is a dynamic pathogen that presents a large repertoire of virulence factors, which enable the microorganism to adapt to the most hostile environments of the host and establish an effective colonization of the intestine. Here, we discussed the oxidative stress defense, bile resistance, nutrient, and micronutrient acquisition systems, protein glycosylation, chemotaxis, secretion systems, the role of flagella and motility, and some aspects of the adhesion and invasion of chicken cells. The understanding of colonization process may allow the implementation of better control strategies of this important and zoonotic pathogen in poultry.

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Chapter 4
Immune Response After *Campylobacter* spp. Infection in Poultry

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Abstract This chapter describes the host immune responses against *Campylobacter* spp. We present an overview about both chicken and human host innate and adaptive immune responses against *Campylobacter jejuni*, with a general description of the pathogen's strategy to disrupt or evade host immune defenses. In addition, host vaccination, as an approach to protect against *C. jejuni* colonization, is reviewed. The mechanisms associated with the development of autoimmune disease after infection are also detailed. In this context, the following questions should be highlighted: Can chickens be used to study the occurrence of autoimmunity? Is innate immune response a borderline between bacteria clearance and autoimmunity? Is the vaccination of chickens effective for food safety? Can *Campylobacter* vaccination induces an autoimmune response in human hosts? How does *C. jejuni* establish long-term intestinal chicken colonization and spread to extraintestinal tissues? Is IgA antibody an immune component for autoimmunity? Is it possible to safely improve the mucosal immune response against *C. jejuni*? Are oral-delivered yolk egg antibodies an alternative to antibiotic therapy? Can enhancing chick protection longer than 3 weeks prevent intestinal colonization and meat contamination?

Keywords Inflammation · Guillain-Barré syndrome · Humoral immune response · Cellular immune response · *Campylobacter* spp

4.1 Introduction

Campylobacter jejuni is a foodborne zoonotic microorganism that infects chickens and human hosts, inducing adaptive immune response and causing intestinal inflammation and human autoimmune Guillain-Barré syndrome (Nyati et al. 2011;

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Humphrey et al. 2014; Kaakoush et al. 2015; Vegosen et al. 2015). Information about the immune response against *C. jejuni* bacteria could clarify the differences detected in clinical manifestations from human and avian hosts. Considering the relevance of the innate immune response to detect and eliminate invader pathogens, we present a review comparing chicken and human innate defense mechanisms against *C. jejuni*. In this chapter, we review the relevant characteristics of adaptive immune response.

4.2 Innate Immunity: Physical Barriers and Pattern Recognition Receptors

Human and chicken innate immune systems have evolved to recognize and eliminate invading pathogenic microorganisms through identification of pathogen-associated molecular patterns (PAMPs) or through physical barriers and bactericidal molecules (Medzhitov 2010; St Paul et al. 2013). Recognition by the host's mucosal innate immune system or evasion of detection and subversion of immune response by the invader pathogen are key mechanisms in host-versus-pathogen interactions (Wigley 2013).

The main challenge is to understand how the bacteria quickly colonize the gut of birds without causing apparent damage. The results of Van Deun et al. (2007) suggest that *C. jejuni* is able to colonize the intestinal cells of chickens by escaping rapid mucosal clearance through rapid epithelial invasion combined with fast replication in the mucus. However, a study by Humphrey et al. (2014) showed that bacteria can cause intestinal inflammation in broiler chickens and that this inflammation is dependent on factors such as the lineage of birds. Instigating questions are highlighted: Is *C. jejuni* a commensal or a pathogenic bacterium? What are the expressed molecules of *C. jejuni* that evade the chicken's innate immune system? How does *C. jejuni* infection modulate the chicken or human innate immune system?

Physical barriers, including epithelial surfaces, bile and mucus are constitutive defenses against invaders microorganism at the intestinal mucosa epithelia. Bacteria overwhelming the innate physical defenses of the host are recognized by pattern recognition receptors (PRRs) in sentinel cells in the mucosa epithelia, resulting in acute inflammation (Murphy 2012).

The epithelial surface separates the body's interstitium from the environment of the intestinal lumen. The apical, lateral and basal intestinal epithelial cell plasma membrane serves as an effective barrier. Additionally, the paracellular space is sealed by tight junctions (TJs) formed by numerous transmembrane proteins, such as claudins and occludin (Clayburgh et al. 2004; Abreu et al. 2005). *C. jejuni*'s motility suggests a paracellular route of colonization with disruption of TJs (Monteville and Konkel 2002). *C. jejuni* disrupts human TJs through modifications in the structure of occludin or by redistributing this protein to the intracellular space (Maccallum et al. 2005; Chen et al. 2006). In chickens, *C. jejuni* alters TJs by

modifications in claudin protein (Lamb-Rosteski et al. 2008). Tight junction disruption results in a lack of epithelium integrity and fluid absorption (Maccallum et al. 2005).

Another physical barrier is bile secreted by the liver and composed of bile acids, such as cholates and deoxychoaltes (DOCs). They are a type of detergent-killing bacteria that disrupting the lipid bilayer of the cell membrane, unfolding and aggregating proteins in cytoplasm bacterium (Gunn 2000; Cremers et al. 2014). The concentration of bile acids in the human intestine ranges from 0.2 to 2 %; in chicken intestines, it ranges from 0.01 to 0.7 % (Zeng et al. 2010; Cremers et al. 2014). Bacterial growth correlates inversely with bile acid concentration. However, invading pathogens avoid DOCs by an efflux pump and the O-antigen from lipopolysaccharide (LPS) (Gunn 2000; Zeng et al. 2010). *C. jejuni* is resistant to the deleterious effects of bile acids (Zeng et al. 2010). In this context, DOCs do not impair *C. jejuni* adherence or motility; bile acids induce the expression of bacterial virulence genes, improving the resistance of the bacteria (Mackichan et al. 2004; Lin et al. 2005; Malik-Kale et al. 2008; Zeng et al. 2010). Besides the resistance to bile acids, gene expression can result a high production of cytolethal distending toxin (CDT) (Van Deun et al. 2007) and accelerate the host cell invasion (Zeng et al. 2010; Stef et al. 2013). *C. jejuni* isolated from human hosts are more resistant to high concentrations of DOCs than chicken isolates (Van Deun et al. 2007). However, the chicken colonization strategy of *C. jejuni* is a short-term cell invasion and evasion to avoid cellular innate defenses, combined with fast replication in the intestinal mucus (Van Deun et al. 2008).

Associated with the top of epithelial barriers, there is a mucus gel layer formed by mucin proteins (MUC). These proteins establish a selective barrier and influence cellular growth, differentiation, transformation, adhesion, invasion and immune surveillance (Hollingsworth and Swanson 2004). There are two groups of mucins: (i) membrane-associated mucins (i.e., mucin-1, MUC1) and (ii) secreted mucins (i.e., MUC2) (Hollingsworth and Swanson 2004; Lang et al. 2006). Mucin is characterized by tandem repeat arrays that are rich in proline, threonine, and serine amino acids (PTS or mucin domain) and are glycosylated (Lang et al. 2007). Protein domains and oligosaccharide structures serve as ligands for bacteria (Hollingsworth and Swanson 2004). The phylogenetic distance between chickens and humans results in evolutionary modifications in mucus structure and glycosylation that could reflect in the pattern of *C. jejuni* intestinal colonization (Verma et al. 1994; Smirnov et al. 2004; Byrne et al. 2007). Host differences in mucus structure may transform the *C. jejuni* behavior from a pathogen in humans to commensal behavior in chickens (Byrne et al. 2007).

Membrane-associated mucin-1 (MUC1) disruption or its inappropriate expression could predispose one to infectious or inflammatory disease (Sheng et al. 2013). MUC1 protein overexpressed in response to *C. jejuni* infection can protect cell hosts against CDT-mediated apoptosis (Mcauley et al. 2007; Lindén et al. 2008). Moreover, mucin has an anti-inflammatory function against Gram-negative bacteria infection (Ueno et al. 2008) and correlates with diminished NF-kappa B activation and decreased interleukin-8 (IL-8) cytokine production (Guang et al. 2010). On the

other hand, a lack of MUC1 increases the antigen presenting cell (APC) expression of co-stimulatory molecules (CD40, CD80 and CD86), increasing secretion of tumor necrosis factor (TNF)-alpha pro-inflammatory cytokine and higher stimulation of naïve CD4+ T cells (Williams et al. 2010).

Phylogenetic differences could be detected in MUC2 PTS domain structures from humans and chickens (Jiang et al. 2013). Human MUC2 PTS domains are glycosylated by O-glycans selecting species-specific bacterial microbiota, resulting in distinct innate immune response by selected microbiota (Johansson et al. 2008). *C. jejuni* interacts with MUC2 by bacterial adhesins and the host's O-glycan, presenting higher tropism for chicken MUC2 than human or murine mucin. Apparently, O-glycan structural differences between bacteria hosts are relevant to intestinal colonization by *C. jejuni* (Naughton et al. 2013).

MUC2 demonstrates regulatory effects on *C. jejuni* growth by upregulating or downregulating bacterial virulence genes (Tu et al. 2008) and increasing intestine colonization (Biswas et al. 2007). A lack of MUC2 or disruption of a molecule's trigger intestinal inflammation is characterized by high production of pro-inflammatory cytokines, such as IL-1beta; TNF-α, and interferon (IFN)-gamma (Heazlewood et al. 2008; Johansson et al. 2008). Curiously, the intracellular protozoa (*Eimeria*)-engineered *C. jejuni* vaccine downregulated chicken MUC2 gene expression and upregulated L-1β, IL-8, and myeloid differentiation primary response gene/protein 88 (Myd-88) (Tan et al. 2014). Apparently, that engineered vaccine elicits local inflammation protecting against *C. jejuni* infection (Clark et al. 2012).

Innate antigen recognition is a pathogen recognition receptor (PRR)-dependent mechanism (Murphy 2012; Schat et al. 2014). Among PRRs, Toll-like receptors (TLR) recognize pathogen molecular associated patterns (PAMPs), activate sentinel cells, and promote phagocytosis. Activated sentinel cells producing pro-inflammatory cytokines trigger acute inflammation (Barton and Medzhitov 2003; Takeda et al. 2003; Lowenthal et al. 2013; St Paul et al. 2013; Wigley 2013). Human immune cells display 10 different TLR molecules (Murphy 2012); similarly, chickens have 10 TLRs (Brownlie and Allan 2011; Schat et al. 2014).

Chicken TLRs are key molecules in the recognition of bacteria, including TLR-2, TLR-4, TLR-5 and TLR-21 (Wigley 2013). PAMP recognition triggers a signaling cascade by adapter proteins, such as Myd88 or TIR domain-containing adaptor inducing interferon (TRIF). Translocated nuclear transcription factors such as NFκB and interferon regulatory factor 3 (IRF3) induce the transcription of pro-inflammatory cytokines (Barton and Medzhitov 2003; Yamamoto et al. 2003).

Human TLR2 (hTLR2) establish a functional heterodimeric conformation with hTLR1 or hTLR6. Chicken have two isoforms of TLR2 (chTLR2t1 and chTLR2t2) and two orthologues of hTLR1 and hTLR6, named chTLR1LA and chTLR1LB (Keestra et al. 2013). Chicken heterodimers, chTLR2t1/chTLR1LA, chTLR2t2/chTLR1LA and chTLR2t2/chTLR1LB, ligands are tri-acylated and di-acylated lipoproteins to hTLR2/hTLR1 or hTLR2/hTLR6 complex. Structurally, hTLR6 presents as two phenylalanine residues in its lipid channel that lack chTLR1. These two amino acids block the interaction of the tri-acylated ligand with the hTLR6

molecule. However, a lack of phenylalanine amino acid residues improves the ligand specificity of chTLR2 complex (Keestra et al. 2013).

Disrupted *C. jejuni* induces a NF-κB-mediated inflammation by hTLR2 or chTLR2 signaling pathways (Al-Sayeqh et al. 2010). TLR2 and IL-10 cytokine-deficient mice have diminished immunopathology post-*C. jejuni* infection (Haag et al. 2012). A TLR2-mediated inflammatory response in *C. jejuni* gastroenteritis could be down- or up-regulated depending on the network interactions between PAMPs, cytokines and PRRs (Friis et al. 2009).

Either human and chicken TLR4 molecules establish a heterodimer with myeloid differentiation protein-2 (MD-2) that recognizes bacterial Gram-negative lipopolysaccharide (LPS) (Hausmann et al. 2002). LPS consists of the lipid A moiety that anchors the molecule in the membrane and a variable number of repeating oligosaccharide units, the O-antigen (Raetz and Whitfield 2002). Lipid A modification might modulate virulence and the resistance of Gram-negative bacteria to innate immune mechanisms (Raetz et al. 2007). The differential expression profiles of innate immune genes in different Gram-negative bacteria infection models (i.e., *Salmonella* Typhimurium and *C. jejuni*) shed light on the tailored responses of the host immune system to specific microbes. It is further evidence that innate regulation of these responses is an important prerequisite to preventing the development of disease (Shaughnessy et al. 2009).

Flagellin from bacterium biding to the chTLR5 and hTLR5 signaling pathway (Keestra et al. 2013) diminished the inflammatory response against either killed or live *C. jejuni* (de Zoete et al. 2010), suggesting a modulatory effect or a TLR5-independent innate response (Andersen-Nissen et al. 2005; Johanesen and Dwinell 2006; Shaughnessy et al. 2009). Additionally, pseudoaminic acid on *C. jejuni* flagella A antigen (FlaA) induces a dendritic cells (DCs) IL-10 cytokine production via Siglec 10-receptor resulting an anti-inflammatory response (Stephenson et al. 2014). Like humans, chickens display a set of Siglec proteins; even so, Siglec human orthologous genes were not described in chickens (de Geus and Vervelde 2013). Figure 4.1 presents a summary of barriers and signaling pathways involved in chicken and human innate immune responses to intestinal colonization by *C. jejuni*.

Other relevant bacterial PAMP is the unmethylated DNA of a ligand to hTLR9 and chTLR21, which have analogous functions. Curiously, chickens and humans lack TLR9 and TLR21, respectively. However, fish display these two PRRs, mediating the antimicrobial activities (Yeh et al. 2013). The ChTLR21 receptor is precociously expressed at 18 embryonic days, predominantly in chick livers (Kannaki et al. 2015). Apparently, *C. jejuni* DNA is unable to trigger an innate immune response by hTLR9 signaling pathway activation, whereas the chTLR21 pathway could be activated by bacteria DNA (de Zoete et al. 2010). Transient chTLR21 gene expression follows *C. jejuni* infection, associated with a low increase in pro-inflammatory cytokine and chemokine production (Shaughnessy et al. 2009).

In conclusion, phylogenetic distance might explain the difference between innate immune response from chickens and human hosts. Physical barriers, such as mucin

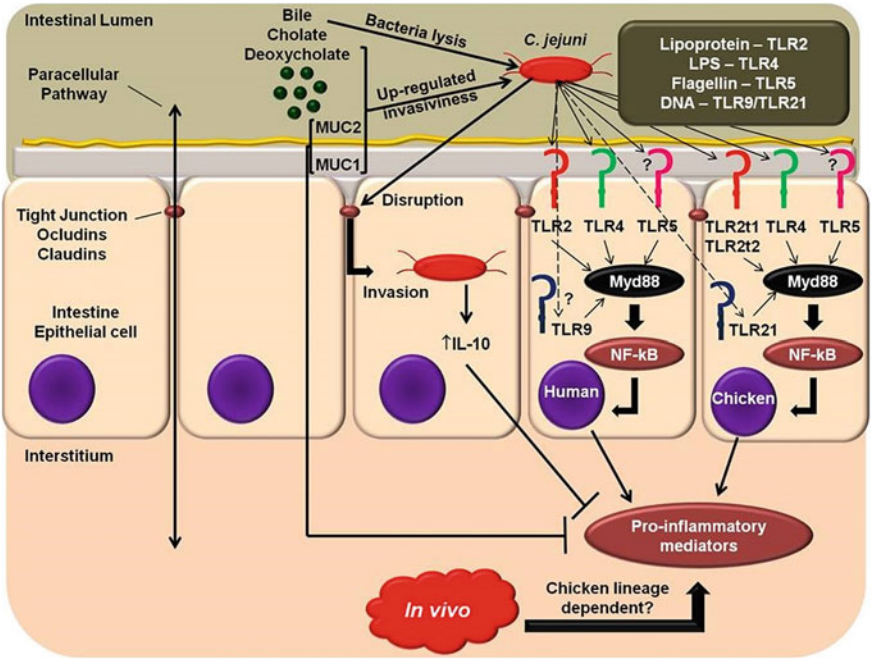

Fig. 4.1 Physical barriers, molecules and signaling activation pathways involved in chicken and human innate immune responses to intestinal colonization by *C. jejuni*. Bile acids, mucus layers, tight junctions and epithelial cells are the major physical barriers to protect hosts against *C. jejuni* colonization. However, in vitro, deoxycholate and mucin composition induce the invasive ability of *C. jejuni* and modulate host innate immune responses. In vitro, bacterial LPS, lipoproteins and DNA can activate TLR signaling pathways and result in pro-inflammatory cytokines and chemokines production

composition, and PRRs molecules might influence the anti-inflammatory response against *C. jejuni* and may be controlling bacterial behavior (i.e., commensal or pathogenic). Further studies about bacterial PAMP ligands to host PRRs are relevant for innovative vaccination strategies.

Intestinal colonization by *C. jejuni* has a controversial behavior in chicken hosts, varying from commensal to pathogenic. Apparently, chicken hosts develop tolerance mechanisms that regulate the inflammatory response against infection. The composition of the mucin layer on intestinal cells modulates *C. jejuni* virulence from commensal to pathogenic bacterium. Bile acids have a bactericidal effect against *C. jejuni*; however, the bacterium evades that innate defense. PAMPs recognition by host PRRs on a cytoplasm membrane of sentinel cells, such as TLR1, TLR2, TLR6, TLR9 and TLR21, triggers local inflammation. The bacterial PAMP-activated TLR signaling pathway may regulate the inflammatory response against *C. jejuni* from clearance to tolerance. Most results have been obtained from in vitro experiments; therefore, in vivo experiments should be carried out. Intriguing questions about innate immune response include the following: (i) Do

intraepithelial lymphocytes have any function on innate immune response post-priming host? (ii) How do bacterial antigens modulate chicken and human TLR signaling pathways? (iii) How do factors that are relevant to *C. jejuni* develop commensal or pathogenic behavior? (iv) Are there TLR ligands to protect against *C. jejuni* colonization? (v) Do intestinal commensal microbiota interfere in *C. jejuni* behavior?

4.3 Adaptive Immunity

Avian and mammal immune systems are an integrated network including many molecules and cells; all of them are organized into lymphoid organs or scattered on epithelial surfaces (Murphy 2012; Schat et al. 2014). Lymphoid organs are divided into primary and secondary organs. The primary lymphoid organ selects adaptive immune cells, such as T and B cells. The thymus selects T cells, while cloacal bursa and bone marrow select B cells. The cloacal bursa is exclusively found in chickens (Schat et al. 2014). T cells are divided into two subpopulations: T helper (Th) cells (i.e., CD4+ T cells) and T cytotoxic cells (i.e., CD8+ T cells). Secondary lymphoid organs, such as the spleen and lymph nodes, are the local developing adaptive immune responses (Murphy 2012). Unlike mammal species, there are no lymph nodes in chickens (Schat et al. 2014). The starting adaptive immune response dendritic cell (DC) recognizes pathogens by PRRs on cell membranes and then engulfs bacteria by the phagocytosis process (Murphy 2012; Liang et al. 2013; Schat et al. 2014). Next, the processing antigen by lysosomes or proteasome (i.e., DC proteolytic organelles) generates pathogen epitopes. In sequence, antigen presenting cells (APC; for example, DC) present epitopes to naïve T cells (i.e., CD4+ T cells or CD8+ T cells) by proteins of major histocompatibility complex molecules (MHC) (Wu and Kaiser 2011). Macrophages and chicken heterophils and thrombocytes cells are APCs (Wu and Kaiser 2011). T cells are MHC-restricted and need epitope presentation by APC (Kroeger et al. 2013). T cell co-stimulatory molecules, such as CD8 and CD4, bind to class I MHC and class II MHC molecules, respectively (Murphy 2012; Schat et al. 2014). The antigen presentation MHC molecule repertoire has a genetic influence, reflecting host resistance or susceptibility to infections (Magira et al. 2003).

APC-produced IL-12, IL-6 and TNF-alpha cytokines (Rathinam et al. 2008; Fig. 4.2) induce a Th cell polarization and secretion of pro-inflammatory cytokines, such as interferon-gamma (IFN-γ) (Edwards et al. 2010). The balance between pro-inflammatory and anti-inflammatory cell cytokines might influence the results of *C. jejuni* infection, such as bacterial clearance, tolerance or autoimmune disease (Nyati et al. 2011). A pro-inflammatory cytokine's profile, named the Th1 profile, presents elevated amounts of IFN-γ cytokine (Tribble et al. 2010; Fimlaid et al. 2014). Recently, the pro-inflammatory Th17 cytokine profile was found to eliminate *C. jejuni* infection (Edwards et al. 2010; Nyati et al. 2011; Maue et al. 2013). The anti-inflammatory cytokine profile, named the Th2 profile, predominates IL-4,

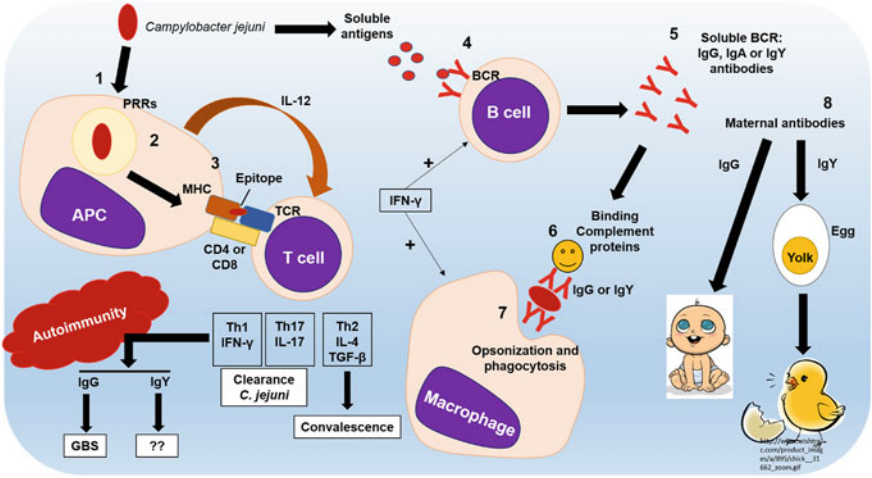

Fig. 4.2 Mechanisms of adaptive immune response against *C. jejuni*: *1* antigen recongnition by Pattern Recognition Receptors (PRRs) and phagocytosis; *2* antigen processing by lysossomal pathway; *3* epitopes presentation by major hystocompatibility complex (MHC) and epitope and MHC recognition by TCR and CD4 or CD8 molecules, respectively; *4* soluble antigens recognition by B cell receptor (BCR); *5* secretion of antibodies isotypes; *6* Complement activation by antibodies; *7* antibodies bacteria opsonization followed by phagocytosis; *8* transference of maternal antibodies, plancentally to human and yolk egg to chicks. Interferon-gamma cytokine (IFN-γ) has protective roles against *C. jejuni* by activation of macrophage and lymphocites. Additionally, IFN-γ and autoantibodies might be associated with autoimmune disease (Guillain-Barré syndrome, GBS) post-*C. jejuni* infection. Antigen presenting cells (APC) IL-12-produced cytokine induces IFN-γ by T helper cells. Recently, IL-17 cytokine demonstrated roles in the elimination of *C. jejuni* infection. T helper 2 (Th2) cytokines may be associated with recovery after bacterial infection. Chickens potentially develop an IgY-mediated autoimmune disease post-*C. jejuni* infection

transforming growth factor-beta (TGF-β) and IL-10 (Tribble et al. 2010; Fimlaid et al. 2014). Different groups of antigens from *C. jejuni* bacteria can trigger a distinct T-helper activation program (Bax et al. 2011).

Campylobacter jejuni infection elicits variable magnitudes for adaptive immune response independent of a live or killed bacterium (Rathinam et al. 2008). Host epithelium cells of mucosal epithelia producing chemokines for DC and lymphocytes are involved in adaptive immune response (Johanesen and Dwinell 2006). Peripheral blood mononuclear cells are an important source for IFN-γ cytokines associated with protection against *C. jejuni* infection (Fimlaid et al. 2014). However, upregulation of the Th1 profile is associated with inflammation and immune-mediated disease progression (Fig. 4.2). Upregulation of the Th2 profile (i.e., IL-4 and TGF-β) correlates to recovery from the disease (Nyati et al. 2011). Autoimmune neuronal disease may be uncommon to infected chickens (Usuki et al. 2006).

Recognition of *C. jejuni* antigens by B cells is not an MHC-restricted mechanism. B cells recognize soluble antigens by the B cell receptor (BCR) (Murphy 2012).

Soluble antigens activate B cells by cross-linking with two BCR molecules (Fig. 4.2). Antibodies are soluble BCRs secreted by B cell-derived plasma cells (Mansfield et al. 2008; Murphy 2012; Schat et al. 2014). The antibody represents the adaptive humoral immune response found in blood, lymph, white egg, yolk egg and mucosal surface (Murphy 2012; Schat et al. 2014). Ancient antibody isotypes are immunoglobulin M (IgM) and IgA found in chicken and human species. Chickens produce the IgY isotype that predominates in blood and yolk egg (Schade et al. 2001). The IgG isotype is abundant in blood and lymph, and the IgA isotype is found on mucosal surfaces (Murphy 2012). IgY antibodies are found only in chicken humoral immune response (Schat et al. 2014).

Maternal antibodies, including IgY and IgG, are protective to offspring against infections (Shoaf-Sweeney et al. 2008). Yolk egg is a source of maternal IgY antibodies; transference from blood for yolk occurs by a specific receptor-dependent mechanism on the ovarian follicle surface (Schat et al. 2014). IgG antibody transference from maternal blood to fetal blood is a transplacental mechanism (Murphy 2012). Chicken vaccination increases the concentration of serum-specific IgY, which results in higher antibody transference to yolk (Garcia et al. 2012; Yeh et al. 2015a). Although the absence of autoimmunity was suggested by incipient studies (Usuki et al. 2006), chicken possibly develops a cross-reactivity antibody-mediated response against host gangliosides on neuronal cells (Nyati et al. 2011).

Contaminated chicken meat is a worldwide-recognized source of *C. jejuni* human infection. Chicken bacterial colonization arises at 3 weeks post-hatching, with overlapping reduction of serum IgY antibody levels (Sahin et al. 2001; Cawthraw and Newell 2010). A specific IgY isotype reduces intestinal colonization (Chintoan-Uta et al. 2015); chicken immunization could improve the specific antibody response against *C. jejuni* antigens, such as flagellar antigens (Yeh et al. 2015a, b), surface-exposed colonization proteins (Neal-Mckinney et al. 2014) and superoxide dismutase protein (Chintoan-Uta et al. 2015). Genetic variations in chickens naturally infected by *C. jejuni* reflect the diversity in the antibody repertory, which is promising to select antigenic targets for vaccines, diagnostics, or therapy (Fernando et al. 2008; Neal-Mckinney et al. 2014; Yeh et al. 2015b).

The antibody-activated bactericidal role of the avian or human complement system is an effective mechanism to eliminate *C. jejuni* infection (Fernández et al. 1995; Sahin et al. 2001). However, lipooligossacharide from *C. jejuni* mimics gangliosides from neuron cells, inducing cellular lysis by complement-binding autoimmune antibodies that are associated with Guillain-Barré autoimmune syndrome (Nyati et al. 2011; Yuki 2015). Commonly, human binding complement antibodies are immunoglobulin M (IgM) and IgG isotypes (Murphy 2012; Schat et al. 2014), while chicken complement binding antibodies are IgM and IgY isotypes (Schat et al. 2014). Interactions between complement proteins and antibodies are species-specific; in the other words, IgY antibodies interact only with chicken complement proteins (Schade et al. 2001). The *C. jejuni* capsule is an evasion mechanism that protects bacteria against the bactericidal complement effect by preventing pathway activation (Keo et al. 2011).

Maternal IgG antibodies are transferred to a human fetus by the placental pathway, while chicken serum IgY antibodies are translocated to offspring by specific receptor uptake into yolk egg (Morrison et al. 2002). Even though maternal IgY antibodies protect young birds during their first three weeks post-hatch, occasional infection at 1 or 3 days of age occurs, especially when environmental exposure to *C. jejuni* is high, such as in free-range birds (Cawthraw and Newell 2010). Although membrane antigens from a different strain of *C jejuni*, such as a human isolate or an avian isolate, elicit different adaptive immune response programs (Shoaf-Sweeney et al. 2008; Waldenström et al. 2010), the vaccination could be a strategy to protect avian hosts and consequently human hosts. Oral human vaccination results in a robust intestine mucosal adaptive immune response against *C. jejuni* with a production of IFN-γ and secretion of high titers of IgA antibodies, demonstrating the importance of mucosal immune response for protecting the host against infection (Tribble et al. 2010).

Serum and mucosal anti-*C. jejuni* IgA antibodies levels are predictive of bacteria protection (Burr et al. 1988). IL-6 cytokine enhanced mucosal protection from *C. jejuni* by increasing IgA production (Baqar et al. 1993). In chicken embryo, oral vaccination at 16 day hatching induces a high titre of anti-flagellin IgA antibody in bile and mucosa, demonstrating a precocious adaptive immune response in oral mucosa (Noor et al. 1995). Apparently, antigens from *C. jejuni* are able to elicit an IgA-based mucosal immune response, independently of mucosal adjuvants (Rice et al. 1997). Intranasal delivery of chitosan-DNA vaccination successfully induced a systemic, mucosal adaptive immune response and might be a promising procedure against *C. jejuni* infection (Huang et al. 2010). Anti-ganglioside IgA antibodies were associated with Guillain-Barré syndrome (Koga et al. 1998).

In conclusion, the adaptive immune response against *C. jejuni* has similar mechanisms in human and chicken hosts. The pro-inflammatory IFN-γ has a protective role in early bacterial infection. Both systemic and mucosal adaptive immune responses have protective roles against *C. jejuni* infection. Bacterial lysis and phagocytosis are important mechanisms in killing and removing *C. jejuni*. Further studies should be necessary to know the pathogeny of Guillain-Barré syndrome, select antigenic targets for vaccination, find oral adjuvants for vaccination, determine procedures to prolong chicken offspring protection by maternal antibodies, and reduce *C. jejuni* intestinal colonization.

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Chapter 5
Campylobacter jejuni in Poultry: A Commensal or a Pathogen?

Lisa K. Williams, Belchiolina Beatriz Fonseca and Tom J. Humphrey

Abstract *Campylobacter* has a long standing association with poultry and is the main cause of cases of human foodborne disease in the developed world, with most cases being linked back to the poultry reservoir, particularly chicken. *Campylobacter* is ideally suited to the poultry niche, and can grow at the increased body temperature of birds of 42 °C. Historically *Campylobacter* was referred to as a commensal of poultry, as it is found in the majority of birds reared for human consumption irrespective of the breed or rearing system used, even though there is experimental evidence from over 30 years ago that certain *Campylobacter* strains harm broiler chickens. More recently there is an increasing body of evidence, supporting this early work, which indicates that *Campylobacter* is not necessarily a commensal of poultry and under certain conditions the bacterium behaves more like a pathogen. Birds mount innate and adaptive immune responses to *Campylobacter*. Recent studies have highlighted the ability of *Campylobacter* to leave the gut and be found in other internal organs and muscle tissue. In addition, *Campylobacter* has been shown to have a negative effect on the health and welfare of the birds, Furthermore evidence of host adaptation and host-specific species leads us to believe that *Campylobacter* is more than just a commensal of chickens. Here we will review the role of *Campylobacter,* host adaption and commensalism within the poultry niche.

Keywords *Campylobacter* · Poultry · Commensal · Pathogen

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5.1 *Campylobacter* as a Zoonotic Pathogen

Campylobacter can be isolated from a variety of sources including companion animals (Brown et al. 2004; Kemp et al. 2005; Andrzejewska et al. 2013), cattle (Busato et al. 1999; Inglis et al. 2004; Gilpin et al. 2008; Kwan et al. 2008), sheep (Zweifel et al. 2004; Açik and Cetinkaya 2006; Oporto et al. 2007; Sahin et al. 2008) pigs (Oporto et al. 2007; Denis et al. 2008; Little et al. 2008; Horrocks et al. 2009), wild animals, including birds (Meerburg et al. 2006; Kwan et al. 2008; Horrocks et al. 2009). However, the major reservoir for human campylobacteriosis is poultry (Levin 2007; Wilson et al. 2010; Hermans et al. 2011; Williams et al. 2014) and this chapter will focus on the bacteria in chickens, in particular.

The genus *Campylobacter* contains 26 species, 2 provisional species and 9 subspecies (Kaakoush et al. 2015). Of these, *Campylobacter jejuni* causes the majority of cases of human infections with most of the rest caused by *C. coli*. Poultry is the main reservoir for both species but we will confine our discussions to the broiler chicken. *C. jejuni* and *C. coli* are widely adaptable for colonisation of chickens and they can be found throughout the gut with most strains of bacteria but predominantly they are isolated from the large intestine and caeca. In many cases, the birds do not exhibit any overt signs of disease and few published studies mention this. In one study birds were colonised with up to 10^8 cfu/g gut contents and still remained asymptomatic (Dhillon et al. 2006) and it is due to this fact that the bacterium is still often referred to as a commensal in poultry (Macpherson and Uhr 2004; Dhillon et al. 2006). However, most studies seemed to have predetermined that *Campylobacter* are chicken commensals and did not examine the birds for health and/or welfare problems. There is now an increasing body of evidence from laboratory and field studies indicating that *Campylobacter* are not what we thought they were and do broiler chickens harm and affect their performance. This will be discussed later.

Thermophilic *Campylobacter,* which includes *C. jejuni* and *C. coli,* are well suited to the chicken body temperature (Hermans et al. 2011; Williams et al. 2014) of 42 °C and the physiology of the bacteria points to a long evolution in, and adaption to, avian hosts (Williams et al. 2014). The bacterium survives within the gut and is associated with the mucus layer rather than the gut epithelial cells. There are two main routes of infection in humans from poultry carcasses, first cross contamination during processing and second via spread of the bacterium from the intestines to other organs. Undercooked chicken muscle and liver are internationally important vehicles for human infection. The chicken gut is colonised with a high number of *Campylobacter* and during processing it is thought that these bacteria cross contaminate the external surface of the carcass. The bacteria can also be found in aerosols and contaminate the carcass in that way.

