Handbook of
Functional
Dairy
Products

EDITED BY

Colette Shortt
John O'Brien

CRC PRESS

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FUNCTIONAL FOODS AND NUTRACEUTICALS SERIES

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**Functional Foods: Biochemical and Processing Aspects
Volume 1**
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Series Editor's Preface

The Functional Foods and Nutraceuticals Series, launched in 1998, was developed to provide a timely and comprehensive treatment of the emerging science and technology of functional foods and nutraceuticals which are shown to play a role in preventing or delaying the onset of diseases, especially chronic diseases. The first five books in the series, *Functional Foods: Biochemical and Processing Aspects*, Volumes 1 and 2; *Herbs, Botanicals and Teas; Handbook of Fermented Functional Foods*; and *Methods of Analysis for Functional Foods and Nutraceuticals* have received broad acceptance by food, nutrition and health professionals.

Functional Foods: Biochemical and Processing Aspects, Volume 1, the first volume of the series, is a bestseller, and is devoted to functional food products from oats, wheat, rice, flaxseed, mustard, fruits, vegetables, fish and dairy products. In Volume 2, the focus is on the latest developments in the chemistry, biochemistry, pharmacology, epidemiology and engineering of tocopherols and tocotrienols from oil and cereal grain, isoflavones from soybeans and soy foods, flavonoids from berries and grapes, lycopene from tomatoes, limonene from citrus, phenolic diterpenes from rosemary and sage, organosulfur constitutes from garlic, phytochemicals from Echinacea, pectin from fruit, and omega-3 fatty acids and docosahexaenoic acid from flaxseed and fish products. Volume 2 also covers solid–liquid extraction technologies for manufacturing nutraceuticals and dietary supplements. The volume *Herbs, Botanicals and Teas* provides the latest scientific and technical information on the chemical, pharmacological, epidemiological and clinical aspects of garlic, ginseng, Echinacea, ginger, fenugreek, St. John's wort, *Ginkgo biloba*, kava kava, goldenseal, saw palmetto, valerian, evening primrose, liquorice, bilberries and blueberries, and green and black teas. The book also contains chapters on international regulations and quality assurance and control for the herbal and tea industry. The volume *Methods of Analysis for Functional Foods and Nutraceuticals* presents advanced methods of analysis for carotenoids, phytoestrogens, chlorophylls, anthocyanins, amino acids, fatty acids, flavonoids, water soluble vitamins and carbohydrates. The fifth volume of the series, *Handbook of Fermented Functional Foods*, provides a comprehensive, state-of-the-art treatment of the scientific and technological information on the production of fermented foods, the microorganisms involved, the changes in composition that occur during fermentation and most importantly the effect of these foods and their active ingredients on human health.

The current volume, *Handbook of Functional Dairy Products*, edited by leading food scientists Drs. Colette Shortt and John O'Brien, addresses the most recent developments in functional dairy ingredients and products, with a clear focus on the effect of these foods and their active ingredients on human health. This book contains 14 outstanding chapters dealing with probiotic lactobacilli and bifidobacteria, lactose hydrolyzed products, trans-galactooligosaccharides as prebiotics, conjugated linoleic

acid and its antiatherogenic potential and inhibitory effects on chemically induced tumors, immuno-enhancing properties of milk components and probiotics, and calcium and iron fortification of dairy products. The potential health benefits of casein-phosphopeptides are also evaluated, and technological opportunities, safety assurance of functional dairy products, developing a dossier to support a functional food, impact of biotechnology for the nutritional enhancement of dairy foods, and communication of the benefits of functional dairy products are addressed.

The book also provides an interesting overview of the market dynamics and the drivers behind the development of functional dairy products. It is noted that there is an opportunity to develop and market dairy products, especially probiotics, that target selected age groups and people in specific disease states and those who have had their microflora compromised (irradiation patients, intestinal surgery patients, post-antibiotic treatment). This and other opportunities will become more real as our understanding of the role that intestinal bacteria play in human health improves, and as new strains of microorganisms with characteristics that make them more effective ingredients in food products are identified and fully characterized.

Drs. Shortt and O'Brien have assembled a group of outstanding international contributors in the forefront of functional dairy products, food science and technology. It is hoped that the effort will be beneficial to food, nutrition and health practitioners, and student researchers and entrepreneurs in industry, government and university laboratories.

G. Mazza, Ph.D., FCIFST
Series Editor

Preface

Milk and other dairy products were recognized as important foods as early as 4000 B.C. During the last two decades, increasing interest has been paid to identifying dairy-derived dietary components that have a measurable impact on biological processes at a physiologically realistic level and thus an impact on body function and health. The range of biologically active dairy-derived ingredients is wide and includes, for example, effects on functions related to digestion, nutrient absorption, functioning of the intestinal flora, immune defenses and effects on blood pressure. The purpose of this book is to explore developments in functional dairy ingredients and products. In addition to focusing on selected ingredients and products, the book also seeks to stimulate thought and consideration about a wide range of inter-related issues, including safety assessment, dossier development, impact of biotechnology and communication of the health benefits of ingredients and products.

An initial overview considers the market dynamics and the drivers behind the development of functional dairy products. Currently, the market for foods claiming gastrointestinal benefits is leading the functional food sector in terms of market size and activity. A wide range of bacteria is used in foods but lactic acid bacteria (LAB), such as lactobacilli and bifidobacteria, tend to predominate in the probiotic food sector. Probiotics are defined as live microbial food ingredients that are beneficial to health. Chapter 2 and Chapter 3 consider the selection, production and benefits of consumption of probiotics. Gut health-associated claims on foods are arguably the most frequently used health claims. Interest in the gut health benefits of "beneficial bacteria" began in the early 1900s and was endorsed by scientists such as Metchnikoff and Tissier at the Pasteur Institute in Europe, Shirota in Japan, and Rettger and Cheplin in the U.S. Probiotic products have dominated activities in the global gut health market and this trend is likely to continue, with the market generally segmenting into yogurts (various kinds) and single-serve delivery drinks.

Chapter 4 and Chapter 5 deal with issues related to dairy-derived carbohydrates and introduce the concept of prebiotics. The functional characteristics of dairy-derived carbohydrates are currently exploited in the development of functional products. For example, synbiotic products are increasing in market presence and contain a probiotic and a prebiotic; the latter is included to enhance viability of the probiotic and to provide additional bifidogenic potential. An estimated 75% of the world's adult population have genetically controlled limited ability to digest lactose, which can lead to gastrointestinal upsets of varying severity. Chapter 4 considers factors influencing lactose intolerance, the treatment of lactose intolerance and the development and marketing of low-lactose products. Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one bacterium or a limited number of bacteria in the colon that have the potential to improve health. The manufacture and health benefits

of prebiotic oligosaccharides and the development of second generation oligosaccharides are addressed in Chapter 5.

Milk-borne bioactive substances are increasingly shown to influence physiological processes and even to have an impact on health parameters, e.g., blood pressure, when consumed in realistic amounts. Chapter 6 looks at the formation of bioactive peptides by enzymatic and fermentation methods. It also covers their occurrence in fermented milk products and highlights the physiologically important bioactive peptides exploited in the development of functional products. Chapter 7 outlines the role of the immune system in health and disease and considers the immunity-modulating potential of milk components and probiotics and their role in maintaining health. Principally found in dairy products, conjugated linoleic acid (CLA) has been associated with the inhibition of chemically induced tumors in rats and to have antiatherogenic potential. Chapter 8 examines the scientific basis for these effects.

Chapter 9 and Chapter 10 deal with calcium and iron fortification of dairy products, respectively. In particular, Chapter 9 describes factors influencing calcium bioavailability; the generation, chemistry and physicochemical properties of casein-phosphopeptides are also addressed. The potential health benefits of caseinphospho-peptides are evaluated as well. In Chapter 10, a novel approach to the fortification of dairy products with iron is described.

Many key factors interplay in ensuring successful functional food products. Chapter 11 and Chapter 12 consider the regulatory environment and the importance of communication. Chapter 11 addresses the development of a health claim dossier for a functional dairy product from a European perspective, while Chapter 12 considers the importance of communicating the science behind the health benefits of dairy products from an American perspective. This chapter also highlights why researchers should care about communicating their research findings and the power of the media, and provides a checklist for communication. In Chapter 13, the role of biotechnology in exploiting and accentuating the activities of LAB to improve the quality and health benefits of functional dairy products is explored. In particular, biotechnological advances in the culture technology production of bioactive peptides by LAB and the exploitation of lactic cultures as cell factories for metabolite production (e.g., CLA, folate, lysine, mannitol, angiotensin converting enzyme inhibitors) are considered. The critical importance of ensuring product safety is highlighted in Chapter 14.

It is hoped that these chapters, compiled by leading international players in the arena of functional dairy ingredients and products, update and provide clear insights for those interested in functional dairy products.

Acknowledgments

Editors

John O'Brien is director of food safety at Danone Vitapole, Groupe Danone's research division, based in Paris. Previously he was a lecturer in food safety at the University of Surrey in Guildford. Dr. O'Brien has B.Sc.Dy. and Ph.D. degrees in food science and food chemistry, respectively, from University College, Cork, Ireland, and an M.Sc. in toxicology from the University of Surrey, U.K. He was founding editor of *Trends in Food Science and Technology* in 1990 and is currently European editor. Dr. O'Brien is also a member of the editorial boards of the *Italian Journal of Food Science*, *Human and Experimental Toxicology* and the *International Journal of Dairy Technology*. He is a member of the Board of Directors of International Life Sciences Institute Europe and a member of the Scientific Committee on Food Safety. He is a member of The Nutrition Society (U.K.), Institute of Biology, Society of Toxicology (U.S.), Institute of Food Technologists (U.S.) and the Society of Regulatory Toxicology and Pharmacology (U.S.). Dr. O'Brien is a fellow of the Institute of Food Science and Technology (U.K.) and of the Royal Society of Chemistry.

Colette Shortt is a food science graduate (B.Sc.Dy.) of the National University of Ireland, Cork, with postgraduate qualifications in nutrition (M.Sc., Ph.D.) and business administration (M.BA., Surrey European Management School, University of Surrey, U.K.). She is a visiting professor to the School of Biomedical Science, University of Ulster. Dr. Shortt has worked as a research nutritionist in Canada and conducted nutritional epidemiological studies while at the Rowett Research Institute, Aberdeen, Scotland. Before joining Yakult (U.K.), she was a research nutritionist with SmithKline Beecham Consumer Healthcare and was involved in the development of functional beverages. Dr. Shortt is a registered nutritionist, a member of The Nutrition Society (U.K.), the Institute of Food Science and Technology (U.K.) and The Society for Dairy Technology (U.K.). She is also a member of the Nutrition Committee of the International Life Sciences Institute Europe and is active in the International Dairy Federation.

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1 Overview of Opportunities for Health-Enhancing Functional Dairy Products

Colette Shortt, Danielle Shaw, and Giuseppe Mazza

CONTENTS

1.1 INTRODUCTION

The concept of functional foods has evolved as the role of food in the maintenance of health and well-being and in the prevention of disease has received increased scientific and commercial interest. The concept first came to light in Japan in the mid-1980s when an ageing population and rising health care costs led the Ministry of Health and Welfare to initiate the regulatory approval of functional foods (Swinbanks and O'Brien 1993). These foods are now officially recognized as foods for specified health use or FOSHU. In Europe, the International Life Sciences Institute has defined functional foods as foods that, by virtue of the presence of physiologically active components, provide a health benefit beyond basic nutrition (Diplock et al. 1999; Salminen et al. 1998). In the U.S., the Federal Food, Drug and Cosmetic Act does not provide a statutory definition of functional foods. However, the American Dietetic Association has stated that functional foods can include those foods that are whole, fortified, enriched or enhanced, while neutraceuticals are isolated components that can then be incorporated into foods to enhance health at

levels not usually obtainable from normal foods (Ross 2000). In general, it is accepted that a functional food provides a health benefit that goes beyond a general nutritional benefit.

1.2 MARKET DYNAMICS

Quantifying the value of the functional food market is a difficult task. Using a strict definition — food and drinks that make some kind of specific health claim on packaging or in advertising — the market in Europe, the U.S., Japan and Australia has been valued at \$5.7 billion (Hilliam 2000). Using a broader definition that includes a wide range of health products (not all making claims but all with the perception of functionality), a market valuation of the global functional food market has been estimated in the region of \$48 billion (Anon. 2000a). Dairy products are significant players in the functional food market; for example, they are estimated to account for approximately 60% of functional food sales in Europe (Young 2000). The U.S. functional food market is developing in a different fashion from that seen in Europe, with its functional food sector more broadly defined as neutraceuticals and consumer interest tending to lie more with botanical dietary supplements rather than fortification of foodstuffs. This is changing, however, as interest in immunity, cancer and heart health grows. The Australian market for functional foods is in its infancy; however, product innovation throughout a number of sectors, such as drinks, bakery and probiotics, is evident, with trends generally following those of the U.S. and U.K. The Australian market size is estimated at \$0.05 billion using a strict definition of functional foods.

Despite considerable market activity, consumer knowledge of functional foods is at a relatively low level; surveys suggest that the majority of consumers have not heard of the term "functional foods" (Anon. 1999). However, as new products are introduced, consumer interest is expected to grow rapidly. This can be witnessed currently as consumers become accustomed to foods supplemented with vitamins and minerals.

The dairy industry is in an excellent position to develop and exploit the functional food market. Growth of the dairy industry is set to continue with total world milk production of 571 million t for the year 2000 (Anon. 2000b). Consumption rates are forecast to increase at an annual rate of 1 to 2% until 2005. Nevertheless, dairy companies within the industry are under constant structural pressure to increase in size and to seek global consolidation opportunities. Countries hosting the world's largest dairy companies — the European Union (E.U.), U.S., New Zealand, Australia and Canada — play a major role in shaping the world dairy market. Together they produce approximately 47% of all cows' milk in the world; the U.S. alone had a market share of 72% in 1999 (Mikkelsen 2000). Within these countries the situation is rapidly changing, with New Zealand and Australia increasing their share of the market, mainly at the expense of the E.U. These two countries are now responsible for more than half of the exports of dairy products to the world market (Jachnik 2000). Indeed the world's top 10 dairy companies together produce approximately 15% of the world milk supply (Mikkelsen 2000) (Table 1.1).

TABLE 1.1
Turnover Estimates of Leading Dairy Companies, 2001

Company	Turnover $ Billion
1 Nestlé	13.5
2 Dean Foods	8.6
3 Dairy Farmers of America	7.8
4 Kraft Foods	6.3
5 Danone	6.2
6 Fonterra	5.8
7 Parmalat	5.6
8 Unilever (estimated value)	5.0
9 Lactalis	4.9
10 Arla Foods	4.6

Source: Adapted from Radobank International, 2002, *World Dairy Trade Map,* Dutch Dairy Commodity Board, The Netherlands.

Trade in dairy products is increasing after leveling off between mid-1998 and mid-1999; the world dairy market is currently worth approximately $300 billion (Anon. 2000b; 2001a). Although milk, cheeses and yogurt account for 60% of this value (Anon. 2001a), significant growth is occurring mainly in added-value products such as milk drinks, yogurts and desserts. As mentioned earlier, the growth of the dairy industry is set to continue. In emerging markets such as Asia, significant volume growth is expected and a shift toward added-value products is predicted. In the mature markets of the E.U. and U.S., value growth will be the key, with diversification set to satisfy consumer demand and the increasingly competitive marketplace. Value can be added to saturated dairy markets by producing new, innovative dairy products; it is here that functional dairy products are expected to excel.

To date, major dairy manufacturers have made serious commitments to development of the functional dairy market (International Dairy Federation 2000; Petit 2000). Table 1.2 illustrates that functional dairy products are the leading sector of the functional food market in many countries (Anon. 2000c).

1.3 FUNCTIONAL DAIRY PRODUCTS

Many distinct drivers are behind the growth of the functional food market; changing demographics is one of the key drivers. Over the next 25 years the population over 65 years of age will more than double worldwide, with the greatest absolute changes occurring in Asia (Figure 1.1). The elderly population of the U.S. and other developed nations is set to increase by 50% during the same period (U.S. Census Bureau 1999; United Nations 2003).

Such major demographic changes present a market opportunity for producers of functional foods, especially because those in this ageing population are entering

TABLE 1.2
Leading Functional Food Sectors by Country

Country	Leading Sector	Sales ($ Million) 1995	1999
Australia	Breakfast cereals	387	331
Austria	Functional dairy	7	47
Belgium	Functional dairy	21	19
Canada	Bakery products	502	561
Denmark	Dairy	22	52
Finland	Probiotic dairy	333	274
France	Dairy products	520	524
Germany	Functional drinks	230	240
Japan	Probiotic dairy	22	823
Norway	Dairy	4	16
Netherlands	Dairy	88	165
Sweden	Dairy	47	79
Switzerland	Probiotic dairy	78	3
U.K.	Breakfast cereals	599	726
U.S.	Bakery products	6552	9755

Source: Adapted from Anon., 2000c, *OTC Bus. News,* 6(146), 8.
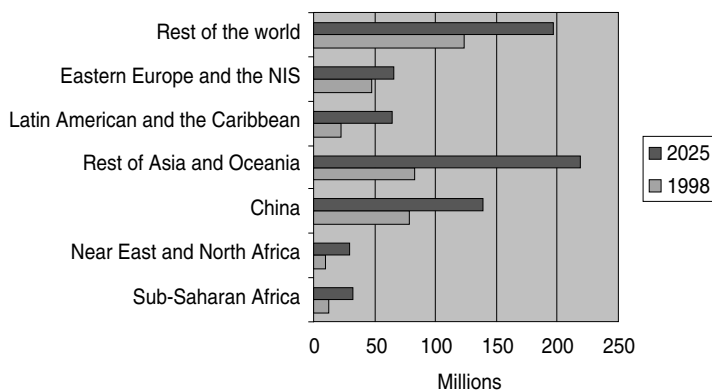
FIGURE 1.1 Growth projection of population over 65 years. (Adapted from U.S. Census Bureau, in *The Official Statistics*, 1999.)

their peak income years. This opportunity is heightened further as people become more health conscious and increasingly aware of the role nutrition plays in their diets. Consumers wish to take more control of their own health (Table 1.3). Socioeconomic evolution and food consumption trends have resulted in food increasingly being a "want" rather than a "need," with a balance between indulgence and health

TABLE 1.3
Some Functional Food Product
Development Health Targets

Condition

Heart disease
Osteoporosis
Gut health
Cancer
Obesity
Ageing
Immune system
Bowel function
Arthritis
Mood/cognitive performance
Neural tube defects
Urinary tract infection

Source: Adapted from Hilliam, M.A. and Young, J., 2000, *Functional Foods Markets, Innovation and Prospects, a Global Analysis*, Leatherhead Food Research Association Publishing, Surrey, U.K.

desired. However, consumers will not compromise on taste or product quality for healthy products and price is also an important determinant in repeat purchase. Although currently only health sophisticates and health-conscious consumers adopt functional foods, these products will eventually be purchased by mainstream consumers (Jay 2000). These factors, along with rising health care costs, are key reasons why opportunities exist for functional dairy foods.

A major advantage of dairy foods is that consumers are already familiar with them and many believe that dairy products are healthy, natural products. Health professionals worldwide encourage consumers to eat varied balanced diets rather than seeking instant solutions, and dairy products are prominent components of balanced diets. Milk and dairy products constitute one of the four major food groups that make up a balanced diet. Cow's milk contains various important vitamins and minerals in substantial quantities. In a varied diet, milk is an important source of protein, calcium and the B-group vitamins, and provides vitamin A, thiamin, niacin, vitamin B_6, folate, vitamin C, magnesium and zinc as well (Holland et al. 1991; Miller et al. 2000; Dairy Council 2001). Carbohydrate is found in the form of lactose, which is generally considered to be of low carcinogenicity. Also, approximately one third of the fat in whole milk is monounsaturated and small amounts of essential fatty acids are provided. Milk is one of the major sources of conjugated linoleic acid (CLA) in the diet, although it is a minor component of milk fat.

Milk has a potential role to play in the prevention of disease. Bone mineral content and bone mineral density increase from infancy to adulthood. Optimizing

TABLE 1.4
Common Gastrointestinal-Related
Reasons for Medical Consultations

Condition

Intestinal disease presumed infective
Functional disorders of the stomach
Irritable bowel syndrome
Duodenal ulcer
Inguinal hernia
Hemorrhoids
Constipation
Hiatus hernia
Other disorders of stomach/small intestine
Anal fissure/fistula
Cholecystitis/cholelithitis
Diverticulitis

peak bone mass and slowing later bone loss are recognized as the most effective ways to reduce the risk of osteoporotic fractures in later life. Nutrients that promote bone synthesis include calcium, vitamin C, vitamin D and vitamin K. In addition to bioavailable calcium, milk also provides phosphorus, zinc and fluoride, which are essential for optimal bone health (Gurr 1999). Studies have confirmed that a milk-drinking habit in early life encourages the establishment of peak bone mass and that milk and dairy product consumption increases bone mineral content and reduces fracture risk (Gurr 1999). Nondigestible oligosaccharides (NDOs), potential ingredients for functional dairy foods, have been found to stimulate various minerals and improve mineralization of the bone. Most studies have been carried out on animals in which the NDOs have increased the availability of calcium, magnesium, zinc and iron (Scholz-Ahrens et al. 2001). Thus, the relationship between functional dairy product and positive bone health becomes stronger, making osteoporosis a definite potential target for functional dairy products. Moderate- or large-sale increases have been predicted for calcium-enriched products, probiotic yogurts and fermented milk drinks (Hilliam and Young 2000).

Because gastrointestinal health is considered a rather taboo subject, many suffer in silence. In a recent study involving 4000 subjects, over a third of the participants considered that the role of the digestive system in general health was not relevant to them even though they had a high prevalence of gastrointestinal upsets, including indigestion, irritable bowel syndrome, diarrhea and constipation (Shortt 2000). Indeed, diet can play a role in modulating many of the common gastrointestinal conditions for which medical advice is regularly sought (Table 1.4). The gut flora plays an important role in the maintenance of health by stimulating the immune system, protecting the host from invading bacteria and viruses, and aiding digestion.

An optimum gut flora balance is one in which beneficial bacteria, such as lactobacilli and bifidobacteria, predominate over potentially harmful bacteria. Many
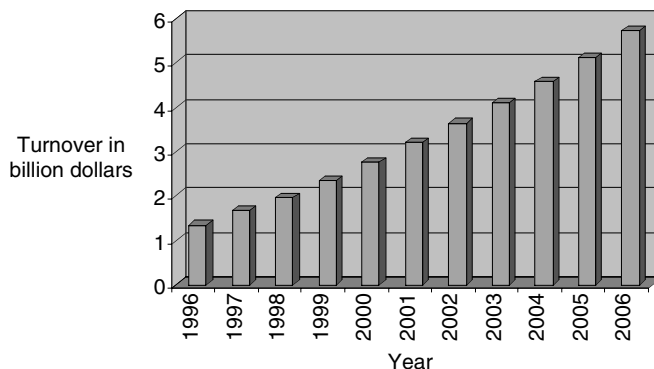
FIGURE 1.2 European market projections for pre- and probiotics. (Adapted from Frost and Sullivan, 2000, Der Markt fur probiotische und prabiotische Milchprodikte, *Milch-Marketing.*)

factors can influence the balance of the gut flora, such as the composition of the diet, antibiotic therapy, infections, food poisoning, stress and ageing (Shortt 1999). Currently, probiotic yogurts and drinks are by far the most developed sector of the market. Figure 1.2 indicates the growth of the European market for prebiotics and probiotics. With probiotic dairy products currently one of the most popular functional food products, it should come as no surprise that a definite association exists between these products and gastrointestinal health. Substantial evidence links some probiotics to the treatment of certain gastrointestinal tract disturbances such as lactose maldigestion, antibiotic-associated diarrhea, *Clostridium difficile*–associated diarrhea and the duration of diarrhea in infants with rotavirus enteritis. Effects on traveler's diarrhea, immunomodulation, *Helicobacter pylori* infection, irritable bowel syndrome, inflammatory bowel disease and some cancers are also being investigated (Marteau et al. 2001; Stephen et al. 2002). Gastrointestinal health is already a target in the development of functional dairy products and should continue as more promising research results are published and strengthen the evidence base.

The mechanisms behind the effects of probiotics need further research. For example, many probiotic effects are due to the mediation of immune regulation and the balance of pro- and anti-inflammatory cytokines. Probiotics can be useful in the alleviation of gut mucosal inflammation, normalization of gut mucosal dysfunction, and down-regulation of hypersensitivity reactions (Isolauri et al. 2001). As mechanisms are more clearly elucidated, more targeted probiotic-based functional dairy foods can and will be developed. Milk-derived bioactive proteins and peptides, for example, α-lactalbumin and lactoglobulin, which have been associated with immunomodulation, also hold considerable promise (Gill et al. 2000; Chapter 7).

In addition to the roles of functional dairy products in bone health, gastrointestinal health and immunity, lactose intolerance is a specific condition that can benefit from functional dairy products. Probiotic bacteria in fermented and unfermented milk products, along with yogurt and other conventional starter cultures, improve lactose digestion and eliminate symptoms of intolerance in lactose maldigesters (de Vrese et al. 2001). Lactose is hydrolyzed by microbial enzyme beta-galactosidase during

TABLE 1.5
Selection of Ingredients and Claims Associated with Functional Dairy Foods

Ingredient	Sources	Examples of Claim Areas
Minerals	Calcium Casein peptides	Optimum growth and development, dental health, osteoporosis
Fatty acids	CLA	Heart disease, cancer prevention, weight control
Prebiotics/carbohydrates	Galactooligosaccharides Lactulose Lactose	Digestion, pathogen prevention, gut flora balance, immunity, lactose intolerance
Probiotics	Lactic acid bacteria Bifidobacteria	Digestion, immunity, vitamin production, heart disease, antitumor activity, remission of inflammatory bowel disease, prevention of allergy, alleviation of diarrhea
Proteins/peptides	Caseins, whey proteins, immunoglobulins, lactoferrin, glycoproteins, specific peptides	Immunomodulation, growth, antibacterial activity, dental health, hypertension regulation (angiotensin inhibitors)

fermentation to produce galactose and glucose; thus, if this bacterial enzyme is released, lactose digestion is improved. This makes fermented products useful for the substantial number of people with a lactase deficiency. Lactose-hydrolyzed dairy products also provide choice to those who suffer from maldigestion.

Calcium in dairy foods has also been associated with a reduction in hypertension risk — a major risk factor in coronary heart disease, stroke and kidney failure. After reviewing research, including the Dietary Approaches to Stop Hypertension (DASH) clinical trial, Miller (1999) concluded that a diet including low fat dairy foods, fruit and vegetables can significantly lower blood pressure while improving other chronic disease risk factors. More recently, fermented milks with specific peptides have been shown to influence hypertension.

Thus, milk is an excellent natural source of nutrients; research also shows that milk-derived components have many beneficial physiological properties (Table 1.5). Functional dairy products, which use milk as a base or use dairy-derived components, are in an excellent position to contribute to the functional food market.

Significant opportunities exist for products whose functionality has widespread appeal. It follows therefore that a product encapsulating the needs of every member of a family is extremely likely to be a success. The potential broad appeal of functional dairy products is an important market advantage. Functional dairy products that affect conditions such as osteoporosis, heart disease and cancer appeal specifically to adults, while products affecting tooth health, bone health and immunity appeal to adults and children alike. The range of sensory characteristics possible with dairy ingredients also allows the production of diverse textures and aromas. Table 1.6 shows the adaptability of dairy products to fulfill consumers' requirements.

TABLE 1.6
Adaptability of Dairy Products to Meet Core Consumer Requirements

Core Consumer Requirement	Milk	Cheese	Butter	Yogurt	Dairy Desserts	Dairy Snacks
Energy	✔	✔	✔	✔		✔
Comfort	✔	✔	✔		✔	
Convenience	✔	✔		✔	✔	✔
Entertainment				✔	✔	
Indulgence		✔	✔	✔	✔	✔
Health	✔	✔		✔		✔

Source: Adapted from Anon., 2000b, *Scand. Dairy Inf.,* 4, 22–23.

Advances in technology and nutrition that add to the opportunities in the functional dairy food market are occurring. Whey proteins are a good source of the branched-chained amino acids; because of their nutritional advantages, specific whey hydrolyzates are set to be included in many milk-based drinks, e.g., infant formula, to aid digestion and absorption (Burrington 2000; Rao 2000). Glycomacropeptide, a whey protein that does not contain phenyalanine, has potential as a rich source of protein for special phenylketonuria diets (Pszczola et al. 2000). Fermented whey, selected as a growth medium for bifidobacteria, can result in the production of antimicrobials that are effective in the inhibition of *Helicobacter pylori*. TOS (*trans*-galactooligosaccharide) is derived from the lactose part of whey and provides another opportunity for adding value to products (Ziggers 2000). Cheddar cheeses containing high levels of probiotic bacteria have been successfully manufactured (Stanton and Ross 1999).

1.4   CONCLUSIONS

A major consideration in the continued development and success of the functional dairy food market is communication. This is linked to other important factors such as development of supporting scientific documentation, a health claims strategy and successful presentation of the product (Young 2000). It is critical to the success of a new functional food product that marketing and consumer education are at the top of the agenda and that the science backs up the product.

Marketing opportunities, like product possibilities, exist across the board for functional dairy foods. The marketing challenge of launching functional dairy foods lies in the many factors that need to be taken into account. Fundamental differences in culture and variance in nutritional knowledge and health priorities of consumers exist. This must be acknowledged in marketing campaigns; to take full advantage of opportunities, it is essential to undertake comprehensive market research before product launch. In saturated markets, the marketing strategy is of paramount importance to broadening the appeal of the product to various consumers and their needs.

Heasman and Mellentin (2001) suggest that messages with large scientific content will not appeal to a mass market, but will attract niche sectors, e.g., blood cholesterol–lowering products. For functional products to appeal to the mass market, a clear, simple, comprehensible message that takes into account consumers' perceptions of nutrition and health is required. Consumers tend to be resistant to dietary change (Childs 1996; de Almeida et al. 1997); indeed, even when consumers understand a food product proposition they may not see the relevance of the food to them. It has been estimated that the number of consumers that are health "uninvolved" far exceeds those that are "aware or active." Thus, even if a functional dairy food is addressing a relevant physiological need, a proportion of potential consumers will not pick up on the message unless communication strategies are specifically developed to address these issues of resistance and relevance (Shortt 2000).

There is, however, an opportunity to develop and market dairy products, especially probiotics, that target selected age groups who have specific requirements (newborns, adolescents, seniors) and individuals with specific disease states (irritable bowel syndrome, inflammatory bowel disease, Crohn's disease, intestinal cancers) or those who have had their intestinal microflora compromised (postantibiotic treatment, gastroenteritis, irradiation or surgery). These opportunities will be realized when more is understood about the role that microflora plays in health and disease and the mechanisms involved in the interaction between bacteria passing through the gastrointestinal tract and the host's gut flora can be characterized. Also, new processes in food manufacturing will allow for the incorporation of microorganisms into a wider range of products; alternatively, new strains of microorganisms will be identified with attributes that render them more effective ingredients for food product development.

Because skepticism toward health claims exists, it is essential that any claim made is scientifically proven in human subjects to ensure consumer trust and acceptance of the product. The key criteria required to establish causation commonly are a temporal relationship, specificity, biological plausibility and coherence (Bradford-Hill 1966). The level of scientific support required varies from country to country. For example, in some countries label claims must be supported by the totality of publicly available scientific evidence and significant scientific agreement among qualified experts must support any claim. Although the demonstration of benefits in apparently healthy individuals is critical to the success of functional dairy products, it is hampered by a dearth of validated biomarkers that are recognized worldwide. It is hoped that results from projects such as PROEUHEALTH (http://www.vtt.fi/virtual/Proeuhealth) and PASSCLAIM (http://europe.ilsi.org/passclaim) will provide guidance in this area. Clear regulatory frameworks will assist market development and foster innovation in the development of functional dairy foods.

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2 Successful Probiotic Lactobacilli: Human Studies on Probiotic Efficacy

Seppo Salminen, Martin Playne, and Yuan Kun Lee

CONTENTS

2.1 INTRODUCTION

This chapter describes key factors that determine the health efficacy for humans of foods containing probiotic lactic acid bacteria. The published data on human studies (including properly designed clinical trials) with the most successful commercial probiotic lactobacilli are summarized. Conclusions are drawn as to which health or disease conditions can be alleviated or cured by probiotic lactobacilli. The chapter describes health conditions in which the proof is strong, in which uncertainties exist, and in which there may be possibilities in the future.

"Probiotic" has been defined by the ILSI Europe (International Life Sciences Institute Europe) working group as "a viable microbial food supplement which beneficially influences the health of the host" (Salminen et al. 1998). This definition implicitly takes into account the fact that safety and efficacy of probiotics must be scientifically demonstrated for each strain and each product. Most probiotics in the food supply have been used in fermented milks and dairy products; in fact, dairy products are the major carriers for the intake of probiotics available today.

"Are all probiotics the same?" is a question often asked by scientists and lay persons. Understanding of probiotic properties has increased dramatically in the last 10 years. It is now realized that even genetically closely related strains can have significantly different properties *in vitro* and *in vivo*.

Until recently, the selection of most probiotic lactic acid bacteria has been primarily based on their technological properties for the manufacture of good and stable dairy products (Jacobsen et al. 1999; Salminen et al. 1999). The trend to include health-promoting properties as well has caused a scientific dilemma as to how to correlate *in vitro* and *in vivo* properties and how to demonstrate health effects. This requires a large body of translational research incorporating laboratory and clinical work and perhaps a deeper understanding of the mechanisms behind proven probiotic effects. It is now important to include dairy technological properties and health properties in the scheme to be able to produce functional dairy products with good sensory characteristics, straightforward technology and proven health effects.

Demonstration of health effects includes research on mechanisms and clinical studies with human subjects. The ILSI Europe working group has also defined probiotic foods as functional if they have been satisfactorily demonstrated to affect one or more target functions in the body beneficially, beyond adequate nutritional effects, in a way relevant to an improved state of health and well-being or to reduction in the risk of diseases (Diplock et al. 1999). These definitions have set the basis for assessment of the health-promoting potential of probiotics. Consequently, it is necessary to demonstrate health efficacy for functional foods containing specific probiotics — not just for the microorganism. It is noted that some of the current selection criteria (discussed later in this chapter) may need to be modified when the mechanisms of probiotic action are understood. Thus, new methods of modeling and selecting novel probiotics are required for further developing dairy-based functional foods with scientifically proven health effects, particularly for specific diseases and target groups. Although probiotic foods are predominantly dairy foods at present, this is changing. Increasingly, probiotics are incorporated into a wide range of foods of nondairy origin — a trend that is expected to continue. Probiotics have also been

available as powders, tablets and capsules in health food stores and pharmacies. To date, much less scientifically critical appraisal of such products has taken place. However, that industry sector is now coming under pressure to market products in which probiotic strains are used that have proven health benefits for humans demonstrated in peer-reviewed scientific publications.

2.2   ASSESSMENT OF HEALTH EFFECTS

It is important to understand that all probiotic strains are unique and that their properties and characteristics should be well defined. Thus, even studies on closely related strains cannot be extrapolated without great caution. It is important that each strain be clearly identified using modern methodology and that the scientific community have access to each commercial strain for research purposes (keeping in mind the protection of intellectual property rights). For that purpose, lodgement in international culture collections is important to make all strains available for all research groups to promote the worldwide assessment on health effects and mechanisms.

The assessment of the health-promoting potential of a probiotic must be based on a valid scientific hypothesis and realistic studies supporting the hypothesis. Knowledge of the mechanisms is an important factor, complemented with information on target functions and validated biomarkers accepted as relevant to the state of health and well-being or reduction of risk of disease. The hypothesis can be supported by studies carried out *in vitro* using cell culture models or *in vivo* using animal models. It is quite demanding to validate the *in vitro* studies and animal models against clinical observations and clinical study results.

The most important studies for proving probiotic health effects are carefully planned and monitored clinical studies in human subjects. All data must be assessed by reference to studies in human subjects, preferably conducted by at least two independent research groups in different locations. Multicenter studies offer a solution for this assessment.

In conclusion, well-designed human studies with requirements similar to those for pharmaceutical studies are required to demonstrate health benefits. Additionally, epidemiological studies or postmarketing surveillance studies are recommended to assess safety and efficacy of probiotics further. Using these criteria, several health-promoting effects can be considered scientifically proven for a few specific strains.

2.3   WELL-CHARACTERIZED PROBIOTIC STRAINS AND THEIR CLINICAL EFFECTS

The strains that have been characterized for their clinical effects and properties have been assessed in recent reviews (De Roos and Katan 2000; McFarland 2000; Salminen et al. 1998). A number of health-related effects are partially established, but some can be considered reasonably well established and clinically well documented. Table 2.1 lists these strain-specific proven health effects; other effects reported for specific probiotic lactobacilli strains are included in Table 2.2.

TABLE 2.1
Established and Proposed Health Effects of Probiotics

Scientifically established effects	Reduction of the duration of rotavirus diarrhea; reduction of the duration of antibiotic-associated diarrhea; reduction of the symptoms of lactose intolerance; prevention of rotavirus and *Clostridium difficile* diarrhea
Future challenges	Studies on treatment of food allergy; colon cancer prevention and treatment; studies on inflammatory bowel disease and irritable bowel syndrome; Crohn's disease; cholesterol control

TABLE 2.2
Current Probiotic Bacteria and Their Reported Effects

Strain	Reported Effects in Clinical Studies	Selected Reviews with Further References
L. johnsonii LA1	Adheres to human intestinal cells; balances intestinal microflora; enhances immunity; adjuvant in *H. pylori* treatment	McFarland, 2000; Salminen et al., 1998
L. acidophilus NCFB 1748	Lowers fecal enzyme activity; decreases fecal mutagenicity; prevents radiotherapy-related diarrhea; reduces constipation	Fonden et al., 2000; Salminen et al., 1998
L. rhamnosus GG (ATCC 53013)	Treats and prevents rotavirus diarrhea; prevents antibiotic-associated diarrhea; treats relapsing *C. difficile* diarrhea; reduces cystic fibrosis symptoms	McFarland, 2000; De Roos and Katan, 2000; Fonden et al., 2000
L. acidophilus NFCM	Lowers fecal enzyme activity; high lactase activity; treats lactose intolerance; produces bacteriocins	Fonden et al., 2000; Sanders et al., 1996
L. casei Shirota	Prevents intestinal disturbances; balances intestinal bacteria; lowers fecal enzyme activities; positive effects on reducing the recurrence of superficial bladder cancer	De Roos and Katan, 2000; Fonden et al., 2000; McFarland, 2000; Salminen et al., 1998
S. thermophilus; *L. bulgaricus*	No effect on rotavirus diarrhea; no immunity-enhancing effect during rotavirus diarrhea; no effect on fecal enzymes; strain-dependent improvement of lactose intolerance symptoms	Fonden et al., 2000; Saavedra et al., 1994
L. acidophilus La-5	Balances intestinal microflora; protects against traveler's diarrhea; enhances immunity	Fonden et al., 2000
Lactobacillus gasseri (ADH)	Reduces fecal enzymes; survives in the intestinal tract	Fonden et al., 2000
L. reuteri	Colonizes the intestinal tract; shortens rotavirus diarrhea	De Roos and Katan, 2000; Fonden et al., 2000

2.3.1 LACTOSE INTOLERANCE

Convincing evidence from several studies indicates that lactose-intolerant individuals suffer fewer symptoms if milk in the diet is replaced with fermented dairy products and functional probiotic-containing foods. Due to partial hydrolysis of lactose during fermentation, the reduced levels of lactose in fermented products relative to milk may contribute to the greater tolerance of yogurt (de Vrese et al. 2001).

Often these effects are due more to the fermentation than the probiotic, but the properties of specific probiotics can also influence the outcome: some probiotic bacteria are rapid users of lactose and other probiotics may have hardly any lactase activity. The lactose hydrolyzing activity varies a lot between strains and forms the basis for studies on milk-containing products. However, the lactase activity and lactose content of the final products are rarely described in reported studies even though their assessment may be critical for interpreting nutritional responses to products. These properties also attest to the applicability of a specific probiotic toward reducing and alleviating symptoms of lactose intolerance; other probiotics may need supporting technology to reduce lactose concentrations in the functional dairy products and within the intestinal tract (de Vrese et al. 2001; Marteau et al. 2001; Sanders et al. 1996).

The mechanisms of action of lactic acid bacteria and fermented dairy products include the following: lower lactose concentration in the fermented product, high lactase activity of bacterial preparations used in the production, and increased active lactase enzyme entering the small intestine with the fermented product or within the viable bacteria able to survive gastric and bile conditions. The bacterial enzyme beta-galactosidase, which can be detected in the duodenum and terminal ileum after consumption of viable yogurt, is thought to be the major factor that improves digestibility by the hydrolysis of lactose, mainly in the terminal ileum. Another factor suggested to influence lactose digestion is the slower gastric emptying of semisolid milk products such as yogurt.

In conclusion, there is good scientific evidence on the alleviation of lactose intolerance symptoms by specific probiotic lactic acid bacteria. However, strain-specific lactase activities may vary over 100-fold. This is especially important in the case of normal yogurt strains *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, in which lactase activities vary considerably. Thus, strains with defined lactase activities should be used in clinical studies. In practice, because different products have varying lactose contents and strains, they vary in their lactase activity when released to the duodenum (Fonden et al. 2000).

2.3.2 ROTAVIRUS DIARRHEA

Lactobacillus GG has been reported effective in the treatment of rotavirus diarrhea, reducing the duration of diarrhea to about half in children with the rotavirus. It has also been reported to be effective in the treatment of watery diarrhea in several studies in Asia with favorable results on colonization. When different lactic acid bacteria were compared for their effects on the immune response to rotavirus in children with acute rotavirus gastroenteritis, differences among various strains were

observed. Serum antibodies to rotavirus, total number of immunoglobulin-secreting cells and specific antibody-secreting cells (sASC) to rotavirus were measured at the acute stage and at convalescence. The treatment with *Lactobacillus* GG was associated with an enhancement of IgA sASC to rotavirus and serum IgA antibody level at convalescence. It was therefore suggested that certain strains of lactic acid bacteria promote systemic and local immune response to rotavirus, which may be of importance for protective immunity against reinfection. For a review on rotavirus diarrhea, see Fonden et al. (2000), De Roos and Katan (2000), Salminen et al. (1998) and Saavedra et al. (1994).

The effects of viable and heat-inactivated lactic acid bacteria have also been compared in a blind randomized clinical study. *Lactobacillus* GG administered as a viable preparation during acute rotavirus gastroenteritis resulted in a significant rotavirus-specific IgA response at convalescence. The heat-inactivated *Lactobacillus* GG was clinically as efficient, but the IgA response was not detected. This result suggests that viability of the strain is critical in determining the capacity of lactic acid bacteria to induce immune stimulation (Kaila et al. 1995). Also, in a study with different preparations of lactic acid bacteria using the recommended doses (1.25-g dose of freeze-dried preparation twice daily for 5 days) in treatment of rotavirus diarrhea, it was shown that *Lactobacillus* GG (cell concentration 5×10^9 cfu/g) was most effective. A preparation containing a mixture of *S. thermophilus* (95%), *L. bulgaricus* (4%), and *L. rhamnosus* (1%) (2.8×10^8 cfu/g) or a preparation containing *L. rhamnosus* (2.2×10^8 cfu/g) did not have a clinical effect on the duration of diarrhea (Majamaa et al. 1995).

It has also been shown that *Lactobacillus reuteri* effectively shortened the duration of watery diarrhea associated with rotavirus (Shornakova et al. 1997); however, both studies were conducted by the same research team, so further confirmation of the effect by other teams in different conditions is required. Shortening of the duration of rotavirus diarrhea using *Lactobacillus* GG is perhaps the best documented probiotic effect. It has been well documented: first, in several studies around the world and also in a recent multicenter study in Europe (Guandalini et al. 2000). Several studies have also used the heat-inactivated *Lactobacillus acidophilus* LB1 and *Bifidobacterium lactis* Bb-12; two studies on *L. reuterii* report shortening of the duration of rotavirus diarrhea in children. As with other documented effects, it is important to remember that the reported studies are specific to the strains used.

The clinical effects of these strains on the duration of diarrhea have been clear and repeatable in different circumstances. Currently, it is not clear whether the clinical effect is due to direct competition between the bacteria and virus particles on adhesion sites or receptors or whether the conjugated bacteria virus complexes result in an inability of the viral particles to adhere to and invade epithelial cells. Another possibility in the case of *Lactobacillus* GG is that it does not remove the viral particle from the surface of the epithelial cells, but rather induces an enhancement in the local production of IgA, thereby neutralizing or removing the viral particles from the mucous membranes. The immunity-enhancing effects are probably mediated by the metabolites of the probiotic because the adhesion of heat-inactivated lactobacilli has been reported to be similar to the viable strains used in *in vitro* adhesion models (Ouwehand et al. 1999). It is also possible that rotavirus diarrhea

is followed by a secondary bacterial diarrhea and that the probiotic may interfere in the early stages of the bacterial diarrhea, thus shortening the total duration of diarrhea.

2.3.3 ANTIBIOTIC-ASSOCIATED DIARRHEA

Antibiotics often cause dysfunction of the intestinal microflora, leading to abdominal cramps, meteorism, diarrhea and other intestinal side effects. Antibiotic-associated diarrhea has been prevented by at least two strains and strain combinations. The best documented studies have been conducted with *Lactobacillus* GG (ATCC 53013) strain in a yogurt form using a dose of two 150-g doses of yogurt daily with about 10^7 cfu/ml (Siitonen et al. 1990) or as a freeze-dried product using doses varying from 10^9 to 10^{10} cfu/day (Arvola et al. 1999; Vanderhoof et al. 1999). All studies reported good efficacy of the yogurt and the freeze-dried strain.

Black and coworkers (1991) reported a double-blind study using 20 healthy volunteers treated with 500 mg of ampicillin who were divided into two groups. Half of the volunteers received 4×10^9 cfu live lyophilized *B. lactis* and *L. acidophilus* La-5. The volunteers receiving lactic acid bacteria were recolonized faster than those receiving placebo and harbored higher counts when compared to the controls. Pathmakanthan et al. (2000) have provided useful tabular compilations of published probiotic studies conducted on antibiotic-associated diarrhea and the related *Clostridium difficile*–associated diarrhea. The most investigation in this area has been done using a strain of *Saccharomyces cereviseae* (*boulardii*). These authors have summarized several studies with large numbers of patients showing the efficacy of *S. boulardii* in the treatment of antibiotic-associated diarrhea, but the yeast is currently not used in food.

Specific strains of lactic acid bacteria have been reported to inhibit many intestinal pathogens, including *Helicobacter pylori*. Surprisingly, there are few reports on the use of probiotics to inhibit bacterial infections caused, for example, by Shigella, Salmonella, and *Escherichia coli* in humans. However, a large number of reports from laboratory experiments and mouse animal models indicate the effectiveness of many probiotic lactobacilli strains in overcoming these infections. Lactic acid bacteria are often able to survive acidic gastric conditions; therefore, it has been proposed that they may have a beneficial influence during the eradication of *H. pylori*, the treatment of which involves multiple strong antibiotics applied simultaneously (Michetti et al. 1999). Thus, eradication therapy offers a good model for studying antibiotic-associated diarrhea and the use of probiotics to alleviate and prevent the intestinal dysfunctions related to antibiotics.

2.3.4 PROBIOTICS AND BLADDER CANCER

In Japan, keen interest has been shown in lactic acid bacteria and their immune effects and cancer-related efficacy. Following several mechanistic studies on the effects of the *Lactobacillus casei* Shirota strain that reported decreased urinary mutagen excretion, studies assessing enhanced immunity and other mechanisms were assessed in experimental animals and in human subjects. Well-performed

human clinical studies were conducted using oral administration of *L. casei* Shirota or a placebo. In one clinical study and another larger multicenter study, the prophylactic effects of oral administration of *L. casei* Shirota (LcS) strain on the recurrence of superficial bladder cancer were reported (Aso and Akazan 1992; Aso et al. 1995).

Recently, a large Japanese case control study on the habitual intake of lactic acid bacteria and reduction of risk of bladder cancer was conducted (Ohashi 2002). Japan has a long history of home delivery of proboitic products and therefore offers a unique setting for this type of study. The results suggested that the habitual intake of fermented milk with the LcS strain reduces the risk of bladder cancer in the Japanese population. This result was valid after consideration of other risk factors such as smoking. When combined with the mechanistic work and the reported human studies, the effect warrants further investigations in other countries. Even though bladder cancer is not a major form of cancer in countries consuming western-style diets, the observation enhances the interest in intestinal microflora-associated characteristics relating to the development of certain forms of cancer.

2.4 EFFECTS NEEDING FURTHER CLINICAL AND NUTRITIONAL ASSESSMENT

2.4.1 INTESTINAL CANCERS, INCLUDING BOWEL CANCER

A number of animal studies have focused on the effect of probiotics on intestinal microecology and cancer (Gill and Rowland 2002; McFarland 2000). *L. acidophilus, L. casei* Shirota and *Lactobacillus* GG have been shown to have inhibitory properties on chemically induced tumors in animals (De Roos and Katan 2000; McFarland 2000; Sanders et al. 1996). However, the data are not consistent and controversial animal studies have been published (McIntosh et al. 1999). It is obvious that the results vary depending on the choice of basal diet for the animals and on the experimental carcinogen used. Other studies indicate that specific strains of probiotic bacteria may be able to down-regulate intestinal microbial enzyme activities (Gill and Rowland 2002; Wollowski et al. 2001). This phenomenon may decrease carcinogen-activating microbial enzymes and have a beneficial effect in the colon, urinary tract and bladder, as shown with the studies on *L. casei* Shirota and superficial bladder cancer. Further studies, especially human studies, are needed; however, human studies are extremely difficult to conduct because of the long period of development of most forms of cancer (Wollowski et al. 2001).