Campylobacter has the ability to leave the poultry gut and colonise other internal organs, the predominant one being the liver. This extra-intestinal spread is a public health concern as there have been several outbreaks of human campylobacteriosis linked to the consumption of chicken livers (Fernández and Pisón 1996;

Cox et al. 2006; Whyte et al. 2006; Medeiros et al. 2008; O'Leary et al. 2009; Simaluiza et al. 2015). *Campylobacter* spp. are frequently isolated throughout poultry production, including rearing and at slaughter (Nebola and Steinhauserova 2006) and their occurrence is well documented (Stern et al. 2001; Wagenaar et al. 2001; Humphrey 2006; Denis et al. 2008). However, little is known about the epidemiology of *Campylobacter* in poultry flocks (Bull et al. 2008), making control measures more difficult to implement and monitor. Controlling *Campylobacter* spp. during poultry processing would reduce the number of the bacteria within the chicken gut and ultimately reduce the number of cases in humans. Many approaches have been tried with limited success and more recently vaccination studies have yielded poor results (Meunier et al. 2015). Despite interventions throughout the poultry rearing process including biosecurity, altering the diet, use of pre and pro biotics and additives (Meunier et al. 2015) at the farm level and others during processing such as rapid chilling, *Campylobacter* spp. are able to survive and as a consequence of this are found on 71 % of chicken carcasses on retail sale in the UK (Food Standards Agency 2015). This is not just a UK problem; across Europe approximately 75 % of carcasses on retail sale contain *Campylobacter* spp. The number of cases in the human population in the UK increases each year, despite many interventions during rearing, processing and giving consumers advice via the packaging and in advertising campaigns.

In the UK it is thought that 82 % of hospital admissions with a diagnosis of food poisoning can be attributed to a *Campylobacter* infection (Adak et al. 2002). In humans, the infectious dose is believed to be low, at around 500 cells in an adult male (Robinson 1981) with an incubation period of up to 10 days, with most people exhibiting symptoms by day four. Symptoms include diarrhoea, which may be bloody, acute abdominal pain and fever. Most infections are self-limiting and those who suffer an infection recover quickly after resting and maintenance of fluid levels; a fatal outcome is rare and would usually occur in the elderly or those already suffering from another serious illness (Skirrow and Blaser 2000). Treatment with antibiotics is rare but resistance to clinically important antimicrobials especially macrolides and fluoroquinolones is increasingly reported (Humphrey et al. 2007).

5.2 *Campylobacter* as a Commensal of Chickens

Campylobacter has long been considered a commensal of chickens. It is found frequently as a component of the caecal microbiome and despite being considered to be a commensal; *Campylobacter* also has properties that could be considered pathogenic, depending on the host immune status, bacterial pathotype, bird type and co-infection with poultry endemic pathogens. It has been generally accepted that almost every bird will be exposed to and colonised with *Campylobacter* at some point during their lifetime. Birds usually become colonised by the third week of life and normally remain so throughout rearing. *Campylobacter* has been found in the various different breeds that are reared for meat and in the different rearing systems.

*Campylobacte*r has been isolated from standard shed reared, barn reared, free-range and organic birds, despite there being a difference in the breed of bird, diet and the way the rearing systems are managed. Historically, *Campylobacter* was considered a commensal of chickens because signs of disease, which we now know can be associated with the bacteria, were ascribed to other causes. Similarly with changes in performance and welfare were associated with other conditions, as were carcass rejections at slaughter. For over 30 years *Campylobacter* were considered to be part of the normal gut microbiota of a chicken despite early evidence indicating otherwise (Neil et al. 1984). With some bird types and with certain strains of *Campylobacter* this is probably still the case but this is not universal. It is surprising; given past and recent evidence many people still seem intent on regarding *Campylobacter* as a chicken commensal.

Further 'evidence' of commensalism, which until recently remained unchallenged, is that chickens were thought not to mount an immune response to *Campylobacter* in the same way as they would not for other commensal pathogens such as *Lactobacillus*. As we develop a deeper understanding of the chicken immune system we begin to realise that it is more complex than previously thought. While many still consider *Campylobacter* to be a commensal of chickens, there is an increasing body of evidence from several research groups (Williams et al. 2013, 2014; Wigley and Humphrey 2014; Humphrey et al. 2015) building up which is paving the way to suggest that *Campylobacter* cannot any longer be considered in this way.

5.3 *Campylobacter* as a Pathogen of Chickens

There is an increasing body of evidence to challenge the view that *Campylobacter* is a commensal of chickens. Several studies have reported the ability of *Campylobacter* to leave the chicken gut and colonise other organs, mainly the liver (Sanyal et al. 1984; Knudsen et al. 2006; Van Deun et al. 2007; O'Leary et al. 2009; Jennings et al. 2011). Muscle contamination has also been reported, the source of this contamination has previously been debated and it is now clear that cross contamination from the intestine during processing is not the only contributing factor to infected meat (Williams et al. 2014). There have been several studies dating back to 1984 (Sanyal et al.1984; Knudsen et al. 2006; O'Leary et al. 2009; Jennings et al. 2011) which have isolated *Campylobacter* from organs other than the gut. Studies have shown that *Campylobacter* can invade the intestinal mucosa (Knudsen et al. 2006) and it is this invasiveness which leads to extra-intestinal spread and colonisation of other internal organs. It is well documented and understood that within the poultry gut *Campylobacter* adheres to epithelial cells and that this adherence is essential for colonisation (Hermans et al. 2011). Extra-intestinal spread, of course, does not indicate disease or that an infectious process is occurring (Williams et al. 2014), but the invasive behaviour of *Campylobacter* suggests that in chickens it should be regarded as either a true or

opportunistic pathogen. It is increasingly being recognised that *Campylobacter* does alter the state of the host (Williams et al. 2014; Humphrey et al. 2014).

There is another dynamic: *Campylobacter* negatively affect bird health and performance. It has long been understood that certain welfare associated conditions such as hock marks and pododermatitis, associated with diarrhoea in the birds, were risk factors for *Campylobacter* infection in commercial flocks (Neil et al. 1984; Bull et al. 2008; Rushton et al. 2009). More recently, Williams et al. (2013) demonstrated that the incidence and severity of hocks marks and pododermatitis were increased when *Campylobacter* was present in artificially infected birds. These leg and foot conditions are caused by prolonged contact with wet litter and this study strongly suggests that *Campylobacter* is causing these leg lesions indirectly by increasing the looseness of the faeces which increases the wetness of the litter (Williams et al. 2013, 2014). Very similar results were obtained by Humphrey et al. (2014), who showed that diarrhoea in certain broiler breeds was associated with high levels of inflammation and much damage to gut mucosa (Humphrey et al. 2014; Fig. 5.1). Figure 5.1 is taken from Humphrey et al. (2014) and shows the breed with high levels of diarrhoea, and raised levels of pododermatitis, has profound damage to the mucosa of the ileum, in particular, whereas in the breed with essentially normal faeces and no pododermatitis the damage was much less (Fig. 5.1). In this study, the different broiler breeds were infected orally with around 10^5 cfu of *C. jejuni* strain M1, which came from a human case of diarrhoea. Williams et al. (2013) also showed marked differences in the incidence of pododermatitis between infected 'slow' and 'rapid' growth breeds when birds were infected with M1. Damage to gut mucosa has been seen in other studies using artificial infection of 'rapid growth breeds' (Awad et al. 2014, 2015). These studies provide evidence to suggest that *Campylobacter* is having a direct impact on the health and welfare of broiler chickens. Awad et al. (2014, 2015) showed that birds

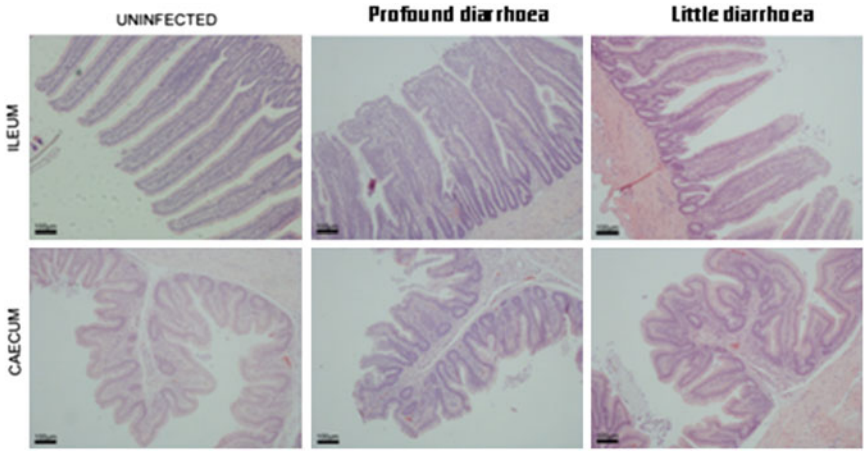

Fig. 5.1 In some broilers, *C. jejuni* M1 damages gut epithelia

infected with *Campylobacter* performed significantly less well in the laboratory. It is almost astonishing that the first study of this type (Ruiz-Palacios et al. 1981), which showed that in chickens given *C. jejuni* isolates from human cases of diarrhoea, over 80 % of the birds got diarrhoea, around 40 % died and there was around a 40 % drop in growth rate in the survivors (Fig. 5.2), has largely been ignored. Figure 5.2 was taken from the paper published by Ruiz-Palacios et al. (1981). Birds were infected orally with around 10^9 cfu of a *C. jejuni* strain isolated from a human case with severe diarrhoea.

Recent field work in the UK and Ireland (Sparks and Whyte personal communications) found that *Campylobacter*-positive commercial broiler flocks had significantly reduced performance than—negative ones although there were bacterial strain to strain differences in the impact of *Campylobacter*. There are now powerful economic and welfare reasons for better *Campylobacter* control on farm, these bacteria threaten the economic sustainability of chicken production and that infection of chickens is likely to cost the international poultry industry much money each year.

Campylobacter being linked to disease in the chicken is not a new phenomenon, in 1954 a link was made between vibrio like organisms which were later identified as *C. jejuni* and vibrionic hepatitis (Tudor 1954). Other authors also found an association between avian vibrio hepatitis (AVH) hepatitis and the presence of *Campylobacter* (Lukas 1955; Hofstad et al. 1958; Moore 1958; Sevolan et al. 1958; Whenham et al. 1961). This disease which causes focal lesions that are greyish-white in colour and 1–2 mm in size (Crawshaw and Young 2003; Burch 2005) persisted

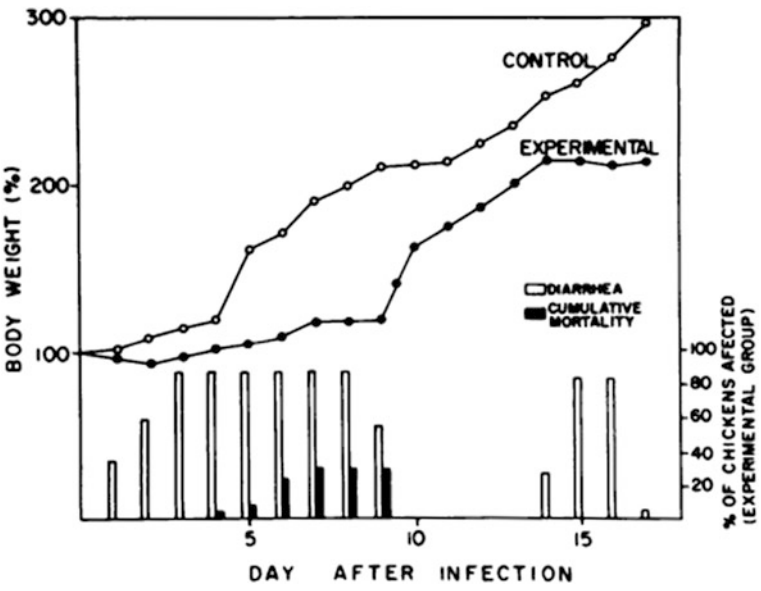

Fig. 5.2 Broiler weight gain and health when given *C. jejuni*

until 1965 and re-emerged in 2000s (Crawshaw and Young 2003; VLA surveillance report 2005). *Campylobacter* has not been conclusively linked with the focal lesions (Jennings et al. 2011) despite being initially linked with the disease (Peckham 1958). Recently, Jennings et al. (2011), found significantly fewer healthy livers contained *Campylobacter* compared to those with greyish-white spots and using FISH they demonstrated that *Campylobacter* was present in higher number in spotty livers, compared to livers without spots which were deemed to be healthy. *Campylobacter* has been found previously in the livers of apparently healthy birds (Cox et al. 2006, 2007, 2009). Another study that leaves us puzzled is about the isolation of bacteria in the bloodstream of commercial broilers (Richardson et al. 2011). As the bacteria may be present in the blood of birds without apparently causing harm to these animals they could lodge in the small blood vessels in chicken muscle when the animals are bred. Undercooked chicken meat is an internationally important vehicle for human infection. Ruiz-Palacios et al. (1981) found that around 15 % of birds in their experimental studies had *Campylobacter* in circulating blood.

Colonisation of the liver is not the only factor leading us to believe that *Campylobacter* behaves like a pathogen in chickens. Other studies report a range of impacts of infection with *C. jejuni*. Damage to gut mucosa has been reported in some studies (Ruiz-Palacios et al. 1981; Sanyal et al. 1984; Gharib Naseri et al. 2012; Awad et al. 2014; Humphrey et al. 2014) but not all (Dhillon et al. 2006; Larson et al. 2008). There are also differences in whether birds suffered diarrhoea. Some report this (Ruiz-Palacios et al.1981; Sanyal et al. 1984; Sang et al. 1989; Humphrey et al. 2014), particularly in young chickens. Diarrhoea has also been reported in turkey and ostrich chicks (Post et al. 1992; Stephens et al. 1998). The bird type could impact on the effects of *Campylobacter* but bacterial strain is also important. The same type of bird has been used in several studies but have been colonised with different *C. jejuni* strains, (Williams et al. 2013; Awad et al. 2014; Humphrey et al. 2014) and diarrhoea was not reported in all. Differences between strains in an ability to cause diarrhoea in chickens have been reported by others (Humphrey et al. 2014). It is clear that certain *Campylobacter* strains cause disease in different chicken types, although few papers looked beyond caecal colonisation.

It is interesting to note that inoculation of *C. jejuni* in chicken embryos also results in high embryonic mortality, even with culture filtrates (Lam et al. 1992; Fonseca et al. 2011). But there are few studies in this area.

5.4 Immune Responses

The immune system of the chicken is highly developed but in comparison to mammals is considered to be smaller and simpler although the functions are the same. The immune response to *Campylobacter* colonisation is complex, and little is known about the interaction between *Campylobacter* and the chicken immune system (Wigley and Humphrey 2014). Like other bacterial species *Campylobacter* is recognised by pattern recognition receptors (PRRs), which lead to the activation

of an immune response. Toll-like receptors (TLR) in particular in terms of *Campylobacter* colonisation TLR4 and TLR21 are activated in the gut, although it has been reported that the increase in expression of these TLRs in response to *Campylobacter* colonisation is short lived (Shaughnessy et al. 2009). Following PRR activation, it has been shown that there is an increase in the pro-inflammatory cytokines IL-6 and IL-1β along with a pro-inflammatory chicken chemokine IL-8 (chIL-8) (Larson et al. 2008; Shaughnessy et al. 2009). After this initial pro-inflammatory response, cytokine levels decrease, suggesting that although initial colonisation is dealt with by the hosts' immune system as an attack it then reaches a level of tolerance (Smith et al. 2008; Hermans et al. 2012; Williams et al. 2014). Previous studies (Poh et al. 2008) have shown that both hetrophils and monocytes are attracted by IL-8 to areas of inflammation. However, there is conflicting evidence whether this happens in IL-8 produced with *Campylobacter* colonisation (Williams et al. 2014). Several studies have shown no heterophilia (Van Deun et al. 2008; Meade et al. 2009) but Smith et al. (2008) observed an increase in hetrophils in the caeca and ileum of experimentally infected birds at 2 weeks of age. TLR5 recognises flagellin and in the case of *Salmonella* Enteritidis colonisation in birds there is a strong inflammatory response to invasion, mainly mediated by the recognition of flagellin through TLR5, leading to the production of CXCL chemokines and pro-inflammatory cytokines including IL-6 and IL-1β (Iqbal et al. 2005; Wigley 2013). This leads to inflammatory damage which is considered to be mild in the chicken and can also lead to immune activation and largely restricts infection to the gut (Withange et al. 2005; Wigley 2013). *Salmonella* that have adapted to causing systemic infection in the chicken, namely serovars Gallinarum and Pullorum lack flagella and so evade recognition allowing systemic infection to occur (Chappell et al. 2009). In a similar way to *Salmonella* Gallinarum and Pullorum, *Campylobacter* can also evade TLR5 recognition by glycosylation of its flagella (Howard et al. 2009), but it is recognised by other receptors including TLR21 (Meade et al. 2009). Several studies (Smith et al. 2008; Meade et al. 2009; Shaughnessy et al. 2009; Humphrey et al. 2014) have shown that there is an inflammatory response to *Campylobacter* in the intestine and whilst it is usually poorly invasive it has been found in other organs including the liver (Sanyal et al. 1984; Knudsen et al. 2006; Van Deun et al. 2007; O'Leary et al. 2009; Jennings et al. 2011) leading to the liver being associated with human cases of *Campylobacter* (Cox et al. 2006; Whyte et al. 2006; Medeiros et al. 2008; O'Leary et al. 2009). Colonisation of the liver in chickens can be asymptomatic (Cox et al. 2009) but it has also been shown to be associated with vibronic hepatitis (Jennings et al. 2011). A number of studies have correlated signs of disease with the numbers of *Campylobacter* present. The more diseased the livers appeared the higher were *Campylobacter* numbers. This and the inflammatory response to *Campylobacter* in the chicken gut suggests that it is misleading to consider *Campylobacter* to be a gut commensal of chickens (Hermans et al. 2012; Humphrey et al. 2014).

As well as mounting an innate immune response to *Campylobacter,* chickens also mount an adaptive immune one, producing antibodies against a number of

Campylobacter proteins including flagellin (Cawthraw et al. 1994; Sahin et al. 2001, 2002; Smith et al. 2008) although our understanding of the adaptive response of chicken to *Campylobacter* is rudimentary (Wigley and Humphrey 2014). Chickens have been shown to produce a humoral response against T-cell dependent and T-cell independent Type 1 antigens, they produce a low response against T-cell independent Type 2 antigens (Jeurissen et al. 1998). The polysaccharide component of the *Campylobacter* capsule is a Type 2 antigen and this has been suggested to contribute to the lack of clearance of *Campylobacter* from the chicken gut (Williams et al. 2014).

There is no doubt that chickens mount a response to infection, whilst an initial response is to be expected to any commensal organism, reports of pathology including heterophilia indicate that *Campylobacter* may be causing a diseased like state, and is behaving more like a pathogen than a commensal (Williams et al. 2014; Humphrey et al. 2015).

5.5 Conclusion

There is no doubt that *Campylobacter* is associated with chickens and causes foodborne illness in humans. In chickens, it is historically being referred to as a commensal but there is increasing evidence to challenge this. Studies have shown that *Campylobacter* is capable of invading the gut cells, causing extra-intestinal infection of other internal organs and muscle. In addition it has been shown that chickens mount innate and adaptive immune response to *Campylobacter* higher than to other commensal bacteria. The evidence suggest that in the right conditions *Campylobacter* behaves more like a pathogen and had a direct effect on the health and welfare of chickens and therefore, should not always be considered to be a commensal of chickens.

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Chapter 6
A Glance at Prokaryotes and Eukaryotes Interplay and *Campylobacter jejuni*–Host Interaction

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Abstract This chapter begins with the historical background to the evolutionary development of the cells. The endosymbiosis theory by which prokaryotes gave rise to the first eukaryotic cells describe the relationship between cell types that may have evolved through predation to mutualism or parasitism. There is also discussion of some interactions between parasitic unicellular organisms and their cell hosts taking into account the dynamic actin cytoskeleton and the immune response resulting from the invasion. Finally, *C. jejuni*, its types of invasion routes, intracellular traffic, virulence factors, and colonization of poultry cells by *C. jejuni* are discussed.

Keywords Intracellular traffic · *Campylobacter*-contaning vacuole · Types of routes

6.1 When Prokaryotes and Eukaryotes Began

The biological universe consists of two types of cells, prokaryotic and eukaryotic, which belong to one of three domains that define three branches of evolution from a common ancestor, the structures and molecules in all cells have so many similarities. Two large groups of single-celled microorganisms can be distinguished on genetic and biochemical grounds, detailed analysis of the DNA sequences from a variety of prokaryotic organisms has revealed two distinct types: the bacteria or eubacteria and archaea. The living world therefore has three major divisions: bacteria, archaea, and eucaryotes (Lodish et al. 2004; Lehninger et al. 2008; Alberts et al. 2008).

As life emerged from the original chemical soup, molecular compartmentation evolved to enhance the efficiency of enzymatic reactions by concentrating enzymes

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together with their substrates, cofactors and products within chemically well-defined cellular and subcellular compartments (Soldati and Neyrolless 2012).

The origin of eukaryotes is still uncertain, but they inherited genes from both Archaea and Bacteria. One possibility is that eukaryotes originated when an Archaea fused with a Bacteria (Pollard and Earnshaw 2007). Eukaryotes contain a defined membrane-bound nucleus and extensive internal membranes that enclose other compartments, the organelles. The region of the cell lying between the plasma membrane and the nucleus is the cytoplasm, comprising the cytosol and the organelles. Eukaryotes comprise all members of the plant and animal kingdoms, including the fungi, which exist in both multicellular forms and unicellular forms, and the protozoans (Lodish et al. 2004). They keep their DNA in an internal compartment called the nucleus. The nuclear envelope, a double layer of membrane, surrounds the nucleus and separates the DNA from the cytoplasm.

Eucaryotes also have other features that set them apart from procaryotes, they have a cytoskeleton, a system of protein filaments crisscrossing the cytoplasm and forming, together with the many proteins that attach to them, a system of girders, ropes, and motors that gives the cell mechanical strength, controls its shape, and drives and guides its movements. It is still a mystery how all these properties evolved, and in what sequence, one plausible view, however, is that they are all reflections of the way of life of a primordial eucaryotic cell that was a predator, living by capturing other cells and eating them (Alberts et al. 2008).

6.2 Endosymbiotic Theory: Predation to Mutualism or Parasitism?

The numerous similarities of free-living bacterial cells with mitochondria and chloroplasts have led scientists to hypothesize that these organelles arose by the incorporation of bacteria into ancestral eukaryotic cells, forming endosymbiotic organelles. Striking evidence for this ancient evolutionary relationship can be found in many proteins of similar sequences shared by mitochondria, chloroplasts, and bacteria including some of the proteins involved in membrane translocation (Lodish et al. 2004).

A predatory way of life helps to explain another feature of eucaryotic cells. Almost all such cells contain mitocondria; molecular evidence has established that eukaryotes acquired mitochondria when a α-proteobacterium became an endosymbiont. Modern-day α-proteobacteria includes pathogenic Rickettsias. When the two formerly independent cells established a stable, endosymbiotic relationship, the Bacterium contributed molecular machinery for ATP synthesis by oxidative phosphorylation. The host cell might have supplied organic substrates to fuel ATP synthesis. Together, they had a reliable energy supply for processes such as biosynthesis, regulation of the internal ionic environment, and cellular motility. Given that some primitive eukaryotes lack full-fledged mitochondria, the singular

event that created mitochondria was believed to have occurred well after eukaryotes branched from prokaryotes (Pollard and Earnshaw 2007).

The acquisition of plastids, including chloroplasts, began when a cyanobacterial symbiont brought photosynthesis into a primitive algal cell that already had a mitochondrion. The cyanobacterium provided both photosystem I and photosystem II, allowing the sunlight to provide energy to split water and to drive conversion of CO_2 into organic compounds with O_2 as a by-product. Symbiosis turned into complete interdependence when most of the genes that are required to assemble plastids moved to the nucleus of host cells that continued to rely on the plastid to capture energy from sunlight. This still-mysterious transfer of genes to the nucleus gave the host cell control over the replication of the former symbiont (Pollard and Earnshaw 2007).

Unfortunately, there is no fossil record to provide an estimate of when some organisms acquired the capacity to survive inside other microbes. If we accept an endosymbiotic origin for mitochondria and other eukaryotic organelles, then we can conclude that the capacity for intracellular residence is ancient and antedated the emergence of eukaryotic organisms as we know them. The emergence of the intracellular lifestyle in ancient microbes appears to have at least three major requirements: (a) size differences between microbes such that one can ingest another, (b) a mechanism for particle ingestion on the part of the host and/or host invasion on the part of the smaller entity, and (c) a capacity for the ingested microbe to survive within the larger host. Such early interactions could have had varied outcomes including survival of both microbes (symbiosis and mutualism), survival of the host (predation), damage to the host (intracellular pathogenesis), or damage to both microbes (incompatibility and antagonism) (Casadevall 2008).

6.3 An Intracellular Lifestyle

Intracellular pathogenic microbes fall into two groups: obligate or facultative. Obligate intracellular pathogens have lost their capacity for living outside of their hosts and these include all viruses, bacteria such as *Rickettsia* and *Chlamydia* spp., and protozoa such as *Plasmodium* spp. In contrast, facultative intracellular pathogens retain the capacity for replication outside their hosts and these include a large number of pathogenic bacteria and fungi. Hence, the designations of obligate and facultative would appear at first glance to represent a clear dividing line for approaching the topic of evolution of intracellular pathogens (Casadevall 2008). Bacteria can be classified according to their association with their host. Obligate intracellular bacteria are unable to grow outside of host eukaryotic cells. While associations can be advantageous to their host (mutualism), other intracellular bacteria can negatively affect the cell (parasitism) (Leroy and Raoult 2010).

The obligate intracelular pathogen has evolved to resist its predators by sheltering in their inside. Intracellular parasitism has been adopted by various

phylogenetically unrelated prokaryotic and eukaryotic microbial species to escape killing by protozoan as well as immune phagocytes, and to convert the hostile environment of the phagocytic vacuole into a permissive niche. Thus, as pathogens learned how to survive inside predatory phagocytic (Soldati and Neyrolless 2012).

To establish infection, pathogenic microorganisms have evolved many strategies to circumvent host defenses and exploit the host cellular machinery. Specific virulence factors disable or subvert vesicular trafficking pathways to and from the host cell surface, which promotes pathogen entry, replication or escape (Guichard et al. 2014).

Many bacterial and eukaryotic parasites trick host cells into providing comfortable living arrangements for their descendants. Some of these microorganisms have similar requirements to viruses, as they cannot grow in extracellular or environmental niches, and must instead establish an intracellular replication cycle. Other intracellular microorganisms can replicate either inside or outside host cells. For these microorganisms, the intracellular lifestyle allows them to gain a competitive advantage relative to other microorganisms, or to facilitate colonization of a host. Life inside cells could either enable evasion of killing mechanisms that are wielded by predatory cells, such some amoebae, or provide a niche to evade host humoral and cellular immune responses (Isberg et al. 2009).

Bacterial pathogens have evolved sophisticated mechanisms enabling them to invade, reside in, and proliferate in a large range of eukaryotic hosts. This often involves hijacking the host phagosomal system, interfering with the host cell signaling and trafficking machinery, and establishing a replication niche to avoid clearance. Whereas some pathogens escape phagosomes and replicate in the host cytoplasm most of the described pathogens replicate in membrane bound, vacuole-like compartments. Such intracellular niches of various pathogens are diverse, and biogenesis often depends on the delivery of bacterial effector proteins into the host cell cytoplasm (Vorwerk et al. 2015).

In all known examples of cytosolic pathogens characterized to date, escape from the vacuole is a bacterially driven process, and some pathogens share common mechanisms for escape that are triggered by specific environmental cues in the vacuole. All cytosolic pathogens adopt the same strategy of entry into the cytosol using mechanisms that rely on the production of secreted enzymes. During entry into the host cell, bacteria are engulfed in a primary vacuole. Once inside the vacuole, bacteria secrete proteins that facilitate escape from the vacuole by disrupting the vacuolar membrane. Bacteria replicate once free in the cytosol, all cytosolic bacteria polymerize actin at the bacterial pole and are therefore capable of intracellular and intercellular motility. During cell-to-cell spread, bacteria are enclosed in a secondary double-membrane vacuole. Bacteria secrete proteins that disrupt both membranes, allowing the bacteria to escape into the cytosol of an adjacent cell. The bacteria then replicate and continue their intercellular spread, disseminating the infection (Ray et al. 2009).

6.4 Innate Immunity: The First Host Response

Host invasion by bacteria initiates an immune response which relies on multiple cell populations and communications between them. This normally results in the clearance of the intruder. However, in the case of pathogenic bacteria, host defenses are challenged with specific attacks on their molecular machineries. Several pathogenic bacteria use different types of apparatus, and various molecules to modulate host cells processes and responses to infection. The pathogenicity of these bacteria is associated with their capacity to survive and replicate within a specialized vacuole or within the cytoplasm of host cells. This can be achieved by avoiding or surviving the phagolysosome formation, escaping the autophagy process of bacteria, a process also known as xenophagy, and interfering with signaling pathways important for immune response, cell survival, and apoptosis. Host cells response to invaders depends on the modulation of key cellular functions, from signals transduction to receptors and vesicles trafficking (Alomairi et al. 2015).

The first important step in innate immunity is the recognition of various infectious microbes as distinct from self, which leads to the induction of the appropriate innate immune response. Activation of innate immune responses in response to pathogens, therefore, relies on the detection of conserved microbial motifs. The recognition of infectious microbes in innate immunity is achieved though the detection of pathogen-associated molecular patterns, the conserved microbial components, including lipopolysaccharide (LPS), peptidoglycan (PGN), flagellin and nucleic acids from bacteria, fungi or viruses, which are essential for the microbial survival, but are not found in higher eukaryotes. This initial recognition is mediated by a set of genome-encoded pattern recognition molecules, which sense the conserved pathogen-associated molecular patterns (Yano and Kurata 2011).

Host cells express a range of receptors that act as microbial sensors. These receptors sense microorganisms and transduce signals that activate immune responses. Host cells use several strategies to recognize specific pathogen-associated molecular patterns (PAMPs) and to alert the immune system. The aim is to activate innate and adaptive immune responses, eliminate the encountered pathogens and establish long-lasting protective immunity against them. PAMPs are sensed by Pattern Recognition Receptors (PRRs), the best-characterized of which are Toll-like Receptors (TLRs). There are 10 TLRs in humans, 13 in mice and 222 in sea urchins, which have evolved to recognize PAMPs from fungi, bacteria, viruses and parasites. TLR3, TLR7, TLR8 and TLR9 detect microbial nucleic acids, whereas TLR2, TLR4, and TLR5 recognize lipoproteins, LPS and flagellin, respectively (Diacovich and Gorvel 2010).

Another family of PRRs is the NOD-like receptors (NLRs), which are C-type lectin receptors that detect bacterial and viral molecules in the cytoplasm, leading to the secretion of interleukin-1β. Members of this family include Nucleotide-binding oligomerization domain-containing protein 1 and 2 (NOD1 and NOD2), NOCHT, leucine-rich repeat (LRR) and pyrin-domain containing protein 1 (NALP1)–NALP14, NLR family CARD domain-containing protein 4 (NLRC4, also known as

IPAF), neuronal apoptosis inhibitory protein (NAIP, also known as BIRC1), class II transactivator, NOD3, NOD9, and NOD27. NOD1 and NOD2 detect peptidoglycan-derived meso-diaminopimelic acid and muramyl dipeptide (MDP), respectively (Diacovich and Gorvel 2010).

Microbial infections activate the host immune system, which aims at eliminating the incoming pathogen, but at the same time may cause harm to the host organism if excessive inflammation is induced. The innate response therefore exerts a rapid first line of defense against the infection, but at the same time also initiates the process leading to eventual development of an adaptive immune response and establishment of immunological memory. Mammalian organisms can be infected by a number of different classes of microorganisms (viruses, bacteria, protozoa, etc.), which have fundamentally different physiologies, structures and mechanisms of propagation. However, because the innate immune system has only a limited number of PRRs available, recognition must be based on something common to infections with these highly different infectious agents. The current concept is that the two main principles in innate microbial recognition are the detection of PAMPs and aberrant localization of specific classes of molecules (Rasmussen et al. 2009).

After internalization by the host cell, several bacterial pathogens reside in intracellular membrane-bound compartments. Bacteria-containing vacuoles provide an enclosed space in which the host cell can direct high local concentrations of reactive oxygen species (ROS), reactive nitrogen intermediates (RNI) and antimicrobial peptides to eliminate intracellular pathogens. Bacteria that escape such membrane-bound compartments can encounter another mechanism of resistance such as ubiquitylation and proteasome degradation or autophagy. Furthermore, channel-forming toxins, virulence factors and other PAMPs derived from pathogens can trigger the activation of the inflammasome. The inflammasome is involved in the activation of caspase 1, which in turn promotes the maturation of several interleukins, the recruitment of inflammatory cells to sites of infection and the activation of a specialized host cell death pathway known as pyroptosis (Diacovich and Gorvel 2010).

The innate immune response constitutes the earliest phase of the host's defense against pathogens and will stimulate and modulate the later onset adaptive response. It operates through PRR that recognize PAMP of viruses, bacteria, fungi and protozoa. PAMPs are conserved within broad classes of pathogens. They are typically products of biosynthetic pathways that are essential for the survival of the pathogen and thus lack the potential for immune evasion through genetic variability. Owing to the panel of PAMPs that is recognized by PRRs, the innate immune system achieves an impressively complete coverage of pathogens despite the genetically limited number of available receptors. Engagement of antiviral PRRs by their cognate PAMPs activates signaling pathways that lead to the production of defense factors such as pro-inflammatory cytokines, type I interferons (IFN-α and IFN-β), or interferon stimulated genes (ISG). ISGs induced by IFN secretion or cell-autonomously upon viral infection collectively establish an antiviral state that limits viral replication and prevents further spread of the infection (Dixit and Kagan 2014).

Adaptation to an intracellular lifestyle offers most pathogens the ability to escape recognition by humoral immune responses such as circulating antibodies and complement. However, within an infected cell, a pathogen is further challenged by intracellular defense mechanisms. Prominent among these is the fusion of pathogen-containing vacuoles with lysosomal compartments. The ability of infected cells to dispose of microbial invaders depends on the cell type and cytokine-dependent activation. Activated macrophages and dendritic cells, for example, provide the least hospitable environment, while nonimmune cells are more permissive. To avoid lysosomal fusion, a pathogen could potentially escape the membrane-bound vacuole. However, NLRs and Rig-like receptors (RLRs) recognize PAMPs in the cytoplasm and induce the production of proinflammatory cytokines and chemokines. These molecules influence adaptive immune response and can trigger host cell death via activation of the inflammasome. Additional antimicrobial responses include autophagosome formation on the surface of cytoplasm-exposed bacteria and their eventual fusion with lysosomes (Kumar and Valdivia 2009).