The reports on the benefits of oral administration of probiotic cultured milks and lactic acid bacteria on tumors have been connected with changes related to tumor induction and promotion. The following mechanisms have been indicated in various studies relating lactic acid bacteria intake and cancer:

- Alteration in intestinal microecology (beneficial microflora effects)
- Altered intestinal metabolic activity (decreased conversion of precarcinogens to carcinogens)
- Normalized intestinal permeability (prevention or delay of toxin absorption)

- Enhanced intestinal immunity (enhanced resistance to chemicals, inflammation and other factors)
- Strengthened intestinal barrier mechanisms (including some or all of the preceding four mechanisms)
- Butyrate supply to villi
- Folate supply in regulation of intestinal cell division

2.4.2 IRRITABLE BOWEL SYNDROME

Streptococcus faecium preparations have been evaluated for treatment of patients with irritable bowel syndrome whose symptoms had been present for an average of 7 years. Although patient-recorded symptoms did not differ significantly in the placebo or *S. faecium* groups, the physician's subjective clinical evaluation of symptoms revealed a significant improvement in the treated group. There is a rationale for investigating the effect of lactic acid bacteria and cultured milks in the treatment of this common disorder in which intestinal motility and dysfunctions in the intestinal microflora are important factors to consider (Madden and Hunter 2002). Further human studies with probiotic bacteria and cultured milks are ongoing in Europe and may provide future strategies for dietary management of this disease.

2.4.3 TRAVELER'S DIARRHEA

Traveler's diarrhea is one of the most common forms of diarrhea for North American and European tourists traveling to African and Asian countries. There has been a long-term interest in the effects of various probiotics and their use in the prevention and treatment of traveler's diarrhea. The few studies on the prevention of traveler's diarrhea show positive outcomes for *Lactobacillus* GG and a combination of *L. acidophilus* La-5 with *B. lactis* Bb-12 (for review, see Fonden et al. 2000; McFarland 2000; Salminen et al. 1998). These studies show some indications of beneficial effects; however, other studies report no effects and information on large human studies using defined strains on traveler's diarrhea is still lacking.

The difficulties in carrying out carefully controlled studies on the prevention of traveler's diarrhea include host and etiological factors. The multifactorial etiology of traveler's diarrhea poses a particular problem. In earlier reports it appears that the elderly population may be less prone to traveler's diarrhea due to more careful food selection and less adventurous travel habits or, perhaps, to acquired immunity against some common agents known to cause this diarrhea. Younger travelers, who are more prone to diarrhea, have perhaps not yet acquired immunity and are more relaxed in their food choices.

Another complicating factor is the etiology, which may vary significantly even within a small community, thus requiring studies that define the causative bacteria and viruses as well as the specified antagonistic properties of each strain. This will also involve studies on competitive exclusion effects between the probiotic and the organism causing diarrhea. Thus, further human studies with known bacterial etiology diarrhea should be conducted to verify the earlier results. The differences between the effects of fermented dairy products or plain freeze-dried bacteria may

have significant effects within the intestinal environment and local colonization influencing competitive exclusion of specific pathogens. These questions must be clarified before any conclusions on the efficacy of probiotics can be made; nevertheless, potential for probiotics or probiotic mixes clearly exists in this area.

2.4.4 LOWERING CHOLESTEROL

Several studies have suggested that ingestion of probiotic microorganisms may lead to lower serum cholesterol levels and, consequently, to a reduced risk of arteriosclerosis and other coronary heart diseases. The data are not consistent and studies have not resulted in long-term reductions in serum cholesterol levels (De Roos and Katan 2000).

2.5 FUTURE CHALLENGES — OTHER POSSIBLE EFFECTS OF MODULATION OF THE GUT FLORA

Probiotic strains together with specific prebiotic carbohydrates may be able to alter the composition of the microflora in different segments of the gastrointestinal tract. This could lead to new understanding of the control of human disease conditions.

2.5.1 ARTHRITIC CONDITIONS, INCLUDING ANKYLOSING SPONDYLITIS

Indications are that symptoms of arthritis are correlated with the composition of the gut microflora and the presence of particular microbial species. If preliminary findings are confirmed, then an opportunity exists for probiotic therapy for such conditions.

2.5.2 INFLAMMATORY BOWEL DISEASES

Preliminary hospital studies have claimed improvement in Crohn's disease by probiotic therapy (Shanahan 2000, 2002). This needs further exploration and the properties of lactic acid bacteria need to be verified in a model system utilizing mucosal tissues and microflora of subjects with inflammatory bowel diseases.

2.5.3 ATOPIC ECZEMA AND FOOD ALLERGY

A long and effective effort exists to modulate the gut microflora by probiotic lactobacilli in order to alleviate the symptoms of food allergy in children and adults as well as to prevent changes leading to the development of allergies. The role of probiotics and gut flora in human allergic disease was first emphasized by the demonstration of differences in gut flora composition between infants developing or not developing atopy within their first year of life. Differences in the neonatal gut microflora appear to be a function that precedes the development of atopy, suggesting a regulatory role of the balance of indigenous intestinal microlfora. Also, a suppressive effect of probiotics on lymphocyte proliferation and interleukin-4 generation *in vitro* has been demonstrated (Sütas et al. 1996a, b). Immunoinflammatory

responses to dietary antigens in allergic individuals have also been shown to be alleviated by specific probiotics. Additionally, distinct differences in the bifidobacteria microflora of allergic and healthy children have been reported; these differences are also seen at the species level when assessing the adhesive properties of the bifidobacteria strains (He et al. 2000, 2001). Thus, the bifidobacteria microflora may be an important target for future research.

In a recent report, the use of specific probiotics has resulted in the first clinical report of allergy prevention. Specific probiotics administered pre- and postnatally for 6 months to children at high risk of atopic diseases reduced the prevalence of atopic eczema to half when compared with that in infants receiving a placebo (Kalliomäki et al. 2001a, b). Future studies are warranted to evaluate the use of probiotics and gut mircoflora modification for prevention of atopic diseases (Kirjavainen et al. 1999).

2.6 SELECTION CRITERIA TO ACHIEVE EFFICACY OF LACTIC ACID BACTERIA IN FUNCTIONAL FOODS

To date, manufacturers of probiotic lactic acid bacteria have concentrated on selecting a strain that is stable, remains viable, is active in the product, and can be delivered economically at a reproducible count over the shelf life of the product. Thus, selection criteria for probiotic strains have concentrated on performance during manufacture of the strain, its incorporation into the food matrix, and viability over the shelf life of the product.

Because evidence shows that the health properties imparted by a probiotic are dependent on the particular strain and are not a property necessarily common to all strains of a particular species, manufacturers are now promoting their products as containing a particular strain. Thus, definitive fingerprinting of strains is becoming part of the selection criteria for probiotics. Additionally, the genetic stability of the strain is now being studied because of indications that some heavily promoted strains are not always genetically similar worldwide.

The probiotic microorganism in functional food must be able to transit the stomach and small intestine and remain viable while subject to low pH conditions and hydrolytic and proteolytic enzymes and bile salts found in that part of the gut. The organism must also be able to impart its health properties in the lower (ileal) segment of the small intestine because this is the site of several diarrheal diseases. It is not yet known if the inhibitory properties some probiotics may show against *H. pylori* infections mean that the probiotic organism must occupy the niches occupied by the pathogen in the lower stomach, or whether the probiotic may function in some indirect immunological manner.

Finally, the probiotic must be able to impart designated health effects in the bowel and must be selected specifically to impart these defined effects. To achieve this, the probiotic must be present in sufficient numbers or be able to grow in the bowel. This may mean that it must be able to adhere effectively to intestinal epithelial cells and to mucosa to avoid being "washed out" by the flow of digesta. Adherence may also be important for "competitive exclusion" — the occupation of surface sites

by the probiotic to prevent occupation by potential pathogens. For immunological effects to occur, it is important that the probiotic cells partially penetrate into the epithelial surface. However, translocation through a permeable cell wall can be undesirable because of the potential danger of cells circulating in the blood system.

In addition to an ability to perform all the preceding functions, the probiotic microorganism must be shown to be safe (Mogensen et al. 2002; Salminen et al. 1998). Furthermore, initial human studies must be conducted to demonstrate its biokinetics. These consist of simple studies to measure its recovery in fecal samples in a quantitative manner, supported by whatever noninvasive measurements can be made with the subject. Following conclusion of these studies, the probiotic strain can then be tested clinically in properly designed clinical trials against the health or disease conditions proposed. It is important also to assess the use of the probiotic strain for other uses, e.g., as a preservation agent, a detoxification agent and so on. Table 2.3 shows selection criteria for development of a new probiotic strain.

2.7 MATHEMATICAL MODELING IN THE SEARCH FOR EFFECTIVE PROBIOTICS

Adhesion of probiotic microorganisms is a factor of great importance to their performance in imparting health effects. Thus, the following section provides some new insights into the study of adhesion in the selection of new probiotics and in the understanding of what is happening in the gut during the consumption of probiotic foods.

2.7.1 MODELING PROBIOTIC PROPERTIES FOR COMPETITIVE EXCLUSION

Adhesion of probiotic bacteria to intestinal mucosa has been considered an important parameter in the competitive exclusion of pathogenic organisms and in stimulating immunological responses in the gastrointestinal tract (Greene and Klaenhammer 1994). An understanding of the kinetics and mechanisms of cell adhesion and competition for adhesion sites would facilitate interpretation of clinical and laboratory data, as well as provide a better insight into the mechanism of competition between probiotic bacteria and pathogens. This would allow selection and development of better probiotics in the future.

The adhesion of bacterial cells on the mucosa surface can be described by a simple dissociation process (Lee et al. 2000) because it is a function of bacteria concentration around the adhesion site and the affinity of the cells (strain dependent) for the adhesion site (Tuomola and Salminen 1998). At equilibrium, the concentration of the bound bacteria (e_x) can be expressed as:

$$e_x = e_m \cdot x/(k_x + x) \tag{2.1}$$

where e_m is the maximum value of e_x at saturated bacterial concentration. The value of e_m is numerically equal to the concentration of adhesion sites on the mucosa

TABLE 2.3
Selection Criteria for the Development of a New Probiotic

Manufacturing
Ability to grow quickly to high numbers in a simple and cheap fermentation medium
Ability to grow and survive in micro-aerophilic or aerobic conditions
Ability to withstand centrifugation, filtration, and freezing/lyophilization without significant loss of
  numbers
Ability to become "active" quickly following application
Ability to survive incorporation into a wide variety of food matrices, including being subject to processing
  temperatures above 45°C and raised concentrations of ethanol and sodium chloride
Testing to be done on the organism and on the organism in the actual food matrix

Shelf Life and Gut Transit
Assessment of viability of probiotic at intervals over shelf life of products, under different storage
  environments (e.g., temperature)
Tolerance to acid–pepsin solution at pH 2 for 2 hours (measurement of survival after exposure)
Tolerance to bile salts at physiological concentrations (measurement of growth), and at higher
  concentrations (measurement of survival after exposure)

Health Properties
Ability to utilize prebiotics (e.g., oligosaccharides, inulin, resistant starch) for growth
Ability to synthesize or utilize vitamins (B-group, folate, vitamin K)
Ability to inhibit pathogens (*in vitro* and *in vivo*) (e.g., *Salmonella typhimurium, Clostridium perfringens,
  Clostridium difficile, Escherichia coli, Candida albicans*)
Beta-galactosidase activity
Bile acid deconjugation properties
Ability to produce hydrogen peroxide
Ability to adhere to Caco2 cells, HT29 cells and fecal and ileostomy mucus
Ability to produce D lactic acid
Autoaggregation of the productive cells

Safety
Dose–response curves in animal models
Platelet aggregation tests
Safe history of origin and/or use

Identification
Phenotypic carbohydrate fermentation profiles (e.g., API tests)
Phenotypic enzyme profile tests (standard kits)
Catalase test
DNA-based tests: PCR based; RAPD, PFGE for strains, and 16sRNA sequences and ITS region tests
  for species
Protein profiles

Other Applications
Aflatoxin removal
Biofilm surface protection
Relative lactic acid production

surface and x is the concentration of bacterial cells in suspension around the adhesion site. k_x is the dissociation constant for the process that determines the affinity of the bacterial cells for the adhesion sites. The term "adhesion site" is used here to refer to the specific receptor and the site at which chemical interactions, e.g., hydrophobic interactions, take place.

If two bacteria (1 and 2) are competing for the same adhesion sites on the intestinal mucosa surface and no interaction takes place between them, the ratio of the two bacteria adhering on the surface can be described as,

$$e_{x1}/e_{x2} = (e_{m1}/e_{m2}) \cdot (x_1/x_2) \cdot (k_{x2} + x_2)/(k_{x1} + x_1) \qquad (2.2)$$

This relationship was found true in the competition between *L. rhamnosus* GG and *E. coli* TG1 for adhesion on an enterocyte-like Caco-2 cell culture model (Lee et al. 2000). The ratio of the two competing bacteria adhered to the Caco-2 cell surface was determined by the concentration and affinity for adhesion sites of the respective bacterium.

Interestingly, the e_m (which is numerically equal to the number of adhesion sites) for *L. rhamnosus* GG and *E. coli* TG1 competing for the same adhesion sites were not equal. The ratio of the number of *L. rhamnosus* to that of *E. coli* was about three to one. This implies that the *E. coli* carried three times the number of adhesions on its surface as that of the *L. rhamnosus*, i.e., every *E. coli* cell occupied three adhesion sites while a *L. rhamnosus* occupied one. Thus, if the probiotic *L. rhamnosus* was competing with a pathogenic *E. coli* for adhesion, and the affinities for the intestinal mucosa surface were equal for the two bacteria, it would take three times the number of the probiotics to exclude one pathogen. Experimental studies suggest that the affinity for adhesion sites correlates with the concentration of adhesion on the bacterial surface (Lee et al. 2000). It may be desirable to select probiotics that have a high concentration of adhesion on their surface and high affinity for the adhesion sites on mucosa surface (Figure 2.1). Some strains of lactic acid bacteria are prone to autoaggregation (Kmet et al. 1995; Lindgren and Jonsson 1999; Perez et al. 1998). Autoaggregation may lead to a false impression of high adhesion in an *in vivo* assay and self-competition for adhesion sites on the mucosal surface. Unfortunately, auto-aggregation is seldom included as a criterion for screening probiotic bacteria.

It has been widely reported that administered probiotic bacteria disappear from the gastrointestinal tract after only a few weeks (Jacobsen et al. 1999). This indicates that these probiotic bacteria are not able to grow in the tract fast enough to maintain a high local cell concentration to colonize the mucosal surface. Lactic acid bacteria make up less than 2% of the intestinal microbial population (Sghir et al. 2000). On the other hand, a bacterium able to establish on the mucosal surface permanently may be tolerated by the host immunological system and would not stimulate host immunity as reported among probiotic bacteria. The relationship in Equation 2.2 suggests that, irrespective of the bacteria's adhesion affinity for mucosal surfaces, pathogens arriving in contaminated food will displace indigenous bacteria as long as the concentration of the pathogen is sufficiently high.

It has been reported that 10 diarrheagenic *E. coli* cells are sufficient to cause an infection in a healthy individual. A recent study has reported that fecal bacteria did
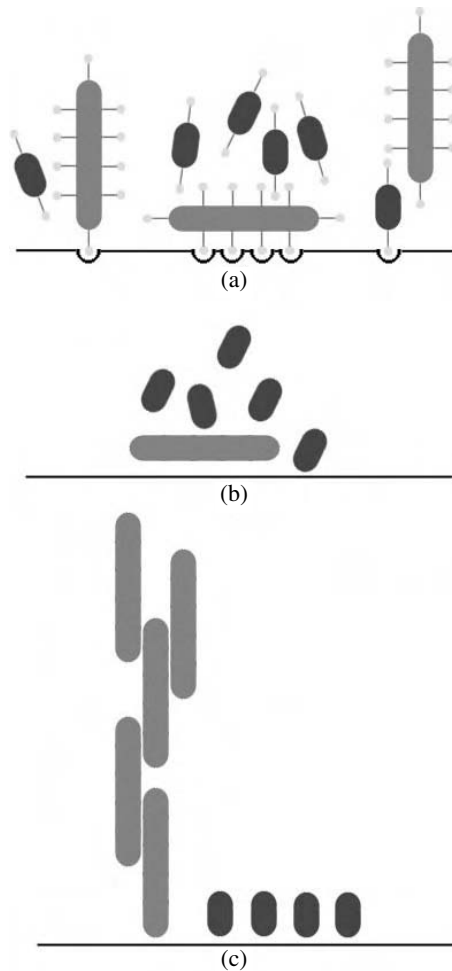
FIGURE 2.1 Mode of cell adhesion on intestinal mucosa and competition for adhesion: (a) specific adhesion–receptor interaction; (b) nonspecific surface interaction; (c) self-competition through autoaggregation.

not affect the adhesion of probiotic bacterium *L. rhamnosus* GG on mucus (Ouwehand et al. 1999), whereas probiotic bacteria are known to compete with pathogenic bacteria for adhesion on a mucosal surface (Heinemann et al. 2000). This raises the question of whether pathogens are binding on adhesion sites outside those occupied by indigenous bacteria. Indigenous bacteria may be ineffective in displacing pathogens, thus lengthening the duration of clinical symptoms compared to in individuals given probiotics. This is often observed. To displace the adhered pathogenic cells from the mucosal surface and to shorten the clinical symptoms, the ratio of the probiotic and the pathogen surrounding the adhesion site needs to be sufficiently high, so that the chance of a probiotic bacterium displacing the dissociated pathogen is high. It was reported that 6×10^9 cfu/g *L. rhamnosus* GG was effective

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TABLE 2.4
Requirements for Nutritional and Clinical[a] Studies of Functional Foods and Probiotic Foods with Health Claims

Well-defined probiotic strains
Each strain documented and tested independently
Extrapolation of data from closely related strains not acceptable
Well-defined study products
Well-defined study populations
Double-blind, placebo-controlled and randomized human studies
Results confirmed by several independent research groups[b]
Publication in peer-reviewed journals

[a] Clinical studies should be conducted according to good clinical practice in human subjects.
[b] Clinical results should be confirmed by at least two independent research groups.

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in shortening the duration of rotavirus diarrhea, whereas 10^8 CFU/g of an *L. rhamnosus* had no clinical effect. This finding may require reconsideration of the minimum desirable dose for a probiotic to impart health properties.

In a study involving competition for adhesion between *L. casei* Shirota and *E. coli* TG1, Lee et al. (2000) suggested that a soluble metabolite of *L. casei* Shirota may be involved in the exclusion of *E. coli* because competition for physical adhesion could not account for the ratio of the adhered number of the two bacteria. Spent culture supernatants of some probiotics have been found to inhibit adhesion of pathogenic bacteria to the mucus surface. Probiotic metabolites may play important roles in the exclusion of pathogens from the gastrointestinal tract when the concentration of the probiotic is at a subsaturation level and is not able to out-compete the pathogen through physical displacement. Carbohydrate–protein and hydrophobicity interactions have also been shown to take part in adhesion of probiotics and pathogens to intestinal mucosa (Granato et al. 1999; Perez et al. 1998).

2.8 SELECTION CRITERIA AND HUMAN STUDIES

Clinical studies with probiotic bacteria need to be developed and conducted further because only a few of the claimed effects are based on a hypothesis and backed by good clinical studies.

It is important to make sure that nutritional and human studies are well defined. Each strain and product should be documented and tested independently because extrapolation of data from closely related strains is not acceptable. Table 2.4 contains suggestions for scientific documentation of health effects in human studies. Protocols for human nutrition studies need to be developed for probiotics and functional foods (Sandström 1995). In some cases even postmarketing surveillance studies on intakes and long-term effects are desirable; such studies have also been used for the safety assessment of current probiotics. The design of clinical studies used in pharmaceutical development should serve as a reference point, but specific protocols and specific criteria relevant to functional foods may also be needed. It is necessary to

identify specific target groups of individuals who may present higher and lower susceptibilities to potential adverse effects. Clarifying the long-term consequences of the interactions between functional food components and functions in the body is also important.

In probiotic studies, it is imperative to ensure that the integrity of the strain used is maintained by regular comparison to a stock culture of the strain stored under stable conditions (e.g., freeze-drying or lyophilization). Knowledge of the genetic stability of the strain is also important. Factory quality control in this area is particularly important if valid clinical studies are to be carried out. New emphasis has recently been placed on the quality control measures for probiotic properties of lactic acid bacteria strains and these certainly need to be developed further for consumer protection purposes, process control and strain stability (Jacobsen et al. 1999; Tuomola et al. 2001).

2.9 MODELING AND GENETIC MODIFICATION OF LACTIC ACID BACTERIA

Modeling may become important as a tool for predicting probiotic properties and required doses when disease-specific, genetically modified probiotic lactic acid bacteria are introduced. Until now, no genetically manipulated probiotic organisms have been used; however, a recent report demonstrates that genetic modification may be a future tool for therapeutic probiotics. Steidler and coworkers (2000) have shown that an engineered *Lactococcus lactis* strain can effectively treat murine colitis by locally secreting interleukin-10 when present in the intestinal mucosa. This offers new views on therapeutic manipulation of the intestinal microecology and mucosal milieu (Shanahan 2000) and may provide future tools for probiotic development. Using such organisms will require even more stringent safety and efficacy assessment and improved modeling for dose–effect relationship characterization.

2.10 CONCLUSION

There is good scientific evidence for some proven strain-specific health effects of probiotic lactic acid bacteria. The efficacy of some strains has been proven in international multicenter studies and/or human studies conducted in different countries. However, mechanistic information and validation of current selection criteria against human studies are lacking. Therefore, more good clinical and nutritional studies in human subjects are required, as well as development of selection criteria and modeling methodologies to achieve improved and more specific probiotics with proven safety and efficacy.

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3 Successful Probiotic Bifidobacteria

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CONTENTS

3.1 INTRODUCTION

Until about the last decade, Western medicine, or indeed Western science, had very little interest in the nature or activity of bacteria within the intestinal tract. It had been accepted that metabolic activity of intestinal bacteria in animals probably played a role in the synthesis of micronutrients that might be of nutritional value to the host animal. However, the perceived wisdom was that absorption of such micro-nutrients depended upon the animal being coprophagic and, consequently, that this activity had little practical interest so far as the human was concerned.

The main interest in the microflora in the alimentary canal in Western medicine had been whether the intestinal bacteria present acted as pathogens, were purely commensal or had a potential to be pathogenic when the host's immunity became compromised. In general terms, if an organism could not be shown to have patho-genic potential within the intestinal tract, it was regarded as commensal and of little or no relevance. In some other countries, on the other hand, and particularly in Japan and parts of the Soviet Union, fermented milk products have been perceived as having health properties for well over half a century (Mitsuoka 1982). It was appreciated that these fermented milk products owed their health-giving properties to the bacteria used for their production and that many of these bacteria could be isolated from the intestinal tract of healthy humans. Thus, the properties of the intestinal bacteria achieved much greater levels of interest in these countries well in advance of that which has developed more recently in the West.

However, over the past decade or so, perhaps as a direct result of the development in interest in alternative forms of medicine and therefore of Eastern ideas, scientists in Western countries have become attracted to the activities of a range of bacteria that can be found within the intestinal tract. An estimated 100 trillion organisms are within the intestinal tract of the average human, comprising something of the order of 400 to 500 different species that have currently been recognized (Salminen et al. 1998); many others may not have been identified so far. These bacteria have a potential metabolic activity that appears to be of the same order as that of the liver of their host.

Of these, perhaps those with the greatest potential benefit for human health have been the Gram-positive lactic acid–producing organisms and, particularly among these, the lactobacilli and the bifidobacteria. These organisms have been termed the probiotic bacteria — a group that appears to have specific health-promoting potential. The term "probiotic" has no specific microbiological significance but recognizes the potential ability of the organism to act in a beneficial way on the host. It therefore represents a group of organisms that can be safely administered to the human subject without fear of producing undesirable sequelae, but with the potential for producing

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TABLE 3.1
Important Features of Probiotics

Must be abundantly safe
Must resist destruction by gastric and intestinal juices
Must show reasonable persistence in the intestinal tract
Should demonstrate antagonism to harmful intestinal bacteria
Should demonstrate desirable enzyme pattern
Should demonstrate immunomodulation activity
Must show desirable characteristics in animal studies

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changes of a beneficial character. There are now several recognized definitions of probiotics, but their essential properties are that they should be available as "live microbial food ingredients that are beneficial to health" (Salminen et al. 1998). The main recognized features of bacteria classed as probiotics are shown in Table 3.1.

At birth the intestinal tract is sterile, but within a matter of days it becomes colonized with various bacterial species (Mitsuoka 1994). Perhaps not surprisingly considering the milk diet of infants, the lactose-fermenting organisms, particularly the bifidobacteria, predominate. During children's and adults' lives, the proportion of the bifidobacteria is reduced steadily, but they still form a substantial proportion of the gut bacteria. This is considered in more detail in Section 3.7 on ecology.

3.2 HISTORICAL OVERVIEW OF BIFIDOBACTERIA

Bifidobacteria were first isolated by Tissier in 1900 from infant feces. He discovered a curious but characteristic Y-shaped anaerobic bacterium and named it *Bacillus* bifidus. It was originally regarded as a member of the family *Latobacillaceae* and it was not until 1924 that Orla-Jensen recognized the existence of a separate genus *Bifidobacterium* (Orla-Jensen 1924). Even then no movement was made in correcting the classification and it was not until as late as 1957 that the existence of several different biotypes of *Bifidobacterium* was accepted (Dehnart 1957); some years later classification based upon their carbohydrate fermentation pattern was first developed (De Vries et al. 1967; Scardovi and Trovatelli 1965). Even then the further developments in the classification did not follow rapidly or easily. By the mid-1980s the number of recognized species within the genus had risen to 24 (Scardovi 1986) and subsequently an additional 8 species have been recognized, bringing the total to 32. Using 16S rRNA analysis, the hierarchical structure of the genus was established as late as 1997 (Stackebrandt et al. 1997). The genus *Bifidobacterium* was collected together with the genus *Gardnerella* into a single family of *Bifidobacteriaceae* within the order of *Bifidobacteriales*.

3.3 SOURCES

Bifidobacteria have been isolated from several different sources, but the prime sources for each of these isolates have been the intestinal tracts or vaginas of animals

TABLE 3.2
The Species of Bifidobacteria Derived from Human Sources

Species	Human Sources	Reference
B. bifidum	Feces of adult and infant; vagina	Orla-Jensen, 1924
B. breve	Feces of infant; vagina	Reuter, 1963
B. infantis	Feces of infant	Reuter, 1963
B. adolescentis	Feces of adult, vagina	Reuter, 1963
B. longum	Feces of adult and infant; vagina	Scardovi and Trovatelli, 1974
B. catenulatum	Feces of adult and infant	Scardovi and Trovatelli, 1974
B. dentium	Feces of adult; appendix; dental caries	Scardovi and Trovatelli, 1974
B. angulatum	Feces of adult	Scardovi and Trovatelli, 1974
B. pseudocatenulatum	Feces of infant	Scardovi et al., 1979
B. gallicum	Feces of adult and infant	Lauer, 1990
B. denticolens	Dental caries	Crociani et al., 1996
B. inopinatum	Dental caries	Crociani et al., 1996

or humans, infants or adults. It appears likely that all are primarily of intestinal origin. The preference, not entirely based upon scientific principles, is to source probiotics from the same species as the potential end-user. In fact, the available evidence suggests that the origin is irrelevant for the use of an organism as a probiotic in the human. The original reason was that bacteria of animal origin would be more likely to be pathogenic in the human, but this does not necessarily apply. Several species originally isolated from animal sources have now been found in human feces. Thus, for example, *B. animalis*, which as its name implies was originally isolated from an animal (chicken), has for many years been used successfully in fermented milk products with no apparent danger to humans.

Perhaps more important than their isolation from the human, some of these organisms have been shown to be capable of adhering to human intestinal cells. Whether the property of cell adhesion is an absolute necessity for activity is still not clear, but if this is accepted as a desirable characteristic, then species with this characteristic would be particularly suitable for probiotic use for the human (Shah 1997a). Because there does seem to be organism specificity for probiotic activity in the human and although safety of some animal-derived organisms may not be a matter of concern, it will be important to establish that they possess the necessary characteristics to have health-potentiating properties in the human.

Thus, because the current account is basically concerned with the development and use of functional foods in humans, it follows that, at least at present, bifidobacteria derived mainly from human sources are the preferred organisms. The sources, designation and original description of each of the human strains are given in Table 3.2. Some of these species have also been isolated from animal species (indicating that species specificity is not absolute) and from sewage, but the important practical aspect is that they have been isolated from the human intestinal tract, whether that is adult, infant or both.

2 Glucose

Fructose-6-P   Fructose-6-P

Erythrose-4-P   Acetyl-P

Glyceraldehyde-3-P   Sedoheptulose-7-P   **Acetate**

Xylulose-5-P   Ribose-5-P

Ribulose-5-P

Xylulose-5-P

2 Glyceraldehyde-3-P   2 Acetyl-P
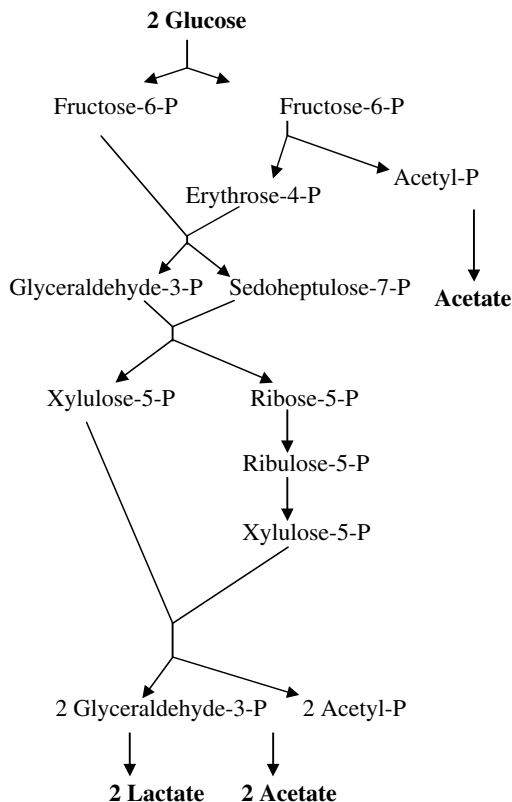
2 Lactate   **2 Acetate**

FIGURE 3.1 Formation of two molecules of lactate and three molecules of acetate from two molecules of glucose by bifidobacteria.

3.4 TAXONOMY

Bifidobacteria can be characterized as anaerobic, Gram-positive, nonmotile, non-sporing, catalase-negative organisms that may occur in a variety of different shapes including short, curved rods, club-shaped rods and Y-shaped bifurcated rods. They are saccharolytic organisms that produce lactic and acetic acid without the generation of carbon dioxide except when they are fermenting gluconate. As shown in Figure 3.1, the fermentation of two molecules of hexose by all bifidobacteria leads to the formation of three molecules of acetate and two molecules of lactate, the key enzyme being fructose-6-phosphate phosphoketolase. This characteristic enzyme activity may be used as the defining taxonomic characteristic of the genus but has no value for differentiation of the constituent species.

In addition to the ability to utilize glucose, bifidobacteria of human origin can utilize lactose and galactose. The majority can also utilize fructose. Besides their ability to metabolize simple sugars, a substantial number of the strains of the various species of bifidobacteria also have the ability to ferment complex carbohydrates, including amylopectins and gums.

Fermentation tests, particularly those involving the differential fermentation of polysaccharides and mucin, have been utilized as a method of identification of the various species of bifidobacteria; however, recent technical developments in DNA identification have made this a more reliable method. The main technique used recently involves a 16S rRNA gene-targeted, species-specific polymerase chain reaction (PCR) method. Alternatively, a pulsed-field gel electrophoresis method can be used that will also determine strain differences (McCartney 2002; McCartney et al. 1996).

The 16S rRNA analysis for the identification of bacteria was first used in 1987 (Woese 1987) and for bifidobacteria with 16S rRNA-targeted, species-specific probes in 1992 (Yamamoto et al. 1992). More recently, the process has been further improved by the development of species-specific probes targeted at different sites of 16S rDNA so that five bifidobacteria species (*B. bifidum, B. infantis, B. breve, B. longum, B. adolescentis*) can be determined simultaneously with PCR amplification (Dong et al. 2000; Kullen et al. 1997). This method can be used to provide a semiquantitative estimate of these species in feces (for example, after oral administration) and to check the identity and quality of commercial products. The technique will clearly benefit from further developments over future years. In addition to these regular species, a substantial amount of genetic modification has been undertaken on the *Bifidobacterium* genus (van der Werf and Venema 2001) and much of the experimental work has been undertaken on genetically modified forms.

3.5 PHYSIOLOGICAL PROPERTIES OF BIFIDOBACTERIA

The optimum temperature for growth lies in the range of 37 to 41°C, with the full growth range extending from about 20 to 45°C, though some strains are capable of growing outside this range. The optimum pH is between about 6.5 and 7.0 with the vast majority of bifidobacteria unable to sustain growth outside the range of 4.5 to 8.5. The genus requires anaerobic conditions for growth; however there is species variation in the sensitivity to oxygen levels.

The primary source of carbohydrate metabolism is through the fructose-6-phosphate shunt, which distinguishes bifidobacteria from the lactobacilli. This pathway generates L(+) lactic acid and acetic acid in the ratio of 3:2, but some species can also generate formic acid and ethanol, which has a profound effect on the growth characteristics. The L(+) form of lactic acid is regarded as an advantage for human infants because this is the physiological form directly utilized by humans. In addition to normal metabolic nitrogen sources, bifidobacteria have the ability (not shared by lactobacilli) to utilize ammonium salts as the sole nitrogen source.

Biavati et al. (1991) have studied the specific nutritional requirements of this genus extensively. Generally, bifidobacteria do not grow well in milk even though they can metabolize lactose; however, this is not due solely to their requirement for anaerobic conditions. In addition to the normal requirements for nitrogen, they need vitamins (some B-complex members that they can synthesize) and various growth factors, of which numerous examples can be found in the literature. Among the most

widely accepted as required are N-acetyl-glucosamines and N-acetyl-neuramic acid, both of which are present in higher concentration in human milk as opposed to cow's milk. The disaccharide lactulose (composed of galactose and fructose) is also an effective factor for bifidobacterial growth and has been used for this property in the preparation of various foods. However, it has a more important role in stimulating bifidobacterial activity in the colon.

In addition to these general and specific growth factors for the bifidobacteria in culture, a group of fructo-oligosaccharides that provide a source of nutrition for bacterial (specifically, bifidobacterial) growth in the colon can be distinguished. This group of compounds has been classed together by many workers in the field as bifidogenic factors, although this term is sometimes given a broader interpretation. This effect, which comes within the activity defined as prebiotic, is considered in more detail later.

The cell wall of bifidobacteria has the typical Gram-positive structure with a thick peptidoglycan envelope. This envelope contains proteins, polysaccharides and techoic acid; the tetrapeptide composition shows variation among the strains (Tamime et al. 1995) in terms of the constituent amino acids and the cross-linkage of adjacent tetrapeptides. Lipoteichoic acids form links with polysaccharide chains within the cell walls, which may be important for the cell adhesion characteristic of most bifidobacteria.

3.6 PRACTICAL ASPECTS OF BIFIDOBACTERIAL FERMENTATION OF MILK

Attention has already been drawn to the fact that, although bifidobacteria grow reasonably well in human milk, cow's milk fermentation by bifidobacteria is slow and far from ideal as a means of producing fermented milk products. Bifidobacteria grow well on various synthetic media, e.g., MRS and TPY broths *inter alia*. Unfortunately, these media are not suitable for large-scale commercial exploitation of bifidobacteria. Quite apart from the costs of such synthetic media for large-scale harvesting of these organisms, they generate off-flavors to the products, making them unsuitable for the production of palatable foods. This particular method for the commercial exploitation of the bifidobacteria has no current merit.

Although milk theoretically contains all the essential nutrients for the growth of bifidobacteria, the level of amino acids and low molecular weight peptides is insufficient to provide the ideal conditions for rapid growth or to maintain prolonged growth of this genus (Gomes et al. 1998; Klaver et al. 1993). In consequence, bifidobacteria have been used only to a limited extent in the commercial milk fermentation process. Indeed, were it not for the potential health-giving properties of the bifidobacteria, it is unlikely that they would be considered at all for milk fermentation.

Nevertheless, fermented milk is the preferred carrier for bifidobacteria; consequently, a substantial number of studies have been undertaken to try to determine methods by which the growth potential can be realistically improved. These studies have included processes such as strain multiple transfer (Kurmann 1998), which in

some cases has resulted in strain adaptation or use of milk from other ruminants (Gomes and Malcata 1998). Unfortunately, neither of these techniques has been appropriate. Better results have been achieved by the addition of various nitrogen sources and redox reduction agents and by specific strain selection of bifidobacteria (Gomes and Malcata 1998; Gomes et al. 1998; Ventling and Mistry 1993). Some of the best nitrogen sources exploited to date have been whey proteins (Petschow and Talbott 1990).

However, in spite of the technical problems involved, the majority of the bifidobacteria-containing probiotic products that are available commercially are based upon fermented milk products. These include fermented liquid milks, yogurts, flavored deserts, ice cream, frozen yogurts, and cheese, though some recent products have tried to exploit the potential additional health-giving property of soy protein by using fermented soya milk or soya yogurt. Increasing use of probiotic products for their reputed health benefits, coupled with the general tightening of regulations governing food safety and reliability of food claims, quite rightly makes the entire question of quality control increasingly important.

The important principles that apply to the production of any probiotic product are:

- The bacteria stated to be included in the preparation should accurately represent the actual content (Hamilton-Miller et al. 1996).
- The number of living organisms at the time of production should not only be equal to the stated content, but this number should also persist throughout the stated shelf life of the product. It has been suggested that the minimum number of viable organisms in any product should be at least 10^5/g (Shah 1997b), that this must be an absolute minimum, and that preference is for somewhat higher levels to be achieved. The advice concerning storage should be such that the shelf life can be achieved reliably. This implies that conditions in relation to storage temperature, temperature variation and light must be defined carefully and that any influence on the exact nature of the food product must be taken into account in determining that storage conditions are appropriate and the advised shelf life can be achieved with the exact food conditions. This will involve studies that take into account the final pH and natural variation that may occur, as well as the influence of each organism on the others in the case of products containing mixed cultures.
- The product must have the required qualitative and quantitative level of the probiotic component and palatability of the whole food must be good at production and maintained throughout the entirety of the shelf life. The fundamental concept of probiotics is that they should be ingested as foods and not as medicines; therefore, consumer acceptance is an important consideration.
- Because the product is consumed not only by normal healthy people (by probiotic definition) but also by other members of the community (intentionally or by accident), it is vital that any and all of the organisms

incorporated, alone and in their combination, be classified as GRAS (generally recognized as safe). The safe consumption level should be substantially above the advised intake levels. Experience shows that whatever the rights or wrongs, medications are frequently ingested at levels higher than the advised levels and that overconsumption of foods with potential health benefits occurs to an even greater extent. Many members of the public believe that if one helping of a food is good for health then ever increasing helpings must be even healthier. Specifically, this must involve consideration of potential problems that may arise in the case of children, e.g., foods should not need to be held in a place inaccessible to children. Even more, it is important to ensure that those with immune deficiency disorders can consume the product without risk.

- For many years, most countries have been very lax over product claims, which have been accepted or even legally permitted for food. Approximately 40 years ago health claims for therapeutic substances were very strictly controlled and subjected to close scrutiny by the appropriate authorities. Claims for natural products and for foods have not been subjected to this scrutiny. Moreover, when they have been examined, the tests applied have been far less rigorous, particularly in terms of the levels of control against bias (Salminen et al. 1998; Sanders and Huis in't Veld 1999). This is now being corrected and those involved in the marketing of these products should welcome these moves. The matter of controlled tests applies to studies in humans and also to those in animals.

Specifically for probiotics, it is vital to demonstrate that when ingested as recommended in the product form, the probiotic bacteria should reach the relevant portion of the intestinal tract at an adequate concentration and in a viable state and should be capable of exerting their effects in the particular host studied.

For probiotics in general and bifidobacteria in particular, there are some technical difficulties in ensuring that good products can be produced on the basis of a fermented milk base. Thus, as already noted, bifidobacteria do not grow readily in milk and fermentation is particularly slow in cow's milk, so it is difficult to develop a reliable product that maintains stability during shelf life. These technical problems are discussed in some detail in reviews by Gomes and Malcata (1999) and in more general terms in Heller (2001). They point out that when there is difficulty with organisms that do not grow rapidly in a milk medium, even those fortified with the appropriate growth factors for the particular strain, the best procedure to consider is the use of a classic culture as a facilitating organism. This is characteristically a synergistic mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp bulgaricus. Alternatively, a single culture of a lactobacillus may be used. It is then possible to consider the addition of the bifidobacteria at the same time as the yogurt culture or to await the completion of the main fermentation, rapidly cool the preparation from the 37°C of the prime incubation to the normal refrigeration temperature, and then add the probiotic at an appropriate level. The main objections to the use of simultaneous early administration of any probiotic are:

- One of the organisms may inhibit the growth of the other. This is particularly the case with the addition of the probiotics because these have the ability, by production of an acid or generation of bacteriocins, to suppress other organisms. Thus a valuable natural property of the organism may act against the ability to produce an appropriate product for administration.
- Starter organisms may develop other chemical substances, e.g., hydrogen peroxide, benzoic acid, or biogenic amines, that can influence the viability of the bifidobacteria.
- The combination of the starter organisms with the bifidobacteria can produce a profound change in the texture or flavor of the fermented milk product that may seriously reduce the palatability of the food.

On the other hand, it may be difficult or even impossible to add the probiotic after the main fermentation has taken place because of the texture of the fermented milk product, e.g., curd formation. The exact technique used depends on studies undertaken by food scientists in the manufacturing–dairy industry. Although general technical characteristics are usually available or can be guessed on the basis of general principles, the exact details of production are usually retained as trade secrets.

3.7   ECOLOGY

The main early study on intestinal ecology relating to the bifidobacteria is that of Mitsuoka (1982). Of the total number of organisms in the intestinal tract, which amounts to some 10^{12} to 10^{14} organisms, approximately 10% can be classified as within the genus *Bifidobacterium*. The actual number and the percentage vary substantially depending upon such factors as age, diet (particularly the polysaccharide content) and lifestyle of the individual.

Mitsuoka demonstrated that bifidobacteria and lactobacilli first appear in the intestinal tract a few days after birth and at that stage they form the major flora until weaning. The exact relationship of the bifidobacteria to the other organisms in the intestinal tract at this stage is largely dependent on whether the infant is breast fed or the exact infant milk formulation used (Benno et al. 1984; Edwards and Parrett 2002; Kawaze et al. 1981). The number of bacteroides, streptococci and eubacteria then increase and the lactobacilli and bifidobacteria become less of a feature. Mitsuoka also suggested that, later in life, the bifidobacteria decrease in number (Mitsuoka 1982). However, a recent study from Finland (Kirjavainen et al. 1998) has cast doubt on any reduction in bifidobacterial number later in life as a physiological phenomenon and has suggested that such a change, if it occurs, represents an adverse response to the lifestyle or diet of the person. According to this suggestion, the bifidobacteria form a numerically and biologically important component of the human intestinal ecology throughout normal life and any reduction in their importance may have a significant influence on health.

3.8 PREBIOTICS AND BIFIDOBACTERIA SPECIES

Attention has already been drawn to the existence of bifidogenic factors. Usually complex carbohydrates, these compounds, when administered by mouth to the host, survive metabolism in the upper part of the intestinal tract, reach the lower intestinal tract unchanged and can encourage the growth of existing bifidobacteria there. They achieve this effect by being specific sources of energy for these organisms. Some of these factors may have a specific growth-promoting effect limited to the bifidobacteria; some also increase some or many lactobacilli. However, it appears that the effects seen with these ingested factors are predominantly the result of an increase in the proportion of bifidobacteria in the lower portions of the intestinal tract.

Because these factors exert health-giving properties by encouraging an increase in the proportion of probiotic bacteria, they have been categorized as prebiotics. Additional activity can theoretically be achieved by the simultaneous ingestion of prebiotics and probiotics (e.g., bifidobacteria) and these have been termed synbiotics (Gibson and Roberfroid 1995).

The major current carbohydrate bifidogenic factors are fructo-oligosaccharides (Gibson and Wang 1994a; Tomomatsu 1994). A substantial number of fructo-oligosaccharides are already known to possess significant bifidogenic activity and these can increase the number of bifidobacteria in *in vitro* and *in vivo* conditions several fold. Many of these oligosaccharides are derived from plant fibers, particularly vegetables; this is probably the reason for some of the desirable (and undesirable) effects in animals of high consumption of vegetables. Some of them are also capable of being metabolized by other enzymes within the intestinal tract to generate substantial amounts of gas. The resulting bloating is certainly one of the substantial disadvantages of this approach to the generation of increased numbers of bifidobacteria in the lower part of the intestinal tract.

In spite of this well-recognized adverse property, prebiotics have been the subject of considerable research during recent years. Apart from the oligosaccharides that occur in natural products, a substantial number of other synthetic compounds, particularly in the range of galacto-oligosaccharides, can act as effective bifidogenic agents. These galacto-oligosaccharides are formed by the bioconversion of lactose using various microbial enzymes; a substantial number of publications now describe their preparation (see Chapter 5). As a result of this interest, synthetic polysaccharides containing monosaccharides other than galactose are now being investigated. It seems very likely that, over the next decade, a substantial number of radically different prebiotics may have interesting commercial possibilities.

In general terms, researchers interested in the possible nutritional and health-giving properties of a balance in the intestinal flora can be divided into those who suggest that the probiotic approach is preferable and those suggesting that the prebiotic approach is preferable. In fact, it appears likely that if the difficulties inherent in the production of palatable synbiotic food products are overcome — and significant difficulties would need to be overcome — this could well be the ultimate preferred solution. Nevertheless, it must be accepted that the use of prebiotics represents a very interesting alternative approach to encouraging increased numbers of bifidobacteria in the intestinal tract.

3.9   NUTRITIONAL VALUE OF BIFIDOBACTERIAL MILK PRODUCTS

Although some doubt about the probiotic characteristics of bifidobacteria-containing products may still exist, the nutritional characteristics are well established. They depend mainly on the nature of the milk product used and whether it has been supplemented before the addition of the starting culture. However, the fermentation can also produce some interesting modifications, depending in part upon which bifidobacterial strain is used, whether it is included in the original starter culture and whether other milk-fermenting organisms have also been employed.

The main changes in the macronutrients occur in the lactose and amino acids. As a result of the fermentation, the level of lactose will be substantially reduced and, depending upon the exact nature of the fermenting organisms, will have been replaced by acetic acid and lactic acid (if bifidobacteria form part of the starter culture) or lactic acid alone, if the bifidobacteria are only added at the end of the fermentation process when the available carbohydrate has been substantially reduced. The lactic acid formed in the fermentation is in the L(+)– if yogurt-type organisms are involved. This is more easily metabolized after absorption (generating almost the same energy as the lactose) than the D(–)– form, which is the end result (with acetic acid) of fermentation with only bifidobacteria. The other main difference is that, by increasing the amount of acid in the product, the production of lactic and acetic acids with bifidobacteria not only produces a very sour flavor and reduces acceptability of the food, but also is capable of producing a metabolic acidosis in young children. However, the fermentation also results in a breakdown of some of the milk protein and an increase in the free amino acids that may assist the digestibility.

Milk fermentation that involves bifidobacteria has been reported to increase the vitamin B-group content of the product, particularly in thiamine, niacin, pyridoxine, folic acid and vitamin K (Tamime et al. 1995). This corresponds to the effects seen with the other main forms of probiotics, namely, the lactobacilli. The results appear to be strain specific, particularly for riboflavin, with *B. bifidum* an especially high accumulator strain for the majority of the B-complex vitamins. However, there must be doubt about the relevance and importance of these results when applied to the administration of bifidobacteria to animals and, even more, to humans.

In the first place, the studies have been undertaken with mixed yogurt-type cultures as opposed to pure cultures of bifidobacteria. Second, these studies with mixed cultures have indicated that probably some of the production will be utilized for bacterial metabolism; thus, absorption of vitamins from the human colon may be slow and variable. Elmadfa and co-workers have shown that the commercially available probiotic yogurt mixture that they examined did not influence the thiamine, riboflavin or pyridoxine status of healthy adult humans (Elmadfa et al. 2001). However, this does not totally preclude the possibility that a pure culture of bifidobacteria might have some effect. Therefore, in nutritional terms, bifidobacteria exert their main potential benefits by reducing the lactose content of the lower part of the intestinal tract, which is particularly beneficial to those with lactose intolerance (see Chapter 4).

3.10 NONCLINICAL STUDIES RELATING TO POTENTIAL HEALTH-PROMOTING AND THERAPEUTIC EFFECTS OF BIFIDOBACTERIAL MILK PRODUCTS

The principle of probiotic activity is that the oral administration of the probiotic organisms allows the specific probiotic bacteria present in the food to enter the intestinal tract. Assuming that they are not destroyed by passage through the stomach or the upper part of the intestine, they will reach the distal intestine. This applies whether concern is for a potential health-maintaining effect in healthy human subjects or the medicinal prophylactic or therapeutic use of such a product in specific clinical disorders. Thus, a vital requirement toward making any health-maintaining, prophylactic or therapeutic claim is the establishment of safe passage of the bifidobacteria into the lower part of the intestinal tract. Although most bifidobacteria are acid and bile resistant, some (e.g., *B coryneforme* ATCC 25911) that lack conjugated bile salt conjugase activity are killed by glycodeoxycholate but not by taurodeoxycholate (Grill et al. 2000).