MHC class II molecules are detected on the surface of professional Ag presenting cells (APCs): dendritic cells, B cells, and macrophages as well as some endothelial, epithelial and tumor cells. The heterodimeric MHC class II molecules fold in the endoplasmic reticulum and are trafficked to the protease rich-endosomal/lysosomal network where they bind to small peptides derived from proteolytically processed protein antigens. These peptide-loaded MHC class II molecules are then shuttled to the cell surface for display to CD4+ T cells. T cell receptors and accessory molecules on CD4+ T cells recognize specific MHC class II: peptide complexes along with co-stimulatory molecules on these APCs. Upon this recognition, CD4+ T cells are activated, secreting cytokines and initiating an immune response. MHC class II molecules display thousands of peptide ligands on the surface of a cell, yet how this spectrum of peptides is shaped by cell stress or metabolic changes is unclear. Classically MHC class II molecules were thought to only present epitopes derived from membrane and exogenous antigens that are internalized via endocytosis. Yet, epitopes derived from cytoplasmic and nuclear sources are also detected bound to these MHC molecules (Deffit and Blum 2015).

6.5 Involvement of the Cytoskeleton

The cytoskeleton and cytoskeletal motors play a critical role in organelle positioning and membrane traffic. Not surprisingly, many pathogens co-opt cytoskeletal functions to maintain and stabilize their intracellular niches (Kumar and Valdivia 2009). Actin is present in a monomeric form, or as filaments derived from the polymerization of several actin monomers. Actin filaments have a defined polarity, which determines the overall direction of filament growth. In cells, actin filaments are often dynamic, assembling or disassembling in response to external stimuli such as growth factors or extracellular matrix components. The regulated assembly or disassembly of F-actin plays critical roles in many cellular processes, remodel

membranes, generating force at actin–membrane interfaces, cell motility, cytokinesis, endocytosis, and vesicular trafficking from the endoplasmic reticulum or Golgi apparatus, contractility. The controlled disassembly of actin filaments also impacts many important biological events, including regulated exocytosis and the engulfment of particles through phagocytosis (Ireton 2013).

Intracellular bacterial pathogens invade non-phagocytic host cells such as intestinal epithelial cells using two mechanisms: zipper and trigger. Bacteria using the zipper mechanism, such as *Yersinia* spp. and *Listeria monocytogenes*, express surface proteins that bind receptors on the host cell membrane on contact, inducing signaling cascades that reorganize the actin cytoskeleton to internalize the bacterium. The trigger mechanism, which is best characterized in *Shigella flexneri* and S. Typhimurium, employs the Tipe III Secretion System (T3SS) to deliver proteins across the host plasma membrane; these proteins directly interact with the cellular components that regulate actin dynamics. Bacteria that escape to the cytosol interact with the actin polymerization machinery to migrate to the plasma membrane, where they can disseminate to neighboring cells. Contrary to intracellular pathogens, extracellular bacteria such as *V. parahaemolyticus* and enteropathogenic *Escherichia coli* (EPEC) adhere to host cells and secrete T3SS effectors that reorganize the actin cytoskeleton in order to manipulate the plasma membrane for effective infection (see Fig. 6.1) (Ham et al. 2011).

Microtubules and actin filaments are required for the transport of vesicles between membrane-bound organelles. The specificity of membrane fusion events is controlled by SNAREs, Rab proteins, and tethering factors. Not surprisingly, many intracellular pathogens modulate Rab recruitment for the establishment of replicative vacuoles (Kumar and Valdivia 2009).

Rab GTPases are central to the organization, maintenance and dynamics of the cellular endomembrane system through their functions in regulating specific membrane transport pathways. In bacterial infection, Rab proteins play a pivotal role in host immunity, internalization by endocytosis or phagocytosis, and directing the transport of phagocytosed pathogens to lysosomes for degradation. The normal transport pathway to lysosomes utilizes numerous Rab proteins to efficiently deliver pathogen containing vacuoles from an early phagocytic compartment to a Rab5-positive early endosomal compartment. Pathogens destined for degradation are then shuttled through a Rab7-positive late-endosome prior to reaching their final destination, the lysosomal compartment. Bacterial pathogens have devised intricate strategies for altering various aspects of the Rab-activation and functional cycle (Stein et al. 2012).

RHO-family GTPases regulate different aspects of actin dynamics: activation of RHOA induces the formation of actin stress fibers, activation of RAC1 induces the formation of lamellipodia, and activation of CDC42 induces the formation of filopodia. Inactivation of RHO-family GTPases leads to a decrease in F-actin and increase in monomeric actin (G-actin), resulting in loss of cell shape, motility and ability to phagocytose or endocytose pathogens. All of these RHO-family proteins (RHOA, RAC1 and CDC42) are common targets of bacterial effectors (Ham et al. 2011).

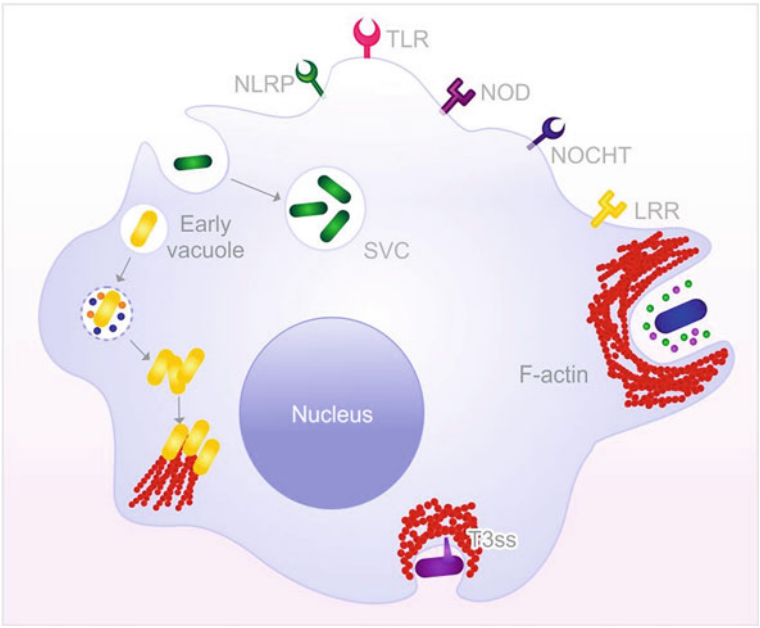

Fig. 6.1 *Bacterial* recognition by *host cells* is fundamental for the initiation of immune responses. Host cells express a range of receptors that recognize microbial products and activate the immunologic system, the well-known are Pattern Recognition Receptors (PRRs) as Toll-like Receptors (TLRs), NOD-like receptors (NLRs), Nucleotide-binding oligomerization domain-containing protein 1 and 2 (NOD1 and NOD2), NOCHT, leucine-rich repeat (LRR). Also actin cytoskeleton modulation of the host-cell is important for many microbial pathogens to enter cells, to disseminate within, to escape from phagocytic cells, or to promote attachment to the cell surface. SVC—*Salmonella* containing vacuole

Arp2/3 is an evolutionarily conserved complex of seven proteins. Two of the seven components (Arp2 and Arp3) have structural similarity to monomeric actin. The Arpc1 component has a WD40 domain that forms a seven-bladed beta propeller. The remaining components (Arpc2, Arpc3, Arpc4 and Arpc5) do not exhibit significant structural similarity to other known proteins. The Arp2/3 complex stimulates polymerization of a new actin filament from the side of an existing ('mother') filament, resulting in a Y-shaped branched actin structure. Studies involving electron tomography suggest that Arpc2 and Arpc4 contact the mother actin filament, whereas Arp2 and Arp3 interact with pointed end of the nascent filament. The Arp2 and Arp3 components are thought to form a dimer on the side of the mother filament, serving as the first subunits of the new actin filament. Thus, the Arp2/3 complex may stimulate actin polymerization by mimicking an actin dimer, whose formation is normally the rate-limiting step in filament assembly (Ireton 2013).

Intracellular membrane fusion events in the eukaryotic secretory pathway depend on a family of membrane bound SNARE proteins to catalyze the bilayer fusion stage. The SNARE family is characterized by a conserved 60–70 amino acid

heptad repeat region, termed SNARE motif, adjacent to a transmembrane or lipidic anchor. Several lines of evidence indicate that specific sets of SNARE proteins form stable complexes through assembly of their SNARE motifs into parallel four-helix coiled coil bundles. During membrane fusion, the regulated assembly of SNARE complexes from opposed membranes in trans is thought to drive bilayer fusion. Post membrane fusion SNARE complexes in cis are disassembled through an ATP and NSF dependent reaction that recycles the SNARE machinery for subsequent rounds of membrane fusion (Flanagan et al. 2015).

In addition to activating the Arp2/3 complex, N-WASP also binds and delivers actin monomers to the nucleation machinery. The WCA domain interacts with monomeric actin, and a proline-rich region binds actin complexed with profilin. N-WASP is itself subject to complex regulation. In the absence of cellular stimuli, N-WASP is autoinhibited due to intramolecular interactions that mask the activity of the WCA domain. The protein WIP (WASP-interacting protein) stabilizes the inactive conformation of N-WASP. In response to growth factors or other stimuli, autoinhibition of N-WASP is relieved through interactions with several cellular factors including the activated (GTP-bound) form of the small GTPase Cdc42, the lipid phosphatidylinositol 4,5-bis phosphate and Src (pronounced "sarc" as it is short for sarcoma) Homology 3 domains from the signaling proteins Toca-1, Nck or Grb2. In addition to these regulatory interactions, activation of N-WASP is also promoted by serine phosphorylation of its WCA domain, which increases the affinity of this domain for the Arp2/3 complex (Ireton 2013).

Given the ability of the Arp2/3 complex and Dia proteins to produce actin filaments that generate force and remodel cellular membranes, it is not surprising that many intracellular microbial pathogens have evolved mechanisms to exploit these two pathways of actin polymerization. For example, Listeria and Shigella manipulate Arp2/3 or N-WASP in order to promote actin-based motility (ABM). The bacterial factor responsible for ABM is a surface protein called ActA, which acts as a structural and functional mimic of the eukaryotic NPF N-WASP. The amino-terminal domain of ActA contains sequences with amino acid similarity to C and A regions of N-WASP. This domain also has an actin monomer binding sequence that is a functional equivalent of the N-WASP W (WH2) region. Like N-WASP, the amino-terminal domain of ActA activates the Arp2/3 complex, stimulating nucleation of branched actin filaments. In addition to this amino-terminal domain, a central proline-rich region of ActA also contributes to ABM by binding the host protein VASP. One possible role of VASP is to recruit profilin, which promotes addition of actin monomers to the plus end of actin filaments (Ireton 2013).

6.6 *Campylobacter jejuni*: Understanding the Enemy

Infections with pathogenic food-borne bacteria constitute one of the leading causes of morbidity and mortality in humans. The World Health Organization suggests that the human population worldwide suffers from about 4.5 billion incidences of

gastroenteritis annually, causing approximately 1.8 million deaths (WHO 2004; Boehm et al. 2012). Various *Campylobacter* spp. are the most common cause of bacterial diarrheal disease worldwide (Young et al. 2007a, b; Nachamkin et al. 2008; Boehm et al. 2012). It is estimated that each year up to 1 % of the western population is infected with *Campylobacter* (Boehm et al. 2012; Young et al. 2007a, b).

Campylobacter jejuni is a wide-spread Gram-negative bacterium and it is considered as a classical zoonotic pathogen, as it is found in the normal intestinal flora in many birds and mammals. Since *C. jejuni* colonizes various food animals, it can contaminate food products during processing and surface water (Friedman et al. 2000; Backert et al. 2013). Curiously, *C. jejuni* displays commensal behavior in chicken while in the human intestine, *C. jejuni* penetrates the mucus and colonizes the intestinal crypts in a very efficient manner. The molecular basis of the difference in pathogenicity of *C. jejuni* in human and chicken still remains to be resolved (Bouwman et al. 2013).

The clinical outcome of *C. jejuni* infection varies from mild, non-inflammatory, self-limiting diarrhea to severe, inflammatory, bloody diarrhea that can continue for few weeks (Young et al. 2007a, b; van Putten et al. 2009; Dasti et al. 2010; Oyarzabal and Backert 2011; Backert et al. 2013). In some cases, *C. jejuni* infections cause autoimmune disease and can be also associated with the development of reactive arthritis and peripheral neuropathies, known as Miller–Fisher and Guillain–Barrè syndromes (Nackamkim et al. 2008; Zilbauer et al. 2008; Backert et al. 2013).

After ingestion by a human host, these bacteria use their flagella-driven motility to colonize the epithelial cells of the ileum and colon (Boehm et al. 2012). The crypts seem to be an optimal growth environment for *C. jejuni* (Stahl et al. 2011; Bouwman et al. 2013). Disease development involves a multifactorial process requiring bacterial adherence to host cells, epithelial cell invasion, secretion of virulence proteins, and bacterial trans/location through the intestinal epithelium. Several studies suggest that after colonization, *C. jejuni* can cross the mucosal barrier and invade intestinal human cells (Park 2002; Konkel et al. 2004; Ó Cróinín and Backert 2012). The publication of numerous complete genome sequences of different *C. jejuni* strains has revealed an organism that displays a large degree of strain to strain variation. This natural heterogeneity has made studying the pathogenicity of this pathogen particularly challenging (Ó Cróinín and Backert 2012).

6.7 *Campylobacter jejuni* Invasion and Traffic in the Host Cell

6.7.1 Types of Routes

To access profound tissues and cause short- or long-term infections in the human body, various pathogenic bacteria, including *Salmonella, Shigella, Listeria* or

Neisseria, must overcome the epithelial barrier (Kazmierczak et al. 2001; Tegtmeyer et al. 2011; Boehm et al. 2012). These important bacterial pathogens are able to cross polarized intestinal epithelial cells by different mechanisms, known as the paracellular and the transcellular routes. Bacteria using the transcellular route enter host cells at apical surfaces followed by intracellular trafficking and leave these cells at the basolateral surface. In contrast, bacteria specialized on the paracellular route cross the epithelial barrier by passage between neighboring epithelial cells and overcome the tight junctions and adherens junctions (Balkovetz and Katz 2003; Boehm et al. 2012). In the case of *C. jejuni*, while some groups reported the paracellular route, others described the transcellular model or a mix of both (Konkel et al. 1992a, b; Monteville and Konkel 2002; van Deun et al. 2008a, b; Hu et al. 2008; Kalischuk et al. 2009; Boehm et al. 2012). In general, the host factors and bacterial factors involved in the transmigration process of *C. jejuni* are still unclear (Boehm et al. 2012).

The intestinal mucosal epithelium in humans is an important cell layer that controls not only digestive, absorptive and secretory functions, but also forms the first barrier against pathogenic microbes (Wessler and Backert 2008; Backert et al. 2013). The intact structure of healthy intestinal epithelial cells is maintained by the integrity of the apical-basal polarity, forming microvilli structures with a well-defined brush border, a highly organized actin-cytoskeleton and proper junctional complexes (Snoeck et al. 2005; Laukoetter et al. 2008; Backert et al. 2013). Importantly, well-established junctions are built up on the lateral cell-to-cell contacts including tight junctions (TJs) and E-cadherin-based adherens junctions (AJs) as well as basally located integrin-mediated cell-matrix contacts such as focal adhesions (FAs) and hemidesmosomes (HDs). While FAs are present both in cultured polarized and non-polarized cells, TJs, AJs, and HDs are only established in polarized and absent in non-polarized epithelial cells. TJs are based on junction adhesion molecules (JAMs), claudins, occludin and other proteins, which represent important structural elements in establishing epithelial cell polarity. They are crucial for the tight sealing of the cellular sheets, thus controlling paracellular ion flux and therefore maintaining tissue homeostasis. The tight apposition of the membranes at TJs, which are localized at the apical end of the lateral membrane, also blocks lateral mobility of membrane proteins and lipids allowing the segregation of membrane components in an apical and basolateral compartment (Backert et al. 2013).

The AJs are positioned basal to TJs and form a network of membrane proteins and associated molecules, which are responsible for the mechanical adhesion between neighboring cells. AJs assemble via homophilic, calcium dependent interactions between the extracellular domains of E-cadherin on the surface of two adjacent epithelial cells. E-cadherin does not only play as an adhesive protein, but also has important functions as a regulator of cell proliferation. By modulating the availability of β-catenin, which binds to the intracellular domain of E-cadherin and helps to connect AJs with the actin cytoskeleton, E-cadherin-based AJs are involved in cell signaling and transcriptional regulation. Therefore, disturbed

E-cadherin signaling is also associated with tumorigenesis (Cavallaro and Christofori 2004; Backert et al. 2013).

The FAs comprise the third group of cell adhesion structures and consist of integrin heterodimers (composed of α and β chains), which are transmembrane receptors that link the extracellular matrix to intracellular FA proteins. FAs modulate multiple signaling cascades to regulate cell attachment, proliferation, migration, differentiation and gene expression events. These processes are controlled by classical "outside in" and "inside out" signal transduction pathways (Hynes 2002; Luo et al. 2007; Backert et al. 2013). The extracellular domain of a given integrin can directly bind to extracellular matrix proteins such as fibronectin, while the cytoplasmic tail is linked to the actin-cytoskeleton via a large number of adapter proteins, including vinculin, paxillin or talin, and signaling enzymes such as focal adhesion kinase (FAK) or Src kinase. These protein complexes continually assemble and disassemble, and this turnover process must be differentially controlled at the leading edge versus the trailing edge of a migrating cell. In addition, HDs constitute adhesive protein complexes that mediate stable attachment of basal epithelial cells to the underlying tissues (de Pereda et al. 2009). Similar to FAs, the organization of HDs relies on a complex network of protein-protein interactions, but in HDs integrin α6β4, laminin and plectin play essential roles (Backert et al. 2013).

Interestingly, many microbial pathogens including *C. jejuni* have adapted mechanisms during evolution to exploit TJs, AJs, FAs, and/or HDs in infected cells in order to proliferate, survive and sometimes persist within the host (Vogelmann et al. 2004; Fasano and Nataro 2004; Backert and König 2005; Wessler and Backert 2008; Backert et al. 2013). A major goal of current *C. jejuni* research is to define the exact role of bacterial adhesion, invasion and transmigration across enterocytes for the induction or absence of pathogenesis in different hosts. Several in vivo studies of human biopsies and infected animal models reported on observations of *C. jejuni* entering gut epithelial cells and underlying subepithelial tissues during infection. For example, electron microscopic studies of biopsies from patients with campylobacteriosis have shown that *C. jejuni* can closely associate to the surface or within the intestinal epithelium, especially in Goblet cells, and was focally present in the lamina propria (van Spreeuwel et al. 1985; Backert et al. 2013). The majority of human patients exhibited the histological picture of acute infectious colitis associated with massive infiltration of immune cells and marked distortion of crypt architecture. Penetration of *C. jejuni* into the intestinal tissue is also supported by the presence of blood and leukocytes in stool samples. Similar observations were obtained during *C. jejuni* infection experiments in monkeys, hamsters, piglets, rabbits and ferrets (Humphrey et al. 1986; Russell et al. 1993; Babakhani et al. 1993; Everest et al. 1993; Nemelka et al. 2009; Backert et al. 2013). In addition, live *C. jejuni* were recovered from other organs in infected animals such as the spleen, liver, mesenteric lymph nodes and blood (Vuckovic et al. 1998; Lamb-Rosteski et al. 2008; Nemelka et al. 2009; Backert et al. 2013). This suggests that *C. jejuni* exhibits the capability not only to adhere to and enter into enterocytes, but can also travel within the host, pass the intestinal epithelial barrier, enter the

lamina propria and even access other organs of various infected hosts (Backert et al. 2013).

Advantages for *C. jejuni* reaching the underlying tissues and submucosa include that the bacteria are no longer subject to peristaltic forces in the intestine and they may gain pronounced access to certain nutrients such as iron. In addition, invasive *C. jejuni* can achieve contact with a set of basal host cell receptors such as fibronectin, which are normally not present at apical surfaces. Another advantage could be that the intracellular environment is better protected to antibiotics as compared to the gut lumen. Finally, by causing inflammatory diarrhea in the intestine, *C. jejuni* can improve its own spread to find a new host (Backert et al. 2013).

This is in agreement with observations that stools from patients are diarrheal and remain *C. jejuni* positive for several weeks (Young et al. 2007a, b; van Putten et al. 2009; Dasti et al. 2010; Oyarzabal and Backert 2011; Backert et al. 2013). Experiments of cultured cell lines with *C. jejuni* have shown that the bacteria can bind to, invade into and survive inside a defined intracellular compartment, called the *Campylobacter*-containing vacuole. These phenotypes, have been reported for both *C. jejuni* infection of non-polarized and polarized epithelial cells. Studies of the translocation capabilities of *C. jejuni* strains across an intestinal epithelium in vitro require tight polarized cell monolayers. Typical chosen cell lines expressing TJs, AJs and FAs include Caco-2, T84, MDCK-I or MKN-28 (Konkel et al. 1992a, b; Everest et al. 1993; Grant et al. 1993; Harvey et al. 1999; Monteville and Konkel 2002; Chen et al. 2006; Hu et al. 2008; Wine et al. 2008; Boehm et al. 2012; Hoy et al. 2012; Backert et al. 2013).

It has been described that while *C. jejuni* can adhere to different cell lines with similar extend the bacterial invasion and transmigration capacities can vary considerably between the different cell lines (Konkel et al. 1992a, b; Brás and Ketley. 1999; Beltinger et al. 2008; Wine et al. 2008; Backert et al. 2013). It was proposed that *C. jejuni* can enter cultured epithelial cell lines of human origin with higher efficacy as compared to non-human cells, suggesting that the pathogen is particularly specialized for disease triggering infection of the human host (Konkel et al. 1992a, b; Backert et al. 2013).

Pathogens utilizing the paracellular mechanism break the TJ and AJ complexes and cross the epithelial barrier by passage between neighboring epithelial cells (Wessler and Backert 2008; Backert et al. 2013). In contrast, some other pathogens specialized on the transcellular mechanism and invade epithelial or specialized M cells at the apical surface followed by intracellular trafficking and exit these cells at the basolateral membrane (Balkovetz and Katz 2003; Bencurova et al. 2011; Backert et al. 2013). Studies on the translocation capabilities of *C. jejuni* across an intestinal epithelium layer in vitro have been performed with multiple strains and polarized cell lines grown in transwell chambers. Migration of various Trans+ *C. jejuni* strains from the apical compartment of transwells through polarized cells was confirmed by determination of colony forming units (CFU) obtained from the lower chamber, gentamycin protection assay (GPA) and other functional assays. Application of chloramphenicol, a well-known inhibitor of bacterial protein biosynthesis, reduced the transmigration potential of *C. jejuni* significantly (Konkel

et al. 1992a, b; Backert et al. 2013). The failure of chloramphenicol to completely abolish translocation may indicate that some of the bacteria possess the factors necessary to facilitate penetration while others may have to synthesize such components *de novo* (Konkel et al. 1992a, b; Backert et al. 2013).

C. jejuni adherence, penetration and transmigration activities were also inhibited at lower temperatures when investigated at 20 and 4 °C as compared to 37 °C (Konkel et al. 1992a, b). These data suggest that adhesion, internalization and translocation of *C. jejuni* require active bacterial and host cell processes at optimal temperature. The current, common opinion is that *C. jejuni* can effectively transmigrate, but the involved mechanisms are controversial in the literature (Backert et al. 2013). *C. jejuni* transmigration did not quantitatively correlate with the intracellular invasiveness of these isolates and a similar repertoire of strains including Inv+/Trans+, Inv+/Trans- and Inv-/Trans+ isolates were found. Taken together, these data suggest that different phenotypic wild-type *C. jejuni* isolates exist in nature and that bacterial transmigration capabilities may correlate with colitis disease outcome. However, more studies are certainly necessary to substantiate this hypothesis (Backert et al. 2013).

The trans-epithelial electrical resistance (TER) of polarized epithelial cells is an indicator of intercellular integrity. Translocation of *C. jejuni* by a paracellular route would be expected to disrupt the junctional complex between epithelial cells. This would increase monolayer permeability and reduce the TER. The ability of enteric pathogens to translocate across the intestinal cell barrier is a prerequisite for infection and is therefore considered an important virulence attribute (Pogacar et al. 2010). To assess the effect of *C. jejuni* on epithelial intercellular integrity, some authors measured the TER immediately after infection of polarized pig small-intestinal (PSI) cells at day 7 (Pogacar et al. 2010). Following attachment of *C. jejuni* to the polarized PSI cells, were observed a decrease in TER in the first 24 h after infection and again at 72 h after infection. The latter effect in TER could be argued to have occurred as a consequence of replication of *C. jejuni* at the cell surface and translocation of these organisms into the intestinal epithelial cells used in this study. A disturbance in TER during the first 24 h after entry of *C. jejuni* into the gut is the result of these bacteria using a transcellular rather than a paracellular route of translocation, as the TER during this interval shows only slight changes. These results imply that *C. jejuni*, once inside the host cell, have no effect on intercellular integrity and could disseminate through the intestinal barrier by a transcellular route (Pogacar et al. 2010).

It has been recently showed that a closely related pathogen, *Helicobacter pylori*, secretes a novel bacterial virulence determinant into the culture supernatant, the serine protease HtrA, which is also present in *C. jejuni*. HtrA proteins constitute a group of heat shock induced serine proteases that influence the adhesion and invasion properties of different bacterial pathogens. HtrA proteins typically consist of a signal peptide, a trypsin-like serine protease domain and one or two protein interaction (PDZ—**P**ost synaptic density protein, **D**rosophila disc large tumor suppressor, and **Z**onula occludens-1 protein) domains. In addition, by binding of the PDZ domain in one HtrA molecule to that in other HtrA molecules, HtrA can

build-up to highly proteolytic active oligomers that also function as a chaperone. The HtrA protease domain consists of an active site, called the catalytic triad, which is formed by the conserved amino acid residues histidine, aspartatic acid and serine. Many bacterial HtrA proteins are suggested to be localized in the periplasm and to be involved in quality control of envelope proteins by degradation of misfolded proteins as well as prevention of formation of aggregates. Thus, it was surprising to find that HtrA exhibits the capability of extracellular transport in *H. pylori*, where it could cleave host surface molecules. It was demonstrated in infected INT-407 or MKN-28 cells that *C. jejuni* HtrA can cleave E-cadherin but not fibronectin as *H. pylori* HtrA. And was also found that the amino acids in the catalytic triad (histidine, aspartate and serine) are conserved and at the expected position among these proteins. These results suggest that HtrA's are highly conserved in various *C. jejuni* isolates (Boehm et al. 2012).

Various in vivo and in vitro studies have shown that this pathogen encodes numerous virulence determinants involved in important disease-associated processes such as bacterial adhesion to, transmigration across, invasion into and intracellular survival within infected intestinal epithelial cells (Backert et al. 2013).

If apical binding of *C. jejuni* to human epithelial cells is a pre requisite for subsequent invasion and transcellular migration is also unclear. There is rapid increase in reports on putative bacterial adhesion factors we have now a list of more than 20 bacterial factors with proposed role in binding and subsequent invasion. In contrast, there is a large gap in our knowledge on corresponding host cell receptors. Thus, there is an urgent need for identifying and characterizing host receptors which can be attributed to certain bacterial factors (Backert et al. 2013).

The only receptor pathway intensively studied and verified by various independent research groups is the CadF → fibronectin → integrin signaling cascade (Monteville and Konkel 2002; Krause-Gruszczynska et al. 2007a, b, 2011; Boehm et al. 2011; Eucker and Konkel 2012; Backert et al. 2013). These studies have presented high resolution pictures of various invading *C. jejuni* strains in multiple non-polarized cell types, but corresponding qualitative and quantitative data for a set of *C. jejuni* strains invading polarized cells from apical or basal membranes are currently not available (Krause-Gruszczynska et al. 2007a, 2011, Boehm et al. 2011). Alternative possibilities include the involvement of ganglioside-like LOS in apical invasion, thus favoring a transcellular route, but this model is in contrast to the paracellular model for HtrA-mediated opening of AJs and basal invasion as triggered by the CadF → fibronectin → integrin complex. How *C. jejuni* can open the TJs after longer coincubation times is yet unclear (Backert et al. 2013).

Several studies exist that could support the apical invasion model, but can *C. jejuni* also enter host cells from basal surfaces? Basal engulfment and entry of *C. jejuni* into non-polarized Chang or polarized Caco-2 cells has been demonstrated by TEM and immunofluorescence microscopy, and this process has been called subvasion (van Alphen et al. 2008; Bouwman et al. 2013; Backert et al. 2013). However, if paracellular transmigration is a prerequisite for subvasion in polarized cells is not yet clear. Furthermore, it is also unclear how the T3SS dependent

injection of certain Cia proteins fits in any of the above models. Thus, more studies are clearly required to unravel the sequence of events that allow *C. jejuni* strains to travel across polarized intestinal epithelial cells, either by a transcellular or paracellular pathway or a mix of both. It should be also considered that individual *C. jejuni* strains might switch from one to the other mode under specific culturing or infection conditions (Backert et al. 2013).

6.7.2 Cytoskeleton

Experimental studies using human cell culture models indicate that *C. jejuni* can enter cells via different routes. Both actin-dependent and microtubule-dependent uptake into eukaryotic cells has been reported. The uptake process may require cellular factors such as caveolin-1 and the small Rho GTPases Rac1 and Cdc42, but not dynamin. The reports of different uptake requirements suggest that *C. jejuni* has evolved multiple mechanisms to gain access to eukaryotic cells, albeit with variably efficiency (Bouwman et al. 2013).

Overall, it has been demonstrated that *C. jejuni* subvasion requires intact microtubules, but that efficient *C. jejuni* invasion into polarized epithelial cells can occur via an actin- and microtubule-independent mechanism. Together, these results suggest that *C. jejuni* efficiently invades an intact layer of polarized epithelial cells from the basal cell side once an access point is available (Bouwman et al. 2013).

Disruption of the polarized Caco-2 actin cytoskeleton dynamics using cytochalasin D or jasplakinolide enhanced rather than blocked *C. jejuni* invasion, similarly, fixation of the microtubules with paclitaxel did not inhibit *C. jejuni* invasion. Disruption of the microtubules with colchicine severely reduced the number of intracellular bacteria, but also the number of subcellular *C. jejuni*. In an attempt to distinguish the effect(s) of colchicine on the subvasion and subsequent invasion process, the islands of polarized epithelial cells were infected for 1 h with *C. jejuni* strain 108 to allow bacterial subvasion to occur, prior to the addition of colchicine. This procedure did not block bacterial invasion, suggesting a role of microtubules in allowing subcellular migration rather than bacterial invasion. To corroborate the actin and microtubule-independent invasion of *C. jejuni*, the polarized Caco-2 cells were treated with the combination of cytochalasin D and colchicine prior to infection with *C. jejuni*. This yielded large numbers of intracellular bacteria for both strains. Overall, this suggests that *C. jejuni* subvasion requires intact microtubules, but that efficient *C. jejuni* invasion into polarized epithelial cells can occur via an actin- and microtubule-independent mechanism. Most enteropathogens trigger their own uptake into eukaryotic cells through activation of cellular endocytic processes that require energy-consuming rearrangement of the actin cytoskeleton and/or microtubule network (Bouwman et al. 2013).

Beside that, it has been showed that different strains of *C. jejuni* efficiently invade polarized epithelial cells via an actin- and microtubule-independent

mechanism, even in the presence of the ATP-depleting compound DNP. The unusual qualities of the described *C. jejuni* invasion mechanism underline the different nature of this pathogen compared to other enteropathogens. Our results indicate that the highly efficient *C. jejuni* entry of the polarized cells involves a different mechanism that does not require gross rearrangement of the cytoskeleton. In fact, most efficient entry of *C. jejuni* was noted in the presence of the actin- and microtubule polymerization inhibitors. The increase in *C. jejuni* invasion in the presence of cytochalasin D may be explained by increased accessibility to the subcellular space due to retraction of cell protrusions. The actin cytoskeleton stabilizing compound jasplakinolide also increased invasion, probably due to inhibition of the turnover of actin filaments which may eventually also result in a loosening of cell attachment. However, when added together with cytochalasin D or at 1 h after inoculation, this effect was overcome suggesting that colchicine merely prevented subvasion rather than the bacterial entry into the cells. We are not aware of other enteropathogens capable of entering mucosal cells via a seemingly actin- and microtubule-independent pathway. For the entry of polarized epithelial cells it was essential to use islands of polarized epithelial cells rather than intact monolayers (Bouwman et al. 2013).

6.7.3 Intracellular Traffic

Campylobacter jejuni is a remarkable foodborne microbe, but by comparison to other well-known enteric pathogens, we know very little about the bacterial and host factors involved in establishing infection and triggering disease. This dilemma is in part due to the clear absence of classical bacterial adhesins, toxins, or typical T3SSs or T4SSs in the sequenced *C. jejuni* genomes. The other enormous handicap is the large amount of highly conflicting data in the literatura (Ó Cróinín and Backert 2012).

For almost every reported factor proposed to be involved in a given host response, there is at least one other study showing the opposite. It is possible that the reported results depend on the specific strains used, how the bacteria were grown, but also on differences in the experimental conditions and applied methodology. In addition, many studies using single mutants lack genetic complementation of the corresponding wild-type gene, which although technically very difficult in *C. jejuni*, would be very useful to restore the phenotypes reported for many of the aforementioned pathogenicity factors (Ó Cróinín and Backert 2012).

It was therefore suggested that *C. jejuni* could translocate across polarized cell monolayers by passing through single cells and/or between two neighboring cells (Backert et al. 2013). The application of pharmacological inhibitors has indicated that the activity of phosphoinositid-3-kinase is necessary for *C. jejuni* transcytosis (Wine et al. 2008; Backert et al. 2013). The role of membrane lipid rafts was assessed by pharmacological depletion of cholesterol and caveolin co-localization using immunofluorescence microscopy. In addition, it was shown that *C. jejuni*

transmigration was enhanced by adding interferon-gamma, probably because of its TER-reducing capabilities during inflammation (Rees et al. 2008; Backert et al. 2013).