The determination of adequate numbers of viable organisms initially involves studies that will examine the composition of the bacterial component of the milk-based product in terms of its nature and quantitative characteristics. This will require study of the product as produced and also determination of the specific storage requirements for maintenance of product stability. This will lead to the definition of the appropriate maximum allowable length of storage under these defined conditions. The main characteristics of the product to be studied are shown in Table 3.3. The studies of Hamilton-Miller et al. (1996) have indicated that many commercially available (in Europe) probiotic preparations fail tests for species and/or quantity of the contained organisms.

In vitro characteristics of the prime strain and any combinations of the strains should be undertaken. Some opinions will diverge on exactly which *in vitro* characteristics are appropriate or ideal from this point of view. Moreover, with further experience the list of such characteristics will change as more is learned about which of these characteristics best mirror the *in vivo* activity and, ultimately, the effects of the products in human subjects. Those regarded as important at present are shown in Table 3.4.

One of the tests over which views diverge concerning the clinical relevance is the study of *in vitro* adherence properties. These cell adherence tests are usually undertaken against defined human intestinal cell lines including HT-29, Caco-2 and HT29-MTX. On balance, the current evidence suggests that some degree of cell adherence is advantageous. Studies have indicated that some bifidobacteria antagonistic to pathogenic bacteria show intestinal cell adherence (Gopal et al. 2001); however, this relationship does not seem to be universal among the probiotics. The mucus binding to the cells of at least some strains of *B. lactis* is increased by the presence of other lactic acid bacteria, showing that the adherence property sometimes involves complicated ecological relationships (Ouwehand et al. 2000).

Studies such as those of Gupta et al. (1996), Collins et al. (1998) and, specifically for strains of bifidobacteria, Misra and Kuila (1995) have indicated substantial

TABLE 3.3
Product Characteristics to Be Checked before a Bifidobacterial Product Can Be Considered as a Potential Probiotic Food or Medicine

Characteristic	Comment
Defined organisms present	Check the organisms by the classic cultural characteristics. Ensure that the strain characteristics are clear by modern gene studies. Make sure that other organisms or strains are not present. Ensure that it is possible to achieve appropriate quantitative characteristics regularly.
Examine taste characteristics at time of preparation	The initial flavor characteristics will depend in part on the strains used and in part on the acid level achieved during the defined conditions of fermentation. It is also important to ensure that the texture of the product is acceptable in relation to the requirements of the market.
Determine optimum storage requirements	This must be undertaken on a trial and error basis. The initial storage tests should be undertaken under conditions that are the easiest to achieve in commercial practice; however, if these do not give an adequate shelf time then more rigorous conditions should be tried until stability is achieved. It should be noted that the stability in storage does not depend only on the temperature at which storage is maintained but also on the characteristics of the product. Thus, it may depend in part on the pH level present after the completion of fermentation, and also on subsequent changes in the pH during any interactions during storage. A further characteristic to examine is whether any precipitation of the bacteria occurs during storage. It is important to ensure that taste and texture are consistent during storage and that any changes in the total number of organisms are small; any variation in the proportion of the different organisms defined as being present should not change over the defined shelf life. From the practical point of view, the stability should be about 4 weeks at normal refrigerator conditions. Reduction below this level produces substantial logistical problems. It should be appreciated that most current studies indicate that it is very difficult to achieve a stable commercial product containing bifidobacteria. Thus, these studies are vital for this particular probiotic.
Determine criticality of storage limitations	The difficulty of ensuring that storage requirements are adhered to absolutely consistently must be appreciated. Thus, it is important to know the level of deterioration that occurs at various suboptimal conditions.

TABLE 3.4
***In Vitro* Characteristics Currently Regarded as Desirable for Probiotics in General and Bifidobacterial Products in Particular**

In vitro Tests	Comments
Survival at pH 2 to 3	This is the first stage in determining whether the product is likely to persist in a viable state during passage through the stomach. Determination of time/content of viable organisms is required.
Survival in aspirated gastric juice	Studies in artificial gastric juices derived from diluted acids do not provide full indication of stability in passage through the stomach, so studies in aspirated gastric juice are highly desirable. This also enables a determination of the potential viability in subjects with different gastric juice characters.
Bile tolerance	The first study that is probably worthwhile is time/content of viable organisms in ox bile. However, human aspirated bile that is reasonably and easily available is preferable. It is arguable whether a study in human bile is *essential* or whether later *in vivo* studies will give more information. This will depend in large measure on the ease with which human bile can be obtained.
Natural gastric juice followed by natural intestinal juice aspirates	The more natural conditions to mimic passage through the intestine are provided by these further studies. However, it can be validly argued that these are by their nature still artificial and that more information can be achieved at less cost in later *in vivo* trials.
Effects on normal stomach/intestinal components	Tests, for example, on amylose activities, are often regarded as valuable at this stage.
In vitro antibacterial activity	It is desirable to have an indication from *in vitro* studies of what range of pathogenic and commensal organisms appear to be sensitive to any antibacterial action of the bifidobacteria. Ideally, the mode of this interference, whether competition with growth substrates or by the production of acid conditions or bacteriocins, is desirable. It is likely that such studies will become increasingly interesting as more is learned about the mechanisms that favor good probiotic activity of different types in humans.
Level and characteristics of intestinal cell adherence	There is still considerable discussion of the relevance of intestinal cell adherence. It is clear that the probiotics do not colonize the intestinal tract; indeed, if they were to do so, this might render them more likely to be unsafe. Studies have indicated a substantial level of adhesion potential among the bacteria that appear to have probiotic value. In theory it is suggested that some adhesion is necessary in order to affect the cells of the intestinal wall. However, it appears that there are different forms of adhesion and that current adhesion tests are not reliable markers of probiotic activity.
Chemical binding characteristics	It is suggested that some of the desirable properties of probiotics, including bifidobacteria, depend upon the ability to bind with intestinal substances (e.g., cholesterol or bile salts). Some groups are now examining these characteristics, whose relevance as markers is far from clear.
Antibiotic sensitivity	This has importance at a later stage relative to clinical safety.

variability in these tests between different species as well as different strains of the same species. Unfortunately, although substantial evidence is available on the *in-vitro* characteristics of different species and strains, virtually no comparable clinical data are available. Consequently, the relevance of these characteristics from the point of view of merits for human use is small. Among the studies that may have some relevance are those of Bernet et al. (1993), Fujiwara et al. (1997) and Romond et al. (1997).

Attempts have been made to develop these *in vitro* techniques into ones that more closely resemble the situation within the intestinal tract. Dynamic computer-controlled models have been developed that mimic not only the various compart-ments of the intestinal tract (stomach, small intestine and colon) but also the natural movements of the chyme through the tract (Minekus et al. 1995). Other similar but more complicated computer-controlled models have also been developed that attempt to mimic activities in the large intestine (Minekus et al. 1999). Such models have been used for the examination of potential probiotics, including bifidobacteria (Mar-teau et al. 1997). Because these models do not allow any interaction with natural epithelium, with active transport of metabolites or with the potential for immune responses, considerable doubt must exist as to whether they offer a great advantage over the previous information from classical *in vitro* studies.

In addition to these *in vitro* observations, several *in vivo* animal studies are currently applied in order to attempt to determine potential probiotic activity. These include studies on normal and gnotobiotic animals. The potential markers of pro-biotic activity sought by these studies are shown in Table 3.5.

The greatest possibility for the use of such animal studies lies in the area of influencing carcinogenic or mutagenic activity. In animal studies it is possible to administer various chemical carcinogens and in gnotobiotic animals to determine the effect of feeding a selection of organisms to represent a defined intestinal flora. The intestinal environment can then be altered by administering putative probiotic organisms. It is possible to determine carcinogenic effects by seeking changes in the crypt cellular development, by determining frank tumors or by mutagenicity tests on cell cultures.

The other tests in which it is important to undertake animal studies are those related to a potential of the probiotic to influence an inhibitory effect on pathogenic organisms. It is possible, of course, to undertake ethical studies on humans infected naturally with a known attenuated pathogen. However, it is valuable to have prior knowledge and understanding of the effect in experimental animals so that the exact conditions of infection and administration of probiotic can be controlled.

Table 3.6 shows studies that can be undertaken in animals and, more importantly, in human volunteers without risk of adverse reactions, which may indicate potential probiotic activity. The important difference is that any studies in which adverse reactions are predictable cannot be undertaken in human volunteers. On the other hand, when no such potential risks are present, studies in the human have clear advantages. Any study in human subjects must follow the Helsinki principles and be approved by an ethics committee. For example, it is important to confirm the validity of the *in vitro* tests for acid and bile resistance by finding bifidobacteria in

TABLE 3.5
Potential Indicators of Probiotic Activity That Can Be Studied in Gnotobiotic and Normal Animal Models[a]

Study	Potential Value
Immune modulation	Substantial evidence indicates that one potentially beneficial activity of a probiotic lies in modulation of immune responses, perhaps as a result of interactions with cells in the Peyer's patches in the intestinal tract (the GALT system). Although some studies can be undertaken in humans, there are merits in being able to control the intestinal bacterial flora (by manipulation of gnotobiotic animals), as well as advantages in being able to study the effects of selected pathogenic bacteria. Such studies cannot, for obvious reasons, be undertaken in humans. The main difficulty lies in interpreting the significance of the immunological changes found.
Antimutagenic activity	The majority of the studies for anticancer activity involve studies that examine the potential of the compound to inhibit cell changes resulting from administration of carcinogenic substances or those that produce cell mutation. These studies clearly cannot be undertaken in humans. The substances involved include azomethoxymethane, N-methyl-N-nitro-N-nitrosoguanidine and 1,2-dimethyl hydrazines. The potential effect of probiotics can be determined by examining crypt changes in the intestinal tract or cell mutations. Other studies have been undertaken on induced tumors transplanted into other sites or animal species.

[a] This table excludes aspects that can be studied in human subjects without risks. These are often initially undertaken in animals during the selection of potential probiotics for human study and are considered in Table 3.6.

the feces or, preferably, in the distal part of the intestine after ingestion in the human. Such tests have indicated that several of the bifidobacteria species are indeed capable of persistence in a viable state during passage through the intestinal tract (Pochart et al. 1992) and could alter some, but by no means all, intestinal flora enzymes (Marteau et al. 1990). Others who have found that the administration of bifidobacteria alters the intestinal flora include Tanaka et al. (1980) using *B. bifidum* 4007 and *B. breve* 4006 in adults and infants and Kan et al. (1977) using *B. bifidum* 4002 in formula-fed infants

However, it is not yet clear whether the presence of exogenous bifidobacteria in the lower intestinal tract as a result of their oral administration in a dairy formulation is of value in normal humans. Amann et al. (1998) have shown that a 12-day feeding program of 10^{10} bifidobacteria daily in five men and eight women (all healthy on a U.S. food intake) altered the number of exogenous bifidobacteria in the feces but did not alter the total number of bifidobacteria nor change the breath hydrogen excretion. A similar effect on the fecal numbers as a result of 5 weeks of feeding *B. longum* were found by Benno and Mitsuoka (1992), but in their study a reduction in the pH, ammonia concentration, β-glucuronidase activity and fecal number of clostridia and bacteroides was seen during the period of administration. A similar

TABLE 3.6
Studies That Can Be Undertaken in Human Volunteers to Determine the Potential of Bacterial Strains to Act as Probiotics

Study	Potential Information
Determination of viability of potential probiotic in the intestinal tract	The entire determination of probiotic potential depends upon the viability of the organism through to the lower intestinal tract in sufficient numbers to show potential activity. At present most of these studies depend upon the demonstration of viable bacteria in the feces during administration of the defined daily intake. Ideally, it would be better if studies were undertaken on samples obtained from different intestinal sites, but such studies are currently rare. Modern genetic techniques should be used to confirm the exact nature of the cultured organisms.
Inhibition of other bacteria	This study will give an indication of which of the intestinal flora will be inhibited by the presence of the presumed probiotic organism. It will not indicate whether the effect is due to interference with the nutritional requirement, the high level of acid generated by the probiotic, or a cytosyn. As with any studies on the intestinal flora, it is desirable that the designation of the bacterial contents should be checked by one of the modern genetic probes. Clearly, except as a matter of chance, it is unlikely that such studies will give any evidence about the effect on known pathogens.
Inhibition of bacterial enzymes	A possible probiotic effect in relation to potential mutagenic effects of the intestinal flora may be indicated by the effect of the probiotic on enzymes generated by intestinal bacteria. Enzymes that have potential for the activation of mutagenic activity include bile salt hydrolase, β-glucuronidase, nitroreductase and azoreductase. The techniques for undertaking the study are clear, but significance of the results is less clear.
Natural bacterial metabolites	Fecal metabolites that may act as indicators of potential health problems arising in the intestinal tract include such substances as ammonia, phenols and cresols. Although it is relatively easy to determine these metabolites and to show the influence of putative probiotics on their levels, considerable doubt about their relevance as biomarkers still exists.
Fecal mutagens	Evidence indicates that the ingestion of fried, barbequed or grilled red meat can give rise to the production of mutagens within the intestinal tract. Some potential probiotic organisms can be shown to reduce the level of these mutagens. The significance of these findings as markers of carcinogenic risk is currently unclear. One of the more recent developments is the study of fecal water in mutagenic studies; however, similar to all these studies, the significance of such studies is unclear.
Influence on immune reactions	Some studies have been undertaken on possible modification of immune reactions to nonpathogenic antigens. This may give an indication of the immune modulation potential of the possible probiotic.

effect on bifidobacterial numbers and the other intestinal flora has been reported in a recent Japanese study (Fujiwara et al. 2001).

It may well be that there is an optimum level of bifidobacteria and possibly other normal organisms in the intestinal tract (perhaps dependent on the nature of the food intake) and that oral administration will only be effective under suboptimal environmental conditions. If this is the case, then it may still be that the ingestion of probiotics has value if the normal level is suboptimal or the exogenous lactic acid bacteria strains have properties that differ from those naturally present in the individual or particular segment of the community. Such an effect is suggested by findings that the daily administration of capsules containing *L. acidophilus* and *B. bifidum* increased the fecal numbers of these organisms in women with premenstrual syndrome, but not in normal controls (Minelli et al. 1996).

A more extensive indication of the details of all these animal and human studies and their relevance to the investigation of potential probiotics has been considered in detail recently by a multinational expert group (Salminen et al. 1998). Their paper includes a critical appraisal of the practical relevance of these tests; for a more extensive appraisal of the topic, see the references at the end of this chapter.

3.11 HEALTH-PROMOTING, PROPHYLACTIC AND THERAPEUTIC POTENTIAL FOR BIFIDOBACTERIA-CONTAINING MILK PRODUCTS

In many countries recent legislation or impending regulations distinguish among the health-promoting effects arising from the ingestion of food products, prophylactic effects in specific disorders resulting from administration and the therapeutic use of these products. This will influence not only the form in which the claim can be made but also the type of study required to demonstrate the effect. This particularly involves the part played by studies in *healthy* individuals, the definition of "health" in this sense and the relevance applied to studies in those who do not fit within these definitions.

The topic of regulation of health claims is currently a very difficult one; different approaches are seen in different countries and the position is likely to change significantly over the next few years or so. It is therefore inappropriate to consider the details of these distinctions in this chapter. One recent discussion of the situation directly related to probiotics is in the paper by Sanders and Huis in't Veld (1999) and readers requiring greater details are referred to this paper. This chapter, therefore, deals with the generality of the potential or known clinical effects of bifidobacteria-containing milk products without considering whether these fit into the detailed consideration of a health-promoting, risk-limiting, prophylactic or therapeutic effect under various current or potential regulations. This chapter considers each of the possible potentially beneficial effects for the bifidobacteria in turn, and defines the current status of studies related to these effects. The potentially beneficial effects are shown in Table 3.7.

TABLE 3.7
Potentially Beneficial Effects of Bifidobacterial Species on the Basis of Current Published Evidence

Reduction of lactose intolerance in those with lactase deficiency
Cholesterol-lowering effect
Regulation of intestinal motility
Modulation of intestinal and systemic immune functions and bacterial balance
   Improvement in the status of premature infants
   Reduction in atopy and food allergies
   Improvement in intestinal viral infections
   Improvement in intestinal bacterial infections
   Prophylaxis of travelers' diarrhea
   Prevention of antibiotic-generated intestinal dysfunction
   Reduction of/protection from radiotherapeutic intestinal dysfunction
Management of irritable bowel syndrome and inflammatory bowel disease
Prevention of carcinogenesis (particularly intestinal)

3.11.1 REDUCTION OF LACTOSE INTOLERANCE

Numerous studies have indicated that various dairy products containing lactic acid bacteria are able to facilitate the digestion of lactose in those who have a lactase deficiency. This lactase deficiency can occur as a result of a genetic abnormality or as a result of short bowel syndrome. A study by Jiang and colleagues indicated that milk supplemented with *B. longum* strains B6 and ATCC 15708 produces a significant improvement of lactose digestion (Jiang et al. 1995). However, a volunteer study in 15 lactase-deficient healthy adults who received a dairy product containing *L. acidophilus* and *Bifidobacterium* species showed no better effect than normal yogurt. It is suggested that any improvements seen might be due to the slow passage from the stomach of the semisolid form of the lactose-containing milk (Vesa et al. 1996).

3.11.2 CHOLESTEROL-LOWERING EFFECT

Some of the now numerous studies with dairy preparations that have examined the potential for cholesterol reduction purport to show a clinically interesting effect (Marteau and Rambaud 1993). One possible mechanism proposed for this was that the presence of organic acids generated by the lactic acid bacteria could inhibit cholesterol production (Fernandes et al. 1987). Alternatively, it was suggested that the effect of lactic acid bacteria on bile acids might inhibit the absorption/reabsorption of cholesterol from the intestinal tract (Klaver and van der Meer 1993; Tahri et al. 1995). A more recent study has indicated differences between the effect of *L. amylovorus* and *B. breve* on cholesterol in the presence of taurocholic acid and that such differences between different organisms may, in part at least, explain the variations seen in experimental studies. However, although there appear to be some successful animal experiments and even some poorly controlled positive human

studies, the long-term results of lactic acid bacteria on lowering cholesterol in humans have not been encouraging (for discussion, see Richelsen et al. 1996).

No convincing evidence exists for any cholesterol-lowering effects by *Bifido-bacterium* species under clinical conditions. At present substantial doubt exists as to whether any of the current probiotics exert such an effect.

3.11.3 REGULATION OF INTESTINAL MOTILITY

Several studies, some of which rely on clinical observations and some that use intestinal transit times, have shown that intestinal motility is improved by adminis-tering lactic acid bacteria. Similar results have been found with preparations con-taining bifidobacteria. An uncontrolled observation in a group of healthy women showed that *B. animalis* DN-173 010 shortened the transit time, particularly in those with a prolonged transit time (Bouvier et al. 2001). The same group subsequently extended their observations with a blind, controlled, crossover trial comparing milk containing *B. animalis* DN-173 010 or a control without bifidobacteria in 36 healthy women (Marteau et al. 2002). Although the pH, fecal weight, bacterial mass and bile acids were not significantly changed, shortening of total and sigmoid transit times was significant. The mechanism of the effect is far from clear. This observation in healthy subjects is supported by different *Bifidobacterium* species studies on constipation, particularly that encountered in bedridden elderly people (Seki et al. 1978; Tanaka and Shimosaka 1982).

Although the number of observations is small and largely anecdotal, the simi-larity to the results with lactobacilli suggests that increased intestinal motility is a valid effect in human subjects following the administration of bifidobacteria.

3.11.4 MODULATION OF INTESTINAL AND SYSTEMIC IMMUNE
FUNCTION AND BACTERIAL BALANCE

The exact mechanism by which any of the probiotics exert their clinical effects, particularly those associated with reduction in pathogenic activity in other bacteria, is still far from clear. Nevertheless, animal and human studies provide evidence that a beneficial effect exists.

3.11.4.1 Animal and Nonclinical Human Studies

Animal and nonclinical human studies with bifidobacteria follow those outlined in Section 3.10 for the general principles applied to all potential probiotic bacteria. These support the contention that many of the bifidobacteria strains tested have characteristics that suggest probiotic potential. These can be divided into those studies showing a direct influence on other organisms (Bartram et al. 1994; Nielsen et al. 1994), which are not very numerous or convincing because of their study design, and those that demonstrate an effect on immune parameters.

On the other hand, a substantial number of studies demonstrate modulation of the immune system, which could potentially indicate an effect of bifidobacteria on pathogenic organisms via immunomodulation. Their problem lies in the fact that a

multiplicity of biomarkers is used and the relevance of most of these is currently far from clear. Thus, they include effects on

- Nonspecific cellular factors such as macrophages (Fernandes and Shahani 1990)
- Polymorphonuclear leucocytes and natural killer cells (Chiang et al. 2000; Hatcher and Lambrecht 1993; Kado-Oka et al. 1991)
- Gamma-interferon production (De Simone et al. 1987)
- Immune response reaction in Peyer's patch cells (Vasui and Ohwaki 1991)
- Production of an immunomodulating polysaccharide (Hosono et al. 1997)
- Cytokine production (Marin et al. 1997)
- Production of IgA antibodies (Lee et al. 1993; Takahasi et al. 1998; Yasui et al. 1992)
- Nonspecific immunophagocytic activity (Schiffrin et al. 1995)

Studies undertaken in congenitally immunodeficient gnotobiotic mice have indicated that the administration of *B. animalis* was very effective in providing immune protection against *Candida albicans* infection, apparently by stimulating the systemic as well as the secretory immune systems (Wagner et al. 1997). With such diversity of findings, the biological significance is difficult to determine.

3.11.4.2 Improvement in the Status of Premature Infants

Reasonable evidence exists that lactic acid bacteria in general play an important role in the health of the very young and, in particular, in premature infants in intensive care units in which the bacterial flora tends to be abnormal and colonization by bifidobacteria may be delayed. This often coincides with the spread of an enterocolitis within the unit. In consequence of the suggested benefit of lactic acid bacteria, recent studies have been undertaken to determine whether this general property is also exerted by bifidobacteria.

It has been shown that several strains of bifidobacteria derived from infants demonstrate antimicrobial activity against *Salmonella typhimurium* SL1344 in *in-vitro* studies using Caco-2 cells. They show a similar antibacterial activity in *in-vivo* axenic C3/He/Oujco mice infected by the lethal *S. typhimurium* C5 strain (Lievin et al. 2000). Thus, some evidence indicates that early colonization with bifidobacteria in premature infants could have value. As might be imagined, it is not easy to organize clinical studies of the deliberate administration of bifidobacteria to very low birth weight infants in intensive care units, but some studies now indicate that the property of improvement of premature infants' intestinal health does extend to bifidobacteria.

In preliminary studies (Akiyama et al. 1993a, b), it was shown that administering *B. breve* appeared to facilitate colonization of the intestinal tract. Consequently, a more formal study to investigate the potential for *B. breve* to encourage colonization was undertaken in 1991 and 1997 in very low birth weight neonates. Prior to this study, 66 very low birth weight infants had received *B. breve* in a preliminary study to confirm that no adverse reactions occurred. Immunohistochemical staining of

stool specimens with a *B. breve* monoclonal antibody showed that, at 2 weeks of age, 73% of the members of the treated group were colonized as opposed to only 12% of the control infants (Kitajima et al. 1997).

In at least one study in an intensive care unit, premature infants given bifido-bacteria had a much lower incidence of enterocolitis than had been encountered previously in the same unit (Hoyos 1999). Such anecdotal findings clearly require confirmation in well-designed, blind-controlled prospective trials.

3.11.4.3 Reduction in Atopy and Food Allergies

Some inconclusive evidence from animal experiments indicates that the effects exerted by probiotics on the immune system may increase the tolerance of the system to some common allergens by reducing their production of immunoglobulin Ig E. This development of tolerance seems to be particularly effective when the probiotic is administered shortly after birth rather than at a later stage, according to studies by Sudo et al. (1997) on the administration of *B. infantis* in germ-free mice. The suggestion is that the development of tolerance to oral antigens may be an important function of bifidobacteria in the early postnatal stage. On this basis, He et al. (2001) studied 50 strains of bifidobacteria isolated from the feces of allergic and age-matched healthy infants. The pattern of bifidobacterial types varied between the two groups with the healthy infants showing a typical infant pattern with predominance of *B. bifidum*, while the allergic infants showed an adult pattern with predominance of *B. adolescentis.* The bifidobacteria from the healthy infants showed a significantly higher adhesion to human intestinal mucus than did those of the allergic group. The same group of researchers from Turku, in Finland, have investigated the effect on the development of eczema in atopic infants of the administration of *B. lactis* (Bb12) or *L. rhamnosus* (GG) during weaning (Kalliomaki et al. 2001; Kirjavainen et al. 2002; Majamaa and Isolauri 1997). They showed that probiotic supplementation pre- and postnatally is effective in prevention of early atopic disease in infants at high risk. The results achieved to date suggest that this could be an interesting area for further clinical study, particularly because the level of atopy seems to be increasing (possibly due to better hygiene) and has resulted in a lower natural level of infant exposure to a variety of antigens in the early postnatal period.

3.11.4.4 Management of Intestinal Viral Infections

In studies in mice, Yasui et al. (1995) showed that young mice born of mothers fed *B. breve* YIT 9018 and immunized orally with rotavirus shortly after birth were protected to a greater extent against rotavirus-induced diarrhea than those immunized with rotavirus alone. A similar effect on rotavirus weanling diarrhea was demonstrated in a piglet model using *B. lactis* (Shu et al. 2001). These results in animal models have been shown to occur in human infants also. In a double-blind, placebo-controlled trial, Saavedra et al. (1994) showed that a milk preparation containing *B. bifidum* and *Streptococcus thermophilus* reduced not only the incidence of acute diarrhea but also the shedding of the organism in rotavirus-infected infants in the hospital. Although similar effects have been reported with other probiotics, to the

author's knowledge, this is the only such study with bifidobacteria reported to date and confirmation is very desirable.

3.11.4.5   Management of Intestinal Bacterial Infections and Diarrhea of Unspecified Origin

In previous sections, attention has been directed to *in vitro* and animal studies demonstrating that administration of bifidobacteria interferes with the growth and adhesion of known pathogenic intestinal bacteria. Additional studies confirming these properties of bifidobacteria are those of Gibson and Wang (1994b), who examined the effect of various bifidobacteria species, including *B. infantis* NCFB 2205, in batch fermentations, Shu et al. (2001) using a piglet model with *Escherichia coli*, and Yamazaki et al. (1982, 1985, 1991) using *E. coli* in mice. One further study that may have relevance in this respect is that of Fukushima et al. (1997), who demonstrated that the administration of *B. lactis* has a beneficial effect on the balance of bacteria within the intestinal tract. They showed that a follow-on milk formula that contained *B. lactis*, when administered to healthy infants, increased the fecal acetic acid content and reduced fecal putrefactive products such as ammonia and indole.

Equivalent beneficial effects have been reported by Tojo et al. (1987) in diarrheal diseases with established pathogens in patients treated with *B. breve* with enteritis caused by infection with *Campylobacter jejuni.* Unfortunately, the clinical literature, in general, abounds in anecdotal evidence of the benefits achieved by numerous substances in various diarrheal states of unspecified etiology. It is impossible to determine whether these have any relevance because, in the vast majority of cases, diarrhea is a self-limiting disorder of relatively short duration. Among such studies are those of Hotta et al. (1987) and Saavedra et al. (1998). Results found with other probiotics and *in vitro* and animal studies suggest an *a priori* reason for the therapeutic value of bifidobacteria in the management of bacterial diarrhea, but the current clinical evidence for such an effect is clearly insufficient.

3.11.4.6   Prophylaxis of Traveler's Diarrhea

On the basis of the evidence (however limited) suggesting that bifidobacteria-containing preparations modified the course of viral and bacterial intestinal disorders, it is not surprising that one clinical study has examined the possible value of a bifidobacteria-containing preparation in travelers' diarrhea (Black et al. 1989). This involved a double-blind, placebo-controlled trial of a mixed culture of *S. thermophilus* and *B. bifidum (lactis* Bb-12) in 81 people traveling the Nile in Egypt. A significant difference was found between the two groups (placebo 71%, active 43% diarrhea). The majority of the studies with probiotics in travelers' diarrhea have not been encouraging and one study with few participants is inadequate to consider this a clear clinical indication for the use of bifidobacteria.

3.11.4.7   Prevention of Antibiotic-Generated Intestinal Dysfunction

For some years a general view has existed, supported by some scientific observations, that intestinal disturbances frequently associated with the ingestion of broad-spectrum antibiotics result from a disturbed balance of the intestinal flora. In some cases *Clostridium difficile* has been shown to be the enteropathogen involved. Several studies have indicated that lactobacilli are effective in the suppression of this enteropathogen. It is therefore no surprise that probiotics in general and bifidobacteria in particular have been tried therapeutically and prophylactically.

In a prophylactic controlled trial against normal yogurt in 10 subjects, the addition of *B. longum* stopped the diarrhea associated with the administration of erythromycin and, in all but one subject, abolished all complaints of intestinal discomfort (Columbel et al. 1987).

In addition, Orrhage et al. (1994) showed in a controlled trial that a milk product containing *B. longum* and *L. acidophilus* reduced the intestinal dysfunction associated with the administration of clindamycin. Another blind controlled study investigated the effect of *B. bifidum I* (correct designation *lactis* Bb-12) together with *L. acidophilus* La-5 after ampicillin treatment and demonstrated that this mixture recolonized the intestinal tract to a normal pattern more rapidly than the placebo (Black et al. 1991).

The entire question of the possible benefit of probiotics in the prevention of antibiotic diarrhea has been the subject of a meta-analysis of nine randomized, double-blind, placebo-controlled trials of probiotics (Ellegaard et al. 1992). Of these, only one involved the use of bifidobacteria (Orrhage et al. 1994). This meta-analysis was not conclusive but suggested that at least some of the probiotics (S*accharomyces boulardii* and lactobacilli are specifically mentioned) can be used to prevent antibiotic-associated diarrhea. They conclude that the efficacy of probiotics in treating antibiotic-associated diarrhea "remains to be proved."

3.11.4.8   Protection and Treatment of Radiotherapeutic Intestinal Dysfunction

It has been held for some time that radiotherapy- and cytotoxic drug therapy–associated bowel dysfunction may be due to a disturbance of the balance of the intestinal flora. It is therefore not surprising that preparations containing bifidobacteria have been tried in such cases. There appear to be two such observations at present. Capsules containing *B. lactis* and *L. acidophilus* (La-5) were administered to those undergoing chemotherapy in a double-blind, placebo-controlled trial. These produced a significant reduction in the duration of symptoms, particularly fever (D'Souza et al. 2002). In another study, preparations containing *B. bifidum* reduced the diarrhea and discomfort of cancer patients undergoing radiotherapy (Ellegaard et al. 1992). Considering that intestinal problems with radiotherapy and chemotherapy are troublesome, it is clear that further studies are warranted.

3.11.4.9   Management of Irritable Bowel Syndrome and Inflammatory Bowel Diseases

Substantial anecdotal information indicates that the administration of lactic acid bacteria is effective in the relief of many of the manifestations in a proportion of the cases of irritable bowel syndrome and inflammatory bowel disease. The proportion, which can be improved, seems to depend primarily on the symptoms involved. Those with alterations in gut motility seem to respond best, corresponding to the effects observed with normal gut motility. To date it appears that there are no published results of use of bifidobacteria in these puzzling disorders; however, limited evidence suggests that they might be effective. Thus, in six patients with ulcerative colitis who each had rectal biopsies from two sites, inflamed mucosa always showed fewer bifidobacteria and more *E. coli* than normal-looking proximal colons. Similar results were also seen in a study in patients with Crohn's disease (Mettler et al. 1983).

In a very recent study (Rayment et al. 2002), it was shown that *B. infantis* UCC35624 administered before colonoscopy in seven volunteers with ulcerative colitis led to the presence of the strain in the feces at levels of 10^5 to $10^{8.7}$ cfu/g recovered from biopsy specimens in the ascending, transverse and descending colon. Evidence also showed that the strain would adhere to inflamed portions of the colon. The investigators also examined a range of other bacteria, but found no indication that these were influenced. A further study examined the merits of a mixture of *B. breve* YIT4065 and *L. acidophilus* YIT0168 in a blind controlled trial in ulcerative colitis (Von Wright et al. 2002) and suggested that this combination had value. The available information can be taken to indicate that carefully planned clinical studies with bifidobacteria would be justified in irritable bowel syndrome and inflammatory bowel disease. Until extensive, long-term studies have been undertaken, it is not possible to determine whether a therapeutic effect exists.

3.11.4.10   Prevention of Carcinogenesis

The development of carcinomas in the human intestinal tract is an important and common phenomenon; for this reason extensive *in vitro* and animal studies, together with a few human trials, have been conducted to try to establish whether the lactic acid bacteria in general have an anticarcinogenic activity. An effect might be exerted by several possible mechanisms (inhibition of bacterial enzymes that produce carcinogens, binding of bile steroids, increase in immune reactions). Several studies have evaluated bifidobacteria administration in the prevention of experimental carcinoma. For a general review of the current situation for lactic acid bacteria in general, see Rafter's (2002) recent article.

Attention has already been directed to the potential of bifidobacteria to influence the bacterial enzymes involved in potential mutagenic and carcinogenic activity (Benno and Mitsuoka 1992; Kirmann and Rasic 1991). Among other *in vitro,* potentially important effects are the demonstration that the carcinogen aflatoxin B, produced when food is contaminated with some strains of the fungus *Aspergillus,* is strongly bound by bifidobacteria and thus made unavailable for intestinal absorption

(Oatley et al. 2000). The fact that nitrites can be destroyed by bifidobacteria (possibly by an effect of the high-acid environment) and that nitrosamine can be metabolized by *B. longum*, reducing each of these toxic food constituents (Grill et al. 1995), is another important effect. In addition to these, many studies have examined the potential for bifidobacteria to affect known colon tumorigenic chemicals in animal models, most of which were undertaken in rats. Thus

- *B. breve* and *B. longum* from human infant stools prevented DNA damage in colonic cells caused by N-methyl-N'-nitro-N-nitrosoguanidine (7.5 mg/kg body weight) in rats after a single dose of 10^{10} viable cells/kg body weight administered orally 8 h before the carcinogen. In a second study using 1,2-dimethylhydrazine (DMH) as the carcinogen, prior treatment with these two strains inhibited the genotoxic effects of DMH. Viable cells were necessary for this effect (Pool-Zobel et al. 1996).
- In another study in weaning rats administered azoxymethane (AOM), *B. longum* administered at a level of 10^8 viable cells/g of feed for 13 weeks suppressed the formation of aberrant crypt foci (a preneoplastic marker) (Challa et al. 1997). The simultaneous administration of lactulose increased the suppression. There was a positive correlation between the higher fecal pH and the number of abnormal crypt foci. Anjana et al. (1997) found similar effects to those of Challa et al. (1997) in terms of the bifidobacteria and lactulose.
- Another rat study, this time in male adults, also used AOM as the mutagenic agent and tested *B. longum*. The endpoint in this study was the presence and size of colon tumors 40 weeks after the last AOM injection. Administration of *B. longum* in the diet suppressed colon tumor incidence, the number of tumors per animal and the tumor volume. The modified diet also reduced ornithine decarboxylase activity and the expression of ras-p21 oncoprotein expression, all indicating a positive effect from the bifidobacteria administration (Singh et al. 1997).
- Similar results with the same carcinogen and probiotic were found by Kulkani and Reddy (1994), who also showed suppression of bacterial glucuronidase.
- In a further adult rat study, Reddy and Rivenson (1993) demonstrated the inhibitory effect of *B. longum* with another mutagenic agent: 2-amino-3-methylimidazo [4,5-*f*] quinoline. *B. longum* produced marked inhibitions in colon, liver and mammary carcinoma in terms of the proportion of animals affected and the number of tumors.

In addition to these rat studies, two studies in the mouse had entirely different hypotheses — namely, a direct anticancer effect within solid tumors not in the alimentary canal and therefore probably acting via a different mechanism. In the first of these studies, Hyung-Young et al. (1991) showed that a variety of lactobacilli and *B. lactis* could exert a direct inhibitory effect on mouse sarcoma 180 and Lewis carcinoma of the lung. Biffi et al. (1997) studied several lactobacilli but also investigated a range of bifidobacteria, namely, *B. infantis, B. animalis* (Bb-12) and

B. bifidum. Compounds generated during fermentation showed an antiproliferative effect against the MCF7 breast cancer cell line. These observations of an apparent direct effect of bifidobacteria on tumor cells may link to the interesting preliminary observations (Bermudes et al. 2002; Fujimori et al. 2002) that live organisms from the genus *Bifidobacterium* may be useful as vectors for anticancer agents and tumor-selective gene therapy. These observations, however, fall outside the fundamental range of this chapter's topic. From the point of view of classical probiotic activity, it is clear that, like so many other lactic acid bacteria, the bifidobacteria show effects that may be biomarkers for anticancer activity — particularly for intestinal cancers. The problem lies in the fact that the relevance of these biomarkers for human disease is not currently known.

3.11.4.11 Other Reputed Beneficial Effects

As with any new potential therapeutic agent, bifidobacteria-containing products have been described as exerting a number of miscellaneous health-giving and therapeutic benefits for which no obvious rationale exists — including the relief of premenstrual syndrome. In a single randomized controlled trial in 21 young women, Minelli et al. (1996) demonstrated an effect of milk fermented with a mixed culture of *B. bifidum* YIT4007. A controlled trial involving the ovariectomized rat osteoporosis model showed that administering *B. longum* and lactulose increases the absorption of calcium and femur strength. The effect appeared to come from a change in the intestinal pH (Igarashi et al. 1994).

3.12 USE OF BIFIDOBACTERIA IN SYNBIOTIC PREPARATIONS

Synbiotics are preparations that combine probiotics and prebiotics in a single product on the basis that their simultaneous administration should encourage growth and persistence of probiotics within the intestinal tract and hence improve the therapeutic value. Although the concept has not currently received the expected support on the basis of the theoretical merit, isolated reports of such products are available. These include a case report of the use of a synbiotic containing *B. breve, L. casei* and galactooligosaccharides with dramatically good results in a 4-year-old girl suffering from short bowel syndrome (Kanamori et al. 2001). Based on one single case, it is clearly not possible to determine if synergy occurred because there are a few isolated reports of similar good effects using a single probiotic. This does encourage further studies of the general value of bifidobacteria containing synbiotics in other disorders even though short bowel syndrome is too rare to be suitable for adequately controlled studies to distinguish synergy.

3.13 SAFETY CONSIDERATIONS

Earlier in this chapter attention was directed to the paramount importance of the safety of any product used as a food for a health-promoting effect, or when the

prophylactic and therapeutic uses are for relatively minor illnesses for which other, safe products are available. As far as bifidobacteria are concerned, the health-promoting and therapeutic potential appears to lie within that which would require safety as a vital component. As a member of the general group of lactic acid bacteria, bifidobacteria fall into the GRAS category. However, in addition to this categorization, they have been subjected to recent observations to confirm their safety. (The entire aspect of safety is dealt with by O'Brien in Chapter 14).

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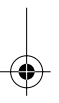
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4 Lactose Intolerance and Low-Lactose Dairy Products

Tuula Tuure and Riitta Korpela

CONTENTS

4.1   INTRODUCTION

Lactose, the main carbohydrate in milk and other dairy products, is found only in the milk of mammals (Figure 4.1). It is not known why milk contains a special carbohydrate; one hypothesis is that lactose solubility may be matched best with milk synthesis and expression and may provide appropriate energy while minimizing osmotic load (Mustapha et al. 1997a). As far as is known, lactose has no special nutritional importance for adults; however, it is the most important source of energy during the first year of a human's life, providing almost half the total energy requirement of infants.
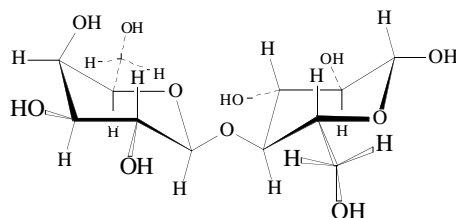
FIGURE 4.1 Lactose is composed of glucose and galactose.

Lactose has several applications in the food industry. It is used, for instance, in sweets, confectionery, bread and sausages because of its physiological properties: lactose provides good texture and binds water and color. Lactose is only about one third as sweet as saccharose and less than half as sweet as glucose.

To be absorbed, lactose needs to be hydrolyzed in the intestine by a β-galactosidase, lactase–phloritzin hydrolase (EC 3.2.1.23/26), generally called lactase. Lactase is found most abundantly in the jejunum (at the beginning of the small intestine); it specifically hydrolyzes lactose. Lactase is one of several disaccharidases contained in the brush border of the small intestine's epithelial cells (enterocytes). It hydrolyzes lactose to its component sugars, glucose and galactose. In most mammals, lactase activity decreases after weaning, but, in some human ethnic groups such as western European Caucasians, lactase activity can persist into adult life, enabling total digestion of large quantities of dietary lactose.

4.2 DESCRIPTION OF LACTOSE INTOLERANCE

4.2.1 HYPOLACTASIA AND LACTOSE MALDIGESTION

Hypolactasia may be primary (i.e., genetic) or secondary. On rare occasions, absence or deficiency of lactase can be congenital, but typically it occurs in early or mid-childhood in populations that experience a genetically determined reduction in lactase secretion and activity after weaning, or transiently due to a disease of the small intestine that damages the intestinal epithelium. Maldigestion of lactose and the associated symptoms known as lactose intolerance result because of hypolactasia (Figure 4.2). Symptoms develop in mid- to late childhood as a result of a progressive reduction in the amount of enzyme produced. Symptoms of lactose intolerance include loose stools, abdominal bloating and pain, flatulence, nausea, and borborygmi. The genetically determined reduction of lactase activity occurs soon after weaning in almost all animals and in many human races (Johnson 1981). The activity drops to about 10% or less of the suckling level. Congenital lactase deficiency is extremely rare (Johnson 1981), with only a few dozen documented cases in the world, most of them in Finland. In the case of secondary lactose maldigestion, the activity of lactase returns when the epithelium heals.

Selective adult-type hypolactasia is inherited through a single autosomal recessive gene (Sahi and Launiala 1977). Pre- and post-transcriptional mechanisms seem to be involved in the expression of the low enzyme activity (Flatz 1995). A
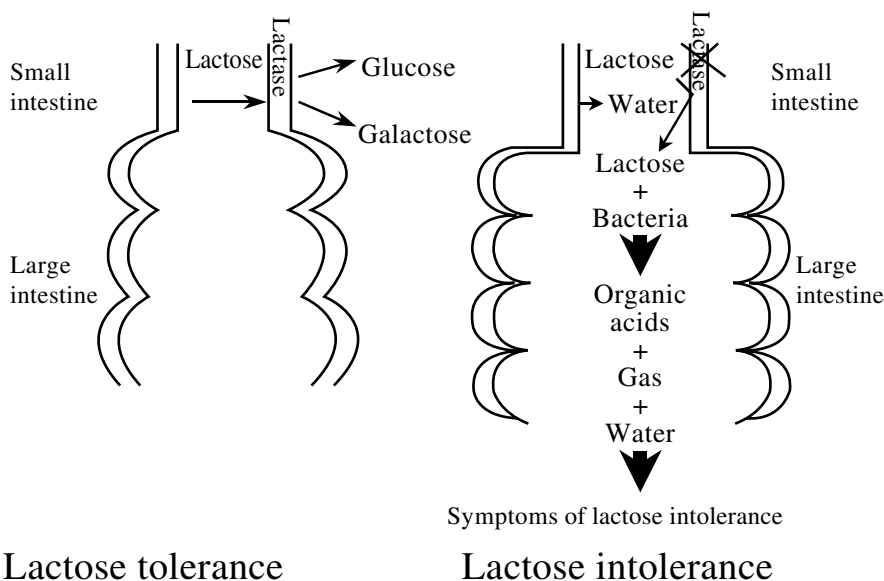
FIGURE 4.2 Intestinal events in lactose tolerance compared to those in lactose intolerance.

cultural–historical hypothesis has been proposed for lactase persistence (Simoons 1981): after the beginning of dairy farming, during periods of dietary stress, individuals who had high levels of intestinal lactase would have had a nutritional advantage. As a result of increased survival, high intestinal lactase activity would have become typical of such a group. Lactase persistence is, indeed, more common in areas with long traditions of dairy farming. However, production of the enzyme does not seem to be induced by lactose consumption.

4.2.2 LACTOSE INTOLERANCE DIFFERS FROM MILK ALLERGY

Lactose intolerance is occasionally confused with milk allergy. They are, however, completely different ailments — if lactose intolerance (present in two thirds of the world's population) can be considered an ailment at all. Lactose intolerance concerns primarily the adult population and is caused by the carbohydrate lactose; milk allergy is almost exclusively limited to infants and caused by milk proteins. The symptoms of each differ as well (Table 4.1).

4.2.3 PREVALENCE OF LACTOSE MALDIGESTION

Scrimshaw and Murray (1988) and Sahi (1994) have reviewed the prevalence of lactose maldigestion in the world, which is above 50% in South America, Africa, and Asia and reaches almost 100% in some Asian countries. In the U.S., the prevalence is 15% among whites, 53% among Mexicans, and 80% in the black population. In Europe it varies from around 2% in Scandinavia to about 70% in Sicily (Figure 4.3). Australia and New Zealand have prevalences of 6 and 9%,

TABLE 4.1
Comparison of Lactose Intolerance and Milk Allergy

	Lactose Intolerance	Milk Allergy
Origin of the ailment	Enzymatic deficiency	Immunologic reaction
Cause	Lactose (carbohydrate)	Milk protein
Symptoms	Flatulence, bloating, diarrhea, abdominal pain	Eczema, nausea, diarrhea, colic, respiratory dysfunction, anaphylactic shock
Occurrence	Mainly in adults	Nearly exclusively in infants and small children
Dietary treatment	Reduction of lactose	Replacement of milk by special formulas
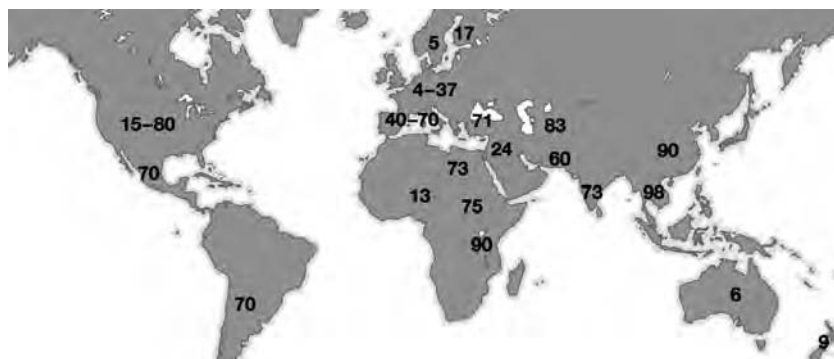
FIGURE 4.3 Prevalence (%) of lactose maldigestion in different parts of the world.

respectively. In general, it can be stated that about two thirds of the world's adult population are lactase nonpersistent.

Gender does not have any effect on the prevalence of hypolactasia in a randomly selected population. However, women seem to be more prone than men to produce symptoms from similar amounts of maldigested lactose (Krause et al. 1996). The effect of age on hypolactasia, especially on symptoms of lactose intolerance, is not yet clear. Although the prevalence of hypolactasia is more common in adults than in children (Caskey et al. 1977; Welsh et al. 1978), some evidence exists that intestinal lactase activity does not continue to decline with age and no differences in the prevalence of hypolactasia between older and younger adults have been observed (Jussila et al. 1970). Studies examining the effect of age on the experience of symptoms have given contradictory results (Jussila et al. 1970; Suarez and Savaiano 1994).

4.2.4 MEASUREMENT OF LACTOSE DIGESTION

Lactose digestion can be studied using direct or indirect methods, both of which have been reviewed by Arola (1994). The direct methods include the measurement

of mucosal disaccharidases using intestinal intubation (proposed as the reference method) and an intestinal perfusion technique for the exact measurement of lactose digestion. The breath tests include the breath hydrogen test and measurement of breath $^{13}CO_2$ after ^{13}C-lactose ingestion and breath radioactivity after ^{14}C-lactose ingestion. The latter is not recommended because of the radioactivity. Among the blood tests are the traditional lactose tolerance test, lactose tolerance test with ethanol, and milk tolerance test. Lactose maldigestion can also be determined by measuring urinary galactose quantitatively, or qualitatively using an enzymatic test strip.

Less reliable stool tests — stool pH, fecal-reducing substances, and paper chromatography for the measurement of sugar in the feces — are not recommended for research purposes. The widely used breath hydrogen test is a fairly reliable method for the diagnosis of lactose maldigestion; the amount of hydrogen excreted correlates with maldigested lactose (Bond and Levitt 1976). Recently, a combination of $^{13}CO_2$ and H_2 breath tests was suggested (Koetse et al. 1999); however, this test would be limited mainly to research use because of the complex equipment it requires.

4.3 FACTORS INFLUENCING SYMPTOMS

4.3.1 AMOUNT OF LACTOSE

Ingestion of 50 g lactose in a clinical tolerance test causes symptoms in 80 to 100% of lactose maldigesters (Bayless et al. 1975; Jussila et al. 1970; Newcomer et al. 1978; Sahi and Launiala 1978); one third to one half of the lactose maldigesters experience symptoms after consumption of 200 to 250 ml of milk (Savaiano and Levitt 1987). In many studies ingestion of hydrolyzed lactose milk has reduced symptoms compared with regular milk (Suarez et al. 1995a).

When the symptom response to smaller doses (around 10 g and less) of lactose was studied, several works showed no dose response (Hertzler et al. 1996; Suarez et al. 1995b; Vesa et al. 1996a). Hertzler et al. (1996) reported a higher mean increase in breath hydrogen excretion after ingestion of 6 g lactose than after ingesting 2 g, indicating at least partial lactose maldigestion with the higher dose. However, the fairly high baseline values of breath hydrogen (approximately 20 ppm) complicate the interpretation of their results. The subjects reported no more symptoms after 6 g lactose than after 0 or 2 g.

Even after ingestion of large amounts of lactose, a small percentage of maldigesters remains free of symptoms (Scrimshaw and Murray 1988). The reason for this is unknown, but the presence of symptom-free subjects is a common observation in connection with other carbohydrate maldigestions as well; only about half of the fructose maldigesters experienced abdominal symptoms after ingesting 50 g fructose (Truswell et al. 1988) and after 25 g fructose and 5 g sorbitol (Nelis et al. 1990).

Most studies have shown low-lactose or lactose-free milk to be tolerated better than lactose-containing milk. However, reconsideration and reinvestigation are necessary due to the controversial results of some well-controlled works that revealed no difference in the tolerance between these milks (Suarez et al. 1995a, 1997; Vesa et al. 1996a) or demonstrated a possible placebo effect (Briet et al. 1997).

4.3.2 ROLE OF FERMENTATION AND ADDITION OF BACTERIA

Lactose maldigesters digest and tolerate lactose in yogurt better than an equivalent quantity of lactose in milk (Dewit et al. 1988; Kolars et al. 1984; Marteau et al. 1990; Rosado et al. 1992), but the importance of lactase activity in yogurt is not clear. Several authors emphasize the importance of the living bacteria of yogurt or other fermented milks in connection with lactose digestion (Gilliland and Kim 1984; Lerebours et al. 1989; Savaiano et al. 1984). However, in two of these studies, the tolerance of heat-treated yogurt was not significantly inferior to that of fresh yogurt with viable bacteria (Marteau et al. 1990; Savaiano et al. 1984). Similarly, digestion and tolerance of lactose were equal after ingestion of three fermented dairy products that had a four-fold difference in their β-galactosidase activity (Vesa et al. 1996b).

In addition, several works have shown that lactose digestion was improved when bacterial cells were destroyed by sonication or by the presence of bile, compared to intact cells (Gilliland and Kim 1984; Marteau et al. 1990; McDonough et al. 1987; Noh and Gilliland 1994). However, contradictory results have also been published (Martini et al. 1987) that may have been due to differences in the tolerance to acid and bile between the bacterial species and strains present in the fermented products.

In direct *in vivo* measurements, maldigestion of 18 g lactose was 9.6% after ingestion of yogurt, 12.5% after pasteurized yogurt, and 39% after milk (Marteau et al. 1990). The gastric β-galactosidase:lactose ratio fell rapidly within 2 h of ingestion of yogurt (Martini et al. 1987), but part of the β-galactosidase obviously survived the passage through the stomach. Yogurt ingestion caused significantly fewer symptoms in lactose maldigesters than did milk (Martini et al. 1991a). When the tolerance of unmodified, low-fat, and lactose-hydrolyzed yogurt was compared, the modification of lactose or fat content did not have any effect on the tolerance of yogurt, but symptoms were significantly and equally reduced with all the yogurts compared with milk (Rosado et al. 1992).

Nonfermented milk containing bacteria grown on lactose also reduced breath hydrogen excretion (Jiang et al. 1996); hydrogen excretion was significantly lower in lactose maldigesters after ingestion of one dose of milk containing a strain of *Bifidobacterium longum* grown on lactose, compared with milk containing the same strain grown on lactose and glucose or another strain of *B. longum* grown on lactose. The former milk contained the highest β-galactosidase activity and the strain was more bile tolerant than the latter one, which probably accounted for the good digestion. The importance of acid and bile tolerance of the strain was suggested also by a study on *Lactobacillus acidophilus* (Mustapha et al. 1997b). A nonfermented milk containing *L. bulgaricus* strain reduced breath hydrogen and symptoms, whereas a strain of *L. acidophilus* only slightly reduced breath hydrogen without an effect on the symptoms (Lin et al. 1998). Bile sensitivity and β-galactosidase activity of these strains were similar, but the cell wall structures of this and other *L. acido-philus* strains tested were tougher than those of *L. bulgaricus* strains. Another strain of *L. acidophilus* also failed to alleviate lactose intolerance when ingested twice a day for 7 days (Saltzman et al. 1999).

Although food ingested simultaneously with milk has been shown to improve lactose digestion (Dehkordi et al. 1995; Martini and Savaiano 1988), this did not

apply to yogurt (Martini et al. 1991b), presumably because lactose digestion from yogurt is already very efficient (Kolars et al. 1984; Marteau et al. 1990). It is not yet clear which properties of yogurt or its bacteria are the most important, but it is evident that yogurt is well tolerated by lactose maldigesters and that the tolerance varies somewhat depending on the strains used.

4.3.3 FOOD COMPOSITION

It has been suggested that full-fat milk causes fewer symptoms in lactose maldigesters than lactose-free milk (Saavedra and Perman 1989; Tamm 1994). Two studies showed that full-fat milk reduced lactose maldigestion and intolerance compared with fat-free milk (Leichter 1973) or with aqueous lactose solution (Solomons et al. 1979); however, other researchers have not been able to confirm these results (Cavalli-Sforza and Strata 1987; Jones et al. 1976; Vesa et al. 1997a). Dehkordi et al. (1995) reported a slight decrease in the maldigestion of lactose in full-fat milk compared to fat-free milk, but no improvement in symptoms. Martini et al. (1987) did not observe any significant difference in the severity of symptoms or the degree of lactose maldigestion in lactose maldigesters who had consumed ice cream and low-fat ice cream with a substantial difference in the fat content between the products (10 and 3% fat, respectively). However, the composition of ice cream and low-fat ice cream differs from that of milk, so the results may not be applicable to milk. Delayed gastric emptying has been proposed as one explanation for improved lactose tolerance after ingestion of full-fat milk compared with skimmed milk or ingestion of milk with a meal instead of milk on its own (Martini and Savaiano 1988; Solomons et al. 1979).

The gastric emptying rate and intestinal transit time alter the time that lactose is exposed to intestinal lactase. After a meal, the stomach contents are progressively emptied into the duodenum over a period of several hours, depending on the energy content and composition of the meal (Bernier et al. 1988). The temperature of a meal or a drink also influences gastric emptying. Ingestion of a cold drink of 4°C slowed down the initial phase of gastric emptying for approximately 10 min after ingestion, compared with a control drink of 37°C (Sun et al. 1988). There was a tendency toward delayed emptying of a drink of 50°C, but the difference was not significant compared to the control drink.

The rapidity of gastric emptying varies depending on many physiological factors. It has been suggested that delayed gastric emptying improves lactose digestion and therefore tolerance. Lactose has been better digested when consumed in milk instead of water (Solomons et al. 1979), in a chocolate milk drink instead of plain milk (Dehkordi et al. 1995; Lee and Hardy 1989), or with solid food (Dehkordi et al. 1995; Martini and Savaiano 1988; Solomons et al. 1985a) or fiber (Nguyen et al. 1982). This alleviation of symptoms is considered to be the result of delayed gastric emptying caused by an increase in the energy content and osmolality. Fat slows down the rate of gastric emptying (Houghton et al. 1990) and increases the jejunal transit time (Spiller et al. 1984). In one study high-energy milk significantly delayed gastric emptying, but had only a borderline effect on lactose digestion and very little

effect on the symptoms (Vesa et al. 1997b). However, pharmacological delay of gastric emptying did improve the tolerance of lactose (Peuhkuri et al. 1999).

Ingestion of yogurt has been shown to lengthen gastric emptying halftime and gastrointestinal transit time compared with regular milk (Arrigoni et al. 1994; Mahé et al. 1994; Marteau et al. 1990). The mechanism of this delay is not known, but it does not seem to be due to differences in lactose digestion because gastrointestinal transit has been lengthened in lactose digesters (Mahé et al. 1994) and in maldigesters (Marteau et al. 1990). An indication of delayed gastric emptying after ingestion of yogurt compared with milk was also obtained in a study on healthy adults with no known lactose digestion status (Gaudichon et al. 1995). The lengthening of the gastrointestinal transit time may be due to the more solid composition of yogurt.

4.4   ADAPTATION TO LACTOSE CONSUMPTION

Continued lactose ingestion reduces breath hydrogen excretion, possibly due to increased colonic acidity (Perman et al. 1981; Vogelsang et al. 1988), and possibly to changes in the colonic flora. Ito and Kimura (1993) provided support for the latter hypothesis by showing that ingestion of lactose for 6 days reduced the total fecal bacteria, specifically bacteroides and *Clostridium perfringens*, while increasing lactobacilli, enterococci, *Candida* ssp, and staphylococci. Fecal short-chain fatty acids were not altered, but the concentration of formic and valeric acid increased, which reflects changes in the composition of the flora.

Increases in intestinal bifidobacteria have been shown in some studies in rats after lactose feeding (Morishita and Shiromizu 1987) and in humans after lactulose (Terada et al. 1992) and yogurt (Bartram et al. 1994) feeding. In the study of Bartram et al. (1994), no additional effect on the intestinal flora was found when yogurt was supplemented with lactulose and *B. longum*, thus suggesting that yogurt has a bifidogenic effect.

Numerous studies have documented an induction of intestinal lactase by lactose ingestion in animals (as reviewed by Peuhkuri 2000), but so far no study has shown this to be the case in humans. However, a 2-week ingestion of *Saccharomyces boulardii*, a yeast that does not contain β-galactosidase activity, increased human intestinal lactase activity without morphological alteration of the mucosa (Buts et al. 1986). The status of lactose digestion in the study group was not given, but the data indicated that the subjects were lactose digesters. It is questionable whether the increase in lactase activity induced by consumption of lactose or probiotic products could be enough to improve tolerance to lactose; changes in the intestinal microflora or other means of adaptation probably play a more important role. This area needs further study.

Several authors have claimed that symptoms of lactose intolerance disappear in some study groups after several weeks of milk supplementation, but the data have not been presented (Habte et al. 1973; Reddy and Pershad 1972). In a more recent study, fecal β-galactosidase increased and breath hydrogen excretion and symptom scores decreased after 2-week dietary supplementation with lactose (Briet et al. 1997). Surprisingly, the symptoms of intolerance decreased as much in a control group on sucrose supplementation, suggesting that the

subjects adapted, perhaps psychologically, to the study protocol. A placebo effect caused by adaptation to the test procedures may thus explain the diminished symptoms. Flatus frequency and ratings diminished in lactose maldigesters after 16 days of lactose ingestion compared with dextrose ingestion (Hertzler and Savaiano 1996). Breath hydrogen excretion also decreased and fecal β-galactosidase activity increased. These results suggest that colonic adaptation to regular lactose ingestion exists. Similarly, in another study a decrease in breath hydrogen was observed during consumption of a dairy-rich diet for a period of 21 days (Pribila et al. 2000).

Lower hydrogen production might have been the result of a proliferation of nonhydrogen-producing bacterial species after continuous lactose ingestion by lactose maldigesters, as was suggested by Hertzler et al. (1997). They showed that decreased fecal hydrogen production, rather than increased hydrogen consumption, was responsible for decreased breath hydrogen excretion; lower fecal pH was not the explanation. Prolonged lactulose ingestion also induced adaptive changes in the colonic function (Flourié et al. 1993); it reduced osmotic diarrhea and fecal outputs of carbohydrates and osmotic moieties and increased orofecal transit time and fecal concentrations of β-galactosidase, lactic acid, and acidity. However, it did not affect other gastrointestinal symptoms of healthy adults.

In an *in vitro* study, lactose was infused to an anaerobic continuous culture inoculated with fresh samples of human feces (Jiang and Savaiano 1997a). The lactose concentration of the samples decreased rapidly within 1 to 2 days of infusion and the β-galactosidase activity increased. When an *L. acidophilus* strain was added to the culture, the decrease in the lactose concentration was significantly greater and increases occurred in acetate and propionate production. These results suggest that colonic bacteria adapt quickly to lactose, which causes efficient utilization of lactose. The same authors evaluated the effects of *B. longum* supplementation on colonic fermentation of lactose in an *in vitro* continuous culture system (Jiang and Savaiano 1997b). At pH 6.7, the reduction of lactose concentration in the sample of fecal culture was greater after supplementation with the bacteria than without them. At lower pH values (6.2 and 5.7), the difference in the reduction of lactose concentration was not marked; β-galactosidase activity was the highest at pH 6.7. The authors concluded that *B. longum* may have the potential to improve lactose fermentation, but it must be noted that gas production increases with augmented fermentation and this may contribute further to gas-related symptoms.

To some extent, lactose maldigesters seem to adapt to continuous lactose consumption. However, the adaptation may not be sufficient for all individuals, especially for the most sensitive ones.

4.5 TREATMENT OF LACTOSE INTOLERANCE

The symptoms of lactose intolerance arise when the amount of lactose arriving in the intestine exceeds the capacity of the intestinal lactase to hydrolyze it. Unlike in allergies, consumption of the substrate causing symptoms is not harmful in lactose intolerance, but it may be very annoying. Table 4.2 lists several options to solve

TABLE 4.2
Options for Treatment of Lactose Intolerance and Their Possible Effects

Solution	Possible Effects
Fewer dairy products	Poorer diet, deficiency of calcium
Low-lactose products	
Fermented	—
Lactose hydrolyzed	Sweet taste in some products
Lactose-free products	
Matured cheese	—
Chromatographically separated lactose	Method not readily available
Lactase preparations	
Added in the product	Sweet taste in some products, expensive
Consumed with the product	Not efficient in all individuals, expensive

TABLE 4.3
Average Content of Lactose in Dairy Products

	Lactose (g/100 g)	Serving Size	Lactose (g/Serving)
Ice cream	6	50–100 g	3–6
Fresh milk	4.8	0.2 l	9.6
Natural yogurt	3.5	0.2 l	7.0
Cottage cheese	1.6	50–100 g	0.8–1.6
Butter	0.7	5–10 g	0.04–0.07
Hard cheese	0		0

the problem; however, some of them have their own disadvantages or do not work for everybody.

The treatment chosen should depend on the severity of the intolerance and often is affected by the options available. Avoiding dairy products is seldom necessary because most lactose-intolerant individuals are able to consume at least some grams of lactose daily. In addition, fermented dairy products like yogurt are very well tolerated, even in larger quantities, and most types of matured cheese do not contain any lactose. Table 4.3 presents the average content of lactose in dairy products.

Pharmaceutical preparations of fungal or yeast-derived β-galactosidase have been developed for the treatment of lactose maldigestion. There is evidence that these preparations increase lactose digestion and alleviate symptoms (Moskovitz et al. 1987; Sanders et al. 1992), but different preparations seem to vary in their effectiveness (Ramirez et al. 1994) and they do not help all subjects (Moskovitz et al. 1987). Compared to lactose in yogurt or in prehydrolyzed milk, these products seem less efficient (Onwulata et al. 1989; Solomons et al.

1985b). One case report on allergy to supplemental lactase enzyme has been published (Binkley 1996).

It has been suggested that consumption of galactose containing carbohydrates would lead to cataract and ovarian cancer. Therefore hypolactasia would protect an individual against these diseases and consumption of lactose-hydrolyzed products would inhibit the protection. In some studies consumption of milk in subjects with lactase persistence has been associated with an increased risk of cataract (Rinaldi et al. 1984; Simoons 1982). Cataract formation has been demonstrated in animals fed large amounts of galactose (Birlouez-Aragon et al. 1989; Kechrid et al. 1986) and in humans with a congenital defect in galactose metabolism (Skalka and Prchal 1980). However, other studies have shown a lack of relationship between lactose digestion and cataract (Bengtsson et al. 1984; Fontana et al. 1995; Lisker et al. 1988). Galactose is suggested as an oocyte toxin for ovarian cancer (Chen et al. 1981; Swartz and Mattison 1988) and with this cancer's link to the ability to digest lactose (Cramer 1989; Cramer et al. 1994; Kushi et al. 1999; Meloni et al. 1999). As in the case of cataract, the results of different studies are contradictory because a lack of association between galactose intake and ovarian cancer has also been demonstrated (Britton et al. 2000; Herrinton et al. 1995).

Milk and milk products are important sources of many nutrients, such as protein, calcium and riboflavin, so avoidance of dairy products is not advised without good reason, and then appropriate dietary modifications need to be made to ensure that nutrient deficiencies do not arise. For example, if alternative sources of calcium are not consumed, intake may be sufficiently low to compromise bone health. Lactose maldigestion is known to be a risk factor for enhanced bone fragility, which may eventually lead to osteoporosis. A study in Italian women (Corazza et al. 1995) demonstrated that bone mineral density and calcium intake were significantly lower in women with lactose maldigestion and symptoms of intolerance than in those with maldigestion alone. This finding supports other studies showing that women with osteoporosis have a significantly higher prevalence of lactose maldigestion, milk intolerance and lower daily calcium intakes than age-matched controls of similar ethnic origin (Lee and Krasinski 1998). Although the etiology of osteoporosis is multifactorial, the importance of achieving an adequate calcium intake in the presence of lactose maldigestion should be emphasized.

4.6 DEVELOPMENT OF LOW-LACTOSE PRODUCTS

Lactose is the main carbohydrate of milk; thus many dairy products contain lactose as the main carbohydrate. However, some dairy products also have naturally low lactose content, e.g., processed soft cheeses and butter. Hard and semihard cheeses are virtually free of lactose. When milk is fermented with lactic acid bacteria, lactose is metabolized to lactic acid; therefore, all fermented dairy products have lower lactose content than fresh milk. Hydrolysis of lactose by β-galactosidases is a technology that produces low-lactose dairy products. Techniques to remove lactose from milk are also available.
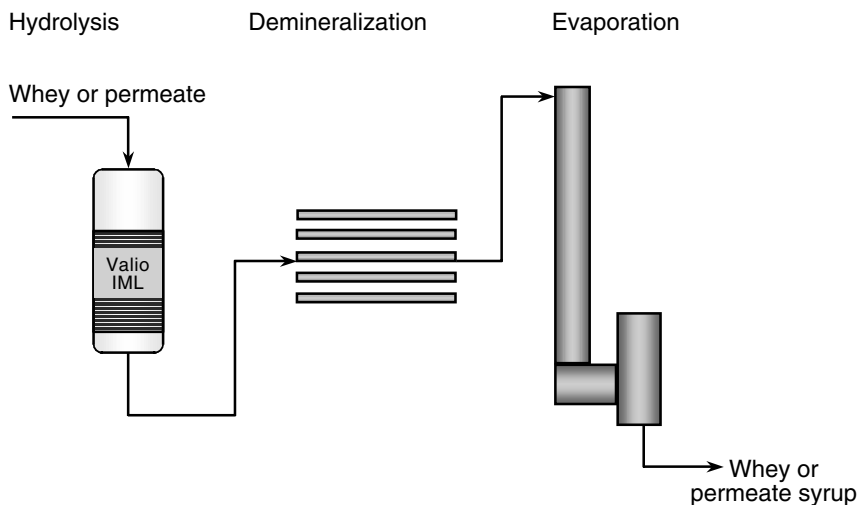
FIGURE 4.4 Valio hydrolysis process.

4.6.1 HYDROLYSIS OF LACTOSE

In hydrolyzed products, lactose is hydrolyzed to glucose and galactose by an enzymatic method (Figure 4.4). The content of lactose is usually guaranteed to be less than 20% of that in normal milk. This means that the content of lactose is lower than 1 g in 100 g of the finished product. This is such a low concentration that most lactose-intolerant people tolerate these products without any problems.

The development of the commercially available Valio hydrolysis process began in the early 1970s. In the beginning most attention was paid to lactose hydrolysis of whey by means of β-galactosidase immobilized to absorption resin by glutaraldehyde (Heikonen et al. 1985). In 1974, the immobilizing techniques were, in principle, solved and in the following year the process parameters of a whey hydrolyzing process were solved. An immobilized β-galactosidase reactor was taken into operation for dairy use on an industrial scale. Since then, the process has operated continuously with good success. The time period for using the immobilized enzyme has normally been at least 1 year. The process also includes ion exchange columns for the demineralization and adjustment of pH. According to Heikonen et al. (1985), the main advantages of inmobilizing the hydrolysis process are:

- The pH optimum of the enzyme used (*Aspergillus niger*) is low enough and the temperature optimum high enough so that hydrolysis can be conducted under microbiologically safe conditions (pH less than 4 and temperature greater than 40°C).
- The carrier (an adsorption resin) is manufactured for industrial use in a packed-bed reactor, so it is mechanically stable even during prolonged use.
- The particle size of the carrier is big enough so that ordinary whey and other proteinaceous substrates can be treated without clogging problems.
- The enzyme and the carrier are accepted for food purposes.

FIGURE 4.5 Valio's low-lactose HYLA® products.

After hydrolysis the whey is concentrated (60% dry matter). In the same way, hydrolyzed permeate syrup can be prepared from the ultrafiltered permeate of the whey. The lactose separated by crystallization has also been used as a substrate. The sweetness of the hydrolyzed lactose is approximately 70% of the sweetness of saccharose. The hydrolyzed whey products are suitable for sweetened milk products such as fruit yogurts, ice cream, and whey drinks. Syrups are also used in processed food products, sweets and bakery products. One reason for using hydrolyzed lactose, besides increasing sweetness and solubility, is that it forms aromatic and color substances due to the Maillard reaction (Heikonen et al. 1985).

Along with immobilized β-galactosidase, the application of soluble enzyme has also been studied. In 1979, as a result of this development, the production of hydrolyzed lactose (HYLA®) skim milk powder began; about 80% of its lactose is hydrolyzed.

The supply of lactose-reduced milk products was increased in Finland when 1.9% fat-containing, UHT-treated and aseptically packed HYLA milk was put on the market. Owing to the soluble β-galactosidase added to the milk, a minimum of 80% of the milk lactose is hydrolyzed after 1 week of storage at 18 to 20°C in the UHT plant. In order to achieve as total a hydrolysis as possible, after distribution it is recommended that hydrolyzed milk be stored at ambient temperature during its 3-month shelf life. Besides milk processing, the same hydrolyzing system can also be utilized in the production of other lactose-reduced UHT products such as flavored milk drinks, soft ice mix and dietary milk-based products (Heikonen et al. 1985). In 10 years Valio has launched over 31 different HYLA products. Today, the selection available covers all the groups of dairy products (Figure 4.5).

4.6.2 REMOVAL OF LACTOSE

A chromatographic separation method has been developed (Harju 1987, 1991) in order to remove lactose from milk (Figure 4.6). Thus, a lactose-free solution of milk proteins and salts can be obtained. This lactose-free (and carbohydrate-free) milk provides totally new possibilities for the development of new types of dairy products.
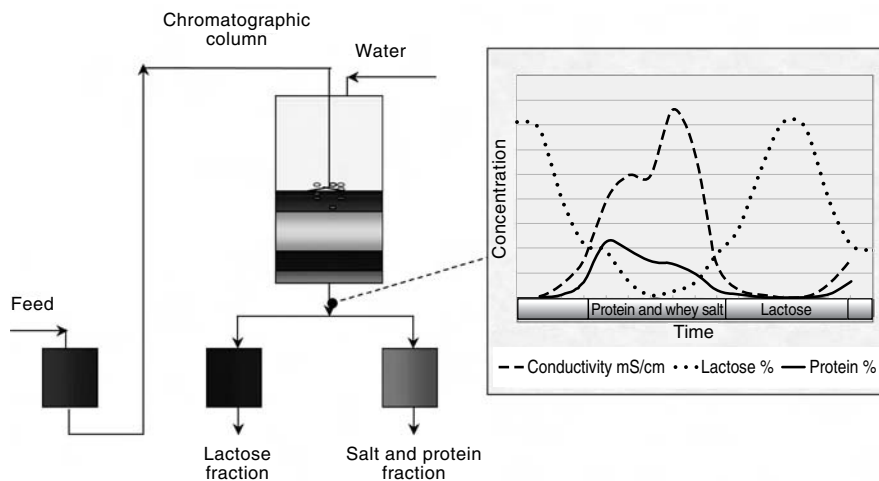
FIGURE 4.6 Valio chromatographic process.

Lactose-free milk has only 40% of the energy value of normal milk and does not have the sweet taste of hydrolyzed milk; other sweeteners can be used to provide sweetness if it is required.

4.7 MARKETING OF LOW-LACTOSE PRODUCTS

The relatively high prevalence of lactose intolerance together with traditionally high milk consumption has contributed to active product development and marketing campaigns for low-lactose products in Finland. Health experts became aware of lactose intolerance in the 1970s. At that time the consumption of fresh milk in Finland was the highest in the world, e.g., in 1975 nearly 230 l per capita per year. People were often advised to give up drinking milk if they suffered from abdominal symptoms. Since then, lactose intolerance has become known to all Finns.

At the same time, an active team at Valio R&D was studying lactose hydrolysis. Initially, techniques to split the lactose in whey were developed and the hydrolysis of lactose in milk was investigated soon after (Tykkyläinen et al. 1990). The marketing executives did not like the idea of launching competing dairy products; however, in 1978 test marketing of a low-lactose milk powder started and results showed a market need for low-lactose products. These products were named HYLA in order to distinguish them from other dairy products. HYLA® Milk Powder was launched in the Valio product range in 1980 and, in 1983, Valio started production of UHT HYLA® Milk. Since 1985 the company has launched a low-lactose product in almost every dairy product group (Tykkyläinen et al. 1990); pasteurized skimmed milk is one of the most recent. Because low-lactose products became successful within a short space of time, Valio has developed new applications to meet the requirements of different types of these products.

HYLA is one of Valio's registered trademarks and the HYLA logo is always printed on the package of low-lactose products; consumers recognize it easily. The

logo is only used on products that have a low content of lactose due to lactose hydrolysis. Many producers of ready-made foods have rights to use the HYLA trademark in their products when they are made with Valio low-lactose products. Today, Valio has the widest selection of different low-lactose products in the world.

4.8   CONCLUSION

Low-lactose and lactose-free dairy products provide new market opportunities. Valio's HYLA products, as well as lactose-free milk, are good examples of such opportunities in the dairy industry. A negative feature in dairy products has been turned into a growing market success. Although some may see low-lactose products as a market niche, Valio Ltd has shown that it is a profitable business area within traditional dairy business.

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5 *trans*-Galactooligosaccharides as Prebiotics

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CONTENTS

5.1 INTRODUCTION

Oligosaccharides are increasingly recognized as dietary-based tools for modulation of the colonic microflora toward a purportedly healthier community structure (Fuller and Gibson 1998). Because they are carbohydrates, their incorporation into foods and drinks like biscuits, cereals, infant formula feeds, carbonated drinks, juices, table spreads and confectioneries has been the chosen way of administration (Crittenden and Playne 1996). Dietary ingredients that can potentially provide beneficial physiological advantages beyond basic nutrition are termed functional foods. For oligosaccharides, this usually involves selectively increasing the levels of gut bifidobacteria and lactobacilli, preferably at the expense of less desirable organisms such as *Escherichia coli*, clostridia and proteolytic bacteroides. This selective fermentation is known as the prebiotic concept (Gibson and Roberfroid 1995).

Prebiotics, therefore, are dietary food ingredients, usually carbohydrates, that are not digested in the upper gastrointestinal tract but transfer to the colon, the most
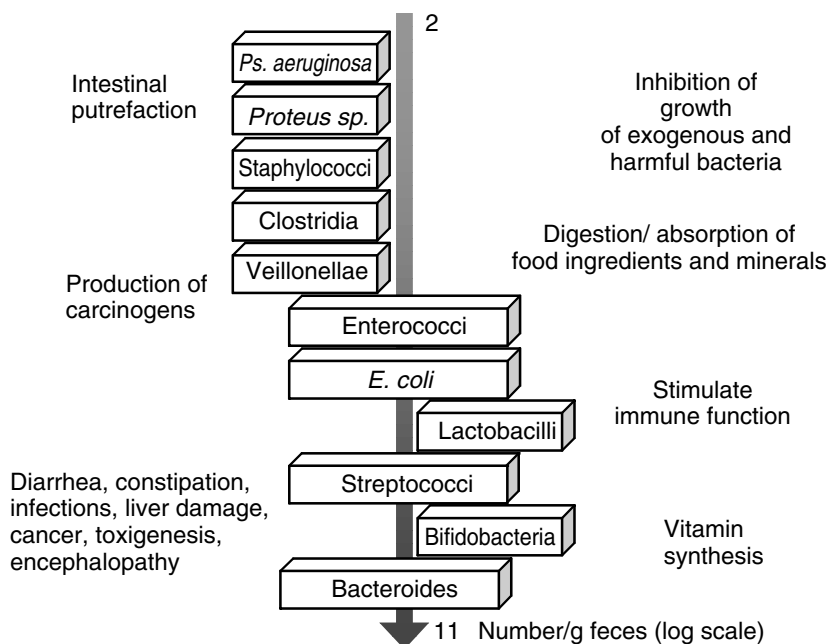
FIGURE 5.1 Principal bacterial genera in the human colon.

heavily colonized microbial area in humans (Cummings and Macfarlane 1991; Gibson and Roberfroid 1999). Therein they are selectively metabolized by the bacterial flora; this specificity should be directed toward beneficial components of that flora, i.e., indigenous probiotics (Gibson and Roberfroid 1995; Figure 5.1). Efficient prebiotics are resistant to stomach acid, bile secretions and mammalian enzymes, but by their structure confer a selective form of bacterial metabolism in the lower gut. In the broadest terms, prebiotics can potentially include any nondigestible food ingredient; however, the selectivity of the fermentation is key to functionality (Van Loo et al. 1995, 1999). Good food processing and sensory characteristics should also be present (Crittenden 1999; Crittenden and Playne 1996).

It is possible to develop a range of such carbohydrates for incorporation into foods in a bid to improve their prebiotic effect. This may be especially useful in the West, where conventional diets have only relatively small quantities of oligosaccharides (approximately 2 to 12 g/day) consumed daily (Macfarlane and Cummings 1991; Roberfroid et al. 1993). In the past, oligosaccharides have been used in the food industry to reduce sweetness and modify viscosity and freezing points of foods, for their preservative properties, to alter food color, lower calorific value, prevent tooth decay, reduce moisture loss in foods and induce dietary fiber-like effects in the large bowel (Crittenden and Playne 1996).

The concept of functional foods as part of a normal diet has made genuine progress in the Far East, with oligosaccharide preparations widely used in countries like Japan (Table 5.1). In Europe, prebiotic use is comparatively less well advanced (Gibson et al. 1999, 2000). Nevertheless, some European manufacturers (Orafti,

TABLE 5.1
Oligosaccharides in Japanese Foods for Specified Health Use

Oligosaccharides	Product Type	Product	Manufacturer
Fructooligosaccharides	Table-top sweetener	Meioligo granules and syrup	Meiji Seika
		Fructo-oligo-saccharide	Nippon Oligo
		Oligo 55, Oligosugar 39	Hakubun
	Coffee drink	Oligo Coffee	Meiji Seika
	Custard dessert	Meioligo Purin	Meiji Milk Products
	Candies	Oligo Yogurette, Oligo Candy	Meiji Seika
Lactulose	Soft drink	Maiasa Sokaina	Morinaga Milk Industry
trans-Galactooligo-saccharides	Table-top sweetener	Cup Oligo Sweet Extra	Nissin Sugar
		Oligomate HP	Yakult Honsha
		Oligotop	Ito Kampo Seiyaku
Isomaltooligosaccharides	Soft drinks	Power Gold	Asahi Beverage
	Table-top sweetener	Oligo Time	Showa Sangyo
Soybean oligosaccharides	Soft drinks	Oligo CC	Calpis Co.
		Nichirei Acerola Extra Blend	Nichirei
	Table-top sweetener	Soy oligosaccharide syrup	Calpis Co.
		Soy oligosaccharide syrup	Dainippon Seito
		Bifiup	Calpis Co.
Xylooligosaccharides	Lactic acid drink	Yogurina	Suntory
	Flavored vinegar	Morishige Genskissu	Morushige Ueda
	Chocolate and candy	Sukkiri Kaicho Jouka	Lotte
Lactosucrose	Soft drinks	Oligo 2400 (apple, carrot, grape)	Taisho Shokuhin Kogyo
	Table-top sweetener	Oligo No Asahi, Nyuka Oligo, Nyuka Oligo granulated	Ensuiko Sugar
	Frozen yogurt	Frozen yogurt	Ezaki Glico
	Candy	Piku Oligo Candy	Ezaki Glico
	Biscuits	Piku Oligo Biscuit	Ezaki Glico

Source: From Gibson, G.R. et al., *Prebiotics: New Developments in Functional Foods*, Chandos Publishing Limited, Oxford, 2000. With permission.

Solvay, Sensus, Beghin-Say, Borculo-Whey) produce prebiotic supplements to be taken as part of the daily diet and new prebiotic-containing foods have emerged. Although the market for prebiotics is constantly expanding, the current status is that three main forms predominate in food ingredients in Europe: fructooligosaccharides, lactulose and galactooligosaccharides (also known as *trans*-galactooligosaccharides or TOS). Because bifidobacteria and lactobacilli

express high levels of β-galactosidase, they are well suited for the directed metabolism of galactooligosaccharides.

This chapter will outline data on the use of galactooligosaccharides as prebiotics and discuss new approaches in their use. For a review on their food-processing characteristics, nondigestibility in the upper gut and other applications, see Schoterman and Timmermans (2000).

5.2 MANUFACTURE OF GALACTOOLIGOSACCHARIDES

Lactose is the precursor molecule for galactooligosaccharides; it is enzymically converted to a mixture of oligosaccharides with $\beta1 \rightarrow 4$, $\beta1 \rightarrow 3$ and $\beta1 \rightarrow 6$ linkages and readily available from cow's milk. Galactooligosaccharides are particularly appropriate for use in dairy products and can also be detected in very low concentrations in human milk (Yamashita and Kobata 1974). They are thus obtainable *in vitro* from lactose by a glycosyl transfer catalyzed by β-galactosidase and occur as complex mixtures with a lactose core and one or more galactosyl residues (Ekhart and Timmermans 1996). Free or immobilized β-galactosidases from several biological sources can be used to manufacture galactooligosaccharides in batch or continuous enzyme reactors to make oligosaccharide products. The oligosaccharides formed have degrees of polymerization values ranging from 2 to 6, depending on the biological source of the enzyme. The technique is one of enzyme reversal and has been applied to oligosaccharide synthesis since the 1950s (Bucke and Rastall 1990).

Beta-galactosidase (EC 32123, β-D-galactoside galactohydrolase) is commonly considered a degradative enzyme associated with the hydrolysis of lactose. It catalyzes the transfer of galactose under very dilute conditions to an acceptor, which is water. The galactose moeity can be transferred to another molecule of lactose acting as the acceptor to build up the higher molecular weight oligosaccharides. This is usually achieved using a concentrated lactose solution and eventually results in the short-chain carbohydrates (Figure 5.2) (Ekhart and Timmermans 1996).

Three products made using β-galactosidases isolated from sources such as bacteria and fungi are commercially available (Crittenden and Playne 1996; Ekhart and Timmermans 1996). First, *trans*-galactosylated oligosaccharides are produced using β-galactosidase from *Aspergillus oryzae* (Tanaka et al. 1983) and consist of tri-, tetra-, penta- and hexa-galactooligosaccharides (Table 5.2). The second, Oligomate 55, is prepared using a β-galactosidase transfer activity in *A. oryzae* and *Streptococcus thermophilus* (Ito et al. 1990) and its configuration is 36% tri-, tetra-, penta- and hexa-galactooligosaccharides, 16% *trans*-galactosylated disaccharides (galactosyl glucose and galactosyl galactose), 38% monosaccharides and 10% lactose. Finally, a preparation containing *trans*-galactosylated disaccharides is manufactured using β-galactosidase from *S. thermophilus* (Ito et al. 1993). *trans*-Galactooligosaccharides have been manufactured by Yakult Honsha and Snow Brand in Japan and by Borculo Domo Ingredients in Europe.
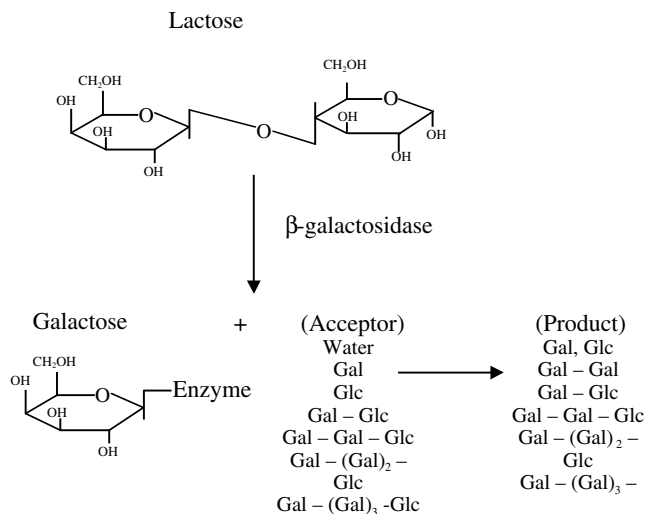
FIGURE 5.2 Transfer reactions of β-galactosidase; Gal, galactose; and Glc, glucose. (After Matsumoto, K. et al., in *Oligosaccharides, Production, Properties and Applications*, Ikoma, T., Ed., Gordon and Breach Science Publishers, Tokyo, 90–106, 1993.)

TABLE 5.2
Constituent Sugar Composition and Configuration of *trans*-Galactosylated Oligosaccharides

Oligosaccharides	Sugar Composition (%)	Oligosaccharide Structure (Major Components)[a]
Trisaccharides	50	Gal (β1 → 6) Gal (β1 → 4) Glc
		Gal (β1 → 3) Gal (β1 → 4) Glc
		Gal (β1 → 4) Gal (β1 → 4) Glc
		Gal (β1 → 4) Gal (β1 → 6) Glc
Tetrasaccharides	35	Gal (β1 → 6) Gal (β1 → 6) Gal (β1 → 4) Glc
		Gal (β1 → 3) Gal (β1 → 6) Gal (β1 → 4) Glc or
		Gal (β1 → 6) Gal (β1 → 3) Gal (β1 → 4) Glc
Penta- and hexa-saccharides	15	Gal (β1 → 6) Gal (β1→6) Gal (β1 → 6) Gal (β1→4) Glc and Gal (β1 → 6) Gal (β1 → 6) Gal (β1 → 6) Gal (β1 → 6) Gal (β1 → 4) Glc

[a] Gal, galactose; Glc, glucose.

Source: After Matsumoto, K. et al., in *Oligosaccharides, Production, Properties and Applications*, Ikoma, T., Ed., Gordon and Breach Science Publishers, Tokyo, 90–106, 1993.
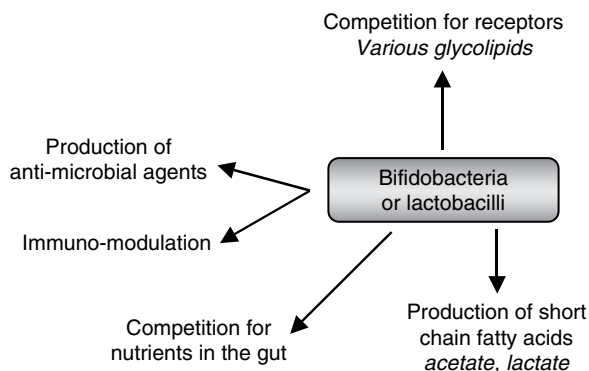
FIGURE 5.3 The probiotic barrier.

5.3 PUTATIVE HEALTH BENEFITS OF PREBIOTICS

Many health benefits have been claimed for prebiotics, although in some cases the underlying mechanisms are not clear. The health benefits with the strongest scientific backing are described in the next sections.

5.3.1 PROTECTION AGAINST INVADING PATHOGENS

Prebiotics selectively increase the population of probiotic bacteria, which have a barrier function against invading gastrointestinal pathogens (Figure 5.3). For this reason, prebiotics might prove a practical means of prophylactically enhancing food safety by inhibiting the chances of organisms such as campylobacters, salmonellae and *E. coli* causing infections in the gut. Fermentation of prebiotics to short-chain fatty acids reduces lumenal pH in the colon, thus inhibiting pathogens such as *E. coli*. In addition, elevated populations of bifidobacteria and lactobacilli mean increased competition with other organisms for nutrients and gut wall receptors. Probiotics are also known to produce antimicrobial agents active against a range of pathogens (Gibson and Wang 1994a).

5.3.2 PROTECTION AGAINST COLON CANCER

Many fecal microorganisms produce carcinogens and tumor promoters from dietary and other components entering the colon. Although the species responsible are not known (with the exception of *Bacteroides* sp and *Clostridium* sp), it is known that bifidobacteria and lactobacilli do not have such activities and dominance of the gut flora with these probiotics results in lower levels of carcinogens (Rowland and Tanaka 1993). Moreover, some of the end products of bacterial fermentation may induce protective capacities. In this case, short-chain fatty acids, principally butyrate, have attracted much attention (Salminen et al. 1998). Butyrate is the preferred "fuel" for colonocyte function in the healthy gut epithelium, but is thought to exert certain effects on preneoplastic or neoplastic colonocytes. These may include the induction of apoptosis, modulation of oncogene expression (e.g., inhibition of *ras* and *src*

proto-oncogenes), induction of certain differentiation markers and regulation of systems involved in cellular adhesion and/or migration (Demigne et al. 1999).

5.3.3 IMPROVED CALCIUM ABSORPTION

The possibility of increasing calcium absorption from the colon by consumption of prebiotics has received much interest recently. The principal site of calcium absorption is the small intestine; however, it is thought that significant amounts are absorbed in the colon. Several mechanisms have been postulated for increased calcium absorption induced by prebiotics (Fairweather-Tait and Johnson 1999), including the reduction in lumenal colonic pH, thus increasing calcium solubility and fermentation of calcium-binding antinutrients such as phytate and thereby liberating calcium.

5.3.4 EFFECTS ON BLOOD LIPIDS

Currently, the food industry is showing intense interest in developing functional foods to modulate blood lipids such as cholesterol and triglycerides. Effects on serum cholesterol levels have been postulated for prebiotics, although the mechanism is unclear (Delzenne and Williams 1999).

5.4 THE PREBIOTIC PROPERTIES OF TOS

trans-Galactooligosaccharides are not hydrolyzed in the upper gastrointestinal tract of humans (Bouhnik et al. 1997; Burvall et al. 1980). As such, they are candidate prebiotics in that they enter the large intestine and are metabolized by the indigenous microflora therein.

These microbiota play a key role in nutrition and health. Through the process of fermentation, gut bacteria metabolize various substrates (principally dietary components) to end products like short-chain fatty acids and gases. This anaerobic metabolism is thought to contribute positively to the host's daily energy requirements. In fact, the human large intestine is probably the body's most metabolically active organ; this is attributable to the resident flora and its activities. Usually, the human host lives in harmony with these complex gut microbiota; however, under certain circumstances, such as antimicrobial intake, stress, and poor diet and living conditions, microbiota balance may be upset. Moreover, the normal fermentative process may produce undesirable metabolites like ammonia, phenolic compounds, toxins, etc. The gut flora is also susceptible to contamination by transient pathogens that serve to upset the normal community structure further. These factors can have serious consequences that may lead to the onset of an acute or chronic gut disorder.

It is clear that microbiota are susceptible to manipulation through dietary mechanisms that target specific bacteria. As such, there is significant current interest in the use of dietary components that help to maintain or even improve the normal gut microflora composition and activities. This is a critical aspect of functional food sciences that targets beneficial gut bacteria. One much used approach is probiotics. Here, live microbial additions are made to appropriate food vehicles like yogurts or other fermented milks. The microorganisms used in this respect are usually lactic

acid bacteria such as lactobacilli and/or bifidobacteria. It is proposed that probiotics exert certain advantageous properties in the gastrointestinal ecosystem that are thought to include improved resistance to pathogens, reduced blood lipids, positive immunomodulatory properties and better protection from chronic gut disorder (Fooks et al. 2000; Fuller and Gibson 1998). To be effective, probiotics should remain viable and stable and be able to survive in the intestinal ecosystem, and the host animal should gain beneficially from harboring the probiotic. The alternative approach, prebiotics, has the same intention in that it is used to alter the gut flora composition.

The key to prebiotic classification is selectivity of the fermentation. Various methodologies may be used to determine this. The simplest *in vitro* fermenters are static batch cultures in which the substrate is added at a known concentration to a vessel. These may contain pure cultures, defined mixed inocula or a complex bacterial flora such as a fecal suspension. Fermenters are incubated anaerobically at 37°C and sampled at intervals (Wang and Gibson 1993). Batch fermenters are closed systems in which the substrate is limited, so the culture follows typical bacterial growth curves and can only be used for short-term course experiments. Moreover, the use of pure cultures does not allow competitive interactions to be investigated. Steady-state continuous culture systems can also be used to simulate the intestinal ecosystem. These may range from single- to multiple-stage chemostats. The latter can be useful gut models, in that physicochemical conditions imposed on each vessel can be made to represent a different region of the intestine. However, although *in vitro* systems may have a degree of complexity and reliability, authentic prebiotic effects are best derived through human volunteer trials.

Various *in vivo* approaches can be used. Animals, usually rats or mice, have been used to determine the effect of substrate on the fecal microflora. Three types of animals have been used: those with their own microflora (Morishita and Konishi 1994; Yamada et al. 1993); gnotobiotic (germ-free) or inoculated with selected types of organisms (Djouzi and Andrieux 1997; Djouzi et al. 1995; Valette et al. 1993), and those associated with a human fecal flora (Mallett et al. 1987; Rowland and Tanaka 1993). Obviously, the ultimate test for the effectiveness of a prebiotic is a human-volunteer, double-blind, placebo-controlled trial that has an appropriately selected group of subjects. However, it should be noted that using feces as test material to determine fermentative interactions is a drawback.

A hierarchy of testing procedures exists for determining prebiotic functionality (Rycroft et al. 1999); these have been variably applied toward determining the prebiotic effect of TOS. The simplest approach is to compare the growth rate of selected pure cultures of gut bacteria, which gives an indication of species involved in the metabolism, but does not determine a true prebiotic effect because of an absence of competitive influences between the microbiota involved. However, the use of pure culture studies is a useful screening approach to determine whether bifidobacteria or lactobacilli have any preferred effect. In this context, Tanaka et al. (1983) compared strains of eight bifidobacteria, five bacteroides, three fusobacteria, six eubacteria, eight clostridia, one *Propionibacterium acnes*, eight lactobacilli, eight streptococci, four enterobacteriaceae and one *Staphylococcus aureus* for their comparative ability to utilize TOS. These studies showed good growth of all the

TABLE 5.3
Animal Studies on the Prebiotic Effect of Galactooligosaccharide

System	Substrate	Dose	Time	Effect	Reference
Rats	TOS[a]	5% (w/v)	7 weeks	Significant increase in bifidobacteria; significant decrease in staphylococci and streptococci	Morishita and Konishi, 1994
Human flora-associated rats	TOS	5 or 10% (w/v)	Unknown	Significant increase in short-chain fatty acids and H_2 for the 10% diet; significant decrease in lactate and succinate; decreased butyrate, valerate and isoacids	Andrieux and Szylit, 1992
Human flora-associated rats	TOS	5% (w/v)	4 weeks	Significant increase in bifidobacteria and lactobacilli; significant decrease in enterobacteria	Rowland and Tanaka, 1993

[a] TOS = *trans*-galactooligosaccharide.

bifidobacteria strains tested, two *Bacteroides fragilis* strains, four lactobacilli strains and four enterobacteria. The indication was that bifidobacteria were the preferred genus stimulated.

For testing in mixed microbial culture, batch fermenters and chemostat type experiments may be employed (Rumney and Rowland 1992). These vary in complexity, with differing conditions of pH, substrate availability, transit time and inoculum used. Unlike the fructooligosaccharides (Gibson and Wang 1994b), galactooligosaccharides have not been extensively researched in this regard. Adding 20 g/d TOS to a continuous culture (Durand et al. 1992) and 10 g/d TOS to a semicontinuous culture containing human fecal bacteria (Bouhnik et al. 1997) increased gas and short-chain fatty acids. Although bacteria were not enumerated, increased lactate and acetate was suggested to be the result of a proliferation of lactic acid bacteria (lactobacilli and bifidobacteria) in response to the addition of TOS.

Galactooligosaccharides have been used in animal models (Table 5.3). One important study showed that galactooligosaccharides had prebiotic properties after feeding to rats associated with a human microflora (Rowland and Tanaka 1993). The model system involves breeding sterile animals and inoculating them with the fecal flora of humans. In this study, six rats were used as controls and six were fed a TOS-containing diet at 5% (w/w). After 4 weeks, the rats were sacrificed and the bacteriology of cecal contents analyzed using plate culture. The data showed a significant increase in bifidobacteria and lactobacilli.

The use of conventional laboratory animals (e.g., rodents) gives useful data on physiological events but the microbiology may not be reflective of the human situation. This is because the gut architecture is vastly different to that of humans and this is bound to influence the nature of microbiota that develop. Thus, the definitive test for prebiotic functionality is a human volunteer trial.

Unlike probiotics, in which recovery in feces may indicate nonpersistence within the gut environment, a change in stool bacteriology in response to prebiotic ingestion is likely to be reflective of events *in situ.* Molecular-based methodologies give an accurate and reliable picture of bacterial changes in biological specimens like stools and have been used to determine prebiotic functionality in the fructooligosaccharides (Tuohy et al. 2001a). Although it is encouraging that data for TOS have been derived through culture-based bacteriology, newer methodologies such as those used by Tuohy et al. (2001a) need to be applied to galactooligosaccharides.