Many other studies have shown that inactivation of flagellar genes in *C. jejuni* resulted in a colonization-negative phenotype in various animal models (Hendrixson and DiRita 2004; Szymanski et al. 2005; Young et al. 2007a, b; Backert et al. 2013). The flagellum does not only have a distinct function in bacterial motility and cell binding, but also acts as a type III secretion system (T3SS) for the delivery of Cia (*Campylobacter* invasion antigens) proteins into the extracellular space or into the host cell. The first described Cia protein member is CiaB (Konkel et al. 1999a, b, c; Backert et al. 2013). The CiaB protein was reported to be translocated into the cytoplasm of host cells, suggesting that it is a T3SS effector molecule facilitating invasion (Konkel et al. 1999a, b, c; Backert et al. 2013). CiaB expression was also shown to be crucial for the secretion of at least eight other Cia proteins, ranging in size from 12.8 to 108 kDa, that were induced upon host cell contact or by the presence of calf serum (Rivera-Amill and Konkel 1999; Backert et al. 2013). However, the exact function of CiaB is not yet clear. Interestingly, the invasion-defective ΔciaB mutant was able to transmigrate across polarized T84 cells like wild-type bacteria suggesting that apical cell invasion is not necessary for *C. jejuni* transmigration, thus favoring the paracellular route (Monteville and Konkel 2002; Backert et al. 2013).

Finally, besides the commonly applied transwell system, a vertical diffusion chamber model system has been recently described, which creates microaerobic conditions at the apical surface and aerobic conditions at the basolateral surface of cultured intestinal epithelial cells, thus producing an in vitro system that probably closely mimics in vivo conditions of the human intestine (Mills et al. 1968; Backert et al. 2013). The use of this vertical diffusion chamber for studying the interactions of *C. jejuni* with intestinal epithelial cells demonstrated the importance of performing such experiments under conditions that converge to the in vivo situation and will allow novel insights into *C. jejuni* pathogenic mechanisms (Mills et al. 1968; Backert et al. 2013). In addition, it should be noted that most of the cell lines used for in vitro studies are already transformed because they originate from cancer patients (Backert et al. 2013).

One of the most effective invasion pathways resulting in nearly 100 % of bacterial uptake at low inocula involves the subvasion entry pathway. This mechanism involves migration of *C. jejuni* underneath cultured cells, followed by bacterial invasion from the basal cell side instead of the apical side (Pryjma et al. 2012; Bouwman et al. 2013). The sequence of events that drive this uptake process remains to be resolved.

Following entry into intestinal epithelial cells, *C. jejuni* appears to localize in a specific compartment in the cytoplasm, which seems to be distinct from the lysosomes. It was found that the *C. jejuni*-containing vacuole (CCV) deviates from the canonical endocytic pathway immediately after host cell entry, thus avoiding delivery into lysosomes. The CCV appears to interact with early endosomal compartments because it associates with early endosomal marker protein EEA-1 and

two trafficking GTPases, Rab4, and Rab5. However, this interaction seems only transient and does not progress inside the canonical endocytic pathway (Ó Cróinín and Backert 2012).

The CCV can be also stained with Lamp-1, a late endosomal marker, although this compartment appears to be unique and clearly distinct from lysosomes. CCVs were not stainable with the lysosomal marker protein cathepsin B and it is also not accessible to certain endocytic tracers. Taken together, the acquisition of Lamp-1 occurring very early during maturation of CCVs appears to proceed by an unusual pathway not requiring the GTPases Rab5 or Rab7, although recruited to the CCV. More studies are required to elucidate in more detail the mechanism by which *C. jejuni* modulates intracellular trafficking and survival (Ó Cróinín and Backert 2012).

CCV are supposed to be a special compartment specifically induced by *C. jejuni*, reminiscent of *Salmonella* that creates its own vacuole Salmonella containing vacuole (SCV). Whether *C. jejuni* survives inside epithelial cells is still under investigation. Intracellular survival may vary dependent on the nature of the *C. jejuni* containing compartment. Furthermore, the procedure to recover the intracellular *C. jejuni* may influence bacterial survival assay results. It has been demonstrated that the *C. jejuni* subvasion entry mechanism is driven by a novel actin- and microtubule-independent process that results in high numbers of intracellular membr/ane-bound bacteria of which a subset survives for up to 48 h (Bouwman et al. 2013).

However, *C. jejuni* still invaded the polarized Caco-2 cells in the presence of 2,3-dinitrophenol (DNP), a ATP inhibitor. This result is consistent with the apparent absence of energy-consuming cytoskeletal changes during the *C. jejuni* entry process. After 1 h of infection *C. jejuni* did not colocalize with any of the labeled cellular compartments and were mainly present at the basal cell Surface (Bouwman et al. 2013).

After 5 h of incubation, the majority (95 %) of the *C. jejuni* resided in CD63-positive membrane-bound vacuoles, a marker of late endo(lyso)somes. *C. jejuni* remained in these compartments for the duration of the infection (24 h). Similar co-localization was observed with the late endosomal marker Lamp-1 consistent with earlier studies. At the times of infection (1, 5, 24 h) investigated *C. jejuni* only rarely co-localized with EEA1-positive early endosomal compartments and did not seem to be specifically localized in close vicinity of the Golgi apparatus. The apparent absence of colocalization of *C. jejuni* with the early endosome marker EEA1 may indicate a rapid intracellular trafficking or perhaps even a bypassing of this route after uptake by this novel invasion pathway. At prolonged infection, a close association of *C. jejuni* with the Golgi apparatus has been reported (Bouwman et al. 2013).

After 48 h of infection, the bacteria did appear absent from the cells. The highly efficient invasion occurred at the basal cell side of polarized epithelial cells and resulted in intracellular bacteria residing in CD63-positive cellular compartments. A novel luciferase reporter-based bacterial viability assay revealed survival of a subset of the intracellular *C. jejuni* for up to 48 h. An unexpected microscopic

observation was the apparent absence of *C. jejuni* in the host cells after 48 h of infection. Control experiments using *C. jejuni*-specific antibodies revealed the presence of *C. jejuni* albeit with changed morphology (Bouwman et al. 2013).

Uptake studies of *C. jejuni* into human epithelial cells demonstrate that viability is important for bacterial entrance. It has been showed that inactivation of protein synthesis reduced the amount of *C. jejuni* capable of entering the cells but not of binding to them. The intracellular presence of *C. jejuni* in human epithelium is in the fact that the majority of *C. jejuni* survived by residing in a vacuolar compartment that did not fuse with lysosomes (Olofsson et al. 2013).

In contrast, in human macrophages which share features with amoebae, *C. jejuni* could not avoid delivery to lysosomes. Previous studies have shown that *C. jejuni* are able to invade, survive and multiply within unicellular eukaryotes, mainly of the genus *Acanthamoeba*. In *A. polyphaga*, the bacteria were able to replicate in co—culture at 37 °C under aerobic conditions and survive for more than 2 months (Olofsson et al. 2013).

The uptake and intracellular trafficking of the *C. jejuni* strain 81–176 in *A. polyphaga* and found that the kinetics of internalization, the total number of internalized bacteria as well as the intracellular localization of internalized *C. jejuni* were dependent on bacterial viability. Furthermore, the number of *A. polyphaga* trophozoites that associated with bacteria was also strongly influenced by bacterial viability. These results suggest that the uptake and intracellular survival of *C. jejuni* in *A. polyphaga* is bacterially induced (Olofsson et al. 2013).

It was found that the kinetics of internalization was quite different between viable and heat killed bacteria in that viable bacteria were taken up at a high rate up to 24 h of co-culture with a decline thereafter, whereas the heat killed bacteria showed a significantly lower initial rate of internalization. This result suggests that *A. polyphaga* can ingest both viable and heat killed *C. jejuni*, but that viable bacteria were taken up more efficiently, indicating a bacterially induced invasion. Studies of intracellular localization using the lysosomal marker dextran showed that the majority of the viable *C. jejuni* were localized in non acidic vacuoles and only a smaller fraction in acidic vacuoles. This difference was not observed for heat killed *C. jejuni* where the numbers of bacteria inside and outside acidic vacuoles were equal. These results indicate that both viable and heat killed bacteria were processed for degradation in acidic vacuoles, but that viable bacteria could to a larger extent escape this degradative pathway and the giant lysosomes in particular (see Fig. 6.2) (Olofsson et al. 2013).

6.7.4 Virulence Factors

Despite the significant health burden caused by *C. jejuni* infections, our present knowledge about the interplay between *C. jejuni* and its various hosts is still very limited. The availability of complete genome sequences from various *C. jejuni* isolates has started to improve our understanding in genetics, physiology,

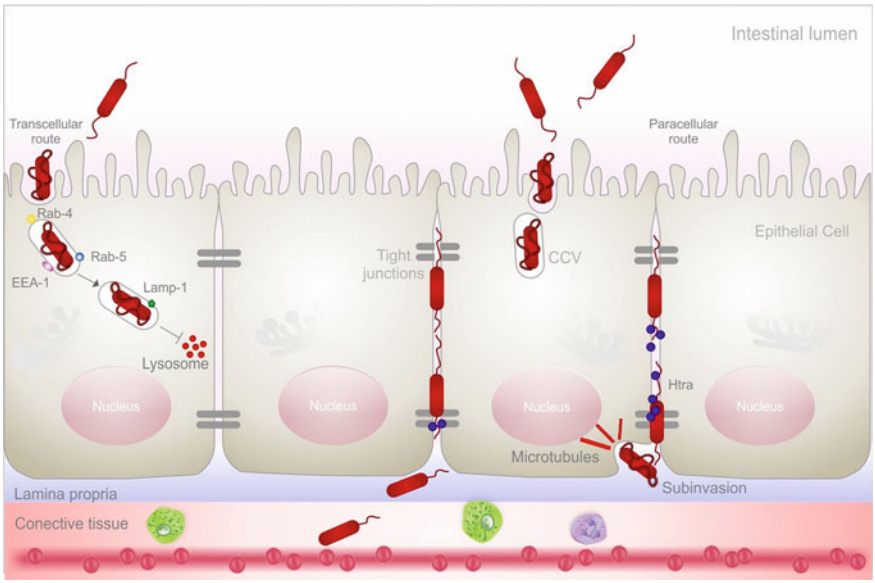

Fig. 6.2 *Campylobacter jejuni* dilemma: paracellular route, transcellular route or both? Transmigration is a mechanism of virulence used by many bacterial pathogens to disseminate within the host, but how *C. jejuni* translocates across epithelial barriers has been known. Maybe two pathways, transcellular and paracellular routes, could be involved. From *left* to *right*, in transcellular route *C. jejuni* is able to translocate across the intestinal epithelium involving endocytic uptake followed by intracellular trafficking, in paracellular route *C. jejuni* may also translocate between disrupted epithelial tight junctions. CCV—*C. jejuni*-containing vacuole

pathogenesis and immunity of *C. jejuni* infections in recent years. *C. jejuni* is the first bacterium reported to encode for both O- and N-linked glycosylation systems, a property that is likely influencing the host-pathogen crosstalk and disease outcome (Backert et al. 2013). In addition, a multitude of infection studies in various animal and in vitro cell model systems revealed the importance of *C. jejuni* motility and chemotaxis as critical features important for establishing successful infections (Szymanski et al. 2005; Young et al. 2007a, b; Janssen et al. 2008; Backert et al. 2013).

Significant progress has been made in recent years which contributed to understanding of the role of several key factors including the cytolethal distending toxin as well as glycosylation and molecular mimicry processes. One of the key differences between infection of humans and chickens by *C. jejuni* is the apparently increased number of bacteria invading epitelial cells in the human host. This suggests that both bacterial adherence to and entrance into epithelial cells may be critical steps that are essential for disease development. Thus, the identification of factors involved in these processes is the key for developing therapeutics to treat infections as well as enhancing our understanding of the pathogenesis (Ó Cróinín and Backert 2012).

There is increasing evidence showing that *C. jejuni* disturbs the normal absorptive capacity of the human intestine by damaging epithelial cell functions, either by cell invasion, the production of pathogenicity-associated factors or indirectly by triggering inflammatory responses. It has been proposed that transmigration across and invasion into intestinal epithelial cells during infection is a major reason of *C. jejuni*-triggered tissue damage. Investigation of gut biopsies obtained from infected patients and in vitro infection experiments of intestinal epithelial cells indicated that *C. jejuni* can enter human host cells. *C. jejuni* expresses various adhesins in the outer membrane including CadF, FlpA, JlpA and PEB1 (Pei et al. 1998; Poly and Guerry 2008; Eucker and Konkel 2012; Boehm et al. 2012). For example, in vitro CadF is a well-known bacterial factor that binds to fibronectin, an important extracellular matrix (ECM) protein and bridging factor to integrin receptors (Moser et al. 1997; Konkel et al. 1999a, b, c; Boehm et al. 2012). Maximal bacterial adherence and invasion of INT-407 intestinal epithelial cells is dependent on CadF and is associated with tyrosine phosphorylation of paxillin, a focal adhesion-based scaffolding factor (Monteville et al. 2003; Boehm et al. 2012). The expression of CadF also seems to be required for the stimulation of the small Rho GTPases Rac1 and Cdc42 via fibronectin and integrin member β1, that are required for *C. jejuni* host cell entry. The signaling pathways involved in the latter process have been described in detail (Krause-Gruszczynska et al. 2007a, b; Boehm et al. 2011, 2012). However, fibronectin and integrin β1 are basolateral receptor molecules and not commonly exposed at apical surfaces in the intestine. It is therefore unclear how *C. jejuni* gains access to these receptors during infection (Boehm et al. 2012).

Mechanisms of bacterial survival under unfavorable environmental conditions, such as the coccoid viable but non culturable state, are known (Park 2002; Murphy et al. 2006), but different virulence properties of cells exposed to stress are still poorly understood (Verhoeff-Bakkenes et al. 2009). Attachment of *C. jejuni*, followed by their invasion of epithelial cells, is a critical stage and a prerequisite for *C. jejuni* pathogenesis. Besides adhesion and invasion, bacterial translocation into intestinal subepithelial tissues is considered an important virulence trait, allowing *campylobacters* to disseminate throughout the host, which may lead to severe systemic disease (Rubesa Mihaljević et al. 2007; Pogacar et al. 2010).

It is still controversial if the role of the flagellum during invasion is restricted to bacterial motility or secretion of bacterial Cia proteins into the medium or even injection into the host cell. This model of Cia protein secretion through the flagellum is very tempting and would support the idea that *Campylobacter* uses a "trigger mechanism" of invasion involving the secretion of effector proteins directly into the cell to induce their uptake, similar to *Salmonella* and *Shigella*. Thus, much more work is required to confirm the role of the flagellum as a secretion system for effector proteins involved in invasión (Novik et al. 2010; Ó Cróinín and Backert 2012).

Confusion also exists as to the exact role played by some of the previously proposed adhesins (Jin et al. 2003; Ó Cróinín and Backert 2012). Proteins such as

JlpA have been described by some authors as being an important adhesin, whereas other groups have been unable to show any significant decrease in invasion in a *jlpA* mutant (Novik et al. 2010). Furthermore, factors such as the PEB proteins which were originally described as adhesins (Pei et al. 1991) now appear to primarily have roles as transporters or as chaperones (Kale et al. 2011), suggesting that they may not directly interact with the host cell but play a more indirect role (Ó Cróinín and Backert 2012).

One protein that is very well characterized and appears to clearly play a role in adherence to host epithelial cells is CadF. This protein along with the recently described FlpA protein appears to bind to fibronectin and specific fibronectin binding sites have been identified in CadF. The importance of CadF has been observed in a large number of strains and using a variety of different experimental approaches. Thus, targeting the fibronectin/integrin receptor could explain why *C. jejuni* may try to reach basolateral surfaces during infection. How the bacteria breach this epithelial barrier, by a transcellular route or a paracellular route, is also under much debate and not yet clears. However, the underlined importance of CadF and the fibronectin/integrin might give support to a "zipper"-like mechanism of invasion as used by *Listeria* or *Yersinia* species (Ó Cróinín and Backert 2012; Lugert et al. 2015).

6.7.5 Campylobacter jejuni *in Poultry and Chickens*

In fact, the intracellular trafficking of *C. jejuni* in poultry and chickens is not fully understood. But, the lower intestine may be the main reservoir *Campylobacter* may be detected in several internal organs such as liver and spleen (Knudsen et al. 2006; Pielsticher et al. 2012). Both meat and laying type chickens are colonized. *C. jejuni* can be also found in other poultry species such as turkeys, Muscovy and Pekin ducks. Beside poultry, a vast variety of wild birds, such as gulls, corvids, waterfowl and passerines are also susceptible for *Campylobacter* spp. and may act as vectors for transmission especially to poultry flocks (Glünder et al. 1988; Craven et al. 2000; Keller et al. 2011; Pielsticher et al. 2012).

C. jejuni isolates can have different colonization potential (Stern et al. 1988; Ringoir and Korolik 2003; Hänel et al. 2009; Hermans et al. 2011). Isolates from humans have been reported to be less successful in colonizing chickens than poultry isolates (Korolik et al. 1998; Ringoir and Korolik 2003; Hermans et al. 2011). Enhanced colonization capacity and increased virulence after in vivo passage through chicks has been shown in several other studies as well (Stern et al. 1988; Sang et al. 1989; Cawthraw et al. 1996; Hermans et al. 2011). This variability in colonization capacity, but the fixedness of the colonization phenotype of a given

strain indicates that *C. jejuni* genes involved in initial and sustained colonization are not identical. However, in contrast to this stable colonization phenotype (Ringoir and Korolik, 2003; Hermans et al. 2011), it has been previously reported that after several in vivo passages a poorly colonizing isolate was able to consistently colonize chicks (Stern et al. 1988; Hermans et al. 2011).

As in the environment, also in the chicken intestine *C. jejuni* is likely to encounter environmental stressors compromising optimal growth (Murphy et al. 2006; Hermans et al. 2011). The persistent colonization of the chicken GI (gastrointestinal) tract by *C. jejuni* indicates that the bacterium harbors regulatory systems that confer protection toward a hostile environment inside, but also outside the host. The mechanism by which the bacterium adapts to this "hostile" environment, resulting in successful and persistent colonization, is poorly understood. It is clear, however, that successful colonization of the chicken GI tract is a multifactorial process (Newell 2002; Hermans et al. 2011) in which genes involved in all areas of the colonization process of *C. jejuni* play a role.

The flagellar apparatus functions as a type III secretion apparatus for the *Campylobacter* invasion antigens (Cia proteins) (Konkel et al. 2004; Hermans et al. 2011), important for in vitro cell invasion (Konkel et al. 1999; Hermans et al. 2011) and chick colonization (Ziprin et al. 2001; Hermans et al. 2011), and secretion is enhanced upon exposure to chicken mucus (Biswas et al. 2007; Hermans et al. 2011). The role of motility of *C. jejuni* colonization in the chicken GI tract is not fully understood. Non-motile *C. jejuni* mutants can colonize chickens, be it at substantially reduced levels and only when chickens are inoculated with high amounts of viable cells (Woster et al. 2004; Hermans et al. 2011).

Probably, motility is needed for *C. jejuni* to pass the GI tract so it can reach its protective niche, the mucus layer of the cecal crypts (Beery et al. 1988; Hermans et al. 2011), and to resist gut peristalsis (Hendrixson and Dirita 2004; Hermans et al. 2011), hence it is important for initial colonization. It is, however, not known if motility is important in the persistence of *C. jejuni* in the intestinal tract, leading to long-term colonization. In any case, it is clear that the specialized flagellum of *C. jejuni* serves multiple functions in the adaptation of *C. jejuni* to the chicken GI tract.

6.7.6 Campylobacter jejuni *Invasion in Cell Lines*

Studies with isolated primary intestinal cells from chickens indeed showed that *C. jejuni* was able to invade chicken cells (Byrne et al. 2007; van Deun et al. 2008a, b; Hermans et al. 2011), an unexpected feature since *C. jejuni* does not associate with chicken crypt epithelium in vivo (Byrne et al. 2007; Hermans et al. 2011). Invasion capacity was largely strain-dependent, but overall no difference was observed between isolates from poultry or human origin. Microtubule- as well as

microfilament-dependent invasion was reported, which is in accordance with results obtained from invasion experiments in human epithelial cell lines (Hu and Hopecko 1999; Hermans et al. 2011).

It was observed that no obvious host tropism occurs: *C. jejuni* isolates from humans, chicken or pigs are capable to adhere to and invade human, avian and porcine cell lines (Biswas et al. 2000; Gripp et al. 2011; Backert and Hofreuter 2013). However, *C. jejuni* isolates adhere to and invade cultured cell lines of certain host or tissue origins with different efficiencies (Poly et al. 2007; Larson et al. 2008; Wine et al. 2008; Backert and Hofreuter 2013), and the adherence and/or invasion capabilities between strains vary significantly (Newell et al. 1985; Fauchere et al. 1986; Biswas et al. 2000; Fearnley et al. 2008; Zheng et al. 2008; Backert and Hofreuter 2013).

The permissiveness by which *C. jejuni* interacts with a wide range of different eukaryotic cell types is striking. While *C. jejuni* adheres to diferente cell lines to similar extend (Konkel et al. 1992a; Backert and Hofreuter 2013), its internalization efficiency varies dependent on the cell lines. It was suggested that *C. jejuni* invades epithelial cell lines of human origin more efficient than cell lines of non-human origin (Konkel et al. 1992b; Backert and Hofreuter 2013). Thus *C. jejuni* infection experiments have been most commonly studied with the human intestinal cell lines Caco-2, T84 and INT-407. In vitro invasion of porcine IPEC-1 and IPEC-J2 small intestinal epithelial cells by *C. jejuni* has been described as well (Naikare et al. 2006; Gripp et al. 2011; Backert and Hofreuter 2013).

Many studies on the genes which are thought to play a role during invasion have been conducted on human epithelial cell lines, but thus far experiments on chicken primary epithelial cecal cells are lacking. While it is tempting to assume that invasion mechanisms in these cells are analogous to those in human cell lines, some diferences do exist*: C. jejuni* can survive in vitro in human T84 epithelial cells by avoiding fusion with lysosomes (Watson and Galan 2008; Hermans et al. 2011), but intracellular survival seems not to be the case in the primary chicken enterocytes (van Deun et al. 2008a, b; Hermans et al. 2011).

The lack of an immortalized chicken intestinal cell line and the complicated handling of primary chicken cecal cells clearly hamper investigation toward invasion (and other) mechanisms in chicken cecal cells. Nevertheless, the recent obtained in vitro and in vivo results described under this section suggest that invasion of *C. jejuni* in gut epithelial cells might be an important colonization determinant in vivo. For survival and optimal colonization in the chick, *C. jejuni* must also be capable of eliciting a suitable response to cytotoxic nitric oxide (NO), a free radical produced by several cells of the host immune system that is bactericidal against *C. jejuni* (Hermans et al. 2011; Shepherd et al. 2011). *C. jejuni* is protected against NO induced nitrosative stress by NO-detoxifying mechanisms, including a nitrite reductase and its single domain *Campylobacter* globin (Cgb) (Pittman and Kelly 2005; Hermans et al. 2011; Smith et al. 2011).

6.7.7 *Microbiota and* Campylobacter jejuni *Colonization*

The intestinal microbiota of healthy mammals is typically dominated by organisms from the phyla Firmicutes (Gram-positive bacteria) and Bacteroidetes (Gram-negative bacteria) (Holmes et al. 2011; O'Loughlin et al. 2015). Collectively, the intestinal commensal microbiota provides the host with numerous physiological benefits, including vitamin synthesis, tissue integrity, digestion, fermentation of proteins and polysaccharides, bile salt metabolism, and stimulation of the immune system (Blaut and Clavel 2007; O'Loughlin et al. 2015). One additional physiological benefit of the intestinal microbiota is the enhancement of host immune defenses by inhibiting growth of potentially pathogenic microorganisms (colonization resistance). Colonization resistance prevents pathogens from establishing a niche and inhibits the outgrowth of opportunistic pathogens (Lawley and Walker 2013; O'Loughlin et al. 2015).

Mice vary in their susceptibilities to *C. jejuni* and can be either completely resistant to colonization or only transiently infected. Mice devoid of intestinal microbiota (germfree) and mice with a defined microbiota (gnotobiotic) have been shown to be more susceptible to *C. jejuni* colonization than mice with normal intestinal microbiota. For example, *C. jejuni* effectively colonizes germfree mice and disseminates to immune tissues, including the mesenteric lymph nodes (MLN) (Lee et al. 1986; Youssef et al. 1987; Jesudason et al. 1989; O'Loughlin et al. 2015). However, germfree mice demonstrate altered lymphoid development, resulting in an impaired immune response (Szeri et al. 1976; Savidge et al. 1991; Shroff and Cebra 1995; O'Loughlin et al. 2015). There are documented instances that mice are susceptible to colonization with *C. jejuni* (Blaser et al. 1983; Chang and Miller 2006; O'Loughlin et al. 2015). However, many researchers have experienced difficulty in obtaining *C. jejuni* colonization of mice unless the animals have been treated with an antibiotic prior to challenge to alter the intestinal microbiota (Lee et al. 1986; Youssef et al. 1987; Jesudason et al. 1989; O'Loughlin et al. 2015). To this end, mice treated with a cocktail of five antibiotics over the course of 6 weeks have been shown to be more susceptible to *C. jejuni* (Bereswill et al. 2011; O'Loughlin et al. 2015).

Transplanting fecal material containing either human or mouse microbiota into these germfree mice demonstrated that mice given human microbiota were more susceptible to *C. jejuni*-mediated disease than mice given mouse microbiota (Bereswill et al. 2011; O'Loughlin et al. 2015). Thus, it is known that the murine intestinal microbiota impacts *C. jejuni* colonization as mice with limited flora are also more susceptible to *C. jejuni* (Chang and Miller 2006; O'Loughlin et al. 2015). Collectively, these results suggest that the murine intestinal microbiota is comprised of microorganisms that specifically inhibit *C. jejuni* colonization. It was demonstrate that ampicillin alters the intestinal microbiota, thereby allowing *C. jejuni* to colonize the intestinal tract. These findings would allow researchers to better explore *C. jejuni*-host interactions, whether the focus is probiotic inhibition or virulence assessment.

6.8 Is *Campylobacter jejuni* Just a Comensal Inhabitant of the Chicken Gut?

C. jejuni is often considered to be a harmless commensal inhabitant of the chicken gut, and the immune response to it in the intestinal tract is thought to be tolerogenic (Hermans et al. 2012; Humprey et al. 2014). However, it has been shown previously that *C. jejuni* is recognized by Toll-like receptor 4 (TLR4) and TLR21, the latter being the functional equivalent of mammalian TLR9. This leads to initiation of innate immune responses in the gut that cause an influx of inflammatory cells, including heterophils, the avian equivalent of the neutrophil (Smith et al. 2008; Meade et al. 2009; de Zoete et al. 2010; Humprey et al. 2014). *C. jejuni* infection may also affect the structure of the chicken intestinal epithelium (Humprey et al. 2014; Awad et al. 2014). The innate responses lead to adaptive responses that can be measured as both mucosal and systemic specific antibodies (Cawthraw et al. 1994; Widders et al. 1998; de Zoete et al. 2007; Humprey et al. 2014). Studies of T lymphocyte function in the gastrointestinal tract during *C. jejuni* infection are very limited, although T cell responses have been shown in the liver during invasive infections (Jennings et al. 2011; Humprey et al. 2014).

Hock marks or hock burn are marks found on the legs of chickens where ammonia from the waste of other chickens within the litter causes burns. Pododermatitis or foot pad dermatitis is a thickening (keratitis) and discoloration of the foot pad and in more severe cases, lesions of the foot pad of the bird caused through poor quality or wet litter with high ammonia content. These conditions are more common in the fast-growing broilers, as is *Campylobacter* infection (Bull et al. 2008; Rushton et al. 2009; Humprey et al. 2014). Colles et al. (2008) also found an association between hock marks and *Campylobacter* infection in a free-range flock in the United Kingdom. Conditions such as hock marks and pododermatitis can be indicative of poor gut health, leading to wet feces and poor-quality litter that in turn lead to damage of the feet and lower legs of the chickens. It was our assumption that the link with *Campylobacter* was associated with the bacterium better colonizing the damaged gut. This may be the case, but previous preliminary studies have shown that *C. jejuni* directly contributes to poor gut health.

In sumary, it was discussed that human and avian *C. jejuni* isolates differ in their ability to colonize chickens (Korolik et al. 1998; Ringoir and Korolik 2003; Hänel et al. 2009; Pielsticher et al. 2012). The possible reasons may be genetic diversity between strains which may also affect the innate and eventually also the acquired immune response in the very early phase of colonization. Overall it may be suggested that *C. jejuni* is non pathogenic for healthy chickens. Other predisposing factors may contribute to the systemic spread of *Campylobacter* in birds and the induction of lesions (Neil et al. 1984; Burch 2005; Prelsticher et al. 2012). Further studies are needed to further understand important host factors responsible for the control of *Campylobacter* in chickens. This may allow the implementation of better control strategies of this important and zoonotic pathogen in poultry.

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Chapter 7
Epidemiology of *Campylobacter* in Farms

Eliane Pereira Mendonça, Belchiolina Beatriz Fonseca and Guilherme Paz Monteiro

Abstract Chickens are recognized as natural hosts of *Campylobacter* and infected poultry carry a high pathogen load in their gastrointestinal tract. There are large gaps in our understanding about factors leading to colonization of *Campylobacter* and subsequent transmission among birds, given that the source of *Campylobacter* in chickens and specific mechanisms entering the flock are not fully clear yet. This chapter summarizes the epidemiology of *Campylobacter* in poultry flocks and considers current issues on the subject.

Keywords Broiler chicken · Campylobacteriosis · Environmental contamination · Infection · Poultry

7.1 Introduction

Chickens are recognized as natural hosts of *Campylobacter* and infected birds carry a high pathogen load in their gastrointestinal tract, especially in the cecum, resulting in contaminated carcasses (Hermans et al. 2011a). There are large gaps in our understanding about factors leading to colonization of *Campylobacter* and subsequent transmission among poultry, given that the source of *Campylobacter* in chickens and specific mechanisms entering the flock are not clear yet. *C. jejuni* was long regarded as a commensal organism in chickens (Manning et al. 2007). However, a study developed by Humphrey et al. (2014) showed that *C. jejuni* cannot be simply considered as a commensal organism in the gut of commercial broilers, since infection in broilers is associated with intestinal inflammation.

The control of *Campylobacter* in chickens is now considered a challenge, given that the establishment of a source of contamination and its spread within a flock are very fast. The majority of the chickens in a farm are colonized in a period of 2–4 weeks after the first poultry has been infected (van Gerwe et al. 2009). After

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the infection, *C. jejuni* rapidly colonizes the cecum in a high level and leads to fecal excretion (Shanker et al. 1990). This high fecal elimination combined with the coprophagy behavior of birds demonstrates that the bacteria is able to spread quickly through the entire flock within a few days (Chaloner et al. 2014) when the first bird of a broiler flock is colonized. These broilers have high numbers of *C. jejuni* in their intestinal tract, especially in the cecum (10^6–10^8 CFU/g), and they remain colonized until slaughter (Beery et al. 1988; Jacobs-Reitsma et al. 1995; Evans and Sayers 2000). A better understanding of the epidemiology *Campylobacter* in poultry flocks is crucial to disease control measures.

7.2 Epidemiology of *Campylobacter* in Farms

Broilers typically start the elimination of *Campylobacter* in the housing age, which may occur due to factors that are not related to environmental contamination, such as changes in the intestinal flora, immunity of the bird, and hormones produced in response to stress, which can influence the starting of bacteria shedding (Humphrey 2006; Cogan et al. 2007).

An example of non-environmental factors is that after colonization by *Campylobacter*, the chick immune system is activated inefficiently and the expression of various antimicrobial peptide genes is reduced, both contributing to the highly persistent colonization of *Campylobacter* in the intestine of poultry (Meade et al. 2009; Hermans et al. 2011b). In the first two weeks of life, maternal antibodies may be present in chicks, triggering a protection for the colonization by *C. jejuni* (Sahin et al. 2001). From two weeks of age, maternal antibodies are no longer present and with three weeks of age, the birds mainly begin to produce their own antibodies against flagellin (Cawthraw et al. 1994; Jeurissen et al. 1998).

Chicken meat is the main foodborne source of human campylobacteriosis (Wingstrand et al. 2006; EFSA 2010). Many countries have established action plans against the occurrence of *Campylobacter* in the chicken production chain (EFSA 2007). A better understanding of the many factors involved in the infection and spread of this pathogen in broilers can prevent and/or reduce the sources of *Campylobacter* in poultry. And this is necessary to prevent the occurrence of numerous campylobacteriosis outbreaks in humans, mainly associated to the consumption and the handling of raw or undercooked chicken.

In general, the occurrence of vertical transmission of *Campylobacter* in poultry has been a controversial issue and it is not recognized by the scientific community as a way that contributes to the colonization of birds (Callicott et al. 2006; O'Mahony et al. 2011). The isolation of *Campylobacter* species in the reproductive tract of breeder hens has been reported (Buhr et al. 2002; Fonseca et al. 2006), however, the transmission of breeder hens to broilers have only been speculated (Cox et al. 2002). Reports suggest that vertical transmission is unlikely or it is not important to epidemiology (Jacobs-Reitsma 1995; Sahin et al. 2003). Studies developed by O'Mahony et al. (2011) and Patriarchi et al. (2011) demonstrated that

none of the genotypes identified in flocks of breeder hens were later identified in broiler chicken farms suggesting that vertical transmission was not involved in the colonization of broiler with *Campylobacter*. However, this subject is still little explored in the literature.

Although considered as a fastidious microorganism, *Campylobacter* is highly prevalent in the environment and it can survive for long periods in and out of a host (Newell 2002; Murphy et al. 2006). Therefore, the transmission from environmental sources is considered the primary route of flocks' colonization by *Campylobacter* (Sahin et al. 2002). The main sources and potential vectors for environmental contamination and infection of birds are cited in (Fig. 7.1).