Several human studies have evaluated the effect of consuming TOS (Table 5.4). Ito et al. (1990) recruited 12 male volunteers and divided them into four groups of three to receive 0, 2.5, 5 and 10 g/d of TOS for 8 weeks. The results showed a significant increase in bifidobacteria as well as lactobacilli at all the test doses. Ito et al. (1993) fed 15 g/d TOS for 6 d and again found a positive effect on bifidobacteria and lactobacilli. Moreover, certain putrefactive enzymes were reduced during the test period. In humans, 10 g/d TOS significantly reduced breath hydrogen (Bouhnik et al. 1997), whereas an increase was seen in human flora-associated rats fed 5 or 10% (w/v) TOS (Andrieux and Szylit 1992). This latter finding is difficult to understand if TOS was acting as an efficient prebiotic because the target populations do not produce gas. As such, if hydrogen excretion is elevated during oligosaccharide ingestion, this suggests that the prebiotic effect has been compromised. This may be the case if high doses are administered. Bouhnik et al. (1997) also reported an increased number of bifidobacteria in feces, which may help to explain the reduction in breath hydrogen.

On the other hand, some negative data have been reported on the bifidogenic effects of TOS. Alles (1998) found that feeding up to 15 g/d TOS had little effect on bifidobacterial numbers of 40 human volunteers when compared to an unsupplemented diet. However, the volunteers had high starting levels of bifidobacteria (>9 $\log_{10}$ CFU/g) in their stools. In this context, it is recognized that the level of prebiotic effect after feeding nondigestible oligosaccharides depends on initial starting levels of bifidobacteria (Tuohy et al. 2001b; Van Loo et al. 1999). Higher initial populations do not elicit as marked an effect as those in which fecal counts are reduced. It must be remembered, however, that a small increase in log population values from a high initial level still means a large increase in bacterial numbers.

It has been suggested that the increased bifidobacterial flora of breast-fed infants may be associated, at least partly, with the presence of galactose-containing oligosaccharides in human milk (Matsumoto et al. 1993). It should be borne in mind, however, that the oligosaccharides in human milk represent a very diverse class of complex oligosaccharides containing several monosaccharides, most of which have little structural resemblance to commercial TOS (Kunz and Rudloff 1993). TOSs are now incorporated into infant formula feeds (Schoterman and Timmermans 2000; Teuri et al. 1998). Other food products that contain galactooligosaccharides include

TABLE 5.4
Human Studies on the Prebiotic Effect of Galactooligosaccharides

Subjects	Substrate[a]	Dose	Time	Effect	Reference
5 men	TOS	3 g/d, then 10 g/d	1 week, then 1 week	3 g/d: little effect; 10 g/d: significant increase in bifidobacteria and lactobacilli and significant decrease in bacteroides	Tanaka et al., 1983
12 men	Oligomate	0, 2.5, 5 then 10 g/d	1 week for each dose	Bifidobacteria increased with dose. For 10 g/d, significant increase in bifidobacteria and lactobacilli	Ito et al., 1990
12 men	TD	15 g/d	6 d	Significant increase in bifidobacteria and lactobacilli; significantly lower bacteroides; significant decrease in indole, p-cresol, NH_3, propionate, valerate, isovalerate and isobutyrate	Ito et al., 1993
8 adults	TOS	10 g/d	21 d	Significantly increased bifidobacteria; significant reduction in breath H_2	Bouhnik et al., 1997
6 adults	TOS	15 g/d	14 d	Significant increase in total count on media for lactic acid bacteria; no change in bifidobacteria	Teuri et al., 1998
44 adults	TOS	0, 7.5, 15 g/d	21 d for each dose	No significant difference in bifidobacteria among the doses tested	Alles, 1998

[a] TOS = *trans*-galactooligosaccharides; TD = *trans*-galactosylated disaccharide.

table-top sweeteners like Cup Oligo Sweet Extra from Nissin Sugar, Oligomate HP from Yakult Honsha and Oligotop from Ito Kampo Seiyaku. These products have been given FOSHU (foods for specified health use) status in Japan on the basis of their bifidogenic effect. Yakult also uses TOS in a lactic acid bacterial drink and yogurt. TOSs have been incorporated into soft drinks, ice cream, chocolate and bread as well (*Japanscan Food Industry Bulletin*, 1998).

The current experimental doses of TOS (approximately 10 to 15 g per day), which have resulted in a bifidogenic effect in human and animal studies, may be

accompanied by side effects of flatulence and bloating due to elevated hydrogen production (Andrieux and Szylit 1992; Bouhnik et al. 1997). However, the target organisms for oligosaccharide use are not gas producers, suggesting a nonselective metabolism of these carbohydrates probably arising from high-dosage forms. Therefore, a useful prebiotic should have a range of low-concentration doses at which it remains active. This will depend upon highly selective fermentation and efficient metabolism of the molecules; these effects must be confirmed by rigorous scientific methodologies.

5.5   BACTERIAL CHARACTERIZATIONS

Critical to the assessment of prebiotic effects is a robust characterization of the gut microbiota involved in fermentation; this applies to *in vitro* and *in vivo* studies. In most studies investigating prebiotics, especially TOS, changes in the intestinal microflora have been detected using classical phenotypic and biochemical methods of identification. It is now apparent that these approaches may not be entirely reliable because they do not recover the nonculturable moiety of the gut flora and allow operator subjectivity. For example, it is important that culturing onto selective agars is consolidated through a diagnostic description of the colonies that form because agars containing antibiotics or other "selective" factors may allow the recovery of phenotypically similar bacterial species to those targeted (Steer et al. 2000; Tannock 1999). Often, however, these latter organisms may be genotypically distinct. Thus, the reliability of many selective agars is questionable and should not be relied upon (Lawson 1999). Gut microbiologists are faced with the challenge of adequate descriptive techniques using this approach.

Studies on probiotics and prebiotics in the past have relied upon agar selectivity for determining bacterial changes. If no follow-up characterization has been carried out, these studies should be seen as (at best) indicative. Following this, various levels of identification can be used. Some authors choose to use microscopic analysis only, but, again, this is not a reliable approach because morphological assessment coupled with staining reactions is not descriptive. One improvement is to look for specific biomarkers to indicate identity and/or biochemical traits such as the production of a specific enzyme profile or fermentation patterns. This is a laborious approach but can be reliable. However, the complexity of the gut flora becomes a further issue. Within these microbiota, a large proportion of unculturable cells exists; these are viable organisms that can be seen under the microscope but do not form colonies on agar plates (Liesack and Stackebrandt 1992). Therefore, as well as being time-consuming, labor-intensive and unable to account for these unculturable organisms, routine microbiological methods have limited value.

With the advent of a range of molecular techniques, this situation is improving. Pure colonies obtained by classical culturing techniques can be identified, to species level by sequence analysis. DNA is extracted from colonies, amplified by the polymerase chain reaction (PCR) and then 16S rRNA is sequenced and compared to an information database (O'Sullivan 1999; Wilson and Blitchington 1996). A range of species- and genus-specific oligonucleotide probes have been developed, using information obtained by sequence analysis (Lawson 1999). Such approaches can be used

in situ where total cells are extracted from a fecal sample and hybridized using labeled probes. Perhaps the most common method is fluorescent *in situ* hybridization (FISH) in which genus-specific fluorescently labeled probes hybridize bacterial rRNA and fluorescent cells are enumerated (Langendijk et al. 1995; O'Sullivan 1999). These procedures have now been applied in human trials to determine the effects of lactulose and fructooligosaccharides as prebiotics, including in food products such as biscuits (Tuohy et al. 2001b, 2002). They now need to be used to determine similar effects with galactooligosaccharides to consolidate the existing data, which indicate prebiotic functionality.

To gain fuller information on the species diversity of a sample that does not rely on culturing, direct community analysis can be used (Suau et al. 1999). This technique involves extracting total DNA or RNA from feces, amplifying by the PCR, cloning into a vector (resulting in many gene clones) and sequencing these to identify the range of species present in the fecal sample (O'Sullivan 1999). The use of FISH in conjunction with direct community analysis allows quantitative and qualitative analyses of the bacterial composition (including unculturable organisms) of the intestinal microflora in response to prebiotic candidates like TOS.

All prebiotics tested *in vivo* and described as bifidogenic indicate that they have an effect on total bifidobacterial (genus level) populations (Bouhnik et al. 1994, 1996; Gibson et al. 1995; Ito et al. 1993; Kleessen et al. 1995; Okazaki et al. 1990). Microorganisms in this genus, together with those belonging to *Lactobacillus,* are designated predominantly as probiotics because they are generally nonpathogenic and have been shown to improve the intestinal microbial balance and/or properties (Table 5.4) when high-enough viable doses are applied to humans or animals. However, lactic acid bacteria may vary in their health-promoting activities. For example, probiotic bacteria like *B. longum, B. bifidum, B. breve* and *Lactobacillus* GG have demonstrable activity in the field of reduction or alleviation of symptoms associated with intestinal disorders such as antibiotic-associated and infantile diarrhea (Fooks et al. 2000). Therefore, it may be advantageous to obtain prebiotic supplements specifically directed toward certain probiotic species, rather than entire genera. An added advantage would be a reduction in the dose and time required to bring about biological activity because any added prebiotic should be preferentially utilized by the target organism. This may be achievable with TOS.

5.6 SECOND GENERATION GALACTOOLIGOSACCHARIDES

Despite the utility of TOSs as prebiotics, it is possible to envisage the development of forms of TOS with enhanced biological properties. One desirable aspect would be TOS development with more species-specific fermentation profiles. An approach to the generation of species-specific TOS that is in its early stages but shows promise is to utilize β-galactosidases from the target microorganisms to generate the oligosaccharides.

Theoretically, all glycosidases can be used to synthesize a variety of oligosaccharides; the extent to which synthesis occurs depends on thermodynamic or equilibrium properties of the enzymes involved (Bucke 1996). A study examining the ability of bacterial β-galactosidases from *Bifidobacterium angulatum*, *Bifidobacterium*

adolescentis, *Lactobacillus plantarum*, *Bifidobacterium infantis*, *Bifidobacterium pseudolongum* and *Bifidobacterium bifidum* Bb12 to synthesize a range of GOS used lactose as a donor of galactosyl residues (Rabiu et al. 2001). At 55°C (37°C for the lactobacilli), β-glycosidases exhibited hydrolytic and galactosyl-transferase activities that yielded mono- and oligosaccharide products, respectively.

The *trans*-galactosylase reaction dominates hydrolysis with increasing lactose concentrations and up to 30% w/w maximum achievable solubility. Under these conditions, production yields reached between 27 to 44% of total sugar concentration in the first 6 to 7 h of incubation, with relative proportions of oligosaccharides generally decreasing as molecular mass increased. The linkages for the oligosaccharides synthesized, when compared to those of the commercial TOS, Oligomate 55 (predominantly 1,4 Gal*p*), were different and had distinct product spectra from other novel preparations. The materials comprising 1,3-Gal, 1,6-Gal, 1,2-Gal + 1,6-Gal and 1,2-Glc and 1,3-Glc type linkages in varying ratios of *B. bifidum* Bb12–, *B. infantis*– and *B. pseudolongum*–derived products had similar bond profiles. *B. angulatum* and *L. plantarum* derivatives resembled the *B. adolescentis* product with a predominance of 1,6 Gal*p* linkages. It was anticipated that the mixture and bond differences obtained for oligosaccharides may confer some selectivity at species level when fermented by colonic microorganisms. In pure cultures, these oligosaccharide mixtures supported larger increases in growth rate on homologous galactooligosaccharide compared to growth on commercial TOS.

These increases in growth rate were seen for *B. angulatum*, *B. bifidum* Bb12, *B. infantis* and *B. pseudolongum*; in the case of *B. angulatum*, *B. infantis* and *B. pseudolongum*, the organisms displayed the highest growth rates of all the bacteria tested on their homologous oligosaccharide mixtures. *B. adolescentis* and *L. plantarum* had a decreased growth rate compared to their growth rate on commercial TOS; however, *L. plantarum* displayed the highest specific growth rate on its homologous oligosaccharide mixture in comparison to the other selected species. To date, this approach has not been tested in mixed cultures; however, the results indicate that β-galactosidases in some lactic acid probiotic bacteria can act as useful catalysts in the nonlinkage-specific synthesis of TOS. This knowledge can be applied to the search for new prebiotic components with species-specific activity, such as increases in total bifidobacteria, rather than simply genus-level activity.

To demonstrate the significance of this approach, purified residues need to be investigated to identify selective entities. In addition, robust and accurate molecular microbiological methods may serve as useful alternatives for detecting specific probiotic species of interest and allow determination of selective capabilities of these novel oligosaccharides to be monitored in mixed fecal culture. Furthermore, this is an excellent approach toward the manufacture of synbiotics, which are combinations of probiotics and prebiotics mixed together in a single product (Gibson and Roberfroid 1995). Generation of the prebiotic through reverse enzyme technology using the probiotic would ensure that it would act in a tailored manner. Therefore, the possibility exists to enhance the survival of probiotic preparations currently used in the gut by linking them with species-effective probiotics.

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6 Milk-Derived Bioactive Peptides: Formation and Prospects for Health Promotion

Hannu Korhonen and Anne Pihlanto–Leppälä

CONTENTS

6.1 INTRODUCTION

It is well known that, apart from nutritional value, proteins also possess biological and physicochemical properties. For example, milk contains a wide range of proteins that provide protection against enteropathogens or are essential for the manufacture and characteristic nature of certain dairy products. Research carried out during the last 10 years has shown that the caseins and whey proteins can be an important source of biologically active peptides (Schanbacher et al. 1998). These peptides are in an inactive state inside the protein molecule and are released during enzymatic digestion *in vitro* or *in vivo*. Bioactive peptides usually contain 3 to 20 amino acid residues per molecule. Biologically active peptides have been found to have specific activities, such as antihypertensive, antioxidative, antimicrobial, immunomodulatory, opioid or mineral-binding activities (Table 6.1). Many milk-derived peptides reveal multifunctional properties, i.e., specific peptide sequences may exert two or more

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TABLE 6.1
Examples of Biologically Active Milk Peptides

Precursor Protein of Bioactive Peptides	Bioactive Peptide Group	Bioactivity Observed
α-, β-casein	Casomorphins	Opioid agonist
α-lactalbumin, β-lactoglobulin	Lactorphins	Opioid agonist
Serum albumin	Serorphin	Opioid agonist
Lactoferrin	Lactoferroxins	Opioid antagonist
κ-casein	Casoxins	Opioid antagonist
α-, β-casein	Casokinins	ACE inhibitory
α-lactalbumin, β-lactoglobulin, serum albumin	Lactokinins	ACE inhibitory
α-, β-casein, lactoferrin	Immunopeptides	Immunomodulatory
Lactoferrin	Lactoferricin	Antimicrobial
α_{S2}-casein	Casocidin	Antimicrobial
α_{S1}-casein	Isracidin	Antimicrobial
κ-casein	Casoplatelins	Antithrombotic
α-, β-casein	Phosphopeptides	Mineral binding

Note: ACE = angiotensin-converting enzyme.

Sources: From Meisel, H., *Int. Dairy J.,* 8, 363–373, 1998, and Clare, D.A. and Swaisgood, H.E., *J. Dairy Sci.,* 83, 1187–1195, 2000.

different biological activities. Due to their physiological and physicochemical versatility, milk-borne bioactive peptides are regarded as highly prominent ingredients for health-promoting functional foods or pharmaceutical preparations. The formation and properties of milk protein-derived peptides have been reviewed in many recent articles (Clare and Swaisgood 2000; Korhonen et al. 1998; Meisel 1998; Meisel and Bockelmann 1999; Xu 1998; Yamamoto 1997).

Peptides with biological activity can be produced from milk proteins in three ways: (1) enzymatic hydrolysis with digestive enzymes, (2) fermentation of milk with proteolytic starter cultures and (3) through the action of enzymes derived from proteolytic microorganisms (Figure 6.1). In this chapter, the current knowledge about milk protein–derived bioactive peptides is reviewed and potential application for promotion of human health is discussed.

6.2 FORMATION OF BIOACTIVE PEPTIDES BY PROTEOLYTIC ENZYMES

The most common way to produce bioactive peptides is by enzymatic digestion using various proteolytic enzymes. Pancreatic enzymes, preferably trypsin, have been used for liberation of many known bioactive peptides from dietary proteins. For example, angiotensin-I-converting enzyme (ACE) inhibitory and immunomodulatory peptides

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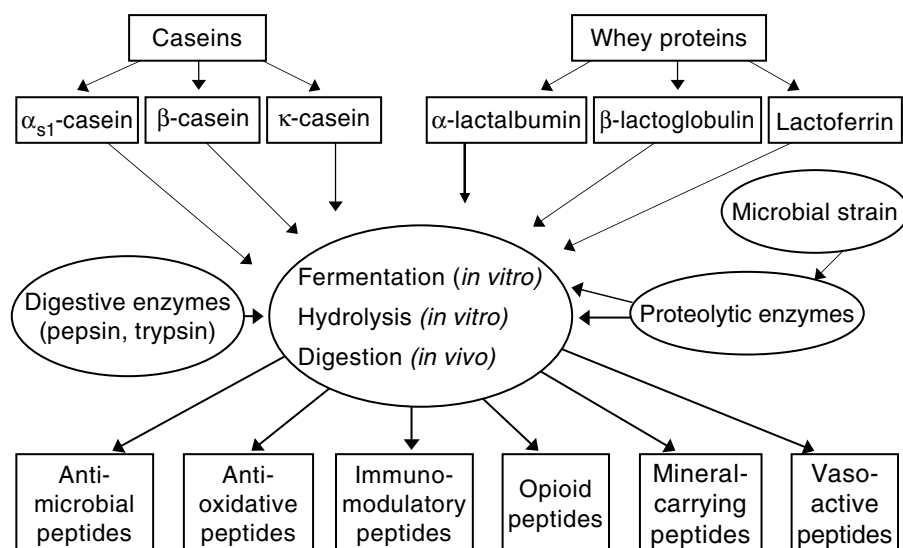
FIGURE 6.1 Potential means of formation of biologically active peptides from major milk proteins.

as well as caseinphosphopeptides (CPPs) were first isolated and identified from a casein hydrolyzate produced with trypsin digestion (Berrocal et al. 1989; Jolles et al. 1981; Maruyama and Suzuki 1982). Later on, other enzymes and different enzyme combinations of proteinases, including alcalase, chymotrypsin, pancre-atin and pepsin, as well as commercial enzymes from bacterial and fungal sources, were utilized to generate bioactive peptides.

Pepsin has been used to produce antibacterial peptides from lactoferrin (Tomita et al. 1994) and opioid peptides from caseins (Pihlanto-Leppälä et al. 1994) and α-lactalbumin (Antila et al. 1991). A pepsin treatment followed by trypsin has been employed to produce an opioid peptide (β-lactorphin) from β-lactoglobulin, opioid peptides from casein and ACE-inhibitory peptides from caseins and whey proteins (Antila et al. 1991; Pihlanto-Leppälä et al. 1994, 1998). Furthermore, higher yields of CPPs have been obtained from casein micelles that were successively digested with pepsin and trypsin rather than from acid-precipitated casein and casein micelles by tryptic digestion alone (Ono et al. 1998). McDon-agh and FitzGerald (1998) have produced CPPs from sodium caseinate using a range of commercial protease preparations of bacterial, fungal, plant and animal origin.

After hydrolysis, the peptides present in hydrolyzates can be fractionated and enriched using methods such as ultrafiltration, nanofiltration, chromatography and ion-charged membranes (Bouhallab et al. 1992; Mullally et al. 1997; Pihlanto-Leppälä et al. 2000; Recio and Visser 1999; Wijers et al. 1998). Further efforts are required in this field with a view to developing industrial-scale techniques for enriching active peptides that have a low molecular mass. Also, research is required to understand the functional and organoleptic properties of these peptides.

6.3 FORMATION OF BIOACTIVE PEPTIDES DURING MILK FERMENTATION

Many dairy starter cultures possess high proteolytic activity. This property is tradi-tionally exploited by the dairy industry because the peptides and amino acids degraded from milk proteins during fermentation contribute to the typical flavor, aroma and texture of the products (Marshall and Tamime 1997). The proteolytic system of lactic acid bacteria, such as *Lactococcus lactis*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* var. *bulgaricus*, is already well known (Law and Haandrikman 1997). This system consists of a cell wall-bound proteinase and several intracellular peptidases. In recent years, many of these enzymes have been identified and characterized genetically (Konings et al. 1999). In fermented milk products, the longer oligopeptides, derived from milk proteins as a result of fermentation, can be a source for the liberation of bioactive peptides when further degraded by intracel-lular peptidases after the lysis of bacterial cells (Law and Haandrikman 1997; Meisel and Bockelmann 1999). In the gastrointestinal tract, digestive enzymes may further degrade long oligopeptides, leading to a possible release of bioactive peptides. Once liberated in the intestine, bioactive peptides may act locally or pass through the intestinal wall into the blood circulation and end up at a target organ, with subsequent regulation of a physiological condition through modulation of the neural, immune, vascular or endocrine system.

Many studies have reported that bioactive peptides are released from caseins and whey proteins during fermentation of milk with various cultures. Nakamura et al. (1995a, b) identified two ACE-inhibitory peptides (Val-Pro-Pro, Ile-Pro-Pro) in milk that was fermented with a starter culture composed of *L. helveticus* and *Saccharomyces cerevisiae*. This enzyme plays a crucial role in the regulation of blood pressure in mammals. Matar et al. (1996) detected immunostimulatory pep-tides in milk fermented with a *L. helveticus* strain. Pihlanto-Leppälä et al. (1998) studied the potential formation of ACE inhibitory peptides from cheese whey and caseins during fermentation with different commercial lactic acid starters used in the manufacture of yogurt, ropy milk and sour milk. The proteolytic activity of all starters was weak and the rate of proteolysis was only 1 to 3% during fermentation. No ACE inhibitory activity was observed in these hydrolyzates. Further digestion of the samples with pepsin and trypsin increased the proteolysis rate to about 45% for whey proteins and 70% for caseins. This resulted in an ACE inhibition rate varying between 35 to 61% for the whey protein hydrolyzates and 86% for the casein hydrolyzates, respectively. ACE inhibitory peptides were released from α_{s1}- and β-caseins and also from α-lactalbumin and β-lactoglobulin. Belem et al. (1999) fermented whey with *Kluyveromyces marxianus* var. *marxianus,* and identified in the hydrolyzate a tetra peptide that had a sequence of β-lactorphin (Tyr-Leu-Leu-Phe). It was suggested that this peptide may have antihypertensive properties. Yamamoto et al. (1999) identified an ACE inhibitory dipeptide (Tyr-Pro) from a yogurt-like product fermented by *L. helveticus* CPN 4 strain. This peptide sequence is present in all major casein fractions.

Microbial enzymes have been used successfully for the production of bioactive peptides from milk proteins. Yamamoto et al. (1994, 1999) reported that casein

hydrolyzed by the cell wall–associated proteinase from *L. helveticus* CP790 showed antihypertensive activity in spontaneously hypertensive rats (SHR). Several ACE inhibitory and one antihypertensive peptide were isolated from the hydrolyzate. Using the same proteinase, Maeno et al. (1996) identified a β-casein-derived antihypertensive peptide from the casein hydrolyzate. The antihypertensive effect of this peptide (Lys-Val-Leu-Pro-Val-Pro-Gln) was dose-dependent in SHR at a dosage level from 0.2 to 2 mg of peptide per kg body weight. This peptide did not show a strong ACE inhibitory activity as such but a corresponding synthetic hexa-peptide, lacking Gln (Lys-Val-Leu-Pro-Val-Pro) had a strong ACE inhibitory activity, as well as a significant antihypertensive effect in SHR. The proline residue in the C-terminus and the amino acid sequence might be important for ACE inhibition of this hexapeptide. Proline is generally known to be resistant to degradation by digestive enzymes.

Milk protein–derived peptides that have been obtained by hydrolysis with digestive and/or microbial enzymes may affect the specific human immune system regulated by peripheral blood lymphocytes. Sütas et al. (1996) demonstrated that digestion of casein fractions by both pepsin and trypsin produced peptides that, *in vitro*, had an immunostimulatory or immunosuppressive influence on human blood lymphocytes. Peptides derived from total casein and α_{s1} casein were mainly suppressive, while those derived from β- and κ-casein were primarily stimulatory. When the caseins were hydrolyzed by enzymes isolated from a probiotic *Lactobacillus* GG var. *casei* spp. *rhamnosus* strain prior to pepsin–trypsin treatment, all hydrolyzate fractions were immunosuppressive, and the highest activity was again found in α_{s1}-casein.

These results suggest that lactic acid bacteria may modulate the immunogenicity of milk proteins prior to or after oral ingestion of the product. This modulation may be beneficial in the down-regulation of hypersensitivity reactions to ingested proteins in individuals with food protein allergy. Indeed, promising results have been obtained in the management of atopic reactions of infants by oral bacteriotherapy with the probiotic *Lactobacillus* GG strain (Majamaa and Isolauri 1997; Kalliomaki et al. 2001). These results are supported by a study by Laffineur et al. (1996) that showed that β-casein hydrolyzed by lactic acid bacteria, e.g., *L. helveticus*, has immuno-modulatory activity, which could be related to interaction with monocyte-macrophage and T-helper cells. Further studies are needed to elucidate the role of immunomodulatory milk protein peptides in the development of oral tolerance or allergy to milk proteins.

6.4 OCCURRENCE OF BIOACTIVE PEPTIDES IN FERMENTED MILK PRODUCTS

Many recent studies have reported on the formation or presence of bioactive peptides in fermented milk products. Such peptides have been identified in sour milk upon fermentation with strong proteolytic starter cultures, e.g., *L. helveticus*, and in ripened cheese varieties (Table 6.2). Calcium-binding phosphopeptides have been identified in fermented milks and various types of cheese (Kahala et al. 1993;

TABLE 6.2
Bioactive Peptides Identified in Fermented Milks and Cheese Varieties

Product	Bioactivity Observed	Reference
Fermented Milks		
Sour milk	Phosphopeptides	Kahala et al., 1993
Sour milk	ACE inhibitory	Nakamura et al., 1995a,b
Fermented milk (treated with pepsin and trypsin)	ACE inhibitory, immunomodulatory, opioid	Rokka et al., 1997
Quarg	ACE inhibitory	Meisel et al., 1997
Yogurt	Immunomodulatory, antihypertensive, antiamnesic, microbicidal, antithrombotic	Dionysius et al., 2000
Cheese		
Parmesan Reggiano	β-casomorphin precursors	Addeo et al., 1992
Comté	Phosphopeptides	Roudot-Algaron et al., 1994
Cheddar	Phosphopeptides	Singh et al., 1997
Edam, Emmental, Gouda, Roquefort, Tilsit	ACE inhibitory	Meisel et al., 1997
Mozzarella, Italico, Crescenza, Gorgonzola (Italian varieties)	ACE inhibitory	Smacchi and Gobbetti, 1998
Cheddar, Edam, Swiss, Feta, Camembert, Blue vein (Australian varieties)	Immunomodulatory, antihypertensive, antiamnesic, opioid agonist	Dionysius et al., 2000
Gouda, Havarti, Emmental	ACE inhibitory	Saito et al., 2000
Edam, Emmental, Turunmaa, Cheddar, Festivo (Finnish varieties)	ACE inhibitory	Korhonen and Pihlanto-Leppälä, 2001

Note: ACE = angiotensin-converting enzyme.

Roudot-Algaron et al. 1994). Beta-casomorphins have been detected in fermented milk (Matar and Goulet 1996), but in ripened cheese only precursors of β-casomorphins have been identified (Muehlenkamp and Warthesen 1996). Rokka et al. (1997) reported that a variety of bioactive peptides are released by enzymatic proteolysis of UHT milk fermented with a probiotic *Lactobacillus* GG strain. Upon fermentation, the product was treated with pepsin and trypsin in order to simulate gastrointestinal conditions. In the hydrolyzate, many bioactive peptides, e.g., ACE-inhibitory, immunomodulatory and opioid peptides, were identified. The formation of bioactive peptides may partially explain the health-promoting properties of milk products containing probiotic bacteria.

Meisel et al. (1997) analyzed a variety of dairy products, sports products and infant formulas for the presence of ACE-inhibitory peptides. Low ACE inhibitory activity was measured in samples having a low degree of proteolysis, e.g., yogurt,

fresh cheese, quarg and sports nutrition–related products. In ripened cheese types, the inhibitory activity increased with developing proteolysis but started to decrease when cheese ripening exceeded a certain level, as measured by the free peptide-bound amino acids ratio. These results are supported by analyses about the occurrence of ACE-inhibitory peptides in Finnish (Korhonen and Pihlanto-Leppälä 2001) and Italian (Smacchi and Gobbetti 1998) cheese varieties. Similar results have been reported with regard to various bioactive peptides identified in Australian yogurt and cheese samples (Dionysius et al. 2000). The most frequently occurring bioactive peptide was derived from β-casein — a peptide reported to have immunomodulatory properties (Coste et al. 1992). Antihypertensive, antiamnesic, antithrombotic, opioid agonist and microbicidal peptides were also identified.

In a recent study, Saito et al. (2000) identified water-soluble ACE-inhibitory peptides from Gouda, Emmental, Edam, Havarti, Blue and Camembert types of ripened cheeses and evaluated their activity by *in vitro* and *in vivo* experiments using SHR. The highest activity was detected in the peptides isolated from an 8-month-aged Gouda cheese. These peptides were derived from α_{s1}- and β-caseins and comprised nine amino acid residues. Upon oral administration of these peptides to SHR, a strong antihypertensive effect was observed. It was suggested that such large peptides require further digestion by intestinal protease or peptidase before they can be absorbed into the blood circulation.

6.5 POTENTIAL PHYSIOLOGICAL IMPORTANCE OF BIOACTIVE PEPTIDES

The potential physiological role of bioactive peptides derived from milk proteins and the *in situ* formation of these peptides in the gastrointestinal tract are still largely unknown. Many *in vitro* studies in which bovine milk proteins were incubated under conditions imitating gastrointestinal digestion have demonstrated the release, for example, of β-casomorphins and opioid antagonists (Brantl et al. 1979; Sato et al. 1986; Zioudrou et al. 1979). The contents of the small intestine have been examined in animal and human studies after the ingestion of milk proteins. A number of studies (Chabance et al. 1995; Masuda et al. 1996; Meisel and Frister 1989; Sato et al. 1986; Scanff et al. 1992; Umbach et al. 1988) have found evidence for the liberation of β-casomorphins, CPPs, and immunostimulatory peptides from casein into intestinal lumen of mammals (rat, minipig, calf, human) or even absorption into plasma after ingestion of milk or a diet containing casein (see Table 6.3 and Table 6.4).

6.5.1 OPIOID PEPTIDES

The absorption and degradation of natural β-casomorphins and their analogues have been studied intensively. Beta-casomorphins are resistant to digestive enzymes and have been detected *in vivo* in weanling pigs (Meisel 1986) and in the small intestine of human subjects (Svedberg et al. 1985). However, no intact transepithelial passage of β-casomorphins has been observed. The physiological influences of opioid peptides may be limited to the gastrointestinal tract; in fact, effects on gastrointestinal transit

TABLE 6.3
Bioactive Peptide Effects in *In Vivo* Animal Studies

Animal	Product Administered	Peptide Precursor/Peptides Identified	Site of Identification/Effect Observed	Reference
Minipig	Casein	β-casein/β-casomorphins	Small intestine content	Meisel, 1986
Minipig	Casein	α_{s1}-casein/CPP	Small intestine content	Meisel and Frister, 1989
Piglet	CPP[a]	α_{s2}-casein, β-casein	Enhancement of intestinal IgA level	Otani et al., 2000
Calf	Skim milk	β-casein/β-casomorphin, immunoactive material	Blood plasma	Umbach et al., 1988
Calf	Skim milk	Caseins/β-casomorphin, CPP immunostimulatory	Small intestine content	Scanff et al., 1992
Rat	Casein	β-casein/CPP	Small intestine/enhancement of calcium absorption	Sato et al., 1986; Tsuchita et al., 2001
Rat	Lactoferrin	Lactoferricin	Intestinal content	Tomita et al., 1994
SHR[b]	Casein	α_{s1}-casein, β-casein/several peptides	Reduction of blood pressure	Yamamoto et al., 1994
SHR	Sour milk	β-casein, κ-casein/Val-Pro-Pro/Ile-Pro-Pro	Reduction of blood pressure	Nakamura et al., 1995b
SHR	Casein	β-casein/Lys-Val-Leu-Pro-Val-Pro-Gly	Reduction of blood pressure	Maeno et al., 1996
SHR	Sour milk	β-casein, κ-casein/Val-Pro-Pro/Ile-Pro-Pro	Aorta/reduction of blood pressure	Masuda et al., 1996
SHR	Whey of yogurt-like product	β-lactoglobulin/Tyr-Pro	Reduction of blood pressure	Yamamoto et al., 1999
SHR	Synthetic α-lactorphin	α-lactalbumin/Tyr-Gly-Leu-Phe	Reduction of blood pressure	Nurminen et al., 2000

[a] CPP = caseinphosphopeptide.

[b] SHR = spontaneously hypertensive rat.

time, antisecretory action and amino acid transport have been shown in some studies. Once the β-casomorphins are absorbed it is likely they are destroyed. However, the situation is different in neonates in which a passive transport across intestinal mucosal membranes occurs. This may lead to physiological responses, such as an analgesic effect resulting in calmness and sleep in infants (Teschemacher et al. 1997).

6.5.2 ANTIHYPERTENSIVE PEPTIDES

A number of studies have been carried out on the antihypertensive effect of ACE inhibitory peptides in SHR (Yamamoto and Takano 1999). Intraperitoneal or oral

TABLE 6.4
Bioactive Peptide Effects Observed in *In Vivo* Human Studies

Animal	Product Administered	Peptide Precursor/Peptides Identified	Site of Identification/Effect Observed	Reference
Adults	Milk	β-casein/β-casomorphin	Small intestine	Svedberg et al., 1985
Adults	Tryptic casein hydrolysate	α$_{s1}$-casein	Reduction of blood pressure	Sekiya et al., 1992
New-born infants	Milk formula/ human milk	κ-casein/antithrombotic	Blood plasma	Chabance et al., 1995
Adults	Sour milk	β-casein, κ-casein, Val-Pro-Pro/Ile-Pro-Pro	Reduction of blood	Hata et al., 1996

administration of casein hydrolyzates and oral administration of ACE inhibitory peptides (α$_{s1}$-casein f(23−34), f(194−199) or β-casein f(177−183)) was shown to decrease blood pressure in SHR but not in normotensive rats (Yamamoto et al. 1994). Tryptic casein hydrolyzate was observed to have an antihypertensive effect in SHR (Karaki et al. 1990). Cheese whey digested with proteinase K had a depressive effect on systolic blood pressure and the highest antihypertensive activity was found with the tripeptide Ile-Pro-Ala derived from β-lactoglobulin (Abubakar et al. 1998). Yamamoto et al. (1999) observed a strong antihypertensive effect in SHR after oral administration of whey from a yogurt-like product where a dipeptide (Tyr-Pro) was formed upon fermentation with *L. helveticus* CPN4 strain. However, this peptide did not exhibit high ACE inhibitory activity.

In a recent study, Nurminen et al. (2000) demonstrated that subcutaneous administration of a synthetic tetrapeptide, α-lactorphin (Tyr-Gly-Leu-Phe), dose dependently lowered systolic and diastolic blood pressure in SHR as well as in normotensive Wistar Kyoto rats. The effecting mechanism was not, however, ACE inhibition. The antihypertensive effect of a Japanese fermented milk (trademark Calpis) has been demonstrated in SHR and in mildly hypertensive humans (Hata et al. 1996; Takano 1998). This product contains two ACE-inhibitory tripeptides (Val-Pro-Pro and Ile-Pro-Pro), which are formed from β-casein and κ-casein by fermentation of milk with *L. helveticus* and *S. cerevisiae*. In SHR, oral administration of 5 ml of sour milk/kg of body weight (BW) significantly decreased systolic blood pressure and the peptides showed a dose-dependent activity up to a dosage of 5 mg/kg BW (Nakamura et al. 1995b). Neither the peptides nor the sour milk changed the systolic blood pressure of normotensive rats. A placebo-controlled study showed that the blood pressure of hypertensive human subjects decreased significantly between 4 and 8 weeks after daily ingestion of 95 ml of sour milk (Hata et al. 1996). In the placebo group, no major changes in blood pressure were observed.

6.5.3 IMMUNOMODULATORY AND ANTIMICROBIAL PEPTIDES

The immunomodulatory effect of many peptides has been demonstrated *in vitro* in a number of studies (Fiat et al. 1993; Kayser and Meisel 1996; Laffineur et al. 1996; Migliore-Samour et al. 1989; Sütas et al. 1996). Immunomodulatory milk peptides affect the immune system and cell proliferation responses. With regard to *in vivo* effects, only a very limited amount of information is available. Parker et al. (1984) observed increased resistance to *Klebsiella pneumoniae* in rats treated intravenously with a hexapeptide derived from human κ-casein. Further research is needed to elucidate the *in vivo* role of immunomodulatory peptides in the immune defense against microbial pathogens and in the regulation of allergic reactions.

The same applies to various antimicrobial peptides that have been shown *in vitro* to inhibit the growth of many pathogenic and nonpathogenic microbes. In particular, lactoferricin, a peptide derived from lactoferrin by pepsin digestion, has been shown to display antimicrobial activity *in vitro* against Gram-positive and Gram-negative microorganisms, including Bacillus*, Escherichia coli,* Klebsiella, Listeria, Proteus, Pseudomonas, Salmonella, Streptococcus, and Candida (Bellamy et al. 1992, 1994; Jones et al. 1994)*.* Alpha$_{s1}$-casein f(1−23), isracidin, obtained from chymosin hydrolysis has been shown to protect mice against *Staphylococcus aureus* and *Candida albicans* (Lahov and Regelson 1996). In a recent study, Pihlanto-Leppälä et al. (1999) demonstrated that a whey protein hydrolyzate obtained by pepsin–trypsin treatment inhibited *in vitro* growth of an *E. coli* strain. This effect was attributed to small peptides derived from α-lactalbumin and β-lactoglobulin. So far, no studies have been reported on the possible antimicrobial effect of the peptides upon oral administration.

6.5.4 CASEINPHOSPHOPEPTIDES

The CPPs possess physicochemical properties that enable the chelation of various minerals, such as Ca^{2+}, Zn^{2+}, Mn^{2+} and Fe^{2+}, thereby enhancing mineral solubility at intestinal pH. Most minerals from food will be dissociated at a low pH in the stomach and subsequently be transferred to the duodenum. These mineral ions may gradually become insoluble as the pH increases. In theory, CPPs exhibit a potent ability to form soluble complexes with Ca^{2+}, preventing Ca^{2+} from precipitation as Ca-phosphate in the intestine.

Considerable controversy exists as to whether CPPs do, in fact, enhance the absorption of dietary Ca^{2+}. CPPs have been shown to enhance calcium absorption in some studies with intact animals whereas most of the balance studies have failed to find any effect of CPP supplementation (FitzGerald 1998). Saito et al. (1998) recently showed that CPP supplementation enhanced calcium absorption in growing rats under conditions of marginal dietary calcium. Accordingly, they suggested that the calcium content of the diet might be an important factor in determining the effect of CPP on calcium absorption. However, in studies that have failed to find an effect of CPP on calcium absorption, high and low levels of dietary calcium have been used (Kopra et al. 1990; Tsuchita et al. 1993). Bennet et al. (2000) have shown that calcium absorption in rats was enhanced by high-casein meals, but in continued

feeding of a high-casein diet, the calcium absorption efficiency was reduced, prob-ably due to adaptation in the active transcellular calcium transport or acceleration in the rate of gastric emptying.

Recently, Tsuchita et al. (2001) showed that the intestinal soluble calcium level and the gastrointestinal calcium disappearance in rats given calcium-fortified milk with extrinsic CPP were significantly higher than the corresponding figures in rats given calcium-fortified milk without CPP. When the rats were given unfortified milk, no significant effect was apparent from the addition of CPP to milk. These results suggest that extrinsic CPP enhanced the calcium absorption mainly from $CaCO_3$ added to the milk. Controversial results have been reported also from studies in human subjects (FitzGerald 1998). For example, Hansen et al. (1997) found that calcium absorption from high- or low-phytate meals in healthy adult subjects was not significantly influenced by the addition of CPP, while Heaney et al. (1994) reported that CPP administration was associated with better absorption of co-ingested calcium by postmenopausal women with low basal absorptive performance.

CPPs have been shown to stabilize amorphous calcium phosphate (ACP) and may be used to localize ACP in dental plaque, maintaining a state of supersaturation with respect to tooth enamel, reducing demineralization and enhancing remineralization (Reynolds 1998). Studies by Rose (2000a, b) showed that CPP-ACP binds well to dental plaque, providing a large calcium reservoir, which is likely to restrict mineral loss during a cariogenic episode and provide a potential source of calcium for subsequent remineralization. Overall, once in place CPP-ACP will restrict the caries process. Another interesting property associated with CPPs is their potential to enhance mucosal immunity. In a recent study, Otani et al. (2000) reported that oral administration of a commer-cial caseinphosphopeptide preparation enhanced intestinal IgA levels in piglets.

6.6 CONCLUSIONS

An increasing number of *in vitro* and *in vivo* studies reveal that biologically active peptides are released from bovine milk proteins upon microbial fermentation and hydrolysis by digestive enzymes. Peptides with different bioactivities can be found in fermented milks and cheese varieties, but the specificity and amount of peptides formed seem to be regulated by the starter cultures used and the rate of proteolysis. Highly proteolytic starter cultures, e.g., *L. helveticus*, produce short peptides with various bioactivities. On the other hand, weakly proteolytic cultures, used regularly in the manufacture of fermented milk products, may not liberate significant amounts of bioactive peptides in the product; however, such peptides could be released *in vivo* after ingestion of the product. This phenomenon has been demonstrated under *in vitro* conditions, but it remains to be shown *in vivo*. Also, the role of proteinases and peptidases isolated from different proteolytic microorganisms needs to be elucidated to facilitate large-scale production of specific peptides. In this context, of special interest are the proteolytic properties of probiotic microorganisms. It is probable that part of the beneficial effects attributed to the foods containing probiotics is associated with bioactive peptides liberated by the proteolytic action of these bacteria.

Some bioactive peptides have proved active in animal models and human studies. So far, the best experimental data are available with regard to antihypertensive peptides that inhibit the ACE system. Further studies are needed to establish the *in-vivo* efficacy of other peptides as well as their long-term effects when administered orally on a regular basis. In conclusion, bioactive milk peptides are a highly diversified source of ingredients that can be exploited in the formulation of health-promoting foods or pharmaceuticals.

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7 Immunomodulation by Dairy Ingredients: Potential for Improving Health

Graham Knowles and Harsharnjit S. Gill

CONTENTS

7.1   INTRODUCTION

Bovine milk is a complex mixture of proteins, carbohydrates, fats, vitamins, minerals
and growth factors. Its primary role is to provide nutrition during early neonatal
development. Milk also helps to protect the newborn from infectious diseases. This
is an important function because, at birth, an infant's immune system has yet to
acquire the ability to protect against infections. Milk achieves protection in two
ways: first by the passive transfer of immunological molecules such as antibodies
that neutralize specific pathogens. This is particularly true for the colostrum of
ruminants, which contains high concentrations of antibodies, thus compensating
for the lack of transfer of immunoglobulins across the placenta. Second, milk
nurtures and orchestrates the development of the immune system so that
responses are effective in dealing with a variety of pathogens while minimizing
the risk of damage to the neonate's own tissues. Thus, apart from supporting
the rapid growth of tissues and providing energy to fuel that growth, milk may
be viewed as a nutrient that establishes a healthy immune system.

   This chapter reviews ingredients found in normal bovine milk that may contrib-
ute to immune health. It discusses opportunities for developing these ingredients
into components of dairy or dairy-based functional foods.

7.2   THE ROLE OF THE IMMUNE SYSTEM IN HEALTH AND DISEASE

To understand how milk-derived molecules may benefit health by affecting the
immune system, it is necessary to understand how the immune system works. The
immune system comprises humoral and cellular defenses. The biologically active
molecules of the humoral defenses comprise soluble factors such as antibodies and
complement. The cellular defenses comprise leukocytes as well as regulatory mole-
cules produced by these cells, such as cytokines and growth factors. Both types of
defenses operate to generate specific and innate immunity, which are defined as:

   • Innate immunity — the immune system responds to pathogens in a manner
     that is nonspecific to the invading pathogen. The magnitude and nature of
     the response are not affected by prior exposure. Innate immunity consti-
     tutes the first line of the body's defense against infectious diseases. Recent
     evidence indicates that molecules secreted by cells of the innate system

also affect the nature of specific immunity. Secretion of lysozyme in tears and events in the phagocytosis of bacteria are two examples of components of innate immunity.

- Specific immunity — the immune system acquires highly specific responses that recognize components of pathogens. Exposure of an individual to a pathogen for a second time results in the rapid production of lymphocytes comprising several subsets. The major types are B-cells that synthesize antibodies, T-helper cells (cells expressing CD4+), which secrete cytokines that regulate activities of the immune system, and cytotoxic T-cells (expressing CD8+) that lyse infected cells. Stimulation of specific immunity is the basis of protective vaccination.

For effective immunity against a range of pathogens, specific and innate components of the immune system are required.

A variety of assays have been used to quantify changes in immune responses. These include:

- Phagocytosis by neutrophils and macrophages of bacteria and yeasts
- Abilities of T- and B-cells to proliferate to mitogens and antigens
- Enumeration of cells secreting cytokines that regulate the function of several types of immune cells
- Amounts of cytokines that are secreted by cells in culture
- Ability of natural killer (NK) cells to lyse other cells
- Expression of surface molecules on various immune cells, including receptors and molecules associated with the major histocompatibility complex
- Enumeration of the number of cells that are secreting antibodies
- Antibody titers after immunization with model antigens (e.g., ovalbumin) and other immunogens (e.g., tetanus and cholera toxin); antibodies include IgG, IgE and IgA in serum and also in secretions
- Delayed type hypersensitivity; a general measure of the competence of CD4+ cells
- Changes in susceptibility to bacteria and viruses, including *Salmonella* sp, *Escherichia coli* and rotavirus

Readers are referred to standard texts for fuller details of these assays and their interpretations (Roitt 1997). Often assays deployed in nutritional research do not determine whether cell numbers have increased or decreased, or whether immune modulation arises from changes in the activity of individual cells. Conspicuous by their absence from this list are assays that detect changes in the intracellular signals that control the activities of immune cells.

The immune system is not infallible and exaggerated responses in specific and innate components caused by failure to regulate the immune system appropriately may damage tissues. Examples include chronic inflammatory diseases (e.g., rheumatoid arthritis), allergies and autoimmune diseases (e.g., some types of diabetes). Sometimes the level of immunity is diminished, e.g., malnutrition, in which case
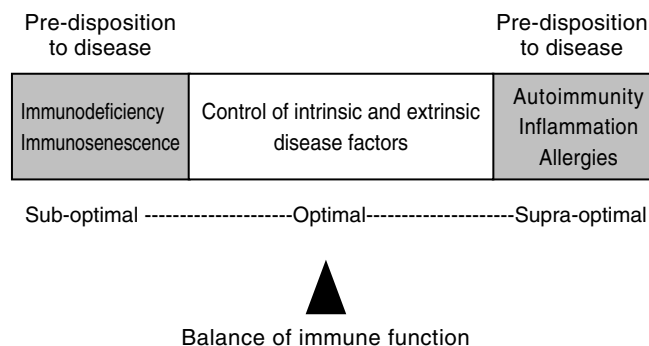
FIGURE 7.1 An overview of the immunologically bioactive components of milk and dairy foods.

recurrent infections occur. Immunomodulators may correct an imbalance in an immune system that is teetering toward disease or enhance the responsiveness of the system when a foreign antigen is encountered (Figure 7.1).

7.3 MILK COMPONENTS AND IMMUNOMODULATION

Milk contains several ingredients that influence components of specific and non-specific immune responses. These ingredients include whey proteins, caseins, lipids, growth factors, hormones, cytokines, nucleotides, glycoproteins/glycopeptides, vitamins, minerals and peptides derived from the protein components (Gill et al. 2001a; Cross and Gill 2001).

7.3.1 EXPERIMENTAL APPROACHES

Evidence that milk ingredients affect the immune system comes from three types of experiments. In the first type, immune cells are isolated from normal animals (i.e., animals that have not been fed experimental diets) and incubated with various ingredients. Data from these types of experiments leave vital questions unanswered. For example, would the bioactive ingredients be stable in the gut? Would they achieve critical concentrations? Are they absorbed? Are the responses of isolated cells a reliable guide to effective immunity? Answers to some of these questions are provided by the second approach, in which diets that contain milk ingredients are fed to animals and the *ex vivo* responses of immune cells are measured. However, it is often difficult to relate these types of measures to protection from infection or other tangible health benefits. In the third (and perhaps best) type of experiment, evidence arises from experiments in which ingredients are incorporated into diets and the effect on infections or disease states is investigated. However, even these types of data require careful interpretation because ingredients could combat infections without affecting the immune system, e.g., binding to pathogens in the gut and thus preventing invasion. This chapter gives examples of all three types of experimental approaches.
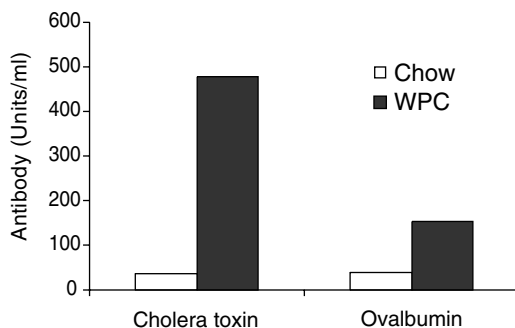
FIGURE 7.2 Whey protein concentrate (WPC) enhances mucosal antibody responses to oral immunization with cholera toxin and ovalbumin.