The estimated prevalence of *Campylobacter* depends on the season, the age of the animal, or the size and type of the flock, diet, management practices and geography (Ellis-Iversen et al. 2009; Näther et al. 2009; EFSA and ECDC 2011; Jorgensen et al. 2011). Studies conducted in Germany and the United Kingdom

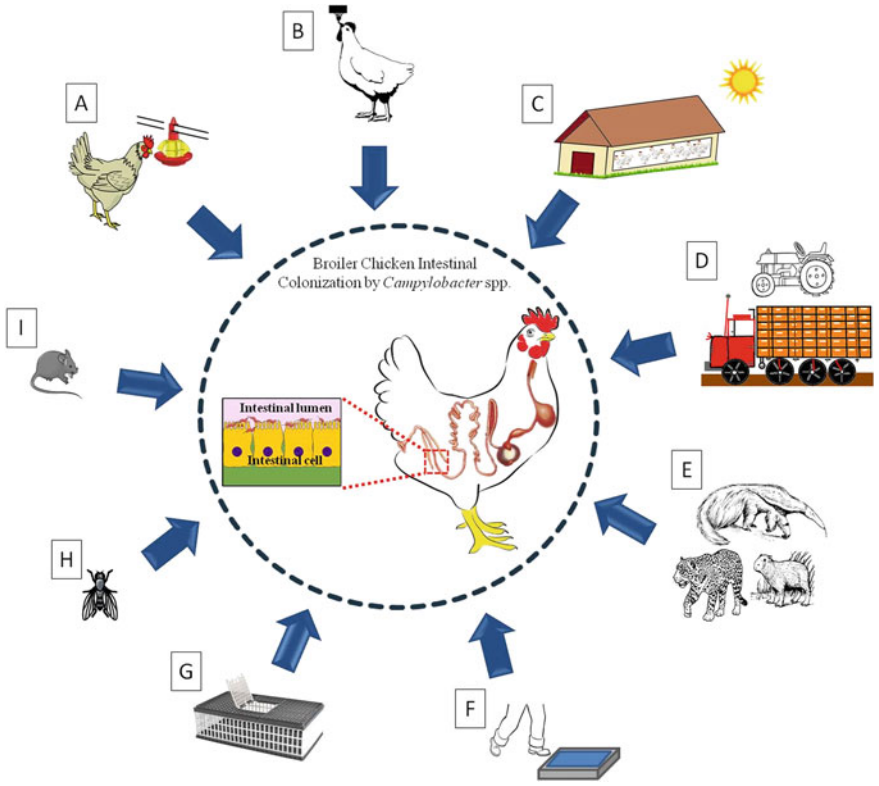

Fig. 7.1 Main sources and potential vectors for infection of birds by *Campylobacter* in the environment. *A* Contaminated feed. *B* Contaminated drinking water. *C* Season of the year (summer months). *D* Contaminated vehicles and equipment. *E* Wild animals. *F* Contaminated footbath. *G* Contaminated shipping boxes. *H* Flies. *I* Rodents

show the influence of the season of the year on the colonization process. In these studies, the authors concluded that there is greater risk of broilers becoming colonized by *Campylobacter* during the summer months (McDowell et al. 2008; Ellis-Iversen et al. 2009; Ellerbroek et al. 2010; Jorgensen et al. 2011).

Thus, there is a significant relation between *Campylobacter* prevalence in broiler flocks and climatic factors, such as ambient temperature, the quantity of sun light, rainfall (Jorgensen et al. 2011), and the risk factors for infection may vary among countries due to weather conditions.

Regarding food, many authors do not consider the participation of the feed in the epidemiology of *C. jejuni* in poultry flocks (Jacobs-Reitsma et al. 1995; Gregory et al. 1997; Carvalho et al. 2001; Zweifel et al. 2008). However, other research considers that the consumption of contaminated feed can be the source of infection (Oliveira et al. 2008; Julien et al. 2013; Sommer et al. 2013). In recent research, we found that *C. jejuni* can survive for 5 days when inoculated with 10^5 CFU g^{-1} at 25 and 37 °C. In this study, *C. jejuni* was able to multiply when inoculated with 10^3 CFU g^{-1}, with greater proliferation observed when the feed temperature of 37 °C was maintained (unpublished data). It is possible that the sample and/or collection method or cultivation technique must be improved for isolation of *Campylobacter* spp in feed.

Contaminated water with *Campylobacter* genotypes is considered an important source of infection in broilers (Bull et al. 2006; Messens et al. 2009). The survival of *C. jejuni* in water is promoted by several factors, including biofilm formation, and possibly viable but not culturable state (VNC), wherein *C. jejuni* presents when it is not in a suitable host (Sparks 2009). Biofilm formation, however, has been associated with decreased potential for colonization in one-day-old chicks (Hanning et al. 2009). It is not believed that *C. jejuni* VNC cells can reduce their binding capacity on the surface and once they set, they can persist undetected, be introduced into the food chain, and come into contact with animals or products (Duffy and Dykes 2009).

Once a flock is colonized, the drinking water is often contaminated with *C. jejuni* strains, isolated from the same broilers, indicating the importance of drinking water in zoonotic transmission of this pathogen throughout the flock (Gellynck et al. 2008; Messens et al. 2009). Studies demonstrate that survival of *Campylobacter* in the aquatic environment is associated with its ability to invade, refuge, and multiply within protozoan vectors, mainly of the genus *Acanthamoeba*. In *A. polyphaga*, the bacteria were able to replicate in coculture at 37 °C under aerobic conditions and survive for more than 2 months (Olofsson et al. 2013).

These protozoans are present in high density in natural and artificial water systems are well suited to hostile environments, such as high temperatures, chlorination, and various disinfectants (Kilvington and Prie 1990; Ahearn and Gabriel 1997). Surviving within amoebae, bacteria cannot only escape the threat of being preyed upon, but can also benefit from protection against conditions that occur outside of the protozoan host, thus allowing their survival in water environments and also in biofilms (Axelsson-Olsson et al. 2005).

Arcobacter butzleri is a microorganism that survives inside amoebae due to its ability to remain inside vacuoles infused with lysosomes, or with the ability to retard the fusion between these structures (Medina et al. 2014). Axelsson-Olsson et al. (2005) demonstrated that *C. jejuni* can survive during diverse periods within *A. polyphaga* and can be transmitted to chicken as endosymbiont of this free living amoeba. In Chile, *Acanthamoeba* sp. genotype 4 was isolated from chicken's drinking water and chicken carcass (unpublished data) and SPF and Broiler chicks experimentally infected with *A. castellanii* containing *C. jejuni* became positive for *C. jejuni* (Fernández et al. 2007; Flores et al. 2009). These results suggest the importance to study *Acanthamoeba* spp. and others protozoan in farms.

Wild and production animals that are colonized are an important risk factor for the transmission of *C. jejuni* to broiler flocks. *C. jejuni* genotypes of cattle, pigs, and breeder hens can also be found in chicken flocks (Ridley et al. 2008; Zweifel et al. 2008; Ellis-Iversen et al. 2009; Hanel et al. 2009; Allen et al. 2011; Ridley et al. 2011; Patriarchi et al. 2011; Hermans et al. 2012). This indicates the importance of horizontal transmission and the risk of *Campylobacter* transmission in multispecies environments (Ridley et al. 2011).

Isolated indistinguishable of clonal origin were found in different flocks during the same period in a breeding poultry rearing system (Ellerbroek et al. 2010; Kudirkiene et al. 2010). This suggests that *Campylobacter* strains can be transmitted from a chicken flock to another or can point to a common external source infecting several chicken flocks on the same farm. Clones persistent in the environment may be responsible for repeated infection of successive broiler flock rotations (Wedderkopp et al. 2003), being some strains of *C. jejuni* very persistent in a confined geographical area (Kudirkiene et al. 2010).

Rodents and flies are potential vectors for transmission of *C. jejuni* for broilers (Hald et al. 2008; Hazeleger et al. 2008; Meerburg 2010). It has been suggested that flies may be the route by which *Campylobacter* enters broilers (Hald et al. 2007, 2008). Hansson et al. (2007) and Ellis-Iversen et al. (2009) have estimated that the peak of *Campylobacter* in the summer is associated with the increase in fly populations and changes in management practices of birds during the summer months.

Personnel, vehicles, and equipment were also identified as potential sources of infection by *C. jejuni* in chickens even after cleaning (Ramabu et al. 2004; Ridley et al. 2011). Furthermore, in broiler farms with three or more aviaries, low-frequency change of the disinfectant footbath and reduced cleaning of the poultry have a greater risk of becoming colonized (McDowell et al. 2008).

Also the thinning of broiler has been implicated as a potential risk factor for colonization of *Campylobacter* for poultry, because of the difficulties in maintaining biosecurity during this procedure (Allen et al. 2008; Patriarchi et al. 2011; O'Mahony et al. 2011). Shipping boxes are still often contaminated with *Campylobacter* when reused, because the decontamination process of the crates is inefficient in most cases (Ridley et al. 2011). Allen et al. (2008) observed an association between *C. jejuni* genotypes present in vehicles and in transport boxes that arrived in a thinning of the farm and those subsequently recovered from poultry after slaughter. Also, during this process, *C. jejuni* specific strains were able to

spread from one farm to another, which was nearby, as they have shared the same staff and/or vehicles to capture the birds.

Possibly, the risk factors mentioned above are closely linked with each other. For example, the temperature rise during the summer months could promote the presence of flies and rodents in the farm, while the increase of precipitation can create reservoirs in water puddle with *C. jejuni* may persist and transmit to other vectors (Jorgensen et al. 2011).

As the broiler colonization by *C. jejuni* has been well documented, little is known about the dynamics of different genotypes in the individual bird if different strains are able to colonize different parts of the gastrointestinal tract. Generally, when more than one genotype colonizes a flock, it was observed that these may coexist over time, rather than one excluding the other (Hook et al. 2005). However, the replacement of a *C. jejuni* strain to another has also been observed in experimental and field studies in chickens, indicating that some are more dominant than others (Konkel et al. 2007; Calderón-Gómez et al. 2009).

A study performed by Chaloner et al. (2014) showed that different genotypes of *C. jejuni* (M1 and 13126) exhibit distinct differences in infection dynamics and ecology in commercial broilers. *C. jejuni* M1 showed biology of classical infection of colonization, rapid elimination and it is widely associated with the cecum. In contrast, *C. jejuni* 13126 is slower to colonize but was most adept at colonizing the upper GI tract being significantly more able to spread among an extra intestinal environment. This understanding of how different strains of *C. jejuni* colonize poultry is important to develop strategies to reduce or eliminate their transmission. Thus, the ability of this microorganism to colonize the digestive tract of the bird is considered to be multifactorial. According to Hanel et al. (2009), the differences in the types of colonization may be due to genetic differences, or differences in gene expression of genes related to colonization/invasion.

The way the organism deals with stresses found in the environment remains poorly understood, but clearly *Campylobacter* developed some coping mechanisms for overcoming these stressors mechanisms (Murphy et al. 2006). It has been shown, in vitro, that the presence of the neurotransmitter norepinephrine stimulates the growth and motility of *C. jejuni* (Cogan et al. 2007). As a result, the stress caused by the thinning process leads to release noradrenaline, contributing to the rapid growth of bacteria in the gastrointestinal tract of the bird, which leads to an increase in the elimination of *Campylobacter* by birds and subsequent rapid spread of bacteria.

The presence of highly mutable sites in the genome of *C. jejuni* is responsible for its rapid adaptation to a new host (Jerome et al. 2011). *C. jejuni* is naturally competent, which means that it can take up DNA from the environment. This leads to recombination between strains, which allow further generation of genetic diversity. The horizontal transfer of both, plasmid and chromosomal DNA, occurs in vitro and during the colonization of the chick indicating that the natural transformation may play an important role in the plasticity of the genome and dissemination of new factors such as resistance to antibiotics, even in the absence of selective pressure (de Boer et al. 2002; Wilson et al. 2003; Avrain et al. 2004).

In vitro, *C. jejuni* displays a marked preference for DNA of *C. jejuni* strains, in contrast to DNA of other species. Moreover, the frequency of natural transformation is affected by carbon dioxide and the density of the bacterial cell, which indicates that horizontal exchange is probably environmentally regulated in vivo (Wilson et al. 2003).

The attribution of a source to the colonization of the chicken's flock by *Campylobacter* is complex. Thus, a combined approach of hygienic measures needs to be implemented properly in all the phases of creation, thus being able to significantly reduce the number of flocks colonized by *Campylobacter*. It may be cited to wash hands before entering the aviary; the use of separate boots for each avian; rodent and insect control; foot disinfection; high standard of cleaning and disinfection of drinking fountain; treatment of drinking water; and decontamination of transport boxes as hygienic measures during the rearing period (Evans and Sayers 2000; Hermans et al. 2012). It has been shown that the prevalence of *Campylobacter* colonization in chickens can be reduced from placing fly screens in aviaries (Hald et al. 2007; Bahrndorff et al. 2013).

Another strategy for preventing colonization by *Campylobactar* is through the introduction of competitive exclusion standard bacterial mixtures (Svetoch and Stern 2010). A *Lactobacillus* strain was isolated from an adult chicken intestine and it showed bactericidal effects against *Campylobacter* in vitro, probably due to production of organic acids and an anti-*Campylobacter* peptide (Chaveerach et al. 2004). A study has shown the possibility of using a *C. jejuni* hyper-colonization strain characterized for the biological control of environmental *Campylobacter* strains not described in commercial poultry flocks, thus reducing the transfer of non-characterized strains to humans via poultry products (Calderón-Gómez et al. 2009).

Furthermore, by improving the health and welfare of animals, colonization can be reduced (Bull et al. 2008). Finally, genetic selection can also help to combat *Campylobacter* colonization in poultry, when poultry strains with better ability of general immune response, and it is more resistant to colonization of this pathogen, are developed (Swaggerty et al. 2009). In conclusion, control of *Campylobacter* in birds faces many obstacles, and probably strategies will have to be combined to develop an adequate, reliable, and efficient strategy to eradicate this human pathogen. (Hermans et al. 2011a).

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Chapter 8
Control of *Campylobacter* spp. in Commercial Poultry Production

Omar A. Oyarzabal and Steffen Backert

Abstract Campylobacteriosis continues to be an important disease worldwide. The most important risk factors associated with the transmission of *Campylobacter* spp. are the consumption of raw milk, undercooked or contaminated chicken, and raw oysters. The number of campylobacteriosis cases associated to raw milk and oysters could be greatly reduced by applying temperature treatments to the product. Although cooking is also the best control measurement for the transmission of *Campylobacter* spp. from chicken meat, the high contamination found in some chicken samples required a more comprehensive approach to reduce the appearance of the pathogen in the final product. This chapter reviews the intervention strategies that have been practically applied by the food industry to reduce the contamination of poultry products with *Campylobacter* spp. The discussion focuses on on-farm interventions and the interventions applied in processing plants. In the USA, chemical interventions have been used for approximately 20 years and have found a commercial niche in the poultry industry.

Keywords *Campylobacter* · Control · Biosecurity · Farm interventions · Plant interventions · Chemical interventions

8.1 Introduction

Campylobacter spp. represents a major gastrointestinal pathogen of humans, while colonizing many poultry species as a commensal. In commercial broiler chickens, these bacteria are present in cecal content up to 8 Log colony forming units

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(CFU) per g and in fecal content in numbers of up to 6 Log CFU per g (Potturi-Venkata et al. 2007a). However, a large proportion of commercial flocks may not carry this organism due to a large variability in the ratio of positive/negative flocks at any given time in a region (Potturi-Venkata et al. 2007a). There are two major species, *C. jejuni* and *C. coli*, that have been isolated from commercial broilers worldwide when confirming isolates to the species level using various molecular techniques (Suzuki and Yamamoto 2009; Gharst et al. 2013). It also appears that commercial turkeys may carry those two species, but unlike the numbers in broiler chickens, turkeys commonly have a higher colonization rate with *C. coli* (Gharst et al. 2013).

The current interventions used to control *Campylobacter* spp. are applied in live animals or during processing of carcasses. During the grow-out period, there have been several attempts to control *Campylobacter* by the addition of antimicrobials in the feed and/or in the water. At the processing level, most of the interventions are antimicrobial substances added after the evisceration of the carcass and throughout the rest of the processing steps. More recently, the research efforts have been focusing on the development of vaccines, both for animals and humans, to help control the prevalence of this bacterial pathogen. Most intervention approaches to control *Campylobacter* spp. in poultry have been tested in vitro under laboratory settings and not too many have become a commercial product. Only a small subset, mainly chemical interventions, has made it into commercial applications that are used in poultry processing plants, primarily in the USA. There are several interventions for which there are no commercial products available yet, such as vaccination, passive immunization, bacteriophages, bacteriocins, etc. For others, such as the application of probiotics and organic acids, there are some commercial products in some countries, but there is little, and in many cases, inconsistent information available on their effectiveness to reduce *Campylobacter* spp. in commercial poultry productions.

This chapter summarizes the intervention strategies that have been applied by the poultry industry to reduce the contamination of poultry products with *Campylobacter* spp. The focus of this chapter is on various interventions that have found a commercial niche and are currently used by the poultry industry. When appropriate, we emphasize in which countries those interventions are used most frequently. Within the next generation of intervention schemes, we will emphasize primarily on the potential use of vaccines as an alternative means to control *Campylobacter* spp. in live commercial poultry.

8.2 On-farm Interventions

8.2.1 Biosecurity Measures

Campylobacter is a particularly difficult organism to control because it is very well-adapted to living in the intestine of live poultry. These bacteria can easily

reach poultry flocks at the farms because they are also naturally present in the surrounding environment. Therefore, biosecurity measures at the farm can never be overemphasized in an overall control program. Contrary to the concept that *Campylobacter* was not a good survivor in the environment, we now have enough information to support the concept that *Campylobacter* tolerates and survives well in a relatively wide range of environmental conditions.

In commercial poultry production, the main transmission route of *Campylobacter* is horizontal spreading. A modern commercial house minimizes the access of this organism to the poultry house via vectors, such as flies, rodents, etc. However, humans, especially farm workers, are still an underestimated carrier in commercial poultry production all over the world (Huneau-Salaun et al. 2007; Newell et al. 2011). The impact of farm personnel on the on-farm transmission of *Campylobacter* spp. has been shown to be important even with the catching personnel, who can easily move *Campylobacter* from one flock to another (Ridley et al. 2011). Other horizontal transmission routes are more difficult to control, such as air, water, and feed. The use of anteroom and designated clothing in chicken farms has helped to reduce the risk of flocks getting colonized by *Campylobacter* spp. by farm workers and visitors.

The most common strategies to counteract *Campylobacter* spp. colonization in poultry farms are designed to prevent the entrance of this pathogen into the flock by installing hygiene barriers and fly screens; the use of disinfected water; and the reduction of slaughter age and thinning attempts (Hald et al. 2008; Bahrndorff et al. 2013). However, the susceptibility of chickens for colonization by *C. jejuni* and their prevalence in the environment have negatively influenced the success of biosecurity-based approaches (Table 8.1). These limitations particularly highlight the need for developing alternative methods to reduce *C. jejuni* in poultry to levels that make a positive impact in public health.

8.2.2 Addition of Organic Acids to Water and Feed

Organic acids have been shown for more than 12 years to have certain bactericidal effects against *Campylobacter* spp. in vitro (Chaveerach et al. 2002; Hilmarsson et al. 2006). However, these encouraging in vitro results have not yet been translated into a consistent reduction of *Campylobacter* spp. in commercial broilers fed with organic acids in feed or water (Hermans et al. 2010). However, organic acids are useful in treating processed broiler products. Currently, a 2 % lactic acid rinse appears effective and will be combined with a multistep antimicrobial intervention under laboratory conditions and validated in small meat plants (Yoder et al. 2012).

The studies to evaluate if water is a source of *Campylobacter* to commercial chickens are difficult to carry out because it is difficult to recover *Campylobacter* from water (Newell et al. 2011). When chlorination of water risk has been studied as a risk factor, in some studies it comes as an important feature to consider reducing changes of colonization. However, it is important to remember that there

Table 8.1 Selected interventions that have been tested to control *Campylobacter* in poultry production

Intervention	Justification	Limitations	References
Biosecurity measures to limit access of humans, domestic animals, and wild animals. For example, anteroom, chlorination of water, etc.	Associated as risk factors in commercial farms	There are very few reports on the quantification of each intervention to assess effectiveness. Many studies have limitations in the experimental design, sampling, and statistical analysis	Ellis-Iversen et al. (2009), Newell et al. (2011)
Addition of organic acids to water and feed	Reduction of *Campylobacter* in live birds and in drinking water	In vitro experiments show a large reduction, but in vivo experiments do not provide consistent results with high reduction in the number of *Campylobacter* per g of fecal material	Byrd et al. (2001), Chaveerach et al. (2002, 2004), Thormar et al. (2006)
Oral administration of antibodies	Immunized chickens for prophylactic and therapeutic control of *C. jejuni*	Limited experimental use. No more than 2 log CFU reduction	Tsubokura et al. (1997)

is no quantification of the impact of chlorination in the reduction of the colonization of chicken with *Campylobacter* spp. Because a few viable *Campylobacter* cells reaching a live chicken is sufficient to start a colonization process that will spread across the majority, if not all, the chickens in a commercial poultry house, it is doubtful that current addition of chlorine will provide a guarantee that no *Campylobacter* cells enters a chicken house.

8.2.3 Application of Probiotics

Several publications have described in vitro the reduction of *Campylobacter* spp. in chickens by feeding with probiotics bacteria (e.g., *Enterococcus faecium*, *Pediococcus acidilactici*, *Lactobacillus salivarius*, etc.), and although some in vivo data in broiler chicks show reduction (Willis and Reid 2008; Robyn et al. 2012), the level of reduction is not consistent using this approach and therefore only few commercial alternatives are available. It has been also shown that application of *Bifidobacterium* spp. reduced the CFU of *C. jejuni* in chicken legs (Melero et al. 2013). In addition, it appears that the combination and concentration of gases used

for packaging doubled the products' shelf-life in comparison with air-packaged samples, resulting in safer products with a longer stability (Melero et al. 2013).

8.2.4 Use of Phages

Several studies have demonstrated the isolation and application of bacteriophages to reduce *Campylobacter* in live broilers. *Campylobacter* reduction by phages varies according to the chosen phage-*Campylobacter* combination, the dose of phage applied and the time after administration, and although in vitro studies help to understand the kinetics of phages, several publications show high variability in vivo results with inconsistent reduction rates (Loc Carrillo et al. 2005). One of the major limitations of using phages, however, is the rapid appearance and spread of *Campylobacter* strains that are phage-resistant. Very recent studies also indicate that successive application of so-called group-II and -III phages significantly enhanced the reduction of *C. jejuni* in broiler chicken, suggesting that phage cocktails have to be carefully composed (Hammerl et al. 2014). To reduce the chances of resistant strains to appear and accumulate, it has been recommended that phages should be applied close to slaughter or on poultry meat (Janez and Loc Carrillo 2013; Kittler et al. 2013, 2014).

8.2.5 Vaccination Strategies

Antibodies generated by the host immune system can be highly effective to clear many disease-causing infections. The generation of specific antibodies against *C. jejuni* has been shown to be associated with resolving the infection in mice and rabbits. Studies using 2–3 weeks old chickens have demonstrated that the presence of maternal α-*Campylobacter* antibodies delays the initial colonization and decreases the frequency of horizontal spread of the bacteria in a given flock (Sahin et al. 2003). This suggests that passive immunotherapy by α-*Campylobacter* antibodies may represent a promising strategy counteracting bacterial colonization in chickens. In fact, a large body of literature has accumulated in the past 15 years, suggesting that vaccination may be an alternative approach to prevent or reduce commercial chickens from *Campylobacter* spp. colonization (Rice et al. 1997; Jagusztyn-Krynicka et al. 2009; Buckley et al. 2010; Garcia et al. 2012). For example, passive immunization with α-flagellar antibodies has been successfully demonstrated to diminish *C. jejuni* infection in mice (Ueki et al. 1987). In addition, the application of hyper-immunized α-*C. jejuni* rabbit sera or α-*C. jejuni* antibodies exhibited pronounced capabilities to reduce the colonization of chicken by *C. jejuni* (Stern et al. 1990). Interestingly, poultry abattoir workers, who developed high titers of *Campylobacter*-specific IgGs, rarely become ill by *C. jejuni* infection (Cawthraw et al. 2000). These results indicate that specific serum IgG responses

induced by endemic exposure to *C. jejuni* might be directed towards a number of protein antigens with apparently conserved epitopes, resulting in resistance to disease due to acquired protective immunity (Cawthraw et al. 2000). However, despite more than a decade of intensive research, an effective α-*Campylobacter* vaccine has not been developed for commercial application.

Various laboratory reports on antibodies as preventive or therapeutic reagents for *Campylobacter* treatment exist, but have not receive much commercial attention. The current limitations of vaccinations are related to (1) considerable production costs; (2) absence of efficient gastrointestinal tract delivery systems; (3) degradation of antibodies by proteases present in the gastrointestinal tract; and (4) high antigenic variation among *C. jejuni* strains, which in turn requires various antibody combinations to target multiple isolates (Jagusztyn-Krynicka et al. 2009; Buckley et al. 2010; Hermans et al. 2011; Connell et al. 2012; Alemka et al. 2013). In addition, we still have an incomplete understanding of the chicken immune system as compared to humans or mice. As we improve our understanding of the interaction of *Campylobacter* spp. with the chicken gut, we may be able to develop effective vaccination procedures and reduce carriage by live commercial poultry.

8.3 Interventions Applied During Processing

The collection of chickens at the farm and the transportation to the processing plants are important events just before processing that may affect the *Campylobacter* status of a given commercial chicken flock. Vehicles, catching equipment, catching personnel can all serve as sources of contamination to *Campylobacter*-free flocks. In addition, the lack of proper cleaning protocols for transport crates has been shown in several studies to serve as a source for contamination to commercial broilers (Ridley et al. 2011).

The antimicrobial interventions during processing comprise the application of chemical compounds, primarily after carcass evisceration. In this category of interventions, there are several commercial applications used in the USA, some of which have been approved for use in other countries. The poultry industry has been using some of these interventions for close to 20 years. Others, such as chlorine, have been used for several decades in poultry processing, but were not assessed for their effectiveness against *Campylobacter* spp. until the past two decades. Yet, the amount of scientific publications assessing the effectiveness of in-plant interventions (Table 8.2) is still quite limited and show, in some cases, a modest reduction (Oyarzabal 2005). For some of these applications, the companies providing the interventions suggest the use of more than one intervention activity on the same facility (e.g., peracetic acid in the chiller and acidifies sodium chlorite post-chill), which complicates further the assessment of the impact of individual treatments.

These chemical interventions include the use of compounds such as chlorine, chlorine dioxide, trisodium phosphate, etc., that have been approved for use on poultry carcasses in the USA. These chemical interventions do not include any

Table 8.2 Commercial antimicrobials commonly used by the poultry industry in the USA to reduce *Campylobacter* spp. during processing

Antimicrobial	Possible mode of action
Acidified sodium chlorite	Broad-spectrum germicides; oxychlorous compounds act by breaking bonds on cell membrane surfaces
Cetylpyridinium chloride	Hydrophilic portion reacts with the cell membrane, resulting in the leakage of the cellular components, disruption of cell metabolism, and ultimate cell death
Chlorine (sodium hypochlorite)	Oxidation of cell components resulting in cell death
Chlorine dioxide	Oxidation of the cellular membrane and cellular constituents; at high concentrations, it breaks the cell wall
Peroxyacetic acid	Strong oxidation of cell membrane and other cell components, resulting in cell death
Trisodium phosphate	Disruption of cell membrane causing leakage of intracellular fluid; details of the antimicrobial mechanism have not been completely elucidated

antibiotics, which are banned from their use in poultry processing or their addition to poultry carcasses. All the chemical interventions used in the USA are comprised of compounds that have been approved as secondary direct food additives by the U. S. Food and Drug Administration (FDA) and are used to control pathogens in raw poultry products. The term "secondary direct food additives" was incorporated in Title 21 of the Code of Federal Regulations Part 173 in 1977, when there was a recodification of the food additive regulations. A secondary direct food additive has a technical effect in food during processing, but not in the finished food, and in many cases it is removed, not found in the final food product (Oyarzabal 2005), or found only as residual traces that are not expected to exhibit any technical effect in the food. Some examples of secondary direct additives include enzyme immobilizing agents, ion exchange resins, and a category of products commonly called "processing aids." Processing aid is a general term that comprises a variety of substances, some of which are added, for example, to help the flow of the product during process, or to prevent the food product from crystalizing, or to strengthen the product, as in the case of strengthening the dough in a frozen pancake. Some of these processing aids fall into the group of antimicrobials and that is where these chemical interventions against foodborne bacteria, including *Campylobacter* spp., are grouped. One chemical compound, cetylpyridinium chloride, may be an exception to this approval, but FDA and the Food Safety and Inspection Service of the U.S. Department of Agriculture (FSIS) have not provided any clarification yet (Kindy 2013).

Most of these chemical compounds were approved by showing reduction in prevalence of non-pathogenic *Escherichia coli* and *Salmonella* pre-versus post-application and, by extension, these products are used to control *Campylobacter* spp., although in some cases there is limited scientific information on the actual reduction of live *Campylobacter* by these chemical compounds. The

mode of action of these agents, however, has never been completely elucidated, but the disruption of the cell membrane appears to be the main mechanism by which these chemical compounds destroy bacterial cells (Oyarzabal 2005).

The actual applications of these products vary, but most are applied pre- or post-chill, as sprays or baths. In a few instances, these compounds are added to the chill water during the chilling of the carcasses after evisceration and cleaning. The FSIS periodically updates directive 7120.1 to provide with the latest on substances that are used in poultry processing for inspection program personnel (FSIS 2014).

There are still limitations in the effect of chemical interventions to reduce *Campylobacter* in poultry carcasses. A recent study has shown that the spraying of some chemical interventions was not effective to significantly reduce the number of *Campylobacter* CFU in chicken carcasses (Meredith et al. 2013). In this study, trisodium phosphate (TSP) at 14 % w/v, lactic acid at 5 % v/v, citric acid (CA) at 5 % w/v, peroxyacids at 200 ppm, and acidified sodium chlorite at 1200 ppm were ineffective as spray applications. However, the immersion of chicken products in 14 % TSP or 5 % CA resulted in *Campylobacter* reductions of 2.49 and 1.44 Log CFU per cm^2 of the product, respectively. In addition, these applications did not appear to negatively affect the sensory quality of the products (Meredith et al. 2013).

The reduction in the numbers of *Campylobacter* spp. in poultry carcasses is usually lower in studies performed in commercial poultry processing plants than studies conducted in laboratory, and most of the studies in commercial processing plants report a limited reduction of *Campylobacter* spp. in poultry products (Burfoot et al. 2015). However, some of the laboratory studies have shown variability in the final reduction according to different *Campylobacter* strains. For instance, in one study capric acid sodium salt showed a different reduction rate for two *C. jejuni* strains, even under the same laboratory conditions (Koolman et al. 2014). There is a large variability within naturally occurring strains and therefore these interventions must be tested for long period of times, in different processing environments and under different circumstances to assess their actual impact in reducing *Campylobacter* spp. in poultry products.

8.3.1 Practical Considerations to Reduce Cross-Contact During Processing

A practical consideration to reduce *Campylobacter* spp. in poultry meat is to organize the processing based on the contamination status of the birds. This term could be called "logistic scheduling of processing," and is based on the results from the testing of live birds few days before processing. In general, the microbiological testing of chicken flocks 5–7 days before the actual processing day provides very good information on the microbial status of the flock. Some flocks are negative

while others are positive. The testing of *Campylobacter* in flocks is relatively simple because when *Campylobacter* is present the organism can be easily detected in fresh fecal material. Once few cells of *Campylobacter* reach a chicken flock and start multiplying, that flock will probably be positive at the time of processing. There may be a variability in the number of *Campylobacter* according to the samples, with some samples having 3 Log CFU $\cdot$ g^{-1} while others may have up to 7–8 CFU $\cdot$ g^{-1}. It is not clear if there is a large variability in the actual number from bird to bird in the same flock, but for all practical purposes if the flocks are positive, it is easy to detect in fecal samples. However, some flocks remain negative up to the time of processing. We do not really understand all the factors that play a role for making a flock ending up contaminated with *Campylobacter* spp. But the fact that there are *Campylobacter*-free flocks has been demonstrated by several studies in different countries and in different seasons (Miwa et al. 2003; Potturi-Venkata et al. 2007b).

Logistic scheduling considers the status (positive vs. negative) of the incoming flocks to the processing plant. It is clear that *Campylobacter* isolates coming with *Campylobacter*-positive flocks can contaminate carcasses of *Campylobacter*-negative flocks during processing (Potturi-Venkata et al. 2007b). These findings suggest that by simply organizing a logistic processing the *Campylobacter*-negative status of broiler flocks could be preserved, which in turn would result in less carcasses contaminated and enhance food safety. Yet, the poultry industry does not have any incentives to incorporate this system. There is no incentive to test and there is no feasible option for the marketing of "less contaminated" *Campylobacter* poultry products.

Some research data provide a strong support for the concept of logistic scheduling. For instance, the detection of *Campylobacter* spp. in boot socks, drag swabs, and fecal samples at the farm had strong, positive associations with the load of *Campylobacter* spp. in carcass rinses in the processing plant, and these positive samples in the farm explained a greater proportion of the variability in the prevalence of *Campylobacter* loads in carcass rinses at the processing plant (Berghaus et al. 2013). In addition, it is known that residual cells from *Campylobacter*-positive flocks will contaminate the flocks that are processed immediately following those *Campylobacter*-positive flocks (Potturi-Venkata et al. 2007b). But which strains are better suited to survive in the processing environment is not known. Nor is it known if any strain that has a better adapted to survive the processing environment is more fitted for human infection and the production of disease.

Some studies, however, do not support the concept of logistic scheduling because of the limited correlation between *Campylobacter* contamination of cecal samples and contamination of finished meat product in the processing plant. In these cases, the research suggests that cecal samples are no good indicator of human exposure to *Campylobacter* (Nauta et al. 2009). Several factors affect the final *Campylobacter* load and type of *Campylobacter* strain in finished poultry products. However, the correlation of *Campylobacter* in live animals and finished products is strong and cannot be ignored in the absence of other type of correlations.

8.4 Future Research

There have been many studies on the evaluation of different interventions to control *Campylobacter* spp. in commercial poultry productions. However, there are relatively few interventions that are commercially available and none of them provides a consistent reduction of more than 3 Log CFU in the number, or a consistent reduction in the prevalence of *Campylobacter* positives, in finished products. Therefore, a successful control program may have to rely on the concept of hurdles, or a series of interventions aiming at reducing bacterial pathogens in foods (Leistner 1978; Cox and Pavic 2010), to take advantage of the sometimes low reduction provided by several different interventions. The hurdle concept has been suggested several times as one of the most comprehensive approaches to control bacterial foodborne pathogens and future studies should aim at quantifying the actual effect of each intervention.

The combination of strategies can also provide some synergistic effect worth discovering. For instance, in laboratory studies some plant extract with antimicrobial properties showed an increased reduction of *Campylobacter* when the product was subjected to freezing temperatures (Piskernik et al. 2011). The effect of lower temperatures in providing synergistic effects of antimicrobials has been known for some time and has provided the reasoning for the application of some chemical interventions after the chilling of carcasses (Oyarzabal et al. 2004). The quantification of these synergistic effects will not be an easy task. But more efforts should be placed on emphasizing holistic approaches with more than one intervention thought the food chain continuum.

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Chapter 9
Campylobacter spp.: Capacity of Biofilm Formation and Other Strategies of Survival and Adaption to Remain in the Poultry Industry

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Abstract By presenting the epidemiology of *Campylobacter* and the main challenges in its control, this chapter reviews the mechanisms used by these microorganisms to adapt and survive in the poultry industry. Despite *Campylobacter*'s apparent fragility to environmental conditions, this agent is flexible and can adapt its metabolism and growth levels to an industrial environment. This chapter addresses mechanisms such as genome modulation, tolerance to high and low temperatures, tolerance to oxidative and nutrition stress, *quorum-sensing* systems, and the capacity of biofilm formation. These abilities are discussed, with consideration of the potential of different behaviors that can explain the organism's ability to survive and multiply in poultry, thus affecting the prevalence of *Campylobacter* in the final product.

Keywords Campylobacteriosis · Genome modulation · Hostile environment · Biofilm formation

9.1 Introduction

Campylobacter is the major microorganism incriminated in foodborne disease outbreaks (EFSA 2014). Its reservoir is the intestinal tract of domestic, wild or production animals, especially chickens (Moore et al. 2005; Keller et al. 2007). Poultry represents a major natural reservoir for this pathogen, because the bacteria have a commensal relationship with this host and can reach densities as high as 1.0×10^8 CFU g^{-1} of cecal contents (Rosenquist et al. 2006). This results in a large number of bacteria being shed via feces into the environment and, as a consequence, rapidly spreading throughout a flock of birds. (Achen et al. 1998).

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The microorganism can be found in poultry industry facilities, where it adapts and uses strategies to survive; thus, its control is a challenge.

It is essential to control the presence of *Campylobacter* in food industries and their facilities and to reduce infection rates in production animal reservoirs. During the production process, sanitary practices should be implemented to prevent cross-contamination, and adequate care should be taken to sterilize facilities and tools (Melo et al. 2013).

9.2 Epidemiology of *Campylobacter* in Food Industries

Many research reports about the isolation of *Campylobacter* in food industries provide an overview of the epidemiology, pathogenicity, resistance and spread of this microorganism. Studies conducted in some member countries of the European Union in 2007 found that the prevalence of *Campylobacter* in fresh chicken meat was 83 % (EFSA 2010). Another study in the EU found an average of 71.2 % of broiler flocks positive at slaughter (EFSA 2010). The prevalence was 63 % in Iran and 45.8 % in Japan (FAO 2009). In Brazil, Franchin et al. (2007) studied 335 samples of chilled carcasses, water and equipment collected at different points inside the broiler slaughter line. They found positivity for *Campylobacter* in 71.3 % of the samples. In Ireland in 2008, a total of 98 % of the 394 broiler chilled carcasses studied were contaminated with *C. jejuni* (EFSA 2010). In Brazil, Melo et al. (2013) isolated *Campylobacter* spp. in 22.38 % of 420 chilled and frozen chicken carcasses produced in slaughterhouses of three different Brazilian states. These data show that the rate of *Campylobacter* isolation in the poultry industry is quite high and can be a source of human infection.

While well adapted to life in the avian host, *Campylobacter* must survive during transit between hosts and on food products under stressful storage conditions, including high and low temperatures and atmospheric oxygen in high levels. The organism must therefore have mechanisms to protect itself from unfavorable conditions (Reuter et al. 2010).

In poultry industries, there are a lot of opportunities that may be a threat to *Campylobacter* survival. In this environment, there are changes in temperature, humidity, pH and nutrient availability. Additionally, after processing, the facilities are cleaned using detergents and sanitizers in order to eliminate the remaining microbiota. It is natural to think that a chicken carcass conveys *Campylobacter* because chickens are natural hosts of these bacteria. To eradicate the organism in the final product is almost impossible.

A few years ago, it was thought that *Campylobacter* was unable to multiply in foods during processing and storage, mainly due to the belief that the bacteria were sensitive to desiccation (Park and Elvers 2002). It would be difficult to believe that these agents could still survive these stressful conditions and still be a source of contamination in the production plant.

9.3 Mechanisms of Adaption to Stressful Conditions

Despite the apparent fragility of *Campylobacter*, it is fastidious and has particular needs, such as a microaerophilic environment; however, it has other features that offset these disadvantages. These features allow the organism's survival in stressful conditions, using mechanisms of adaption such as a genome that supports low and high temperatures, oxidative and nutritional stress, and the capacity of biofilm formation. It is therefore essential for *Campylobacter* to possess the ability to respond quickly to changes in environmental conditions through a series of specialized systems. These systems work by promoting an adjustment of their transcriptome in order to modify and facilitate a response to the subject condition, translating into physical changes (Lodge 2015).

Campylobacter genomically have the capacity to support low temperatures, and they also can survive for four weeks or more in water at 4 °C (Germano and Germano 2001). However, in this condition, according to Stintzi and Whitworth (2003), the expression of about 13 % of the bacterial genome is significantly changed by cold shock. Despite this, *Campylobacter* presents acquisition or the biosynthesis of cryoprotectant molecules, leading to changes in the lipid composition of the membrane in order to keep it viable in these conditions.

Dimitraki and Velonakis (2007) pointed out that freezing inhibits the growth of *Campylobacter*, reducing their numbers but not eliminating them completely in frozen foods, such as chicken carcasses (Humphrey et al. 2007). Lee et al. (1998) explains that *Campylobacter* spp. can survive in this extreme condition during the cycle of broiler production, fixing themselves deeply into the chicken's skin. This strategy probably creates an ideal environment for bacteria to adhere to and survive stress during the slaughter. Jang et al. (2007) showed that *Campylobacter* could persist in the feathers' follicles, as well as penetrate the skin and be protected, keeping the spiral shape of this bacteria.

The release of superoxide radicals, which occurs in conditions of stress by cold, causes damage to the bacterial cells. Therefore, the expression of superoxide dismutase (SOD) proteins, present during cold shock, is a response to this stressful situation used to guarantee the survival of *Campylobacter* under freezing conditions. Those SOD enzymes act as catalyzing superoxide molecules in order to protect the cellular components (cytoplasmatic enzymes, DNA, and membrane factors) against oxidative stress (Stintzi and Whitworth 2003).

Campylobacter also features potential tolerance to high temperatures and heat stress, inducing the synthesis of proteins related to variations in temperature (Reid et al. 2008). However, during the stress of cold shock, these genes, such as *dnaJ,* repress their translations (Beales 2004).

The *dnaJ* gene expression allows the bacteria to grow at temperature of 40 °C (Konkel et al. 1998). Proteins derived from *dnaJ* overcome rapid changes in temperature, letting the microorganism survive and adapt to that thermal condition. These proteins can suffer deformations in order to prevent the vital processes of *Campylobacter* from being compromised. In that way, the expression of genes such

as *dnaJ*, *dnaK* and *htrA* are important to degrade deformed or damaged proteins, promoting proper conformation of the proteins and protecting them against denaturation (Miller et al. 2009).

The quick response to temperature changes may be explained because *Campylobacter* can be found in a wide range of environmental temperatures from frozen/refrigerated foods (−20 °C/4 °C), such as the gastrointestinal tract of poultry (42 °C) (Melo et al. 2013).

9.4 Capacity of Biofilm Formation by *Campylobacter*

Another strategy adopted by bacteria to survive in hostile conditions is the production of biofilms. A biofilm is defined as a mono or multi-species population of bacterial cells, which is attached to a surface and connected by an extracellular polymeric substance. The composition varies depending on the microbial species involved, but it generally contains nucleic acids, proteins and polysaccharides (Donlan 2002; McCrate et al. 2013; Brown et al. 2014).

The capacity of biofilm formation involves the interaction of genetic and environmental factors (Pascoe et al. 2015). It is a bacterial mode of growth and a strategy of survival, where the surface-attached and matrix-encased bacteria are protected from stressful environmental conditions, such as ultraviolet radiation, predation, desiccation, disinfectant and antimicrobials. In this interaction, the pathogens are shown to be more resistant when they form biofilm than when they live free as planktonic cells (Elasri and Miller 1999; Fux et al. 2005; Matz et al. 2005; Chang et al. 2007; Reuter et al. 2010).

A study performed by Plummer (2012) discussed a *quorum-sensing* mechanism as having an important role in *Campylobacter* adaptation to the environment and also as a stimuli in producing biofilms. They discussed that several intercellular bacterial communication mechanisms have been identified in many bacterial species. These systems are known as *quorum-sensing*. They have been demonstrated to influence a variety of bacterial processes including motility, biofilm formation, expression of virulence genes, and animal colonization. Some species of *Campylobacter* possess a LuxS/autoinducer-2 (AI-2) mediated system. AI-2 is formed by a product of the activated methyl recycling pathway, specifically by the LuxS enzyme. This gene is involved in a variety of physiologic pathways of *Campylobacter* including motility, autoagglutination, cytolethal distending toxin (CDT) expression, flagellar expression, oxidative stress, and animal colonization.

Van Houdt and Michiels (2010) said that the ability of many bacteria to adhere to surfaces and to form biofilms has major implications in a variety of industries, including the food industry. On these sites, biofilms create a persistent source of contamination. It depends on an interaction between three main components: the bacterial cells, the attachment surface and the surrounding medium (Donlan 2002; Dunne 2002; Stoodley et al. 2002).

Biofilm formation may play a role in the epidemiology of *Campylobacter* infections. Knowledge of the conditions that may favor its formation is essential to establish control measures for this agent in poultry farms and processing plants in order to reduce the reservoirs of contamination and the incidence of campylobacteriosis (Teh et al. 2010).

In laboratory conditions, *Campylobacter* is a fastidious organism that needs specific conditions for growth, like temperatures of 34–44 °C and microaerobic conditions. However, during food chain production, there are many stressful obstacles that *Campylobacter* has to overcome, such as temperature variations, lack of nutrients, and exposure to high levels of oxygen. Research has given some understanding of *Campylobacter*'s stress responses; however, there is more to know of how these factors work together to allow survival of this bacteria in the human food chain. One possible contributor to this survival is the capacity of biofilm formation (Ica et al. 2011; Siringan et al. 2011).

The motility of bacteria has been pointed out as an important virulence factor that potentiates the colonization of host organisms and also biofilm formation. Flagellar motility is crucial for initial cell-to-surface contact and biofilm formation under stagnant culture conditions for many species of bacteria (Van Houdt and Michiels 2010).

Initially, the motility of *Campylobacter* can be useful to reach the surface by allowing the cell to overcome the repulsive forces between the cell and the surface. This mechanism is possibly more important under stagnant than under flow conditions. In addition, motility can be required to move along the surface, facilitating the growth and spread of a developing biofilm (Van Houdt and Michiels 2010).

Reuter et al. (2010) studied biofilm formation, comparing nonmotile and motile strains after static incubation for 2 days at 37 °C. Under microaerobic conditions, the motile strain formed 50 % more biofilm than the nonmotile one. The authors also concluded that the divergence was not because of the differences in viability, once the equivalent numbers of viable cells were recovered from the culture supernatants.

Most biofilms are composed of more than one species of microorganisms, which leads to interspecies and intraspecies interactions, and to the general complexity of the macromolecular mixture (Sutherland 2001). Often, these interactions result in enhancing the resistance of the microbial population to environmental stress.

Research about this theme was performed by Hilbert et al. (2010), in which the authors showed that *Campylobacter* inoculated with strains of *Pseudomonas*, both isolated from chicken meat and humans, showed prolonged survival in vitro, supporting the tension of atmospheric oxygen for up to >48 h probably due to this interaction of metabolic commensalism with *Pseudomonas* spp. This bacterium–bacterium interaction might set the basis for the survival of *C. jejuni* on chicken meat and thus be the prerequisite step in the pathway toward human infection.

Several reports have shown that *Campylobacter* species are capable of forming a monospecies biofilm (Figs. 9.1 and 9.2) and can colonize a preexisting biofilm (Sulaeman et al. 2010; Teh, Flint and French 2010; Ica et al. 2011; Siringan et al. 2011). This adaption is useful to support the suboptimal conditions and in that way

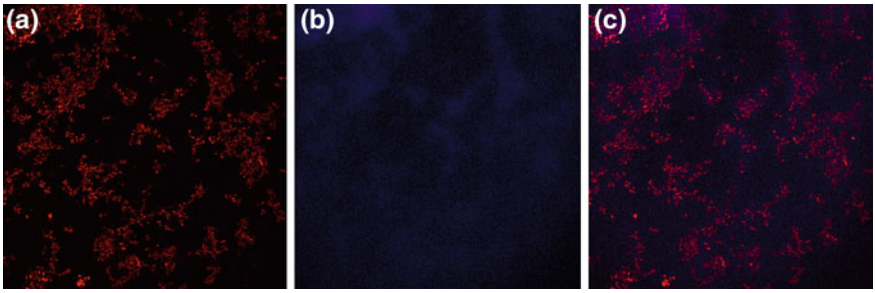

Fig. 9.1 Fluorescence micrographs of *C. jejuni* grown for 72 h, objective 63x/1.40. **a** *C. jejuni* were stained with Propidium iodide (PI); **b** Biofilms were stained with *Calcofluor white*; **c** Overlapping of the images **a** and **b**

Fig. 9.2 Three-dimensional reconstructed image showing the arrangement of *C. jejuni* biofilm grown for 72 h

guarantees the survival of bacteria by the increase of resistance to disinfectants, antimicrobials, and antibiotics (Reuter et al. 2010; Sofos and Geornaras 2010; Brown et al. 2014).

A study of *Campylobacter* in multispecies biofilms showed that the changes in the species compounding the biofilm are continuous, changing up to 40 % every day. This demonstrates the role of cells shed as descendant from the biofilm (Hanning et al. 2008; Reuter et al. 2010).

A serious challenge for food processing industries is the difficulty of removing organic residues that run off from carcass eviscerations and are rich in nutrients like carbohydrates, proteins and lipids. This is an ideal medium for a pathogen to remain and survive in this environment (Chmielewski and Frank 2007; Brown et al. 2014).

Biofilm formation can be simulated under laboratory conditions. Environmental biofilms from poultry facilities have been shown to contain *Campylobacter* (Pearson et al. 1993; Zimmer et al. 2003; Bull et al. 2006). *Campylobacter* biofilms allow the organism to survive up to twice as long under atmospheric conditions and in water systems (Joshua et al. 2006; Lehtola et al. 2006; Asakura et al. 2007; Reuter et al. 2010).

One assay that has been used in laboratory experiments that can simulate this is known as the "chicken juice" model—that is, nothing other than the exudate collected from defrosted carcasses, followed by supplementation or replacement of standard laboratory media with this sterile-filtered liquid. Research has shown that *Brucella* broth supplemented with chicken juice increases the survival of planktonic

cells of *Campylobacter* submitted to chilled and frozen storage (Lehtola et al. 2006; Reuter et al. 2010).

Brown et al. (2014) investigated the effect of chicken juice on the attachment of *Campylobacter* to surfaces and evaluated the capacity of biofilm formation. They showed that in the presence of chicken juice, biofilm formation is increased, considering the cell number and the attachment to abiotic surfaces. The authors concluded that the chicken juice provides a conditioned surface for the pathogen to adhere to.

Campylobacter is ubiquitous in the aerobic atmosphere and has regulatory systems to sense and adapt to external stimuli, such as aerobic and oxidative stress (Van Houdt and Michiels 2010; Gundogdu et al. 2011; Pascoe et al. 2015). Gonzáles-Hein et al. (2013) suspected that the gene encoding the regulatory protein CsrA might play a vital role in the regulation of stress responses and virulence determinants in this pathogen. They demonstrated that the global posttranscriptional regulator *csrA* (*c*arbon *s*tarvation *r*egulator) favors biofilm formation, adherence of intestinal epithelial cells and survival to oxidative stress (Fields and Thompson 2008; Gonzáles-Hein et al. 2013).

Campylobacter strains have different capacities to produce biofilms (Asakura et al. 2012). This could promote survival outside of the host, transmission and the colonization of multiple host species (Sheppard et al. 2009). Research performed by Pascoe et al. (2015) suggests that genetic determinants of biofilm formation differ between species, but they focused their study in how this works in strains of the same species with different genetic backgrounds. The authors investigated the genetic basis of biofilm formation in 102 *Campylobacter jejuni* isolates from different hosts, quantified biofilm formation, and identified hotspots of genetic variation in homologous sequences that correspond to variation in biofilm phenotypes. They found 46 biofilm-associated genes in total, including those involved in adhesion, motility, glycosylation, capsule production and oxidative stress. The genes associated with biofilm formation were different in the host generalist, suggesting the evolution of enhanced biofilm from different genetic backgrounds and a possible role in the colonization of multiple hosts and transmission to humans.

The research also highlighted that genes responsible for general bacterial characteristics can also stimulate biofilm production. Genes related to motility (Svensson et al. 2009), chemotaxis (Golz et al. 2012), capsule production (Malde et al. 2014) and protein glycosylation (Joshua et al. 2006; Guerry 2007) include genes putatively involved in biotin biosynthesis, cell wall biosynthesis, nickel transport, heat shock, and iron or zinc uptake. At least four genes thought to be involved in sensing oxidative stress were also associated with biofilm production, including *trxA*, *trxB*, *ilvE* and *nuoC* (Pascoe et al. 2015). This same study suggested that microaerophilic organisms, such as *Campylobacter*, can produce biofilms as an adaptation aimed to be succeeded in transmission to humans as the bacteria are exposed to high atmospheric oxygen concentrations when leaving the reservoir host gut (Pascoe et al. 2015).

A study performed by Reuter et al. (2010) correlated the biofilm formation with aerobic conditions. The authors found out that the capacity of biofilm formation

increases in this condition. These scientists examined biofilm formation under aerobic conditions and compared to a control group submitted to microaerobic atmosphere, considering that during food processing and also during the host transfer *Campylobacter* is exposed to high levels of oxygen and still remains viable enough to cause infection. The researchers found that the level of biofilm formation under aerobic conditions was double that observed under microaerobic conditions. They highlighted that survival in a biofilm would be an explanation of how the bacteria can support exposure to high levels of oxygen, demonstrating that the level of biofilm formation by *Campylobacter* increases considerably under aerobic conditions.

This study concludes that *Campylobacter* may have viable cells recovered after 50 days of culture. Continual shedding of *Campylobacter* cells into the environment produces populations of free living cells, which under stressful conditions may die or reattach to a preexisting biofilm. However, under favorable conditions, *Campylobacter* are able to colonize other niches (Reuter et al. 2010), such as chicken carcasses or even the human consumer of the final product.

Considering the difficulty of controlling or eliminating biofilm production and the other strategies that *Campylobacter* may use to remain viable, it is important to recognize the behavior of this pathogen. Brown et al. (2015) found out that extracellular DNase (eDNase) activity can inhibit or even degrade biofilms of *Campylobacter*. Because eDNase treatment has proved to be so effective against biofilms, extraction of eDNase enzymes from *Campylobacter* strains, it could in the future provide a cost-effective alternative source of DNase enzymes. This would assist in developing applications to improve food safety by the prevention of biofilm-assisted transmission of foodborne pathogens, such as *Campylobacter*.

As Van Houdt and Michiels (2010) highlighted, biofilm prevention and control have to be a priority in industries, stimulating cleaning and disinfection programs that prevent or eradicate biofilms. These include the biofilm-supporting properties of food contact materials, in addition to their thermal, mechanical and chemical resistance, as an element of the hygienic design of equipment and utensils; identifying biofilm-prone areas in existing process lines; and monitoring organic and microbial load in these areas. Research should continue on the efficacy of cleaning agents and disinfectants, the factors involved in attachment and biofilm formation, the decreased sensitivity of biofilm bacteria to disinfectants, and the development of novel biofilm prevention or control strategies.

9.5 Biofilms of *Campylobacter* Versus Viable but Nonculturable (VNC) Forms: Molecular Association and Implications in Public Health

Despite the high prevalence of *Campylobacter* in food, little is known about the mechanisms that *Campylobacter* uses to adapt and survive the stresses. *Campylobacter* is a major cause of bacterial food-borne diseases. The status of

Campylobacter is due to its ability to make the transition to the VNC state, particularly within the biofilm, and thus not be detected by traditional techniques.

The knowledge that biofilms provide protection for micro-organisms is well described by the fact that biofilm on surfaces in contact with food is more resistant to removal and acts as a constant source of contamination and recontamination. A study demonstrated the ability of *C. jejuni* in a sessile form to gain the VNC form in vitro and to remain in this condition for extended periods (60 days) in elevated numbers (10^6 viable cells) under refrigeration (4 °C) (Magajna 2014). The possibility of development of these VNC forms within a biofilm has major implications for food security and justifies the risks that go beyond the simple detection of the agent in laboratory, but also to research various forms of camouflage. This points to the need for a more rigorous and precise control in food, with more efficient routines by monitoring of methods in food and contact surfaces where biofilms may be present.

Campylobacter does not have the stress response systems commonly found in other enteric pathogens, such as *Salmonella* spp. and *Escherichia* spp.. However, it is able to withstand the stresses in the form of biofilms or VNC form (Rollins and Colwell 1986; Trachoo and Frank 2002). Although there are few studies on the relationship between these phenotypes, there are data showing the interference of some genes on the VNC stage and on biofilms, suggesting that these two systems may be related on a molecular level (Gangaiah et al. 2009; Drozd et al. 2011).

Four genes in particular, kinase polyphosphate 1 (*ppk1*), an alkaline phosphatase (*phoX*), a rapid response regulator (*spoT*) and a nutritional stress regulator (*csrA*) appear to have interference on both biofilm and in stress under adverse conditions in *Campylobacter* (Gaynor et al. 2005; Candon et al. 2007; Fields and Thompson 2008; Gangaiah et al. 2009; Drozd et al. 2011). The *ppk1* catalyzes the synthesis of inorganic polyphosphate (Poly-P), which consists of a long chain of phosphate residues linked by high energy; it acts as a reservoir of energy and phosphate. In *C. jejuni*, Poly-P builds up during the transition from the exponential to stationary phase and plays a role in the survival of low nutrient levels, in the biofilm formation in a natural way, in osmotic tolerance, resistance to antibiotics, on the intracellular survival and colonization (Candon et al. 2007; Gangaiah et al. 2009). Mutant strains of *ppk1* present deficiencies in the levels of Poly-P and are less able to enter the VNC state, indicating that the Poly-P is required to maintain viability and allow the cells to enter the VNC state during stress (Gangaiah et al. 2009).

There is evidence showing that there is also a relationship between other genes cited with *Campylobacter* ability to acquire the VNC form as the direct relationship of these genes with the polyphosphate and biofilm formation (Magajna 2014). Alkaline phosphatase (*phoX*) provides to the cell of *Campylobacter* inorganic phosphate (Pi) by hydrolysis of phosphate groups. Pi, which is typically low in the environment, is necessary for the formation mediated by ppk1 and Poly-P (Drozd et al. 2011). The response mediated by *C. jejuni* associated with *spoT* is linked to overall stress, usually triggered by starvation of amino acids, modifying expression of the gene to promote their survival during growth. *C. jejuni* is able to accumulate large amounts of Pi in response to carbon and phosphate starvation in the presence

of this gene (Wells and Gaynor 2006). The post-transcriptional regulatory overall *csrA*, which activates or represses mRNA translation into protein, plays a role in motility, biofilm formation, adherence to epithelial cells and defense oxidative stress in *C. jejuni* (Fields and Thompson 2008; Timmermans and Van Melderen 2010).

Several studies have shown that expression of these four genes is significantly higher when *C. jejuni* is presented in a sessile manner and that the acquisition of the VNC stage form biofilms is more evident when compared with planktonic life due to direct action of these genes (Wells and Gaynor 2006; Gangaiah et al. 2009; Timmermans and Van Melderen 2010; Drozd et al. 2011; Magajna 2014). Thus, the difficulty in the control of this pathogen is reinforced once the agent in the VNC form is not detectable in the food production process and can become viable in vivo and initiate the disease in the host.

These data provide the basis for the need to improve detection methods used in the control of food safety, which should be more accurate in identifying the number of viable cells in biofilms of *Campylobacter*. It must be considered that the VNC forms are potentially infectious, represent imminent risk to consumers and are hidden by traditional identification techniques.

9.6 Conclusions

As discussed in this chapter, *Campylobacter* have different mechanisms of adaptation and virulence factors, which allow the pathogen to support hostile conditions during the process of food production. Understanding how this agent regulates these mechanisms is extremely important for the use of this pathogen in industries; the potential threats during food processing, the sources of infection, and how to avoid the infection of other hosts, especially the human consumer, should be known. The adaptation strategies that guarantee their viability during the stressful situations in food processing are regulated by genes that tolerate a diversity of obstacles, such as low and high temperatures, resistance to disinfectants and sanitizers, tolerance to high levels of oxygen, nutritional scarcity and the capacity to produce biofilms, which are abilities that make *Campylobacter* a potential threat against public health.

Studies on the mechanisms of *Campylobacter* resistance throughout the food processing chain are still essential to monitor their prevalence in food industries and also understand their epidemiological behavior. Continued research on their adaption mechanisms, virulence and resistance genes are important pathways to an understanding of the spread of *Campylobacter* in the food chain, to identify sources of infection and to create strategies to control this pathogen.

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Chapter 10
Antimicrobial Resistance in *Campylobacter* spp.

Lisette Lapierre, María Laura Arias and Heriberto Fernández

Abstract The resistance of infectious agents to a broad range of antimicrobial drugs is a growing public health problem, which raises significant social concerns. This chapter describes the main antimicrobial resistance problems in developed and developing countries and the possible relationship between the resistances of *Campylobacter* strains found in poultry and humans. Determining the level of the problem is essential for control, formulating, and monitoring an effective response to antimicrobial resistance.

Keywords Antimicrobial resistance · Antimicrobial surveillance systems · Antimicrobial genotypic resistance · *Campylobacter*

10.1 Introduction

Campylobacter is recognized as one of the most important pathogens associated to the production of enteritis in human beings (Idris et al. 2006; Nelson and Harris 2006; CDC 2013). *Campylobacter jejuni* and *C. coli* are the species that produce most of the infections in the population, estimating that these may cause disease to up to 400–500 millions of persons/year (Allos 2001; Moore et al. 2005).

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Transmission ways to population include the ingestion of water, non-pasteurized milk and/or the consumption of raw or undercooked meat. Poultry is being the most implicated one due to the fact that birds are the principal reservoir of these bacteria.

The enteritis produced by *Campylobacter* is known as campylobacteriosis and is characterized for being an auto limited aqueous to bloody diarrhea, which lasts in average 5 to 7 days and does not require antimicrobial treatment. Antimicrobial treatment is just required when the disease shows extraintestinal manifestations or when patients are small children, pregnant women, or immunocompromised persons. Macrolides, including classic erythromycin or the new ones as clarithromycin and azithromycin are the drugs of choice to be used when the diagnosis of this pathogen is confirmed by culture at the laboratory (Payot et al. 2006a). Fluoroquinolones are second choice of drugs and are generally used as empirical treatments for enteritis that have no pathogenic microorganism diagnosed (Aarestrup et al. 2008; Ge et al. 2013). A metaanalysis study has shown a reduction of 1.32 days in the duration of *Campylobacter* enteritis in those patients with erythromycin or fluoroquinolone treatment (Ternhag et al. 2007). Other therapeutically alternatives include the use of tetracycline, doxycycline, and chloramphenicol. Serious systemic infections are treated with aminoglycosides as gentamicin or even a carbapenem as imipenem (Okada et al. 2008). Third generation cephalosporins are not used since they are not effective against *Campylobacter* (Pacanowski et al. 2008).

Nevertheless, since the 1980s and as it occurs with most pathogens, resistance to the antimicrobials used in the treatment of complicated enteritis has emerged. This resistance has also been reported for other antimicrobial families (Lehtopolku et al. 2010). In many countries, the emergence of resistance in *C. jejuni* and *C coli* strains, especially to fluoroquinolones, has made the treatment of complicated infection of critical patients difficult. Erythromycin resistance has increased significantly in the last years, especially in *C. coli* strains.

Although the resistance of human origin strains to macrolides is low, it increases every year, especially in bacteria isolated from poultry (Kim et al. 2006). A 14-year study in Cape Town, South Africa, indicated that there was a significant rise in the antibiotic resistance of erythromycin and other antibiotics by *C. jejuni* and other *Campylobacter* species isolated from pediatric diarrhetic patients (Bester et al. 2011). This situation might complicate the future use of erythromycin as a first choice for treatment. Resistance of *Campylobacter* strains to different antimicrobials including ciprofloxacin and other fluoroquinolones, macrolides and lincosamides, chloramphenicol, aminoglycosides, tetracycline, ampicillin and other β-lactams, cotrimoxazol, and tilosine has been previously reported, therefore decreasing the available therapeutical alternatives (Padungtod et al. 2003). The level of antimicrobial resistance observed for *Campylobacter* strains especially against ciprofloxacin and tetracyclines is high, and the actual emergence of new resistance to macrolides and other antimicrobials is worrying (Fernández 2011; Ge et al. 2013). This increasing antimicrobial resistance trend noticed many decades ago has been indicated as a public health problem (Skirrow 1994).

The antimicrobial resistance of zoonotic pathogens is an important subject to be studied, in both developed and developing countries. Actually, this situation is

worrying since many drugs have lost their clinical efficacy, making diseases longer and/or increasing the treatment costs and even the mortalities associated. It has been shown that the use of antimicrobials as prophylactic treatment and/or antimicrobial growth promoters in veterinary medicine has contributed to the increase of bacterial resistance, a fact of great importance in zoonotic bacteria (Angulo et al. 2004). Under this context, the World Health Organization (WHO) the World Organization for Animal Health (OIE) and the Codex Alimentarius have shown that good practices for the use of veterinary drugs in food production animals must be implemented. Various recommendations and measures shall be considered and all the interested parts must be involved, since the actions taken in veterinary medicine may directly or indirectly affect human health. For example, tracking the susceptibility of animal isolated *Campylobacter* strains to fluoroquinolones might predict what will happen with the susceptibility of these drugs in human's isolated strains (Smith et al. 1999; Van Looveren et al. 2001). This is why it is so important to introduce antimicrobial resistance surveillance systems, in order to adopt control measures and evaluate its impact through time, having as main objective the protection of human, animal, and ecosystem's health.

Following this idea, different countries have instituted surveillance systems for the resistance to antimicrobials with a "One Health" focus, following strains isolated from humans, animals, and food (CIPARS 2007; FDA 2012 [NARMS]; DANMAP 2012). Surveillance data are integrated in order to develop actions that trend to diminish the problem from several parts. The results of these programs are used for observing trends, generating reports, establishing comparisons, creating surveillance platforms, and taking decisions that benefit human and environmental health. These countries also have to apply a legislation that covers aspects related to the use of antimicrobials in veterinary and human medicine, knowing the quantities of drugs used, the form in which they are used, as well as the target species. Unfortunately, few countries have this kind of data. The susceptibility/resistance monitoring of antimicrobials, especially those used for the treatment of *C. jejuni* and *C. coli* in critical patients and those used in the food chains, especially in poultry, might help to establish preventive and control measures of the resistance emergency in the medium or long term.

10.2 Phenotypical Resistance to Antimicrobials in *C. jejuni* and *C. coli* Strains

Campylobacter was recognized as an important human pathogen in 1972. Nevertheless, the standardized susceptibility analysis methods were not available until 2004 (McDermontt et al. 2004, 2005). So, before this date, the laboratories that made antimicrobial susceptibility analysis in *Campylobacter* strains could use different protocols. There are different protocols for the growth of the strain, in which the conditions or the culture media used are different, also each lab could

apply different criteria for the interpretation of the susceptibility results obtained. In order to compare the obtained results in different laboratories, these criteria and protocols have to be harmonized. The harmonization of criteria has to be done by institutions such as the Clinical and Laboratory Standards Institute (CLSI) in United States and/or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) in Europe. EUCAST is an institution conformed by several national committees of the countries forming the European Union, they standardize the norms of this matter. Both CLSI and EUCAST develop guidelines for the standardization of the susceptibility to antimicrobials analysis techniques in different pathogens and publish the criteria for the interpretation of the results for each bacterial category (indicator or pathogen), qualifying them as sensitive, intermediate or resistant; also they publish and actualize the cutoff points.

Most of the official laboratories that realize resistance surveillance use the protocols and guidelines recommended by CLSI and/or EUCAST (Ge et al. 2013). Most of the European countries, as well as the developed ones and US, Canada and Australia have resistance surveillance programs for zoonotic pathogens, which include *Campylobacter* isolated from poultry and or poultry meat. In Latin America, Mexico and Colombia (COIPARS) have surveillance programs that include *Campylobacter* strains isolated from poultry and its products (Donado-Godoy et al. 2015).

Since 1995, surveillance programs for monitoring *Campylobacter* resistance were designed followed by integrated programs. Within the most outstanding programs in North America, the US National Antimicrobial Resistance Monitoring System (NARMS) established in 1996, as well as the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), which initiated in 2002, have to be mentioned. Both programs follow the guidelines and protocols recommended by CLSI for the growth of *Campylobacter,* use the broth microdilution technique and realize trials against a panel composed of 9 antimicrobials: azithromycin, ciprofloxacin, clindamycin, erythromycin, chloramphenicol, gentamicin, nalidixic acid, tetracycline, and telithromycin. Historically, both surveillance programs use the cutoff points from CLSI, nevertheless, actually and looking forward for a harmonization of the protocols, both programs use for data interpretation the epidemiological cutoff values (ECOFFs) given by EUCAST (Ge et al. 2013). In the case of Europe, there are several surveillance programs for resistance, as the Danish Integrated Antimicrobial Resistance or DANMAP, initiated in 1995. Other member states of the European Community (EU) also have their own monitoring programs and collect antimicrobial resistance data from foodborne pathogens including *Campylobacter*. All data are further analyzed by the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC). These organisms generate reports on antimicrobial resistance trend and prevalence (EFSA/ECDC 2015). Some reports on the resistance trends of *Campylobacter*, as the ones realized by NARMS between 2007 and 2011 show that strains are generally resistant to tetracycline, being this one the most common resistance registered, followed by the resistance to quinolones, specifically to ciprofloxacin and nalidixic acid. Resistance frequency to other antimicrobials including gentamicin, clindamycin, azithromycin, erythromycin, telithromycin, and

chloramphenicol is significantly lower. NARMS reported in 2011 resistance percentages to ciprofloxacin and nalidixic acid for *C. coli* and *C. jejuni* strains isolated from chicken broiler of 27.9 and 19.2 %, respectively. Tetracycline resistance was 42.1 % for *C. coli* and 45.1 % for *C. jejuni (*FDA NARMS 2012).