7.3.2 WHEY AND THE WHEY PROTEINS

7.3.2.1 Whey

Whey is a complex mixture of proteins that enhances a range of innate and acquired immune responses. The immunomodulatory effects of whey have been demonstrated in the three experimental approaches mentioned previously.

The proliferation of lymphocytes is increased when they are incubated with whey (Wong et al. 1998). Whey is thought to boost intracellular levels of glutathione (Bounous 2000), a molecule known to increase proliferation of lymphocytes. In contrast, whey reduces some functions of neutrophils (Wong et al. 1997a).

Diets containing whey have been tested extensively for effects on innate and acquired immune responses. For example, a diet containing whey protein concentrate (WPC) elicited higher antibody responses to specific antigens than diets containing other protein sources (Bounous et al. 1988). Studies in our laboratory have demonstrated that diets containing WPC also increased phagocytic cell function (Gill et al. unpublished) and mucosal antibody responses to oral immunizations (Figure 7.2) (Low et al. 2002). Whey-based diets also increased the proliferation of murine lymphocytes to mitogens (Wong et al. 1997b). The immunity-enhancing activities of WPC are also affected by the conformational state of the proteins (Bounous and Gold 1991). Whey improved liver function tests in people with chronic hepatitis B infections, but had no effect on those with hepatitis C infections (Watanabe et al. 2000).

Whey favors the growth of *Bifidobacteria* sp (Romond et al. 1998) and in some situations, such as extended feeding trials in man, it might be difficult to ascertain whether changes in immune responses are due to direct action of bioactive molecules or, possibly, to altered gut flora. Whey comprises a variety of major and minor components, including β-lactoglobulin, α-lactalbumin, albumin, immunoglobulins (Igs), lactoferrin and milk growth factor. Many of these components, when tested individually, are immunoactive.

7.3.2.2 Alpha-Lactalbumin

Diets enriched with α-lactalbumin increase lymphocyte function and the responses of spleen lymphocytes to T- and B-cell mitogens (Morley et al. 1997; Peterson et al. 1998; Wong and Watson 1995). Perhaps more importantly, these *ex vivo* effects corresponded with resistance to challenge with *Salmonella typhimurium* (Kutzemeier 1998).

In vitro studies have shown that the cells in sheep lung lavages increased their secretion of the cytokine IL-1β when incubated with α-lactalbumin (Wong et al. 1997a). IL-1β has broad proinflammatory properties and might be expected to increase antigen presentation to immune cells.

Many milk proteins contain immunologically active peptides. Sometimes the immune properties only manifest in the peptide and are absent from the intact molecule. Peptides produced by enzyme hydrolysis of α-lactalbumin enhance antibody response to sheep red blood cells (Bounous and Kongshavn 1982; Bounous et al. 1981). Hydrolyzed α-lactalbumin also enhanced humoral immune responses to the antigen TNP-Ficoll (Bounous et al. 1985). This is interesting because humoral responses to TNP-Ficoll are independent of T-cells and comparable responses may assist in immune responses to the capsules of some bacteria. Lactorphin is a tetra-peptide (Tyr-Gly-Leu-Phe) arising from digestion of α-lactalbumin and has opioid-like properties (Bordenave et al. 1999).

The preceding examples indicate that α-lactalbumin may increase immune responses; however, the molecule also has properties with the potential to blunt immune responses. These properties have only been demonstrated by *in vitro* techniques and may not occur when tested in feeding trials. Apoptosis regulates some aspects of immune responses and multimeric human α-lactalbumin induces apoptosis in some cells (Ho and Baxter 1997; Svensson et al. 2000). Human α-lactalbumin also inhibits complement activation (Ogundele 1999); whether bovine lactalbumin has the same properties is unknown.

Alpha-lactalbumin prevented chemical injury to gastric mucosa (Matsumoto et al. 2001) and if this property also prevents tissue damage caused by pathogens, then α-lactalbumin may aid recovery. Chemical modification of α-lactabumin by the incorporation of negative carboxyl charges reduced replication of HIV (Berkhout et al. 1997). Some milk components may have special features that are only manifest in the presence of other molecules. Thus it has been suggested that fatty acids may interact with human α-lactabumin and confer bacteriocidal properties (Hakansson et al. 2000).

7.3.2.3 Beta-Lactoglobulin

The best-known immunological property of β-lactoglobulin is its ability to induce allergic immune responses in susceptible individuals. Therefore, any immunological exploitation of β-lactoglobulin could depend on isolating fragments of the molecule that retain beneficial bioactivity but are nonallergenic. For example, some peptide fragments of β-lactoglobulin induce immune tolerance when fed to mice (Pecquet

et al. 2000). These peptides might be useful in several contexts, e.g., additives to foods that sometimes contain known allergens.

Beta-lactoglobulin is a carrier of small hydrophobic molecules, including retinoic acid, which is a potential modulator of lymphocyte responses (Elitsur et al. 1997). Trans-retinoic acid enhances mitogen-stimulated proliferation of colonic lamina propria–derived lymphocytes — an effect dependent on the presence of macrophages (Elitsur et al. 1997). A chemically altered form of β-lactoglobin blocked HIV binding to CD4 receptors on T-cells (Neurath et al. 1996).

7.3.2.4   Lactoferrin

A remarkable number of immunological and bacteriostatic properties have been ascribed to lactoferrin (Nuijens et al. 1996). Fed to mice, lactoferrin increased total gut Ig and secretion of IgA from Peyer's patches. However, no changes were found in levels of serum IgA (Debbabi et al. 1998), which suggests that lactoferrin increases Ig synthesis specifically within the gut compartment or increases the transport of IgA across the epithelia. Other experiments in which lactoferrin was given by mouth suggested that it suppresses IgE-mediated hypersensitivity (Otani and Hata 1995), induces neutrophilia (Zimecki et al. 1999), reduces secretion of TNFα and IL-6 (IL-6 is responsible for the maturation of B-cells and production of antibodies), and increases delayed type hypersensitivity (Zimecki and Kruzel 2000). In guinea pigs, lactoferrin hastened resolution of skin lesions caused by infection of the fungus *Trichophyton* sp.

All these experiments show that lactoferrin retains biological activity when given by mouth. However, the doses (sometimes approximately 1 mg/g body weight) required to achieve some of these effects are high. Activated lymphocytes and cells of the gut mucosa express lactoferrin receptors (Gislason et al. 1995; Mincheva-Nilsson et al. 1997). Thus, if the human receptor will bind the bovine molecule then these cells could be competent to respond to lactoferrin in the diet and initiate the effects described previously.

Incubation of cells with lactoferrin has diverse effects. Thus, bovine lactoferricin B (amino acids 17 to 41 of lactoferrin) suppressed the *in vitro* phagocytosis by murine macrophages (Otani 1994) and the production of IL-6 by a human monocytic cell line (Mattsby-Baltzer et al. 1996). Bovine lactoferrin stimulated the release of the chemokine IL-8 from polymorphonuclear leukocytes (Shinoda et al. 1996). Lactoferrin decreased proliferation of splenocytes, but when the molecule was hydrolyzed, proliferation of these cells increased (Miyauchi et al. 1997). Hydrolyzed lactoferrin also decreased the blastogenesis caused by mitogens (Miyauchi et al. 1997).

It is not easy to draw a unifying explanation for these results, although the authors speculate that different parts of the lactoferrin molecule have different effects upon T- and B-cells. Lymphocytes bind lactoferrin and the affinity progressively falls as arginine residues are removed from the molecule (Legrand et al. 1997). One wonders whether this pattern of degradation occurs in the gut and affects the ability of lactoferrin to bind to enterocytes. In some assays, lymphocytes may respond to lactoferrin by secreting cytokines and these cytokines inhibit proliferation or make

cells refractory to other stimuli. Whatever the explanation, this illustrates the need to undertake feeding trials to establish the dominant effect of an ingredient.

Human lactoferrin has been shown to cause the *in vitro* differentiation of CD4$^-$/CD8$^-$ lymphocytes to CD4$^+$ T-cells (Dhennin-Duthille et al. 2000). The switch from CD4$^-$/CD8$^-$ to CD4$^+$/CD8$^-$ is a crucial step in the emergence of T-cells that have become fully functional components of the developing immune system. The structural homology between bovine and human lactoferrin, together with the proven ability of native bovine lactoferrin to suppress lymphocyte blastogenesis *in vitro*, predicts a lymphocyte receptor-binding region for bovine lactoferrin similar to that of human lactoferrin (Schanbacher et al. 1997).

Lactoferrin inhibited replication of hepatitis C virus (Ikeda et al. 2000) and rotavirus (Superti et al. 1997) and also had antiviral properties when fed to mice. Thus mice were protected against cytomegalovirus due to increased NK cell activity (Shimizu et al. 1996). Acylation and other charge modifications of lactoferrin increased inhibition of binding of human immunodeficiency viruses to cells (Swart et al. 1999). The N-terminal fragments of lactoferrin have antibacterial properties (van Hooijdonk et al. 2000). Whether milk products can be supplemented with these modified proteins and the dose required to affect pathogen infectivity is unknown.

The bovine gene for lactoferrin is genetically polymorphic (Martin-Burriel et al. 1997). It is not known whether these polymorphisms affect its immunomodulatory properties; however, polymorphisms in goat lactoferrin affect bactericidal properties (Akin et al. 1994). The potential of lactoferrin as a beneficial ingredient has led to its inclusion in some follow-on milks containing milk powder.

7.3.2.5 Immunoglobulins

Colostrum and early postpartum milk contain high levels of Igs (predominantly IgG). Studies have shown that preparations of Ig concentrated from colostrum containing high antibacterial antibody titers offer clinical benefit in combating some bacterial infections (Stephan et al. 1990). Among HIV-infected individuals, the incidence of chronic diarrhea can be significantly reduced following consumption of bovine colostral Ig concentrate (Rump et al. 1992). Clinical trials have been carried out using hyperimmune bovine colostrum containing antibodies against *Shigella flexneri*, *Helicobacter pylori, Vibrio cholerae* and rotavirus (Boesman-Finkelstein et al. 1989; Casswall et al. 1998; Michalek et al. 1978; Tacket et al. 1992). Although results have been variable, the evidence has generally indicated that if colostrum preparations have high antibody titers against defined pathogens, they can provide protection against gastrointestinal disease in humans. In particular, hyperimmune colostrum containing high titers of antibody against rotavirus was effective in the treatment of infant diarrhea (Ebina et al. 1985). These studies should encourage the development of marketable Ig-containing dairy foods. However, the industry must be aware of the continued need for safety monitoring of bovine products and must also be sensitive to the ethical considerations of consumers when promoting products derived from hyperimmunized cows.

Derived from the cleavage of the F_c region of IgG, tuftsin is a tetrapeptide (Thr-Lys-Pro-Arg) likely to arise from the digestion of milk or colostrum; it has a range of immune properties. Human tuftsin stimulates leukocyte chemotaxis and increases phagocyte motility, oxidative metabolism, antigen processing, and the cytotoxicity of NK cells (Werner et al. 1986). Tuftsin induces blood monocytes to secrete IL-1 (Spirer et al. 1989). In one report (Wieczorek et al. 1994), tuftsin suppressed humoral responses but had no effect upon cellular responses; however, removal of the N-terminal amino acid yielded a tripeptide that potentiated humoral and cellular responses. Although the immunoregulatory role of bovine tuftsin remains to be determined, the high Ig content of bovine milk and colostrum would suggest that this might be a fruitful area for future study.

7.3.2.6 Glycomacropeptide

Glycomacropeptide (GMP) is referred to as a caseinomacropeptide and a caseinoglycopeptide. It is formed during the digestion of κ-casein by chymosin. The chemical properties of GMP are responsible for its separating with whey, rather than curd. Its immunological properties are dealt with under the casein group of molecules.

7.3.3 CASEINS

7.3.3.1 General

Caseins constitute about 80% of total milk protein and comprise four types: α_{S1}, α_{S2}, β and κ. Alpha- and κ-caseins suppress the *in vitro* phagocytic responses of murine macrophages (Otani 1994); in contrast, β-casein enhances the activities of neutrophils and macrophages (Wong et al. 1996). Peptides derived from the enzymatic cleavage of α- and β-caseins suppress mitogen-stimulated *in vitro* proliferation of lymphocytes derived from peripheral blood and lamina propria (Elitsur and Luk 1991; Kayser and Meisel 1996; Morgan et al. 1999; Schlimme and Meisel 1995; Wong et al. 1997b). In contrast, peptides derived from the enzymatic cleavage of κ-casein enhance human lymphocyte function *in vitro*. Similar to whey, a diet enriched in caseins affects the gut flora; in contrast to whey, caseins favor colonization by *Bacteroides* sp. and *Enterococci* sp. (Balmer et al. 1989).

7.3.3.2 Alpha$_{S1}$-Casein

Hydrolyzates of α_{S1}-casein have different effects depending on the enzyme used in digestion. Thus, pancreatin and trypsin digestion of α_{S1}-casein inhibited the mitogen-stimulated proliferation of murine splenic lymphocytes and rabbit Peyer's patch cells, but the products of pepsin and chymotrypsin treatment of α_{S1}-casein had no effect on these cells (Otani and Hata 1995). Human immune cells may be different from mouse in this respect because pepsin and trypsin hydrolysis of α_{S1}-casein suppressed mitogen-induced proliferation of peripheral blood mononuclear cells *in vitro* (Kayser and Meisel 1996). Some of these peptides have been characterized further. For example, trypsin digestion of bovine α_{S1}-casein released Thr-Thr-Met-Pro-Leu-Tyr, which promoted antibody formation and phagocytosis

(Yun et al. 1996a) and also reduced the severity of infections of *Klebsiella pneumoniae* in mice (Migliore-Samour et al. 1989). Tripeptides of casein have also been reported to be immunomodulatory (Berthou et al. 1987). Because trypsin is a major proteinase, its specificity may be important in forming immunity-enhancing or -suppressing peptides from casein.

Chymosin digestion of α_{S1}-casein produces isracidin (residues 1 to 23), which has a variety of potential therapeutic properties. These include protection of mice against infection by *Staphylococcus aureus* when the peptide is injected intramuscularly, stimulation of phagocytosis in mice infected with *Candida albicans* and protection of cows and sheep against mastitis (Lahov and Regelson 1996).

Residues 90 to 96 (Arg-Tyr-Leu-Gly-Tyr-Leu-Glu) and 90 to 95 (Arg-Tyr-Leu-Gly-Tyr-Leu) of α_{S1}-casein have opioid-like properties. Opioid peptides enhance lymphocyte proliferative responses, NK cell activity and neutrophil locomotion. T-cells and human phagocytic leukocytes express opioid µ-receptors and thus it is possible that absorbed milk peptides with opioid-like activities may bind to these cells (Migliore-Samour and Jolles 1988). Whether this occurs physiologically would depend on the binding constants of the receptors for the ligand and whether the ligand achieves adequate concentration.

Residues 23 to 34 (Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys) of α_{S1}-casein stimulate phagocytosis of murine peritoneal macrophages; this effect may be responsible for inducing protection against infections of *Klebsiella pneumoniae* (Meisel 1997). This peptide also inhibits angiotensin I converting enzyme and would prevent the cleavage of bradykinin, which promotes acute inflammatory responses.

7.3.3.3   Beta-Casein

Pancreatin and trypsin digests of β-casein inhibit mitogen-stimulated proliferative responses of murine splenic lymphocytes and rabbit Peyer's patch cells (Otani and Hata 1995). Digestion with pepsin and chymotrypsin did not have comparable effects (Otani and Hata 1995). In contrast, the C-terminal part of β-casein (residues 193 to 209) stimulated proliferation of rat lymphocytes in the absence of extraneous mitogens or antigens (Meisel 1997). Peptides derived from pepsin/trypsin hydrolysis of β-caseins suppressed the mitogen-induced proliferation of human lymphocytes (Kayser and Meisel 1996). Residues 63 to 68 (Pro-Gly-Pro-Ile-Pro-Asn) and 191 to 193 (Leu-Leu-Tyr) of β-casein promoted antibody formation and phagocytosis. The tripeptide also enhanced antigen-dependent T-cell proliferation (Jolles 1986).

The β-casomorphins derived from β-casein (residues 60 to 70) have opiate-like properties and suppress proliferation of lymphocytes isolated from the human lamina propria. The opiate receptor antagonist naloxone reversed this effect, suggesting the involvement of opioid receptor-associated signaling pathways (Elitsur and Luk 1991). The effects of β-casomorphin-7 (residues 60 to 66) depend on the concentration of the peptide. Thus low concentrations suppress mitogen proliferation, but high concentrations enhance it (Kayser and Meisel 1996). Following ingestion of normal milk, β-casomorphin was found in intestinal aspirates, thus demonstrating that the peptide may arise *in vivo* (Svedberg et al. 1985).

In contrast to observations on isolated immune cells, experiments with β-casomorphins *in vivo* showed that the peptide enhanced the resistance of mice to

Klebsiella pneumoniae infections. Possibly the immune effect may be concentration dependent. Opiates have several physiological effects in a variety of organs, but in general they are considered immune suppressive when taken orally (Eisenstein and Hilburger 1998; Moore 2000).

7.3.3.4 Kappa-Casein

Digestion of κ-casein yields several peptides with immunomodulatory potential. For example, tryptic digest of κ-casein yields casoxin C (Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg), whose properties have recently been reviewed (Meisel and FitzGerald 2000). Casoxin C may be involved in the complement activation. It has structural homology to the compliment component C3a, binds to C3a receptors, and has comparable but less potent biological activity to C3a (Takahashi et al. 1997). In addition, casoxin C showed phagocyte-stimulating activities.

Digestion of κ-casein by chymosin yields glycomacropeptide GMP (residues 106 to 169); GMP suppresses the mitogen-induced proliferation of B- and T-cells (Ikemori et al. 1997; Otani and Hata 1995). Suppression may be caused by GMP preventing IL-1 from binding to its receptor on immune cells (Monnai and Otani 1997). Although these reports claim GMP suppressed B-cell proliferation, another report claims that a similar molecule increased lymphocyte proliferation (Yun et al. 1996b; Radogna, 1997) and the *in vitro* secretion of IgA (Yun et al. 1996a). All of these reports refer to effects on cells incubated *in vitro*. GMP in the diet of mice enhances lymphocyte function (Debabbi et al. 1998). In contrast, dietary GMP has been shown to suppress serum antibody responses against orally and systemically administered foreign antigens in mice (Monnai et al. 1998). The extent of glycosylation of this peptide is variable and may affect its properties (Pisano et al. 1994). GMP has oligosaccharide residues similar to cholera toxin (Kawasaki et al. 1992), which is known to be a powerful mucosal adjuvant.

Pepsin-trypsin digest from *Lactobacilli* bacteria yields peptides from κ-casein that have different immune properties from the peptides produced by chymosin digestion. Normally, κ-casein peptides enhance the mitogen-induced proliferation of human lymphocytes and the secretion of cytokines; however, the enzymes from *Lactobacilli* produce peptides from κ-casein that suppress these types of immune responses (Sutas et al. 1996). This is of interest because it is a potential indication of how bacteria in the gut could give rise to immunomodulators that oppose the effects of normal digestion. Also, the κ-casein peptide Phe-Phe-Ser-Asp-Lys (residues 17 to 21) promotes phagocytosis and antibody production formation (Jolles 1986, 1988). In addition, Tyr-Gly (residues 38 and 39 of κ-casein) enhanced cellular proliferation of human lymphocytes induced by concanavalin A (Kayser and Meisel 1996; Meisel 1997; Solenberger 1997).

7.3.4 GROWTH FACTORS

Milk contains several growth factors. Whether their primary purpose is maintenance of the lactating mammary gland or for the benefit of the suckled is unclear. Several growth factors have immunological properties.

7.3.4.1   Betacellulin

Betacellulin was recently isolated from bovine colostrum, milk and whey (Bastian et al. 2001). It is a member of the epidermal growth factor (EGF) and binds to the same receptors as EGF.

7.3.4.2   Transforming Growth Factor-β

TGF-β is an immunomodulator present in milk (Goldman et al. 1997; Kim et al. 1993; Wieczorek et al. 1995). TGF-β is activated by low pH (Ishizaka et al. 1998) and secreted in different forms during lactation (Plath et al. 1997). When given to mice, TGF-β in human milk inhibited responses to sheep red blood cell (SRBC) following oral immunization with SRBC; however, responses elicited by systemic immunization with SRBC were enhanced (Ishizaka et al. 1994). The findings suggest that TGF-β in human milk functions as a specific adjuvant for protective antibody responses against systemic infections. Based on these observations TGF-β could reduce mucosal immunity to gut acquired infections. TGF-β also increases NK cell activity (Ishizaka et al. 1998) and the production of IgA by lymphocytes (Kim et al. 1998). TGF-β undoubtedly has the potential to modify the immune system. Perhaps the biggest challenge to its eventual addition to diets and supplements is to identify the circumstances when consumption would be acceptable and cost effective.

7.3.4.3   Milk Growth Factor

Milk growth factor (MGF) is a peptide that has complete N-terminal sequence homology with bovine TGF-β2. MGF suppresses *in vitro* proliferation of human T-cells, including proliferation induced by mitogen, IL-2 and exposure of primed cells to tetanus toxoid antigen (Stoeck et al. 1989).

7.3.5   OTHER MOLECULES

7.3.5.1   Defensins

Defensins are small bacteriocidal peptides present in neutrophils and secreted by epithelia, including mammary tissue; they are present in milk (Jia et al. 2001). It was recently reported that defensins at concentrations 10- to 100-fold below that required for bactericidal activity induce CD45RA+ and CD8 T-cells to migrate (Chertov et al. 2000) and also form chemotactic gradients for immature dendritic cells (Tani et al. 2000).

7.3.5.2   Nucleotides

The nucleoside and nucleotide content of human and bovine milk was reviewed recently (Schlimme et al. 2000). Nucleotides are essential for the immune system and adequate supply is provided in a normally balanced diet. When diets are restricted in nucleotides (e.g., parenteral nutrition), immune responses are adversely affected and supplementation restores lost immunity (Martinez-Augustin et al.

1997). In weanling mice fed infant milk formulae, nucleotide supplementation enhances NK cell activity and secretion of cytokines by macrophages (Carver 1999).

7.3.5.3 Pattern Recognition Molecules

Toll-like receptors on cells detect microbial products and link the presence of likely infection to induction of immune and inflammatory responses by immune cells (Aderem and Ulevitch 2000). Perhaps the best-known example is the response of B-cells, through toll-like receptors, to lipopolysaccharide (LPS) from Gram-negative bacteria (Ulevitch 1999). CD14 is a co-receptor for LPS and acts through toll-like receptors; it has been detected in soluble form in milk and colostrum (Filipp et al. 2001). Soluble CD14 in milk may be beneficial in two respects: first by binding LPS in the gut lumen and thus prevent attachment of LPS to cells of the gut and, second, by the ability of CD14 to induce proliferation of B-cells. Whether CD14 survives passage through the stomach is unknown.

7.4 THE EFFECT OF MILK COMPONENTS ON THE IMMUNE SYSTEM

The preceding sections, to some extent, catalogue those milk ingredients that modify the ability of intact animals, and in some cases isolated immune cells, to respond to various stimuli (mitogens, antigens and immunizations etc.). In many instances, the molecular events responsible for immunomodulation are still ill-defined.

The benefits of dietary consumption of immunoglobulins are probably due to antibodies binding to pathogens in the lumen of the gut. Passive transfer of antibodies probably does not lead to changes in the activities of cells of the immune system and is not true immunomodulation. However, as mentioned previously, *in vitro* experiments have demonstrated several effects of tuftsin, a peptide derived from Ig, on immune cells. However, experiments in which nonspecific Ig has been fed to animals have not revealed effects on the cells of the immune system, although such a possibility should not be wholly discounted. Whether the immune benefits of consuming Ig can be truly considered due to the modulation of the immune system is a moot point.

No doubt several events occur before the bioactivity of a milk ingredient manifests itself as true immunomodulation. Some of these steps have been ignored, but they may yield crucial information on how dietary components affect immunity. For example the digestion and absorption of bioactive milk ingredients in the gut are poorly understood. These steps are likely to be the first in the initiation of immunomodulation. Although some dietary antigen appears antigenically intact in the portal circulation, most of these molecules are cleared by the liver. However, fragments of ingested lactoferrin and casein have been recovered in peripheral blood (Harada et al. 1999).

Ingredients may affect immune responses by binding to receptors on enterocytes. For example, enterocytes express receptor molecules that are typical of immune presenting cells (Martin-Villa et al. 1997). The tips of the villi in the gut contain the differentiated enterocytes that express receptors for TGF-β (Lionetti

et al. 1999). Milk ingredients that affect the expression of these and other receptors may affect antigen presentation and thus trigger changes in immune responses. Ingredients may affect membrane architecture in general; for example, the milk component conjugated linoleic acid (CLA) is incorporated into cell membranes (Ip et al. 1994). In examples of receptor binding, one may expect the subsequent intracellular events to be similar to those that occur when the natural ligand binds to its receptor.

The immunity-enhancing abilities of milk proteins could be due to their unique amino acid profile. Thus, substitution of intact bovine whey protein with an equivalent free amino acid mix, which duplicates the specific amino acid profile of whey, was found to enhance humoral immune responses in mice. Milk proteins have also been reported to exert their immunoenhancing effect by increasing the glutathione levels in the immune cells. Treatment of mice fed diets containing whey protein with L-buthionine-sulfoximine (an inhibitor of glutathione synthesis) resulted in a significant reduction in immune responsiveness (Bounous et al. 1989).

The effects of milk ingredients on molecular events within immune cells are also poorly understood. Consider the enhancement of mitogen-stimulated proliferation of lymphocytes by a fragment of α-lactalbumin. There are several subsets of T-cells, each of which has a different immunological function. Are cells from different subsets equally affected by fragments of α-lactalbumin? Are the mechanisms that are triggered by the fragment comparable to the normal stimuli that regulate lymphocyte proliferation? Is such proliferation subject to the expected regulatory mechanisms? Much remains to be learned, especially because the control and consequences of cell signaling in the immune system are the subject of intense research. DNA microarray is used to profile the expression of different genes in different physiological and disease states and has been applied to cells of the immune system (Galon et al. 2002). The same technique could be applied to identify genes affected by milk ingredients and determine whether they are part of the normal regulatory processes of the immune system.

7.5 MILK INGREDIENTS IN HEALTH AND DISEASE

7.5.1 Improving Impaired Immunity

Malnutrition, burns, cancer, surgery and severe stress all impair immunity. Diets containing supplements that restore immune competence faster than nonsupplemented diets have formed part of the treatments for these conditions. For example, in severely malnourished children, special diets restored anthropometric measures in about 1 month, but took twice as long to restore immune measures. Zinc supplementation has been shown to hasten recovery of immune responses (Chevalier et al. 1996). Several studies have shown that feeding with special diets or providing supplements of glutamine or vitamin A improved health (Houdijk et al. 1998; Shankar et al. 1999). However, whether such health benefits can be attributed to alteration of particular immune responses is less clear. Comparable evidence that supplementing diets with specific milk ingredients beneficially affects immune status in man

is lacking, although a small pilot study showed that dietary whey proteins may promote weight gain in HIV-positive men (Bounous et al. 1993).

Intense physical exercise and prolonged athletic training depress several immune parameters. People who engage in such activities are often well nourished and receive professional advice on diets, yet their immune systems often become compromised by their choice of profession or lifestyle. Thus it seems that in special circumstances, "normal" nutrition is inadequate to sustain a stressed immune system. These people would seem to be ideal recipients for dietary supplements to sustain their immune systems.

Carriers of bacterial and viral pathogens often harbor infections without symptoms of disease. However, episodes of illness often arise from these asymptomatic infections and carriers are often the source of infection for others. Examples are nasal carriage of *Staphyloccoccus aureus* and the development of carrier status of individuals infected with hepatitis B virus. We have already commented on the effect of α-lactalbumin on liver function tests in hepatitis B infections (Watanabe et al. 2000). Close communities in hostels and hospitals are particularly vulnerable to outbreaks of certain types of bacteria. Immune status partly affects carrier status (Peacock et al. 2001) and one may speculate that diet may be an acceptable and practical prophylactic intervention.

The age structure of consumer-oriented societies is changing. We live longer and the elderly have an increased risk of developing cancers and autoimmune diseases (e.g., rheumatoid arthritis). The elderly manifest a general decline in immune function, although the age at which the impetus for this decline occurs is far from clear. The utilization of immunologically active components from milk incorporated into the diet may offer benefits to these groups.

7.5.2 ANTICANCER BIOACTIVITY OF MILK

Modulation of the immune system may offer significant improvements in the control of cancers. Components of milk could affect immune cells that may affect the growth of tumors; these include NK cells (Shimizu et al. 1996) and cytotoxic lymphocytes (Bounous and Kongshavn 1985; Monnai et al. 1998). The effect is not restricted to isolated cells because whey proteins in the diet of rats reduced the growth of chemically induced tumors (McIntosh et al. 1995). WPC also displayed tumor-regressing effects against some forms of human cancer (Kennedy et al. 1995).

Some lipid components of milk, in particular CLA and sphingomyelin, are known to possess antitumor properties. CLA has been shown to regress chemically-induced tumors (Ip et al. 1994) and recent research in our laboratory has shown that milk-derived CLA enhances the function of immune cells (Figure 7.3).

Experimental vaccines are being developed against skin cancer (melanomas and those caused by viral infections) lymphomas and colorectal cancer (Armstrong et al. 2001). Cancer vaccines, especially therapeutic vaccines, are difficult to develop for a number of reasons. For example, cancer is often accompanied by immunosuppression and tumors are poorly immunogenic. A solution to these problems may, in part, be addressed by dietary modulation of the immune system.
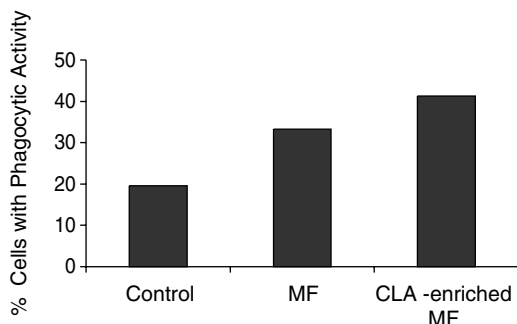
FIGURE 7.3 The effect of diets supplemented with milk fat (MF) or MF enriched with conjugated linoleic acid (CLA) on phagocytic activity of peripheral blood leukocytes in mice.

7.5.3 ANTI-INFLAMMATORY POTENTIAL OF MILK

Occasionally, the immune system becomes aggressive and directs its attentions toward the body's own tissues. Immunoregulatory components of milk may be useful in combating such conditions. Bovine milk contains anti-inflammatory cytokines and growth factors, including TGF (Stoeck et al. 1989). Neonatal mice genetically deficient in TGF developed chronic inflammation of the lower intestine and survived only as long as they received maternal milk containing immunosuppressive TGF analogues (Kulkarni and Karlsson 1993). A diet containing anti-inflammatory factors derived from bovine milk reduced mammary inflammation of rats caused by pyogenic infections of *Staphylococcus aureus* (Owens and Nickerson 1989). A casein-based diet containing high levels of TGF-β fed to children with Crohn's disease ameliorated chronic inflammation of the intestine; the effect was due to the ability of TGF to suppress inflammatory cytokine production (Fell et al. 2000).

Allergic diseases represent an imbalance in a specific compartment of the immune system, resulting in increased levels of reaginic antibody (IgE) and mucosal inflammation via the release of vasoactive amines from mast cells. Dietary interventions with bioactive ingredients may be a suitable strategy for this chronic condition. Thus bovine colostral whey has been shown to suppress systemic IgE responses in mice (Watson et al. 1992). Also, lactoferrin and κ-casein inhibited the release of histamine from mast cells in an *in vitro* study, suggesting that these proteins might play a role in limiting allergic inflammation (Otani and Yamada 1995).

7.6 IMPROVING INTESTINAL MICROFLORA AND THE IMMUNE SYSTEM

Diet affects intestinal microflora (Dai and Walker 1999); breast-fed babies have a different flora compared to formula-fed babies (Balmer et al. 1989). Milk and dairy foods can contribute to establishing a healthy gut flora by providing a favorable environment for the colonization of *Lactobacilli* sp. Several strains of *Lactobacilli* have been developed into probiotics.

TABLE 7.1
Immunomodulatory Properties of Some Strains of *Lactobacilli* Species That Are or Are Nearly Commercially Available

LAB strain	Effect on Immune System	Related Health Benefit
L. rhamnosus GG	↑[a] Antibody responses (humans) ↓[b] Proinflammatory cytokines Cleaves allergenic food molecules	↓ Rotavirus infection (children) ↓ Traveler's diarrhea (adults) ↓ Antibiotic-associated diarrhea (adults and children) ↓ Food allergy and eczema (infants)
L. rhamnosus HN001	↑ Antibody responses (mice) ↑ Cellular immune responses (mice and humans)	↓ Intestinal tract pathogens (mice and other animal models)
L. casei Shirota	↑ Antibody responses (mice and humans) ↑ Cellular immune responses (mice and humans)	↓ Infant diarrhea ↓ Tumor growth (adults) ↓ IgE and allergies
L. johnsonii La1	↑ Cellular immune responses (humans)	
B. lactis HN019	↑ Antibody responses (mice) ↑ Cellular immune responses (mice and humans)	↓ Intestinal tract pathogens (mice and other animal models)

[a] ↑ Immune response elevated.

[b] ↓ Immune response lowered, or animals less susceptible to infection.

Source: Adapted from Gill, H.S., 2000, Dairy products and immune health, in *Functional Foods 2000*, Angus, F. and Miller, C. (Eds.), Leatherhead Publishing, Surrey, U.K., 268–284.

7.6.1 PROBIOTICS

Probiotics are microbes (traditionally bacteria) that, when consumed, benefit the well-being of the consumer (Fuller 1997). Certain well-defined strains of *Lactobacilli* sp. and *Bifidobacteria* sp. modulate the immune system and affect the course of disease (Table 7.1). For example, *L. rhamnosus* GG and *L. casei* Shirota increased antibody responses to vaccination and reduced the severity and duration of diarrhea (Isolauri et al. 1995; Kaila et al. 1992; Majamaa et al. 1995; Oksanen et al. 1990; Yasui et al. 1999). Consumption of strain *L. johnsonii* La1 enhanced the phagocytic capacity of peripheral blood leucocytes (Schiffrin et al. 1997). Other strains of *L. rhamnosus* HN001 and *B. lactis* HN019 (Prasad et al. 1999) enhanced immune responses in humans and in animals (Arunachalam et al. 2000; Gill 1998; Gill et al. 2001b; Sheih et al. 2001). For the strain *B. lactis* HN019, the greatest changes in immune responses were found in those subjects whose pretreatment levels were poorest (Gill et al. 2001c) and the greatest improvement was observed in the older participants in the study (Gill et al. 2001b). Infections due to *S. typhimurium* and enterotoxigenic *Escherichia coli* were also reduced by feeding *L. rhamnosus* HN001 and *B. lactis* HN019 (Gill et al. 2001d; Shu and Gill 2001; Shu et al. 2000).

Interestingly, the prebiotic, galacto-oligosaccharide further enhanced the innate immune responses induced by *B. lactis* HN019 (Chiang et al. 2000). These observations suggest that immune benefits can be optimized by targeting sectors of the population and incorporating substrate that favor the appropriate colonization of the gut.

The effects of consuming lactobacilli are not restricted to immunity enhancement, but rather seem to improve immune health. Thus, some *Lactobacillus* strains suppress IgE antibody responses in mice (Matsuzaki and Chin 2000; Shida et al. 1998). In human studies, chronic consumption of yogurt or fermented milk has been shown to lower serum levels of IgE (Trapp et al. 1993). *L. rhamnosus* GG reduced symptoms of atopy in children from families with histories of diseases such as atopic eczema, allergic rhinitis and asthma (Kalliomaki et al. 2001). The details of this recent study are important because they illustrate the parameters that might affect success or failure of a trial in humans. Thus babies with genetic predisposition to atopy were studied whose immune imbalance could have been particularly amenable to correction. Probiotics were given to mothers in capsules before delivery and their babies were subsequently spoon-fed the same probiotic. Thus the probiotics were not consumed as part of the diet. The timing of probiotic intervention (before birth and neonatal) may be important from the perspective of successful outcomes. Finally, the probiotics had no effects on levels of IgE or on allergic responses to antigens pricked into the skin.

The conclusion was that the regimes affected clinical conditions without discernable effects on IgE and mast cells. However, in a similar study undertaken in adults (60 to 70 years of age), chronic consumption of yogurt reduced not only allergic symptoms reported by participants but also total IgE (Van de Water et al. 1999). Allergy in children was also reduced by consumption of whey supplemented with *L. rhamnosus* GG and the children consuming the probiotic had lower indices of intestinal inflammation than those whose diet did not include the probiotic (Majamaa and Isolauri 1997).

The dendritic cell (DC) is the heart and brains of the immune system. These cells take up antigens and present them to other cells of the immune system. They also secrete a variety of signals that, depending on the "mood" of the DC, may enhance or suppress immunity. Different strains of *Lactobacilli* sp. caused DCs to secrete different cytokines (Christensen et al. 2002). Thus *L. casei* subsp *alactus* caused DCs to secrete TNF-α and IL-12 while *L. reuteri* had the interesting property of inhibiting the abilities of *L. casei* to secrete these cytokines. This study highlights the opportunity to use probiotics to instruct the immune system to bolster only those functions that are appropriate. At birth we may use probiotics to prevent allergies and protect against infection, but in later life, other types of probiotics may be used to minimize the risk of cancers and perhaps immune senescence. Specific genes responsible for probiotic properties have yet to be identified, but the potential to transfer genes between strains of *Lactobacilli* has been demonstrated (Hickey et al. 2001).

Some strains exhibit antitumor properties that may be achieved by immunoregulation. Thus, orally administered *L. casei* Shirota reduced the growth of secondary tumors after resection of the primary tumor; immune cell function in mice was also

enhanced (Kato et al. 1994). The strain also reduced recurrence of secondary tumors in humans (Aso et al. 1995) and promoted antitumor immune responses in patients (Sawamura et al. 1994).

Because the ecology of lactobacilli is complex, several factors may affect the success of probiotic strains when tested in diverse human populations. Only one example is given to illustrate this concept. Lactobacilli produce small peptides called lactacins that are bacteriocidal to other *Lactobacilli* sp. (Allison and Klaenhammer 1996). Susceptibility to this molecule varies between strains and may be important because some probiotic strains fail to compete with naturally occurring lactobacilli already resident in the gut (Tannock et al. 2000). Not all immunological applications of probiotics require the bacteria to enter the gut; some have been used as vectors to deliver tetanus toxoid to the nose (Grangette et al. 2001). Whether the simultaneous exposure to immunogen- and immunity-enhancing agents presents any significant benefit over conventional vaccine adjuvants has yet to be established.

7.7 CONCLUSIONS

Bovine milk contains bioactive molecules that have the potential to increase or decrease immune responses. Whey, its composite proteins, and their hydrolysates have been shown to enhance aspects of humoral and cellular immunity and offer promise as ingredients in functional foods. More importantly, some of these molecules are effective in combating infections, cancers and immune pathologies. Many bioactive molecules (but by no means all, e.g., caseins) are present at low levels in normal milk, although higher levels may be encountered in colostrum and early postpartum milk. With high titers of antibodies against defined specificities, colostrum offers significant advantages in combating pathogens that invade through the gut. Many bioactive molecules will lose potency as they transit the gut; however, such molecules may become useful if their biological activity can be protected by chemical modification or encapsulation.

Recent technological advances (e.g., proteomics and gene expression profiling) will continue to reveal biologically active components of milk. Arguably, most research on immunomodulation by milk ingredients has been conducted on *ex vivo* aspects of the immune system. Although tantalizing, such data are not easily related to health benefits. Comparatively little is known about the intracellular events (especially gene expression), which are causally related to the consumption of immunity-enhancing milk ingredients and probiotics. For the present, progress may continue, providing criteria such as efficacy and safety are satisfied. However, understanding the intracellular molecular mechanisms that accompany immunomodulation by dietary ingredients could reveal new opportunities. The mechanisms that result in enhancement of antibody responses by dietary ingredients could be different from those caused by adjuvants. Incorporating probiotic strains of *Lactobacilli* sp. into functional foods and other products is arguably more advanced than the development of products that contain bioactive molecules derived from milk.

Immune health is threatened in many situations; sometimes it is a corollary of accompanying diseases and sometimes the consequence of life choices. However,

functional foods are unlikely to acquire the same status and role as traditional drugs in treating overt disease. Consumers may take them to reduce (rather than remove) their risk of contracting infections, lessen their symptoms once infected, or speed recovery. They may be used as supplements to improve therapy of established treatments, especially those for chronic conditions in which people seek to reduce their dependency on drugs. Perhaps diseases or circumstances exist in which drugs are only partially effective, but an extra boost to immunity may improve efficacy. These different scenarios present individual challenges in relation to efficacy testing. Regulations pertaining to food labeling, safety and health claims grow ever more demanding; therefore, to satisfy anticipated regulations, future research should focus on defining the abilities of ingredients to impact the health and wellness of consumers.

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8 Conjugated Linoleic Acid

David Kritchevsky

CONTENTS

8.1   INTRODUCTION

Conjugated linoleic acid (CLA) is an inclusive designation of a number of naturally occurring octadecadienoic acids. CLA is a product of the biohydrogenation of linoleic acid that occurs in ruminant animals. It is found principally in dairy products and the meat of ruminant animals; the major isomer in these foods is the *cis*9, *trans*11 modification. The concentration of CLA in food sources is about 1 to 10 mg/g fat. Chin et al. (1992) have published a summary of the levels of CLA found in foods and Fritsche and Steinhard (1998) estimate that daily intake in man ranges from 0.3 to 1.5 g per person.

Parodi (1999) has described the early history of CLA beginning with its discovery in milk fat in 1932 and discusses the chemistry leading to proof of its structure. The conjugated diene present in milk fat was shown to be an intermediate in the course of hydrogenation of linoleic acid by the rumen bacterium *Butyrivibrio fibrisolvens* (Kepler et al. 1966; Kepler and Tove 1967). CLA might have remained an interesting component of rumen chemistry if it had not been for the discovery by Pariza et al. (1979) of an antimutagen in cooked hamburger and the demonstration by Ha et al. (1987) that the active material was a mixture of CLA isomers, namely, the *cis*9, *trans*11; *trans*9, *trans*11; *trans*10, *cis*12, and *trans*10, *trans*12 modifications. These observations and the commercial availability of CLA isomers led to a decade

TABLE 8.1
Influence of Mutagenesis Modulator on
Dimethylbenz(a)anthracene-Induced Skin Tumors in Mice

	Papillomas/Mouse		
Group[a]	Control	Test	P
A) SENCAR mice	30.2 ± 3.4	13.4 ± 2.4	0.01
B) SENCAR mice	9.6 ± 0.8	4.5 ± 0.9	0.01
C) CD-1 mice	10.0 ± 1.7	3.2 ± 1.1	0.01

[a] A) 20-week study; modulator prepared by ion exchange. B) 19-week study; modulator prepared by extraction. C) 10-week study; modulator prepared on florisil.

Source: From Pariza, M.W. and Hargraves, W.A., *Carcinogenesis,* 6, 591–593, 1985. With permission.

of intense research into the anticarcinogenic effects of CLA and, predictably, into its effects on other diseases. At this writing the bulk of CLA literature still relates to its anticarcinogenic effects.

8.2 CLA AND CANCER

The material that Pariza et al. (1979) called "mutagenesis modulator" was found to be present in uncooked as well as in cooked hamburger and shown to inhibit isoquinoline-induced mutagenicity in the Ames test (Pariza et al. 1983). Pariza and Hargraves (1985) showed that mutagenesis modulator could inhibit 7,12-dimethyl-benz(a)anthracene (DMBA)-induced epidermal tumors in mice (Table 8.1). This was 2 years before Ha et al. (1987) established its structure.

Base-catalyzed isomerization of linoleic acid-rich oils produces a mixture of CLA forms, principally the c9,t11 and t10,c12 isomers. The commercial preparations used in most of the studies described next contain 40 to 45% of each of the major isomers. This isomer mixture was compared with linoleic acid for ability to inhibit benzo(a)pyrene (BP)-induced forestomach tumors in mice. CLA inhibited tumor formation by 40 to 50% whereas linoleic acid was without effect (Ha et al. 1990).

The early studies showed that CLA could affect tumorigenicity when applied directly to the tumor site. Ip and his colleagues showed that dietary CLA also inhibited chemically induced mammary tumors in rats independently of type or amount of dietary fat and independently of type of carcinogen. Ip et al. (1991) fed female Sprague-Dawley rats a basal diet or one augmented with 0.5, 1.0 or 1.5% CLA. The dietary regimen was instituted 2 weeks prior to administration of DMBA and continued to the end of the experiment (24 weeks); even the lowest level of CLA exerted some antiumorigenic effect (Table 8.2).

To address the possibility that the CLA effect was due to interference with the metabolic conversion of DMBA to an active form, Ip et al. (1994) tested CLA effects

TABLE 8.2
Effect of Different Levels of Dietary CLA on 7,12-Dimethylbenz(a)anthracene-Induced Mammary Tumors in Sprague-Dawley Rats (30/group)

| Group | CLA (%) | DMBA | Tumors | | |
			Incidence	No.	Multiplicity
1	–	+	80.0	81	2.7 ± 0.3
2	0.5	+	66.7	55[a]	1.8 ± 0.2[a]
3	1.0	+	46.7[a]	36[a]	1.2 ± 0.2[a]
4	1.5	+	40.0[a]	32[a]	1.1 ± 0.1[a]
5	1.5	0	0	0	0

[a] $p < 0.05$ compared to control.

Source: After Ip, C. et al., *Cancer Res.,* 51, 6118–6124, 1991.

TABLE 8.3
Effect of CLA on Mammary Tumors in Sprague-Dawley Rats Induced by 7,12-Dimethylbenz(a)anthracene (DMBA) or Methylnitrosourea (MNU)

| Carcinogen | CLA[a] (%) | Tumors | |
		Incidence	Number
DMBA	—	80	62
DMBA	1	52[b]	38[b]
MNU	—	88	76
MNU	1	60[b]	50[b]

[a] CLA fed from weaning to 1 week postcarcinogen administration (5 weeks).
[b] $p < 0.05$ compared to control.

Source: After Ip, C. et al., *Nutr. Cancer,* 24, 241–247, 1995.

on the carcinogenicity of methylnitrosourea (MNU; a direct acting carcinogen). In a 36-week study they found that dietary levels of CLA as low as 0.1% decreased significantly the carcinogenicity of DMBA. They also found that 1% dietary CLA reduced the incidence of DMBA-induced mammary tumors by 35% and the incidence of MNU-induced mammary tumors by 32% (Table 8.3). CLA was shown to reduce the proliferative activity of ductal and lobuloalveolar mammary epithelial cells by 15 and 23%, respectively. This observation offered a suggestion that CLA might affect mammary tumorigenesis by a direct effect on the target organ. CLA feeding for only a short period in the rat's life corresponding to postweaning and puberty was enough to reduce tumorigenesis caused by subsequent administration

of MNU by about 25 to 30%, depending on time of MNU treatment (Ip et al. 1995). The same study showed that feeding 1% CLA as a triglyceride or as the free fatty acid had virtually the same effect on MNU-induced tumorigenesis.

The amount and type of dietary fat can influence the course of experimental carcinogenesis; linoleic acid enhances experimental carcinogenesis. The possibility that elevated levels of linoleic acid might swamp the CLA effect was tested in rats fed a fat blended to reflect the fatty acid composition of the American diet. The ratio of saturated to monounsaturated to polyunsaturated fatty acids in the blended fat was 1:1:1. Inhibition of DMBA-induced mammary tumorigenesis was virtually the same in rats fed 1% CLA together with 10, 13.3, 16.7 or 20% of the blended fat (Ip et al. 1996). The amount of linoleic acid in the diet (2 or 12%) did not affect inhibition of DMBA-induced mammary cancer by CLA.

Liew et al. (1995) studied the effect of CLA on colon carcinogenesis induced by 2-amino-3-methylimidazo[4,5f]quinoline (IQ) in F344 rats. IQ alone yielded 100% incidence of aberrant crypt foci (4.3 ± 2.4 ACF per rat); when CLA (0.5%) was added to the diet, ACF incidence was reduced by 40% and ACF per rat was reduced to 1.1 ± 1.3 ($p < 0.05$). When the diet contained safflower oil, ACF incidence was 100% but ACF per rat was reduced by 26% (3.2 ± 1.7). Dietary CLA reduced IQ–DNA adducts in the liver by 27% and in the colon by 41%.

Belury et al. (1996) showed that increasing levels of dietary CLA reduced promotion of skin tumors in mice promoted by phorbol ester (12-0-tetradecanoyl phorbol-13-acetate). Papilloma yield after 24 weeks in mice fed 0, 0.5, 1.0 or 1.5% CLA was 6.71, 5.92, 4.83, and 4.67, respectively. Level of inhibition by the two higher concentrations of CLA was statistically significant ($p < 0.05$).

The SCID (severe combined immunodeficient) mouse provides a vehicle for examining effects of human tumor cells in an experimental animal model (Cesano et al. 1992). Subcutaneous injection of tumor cells into this model results in tumor growth at the site of injection as well as metastatic proliferation of the tumors. SCID mice were fed 1% CLA for 2 weeks prior to subcutaneous inoculation of 10^7 MDA-MB468 cells (human breast adenocarcinoma). The mice were followed for 14 weeks. After 9 weeks, the weight of tumors in the treated mice was 74% ($p < 0.01$) lower than in the controls and tumor area (mm^3) was reduced by 87% ($p < 0.01$). At 14 weeks, tumor weight in treated mice was reduced by 30% ($p < 0.02$) and tumor weight by 62% ($p < 0.05$). No breast cancer cells spread to lungs, peripheral blood or bone marrow (Visonneau et al. 1997).

SCID mice were also fed a semipurified diet containing 1% CLA for 2 weeks prior to injection with 5×10^6 DU-145 cells (human prostatic carcinoma) and followed for 12 weeks. In addition to a control group maintained on laboratory ration, a third was fed a semipurified diet containing 1% linoleic acid. Food intake (1 g/day) at week 12 was similar in the control and linoleic acid-fed groups (about 4 g) and significantly lower in the CLA group (about 3 g). Tumor volume measured at 4 weeks was similar in all three groups; by 8 weeks tumor volume in the linoleic acid-fed mice was slightly larger than in the controls and significantly larger ($p < 0.05$) than in CLA-fed group. By 12 weeks, tumor growth in the CLA-fed group was only slightly greater than it was at 4 weeks (about 400 mm^3), whereas in the linoleic-acid and control groups it had risen several fold. Tumor mass was lowest

in the CLA-fed group (p < 0.001) against both controls and highest in the mice fed linoleic acid (p < 0.05) against commercial diet). CLA-fed mice exhibited a dramatically reduced number of lung metastases compared to the other two groups; they showed no metastases to any other tissue (Cesano et al. 1998).

8.2.1 TISSUE CULTURE STUDIES

A number of *in vitro* studies of CLA have reported on the growth of cells in culture. Visonneau et al. (1996) examined the comparative effects of different concentrations of CLA or linoleic acid (10^{-4}, 10^{-5}, and 10^{-6} *M*) on a number of cell lines. Five breast carcinoma cell lines were tested: MBA-MB468, MCF7, MDA-MB 231, HS-578T, and BT-474. Except for the BT-474 line, CLA inhibited growth at every concentration. Linoleic acid at a concentration of 10^{-4} *M* stimulated cell growth. At the lowest concentration, linoleic acid was generally inhibitory. CLA inhibited growth at all concentrations and inhibited growth of a prostatic carcinoma cell line (DU145) as well as a melanoma cell line (WM451) at all three concentrations. CLA at 10^{-4} *M* inhibited growth of a colon carcinoma cell line (HT-29) and a glioblastoma cell line (U87-MG).

Shultz and his co-workers (1992a, b) and Cunningham et al. (1997) studied three human cell lines. When CLA was incubated with human malignant melanoma (M21-HPB), colorectal (HT-29), or breast cancer (MCF-7) cells, reduction in growth, depending on time and dose, was observed. All three cell lines, when incubated with CLA, incorporated less tritium-labeled leucine than did controls, suggesting cytotoxicity. Linoleic acid and CLA were used at concentrations of 1.78 to 7.14×10^{-5}. Linoleic acid stimulated MCF-7 cell growth initially but became inhibitory at 8 to 12 days. CLA, on the other hand, was inhibitory at all concentrations; inhibition reached 100% after 12 days. These researchers investigated the possibility that the CLA effect involved inhibition of eicosanoid synthesis. Their findings suggested that CLA activity was mediated through inhibition of lipoxygenase activity. Linoleic acid, but not CLA, increased peroxide concentrations in normal human breast cells (HMEC) and MCF-7 cells. Treatment of MCF-7 cells with CLA and a cyclooxygenase inhibitor (indomethacin) stimulated cell growth, but when CLA was used together with a lipoxygenase inhibitor (nordihydroguaiaretic acid, NGDA) growth was inhibited.

Durgam and Fernandes (1997) attributed the inhibitory effect of CLA on growth of human breast cancer cells to interference with the hormone-regulated mitogenic pathway. They compared CLA effects on estrogen responsive (ER) MCF-7 cells and ER negative MDA-MB-231 cells. They found that CLA (3.5×10^{-5} *M*) decreased MCF-7 cell growth significantly but had no effect on the growth of the MDA-MB-231 cells.

Schonberg and Krokau (1995) suggested that the CLA effect involved lipid peroxidation. In contrast, using a human hepatoma cell line (HepG2), Igarashi and Miyazawa (2001) attributed growth inhibition by CLA to alterations in fatty acid metabolism but not lipid peroxidation. Their indication of peroxidation was formation of thiobarbituric acid-reactive substances (TBARS), which was 6% higher in cells incubated with linoleic acid than in those incubated with CLA. The level of TBARS production was about the same as that seen in control cells and in every

case was strongly inhibited by addition of α-tocopherol. Total fatty acid content of HepG2 cells incubated with CLA was 32% greater than controls and 53% greater than cells grown with linoleic acid. The major differences between the fatty acid composition of control cells and those grown with CLA was in the higher content of palmitic acid (+35%), palmitoleic acid (+83%) and stearic acid (+37%). Cellular lipids were increased significantly in cells supplemented with CLA. The data suggest stimulation of *de novo* lipid synthesis.

Studies of the inhibitory effects of CLA on DNA adduct formation with IQ or PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) show that, in general, CLA reduces adduct formation so it may act as a blocking agent under certain conditions. The mechanism by which CLA blocks DNA adduct formation has not been elucidated (Liew et al. 1995; Schut et al. 1997; Josyula and Schut 1998).

An active area of research and speculation concerns effects of CLA on eicosanoid metabolism. Many investigators have shown that CLA is readily incorporated into cell membrane phospholipids; thus, CLA may displace arachidonic acid in phospholipids. In a study of effects of CLA in MCF-7 and SW480 cancer cells, Miller et al. (2001) found that the c9, t11 isomer significantly decreased conversion of arachidonic acid to PGE2. The underlying finding was that CLA reduced incorporation of arachidonic acid into the phosphatidyl choline (lecithin) that is the preferential substrate for phospholipase A2, which, in turn, releases arachidonic acid for eicosanoid synthesis. Arachidonic acid is released from phospholipids by the action of phospholipase A2 and converted to eicosanoids along the cyclooxygenase pathway (yielding prostaglandins and thromboxanes) as well as the lipoxygenase pathway (yielding leukotrienes). Eicosanoids affect cell proliferation, inflammation and immunity, all of which may influence carcinogenesis.

8.2.2 HUMAN STUDIES

A basic interest lies in relating experimental findings to possible CLA effects on human cancers. Knekt et al. (1996) reported that a high intake of milk is correlated with a reduced risk of breast cancer and Knekt and Järvinen (1999) have reviewed the field. Analysis of epidemiological evidence relating to dairy products and risk of breast cancer is confusing. High consumption of dairy products has been linked to both reduced and elevated risks. Many modifying factors are in these studies, such as age, body weight, menopause, components of the specific dairy products under consideration, other components of the diet and caloric intake.

Lavillonniere and Bougnoux (1999) report preliminary data from a study of the CLA content of breast adipose tissue from women with breast cancer and controls. CLA content of control tissue was elevated compared to that in afflicted tissue. Aro et al. (2000) studied serum and dietary CLA in Finnish women with breast cancer. They examined 195 cases (35% premenopausal) and 208 controls (36% premenopausal). In postmenopausal women, dietary CLA, serum CLA, myristic acid and *trans*-vaccenic acid were significantly lower in cases than in controls.

Animal data are almost unanimous in finding CLA to inhibit experimental tumorigenesis and tumor cell proliferation *in vitro*. There are few data relating CLA in the human diet to anticarcinogenesis. CLA is a component of a class of dietary

foods (animal products) that have borne the brunt of the epidemiological blame for human carcinogenesis. Continuing experimental evidence from CLA research may lead to reconsideration of dietary effects on carcinogenesis.

8.3   CLA AND EXPERIMENTAL ATHEROSCLEROSIS

Reports that CLA might exhibit antioxidant activity *in vivo* and *in vitro* and knowledge that oxidized cholesterol (Imai et al. 1976) and oxidized low density lipoprotein (LDL) (Steinberg et al. 1989) significantly promoted atherogenesis prompted investigation of the effects of CLA on experimental atherosclerosis.

In the first study, control and test groups of six rabbits each (three male, three female) were fed a semipurified diet containing 0.1% cholesterol for 22 weeks. The diet of the test rabbits was augmented with 0.5 g/d of CLA. At necropsy, plasma total cholesterol, LDL-cholesterol, and triglyceride levels were lower than those of controls but not significantly so. The plasma LDL/high-density lipoprotein (HDL) cholesterol ratios were 16.5 for the control group and 10.9 for the CLA-fed group. Histological examination of the aortas showed maximal plaque thickness (mm) in the thoracic aorta to be the same in both groups; however, maximal plaque thickness in the abdominal aorta of CLA-fed rabbits was 27% smaller. Lipid deposition and connective tissue development were less severe in the aortas of the CLA-fed rabbits (Lee et al. 1994). On the basis of the thiobarbituric acid-reactive substances' assay, levels of plasma peroxides were the same for both groups.

A second experiment was designed to confirm the apparent antiatherogenicity of CLA and also to examine its effects on progression or regression of established atherosclerotic lesions. Once established in rabbits, atheromata do not regress after removal of the atherogenic stimulus and may become slightly more severe. Some hypolipidemic drugs added to a cholesterol-free diet after lesions have been established may cause a slight reduction in severity of atherosclerosis. A group of 30 rabbits was fed a semipurified atherogenic diet containing 0.2% cholesterol (control); 10 rabbits were fed the same diet augmented with 1% CLA. After 90 days, the rabbits receiving the control diet were bled and randomized into three groups of 10 rabbits each of equal serum cholesterol levels. One group of control rabbits and the CLA-fed rabbits were necropsied. The two remaining groups were placed on a cholesterol-free diet with or without 1% CLA. These rabbits were necropsied 90 days later.

No significant differences were found in weight gain or serum lipids. However, the rabbits fed CLA exhibited 31% less severe atheromata in the aortic arch and a 40% lower severity in the thoracic aorta (Table 8.4). After rabbits were on the regression regimen for 90 days, serum cholesterol had fallen by 83% on the control regimen and 67% on the CLA diet. The percentage of HDL-cholesterol had risen by 121 and 74%, respectively. Serum triglycerides were unchanged in the control group but were 58% lower in the CLA-fed animals. Examination of the level of atherosclerosis revealed practically no change in the control rabbits; however, severity of atherosclerosis in the aortic arch and thoracic aorta of the CLA-fed rabbits had been reduced by 31 and 30%, respectively. The involved area had been reduced by 31% (Table 8.5).

TABLE 8.4
Necropsy Data for Rabbits Fed 0.2% Cholesterol with (Control) or without 1% CLA for 90 Days

	Group (n = 10)	
	Control	**CLA**
Weight gain (g)	822 ± 1.22	742 ± 87
Liver weight (g)	109 ± 5	111 ± 7
Liver (% body weight)	3.13 ± 0.14	3.24 ± 0.18
Serum (mg/mL)		
Cholesterol	430 ± 40	559 ± 53
% HDL-C[a]	6.1 ± 0.44	3.6 ± 0.51
Triglyceride	77 ± 6	135 ± 36
Aorta (0–4 scale)		
Arch	2.39 ± 0.47	1.65 ± 0.37
Thoracic	2.35 ± 0.36	1.40 ± 0.40
Area (%)	49 ± 1	30 ± 10

[a] HDL-C = high-density lipoprotein cholesterol.

Source: After Kritchevsky, D. et al., *J. Am. Coll. Nutr.,* 19, 474S–477S, 2000.

TABLE 8.5
Necropsy Data for Rabbits with Established Atherosclerosis Fed Corn Oil with (Control) or without 1% CLA for 90 Days

	Group (n = 10)	
	Control	**CLA**
Weight gain (g)	461 ± 65	246 ± 37
Liver weight (g)	99 ± 9	83 ± 6
Liver (% body weight)	2.33 ± 0.20	2.31 ± 0.13
Serum (mg/mL)		
Cholesterol	73 ± 10	140 ± 24
% HDL-C[a]	13.5 ± 1.02	10.6 ± 0.81
Triglyceride	77 ± 13	57 ± 5
Aorta (0–4 scale)		
Arch	2.35 ± 0.35	1.65 ± 0.26
Thoracic	2.30 ± 0.40	1.65 ± 0.22
Area (%)	51 ± 11	34 ± 6

[a] HDL-C = high-density lipoprotein cholesterol.

Source: After Kritchevsky, D. et al., *J. Am. Coll. Nutr.,* 19, 474S–477S, 2000.

TABLE 8.6
Necropsy Data for Rabbits Fed 0.2% Cholesterol with 0.0 (Control), 0.1, 0.5, or 1.0% CLA for 90 Days

	Group (n = 8)			
	Control	**0.1% CLA**	**0.5% CLA**	**1.0% CLA**
Weight gain (g)	104 ± 46	3 ± 108	67 ± 43	50 ± 79
Liver weight (g)	68 ± 5	77 ± 6	66 ± 4	78 ± 6
Liver (% body weight)	2.73 ± 0.16	3.22 ± 0.35	2.63 ± 0.12	3.35 ± 0.32
Serum (mg/dL)				
Cholesterol (%)	983 ± 118	1281 ± 116	1263 ± 104	1103 ± 134
% HDL-C[a]	5.0 ± 0.9	3.3 ± 0.54	3.3 ± 0.58	5.0 ± 1.14
Triglyceride	190 ± 32	246 ± 47	205 ± 48	216 ± 38
Aorta (0–4 scale)				
Arch	2.36 ± 0.39[b,c]	1.69 ± 0.23[d]	0.88 ± 0.20[b,d]	1.00 ± 0.28[c]
Thoracic	2.21 ± 0.42[e,f]	1.31 ± 0.28	0.75 ± 0.21[e]	0.94 ± 0.27[f]
Area (%)	44 ± 12[g]	32 ± 7[h]	11 ± 4[g,h]	18 ± 6
% Ester cholesterol	74.7	52.0	34.1	44.3

[a] HDL-C = high-density lipoprotein cholesterol.

[b–h] Values in a horizontal row bearing the same letter are significantly different.

Source: After Kritchevsky, D. et al., *J. Am. Coll. Nutr.,* 19, 474S–477S, 2000.

The next study was designed to investigate different concentrations of CLA for their antiatherogenic potential as well as their effects on pre-established lesions. Accordingly, one large group of 40 rabbits was fed the same diet augmented with 0.1, 0.5, or 1.0% CLA. The postcholesterol-feeding phase of the study involved feeding the cholesterol-free diet or the same diet containing 0.1, 0.5 or 1.0% CLA to rabbits with established lesions. The results of the first phase of the study are summarized in Table 8.6. As in the earlier study, no differences in serum lipids were present. The effects of CLA on atherogenesis were striking. Even at 0.1% of the diet, CLA reduced severity of atherosclerosis in the aortic arch and thoracic aorta by 28 and 41%, respectively. When fed as 0.5 or 1% of the diet, CLA significantly reduced atherosclerotic involvement in the aortic arch and thoracic aorta. The percentage of esterified cholesterol present in the aortic tissue is a value that can also be used as a measure of severity of atherosclerosis. This parameter paralleled the other findings.

Rabbits with pre-established atherosclerosis were placed into four groups of seven rabbits each. One group was fed the control diet and the others were fed the same diet plus 0.1, 0.5 or 1.0% cholesterol. As in the earlier study, after 90 days of consuming a cholesterol diet, the severity of lesions in the aortic arch and thoracic aorta was increased (by 12 and 4%, respectively). Addition of 0.1 or 0.5% CLA to the diet had virtually no effect on the severity of atherosclerosis (Table 8.7). However, consistent with the earlier study, at 1% of the diet, CLA reduced the severity of aortic arch atheromata by 27% and that in the thoracic aorta by 45% ($p < 0.01$) (Kritchevsky et al. 2000).

TABLE 8.7
Necropsy Data for Rabbits with Established Atherosclerosis Fed Corn Oil with 0.0 (Control), 0.1, 0.5, or 1% CLA for 90 Days

	Group			
	Control **(n = 7)**	**0.1% CLA** **(n = 6)**	**0.5% CLA** **(n = 7)**	**1.0% CLA** **(n = 6)**
Weight gain (g)	312 ± 99	265 ± 104	298 ± 84	242 ± 73
Liver weight (g)	52 ± 3	58 ± 6	64 ± 5[a]	47 ± 3[a]
Liver (% body weight)	1.82 ± 0.07[b]	2.05 ± 0.16	2.28 ± 0.20[b,c]	1.75 ± 0.13[a]
Aorta (0–4 scale)				
Arch	2.64 ± 0.28	2.25 ± 0.28	2.50 ± 0.29	1.95 ± 0.40
Thoracic	2.29 ± 0.36[d]	2.33 ± 0.44	2.00 ± 0.15[e]	1.25 ± 0.17[d,e]
Area (%)	53 ± 7	53 ± 10	49 ± 5	30 ± 10

[a–e] Values in a horizontal row bearing the same letter are significantly different.

Source: After Kritchevsky, D. et al., *J. Am. Coll. Nutr.,* 19, 474S–477S, 2000.

The commercial availability of the two major CLA isomers has made it possible to test them individually for their effects in experimental atherogenesis. Rabbits were fed an atherogenic diet containing 0.2% cholesterol and the same diet augmented with 0.5% of the isomer mix, c9,t11-CLA or t-10,c12-CLA. All three test diets inhibited atherogenesis to the same extent (about 55 ± 4%).

Administration of cholesterol and saturated fat to hamsters leads to aortic sudanophilia (Kowala et al. 1991). Nicolosi et al. (1997) fed male hamsters (10 per group) an atherogenic diet consisting of 88.9% commercial ration, 10% coconut oil, 1% safflower oil, and 0.12% cholesterol or the same diet augmented with CLA (0.25 to 5.0%) or linoleic acid (5%). Total cholesterol levels were reduced in all of the test groups. The fatty streak area was reduced by 19, 26, and 30%, respectively, in hamsters fed 0.25, 0.50, or 1.0% CLA. Linoleic acid feeding reduced fatty streak area by 25%. It was determined in a subsequent study (Wilson et al. 2000) in which hamsters (12 per group) were fed the atherogenic diet alone, or the diet plus 1% CLA or 1% linoleic acid, that CLA and linoleic acid significantly lowered total plasma cholesterol levels (control, –327 ± 16; CLA, –285 ± 11; LA, –264 ± 8 mg/dL). The aortic fatty streak area ($\mu m^2/mm^2$) was 19.4 ± 2.25 in the controls, 10.3 ± 1.55 in the hamsters fed CLA and 17.1 ± 2.62 in those fed linoleic acid. The difference between the CLA and control groups was significant. LDL oxidation was reduced significantly in the CLA-fed hamsters.

8.4   OTHER EFFECTS OF CLA

8.4.1   EFFECTS ON BODY FAT

When CLA is fed to growing animals it leads to reduced fat deposition and increased body mass. Park et al. (1997, 1999a) showed that when CLA (0.5 to 1.0%) was fed to growing mice, body fat was severely reduced and weight gain could be accounted

for by increased body protein. The effect was seen in mice fed a CLA mix but it has been shown that the t10,c12 isomer is the active entity vis-à-vis fat loss and protein accretion (Park et al. 1999b). The effect on fat loss and protein accretion has also been seen in growing pigs (Dugan et al. 1997). Studies in tissue culture have shown CLA to enhance lipolysis (Park et al. 1999b), suppress triglyceride accumulation and enhance apoptosis (Evans et al. 2000).

8.4.2 DIABETES

CLA (1.5%) fed to the Zucker diabetic fatty (fa/fa; ZDF) rat results in significant reductions in fasting glucose (24%), insulin (47%), triglycerides (51%) and free fatty acids (49%). In contrast, a diet containing thiazolidinedione reduces the same parameters by 24, 82, 86, and 60%, respectively. The study was suggested by the observation that CLA was a PPARγ activator (Belury and Vanden Heuvel 1999).

8.4.3 IMMUNE SYSTEM

Sugano et al. (1999) have reviewed the effects of CLA on immune function. CLA (0.5 or 1.0% in the diet) lowers the levels of prostaglandin E2 in the serum of rats significantly (by 14 and 24%, respectively). Dietary CLA (1%) will lower leukotriene (LT) B_4 levels in spleen and lung by 19 and 35% and LTC_4 levels in these organs by 8 and 68%. Levels of immunoglobulins A, G, and M in rat spleen lymphocytes are increased significantly by dietary CLA even when fed at levels as low as 0.05%. Immunoglobulin production in mesenteric lymph node lymphocytes is reduced significantly by dietary CLA. CLA may exert its antiallergic effects through its regulation of eicosanoid production and enhancement of immunoglobulin production.

8.5 MECHANISMS OF ACTION

The mechanisms of CLA action are moot. CLA blocks DNA adduct formation, which may be one way in which it affects carcinogenesis (Liew et al. 1995). CLA is incorporated into cell membrane phospholipids (Sugano et al. 1997). CLA may displace arachidonic acid, reducing its incorporation into lecithin, which is the preferential substrate for phospholipase A2, which releases arachidonic acid for eicosanoid synthesis. Thus, the cyclooxygenase pathway to prostaglandins and thromboxanes and the lipoxygenase pathway to leukotrienes are inhibited. The interactions of CLA with the PPAR family may also be a key to its action. At present we have a wide array of potential mechanisms that may be different for every different type of CLA activity. The relatively easy work of cataloging CLA effects is almost finished. We now are entering the more complex and difficult phase of CLA research — how does it do what it does?

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9 Calcium Bioavailability of Dairy Components

David D. Kitts and Wendy Kwong

CONTENTS

9.1 INTRODUCTION

Dairy products are well recognized as excellent sources of essential nutrients in general and of dietary calcium in particular. In the North American diet, dairy products represent about 75% or more of the daily calcium intake (equivalent to approximately 75% RDNA) with the remaining portion coming from vegetables, grains and fruits. Table 9.1 lists the mineral content of different dairy products as well as a few plant products. The calcium content of different dairy products varies considerably, from approximately 60 mg/100 g for cottage cheese to 110 to 120 mg/100 g for various fluid milks and yogurt and 500 to 700 mg/100 g for various cheese products. Cottage cheese prepared from skim milk without the addition of calcium chloride ($CaCl_2$) has only 9 mg/100 g. Adding $CaCl_2$ to fresh milk will result in a shift of the soluble calcium into the casein micelles; therefore, adding $CaCl_2$ for the purpose of promoting rennet action increases the calcium content of cottage cheese. In comparison, soybean milk and tofu products

TABLE 9.1
Mineral Content of Some Dairy and Plant Products[a]

Constituent	Ca	P	Ca/P	Fe	Na	Na/K
Fluid milk	119	93	1.3:1	0.04	49	0.3:1
Skim milk	129	104	1.2:1	0.04	53	0.3:1
Evaporated skim milk	261	202	1.3:1	0.12	106	0.3:1
Buttermilk	116	89	1.3:1	0.04	105	0.7:1
Yogurt	121	95	1.3:1	0.04	46	0.3:1
Cottage cheese	60	132	0.5:1	0.24	405	4.8:1
Cheddar cheese	729	521	1.4:1	0.28	629	6.3:1
Processed cheese	621	754	0.8:1	0.56	1450	8.8:1
Soybean milk	21	48	0.4:1	0.9	21	0.2:1
Tofu (soycurd)	105	97	1.1:1	6.3	7	0.1:1
Kale	82	26	3.0:1	20	18	0.4:1
Spinach	20	11	1.8:1	16	15	0.1:1
Soybeans	52	129	0.4:1	7	1.6	0.1:1

[a] Values are given as milligrams per 100 gm.

Source: From *Nutritive Value of American Foods*, Agriculture Handbook No. 456, Washington, D.C., 1975.

contain 21 and 105 mg/100 g, respectively. This example is important in under-
standing how the distribution of calcium varies in different dairy products and,
furthermore, whether the distribution of calcium can influence the bioavailability
of this mineral.

In addition to the fact that dairy products represent excellent sources of dietary
calcium, it is also important to note that the bioavailability of calcium is also high
in these food systems. Balance studies conducted in rats have reported apparent
absorption efficiencies of calcium that range from 54 to 75%, which is equivalent
to or higher than efficiencies obtained with calcium supplements (Buchowski et al.
1989; Buchowski and Miller 1991; Greger et al. 1987). Calcium from nonfat dry
milk powder and yogurt products has been reported to be utilized in rats 118 and
109%, respectively, compared to that from calcium added as a calcium carbonate
source (Wong and LaCroix 1980). In human adults, the absorption of radioisotopic
calcium tracers ranges from 25 to 35% (Heaney and Recker 1985; Nickel et al. 1996;
Recker et al. 1988). Calcium absorption in preterm infants can reach as much as
82% of the dietary calcium intake (Lui et al. 1989). A "milk factor" has been reported
to facilitate calcium, zinc and iron solubility in the presence of cereal fiber and
sodium phytate (Clydesdale and Nadeau 1984; Platt et al. 1987). This finding par-
tially explains the improved fractional absorption of calcium observed in subjects
consuming a wheat bran cereal containing milk (Weaver et al. 1991). Moreover,
other human balance studies concerned with examining the relative absorption of
calcium from different foods have reported that dairy products such as milk and
cheese provide relatively higher bioavailability of calcium than spinach (Heaney
et al. 1988; Landis et al. 1987).

These results collectively indicate the importance of components present in milk that may include lactose or associated hydrolysis products (Andrieux and Sacquet 1983; Buchowski et al. 1989, Buchowski and Miller 1991; Yuan et al. 1991a), milk fat (Delisle et al. 1997) and milk proteins (Hansen et al. 1996; Sato et al. 1983, 1986; Yuan et al. 1991a); these components may contribute to enhanced calcium absorption by facilitating solubility. Caution is required in the extrapolation of these findings to specific recommendations for the consumer because primary limitations exist for confirming findings between distinct studies as a result of the different methodologies used to assess calcium bioavailability (Table 9.2).

Milk proteins have been shown to contain a number of potentially biologically active peptides that contribute to, or modulate, a number of specific physiological mechanisms that regulate metabolic homeostasis. Studies have shown that casein peptides released in the duodenum have a physiological function that will potentially regulate gastric and pancreatic secretions (Aleinik et al. 1984), provide immunostimulating (Otani et al. 2000) and opiod (Brantl 1985) activities, and play a role in calcium transport (Mellander 1950; Naito et al. 1972). In the latter example, considerable work has been dedicated to the discovery and characterization of bioactive phosphopeptides from milk, which are considered to facilitate intestinal absorption of calcium by solubilizing calcium phosphate (Kitts and Yuan 1992). Characterizing the primary and secondary structures of phosphopeptides derived from caseins has been an important step in understanding these peptides' biological significance to calcium metabolism. Moreover, this information is critical for generating food-derived physiologically active components from parent milk protein sources that have commercial applications. A number of food and dental products designed to contain caseinophosphopeptides have been developed in Europe and Japan with the intention of providing a nutraceutical designed to enhance the bioavailability of mineral-supplemented foods (FitzGerald 1998).

The purpose of this chapter is to review the role of dairy protein constituents in faciliitating calcium bioavailability. In particular, emphasis will be placed on the interactions between caseinates and calcium ions, which largely depend on the physicochemical conditions that enable optimal solubility of calcium in the gastrointestinal tract. Specific examples of potential health benefits attributed to calcium enhancement by milk proteins or products of milk protein digestion are also reviewed.

9.2 CALCIUM ABSORPTION: MECHANISMS AND CONSIDERATIONS

The transport of calcium in the intestine involves translocation of calcium ions from the intestinal lumen to the lateral space occupied by the lamina propria (Figure 9.1). In general, intestinal calcium transport is essentially transmucosal transport because the mucosal layer of the intestine is the only barrier that calcium must cross to reach the portal circulation. It is important to note that the absorptive surface of the intestine is a monolayer of differentiated epithelial cells organized

TABLE 9.2
Procedures Used and Results of Casein/Caseinophosphopeptide-Induced Calcium Bioavailability Studies

Procedure	Measurement	Result	Reference
A. Calcium excretion patterns			
1. Balance studies	Apparent Ca absorption and Ca balance under steady-state conditions	No effect of dietary protein source; no effect of synthetic or natural CPP	Yuan and Kitts, 1994 Scholz-Ahrens et al., 1990 Shah et al., 1990 Yuan and Kitts, 1992
B. Monitoring radiolabeled calcium excretion on deposition patterns			
1. Oral dose of tracer (extrinsic or intrinsic labeling and fecal excreta collection)	True absorption	Casein > soya; CPP: no effect	Sato et al., 1986 Buchowski et al., 1989 Bennett et al., 2000
2. Fractional absorption (^{45}Ca or ^{47}Ca)	Net absorption from entire intestinal tract	Casein-induced effect on Ca absorption; CPP: no effect	Brommage et al., 1991 Bennett et al., 2000
3. Deposition of radioactivity in bone	Femur–tibia ^{45}Ca activity as index of Ca utilization	Casein > soya; CPP: no effect	Yuan and Kitts, 1991, 1994 Bennett et al., 2000
C. Intestinal segmentation and ^{45}Ca per fusion			
Ligated duodenal loop	Net absorption of ^{45}Ca (transcellular)	Positive CPP effect	Mykkanen and Wasserman, 1980
In vitro everted gut sac	Transport of ^{45}Ca	Positive CPP effect	Mykkanen and Wasserman, 1980

Ligated ileal loop	Net absorption of ^{45}Ca (paracellular)	Positive CPP and casein effect	Naito et al., 1972 Lee et al., 1980, 1983 Nagasawa et al., 1991 Kitts et al., 1992 Yuan and Kitts, 1991, 1994
D. Bone endpoint measures			
Mineralization composition	Calcium utilization	Casein > soya; CPP: no effect	Yuan et al., 1991a Yuan and Kitts, 1994 Yuan and Kitts, 1991 Scholz-Ahrens et al., 1990
Biomechanical testing	Bone strength, compression endpoint measures of Ca utilization	Casein > soya; CPP: no effect	Yuan et al., 1991a Yuan and Kitts, 1994 Scholz-Ahrens et al., 1990
Bone loss	X-ray bone densitometry	CPP: positive effect	Yamaguchi et al., 1998

Notes: Method A does not test for absorbability of calcium from foods or calcium preparations, but gives important information on the physiological regulation of whole body calcium retention (e.g., hormonal activity and metabolic activity of skeleton). In method B, simultaneous kinetic analysis of ^{45}Ca radioactivity recovery in blood, urine and feces will enable determination of luminal–serosal flux and endogenous fecal calcium (not secreted calcium). The bone ^{45}Ca deposition data do not indicate the net secretion or exchange diffusion of calcium and may overestimate calcium utilization. Method C can yield important information on the physiology of calcium transport. Gives data on kinetics of calcium transport in different intestinal locations of the gastrointestinal tract. (e.g., involvement of transcellular and paracellular absorption). Gives no evidence for bioavailability because of lack of involvement of physiochemical factors that may influence solubility or reprecipitation of calcium salts. Method D gives good evidence of potential bioavailability (utilization) of calcium only with experimental durations of >8 weeks. Correlates well with bone mass and factors associated with bone loss. Related more to quality of whole diet and physiological status than to calcium status only.
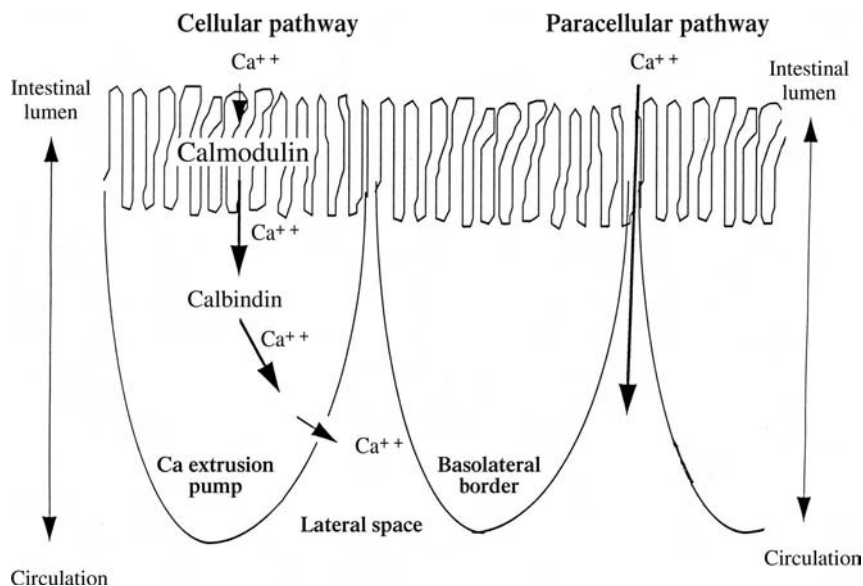
FIGURE 9.1 A diagrammatic representation of calcium uptake mechanisms in the intestine cell.

into morphologically and functionally distinct cellular compartments of villi and crypts of Lieberkuhn. The enterocytes, which are the absorptive cells, come into contact with calcium from the brush border-bearing lumenal membrane to the basolateral membrane. At the basolateral membrane, nutrients, including calcium, exit to the extracellular fluid of the lamina propria and ultimately diffuse into the general circulation. Tight junctions represent forms of membrane contacts between adjacent epithelial cells, which, on the basis of number and complexity, will influence the permeability of the intestinal epithelia (Claude 1978).

The pathways for calcium absorption involve the translocation of calcium from transcellular and paracellular mechanisms of ion absorption (Figure 9.1). This can be confirmed by plotting the intralumenal concentration of calcium against the rate of intestinal calcium transport; the result produces a curvilinear line and thus suggests the coexistence of a nonsaturable and saturable component for intestinal calcium transport (Pansu et al. 1981, 1993). From this consideration, the rate of net calcium transport flux from the mucosal to the serosal side of the intestine can be mathematically expressed as a product of the saturable and nonsaturable components according to:

$$Jm \rightarrow s = A + P[Ca_l^{++}]$$

where $Jm \rightarrow s$ refers to the net transport of intestinal calcium, A is the net saturable Ca^{++} flux, P is the rate (slope) of the net nonsaturable calcium flux as a function of $[Ca_l^{++}]$, and $[Ca_l^{++}]$ is the intralumenal calcium concentration.

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Four major physicochemical factors need to be considered in evaluating calcium absorption efficiency for potential deposition in body calcium tissue pools. These include:

- Solubility of calcium salt in the acid medium of the stomach contents
- Reprecipitation of calcium salts following neutralization by bicarbonate ions from pancreatic secretion
- Calcium complexation with anions while in the neutral environment of the small intestine
- Rate-limiting reduction of calcium salt exposure to the absorptive site of the small intestine due to gastric emptying rate or trapping in fecal material

Because the mechanisms underlying the cellular pathway are potentially rate limiting, an upper limit to the capacity of calcium transport via the cells explains the saturable component that represents the transcellular pathway. In contrast, the nonsaturable component represents transport via a paracellular pathway (Pansu et al. 1981). The rate of calcium transport via the saturable route attains a constant value at different intralumenal calcium concentrations, whereas the rate of calcium transport from the nonsaturable route increases as a linear function of the intralumenal calcium concentration.

Intestinal calcium transport from the transcellular pathway is primarily dependent on vitamin D (DeLuca and Schnoes 1983; Wasserman et al. 1992) and occurs at the greatest extent in the duodenum. The finding that $1,25-(OH)_2D_3$ receptor occupancy is correlated with the expression of the cytosolic calbindin production, which in turn is correlated with the rate of intestinal calcium transport, is evidence that the role of vitamin D in regulating intestinal calcium transport involves biosynthesis of calcium-binding proteins (Hunzikar et al. 1982). Other researchers have also indicated a role of vitamin D action on intestinal calcium transport that goes beyond the induction of calbindin biosynthesis (Wasserman et al. 1982).

In contrast, the paracellular transport mechanism of calcium occurs primarily throughout the distal segment of the small intestine, and possibly the large intestine, and is independent of vitamin D. Instead, mucosal sodium and lactose concentrations can greatly enhance calcium absorption. This pathway represents a solvent drag effect that facilitates calcium absorption by an active extrusion of calcium in the basolateral membrane. This results in a convective transfer of lumenal calcium, which increases calcium concentration in the extracellular space otherwise occupied by the lamina propria (Karbach 1992). This nonsaturable, energy-independent (and therefore passive) absorption of calcium (Bronner 1992) occurs by an osmotic gradient that attracts lumenal water along with solutes and is regulated by the permeability of tight junctions.

The duodenum and ileum have the potential to absorb calcium against an electrochemical gradient, even though a greater rate has been observed in the duodenum (Behar and Kerstein 1976). Notwithstanding this finding, dietary calcium is considered to be absorbed mainly in the distal part of the lower intestine when a normal level of available dietary calcium is present in this intestinal section. Early studies by Marcus and Lengemann (1962) demonstrated an equivalent proportional

absorption for strontium and calcium in various segments of the intestine that represented 62 and 86%, respectively, for liquid and solid forms of calcium. Relative efficiencies of calcium absorption from stomach, duodenum, jejunum and ileum were estimated to be 0, 15, 23 and 62%, respectively, from a liquid diet.

Cramer and Copp (1959) also reported regional intestinal intralumenal calcium concentrations in rats fed a solid diet to be 2.4, 2.0 and 8.1 m*M* for duodenum, distal jejunum and distal ileum, respectively. Moreover, the level of calcium intake will potentially influence the preferential route of absorption as evidenced by the results of Pansu et al. (1981), which showed increasing calcium intake could result in an increase in the transcellular and a decrease in the paracellular absorption calcium. Therefore, part of the redistribution of calcium absorption is explained by the down-regulation of intestinal calcium-binding protein when calcium intake is high or when absorption is enhanced by a co-nutrient such as lactose (Pansu et al. 1979). Whether this is the case for bioactive caseinophosphopeptides derived from milk protein has not been determined.

Finally, it is well recognized that mineral solubility is a primary prerequisite for optimal bioavailability (Ait-Oukhatar et al. 1997; Duflos et al. 1995). For calcium to be absorbed efficiently it must be present in a free and soluble form in the luminal contents of the gastrointestinal tract. Physicochemical factors influencing the degree of absorbability of dietary calcium or calcium supplements include the solubility of calcium in the gastric acid and the intestinal luminal contents, as well as the chelation or complexing of calcium by reactive anions in the distal part of the small intestine. Thus, foods that enhance gastrin and gastric acid secretion, such as high-quality protein sources, could contribute in part to a modification in calcium absorption. In contrast, foods that contain reactive substrates, such as oxalate in spinach or phytates in bran products, may reduce calcium bioavailability. The studies of Heaney and co-workers (1989) provide clear evidence of a meal effect on calcium bioavailability. This concept was based on the hypothesis that the co-ingestion of a meal with calcium from milk or from calcium supplements in the form of pills will reduce the rate of gastric emptying and increase gastric acid secretion. The result is a slower release of calcium into the intestinal tract in a form that is optimal for absorption and exposure to absorption sites located on the intestinal mucosa (Duflos et al. 1995).

In relative terms, the large intestine has a minor role in calcium absorption in the rat (Urban et al. 1980) and humans (Sandstrom et al. 1986). Certain evidence shows that colonic fermentation of soluble fibers producing short-chain fatty acids (SCFA), namely, acetate and propionate, can facilitate a nonsaturable diffusion of calcium ions. This occurs from the release of hydrogen ions derived from the uptake of SCFA, with low pKa values, that enhance the production of hydrogen ion required for calcium absorption (Lutz and Scharrer 1991). It is doubtful that products of milk proteins would have a direct role in facilitating calcium absorption at this site of the intestine.

Numerous experimental approaches have been used to assess or compare the calcium absorption efficiencies of different dairy and food products. These methods vary from *in situ* methods that involve infusion of radiotracers into isolated segments of the intestine to whole-animal calcium balance studies involving longer-term studies conducted under steady-state conditions. Various researchers have also

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attempted to relate the relative bioavailability of different calcium preparations in foods with bone mass and bone mechanical activity (Yamaguchi et al. 1998; Yuan and Kitts 1994). It is important to recognize the advantages and limitations of different methods used to assess calcium bioavailability before drawing specific conclusions or comparisons.

9.3 MILK PROTEIN CONSTITUENTS AND CALCIUM BIOAVAILABILITY

9.3.1 MILK PROTEIN CONSTITUENTS

Bovine milk contains approximately 33 g/l total protein and is a complex mixture of caseinates and whey proteins. Caseinates precipitate from milk at pH values of 4.6, whereas whey proteins are soluble at pH 4.6. The caseinates represent a range of 75 to 85% of the total protein; whey proteins constitute the remaining 15 to 25%. The dominant caseinates in bovine milk are the α_s-caseins, which are present at approximately twice the concentration of β-casein (Table 9.3). In comparison, human milk contains approximately half the total protein found in bovine milk and has only a trace amount of protein that corresponds to α_s-casein; the main component is β-casein (Rasmussen et al. 1995). Another characteristic difference between bovine and human milk involves the amount and composition of whey proteins. These proteins comprise approximately 5.5 to 7.0 g/l for human and bovine milk; however, unlike bovine milk, human milk is devoid of β-lactoglobulin, but contains proportionally more α-lactalbumin (Table 9.3).

TABLE 9.3
Distribution of Principal Milk Proteins in Bovine and Human Milk

Protein Component	Bovine Total Protein (%)	Conc. (g/L)	Human Total Protein (%)	Conc. (g/L)
Caseins	75–85	25.0	25	2.0
αs_1-Casein	45–65	8–10		
β-Casein	30–40	8–10	65	1.3
κ-Casein	12	3	27	0.5
ν-Casein	4.1	1		
Whey proteins	20–30	7	75–80	6
α-Lactalbumin	33	1	50	35–37
β-Lactoglobulin	13	4		
Immunoglobulins	20	6.6	13–15	1.1
Serum albumin	1.2	0.4	6.3	0.5
Essential amino acids (g)		16		4
Amino acids		33		13

An important aspect of these characteristic differences in protein content in human and bovine milk involves the distribution of minerals. For example, the casein fraction of bovine milk contains the major portion of the total minerals (e.g., Zn, 84%; Cu, 44%; Ca, 41%; Mg, 25%; Fe,24%), compared to a relatively smaller amount of minerals present in the human caseinate fraction (Zn, 8%; Ca, 6%; Cu, 7%; Mg, 6%; Fe, 9%) (Fransson and Lonnerdal 1983). In addition, numerous bioactive peptides, consisting of prolactin, insulin, epidermal growth factors, somatostatin and peptide hormone-releasing factors, secretalogues and calcitonin, exist in mammalian milk. Although present in very small amounts in milk, these biologically active peptides not only can affect calcium bioavailability directly, but also can modulate gastrointestinal tract motility, which will indirectly influence calcium bioavailability. For example, prolactin is known to increase intestinal calcium absorption by possibly regulating the biosynthesis of $1,25\text{-}(OH)_2D_3$ (Sponos et al. 1981).

9.3.2 Calcium Distribution and Binding to Milk Proteins

Studies have shown that 60 to 65% of the total calcium (e.g., 32 m*M*) present in milk is complexed in colloidal form with phosphate or citrate amorphous salts associated with the native casein micelle or located in a network system as micellar calcium phosphate (McMahon and Brown 1984). The casein subunits are a hydrophobic core surrounded by κ-casein molecules bound together by colloidal calcium phosphate clusters to form micelles. Additional interactions coming from hydrophobic and hydrogen bonding further contribute to the colloidal dispersion in milk. A stabilized quaternary structure is derived from the calcium–phosphate linkages and removal of the colloidal calcium phosphate will result in disintegration of the native casein micelles. The micelles are composed of spherical subunits of 10 to 20 nm in diameter, with a molecular weight of approximately 2×10^6 Da. The subunits contain cores of calcium-sensitive α_{s1}- and β-casein that are stabilized by an outer core of κ-casein, the least sensitive of the different caseinates to the intestinal calcium mileau content because of its lower phosphoseryl content.

Micellar calcium phosphate represents calcium bound to phosphate ester moieties of phosphoseryl residues positioned nonhomogenously on the casein molecule. The phosphoserine residues are present in all major casein molecules and together with colloidal calcium phosphate contribute to the stability of the casein micelles. The level of phosphorylation will determine the calcium-binding affinity of the components, as evidenced by the increased capacity of α_{s1}- and β-casein to bind calcium following chemical phosphorylation (Yoshikawa et al. 1981). Casein polymorphism will also influence the overall extent of phosphorylation on the molecule (Mercier 1981). Phosphorus is bound to four different caseinates, including α_{s1}-, α_2-, β- and κ-casein, through monoester linkages to seryl residues located within the primary structure of the protein or peptide.

A significant proportion (e.g., 30 to 35%) of calcium in bovine milk is present as soluble calcium. From this fraction, approximately 70% is complexed with phosphate, citrate, or serum proteins and the remaining 30% is present in a free ionic form. The distribution of calcium corresponds to the distribution of phosphorous, which is also dispersed as colloidal (approximately 63%) and free (approximately

16%), and bound as a calcium phosphate salt (approximately 20%). Calcium is not homogenously distributed with the casein micelle and changes in temperature or pH can alter the exchangeability or distribution of calcium between colloidal and soluble forms in accordance with the extent of phosphate ionization (Pierre et al. 1983). Soluble caseins, although in comparatively small amounts in dairy products, are not present in the micellar state but increase with a reduction of temperature (McMahon and Brown 1984). With an increase in temperature, however, the calcium bound to casein decreases and a linear increase in exchangeable calcium from bound ligand to free ionic form is observed over a temperature range of 20 to 90°C (Pierre et al. 1983).

This event is in contrast to cooling of the dairy product, which results in an increase in Ca^{+2} activity as a result of dissociation of colloidal calcium phosphate and in a change in the net negative charge on the surface of the micelle. The stability of the casein micelle is in part affected by the negative charge residing at the surface. For example, at the normal pH of milk (e.g., 6.8), the colloidal calcium phosphate is insoluble; however, lowering pH, as is the case in dairy product fermentation practices (e.g., yogurt) or acid-coagulated products (e.g., buttermilk), will result in the release of calcium phosphate as calcium in ionic form due to the solubilizion. Moreover, the addition of rennet will also result in a decrease in the net negative charge on the macropeptide. Some investigators have considered these events to be the cause for reduced bioavailability of calcium (Kansal and Chaudhary 1982; Wong and LaCroix 1980) because calcium in a soluble free ionic form is more susceptible to forming nonabsorbable complexes with organic or inorganic ligands.

Using intrinsic and extrinsic radiolabeled calcium dairy products, Buchowski et al. (1989) reported that 25 to 30% of the calcium tracer was partitioned in the soluble fraction. Following acidification of the milk with hydrochloric acid or by fermentation to produce a yogurt product, the calcium solubility and exchangeability increased dramatically. However, returning the acidified dairy product to the original pH lowered bioavailability of the calcium tracer, which corresponded to reduced exchangeability of the calcium ion. Employing a similar fractional tracer absorption technique in nutritional studies with premenopausal women subjects, Nickel et al. (1996) also showed that calcium bioavailability was lower in yogurt and a cheese analogue compared to milk, cheddar cheese or processed cheese sources. This finding is in contrast with studies performed in rats fed varying dietary intakes of calcium in the form of calcium carbonate or yogurt. Despite the lack of differences in bone calcium levels from these animals, stronger bones, as indexed by a biomechanical parameter for measuring peak force to fracture bones, were observed in rats fed the yogurt calcium source (Kaup et al. 1987)

In human milk, the small amount of α_{s1}-casein and the presence of β-casein make self-association properties of the phosphoryl groups of β-casein especially important for the formation and function of human casein micelles. There is evidence that a relatively small amount of calcium binding can occur with α-lactalbumin in human milk (Lonnerdal and Glazier 1985) and studies conducted in rats have reported that α-lactalbumen was very effective at contributing to increased calcium absorption (Pantako et al. 1992). The calcium-binding activity of this particular whey protein is likely associated with the structure of the protein and the fact that calcium is required to stabilize the protein (Bernal and Jelen 1984; Hiraoka et al. 1980).

TABLE 9.4
Principal Caseinophosphopeptides and Peptide Fragment Hydrolysis Characteristics from Bovine Casein

Caseinate Fraction							Fragment Example				

β-casein

12			15		17	18	19		
Ile-	Val-	Glu-	Ser-	Leu-	Ser-	Ser-	Ser-	Glu-	Glu-
			|		|	|			
			p		p	p			

main peptide fragments

β-	(1-25) - 4P	3.9% P,	MW = 3123
β-	(1-28) - 4P	3.6% P,	MW = 3469
β-	(33-48) - 1P	1.5% P,	MW = 2062

αs_1-casein

45					60		64		66	67	68		
Gly-	Ser-	Glu-	Ser-	Thr...	Met-	Glu-	Ala-	Ser-	Ile-	Ser-	Ser-	Ser-	Glu-	Glu-
	|							|		|	|	|		
	p							p		p	p	p		

αs_1-	(59-79) - 5P	5.7% P,	MW = 2721
αs_1-	(43-58) - 2P	3.2% P,	MW = 1928

αs_2-casein

7						35							
Val-	Ser-	Ser-	Ser-	Gly-	Glu...	Gly-	Ser-	Ser-	Ser-	Glu-	Glu-	Ser-	Ala
	|	|	|				|	|	|			|	
	p	p	p				p	p	p			p	

main peptide fragment

αs_2-	(35-70) - 4P	4.1% P,	MW = 3009

9.4 GENERATION OF CASEINOPHOSPHOPEPTIDES (CPPs)

Bovine casein consists of caseinate subunits designated as α_{s1}-casein, α_{s2}-casein and β-casein (ratio of 3:0.8:3) possessing uniquely different amounts of positional phosphoseryl residues (e.g., ranges of 8 to 9:10 to 13.5: and 5, respectively; see Table 9.4). These components of the native protein are directed at stabilizing the amorphous micellar calcium phosphate structures of casein, which in turn provides the required micelle size in milk for curd formation. CPPs are smaller peptides present in an inactive site of a much larger polypeptide chain, casein, and are liberated following protease digestion of casein.