The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS 2012) reported resistance to tetracycline in 39.3 % of the strains isolated from broiler chicken. This is followed by quinolone resistance being 9.4 %, macrolides 4.3 %, telithromycin 1.7 %, and clindamycin 0.9 %. No resistance to gentamicin or chloramphenicol was found on these isolates (CIPARS 2012). The analysis of data provided by member countries allowed EFSA/ECDC to inform in 2015 that the antimicrobial resistance in *C. jejuni* strains isolated from broiler chicken was 54.5 % for ciprofloxacin, 52.3 % for nalidixic acid, and 41.4 % for tetracycline. For *C. coli* strains, several reports showed resistance to ciprofloxacin (68.8 %), nalidixic acid (63.9 %), tetracycline (70.4 %), and erythromycin (13.7 %). Data from COIPARS (Colombia) show that *Campylobacter* spp. strains isolated from chicken and subproducts are very resistant to ciprofloxacin (50–97 %); for erythromycin the reported resistance is 48 %, being most strains isolated from meat from supermarkets whereas 83 % of the strains isolated from carcasses at a slaughtering plant were tetracycline resistant (Donado-Godoy et al. 2015). It is important to mark that in most of the reported data, *Campylobacter* is susceptible to gentamicin and contrary to other Gram negative pathogens does not show high multiresistance levels.

When comparing the resistance rates of *Campylobacter* strains found in US or Canada surveillance programs with that from the European Union or Colombia, the last ones are higher. This may be due to the fact that there is a very strict legislation on the use of antimicrobials for poultry production in the countries with lower resistance levels. For example, in 2005 the Food and Drug Administration (FDA) in the United States, based on active surveillance results, banned the use of enrofloxacin in poultry, being one of the reasons to achieve a reduction in the resistance rate of quinolone-resistant *Campylobacter* strains isolated from human beings (FDA 2005). Since this measure was taken, resistance levels to ciprofloxacin in this country have remained stable (Zhao et al. 2010).

In Colombia, for instance, there is no legislation for the prudent use of antibiotics and the surveillance program is recent, done with private funding as a pilot plan with capacity to analyze a low number of samples. Thus making not possible to reduce in a short term the high resistance levels reported.

Different authors have studied the resistance emergency in *Campylobacter* strains, and the resistance percent data are median or high. Di Giannatale et al. (2014) did an antimicrobial susceptibility study in Italy in 145 *Campylobacter* strains from different origins, including raw milk, poultry feces, poultry meat, milk cow feces, and human feces. Authors found high resistance levels to ciprofloxacin (62.76 %), tetracycline (55.86 %), and nalidixic acid (55.17 %).

With regard to ciprofloxacin, in South America, Pollet et al. (2012) reported that in a period of ten years (2001 to 2010), *C. jejuni* ciprofloxacin resistance increased in three Peruvian regions in more than 10 %, reaching 89.8 % in Lima, 82.8 % in

Cusco, and 48.9 % in Iquitos. In Argentina, Tamborini et al. (2012) found ciprofloxacin resistance in 65 % of *Campylobacter* strains isolated from humans, dogs, and poultry showing by PFGE that some human strains genetic subtypes matched with genetic subtypes isolated from dogs and chickens. In Chile, ciprofloxacin resistant *Campylobacter* were isolated from humans with gastroenteritis (60 %), poultry (58.2 %), and bovines (18.2 %). Some of these strains shared the same PFGE patterns and the same resistance profiles. Moreover, some human isolates showed indistinguishable PFGE profiles with strains isolated from poultry and bovine (González-Hein et al. 2013). A recent study carried out in Ecuador reported high frequency of ciprofloxacin resistant *C. jejuni* and *C. coli* strains (98.3 %) isolated from chicken livers for human consumption (Simaluiza et al. 2015).

In the specific case of strains isolated from poultry or poultry meat, there are several authors that describe high antimicrobial resistance frequencies, specifically for fluoroquinolone and tetracycline families. A study realized by Fraqueza et al. 2014 in which the susceptibility of *C. jejuni* and *C. coli* strains isolated from different chicken broiler production systems was analyzed to 11 antimicrobials, they found high resistance rates (higher than 80 %) to fluoroquinolones. Also in this study, it was found that strains were resistant to tetracycline (58 % for *C. jejuni* and 76 % for *C. coli)*. Wieczorek et al. 2015, analyzed 1151 strains of *Campylobacter* (*C. coli* and *C. jejuni)* isolated from broiler carcasses in Poland and they reported that 81.6 % of isolates were resistant to ciprofloxacin, 56.1 % to tetracycline, and 2.4 % to erythromycin. Only 1.7 % *Campylobacter* isolates displayed a multiresistance pattern. In Southern Ecuador, *Campylobacter* isolated from commercial chicken livers showed high resistance to tetracycline (78.1 %) but lower to ampicillin (25.0 %) and erythromycin (12.5 %), while multiresistance was observed in 28.1 % of these strains (Simaluiza et al. 2015).

It is important to consider the impact that environment might have over the resistance rates reported. Actually, there is an increase in the presence of antimicrobial residues and/or resistant bacteria present in the environment; this increase is related to soil and/or water contamination with discharges coming from animal husbandry facilities, hospitals, and pharmaceutical industries, transforming it into an ecological type problem (Moore et al. 2006). This situation is much more important when the changes and trends of *Campylobacter* resistant strains are observed. The use of fluoroquinolones as enrofloxacin in the production of broiler chicken has had an effect in the emergence of resistance to ciprofloxacin both in strains isolated from animals as well as the ones isolated from human patients (Fraqueza et al. 2014). This situation is evidenced when the resistance rates to these drugs are observed especially in countries where there is no restriction on their use. Nevertheless, for the time being the contribution to the emergence of resistance to fluoroquinolones in *Campylobacter* strains isolated from human beings due to the use of this antibiotic in poultry industry and human medicine has not been quantified. A fact that has been demonstrated is that resistance genes can be interchanged between bacterial genres and the barriers that avoid this transfer between different organisms and environment are decreasing. An example of environmental contamination is the presence of dogs fecal material in a public park. A study realized

Table 10.1 *Campylobacter* resistance (%) to four antimicrobials in five South American countries (adapted from Fernández 2011)

Country	Ampicillin	Erythromycin	Tetracycline	Fluoroquinolones
Argentina	47.2	6.3	40.8	49.1-59.6
Bolivia	NI	61.4	65.9	47
Brazil	18–26.9	9.1–38.9	9.1–43	14–72.2
Chile	4.6–25	5.6–58.6	1.8–15.1	5.3–50
Peru	37	17	NI	63–78

NI Not informed

in the Southern part of Ecuador shows that 87.5 % of the *Campylobacter* strains isolated from this kind of samples were resistant to tetracycline and 100 % to ciprofloxacin (Toledo et al. 2015).

Most of the data originated in Latin America on antimicrobial resistance in *Campylobacter* are mostly sporadic efforts done by academic research groups (Fernández et al. 2000; Ruiz-Palacios et al. 2007; Fernández 2011; Notario et al. 2011; Pollet et al. 2012; Zaidi et al. 2012). As shown in Table 10.1, in South American countries antimicrobial resistance in *Campylobacter* strains isolated from different sources seems to be an important but not well-dimensioned problem (Fernández 2011). On the other hand, *Campylobacter* isolation, identification, and antimicrobial susceptibility testing from the different Latin American countries have been performed with different analytical methods, which make comparison of results more difficult, emphasizing the need for harmonization and standardization of diagnostics methods (Fernández 2000). Besides harmonization and standardization of diagnostics methods strengthening, *Campylobacter* antimicrobial resistance surveillance programs and capacity building with the association between public health services and the academic world are necessary to implement in developing countries.

Because of research work done in the field, and the data reported from the resistance surveillance programs, today we have a better understanding of how antimicrobial resistance is initiated, acquired, and kept in *Campylobacter* strains. Nevertheless, in addition to the phenotypic data of susceptibility/resistance, more information on the molecular mechanisms associated to resistance and on the transfer of resistance genes to different organisms must be acquired in order to have a complete vision of this problem.

10.3 Antimicrobial Genotypic Resistance in *C. jejuni* and *C. coli* Strains

10.3.1 Generalities

Campylobacter is a bacterium that has the intrinsic capacity for transforming and acquiring resistance genes from other organisms. This condition has favored the

acquisition of antimicrobial resistant strains, a worrying situation especially referred to the resistance to fluoroquinolones and erythromycin.

C. jejuni's antimicrobial resistance has been related to chromosomal and plasmid genetic elements, representing a combination of endogenous and acquired genes (Lovine 2013).

Resistance mechanisms present in *Campylobacter* strains can be summarized in the following ones:

1. Modification and/or alteration of target site (DNA gyrase mutation)
2. Antibiotic's impossibility to reach target site (major protein of external membrane)
3. Antibiotic's elimination using efflux pumps (multidrug efflux pumps as CmeABC)
4. Modification and/or alteration of the antibiotic (β lactamase production)

In *Campylobacter* spp. it is usual to detect a synergism between the resistance mechanisms, where generally multidrug efflux pumps are present with an additional secondary mechanism. Efflux pumps are associated to intrinsic resistance mechanisms against a wide variety of antibiotics, it is common to find different types such as CmeDEF, CmeG, nevertheless, the better described efflux pump in this specie is the CmeABC.

Campylobacter spp. presents intrinsic resistance against novobiocin, bacitracin, vancomycin, and polymyxin/colistin possibly mediated by the absence or low affinity of target to antibiotics. For the intrinsic resistance to trimethoprim, several forms of the dihydrofolate reductase enzyme, codified by *dhfr1* and *dfr9* genes have been described in more than 90 % of the *C jejuni* strains where the presence of these genes has been studied. As indicated before, *Campylobacter* strains show high rates of resistance especially to drugs of the fluoroquinolones and tetracycline families, and low but emergent rates to macrolides, especially erythromycin. The presence of these high rates of resistance to the two classes of antimicrobials mentioned before, depends on several factors such as the abuse of these drugs in different environments, but also has been related to the presence of the most common resistance molecular determinants that confer this characteristic to strains of the *Campylobacter* genus.

10.3.2 Fluoroquinolones Resistance Determinants

Fluoroquinolones (FA) are synthetic antibiotics with a strong bactericidal activity against Gram negative and Gram positive bacteria (Zhang et al. 2003); they cause cellular death due to the inhibition of the bacterial DNA synthesis. The target spots of these antibiotics are two big enzymes: DNA gyrase (codified by *gyrA* and *gyrB* genes), the topoisomerase IV (codified by gens *parC* and *parE*). Fluoroquinolones form a stable complex with these enzymes altering the replication, transcription,

recombination, and repair of bacterial DNA (Drlica and Malik 2003; Lovine 2013). In bacteria, the DNA gyrase catalyzes negative super coil of ATP dependent DNA and releases the accumulated stress in DNA's torsion and the transcription and replication complexes. By the other way, topoisomerase IV is a decatenating enzyme, implicated in the rupture and reunion processes of the DNA double strand and also required for the energy transduction through the ATP hydrolysis (Hooper 2001).

Three different mechanisms have been described for *Campylobacter* resistance to fluoroquinolones, including pump efflux, target site modification, and membrane permeability alteration (Zhang et al. 2003).

The most frequent one is the modification of the target site and it takes place thanks to the substitution of amino acids through punctual specific mutations in the "Quinolone Resistance Determinant Region" (QRDR) of the *gyrA* gene (Lovine 2013). The most common punctual mutation in *Campylobacter* is the change in C257T of *gyrA* gen, which implies the substitution on Thr-86-Ile giving the bacteria the ability to grow in high ciprofloxacin concentrations (high minimal inhibitory concentration MIC). Less common mutations, as the substitution in Thr-86-Ala are responsible of the high resistance level to nalidixic acid (high MIC) and low resistance levels to ciprofloxacin (low MIC) (Payot et al. 2006b). Although the Thr-86-Ile punctual mutation is the most common one and confers high resistance levels, there are other less common punctual mutations that give intermediate resistance levels to fluoroquinolones, including Asp-90-Asn, Ala-70-Thr, Thr-86-Lys, Thr-86-Val, and Asp-90-Tyr (Luo et al. 2003). It has been shown that Thr-86-Ile mutations do not confer an increase in the fitness of *Campylobacter* spp. It has been observed in an avian model that the fluoroquinolone resistant strains persist in the farm, even after the antibiotics retirement because of this mutation (Lovine 2013). Mutation events are a fundamental factor for the development of resistance in *Campylobacter*, especially in *C. jejuni*. The absence of many genes present in other bacteria that codify for DNA repair elements such as *mutH* and *mutL* (mismatch repair system), *sbcB* (repair during recombination) *phr* (pirimidinal dimer reparation), and *vsr* (very short patch repair) has been demonstrated in this species, as well as the absence of genes needed to repair UV-induced damage as UmuCD, alkylating agents (*ada* gene), facilitating the appearance of mutations (Zhang et al. 2006).

It has been demonstrated that *C. jejuni* and *C. coli* do not have *parC* and *parE* genes, so a unique modification of the *gyrA* sub unit is enough to confer high resistance levels to fluoroquinolones (Payot et al. 2006b). Contrasting with what happens in *Campylobacter*, where a punctual mutation can generate relevant resistance levels, in other Gram negative bacteria as *Escherichia coli* or *Salmonella* spp, the accumulation of mutations in the QRDR is needed in order to acquire resistance to fluoroquinolones, so the expression of high drug resistance levels is less frequent (Lovine 2013). This situation may partially explain the emergence of fluoroquinolones resistance in *Campylobacter* strains.

A second resistance mechanism present in *Campylobacter* strains against fluoroquinolones is the drug elimination through the action of efflux pumps. The

most common efflux system in this species is the multidrug CmeABC efflux pump, which is constitutive in several *Campylobacter* strains (Lin et al. 2002). It has been described that it is implicated in the resistance to fluoroquinolones and macrolides as well as the intrinsic resistance to bile salts (Pumbwe and Piddock 2002). The multidrug efflux pump is codified by an operon constituted by three genes: *cmeA*, *cmeB*, and *cmeC*, that codify for a periplasmic fusion protein, an internal membrane transporter protein and an external membrane protein, respectively (Lin et al. 2002). The CmeABC efflux pump has an important role in the antimicrobial resistance of *Campylobacter* since the inactivation of *cmeB* or the use of efflux pump inhibitor generate an increase in the susceptibility to different antibiotics, even the ones that *Campylobacter* is intrinsically resistant (Akiba et al. 2006).

The reduction of the fluoroquinolones intracellular concentrations due to the CmeABC efflux pump together with the punctual mutations on the QRDR of *gyrA* gen (particularly in Thr-86-Ile), generate high resistance levels to these antibiotics, due to the fact that these mechanisms act synergically. This synergic effect can be seen in strains that present intermediate resistance to fluoroquinolones, showing higher resistance levels when the CmeABC efflux pump is expressed (Cagliero et al. 2006). There are evidences that show that the resistance to fluoroquinolones has emerged in *Campylobacter* strains isolated from broiler chicken, even in the absence of the administration of these antibiotics in production farms. As showed before, the principal resistance mechanisms is due to punctual mutations in *gyrA*, so it is difficult to attribute this resistance emergence to the presence of mobile elements that confer multiresistance to antimicrobials. More studies are necessary in order to clarify in which way these strains acquire resistance and how the resistance determinants are disseminated. It is believed that in productive systems where no fluoroquinolones have been used, the administration of other antimicrobials of different families could select the resistance to fluoroquinolones in *Campylobacter* strains; this could happen by the overexpression of CmeABC way (Asai et al. 2007).

10.3.3 Macrolides Resistance Determinants

Macrolides are natural antibiotics and most of them are derived from the metabolism of *Streptomyces*. They are widely used and are effective against infections produced by Gram positive and Gram negative bacteria (Wieczorek and Osek 2013). The antimicrobial activity of macrolides is based on the inhibition of the bacterial protein synthesis, due to the reversible union with the ribosomal 50S subunit (Mankin 2008). Macrolides prevent the formation of peptide bonds through its interaction with the 23SrRNA, major component of ribosomal 50S sub unit. It is postulated that these antibiotics cause a dissociation of the peptidyl-tRNA blocking the way used by new peptides for leaving the ribosome, thus interfering with polypeptide chain elongation (Tenson et al. 2003). *Campylobacter* resistance to

macrolides is based on the modification of the target site of union of these antibiotics through mutations: the ribosomal 23S rRNA subunit and in ribosomal protein (Batchelor et al. 2004). *Campylobacter*'s chromosome has three copies of the *rrn* gene that codify for the 23S rRNA, substitutions of the adenine residues at the positions 2074 and 2075 confer resistance to erythromycin; at least two of these genes must mutate in order to confer resistance to these drugs (Jeon et al. 2008).

In *C. jejuni* and *C. coli*, the most common substitution is the transversion of A2075G (Gibreel et al. 2005), and less frequent the modifications of A2074C and A2074G. These modifications confer high resistance levels against erythromycin (MIC > 128 mg/L) (Avrain et al. 2004). The mutations on genes *rplD* and *rplV,* that codify for ribosomal proteins L4 and L22 confer low resistance levels against these antibiotics. Mutations of these genes only affect the regions between amino acids 55 and 77 of L4 and between 109 and 142 of L2 (Caldwell et al. 2008); exact role of these modifications, due to mutations, insertions, or deletions is not clearly defined yet (Cagliero et al. 2006). Additionally, efflux pumps are other resistance mechanism present in *Campylobacter* against these antimicrobial. In this species, at least eight different efflux systems have been described, being the most important one the CmeABC efflux pump, as mentioned before. As with fluoroquinolones, this efflux system acts synergically with the mutations in 23S rRNA, conferring high resistance levels against macrolides (Corcoran et al. 2006).

It has been described that *Campylobacter* strains with high resistance levels and that present mutations in A2074G and A2075G reduce significantly the MIC when the CmeABC efflux system is inactivated, what might confirm the synergic activity between these two mechanisms (Cagliero et al. 2006, Lin et al. 2007), as well as the potential role of efflux system in the intrinsic resistance of *Campylobacter* (Cagliero et al. 2006).

Concerning resistance to macrolides, *Campylobacter* strains isolated from broiler poultry do not present high frequency of phenotypical resistance, possible due to the fact that macrolides are not habitually used in poultry.

10.3.4 Tetracycline Resistance Determinants

Tetracyclines are antibiotics either from natural or semisynthetic origin, frequently used in human and veterinarian medicine. They have a broad spectrum and are active against Gram negative and Gram positive bacteria. Its activity is bacteriostatic; nevertheless they can reach a bactericidal activity in high doses (Chopra and Robert 2001). These antibiotics act by binding to the minor unit of the bacterial ribosome, specifically under helix 34 of the 16S rRNAS, in a pocket between the head and platform of the 30S ribosomal subunit. In this union site, tetracyclines occupy the A site of the 30S ribosome, preventing the union of the aminoacyl tRNA (aa-tRNA), so the ribosome cannot complete the elongation process of the peptides (Brodersen et al. 2000).

Campylobacter resistance to tetracyclines is done because of the expression of one of the following mechanism: efflux pumps, protection by cytoplasmic proteins of the specific union site between the antimicrobial and the ribosome, drug modification, and modification of the union site between tetracycline and 16Sr RNA (Connell et al. 2003a). The most widely distributed one between *C. jejuni* and *C. coli* strains is the protection of union site between the antimicrobial and the ribosome mechanism, that is mediated by the *tet(O)* gene, which codifies for protection proteins for ribosome (PPRs). These proteins join to the A site of 30SD ribosome generating a conformational change that results in the liberation of the tetracycline molecules from their action site and that is dependent on the presence of GTP (Connell et al. 2003a).

Additionally, this conformational change persists for a long period, allowing the protein elongation process to continue efficiently (Connell et al. 2003b).

tet(O) gene is codified in an autotransferable plasmid of 45–58 kb of size, its presence confers high resistance levels to tetracyclines, achieving even MIC of 512 mg/L (Gibreel et al. 2004). This plasmid may be transferred between *C. jejuni* and *C. coli* strains but cannot be transferred to *E. coli*, suggesting that it is restricted just to *Campylobacter* species. The presence of gene *tet(O)* in conjugative plasmid may play a substantial role in the dissemination of resistance against these antibiotic and in the high resistance rates reported (Wieczorek and Osek 2013). The presence of this gene has been reported at chromosomal level, it has been detected in 76 % of *C. jejuni* tetracycline-resistant strains that did not present plasmids (Pratt and Korolik 2005). Considering the guanine and cytosine content (G + C), sequence homology, codons use, and hybridization analysis, it is postulated that the *Campylobacter* gene *tet(O)* was probably acquired by horizontal transfer from *Streptomyces, Streptococcus* or *Enterococcus* spp. (Batchelor et al. 2004). There is a 75 % homology between the *tet(O)* gene present in *Campylobacter* and the *tet(M)* present in *Streptococcus pneumoniae,* as well as a relation %G + C of 40 % (Wieczorek and Osek 2013). Tetracycline resistance in *Campylobacter* strains isolated from different origins is widely spread, and in *Campylobacter* strains isolated from broiler poultry the resistance rates reported in different countries is over 50 %.

10.3.5 Aminoglycoside Resistance Determinants

The aminoglycosides are a group of antibiotics of natural origin, most of them with a bactericidal effect. Its action mechanism is based in the union to the site A of the 30S subunit of the bacterial ribosome; this interaction leads to the production of aberrant proteins since it interferes in the precise recognition between codon and anticodon. Also, a disruption in the protein elongation is produced by the inhibition of the translocation of tRNA at the P site of the bacterial ribosome (Jana and Deb 2006). Resistance to aminoglycosides present in *Campylobacter* is produced because of the synthesis of proteins that modify and inactivate these antibiotics. In

this species, three enzymes that can inactivate aminoglycosides have been described: aminoglycoside phosphotransferase I, III, IV and VII, aminoglycoside adeniltrasferase, and 6 aminoglycoside adeniltrasferase (Zhang and Plummer 2008). The genes that codify for these enzymes are widely distributed, both in Gram negative as in Gram positive bacteria. The enzymatic modification produced in aminoglycosides generates a decrease in its affinity for the ribosomal A site, due to the fact that these enzymes stimulate the synthesis of 30-O-aminoglyzoside phosphotransferase (Aarestrup and Engberg 2001).

Resistance to gentamicin is the most studied one. There are different genes and mechanisms that confer this resistance, including: the presence of the *aacAr* gen in *C. jejuni* (Lee et al. 2002), the presence of *aph (2″)-If* gene and of plasmids that take the *aacA/aphD* genes and a genomic island found in *C. coli* that present the *aac (6′)-IeI-aph (2″)-Ia* genes (Qin et al. 2012; Toth et al. 2013). A 2012 Chinese publication reports high resistance levels to gentamicin in *C.coli* strains isolated from production animals in China. When the strains analysis is done, a high prevalence of isolates with a new genomic island that presents resistance genes to multiple antibiotics of the aminoglycoside family was found (Qin et al. 2012). This contrasts with most of the publications and reports of the resistance surveillance programs, where generally the aminoglycoside resistance rates for broiler poultry strains are close to zero. Low gentamicin resistance prevalence might be due to the small use of this antimicrobial in animal production, since its administration is injectable and oral administered antimicrobials are preferred.

10.3.6 β-Lactamic Resistance Determinants

β-lactamics are a group of antibiotics that inhibit the bacterial cell wall biosynthesis. This group includes: penicillins, cephalosporins, carbapenems and monobactams. Several resistance mechanisms to β–lactamics have been characterized, including the expression of new proteins that join the penicillin, mutations in porins and the efflux pumps production. In Gram negative bacteria, the most extended mechanism is the production of enzymes that breaks the β–lactamic ring (β-lactamases). Since the use of antimicrobials of this family is not usual for the treatment of campylobacteriosis, there is little information about the resistance mechanisms presented in these strains. The expression of β-lactamases in *Campylobacter* strains confers resistance to amoxicillin, ampicillin, and ticarcillin that might be counteracted by tazobactam, clavulanic acid and sulbactam. These enzymes do not affect the susceptibility to carbapenem or cephalosporins (Lovine 2013). Recently, the OXA61 β-lacatamase has been identified in *C. jejuni* (Alfredson and Korolik 2005; Griggs et al. 2009). This enzyme confers resistance to penicillin, oxacillin, ampicillin, amoxicillin-clavulanate, piperacillin, and carbenicillin (Loveni 2013).

On the other hand, the MOMP external membrane protein does not allow the entrance of antibiotics with a molecular weight over 360 MW or that are not anionic, so some drugs of the β-lactamic family cannot make their action and the

strains present an intrinsic resistance to them. The CmeABC efflux pump might also contribute to the resistance to β-lactamics in *Campylobacter*. This antimicrobial family is used in animal production; nevertheless, *Campylobacte*r strains isolated from broiler poultry present very low resistance rates, so this might represent a treatment option for patients with multiresistant strains.

10.3.7 Transmission of Resistance Determinants

Mutation events are the principal factors responsible for the development of resistance to antimicrobials in *Campylobacter* strains. Nevertheless, the acquisition of antimicrobial resistance determinants by gene horizontal transfer (GHT) might play an important role in the dissemination of some genes. Some of the resistance determinants might be acquired by transduction, transformation, and conjugation (Jeon et al. 2008). In this sense, conjugation process plays a key role in the resistance to tetracyclines, through the transfer of *tet(O)* gene through plasmids (Luangtongkum et al. 2009).

Natural transformation might be important in the transmission and dissemination of resistance determinants against fluoroquinolones between different populations, strains, and species. Nevertheless, it is suggested that this phenomena has not contributed significantly to the emergence of resistance mutants (Jeon et al. 2008). Thanks to the use of *Campylobacte*r strains deficient on the natural transformation process, it has been demonstrated that the development of resistant mutants from a sensitive population is not widely influenced by natural transformation and is regulated principally by selection and enrichment processes of fluoroquinolone resistant mutants in a spontaneous way (Luangtongkum et al. 2009).

Several transmissible plasmids for conjugation in *Campylobacter* have been described, most conjugative plasmids have tetracycline and aminoglycoside resistant genes. Although the interspecies transfer of this kind of plasmid has been demonstrated with *Campylobacter*, this process is more relevant in the intraspecies transmission (Avrain et al. 2004; Gibreel et al. 2004; Pratt and Korolik 2005). The most frequent case of horizontal transmission of resistance in *Campylobacter* is the *tet(O)* gene. Due to the high prevalence of conjugative plasmids that have this gene, it is possible that this process has had an important role in the dissemination of resistance to tetracycline (Luangtongkum et al. 2009). The transfer of conjugative plasmids carrying the gene *tet(O)* between *C. jejuni* strains present in the intestine of poultry has been reported (Avrain et al. 2004). Oyarzabal et al. (2007) detected the transfer, through conjugation, of chromosomically codified genes that confer resistance to streptomycin from *Helicobacter pylori* to *C. jejuni*. Integrons and mobile genetic elements such as transposons and insertion sequences have an important role in the transmission and dissemination of antibiotics resistance genes, especially in Gram negative bacteria. Nevertheless, these elements seem to have no importance in the dissemination of resistance in *Campylobacter* strains (Kim et al. 2006). Only class I integrons have been detected in *C. jejuni* and *C. coli* strains,

they carry resistance genes for aminoglycosides *aadA2* and *aaA4*; integrons are not found in most of the strains of the genus *Campylobacter* (Zhang and Plummer 2008; Luangtongkum et al. 2009).

10.4 Conclusions

Antimicrobial resistance is an important public health problem that has increased with time and has a global dimension. We are all immersed in a big ecosystem and any measure that is adopted in order to control or diminish resistance emergence will have a repercussion in the ecosystem and in the health of animals and people.

The levels of resistance to fluoroquinolones reported in *Campylobacter* strains isolated from poultry and derivatives are worrying. Specifically with *Campylobacter*, there is an association between the use of fluoroquinolones in poultry industry and the high resistance levels in these strains. Also *Campylobacter* is a microorganism that has an important genomic plasticity so mutant strains might generate easily and probably keeping the drug resistance does not represent an important fitness for the bacteria, so in free antibiotic environments the resistance might persist. So, the emergence and dissemination of resistance to fluoroquinolones in *Campylobacte*r in animal reservoirs are influenced by multiple factors, including the antimicrobial treatments realized and the environment where productive systems are developed. More studies have to be done and more control measures must be implemented in all countries to control the emergence of resistance in zoonotic pathogens, in order to protect public and animal health.

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Chapter 11
Non-*jejuni/coli Campylobacter* Species and Related Organisms in Poultry, and Their Relevance in Human and Animal Disease

Albert Joseph Lastovica

Abstract This chapter provides a survey of research on non-*jejuni/coli Campylobacter* spp. and related *Arcobacter* and *Helicobacter* species in poultry. The isolation and identification, clinical and veterinary relevance, and the pathogenicity and antibiotic sensitivity of the organisms are reviewed. Most studies done on poultry, especially chicken, have focused on *C. jejuni* and *C. coli.* Increasingly, other species, such as *C. lari*, *C. upsaliensis*, and *C. avium*, as well as species of the related genera *Arcobacter* and *Helicobacter* (for example, *A. butzleri* and *H. pullorum*), are being found in poultry. These fastidious bacteria may be undetected, or under detected, due to unsatisfactory isolation procedures. Investigations to understand the role of the non-*jejuni/coli Campylobacter* spp. in poultry infection and their disease potential in humans and animals are still on-going.

Keywords Poultry · Non-*jejuni/coli Campylobacter* · *Arcobacter* · *Helicobacter*

11.1 Introduction

Poultry (broilers, laying hens, ducks, turkeys, quails and ostriches) can easily become colonized by *Campylobacter* spp. (Robino et al. 2010). Most research on *Campylobacter* in poultry has been done on *C. jejuni* or *C. coli* in chickens. Chicken is a major human food and the handling and consumption of chicken is a significant source for human *Campylobacter* infection. *C. jejuni* and *C. coli* are ubiquitous in the environment. They have recognized virulence properties, and can be grown relatively easily in laboratories (Lastovica et al. 2014). Other emerging

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Campylobacter spp., such as *C. upsaliensis* and *C. concisus* as well as species of *Arcobacter* and *Helicobacter* have fastidious growth requirements and are usually under isolated, or not isolated, due to lack of appropriate growth conditions. The pathogenic potential of these organisms in human and animal health is increasingly being recognized.

11.2 Non-*jejuni/coli Campylobacter* Species

Campylobacteraceae is the largest and most diverse family in the phylogenetically distinct class Epsilonproteobacteria, which is comprised of the genera *Campylobacter* (33 taxa), *Arcobacter* (21 taxa), and *Sulfospirillum* (7 taxa) (Lastovica et al. 2014) These bacteria are Gram-negative, nonsaccharolytic, non-spore forming, with a low G+C content. Individual species are able to grow in microaerobic, anaerobic, and/or aerobic conditions at temperatures from 25 to 42 °C. They can be free-living, commensal, or pathogenic, motile or aflagellate. Members of the *Campylobacteraceae* are curved, spiral or S-shaped rods that are 0.2 to 0.9 μm wide and 0.5 to 5.0 μm long (Fig. 11.1). Most research done on *Campylobacter* spp. has been restricted to *C. jejuni* and *C. coli.* There have been only a few studies on non-*jejuni/coli Campylobacter* spp. and the related species of *Arcobacter* and *Helicobacter* in chicken (Lynch et al. 2011; Manfreda et al. 2011; Kaakoush et al. 2014). The role of these species in human and animal diseases is not fully understood (Man 2011; Lastovica et al. 2014).

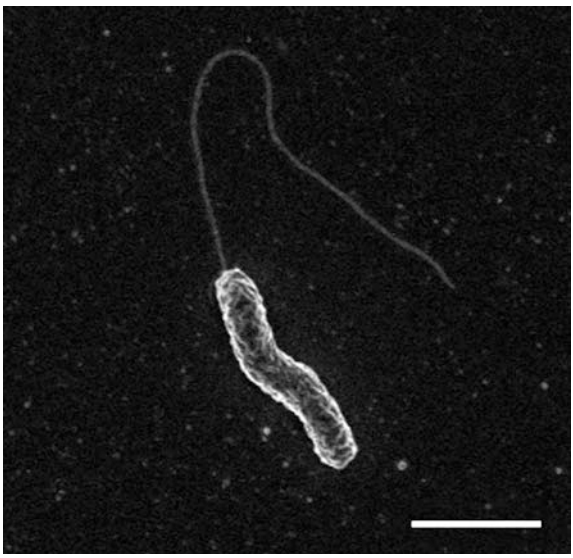

Fig. 11.1 Electron micrograph of *Campylobacter concisus*. Bar marker = 1.0 μ. Courtesy of Dr. L. Zhang, University of New South Wales, Sydney Australia

11.2.1 Isolation and Identification

In a recent study of 176 clinical laboratories testing for *Campylobacter* spp., most used culture-based methods. Lack of standardized procedures may possibly result in under or missed diagnoses (M'ikanatha et al. 2012). Enrichment media for *Campylobacter* use selective antimicrobials such as vancomycin, cefoperazone, and cyclohexamide. However, some *Campylobacter* species may be sensitive to the antibiotics present in selective media, or have special atmospheric or temperature requirements. The Cape Town Protocol (Lastovica 2006) overcomes these difficulties by using membrane filtration onto antibiotic-free Tryptose Blood agar plates incubated in a hydrogen-enriched atmosphere at 37 °C. Essentially all known species of *Campylobacter*, *Helicobacter*, and *Arcobacter* can be isolated by this protocol. The Cape Town Protocol has been successfully used by several laboratories to isolate *Campylobacter*, *Arcobacter*, and *Helicobacter* spp. from human, chicken and gull stools, human blood, and human biopsy samples (Lastovica and Le Roux 2003; Lastovica 2006; Kinzelman et al. 2008; Jacob et al. 2011). Fastidious non-*jejuni/coli Campylobacter* spp., *Helicobacter*, and *Arcobacter* species may be under isolated or missed entirely when isolation protocols specifically designed for robust *C. jejuni/coli* isolates are used. *Campylobacter jejuni* and *C. coli* isolates tend to be vigorous growers and appear on a culture plate usually by day two or three of incubation, while the more fastidious non-*jejuni/coli Campylobacter* spp., *Arcobacter* and *Helicobacter* strains usually appear days later. In a study of human enteric and oral isolates of *C. concisus*, Lee et al. (2014) observed that the presence of hydrogen greatly increased the growth of *C. concisus*. It would be a consideration for a diagnostic microbiology laboratory to increase the incubation time of primary isolation plates to detect slower growing strains. Mixed infections of *Campylobacter* and related organisms in chickens have been detected by culture (Lastovica et al. 2014), and by molecular methods (Wainø et al. 2003; Robino et al. 2010).

In a 17-year survey (Lastovica and Allos 2008; Lastovica unpublished data) of the prevalence of *Campylobacter* in the diarrhetic stools of South African pediatric patients, 18 % of them had coinfections of two to five species of *Campylobacter*, *Arcobacter*, and/or *Helicobacter*. The same study indicated that ~35 % of the isolates were *C. jejuni/coli*, the rest were other *Campylobacter*, *Arcobacter*, or *Helicobacter* species (Table 11.1).

A number of species-specific PCR assays are available for the detection of non-*jejuni/coli Campylobacter* spp. The PCR-RFLP assay developed by Kamei et al. (2014) can detect and differentiate *C. jejuni*, *C. coli*, *C. fetus*, *C. hyointestinalis*, *C. lari*, *C. helveticus*, and *C. upsaliensis.* Chaban et al. (2010) utilized a quantitative PCR (qPCR) assay that they developed based on the *cpn60* gene. The lower detection limit of this assay is 10^3 copies/g of feces. These researchers were able to detect in addition to *C. jejuni* and *C. coli*, 12 additional *Campylobacter* species. Miller et al. (2005) developed a multilocus sequence typing (MLST) system for the

Table 11.1 Distribution of *Campylobacter* and related species isolated from diarrhetic stools of pediatric patients at the Red Cross Children's Hospital, Cape Town, South Africa, October 1, 1990 to September 30, 2007[a]

Species or subspecies	No.	%
C. jejuni subsp. *jejuni*	1985	32.66
C. concisus	1526	25.19
C. upsaliensis	1435	23.56
C. jejuni subsp. *doylei*	438	6.38
H. fennelliae	339	5.62
C. coli	190	3.15
C. hyointestinalis	57	0.94
H. cinaedi	51	0.74
CLO/HLO[b]	31	0.51
A. butzleri	21	0.34
C. fetus subsp. *fetus*	9	0.15
C. curvus/C. rectus/H. rappini	9	0.15
C. sputorum biovar *sputorum/C. lari*	7	0.12
Total	6098	100.00

[a]Data from Lastovica and Allos (2008) and unpublished results
[b]CLO/HLO *Campylobacter* or *Helicobacter* organisms that could not be fully characterized

detection of *C. coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus*. MLST will prove useful in differentiating strains of *Campylobacter* spp., identifying mixed cultures and detecting genetic exchange within the genus.

11.2.2 Habitat

Similar to *C. jejuni/coli*, other *Campylobacter* spp. are able to colonize the oral cavity, intestine, stomach, and reproductive tracts of humans, large animals (cattle, sheep, deer), small animals, birds, and reptiles. Non-*jejuni/coli Campylobacter* spp. have been isolated from water and soil (Lastovica and Allos 2008).

11.2.3 Poultry

In an examination of fastidious *Campylobacter* spp. in fresh meat and poultry, Lynch et al. (2011) used a modification of the Cape Town Protocol (Lastovica 2006) to isolate, and then identify by genus and species-specific PCR assays, and by biochemical testing. *C. jejuni/coli* as well as the fastidious *Campylobacter* spp., *C. concisus*, and *C. mucosalis* were identified. These species, plus *C. curvus*, *C. sputorum*, and *C. upsaliensis* were also identified from minced/ground beef. These authors confirm that the diversity of *Campylobacter* spp. in meat for human consumption is greater than previously reported. Kaakoush et al. (2014) studied the

fecal microbiota of 31 market-age (56-day old) broiler chickens, from two different farms, and samples were analyzed using high-throughput sequencing. The samples were then screened for two emerging human pathogens, *C. concisus* and *H. pullorum*, using species-specific PCR. The prevalence of *Campylobacter*, and *Helicobacter* was also determined in the 31 chicken samples using pyrosequencing data. *C. jejuni* subsp. *jejuni* was detected in 41.9 %, *C. jejuni* subsp. *doylei* in 61.3 %, *C. concisus* in 6.4 %, *C. upsaliensis* in 9.7 %, *H. pullorum* in 22.6 %, and *H. brantae* in 64.5 % of chicken samples. Rossi et al. (2009) isolated 3 strains of *C. avium* sp. nov. from the cecal contents of broiler chickens and a turkey. There have been no subsequent reports of this *Campylobacter* spp.

11.2.4 Clinical Relevance

Over a 17-year period from 1977–1995, examination of 6098 pediatric diarrhetic stools in Cape Town, South Africa, indicated that *C. jejuni* subsp. *jejuni* and *C. coli* formed 35.3 % of the isolates, The remainder were non-*jejuni/coli Campylobacter* spp., *Arcobacter*, or *Helicobacter* (Table 11.1). Isolation of these fastidious species was only possible with the introduction of the Cape Town Protocol (Lastovica 2006). These organisms were not detected previously when using antibiotic-containing selective media (Lastovica and Allos 2008).

C. jejuni subsp. *doylei* causes gastritis in humans, but this species is more commonly isolated from human blood cultures than stool cultures. In this study of pediatric patients, *C. jejuni* subsp. *doylei* was found in 6.4 % of diarrhetic stools (Table 11.1), but occurred in 24 % of the blood cultures (Lastovica et al. 1996; Lastovica et al. 2002). Morey (1996) reported that *C. jejuni* subsp. *doylei* was isolated from 85.2 % of *Campylobacter*/*Helicobacter*-related bacteremia cases in Australia over a five-year period.

C. concisus and *C. upsaliensis* were detected by Kaakoush et al. (2014) in chicken feces and processed chicken meat. This suggests that chickens may serve as a reservoir for these species. *C. upsaliensis* has been isolated from the blood cultures of pediatric patients (Lastovica et al. 1989), and has been associated with persistent bloody diarrhea (Couturier et al. 2012) and fatal sepsis (Nakamura et al. 2015). Gurgan and Diker (1994) documented the isolation of *C. upsaliensis* in the blood and fetoplacental specimens of a woman experiencing spontaneous abortion at 18 weeks gestation. A high prevalence of *C. concisus* and *C. ureolyticus* in biopsy samples taken from adults with ulcerative colitis suggests that they contribute to the disease (Mukhopadhya et al. 2011). *C. lari* has been linked to enteritis and septicemia. Additional details of the clinical features of other non-*jejuni/coli Campylobacter* spp. are detailed in Lastovica and Allos (2008).

11.2.5 Veterinary Relevance

C. upsaliensis and other non-j*ejuni/coli Campylobacter* spp. have been associated with diarrhetic dogs. Chaban et al. (2010) by means of PCR assays examined 70 healthy and 65 diarrhetic dogs. It was found that 58 % of the healthy and 97 % of the diarrhetic dogs shed detectable levels of non-*jejuni/coli Campylobacter* spp. Healthy dogs had 0–7 detectable *Campylobacter* spp. and diarrhetic dogs had 1–12 detectable *Campylobacter* species. Lawson et al. (1988) presented serological data on the association of *C. mucosalis* and *C. hyointestinalis* with proliferative enteropathy in pigs.

11.2.6 Pathogenicity

Sylvester et al. (1996) reported that *C. upsaliensis* bound in a concentration-dependent fashion to purified human small intestine mucin. A lipid-silica affinity column detected at least five bacterial surface proteins (50 to 90 kDa) capable of binding phosphatidylethanolamine. These researchers speculate *C. upsaliensis* expresses a specific mucin epitope(s). Mucin binding could influence access of the bacteria to cell membrane receptors and thus influence host resistance to infection. Mooney et al. (2003) demonstrated invasion of human epithelial cells by *C. upsaliensis* using confocal, immunofluorescence, and transmission electron microscopy. *C. upsaliensis* is capable of invading epithelial cells and interacts with the cytoskeletal structures of host cells in order to gain entry. Cytolethal distending toxins (CDTs) cause damage to the DNA of mammalian cells. CDTs are produced by Gram-negative bacteria including *Campylobacter* and *Helicobacter* species. The CdtA and CdtC subunits of CDT are required to facilitate cell surface binding to target cells to allow internalization of the active CdtB subunit that is functionally homologous to mammalian deoxyribonuclease. This toxin is translocated into the nuclear compartment to exert its toxic action. The toxin induces DNA damage and the disruption of DNA damage responses. This results in the blocking of the target cells in the G1 and/or G2 phases of the cell cycle and the initiation of DNA repair mechanisms. Cells that fail to repair damaged DNA will experience apoptotic cell death. A recent review by DiRienzo (2014) provides detailed information on CDT. Mooney et al. (2001) demonstrated that *C. upsaliensis* whole-cell preparations and extracts produce a cytolethal distending toxin (CDT)-like effect on HeLa cells resulting in progressive distention, nuclear fragmentation, and cell death. Additional experimentation demonstrated that when HeLa cells and human T lymphocytes cells were treated with *C. upsaliensis* lysate, cell division arrest occurred in G1 or G2 phases. Fouts et al. (2005) undertook a sequencing and comparative analysis study of a clinical isolate of *C. upsaliensis* RM3195. Sequence analysis revealed a putative virulence *licABCD* locus with partial, but significant, similarity to genes present in *Haemophilus influenzae* (Weiser et al. 1989) and commensal *Neisseria* species

(Serino and Virji 2002). *LicABCD* genes in these microorganisms encode proteins involved in the acquisition of choline (*licB*), synthesis of phosphorylcholine (PCho) (*licA*), (*licC*), and transfer of PCho (*licD*) to lipopolysaccharide or teichoic/lipoteichoic acids to facilitate attachment to host cells (Fouts et al. 2005). Parker et al. (2007) in a comparative molecular analysis of *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei* observed the total absence of the virulence gene *cdtA* and near total absence of *cdtB* among the *C. jejuni* subsp. *doylei* strains. As these *cdt* genes encode subunits of the CDT, this indicates that *C. jejuni* subsp. *doylei* strains do not produce this toxin. Samosornsuk et al. (2015) have demonstrated a new variant of CDT in a clinical isolate of *C. hyointestinalis* isolated from pigs by sequencing a 13,965 bp genomic region which codes for the three subunits of CDT: CdtA, CdtB, and CdtC. Amino acid sequence data of CdtA of *C. hyointestinalis* showed ~39 % homology with the CdtA of *C. coli.* Sequences of CdtB and CdtC of *C. hyointestinalis* were homologous to CdtB (65.7 %) and CdtC (33.1 %) of *C. upsaliensis*. Filter-sterilized sonic lysate of *C. hyointestinalis* demonstrated distention and cell death arresting the cell cycle at the G2/M phase.

11.2.7 Antibiotic Sensitivity

Forty-one strains of *C. upsaliensis* were tested against 24 antimicrobial agents with the use of a broth microdilution assay. Most isolates were susceptible to the fluoroquinolones and β-lactams tested. All strains were resistant to trimethoprim and teicoplanin (Preston et al. 1990).

Fouts et al. (2005) present antibiotic sensitivity data on a strain of *C. upsaliensis* that was found to be sensitive to cefoperazone, erythromycin, and ciprofloxacin, but resistant to oxytetracycline. Scanlon et al. (2013) examined the cecal contents (n = 402) and carcass swabs (n = 401) of pigs. While *C. coli* (37 %) was the most common isolate, other *Campylobacter* spp. were also isolated—*C. concisus* (10 %), *A. butzleri* (8 %), *C. helveticus* (8 %), *C. mucosalis* (6 %), *A. cryaerophilus* (3 %), *C. fetus* subsp. *fetus* (1 %), *C. lari* (0.5 %), and *C. curvus* (0.5 %). All the non-*jejuni/coli Campylobacter* spp. were sensitive to ciprofloxacin. The level of resistance to erythromycin was up to 100 % in *C. concisus* and *C. helveticus.* This is of concern, as erythromycin is the drug of choice in the treatment of severe gastroenteric *Campylobacter* infections. The study of Scanlon et al. (2013) shows that there is a much wider range of *Campylobacteraceae* present in porcine samples than previously assumed.

11.3 *Helicobacter*

Helicobacter is a genus of the family *Helicobacteraceae* that is a member of the class Epsilonproteobacteria. These Gram-negative bacteria possess a characteristic helical shape. They were initially considered members of the *Campylobacter* genus,

and were known as "*Campylobacter pylori*". Subsequently they were reclassified into a new genus, *Helicobacter*, as *Helicobacter pylori* (Goodwin et al. 1989). The *Helicobacter* genus currently contains 33 recognized species, as detailed by Mitchell et al. (2014) in their comprehensive summary of the *Helicobacteraceae* family. The most well studied (and the type strain) of *Helicobacter* is *H. pylori*. This organism is a cause of peptic ulcers, gastric cancer, duodenitis, and other diseases in man. A recent review article by Mégraud et al. (2015) summarizes aspects of the pathogenicity of *H. pylori.*

11.3.1 Isolation and Identification

Isolation of *Helicobacter* spp. is similar to that of non-*jejuni/coli Campylobacter* spp. The isolation of *H. pullorum* from chicken intestine, feces, and other specimens, using filtration and incubation on blood agar plates in a hydrogen-enriched atmosphere as recommended by the Cape Town Protocol (Lastovica 2006), was successfully done by Ceelen et al. (2006), and Borges et al. (2015). Stanley et al. (1994) developed a species-specific *H. pullorum* PCR assay. A novel real-time PCR assay has been developed for the direct detection of *H. pullorum* in food. The assay was specific, reproducible, with a detection limit of one colony-forming unit (CFU)/g (González et al. 2008). Various researchers have used PCR assays to detect *H. pullorum* (Ceelen et al. 2006; Manfreda et al. 2011; Borges et al. 2015).

11.3.2 Habitat

Helicobacter species are specialized bacteria that have adapted to the ecological niche presented by mucus. *H. pylori*, *H. mustelae*, and *H. felis* have a predilection for the human gastroenteric tract. Other *Helicobacter* spp. such as *H. fennelliae*, *H. cinaedi*, *H. canadensis*, and *H. pullorum* may be isolated from intestine, the hepatobiliary system, or, the blood of humans (Smuts and Lastovica 2011; Mateos-Muñoz et al. 2013). *Helicobacter* species have been isolated from the intestinal tract of a variety of animals—*H. pullorum* from chicken, *H. cetorum* from whales and dolphins, *H. suis* from swine, *H. bilis* from mice, *H. canis* from dogs, *H. cinaedi* from humans and hamsters, and *H. equorum* from horses (Mitchell et al. 2014). Sasaki et al. (1999) detected *H. pylori*-specific DNA from water, field soil, flies, and cow feces by using nested PCR assay. These reservoirs may also harbor other *Helicobacter* spp. such as *H. pullorum.*

11.3.3 Poultry

Helicobacter pullorum is currently the only recognized *Helicobacter* spp. commonly isolated from chicken. *H. pullorum* is a nongastric urease-negative *Helicobacter* species colonizing the lower bowel in humans. A detailed description of *H. pullorum* is given in Mitchell et al. (2014). Stanley et al. (1994) were the first to describe *H. pullorum* as a new species. They isolated it from the duodenum, cecum, and liver of broiler and layer chickens with hepatitis and enteritis, and from human patients with gastroenteritis and liver disease. On the basis of DNA homology studies, 16S rRNA analysis, biochemical and electron microscopic observations, these strains were identified as belonging to an unnamed species of *Helicobacter* for which they proposed the name *H. pullorum* sp. nov. Nebbia et al. (2007) investigated the presence of enteric *Helicobacter* spp. in poultry (n = 130) by PCR sequencing. About 80 % of the chickens, laying hens, and guinea fowl tested were positive for *Helicobacter* DNA. *H. pullorum* was the most frequently identified (62.1 %) *Helicobacter* species. *H. pullorum* may infect the intestinal tracts of various avian species. It has been detected in the intestinal contents and livers of broiler chickens, and laying hens (Atabay et al. 1998; Ceelen et al. 2006; Zanoni et al. 2011). *H. pullorum* has also been found in turkey (Zanoni et al. 2011) and in a psittacine bird (Ceelen et al. 2006). Manfreda et al. (2011) studied the prevalence of *H. pullorum* isolated from the ceca of chickens from 34 conventionally reared flocks raised in poultry houses, eight organic flocks, and seven flocks of free-range chickens. PCR assays identified *H. pullorum* in 93 % of the farms tested when the ceca of healthy broilers were examined. The free-range chickens were 54.2 % *H. pullorum*-positive, while both the conventional and organic farms were 100 % positive. A high level of genetic variability was noted in isolates from *H. pullorum*-positive chickens. Gibson et al. (1999) examined 13 human and seven poultry isolates of *H. pullorum* (confirmed by a species-specific PCR) from four countries by two fingerprinting techniques: amplified fragment length polymorphism (AFLP) and pulsed field gel electrophoresis. Both human and poultry isolates had distinct genotypes, and most strains showed a high degree of genetic diversity. Ceelen et al. (2007) inoculated by gavage, four groups of one-day-old broiler chicks with different *Helicobacter* strains. Fecal samples were collected and analyzed for *H. pullorum* DNA by PCR assay. The chicks were euthanized and the liver and intestinal tracts were examined histologically and bacteriologically. These researchers detected *H. pullorum* DNA in fecal samples until 42 days post inoculation, and found that the bacterium was closely associated with cecal epithelial cells. This implies that chicken meat could constitute an infective source for humans. Borges et al. (2015) using membrane filtration, isolated four strains of *H. pullorum* from 17 samples of chicken meat. From these observations, they suggested that *H. pullorum* could be transmitted to humans by the consumption of chicken meat.

11.3.4 Clinical Relevance

H. pullorum has been isolated from stool samples of humans with gastroenteritis (Stanley et al. 1994; Steinbrueckner et al. 1997; Young et al. 2000). *H. pullorum* DNA was isolated from the biliary trees and gallbladders of patients suffering from chronic cholecystitis by Karagin et al. (2010). *H. pullorum* DNA was isolated from the livers of patients suffering from autoimmune liver disease, acute liver failure, and hepatocellular carcinoma by Stanley et al. (1994), Nilsson et al. (2003), Pellicano et al. (2004), Casswall et al. (2010). A case of bacteremia in a 35-year-old man with pyrexia was reported by Tee et al. (2001). 16S rRNA gene sequencing identified the organism as *H. pullorum*-like.

11.3.5 Veterinary Relevance

Stanley et al. (1994) described *H. pullorum* that was isolated from the livers of chickens with vibrionic hepatitis and enteritis. Ceelen et al. (2007) inoculated one-day-old broiler chickens with *H. pullorum* strains isolated from humans or poultry. Mild lesions in the ceca were present in broilers. Immunohistochemical examination revealed that the bacterium was closely associated with cecal epithelial cells. Cacioppo et al. (2012) observed that Brown Norway rats were persistently colonized by *H. pullorum* and had a sustained *H. pullorum*-specific IgG response measured by ELISA. Cacioppo et al. (2012) did not find intestinal or hepatic pathology associated with *H. pullorum*.

11.3.6 Pathogenicity

Ceelen et al. (2006) examined 10 poultry and three human isolates of *H. pullorum* for the presence and activity of CDT. A PCR assay was used to detect the *cdtB* gene, and, as well, Hep-2 cells were inoculated with sonicates of all strains and observed microscopically. Their conclusion was that all the *H. pullorum* isolates examined, possessed the *cdtB* gene but functional CDT activity was only detected in one of the human strains. Varon et al. (2014) presented data on the activity of CDT from 10 strains (5 avian and 5 human) of *H. pullorum* on the human colonic cell lines Caco-2 and HCA-7 in coculture experiments. All *H. pullorum* strains induced morphological changes in the Caco-2 and HCA-7 cells that were enlarged with multiple or distended nuclei after 72 h in coculture. Cell cycle analysis indicated an increase in the percentage of cells in the G2/M phase. The distended phenotype was also observed after treatment with a filtered bacterial culture supernatant. The presence of the *cdtB* gene in the 10 *H. pullorum* strains was confirmed by sequencing. *H. pullorum cdtB* was also responsible for a dramatic remodeling of the actin cytoskeleton to form actin-rich, large lamellipodia, and

decreased cellular adherence. These researchers conclude that the CDT is a major virulence factor of *H. pullorum.*

By using adherence, gentamicin protection assays, and scanning electron microscopy, Sirianni et al. (2013) demonstrated that *H. pullorum* could adhere to host cells through flagellum–microvillus interaction. Proteomics coupled with mass spectrometry characterized the secretome of *H. pullorum.* Functional classification revealed six putative virulence and colonization factors: cell binding factor 2, flagellin, secreted protein Hcp, valine-glycine repeat protein G, a type VI secretion protein, and a protease. Additional observations suggested the type VI secretion system of *H. pullorum* might interact with endocytic vesicles and secrete pathogenic factors. Borges et al. (2015) used whole genomic sequencing and comparative genomics on 4 strains of *H. pullorum* isolated from chicken meat. They found 18 highly polymorphic genes, plasmids, prophages, and a complete type VI secretion system. This secretion system was found in 3 of 4 isolates, suggestive of a pathogenic role. Guidi et al. (2013) suggested that chronic infection by CDT-producing bacteria might promote genetic instability and an altered DNA damage response, possibly leading to malignant transformation and cancer.

11.3.7 Antibiotic Resistance

Bascuñana et al. (2011) tested five *H. pullorum* isolates and found that they were all resistant to tetracycline, ciprofloxacin and levofloxacin, but sensitive to amoxicillin, erythromycin, and gentamicin. Pasquali et al. (2007) sequenced the 2490 bp *gyrA* gene of *H. pullorum* strain CIP 104787T. The nucleotide sequences of the quinolone resistance-determining regions (QRDRs) of nine isolates either sensitive or resistant to ciprofloxacin were compared. All ciprofloxacin-resistant poultry isolates tested showed an ACA → ATA (Thr → lle) substitution at codon 84 of *gyrA*. This substitution was functionally confirmed to be associated with the ciprofloxacin-resistant phenotype of poultry isolates.

11.4 *Arcobacter*

Arcobacter spp. are spiral-shaped, nonspore forming, Gram-negative organisms belonging to the family *Campylobacteraceae*. These bacteria possess a characteristic helical shape and were initially considered to be atypical (aerobic) members of the *Campylobacter* genus. They were then known as "*Campylobacter cryaerophila*" but were later reclassified as *Arcobacter cryaerophila* in the new genus, *Arcobacter*, by Vandamme et al. (1991). Two recent review articles provide an overview of the current knowledge on *Arcobacter* (Collado and Figueras 2011; Lastovica et al. 2014). *Arcobacter* are morphologically similar to *Campylobacter* and *Helicobacter*, but unlike them, *Arcobacter* grow in aerobic or microaerophilic

conditions and at a lower temperature. At present, 21 *Arcobacter* species are recognized or proposed. *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. thereius*, and *A. cibarius* are considered the only *Arcobacter* spp. of relevance for animals and humans (Collado and Figueras 2011; Lastovica et al. 2014).

11.4.1 Isolation and Identification

Fallas-Padilla et al. (2014) tested six different methods for isolating *Arcobacter* by using combinations of enrichments in de Boer or Houf selective broths with subsequent isolation on blood agar (directly or with a preceding filtration step), or on *Arcobacter* selective agar. Suspect colonies were identified with a genus-specific PCR. Species-level identification was obtained with a multiplex PCR. For *Arcobacter* isolation, enrichment in Houf selective broth followed by filtration on blood agar showed the best performance with a sensitivity of 89 % and a specificity of 84 %.

A rapid and accurate test for preliminary identification of *Arcobacter*, *Campylobacter*, and *Helicobacter* species is the Oxoid Biochemical Identification System (O.B.I.S.) that differentiates bacteria of the *Campylobacteraceae* family and *Helicobacter* species from all other Gram-negative and Gram-positive bacteria (Hoosain and Lastovica 2009). González et al. (2014) developed an *Arcobacter* genus-specific PCR assay designed to amplify an 85 bp DNA fragment on the 16S rRNA gene. A PCR assay using species-specific primers has been developed for the detection of *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, and *A. cibarius* in chicken meat (Pentimalli et al. 2009). Levican and Figueras (2013) compared the performance of five PCR methods that target regions of 16S rRNA, 23S rRNA, or *gyrA* genes to identify *Arcobacter* species. Ninety-five type, reference and field strains of *Arcobacter* were tested with all five PCR methods. They conclude that the five tested methods were not reliable and misidentified between 16.8 and 67.4 % of the studied strains. The PCR assays with the least misidentification were the methods of Figueras et al. (2008) and that of Douidah et al. (2010). Quiñones et al. (2007) used a different approach for the detection of *Arcobacter* spp. and *Campylobacter* spp. from chicken samples. These researchers used DNA oligonucleotide arrays to determine simultaneously the presence of *Arcobacter* and *Campylobacter* in retail chicken samples. Probes were selected that target housekeeping and virulence-associated genes in both *Arcobacter* and *Campylobacter*. Specific identification of *A. butzleri* and *C. jejuni* was achieved without the need for a PCR amplification step.

11.4.2 Habitat

Arcobacter spp. have been identified in the intestines and feces of animals (poultry, cattle, pigs, sheep, rabbits), in foods derived from these animals, and from food

processing environments. *Arcobacter* has also been isolated from cheese and other dairy products, seafood, vegetables, and used dishcloths (Collado and Figueras 2011; Chavatte et al. 2014; Lastovica et al. 2014; Giacometti et al. 2015). Other sources are wildlife, zoo and pet animals, zooplankton, human blood, and human feces. They have also been found in seawater, river and lake water, wastewater, ground water, and drinking water (Lastovica et al. 2014; Hsu and Lee 2015).

11.4.3 Poultry

Fernandez et al. (2015) in their phenotypic and PRC characterization of *Arcobacter* isolates from a variety of sources in Chile, found *A. butzleri* in 10 % and *A. cryaerophilus* in 20 % of 20 chicken fecal samples. The low prevalence of *Arcobacter* species in chicken gut might be an indicator that this organism is not a natural intestinal commensal and is only a transient organism incapable of colonizing the chicken gut. A more probable interpretation of these observations is that the normal corporal temperature in chicken (40.5–42 °C) is a limiting factor for the colonization of chicken by *Arcobacter* species whose optimal growth temperature range is from 26 to 30 °C. In contrast, *Campylobacter* with its optimal growth temperature of 42 °C is recovered in high numbers from chicken gut.

Rahimi (2014) by culture and PCR assay found 28 % of 100 chicken meat samples were positive for *Arcobacter* spp., 11 % of the 60 duck samples were contaminated, as were 11 % of 100 turkey samples, 8 % of 50 geese samples and 3 % of 60 ostrich meat samples. *A. thereius* has been isolated from duck (Houf et al. 2009). Zacharow et al. (2015) examined by culture and multiplex PCR, 210 retail meat samples. They isolated 79 *A. butzleri* and six *A. cryaerophilus* from pork, beef, and chicken meat. Incidence of *A. butzleri* was highest in chicken meat (83 %) followed by beef (16 %) and pork (14 %).

The recovery of *Arcobacter* from processed poultry meat ready for human consumption is high (chicken meat 92 %, muscular stomach 32 %, chicken liver 92 %) (Fernandez et al. 2015). Poultry may be contaminated by *Arcobacter* spp. in processing plants due to the storage temperature (4 °C/or at room temperature—conditions that *Arcobacter* spp. can tolerate). *Arcobacter* species are viable for several weeks at 4 °C under aerobic conditions (Lastovica, unpublished). Khoshbakht et al. (2014) present PCR evidence for the detection of several *Arcobacter* spp. in the environment and rinse water of chicken processing plants in Iran.

11.4.4 Clinical Relevance

Arcobacter spp. have been isolated from asymptomatic humans, but *Arcobacter* may cause persistent watery diarrhea and bacteremia in susceptible humans.

Patients infected with *Arcobacter* might experience nausea, abdominal pain, chills, vomiting and fever, clinical symptoms similar to those caused by *C. jejuni* (Lastovica and Allos 2008). The transmission of *Arcobacter* spp. can occur by ingestion of food of animal origin, by direct contact with animals, by fecal–oral or waterborne routes (Collado and Figueras 2011). On et al. (1995) isolated *A. butzleri* from a neonate with bacteremia. *A. butzleri* was detected by 16S rRNA gene sequencing of the blood culture of a 69-year-old woman with acute appendicitis (Lau et al. 2002), and was also isolated from the blood culture of a 60-year-old man with liver cirrhosis and a high fever (Yan et al. 2000). Arguello et al. (2015) described a case of an 85-year-old man with fever, hypotension, and a chronic, persistent diarrhea. In this case, *A. butzleri* was identified by 16S rRNA sequencing. *A. butzleri* has been isolated from the diarrhetic stools of children in South Africa (Table 11.1). Taylor et al. (1991) described *Arcobacter* (then known as aerotolerant *Campylobacter* spp.) as the third most common organism isolated from the diarrhetic stools of 631 children in Bangkok, Thailand. Over an 8-year period, *A. butzleri* was the fourth most commonly isolated *Campylobacter*-like organism from 67,599 diarrhetic stool samples in Belgium and was frequently associated with a persistent watery diarrhea (Vandenberg et al. 2004). An outbreak of *A. butzleri* occurred in 10 Italian children, 2 to 5 years of age, all of whom had episodes of recurrent abdominal cramps, lasting up to 2 h several times a day. All the *Arcobacter* strains belonged to a single serogroup with identical protein profiles. The successive timing of the cases suggested person-to-person contact (Vandamme et al. 1992). Lappi et al. (2013) reported on an outbreak of foodborne illness among attendees at a wedding reception. Forty-seven patients and 43 healthy attendees were included in a case-control study. Eating chicken was the only factor associated with the illness. Culture did not detect common bacterial or viral pathogens. Subsequent testing with PCRs targeting the 16S rDNA/23S rDNA of several *Arcobacter* species, and the *rpoB* and *rpoC* gene of *A. butzleri*, confirmed *A. butzleri* in four patients and *A. cryaerophilus* in one.

11.4.5 Veterinary Relevance

A. skirrowii has been isolated from sheep and cattle with diarrhea, hemorrhagic colitis, and an aborted pig fetus (Ho et al. 2006). *A. butzleri* and *A. cryaerophilus* have been associated with abortion and enteritis in pigs (de Oliveira et al. 1997). *A. cryaerophilus* has been isolated from the feces and aborted fetuses of sheep and cattle, and from the milk of cows with mastitis (Neill et al. 1985). It was isolated from naturally infected rainbow trout (*Oncorhynchus mykiss* Walbaum). Experimental infection of other rainbow trout caused deaths with clinical abnormalities such as degenerated opercula and gills, hemorrhagic kidney, liver damage, and serous fluid in swollen intestines (Yildiz and Aydin 2006). *A. butzleri* has been isolated from primates with diarrhea (Anderson et al. 1993).

11.4.6 Pathogenicity

Johnson and Murano (2002) investigated *Arcobacter* isolates from poultry, cattle, irrigation water, and human diarrheal cases for the presence of CDT. A PCR assay for CDT was used, and also *Arcobacter* cell filtrates and sonic extracts were tested for CDT-like activity on Chinese hamster ovary, HeLa, and Intestinal 407 (INT407) cells in culture. No CDT amplimers were observed in any of the *Arcobacter* isolates tested. Toxicity to HeLa and INT407 cells was observed, and subsequently analyzed for cell cycle arrest in the presence of *Arcobacter* extracts by flow cytometry. No disruption of cell cycles was noted, suggesting that CDT is not expressed by *Arcobacter* which produces a toxic entity different from than that of CDT.

Karadas et al. (2013) demonstrated the presence of 10 putative virulence genes in 52 *A. butzleri* strains by PCR. The genes *ciaB*, *mviN*, *pldA*, *tlyA*, *cj1349*, and *cadF* were in all strains. The genes *irgA* (15 %), *iroE* (60 %), *hecB* (44 %), and *hecA* (13 %) were detected in only a few strains. With HT-29 cells, four of six isolates adhered and three of them invaded. All six strains tested adhered to, and invaded, Caco-2 cells. The genes *ciaB*, *cadF*, and *cj1349* of all six isolates were sequenced, but no significant changes of the amino acids in the putative functional domains were observed. These observations have shown some *A. butzleri* strains are capable of invading cell lines therefore strengthening the pathogenic potential of *Arcobacter* species. Ferreira et al. (2014) in a separate study of *A. butzleri* isolated from human and nonhuman sources observed high levels of adhesion of *A. butzleri* on Caco-2 cells. *A. butzleri* isolates were able to survive intracellularly in Caco-2 cells and induce a significant upregulation of interleukin-8 secretion and structural cell rearrangements.

11.4.7 Antibiotic/Biocide Sensitivity

Zacharow et al. (2015) tested *A. butzleri* strains isolated from chicken, beef, and pork. Most *A. butzleri* isolates were resistant to β-lactams, like ampicillin (85 %), amoxicillin with clavulonic acid (63 %), and macrolides, that is, erythromycin (62 %). All but one of the *A. cryaerophilus* isolates was susceptible to erythromycin. Almost 80 % of the *Arcobacter* isolates were susceptible to tetracycline and aminoglycosides. Multi-resistant isolates were found in 53 % *A. butzleri* strains, and 16 % of the *A. cryaerophilus* isolates. Eight *A. butzleri* isolates were resistant to all antimicrobials tested. This indicates a substantial incidence of multi-resistant foodborne *Arcobacter* in meat for human consumption. Rasmussen et al. (2013) evaluated the occurrence and persistence of *Arcobacter* spp. in a Danish broiler slaughterhouse. Of 235 swabs taken from the production line, 13.6 % of samples were positive for *A. butzleri*, 29 out of the 32 isolates originating from the evisceration machine. *A. butzleri* isolates were confirmed by PCR assay, and were typed by MLST, which indicates high strain variability. When

tested, most *Arcobacter* strains tolerated 0.5 % hypochlorite biocide that is routinely used in slaughterhouse sanitizing. Abdelbaqi et al. (2007) determined the nucleotide sequence of the *gryA* gene of *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, and *A. cibarius.* The deduced GyrA proteins are more closely related to *H. pullorum* than *Campylobacter* species. A (Thr → lle) mutation at amino acid 85 in the quinolone resistance-determining region (QRDR) was associated with ciprofloxacin resistance for two *A. butzleri* strains and one *A. cryaerophilus*-resistant strain.

11.5 Conclusion

The understanding of the epidemiology, pathogenicity, clinical, and veterinary significance of *Campylobacter* spp. other than *C. jejuni* and *C. coli* and of the related genera of *Arcobacter* and *Helicobacter* has grown dramatically in recent years. Our knowledge of the diversity, prevalence, and persistence of these microorganisms has increased substantially. A better awareness of the genomic diversity of *Campylobacter* species other than *C. jejuni/coli* is largely due to advances in molecular biology, together with the development of novel culture methodologies which has led to the detection, isolation, and comparative analysis of a variety of under-recognized, fastidious *Campylobacter* spp. including *C. upsaliensis*, *C. hyointestinalis*, *C. concisus*, and *C. ureolyticus*, as well as *Arcobacter* and *Helicobacter* species. Although there has been progress in the understanding of the pathogenic potential of these microorganisms, a great deal of additional research remains to be done. There is an increased awareness of these organisms in the food chain. Transmission of antibiotic resistant *Campylobacter* spp. and related organisms from food animals to humans requires on-going surveillance. Antimicrobial resistance continues to increase globally. Government regulatory bodies have recognized this public health concern, and the use of antimicrobials in food animals has been regulated, or even banned. Although some progress has been made, on-going research is required to fully understand the pathogenic role that non-*jejuni/coli* *Campylobacter* spp. and related organisms play in human and animal disease.

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