Phosphopeptides are derived from tryptic digests of casein. They contain a cluster sequence of Ser-P-Ser-P-Ser-P-Glu-Glu on the N-terminal fragment of the casein peptide, which represents the active sites of the bioactive peptide believed to stabilize amorphous calcium phosphate at neutral and at alkaline pH. This property has been
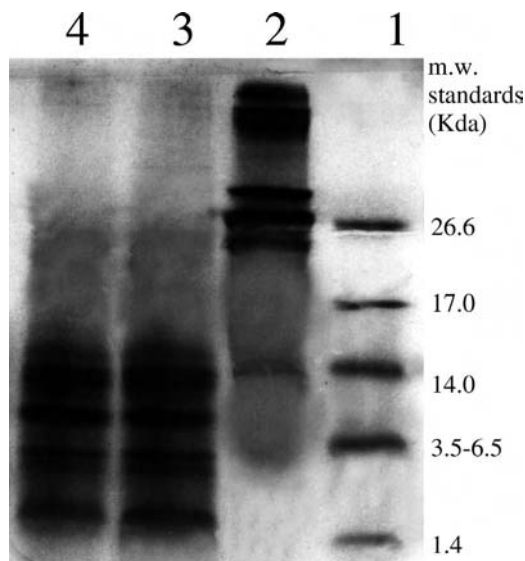
FIGURE 9.2 An electrophoretigram of casein and peptide digestion products following tryptic digestion. Lane 1 = molecular weight standards; Lane 2 = casein; Lanes 3 and 4 = casein tryptic digestion products.

related to the efficacy of these peptides to potentially influence calcium solubility for optimal bioavailability (Kitts and Yuan 1992; Park et al. 1998). Kinetic analysis of isolated phosphopeptides following early stages of trypsin hydrolysis of β-casein have shown that the generation of the three phosphoseryl residues followed by two glutamic acid residues, which characterizes the N-terminal fragment of β-casein (amino acid residues 1–28), occurs through the cleavage of two lysine residues, namely, Lys[28] and Lys[29]. Subsequent hydrolysis follows through cleavage at Arg[25] and Ile[26] and yields the β-casein (amino acid residues 1–25) peptide residue (Leonil et al. 1988). A number of studies have reported recovery yields of CPPs that range from 6 to 16% (Gerber and Jost 1986; Juillerat et al. 1989; McDonagh and FitzGerald 1998). The principal milk protein and associated tryptic digests are shown in Figure 9.2.

A mixture of individual phosphopeptides has been isolated from tryptic digests of β-casein (Kitts et al. 1991; Manson and Annan 1971; Osterberg 1960) as well as cheese products (Roudot-Algaron et al. 1994). Other researchers have recovered CPPs following enzymatic hydrolysis of casein using alcalase (Adamson and Reynolds 1996) and immobilized glutamic acid-specific endopeptidase (Park and Allen 1998). Tryptic digests of α_{s1}-casein and β-casein generate phosphopeptides with greater Ca^{2+} binding activity compared to enzymatic hydrolysis using glutamic acid-specific endopeptidase (Park and Allen 1998). This is attributed to the shorter peptides containing fewer phosphoseryl residues following endopeptidase activity compared to trypsin activity. Iron is not released in association with a low molecular mass peptide by pepsin or typsin digestion; rather, the concentration of charged

phosphates present on the β-casein (amino acid residues 1–25) peptide may protect against further digestion by pancreatic endopeptidases (West 1986).

CPPs have also been recovered from intestinal contents of rats and weanling pigs fed casein diets, thereby confirming that they can be a product of casein digestion *in vivo* (Hirayama et al. 1992; Kasai et al. 1992; Meisel and Frister 1989; Nagasawa et al. 1991; Naito et al. 1972). A peptide chain of approximately nine amino acid residues with a serine to organic phosphorous molar ratio of 1:1 has been recovered from the intestinal chyme of weanling pigs fed a casein diet (Meisel and Frister 1989). A sequence of three phosphoseryl followed by two glutamic or glutamine residues characterized the peptide, which was found to have all serine residues phosphorylated. Showing close similarity between the endogenously derived CPP with exogenously enzymatic hydrolysis of CPP, these results strongly suggest that the highly polar acid domains that represent mineral binding sites for CPP derived *in vitro* also can exist for naturally endogenous-derived CPP. Although relatively resistant to further enzymatic proteolysis, the phosphate moieties present on these particular oligopeptides can be hydrolyzed by acid and alkaline phosphatases. This is particularly true with the N-terminal O-phosphoserine groups that are dephosphorylated at a faster rate by intestinal alkaline phosphatases than phosphoserine groups bound by peptide linkages.

9.4.1 CHEMISTRY OF CPPS

The amino acid sequences of different major casein peptide hydrolysis products derived from bovine milk are summarized in Table 9.4. Tryptic hydrolysis of β-casein contains a peptide with 209 amino acids and 5 phosphoseryl residues in total, which yields two N-terminal phosphopeptide fragments; namely, 1–25 and 1–28 β-casein fragments. These two peptide fragments contain four phosphoseryl residues located at positions 15, 17, 18 and 19 on the peptide. In contrast, α_{s1}-casein has 199 amino acid residues and a total of 7 to 9 phosphoseryl groups, which enables it to potentially bind more Ca^{2+} than β-casein. The primary structures of different CPPs have in common an acidic and highly polar domain sharing a common motif that has a sequence of three phosphoseryl residues. Park et al. (1998) have shown that the higher overall binding of Ca^{2+} to α_{s1}-casein compared to β-casein is due to the lower number of phosphoseryl residues present in β-casein. This is in contrast with other researchers who have shown that calcium-binding affinity for α_{s1}-casein (e.g., 17 mol/mol CPP) is different from β-casein (e.g., 24 mol/mol CPP) at typical intestinal pH (9.0) conditions (Reynolds 1993).

An explanation for the different findings likely involves the stiochemical Ca/P ratio of principal phosphoseryl binding sites on the phosphopeptides as well as the specific conformation structure of the peptide (Baumy et al. 1989). Additional factors such as the content of calcium, pH of the reaction mixture, ionic strength and disassociation constant values of individual phosphoseryl residues on the peptide may also influence the binding pattern. This may be attributed to reduced cation binding by casein molecules due to lowering of pH, which reflects a decrease in the ionization state of the phosphoseryl residues and, to a lesser extent, the gluamyl, aspartyl, tyrosyl and histidyl residues. Increasing ionic strength by adding NaCl will

decrease the pK values by modifying the amino acid charge distribution on the phosphoseryl residues. This will increase dissociation of these groups and lower the affinity to cations present, in addition to potentially forming a competition between Ca^{2+} and Na^+ on the peptide–phosphate moiety. Thus, electrostatic interactions account for most of the calcium binding to CPP (Dickson and Perkins 1971; Waugh et al. 1971).

Moreover, calcium-binding affinity is enhanced by the location of the phosphoseryl residues that are in a close cluster, as well as the presence of certain neighboring amino acid residues. The characteristic glutamic and aspartic acid residues in the peptide also provide free carboxyl groups, in addition to phosphoseryl residues of caseinates (Kitts et al. 1991) for bivalent metal binding; however, these sites are relatively less important for calcium binding (Dickson and Perkins 1971). The significance of these physicochemical criteria for calcium binding relative to enhancing calcium bioavailability has not been fully ascertained. Lee et al. (1980) estimated that 1 mol of CPP can bind to 40 mol of calcium ion, with binding constants in the order of 10^2 to 10^3 M^{-1}. These relatively low binding affinities would not be expected to interfere with intestinal absorption. The general agreement is that phosphopeptide fractions have greater Ca^{2+}-binding affinity than the nonphosphopeptide fractions generated from tryptic hydrolysis of β-casein and α_{s1}-casein.

It is important to note that the affinity of CPP to sequester divalent ions is not limited to calcium. The strong affinity of CPP for calcium ions is also seen with other bivalent ions, including Fe^{2+}, Zn^{2+} and Mn^{2+} (Baumy et al. 1989; Emery 1992; Gaucheron et al. 1997; Hurrell et al. 1989; Reeves and Latour 1958). Beta-casein (1–25) has also been shown to bind to iron through the phosphoseryl residues present in CPP and this binding activity is sufficient to keep the iron soluble within the duodenal alkaline pH range (e.g., pH 9.0; Bouhallab et al. 1991). The four phosphoseryl residues present in the N-terminal fragment of the peptide bind to four iron molecules and have been reported to enhance iron bioavailability in the rat (Ait-Oukhatar et al. 1997; Peres et al. 1997). Evidence indicates as well that the β-casein (1–25) peptide binding to iron is resistant to low gastric pH. On the other hand, intestinal phosphatases (e.g., derived from apical membrane of the enterocytes present in the brush border membrane) release free iron from the peptide. This can alter subsequent digestion of the peptide because binding of iron to phosphoseryl residues results in partial denaturation of the peptide and thus acts to reduce the activity of intestinal phosphatases to iron bound to β-casein (1–25) peptides (Bouhallab et al. 1991).

9.4.2 PHYSICOCHEMICAL PROPERTIES OF CPP AND CALCIUM BIOAVAILABILITY

The potential usefulness of CPPs for nutritional purposes was initially reported by Mellander (1950), who showed that a trypsin digest of casein was effective at promoting bone mineralization in ratchitic children without the requirement for vitamin D therapy. Reeves and Latour (1958) reported that an empirical formula for a phosphopeptide derived from the *in vitro* tryptic digestion of casein approximated 9.9 N:6.6 PO_4:12.2 Ca, without the presence of inorganic phosphate. This

material was effective at maintaining calcium hydrogen phosphate ($CaHPO_4$) in solution within a pH 7 to 10 range. CPPs are effective at completely inhibiting the formation of insoluble calcium phosphate at concentrations as high as 10 mg/100 mL, although the efficiency of inhibition greatly depends on the Ca/P ratio (Berrocal et al. 1989). Ideally, the serine:phosphorus ratio content of the CPP should be 1.0, thus indicating that all serine residues are phosphorylated. Dephosphorylation of milk proteins or CPPs results in loss of mineral-binding activity (Berrocal et al. 1989; Gerber and Jost 1986; Sato et al. 1983), thus providing an additional line of evidence for the importance of the phosphorous component in eliciting bivalent metal-binding activity.

The physiological significance of CPPs derived from milk caseins, α_{s1}-casein and β-casein concerns the affinity in which they compete for ionized calcium and inhibit the precipitation and subsequent formation of insoluble calcium salts in the small intestine (Mellander 1963; Naito et al. 1972; Naito and Susuki 1974; Sato et al. 1998). This property of CPP has not been confined to the intestinal mileau (Sato et al. 1983, 1986), but also has been examined in salivary and anticariogenic situations (Hay et al. 1979; Moynihan et al. 1999; Reynolds 1987), and embryonic (Gerber and Jost 1986; Landis et al. 1984) and adult (Reynolds et al. 1982) bone mineralization. The *in vitro* results of Termine and Posner (1970) demonstrated a rapid phosphate-induced precipitation of calcium occurring at a pH that was similar to the lower intestine (pH 7.5). Various macropeptide species can be generated from enzymatic hydrolysis of milk proteins, but no correlation exists with a presence in the luminal contents and associated function to make calcium salts that will assist with paracaellular calcium absorption soluble. A common finding in many studies using a variety of different methodologies has been the increased concentration of soluble calcium in luminal contents of the lower intestine of rats fed casein compared to alternative protein sources (Lee et al. 1983; Nagasawa et al. 1991; Naito and Susuki 1974; Yuan and Kitts 1994). Administering CPP with a calcium source has been shown to result in improved calcium bioavailability in postmenopausal women that exhibit low calcium absorptive performance (Heaney et al. 1994). General agreement for these observations involves the affinity of phosphopeptides to stabilize amorphous calcium phosphate at neutral and alkaline pH conditions of the intestine, thus inhibiting precipitation of calcium to form insoluble calcium phosphate or calcium carbonate salts.

Studies from our laboratory have shown that rats fed heat-denatured casein exhibit lower *in situ* calcium bioavailability compared to counterparts fed native casein. This result was interpreted to indicate a reduced generation of bioactive phosphopeptides as a result of poor digestion caused by heat-induced denaturation of the casein (Yuan and Kitts 1994). Employing the inverted ileal gut sac and *in situ* ligated intestinal loop techniques, Mykkanen and Wasserman (1980) reported an increased transfer of radiolabeled calcium into the mucosal tissue and the appearance of radioisotope in the serosal solution that was proportional to the peptide concentration of selective, although unknown, CPPs. Futhermore, nonlinearity of the radionucleotide flux across the intestinal membrane was interpreted to represent at least two types of transport processes promoted by the peptide. These processes included

a saturated reaction of the peptide, when the peptide was limited, and an enhanced peptide-mediated translocation of the radiolabeled calcium at higher concentrations of phosphopeptide. An enhanced absorption of calcium tracer attributed to the presence of CPP was also reported in the duodenal segment of the intestinal tract in normal and rachitic chicks. This finding was the first report that CPP stimulated calcium bioavailability and was not confined to the distal small intestine, even though the mode of action is undoubtedly independent of vitamin D. The role of CPP in directly initiating increased Ca flux across the intestinal lumen is doubtful, however, because other workers have shown that, in fact, the CPP–Ca complex is not absorbed but instead remains on the mucosal side of the intestinal tissue (Li et al. 1989).

Lee and co-workers (1983) also reported that the oral administration of radio-labeled calcium to rats shortly after feeding a casein-based diet resulted in peak radioactivity of the calcium tracer detected in the portal blood after only 30-min postisotope administration and at a greater concentration than that observed in rats fed soya protein-based diets. Conducting a balance study in vitamin D–normal and vitamin D–deficient rats has also produced results that do not suggest a marked effect of a CPP-mediated increase in apparent calcium absorption efficiency or retention (Kopra et al. 1992; Yuan and Kitts 1991). It is noteworthy, however, that in both studies, the employment of CPP-fortified diets in the experimental design resulted in an increase in urinary calcium excretion, although no differences in femur calcium uptake were reported. An increased urinary calcium excretion could in part represent enhanced absorbed calcium that was excreted rather than used for bone mineralization in response to adaptation-mediated responses triggered by hormonal control in response to maintaining whole-body calcium homesostasis.

Reported by Bennett et al. (2000), true fractional calcium absorption studies using a fecal ^{47}Sc:^{47}Ca tracer ratio procedure in normal rats have demonstrated enhanced calcium absorption only at a minimal dietary casein intake of 500 g/kg, or an equivalent amount of 50 g CPP (assuming a theoretical yield of CPP derived from casein is 10 g/kg; Kopra et al. 1992). However, the addition of CPP derived from enzymatic hydrolysis of casein, at twice the equivalent concentration of feeding 500 g casein/kg diet, proved to have no effect on enhancing calcium bioavailability. Also, no dose relationship between the level of CPP added to the meal and the change in calcium bioavailability was noted.

This result is contrary to similar studies using fractional Ca tracer kinetics and casein feeding in rats (Brommage et al. 1991). The discrepancy in results between the two studies may be attributed to the noted differences in dietary calcium status relative to calcium requirements of the test animals used in the studies. Moreover, the noted effects of the importance of controlling the level of dietary protein and other constituents fed to the animals on rate of gastric emptying should not be discounted because these factors can influence calcium absorption efficiency (Shi et al. 1997). It is also noteworthy that adaptation of the active transcellular calcium transport pathway was offered as an additional explanation for the reduced calcium absorption efficiency noted with feeding high amounts of CPP in a single meal (Bennett et al. 2000).

9.5   POTENTIAL HEALTH BENEFITS OF CPP–CALCIUM INTERACTIONS

9.5.1   BONE

Dairy products, especially milk and cheese, have been shown to have beneficial properties on mineralization of bone in prepubertal girls (Chan et al. 1995) and postmenopausal women (Nickel et al. 1996). Early studies by Mellander (1950) first reported that CPP increased bone calcification of rachitic patients; a potential for CPP-induced calcium bioavailability resulting in enhanced uptake of calcium in bone has also been reported by Sato et al. (1986). These researchers administered a crude CPP extract in the form of a tryptic digest of casein simultaneously with a calcium tracer *in situ* and reported a significant uptake of the tracer into rat femur. A similar finding using calcified, and decalcified CPP preparations has been reported in ovariectomized rats (Tsuchita et al. 1996). Moreover, a formulated calcium tofu containing CPP has also been shown to prevent bone loss in ovariectomized rats, relative to sham-operated controls (Yamaguchi et al. 1998).

The general understanding that ovarian hormone deficiency can induce bone loss (Riggs et al. 1969) makes this a particularly important finding. Significant increases in the calcification of the diaphyseal area of explanted and cultured embryonic rat femora, tibiae and metatarsal rudiments have also been attributed to CPP *in vitro* (Gerber and Jost 1986). *In vivo* studies employing bone calcification indices and different biomechanical characteristics have also been shown to be affected adversely by feeding diets containing reduced amounts of casein, or denatured casein, by excessive thermal processing (Yuan and Kitts 1994). The conclusion that CPP may have a role in bone mineralization and biomechanical strength is based on the fact that reducing the level of dietary protein intake from casein, as well as feeding similar levels of denatured protein, resulted in a calculated 35 to 65% reduction in apparent CPP content of ileal contents. The latter can be explained by reduced digestibility of protein source. Brink et al. (1992) reported that higher bone turnover was observed in rats fed soybean-based beverages compared to cow's milk.

Other studies have not been able to confirm the enhanced utilization of calcium from CPP when normal rats are fed intact casein (Bennett et al. 2000; Forbes et al. 1979; Yuan et al. 1991b) or, for that matter, when rachitic chicks are fed the intact protein source (Mykkanen and Wasserman 1980). Similarly, specific phosphopeptide preparations fed to normal (Bennett et al. 2000; Kopra et al. 1992) and calcium-deficient (Kopra et al. 1992) rats failed to show enhancement of calcium utilization. The lack of differences in calcium balance or bone parameters in these studies has been suggested to involve the limited presence of intact, active caseinophosphopeptides in the gut shortly after intake. This restriction is principally attributed to CPP-induced intestinal phosphatase (E.C. 3.1.3.1) activities, which in rodents could render the peptides more susceptible to proteolysis.

However, the findings of other workers do not support this explanation. For example, Hirayama et al. (1992) reported a recovery of 8.3% w/w of the original amount of CPP fed to rats, which corresponded to α_{s1}- (61–74) and β-casein (7–24) and elevated concentrations of luminal soluble calcium in the distal small intestine.

A similar α_{s1}-casein peptide (66–74 genetic variants A–E) has also been recovered from weanling pigs fed casein-containing diets (Meisel and Frister 1989). Moreover, Brommage et al. (1991) recovered CPP in the ileum after 60 min following oral administration of the phosphopeptide to rats, suggesting that different CPP preparations between studies may be an underlying cause for the discrepancy of findings. It is also possible that a requirement for a threshold concentration level of CPP is generated from casein digestion for enhancing calcium bioavailability. This conclusion is supported by the findings of others that CPP had no effect on calcium absorption (Shah et al. 1990) or femur calcification (Yuan and Kitts 1991) and that bone mineralization is influenced more by dietary calcium intake than variation in protein source (Yuan and Kitts 1992).

More recently, Bennett et al. (2000) have reported that CPP has no direct acute effect on Ca uptake in bone at low levels, but in fact may actually reduce bone mineralization if administered at higher concentrations that range from 200 to 500 g/kg diet. The explanation for this finding is based on the compositional properties of CPP that may alter intestinal Ca transport as well as produce a shift in the intestinal phosphorous to calcium ratio to an extent that would potentially reduce calcium bioavailability. This conclusion is based on the assumption that proteolysis of the CPP by intestinal phosphatases would take place, thus yielding more free phosphate, which in turn is known to decrease calcium absorption (Mahoney and Hendricks 1978).

Finally, the fact that other milk proteins may be involved in bone health should not be ignored; the findings of many, including Ranhota et al. (1997), have indicated that calcium present in a milk protein whey fraction can provide greater calcium deposition into femoral tissue compared to calcium supplements such as calcium carbonate, calcium lactate or even calcium citrate. That milk has an obvious functional role in the skeletal growth of newborn animals likely explains why others have been able to demonstrate that milk whey protein is effective for osteoblast cell proliferation and differentiation (Takada et al. 1996).

9.5.2 DENTAL HEALTH

Milk proteins have been proposed to have potentially important roles in the prevention of dental caries by three principal mechanisms: (1) a topical mechanism substantially increasing the level of calcium and phosphate in dental plaque; (2) binding strongly to hydroxyapatite and reducing the rate of dissolution of the mineral; (3) increasing dental plaque pH to retard demineralization of enamel (Harper et al. 1986, 1987; Herod 1991; Reynolds 1987). A summary of the various research findings is given in Table 9.5. The primary CPPs with anticariogenic properties are derived from α_{s1}-casein $\{\alpha_{s1}$-f(59–79)$\}$, β-casein $\{β$-csn-f(1–25)$\}$ and two peptides derived from α_{s2} casein $\{\alpha_{s2}$-f(1–21) and α_{s2}-f(46–70)$\}$.

The mechanism of dental caries formation is well understood. Cariogenic bacteria, including the mutans streptococci and lactobacilli, produce organic acids within dental plaque on fermentation of carbohydrates, which diffuse through the plaque and into the porous subsurface enamel. The dissociation of these organic acids will lower pH, and in turn make hydroxyapatite $(Ca_{10}(PO_4)_6(OH)_2)$ crystals soluble,

TABLE 9.5
Evidence for the Anticariogenic Properties of Milk Proteins

Milk Constituent	Model System	Observations	Reference
Milk minerals (Ca, PO_4)	Human subjects	Protection against cavities	Jenkins and Ferguson, 1966
	In vitro demineralization	Protection against enamel demineralization	Grenby et al., 2001
Casein/Whey Proteins			
Caseinate	Rat caries model (2% w/v drinking water)	Reduced cavities	Reynolds and del Rio, 1984
	Human subjects (chocolate confectionary)	Reduced cavities	Reynolds and Black, 1987
αs_1 (casein tryptic digest)	ICT[a], human subject	Incorporate into plaque and prevent demineralization of enamel	Reynolds, 1987
Casein polypeptide proteose–peptones fragments	*In vitro* demineralization	Inhibit demineralization of enamel	Grenby et al., 2001
Cheese			
Casein fraction	Rat caries model	Cariostatic activity	Harper et al., 1987
	Desalivated rat caries model	Cariostatic activity	Krobicka et al., 1987
Whey fraction	Rat caries model	Protection at buccal surface	Harper et al., 1987
H_2O-soluble components	ICT, human subject	Depressed demineralization; enhanced remineralization	Silva et al., 1987
Cooked cheese	Human subjects	Increase plaque Ca^2 concentration	Moynihan et al., 1999

[a] ICT = Intra-oral cariogenicity test.

leading to demineralization of the tooth enamel and dentin. Without remineralization from the saliva, dental caries occur.

CPPs contain a cluster of phosphoseryl residues that markedly increase the solubility of calcium phosphate by forming colloidal casein–phosphopeptide amorphous calcium–phosphate complexes (CPP–CaP); the result is an increase in the level of calcium phosphate in plaque facilitated by the CPPs acting as a reservoir of calcium phosphate. Studies have shown that the increase in calcium and phosphate content of plaque corresponds with the incorporation of casein into plaque (Reynolds

1987). The sequestering and localization of amorphous calcium on the tooth surface acts to buffer the free calcium phosphate ions and maintain a state of super saturation with respect to its close association with hydroxyapatite, thereby depressing enamel demineralization and enhancing the potential for remineralization. Once incorporated into plaque, α_{s1}-casein has been shown not only to act as a calcium phosphate reservoir, but also to buffer against bacterial acid production via a proton-accepting affinity at pH 7.0. These findings explain earlier reported observations by Weiss and Bibby (1966) that the solubility of enamel could be reduced by more than 20% if treated with milk. Similarly, Harper et al. (1986) have reported anticariogenic activity of different cheeses that were attributed to the casein and calcium phosphate contents.

Clarifying these observations, more recent studies have reported that a CPP–CaP complex could significantly reduce caries activity in a dose-dependent manner, with 0.1% CPP–CaP producing a 14% reduction and 1.0% CPP–CaP producing 55 and 46% reductions in smooth-surface and fissure caries activity, respectively (Reynolds et al. 1995). These results were found to be equivalent to the use of 500 ppm fluoride and, moreover, to produce an additive anticariogenic effect when CPP–CaP and fluoride were combined in the treatment. The anticariogenic activity of the CPP–CaP was not associated with a reduction in the level of total streptococci in plaque (Reynolds et al. 1995).

The potential of minor proteins or peptides of milk associated with anticariogenic activity has recently been investigated with proteose–peptone fractions (Grenby et al. 2001). A major proportion of this fraction is believed to be derived from β-casein and not from κ-casein (e.g., glycoprotein). Proteose–peptones are water-soluble components present in low concentrations in milk and can contain phosphoseryl residues or other amino acids with ionic and neutral polar groups that may act to sequester calcium ions and thus protect against demineralization. Moreover, these same components may also form hydrogen bonds with the enamel surface as well as react with plaque matrix molecules or bacteria in facilitating anticariogenic properties.

From a dental standpoint, use of cheese as the last food in a meal will help to reduce caries. Even following substantial dilution of cheese with other meal components and heat treatment, cheese remains effective in significantly increasing plaque calcium concentration. The promotion of meals and snacks containing cheese as a means of caries prevention represents a positive approach to improved oral health (Moynihan et al. 1999).

9.5.3 HYPERTENSION

Evidence from epidemiological and experimental animal studies is sufficient to indicate a role of calcium ion in the regulation of vascular smooth muscle tone and thus blood pressure by influencing the sympathetic nervous system or, alternatively, the rennin–aldosterone axis. Studies conducted in the spontaneously hypertensive rat have demonstrated that dietary calcium restrictions can induce hypertension, whereas calcium supplementation can attenuate the onset of hypertension (Kitts et al. 1992; McCarron et al. 1981; McCarron and Morris 1987). This, along with the excellent bioavailibility of calcium from dairy products, corresponds with the

reported negative correlation between blood pressure and the consumption of dairy products (Ackley et al. 1983). However, other studies focused on the potential role of casein in facilitating calcium bioavailability have not been able to confirm a reduction in hypertension in the normal or hypertensive rat fed different dairy proteins, lactose or CPP, despite induced positive changes in paracellular calcium uptake as measured by the *in situ* ileal loop technique (Kitts et al. 1991, 1992; Nagasawa et al. 1991). Rather, it is doubtful that enhanced calcium bioavailability from consumption of dairy products can sufficiently modify whole-body calcium utilization in a manner that will influence the calcium homeostatic control of hypertension (Kitts et al. 1992).

9.6 CONCLUSIONS

Despite the relatively good agreement in the literature from *in situ* studies using the ligated ileal loop protocol that indicate a potential role for CPP to enhance paracellular and, to a lesser extent, transcellular calcium bioavailability, controversy exists as to whether these experimental methods can be extrapolated to the intact intestine. Confirmation of results has not been successful from studies conducted with the ligated intestinal segments *in situ* relative to whole-animal tests using balance studies, fractional absorption of radiolabeled calcium tracers and bone composition and functionality tests (Table 9.2). Moreover, additional studies that pay close attention to the precise composition of CPP generated through well-controlled enzymatic hydrolysis methods are required.

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10 Iron Fortification of Dairy Products: A Novel Approach

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CONTENTS

10.1 INTRODUCTION

Micronutrient malnutrition is a term commonly used to refer to mineral and vitamin nutritional deficiency diseases. Iron deficiency anemia, vitamin A deficiency and

iodine deficiency disorders are among the most common forms of micronutrient malnutrition. Although the major malnutrition problems are found in developing countries, micronutrient deficiencies also exist in some vulnerable groups in developed countries (e.g., children, adolescents, pregnant and lactating mothers). Deficiency in iron, vitamin A, or iodine affects 30% of the world's population. Some 735 million people suffer from clinical forms of these deficiencies and another 2 billion from subclinical forms. Iron deficiency anemia affects 60% of Asian women of reproductive age and 40 to 50% of children enrolled in preschool and primary grades (Joseph 2000). This deficiency causes more than half the maternal deaths in the world and depresses academic achievement in language and reading skills of young students.

It is estimated that up to half of all anemia is caused by dietary iron deficiency (MacPhail and Bothwell 1992). In some areas, over half of the members of the previously mentioned groups may be anemic, but the disorder is also seen in older children and men (United Nations 1991; World Health Organization 1992a). Anemia in infants and children is associated with retardation of physical growth and intellectual and psychomotor development, as well as reduced work capacity, and exacts a high economic burden on society (Levin 1986). Blood loss in childbirth can be dangerous for anemic women and is the primary cause of many maternal deaths. Maternal anemia may also lead to fetal growth retardation, low-birth-weight infants and increased rates of early neonatal mortality.

Young children are most susceptible to iron deficiency because they require relatively high amounts of iron for rapid growth during the first 2 years of life; usually, their diet is low in iron unless it is added as a nutritional supplement (Dallman et al. 1980). Iron deficiency anemia is a major cause of low birth weight and maternal mortality (DeMaeyer et al. 1989) and has recently been recognized again as an important cause of cognitive deficit in infants and young children. Iron deficiency also has a profound affect on productivity and therefore has economic implications for countries in which it is a significant public health problem (McGuire and Galloway 1994; Scholz et al. 1997).

Although iron has the potential for use in more food vehicles than iodine or vitamin A, fortification with iron is technically more difficult than with other nutrients because iron reacts chemically with several food ingredients. The biggest challenge with iron is to identify a form that has adequate bioavailability and yet does not alter the appearance or taste of the food vehicle. The buff-colored, insoluble, iron phosphate compounds are stable under a variety of storage conditions, but are poorly bioavailable. Soluble iron salts like ferrous sulfate are well absorbed but easily discolored by other food components like tannins, etc.

Many countries have launched national projects to enrich iron in daily foods. In response to public health goals of reducing deficiency, the U.S. Food and Drug Administration (FDA) in the 1940s and 1950s established standards of identity for enriched staple foods (e.g., flour, bread, rice, cornmeal) and specified levels of iron, in addition to other vitamins to be added (Crane et al. 1995). Chile in the 1950s was the first Latin American country to fortify with iron, although not explicitly for anemia control. In 1993, Venezuela legislated for fortification of wheat flour (Layrisse et al. 1996); at least 18 governments in the region are now implementing

fortification through wheat flour. The Philippine government is trying to establish a supply of iron-enriched rice. Recently, demand for nutritionally fortified foods (iron, calcium, vitamins, carotenoids, etc.) has increased as more consumers look for fortified foods to avoid taking dietary supplements or altering their eating habits (Hallberg et al. 1966a, b; Hurrell and Cook 1990; Hallberg 1995; Hurtado et al. 1999; Allen 1998).

Iron deficiency is a common nutritional deficiency worldwide, affecting mainly older infants, young children, and women of childbearing age (Pilch and Senti 1984; Dallman et al. 1984). In developing countries it is estimated that 30 to 40% of young children and premenopausal women are affected by this deficiency (DeMaeyer et al. 1985). Increasing the content and bioavailability of iron in the diet can prevent iron deficiency and fortification of daily foods like dairy products is one of the most effective solutions.

Many iron compounds that exhibit high bioavailability, such as ferrous sulfate, adversely affect food quality by accelerating lipid oxidation or by producing an unfavorable color or flavor (Bothwell and McPhail 1992; Disler et al. 1975; Hurrell 1985, 1992; Douglas et al. 1981; Edmonson et al. 1971; Viteri et al. 1995). In dairy products, compatible and nonreactive iron compounds are attractive as fortifiers because they have less of an "iron taste" compared with soluble iron. Ferric pyrophosphate is considered one of these compounds because of its nonreactivity; unfortunately, due to its low bioavailability and insolubility, using it to fortify dairy products is impractical. However, a superdispersed formulation of ferric pyrophosphate has been developed for use in dairy products such as milk and yogurts and other food products outlined in this chapter.

10.2 PHYSICOCHEMICAL PROPERTIES OF SUPERDISPERSED FERRIC PYROPHOSPHATE (SDFᴇ)

Ferric pyrophosphate was prepared by adding sodium pyrophosphate solution to ferric chloride solution. The precipitate collected was washed and a ferric pyrophosphate suspension was dispersed with lecithin and other emulsifiers. Conventional ferric pyrophosphate, which is insoluble, was made soluble in water by this technique (Figure 10.1). SDFe has been commercialized under the name "SunActive Fe" by Taiyo Kagaku Co., Ltd., Japan. (SunActive Fe-12 is a liquid preparation of 12 mg Fe/g; SunActive Fe-P80 is a dried powder preparation of 80 mg Fe/g.) The physicochemical properties of stability, bioavailability and safety of SDFe have been evaluated.

10.2.1 Particle Size Distribution of SDFe

The particle size distribution of SDFe (diluted with water 1:50) and ferric pyrophosphate (diluted with water 1:1250) was measured using a laser diffraction particle size distribution analyzer (Helos Sympatec Co., Ltd.). SDFe showed a sharp particle distribution size (particle size distribution: 0.1 to 2.6 μm, with an average particle size distribution of 0.5 μm) that is several times smaller than that of commercial
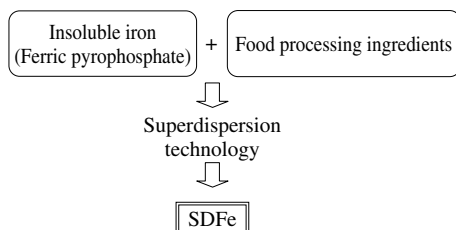
FIGURE 10.1 Superdispersed ferric pyrophosphate (SDFe; commercial name: SunActive Fe™).
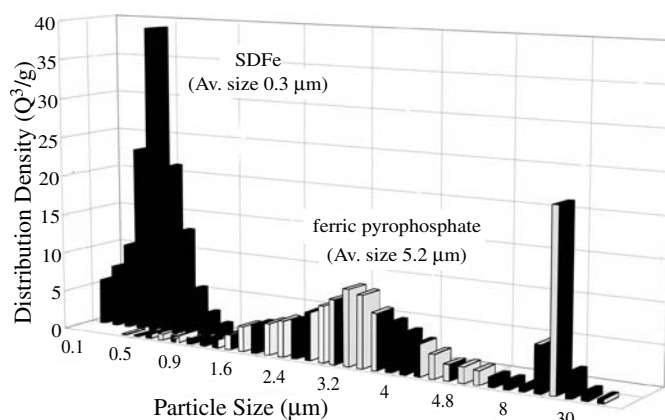
FIGURE 10.2 Particle size distribution of SDFe and ferric pyrophosphate.

SDFe   Ferric pyrophosphate
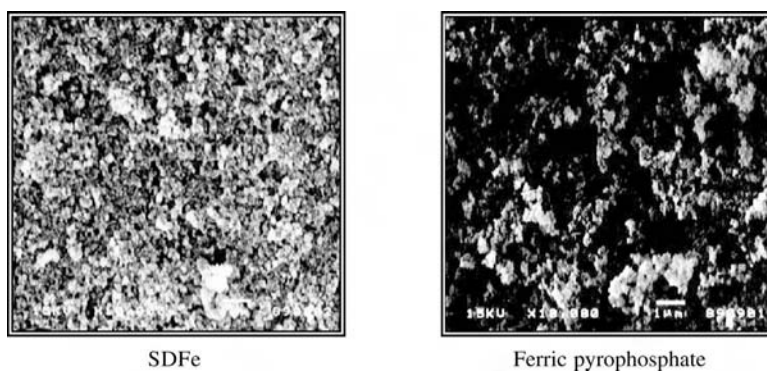
FIGURE 10.3 Photographs of scanning electron microscope of SDFe and ferric pyrophosphate.

ferric pyrophosphate with an average particle size distribution of 5.2 μm (Figure 10.2). Scanning electron microscopy showed that SDFe did not show aggregation as with commercial ferric pyrophosphate; results showed that SDFe is uniformly dispersed in water (Figure 10.3).

SDFe solution
(Fe 12 mg/g)

Ferric pyrophosphate solution
(Fe 12 mg/g)
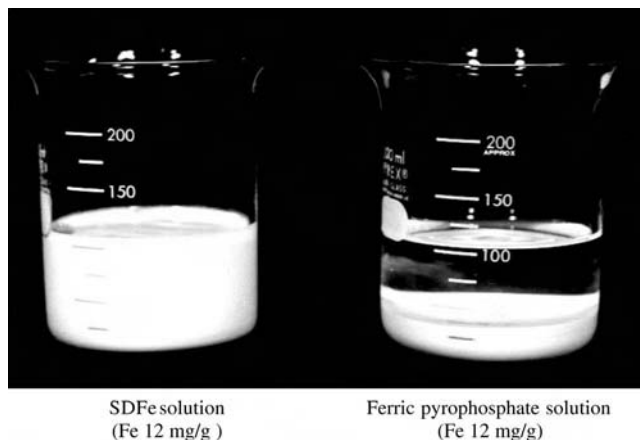
FIGURE 10.4 Solubility of SDFe and ferric pyrophosphate.

10.2.2 STABILITY OF SDFE

Although commercial ferric pyrophosphate solutions contain 12 mg/g iron precipitate, SDFe maintains ferric pyrophosphate in suspension (Figure 10.4).

10.2.2.1 Stability of Vitamin C with SDFe and Other Test Iron Compounds

The stability of vitamin C was higher with SDFe compared with other iron sources. The test was performed using SDFe, ferric pyrophosphate, sodium ferrous citrate, and ferrous sulfate as 1 g Fe was mixed with 100 g ascorbic acid, respectively. The samples were stored in a dark place at room temperature for 0, 1, 2, and 4 weeks. After storage, residual vitamin C was measured. SDFe showed the highest stability of vitamin C and the lowest with residual vitamin C. SDFe (1.6 mg Fe/100 ml iron) and ascorbic acid (24 mg/100 ml) were mixed with a commercial whey drink (pH 4.2) or a commercial soft drink (pH 3.3), or water as control. The samples were pasteurized at 80°C for 30 mins and stored in a dark place at 5°C for 0, 2, and 4 weeks. SDFe was found to be stable in low pH drinks (Figure 10.5).

10.2.2.2 Color Stability of SDFe

The iron concentration of the various iron compounds used in the study, SDFe, ferric pyrophosphate, ferrous sulfate, and sodium ferrous citrate, was adjusted to 5 mg iron per 100 g of distilled water, heated at 70°C for 10 min and then kept at 40°C. There was no precipitation of SDFe solution after storage for 3 months. By contrast, commercial ferric pyrophosphate sedimented immediately; a brownish precipitate was formed in the case of ferrous sulfate and the solution containing sodium ferrous citrate turned brown after 2 days (Figure 10.6). Experimental preparations were prepared that contained 100 ml of the liquid samples, 2.6 g of fat, 3.7 g of protein,
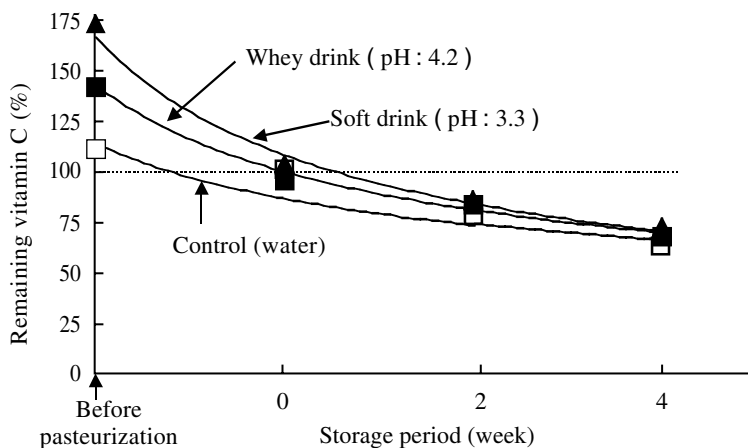
FIGURE 10.5 Stability of SDFe with ascorbic acid in dairy and other drinks.

SDFe	Ferric pyrophosphate	Ferrous sulfate	Sodium ferrous citrate
(Clear)	(White precipitation)	(Brown precipitation)	(Yellowish-Brown)
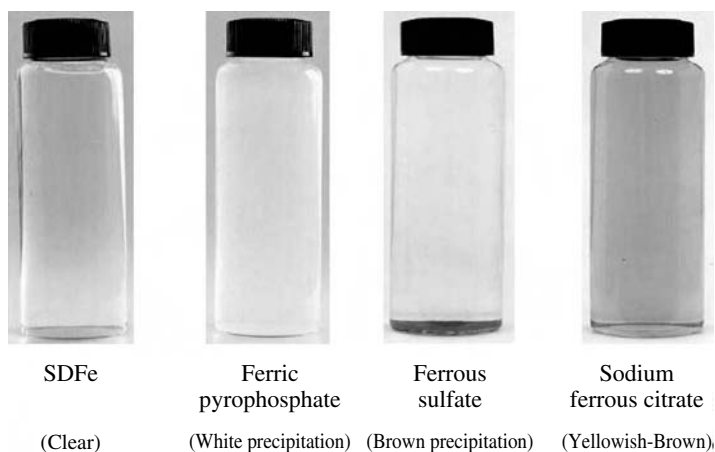
FIGURE 10.6 Stability of SDFe and other iron solutions.

17.2 g of carbohydrate, 2.5 mg of iron (sodium ferrous citrate or SDFe) and a small amount of vitamins and minerals. The samples were pasteurized at 120°C for 20 min and stored in a dark place at 50°C for 0, 1, 2, 3, and 4 months. After storage, the color change (ΔE) of the samples was measured. SDFe was the most stable and experienced little color change (Figure 10.7).

10.2.2.3 Stability of SDFe with Salt and Sugar

Iron is very reactive with NaCl or edible salts or sugars. SDFe, ferric pyrophosphate, ferrous sulfate, or sodium ferrous citrate as 6 and 12 mg iron were mixed

Fe: 1.6mg / 100g , Brix: 21,   pH:6.6

Heat treatment: 120°C, 20 min
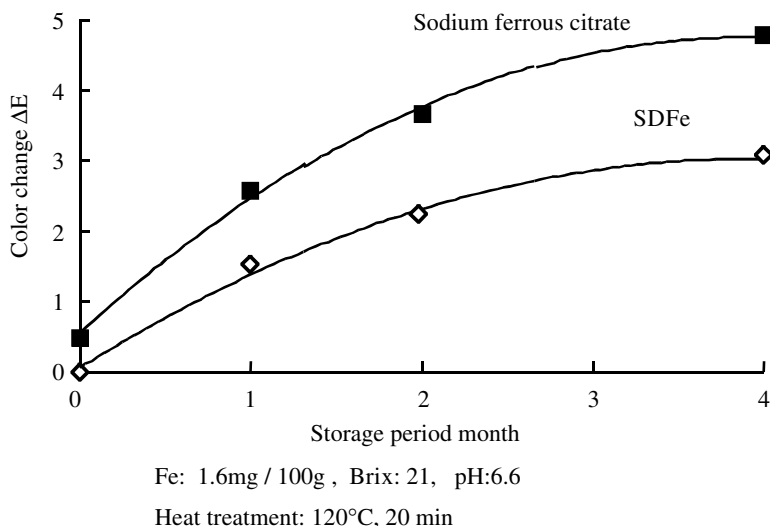
FIGURE 10.7 Color stability of SDFe in nutrient solution.

with 10 g of salt and 10 g of sugar, respectively. After storage at room temperature, the color of the mixtures was observed. SDFe was quite stable with salt and sugar and did not show reactivity compared with ferrous sulfate and ferrous citrate (Figure 10.8).

10.2.2.4 Heat Stability of SDFe

SDFe (1.6 mg as iron) was mixed with 100 g of concentrated prune juice. Then, heat treatment was given one to three times at 121°C for 30 min. After cooling, each sample was centrifuged at $8000 \times g$ for 30 min. Free-iron concentration in the supernatant was determined by atomic absorption. SDFe released almost no free iron. SDFe was also found to be heat stable and maintained its emulsifying and flavor-masking properties during baking and retorting.

10.2.3 SENSORY EVALUATION STUDIES

Sensory evaluation tests for unpleasant flavors were conducted by 10 panelists on 5% fructose–glucose–liquid sugar solutions with added SDFe, ferric pyrophosphate, ferrous sulfate or sodium ferrous citrate, respectively. SDFe showed no flavor compared with other iron sources (Table 10.1). SDFe was mixed with a yogurt drink and then a 10% vitamin C solution was added. Final concentrations of iron and vitamin C ranged from 1.0 to 10 mg and 5.0 to 100 mg in the yogurt drink, respectively. No unpleasant tastes were reported for the yogurt drink containing 10 mg of SDFe (10 mg as iron) (Figure 10.9).

(a) 6 mg Fe / 10 g salt
(b) 12 mg Fe / 10 g sugar
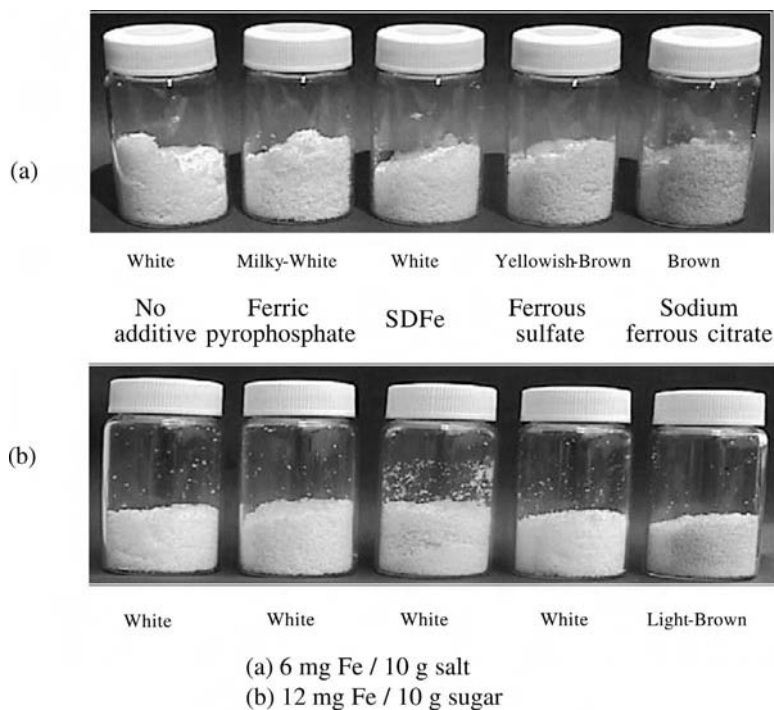
FIGURE 10.8 Stability of SDFe with sugar and salt.

TABLE 10.1
Sensory Evaluation of Different Iron Solutions

Iron Sources	Evaluation
SDFe	1.2 ± 0.1[a]
Ferric pyrophosphate	1.6 ± 0.2[b]
Sodium ferrous citrate	2.7 ± 0.2[c]
Ferrous sulfate	3.3 ± 0.1[d]

Notes: Sample: 5 mg Fe/100 ml, 5% glucose solution. Data are expressed as means ± SE. Evaluation method: 0: no odd flavor or taste; 1: no iron flavor and taste; 2: iron flavor and taste; 3: strong iron flavor and taste; 4: extremely strong iron flavor and taste.

[a–d] Values with different superscript letters are significantly different ($p < 0.05$).
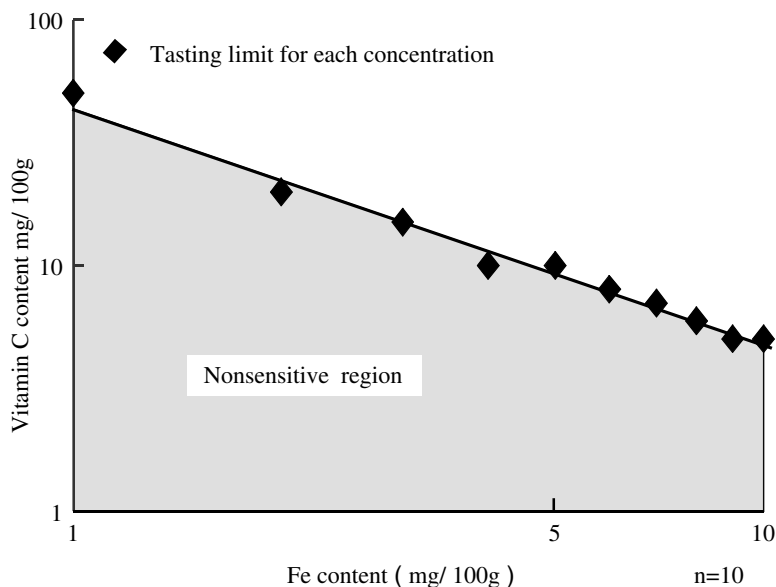
1-10 mg Fe and 5-100 mg Vitamin C / 100 g yogurt drink

FIGURE 10.9 Flavor stability of SDFe in yogurt drink.

10.3   SDFe — IRON BIOAVAILABILITY

Absorption and bioavailability of SDFe was evaluated by the serum iron (Pagella et al. 1984), hemoglobin regeneration efficiency (HRE) (Forbes et al. 1989) and the modified AOAC methods in rats.

10.3.1   IRON BIOAVAILABILITY EVALUATED BY SERUM IRON CURVES OF NORMAL RATS

This method was based on serum iron concentration (SIC) in normal rats (Ekenved et al. 1976). The test was carried out to verify the increase of SIC by the adminis-tration of SDFe. A standard diet was fed to 10-week-old rats for 5 days. All rats were randomized into five groups of ten rats each with approximately equal average body weight among the groups and were fasted for 18 h prior to the examination. SDFe, ferric pyrophosphate, sodium ferrous citrate, ferrous sulfate and commercial heme iron dispersed or dissolved in distilled water at 2 mg Fe/kg body weight were orally administrated to the rats by gavage. After 0.5, 1, 2, 4 and 8 h of oral administration, blood samples were taken from the carotid section for determination of SIC.

Iron absorption determined by SIC curve showed a mean value of 113.4 µg/dl in the control, which remained practically unchanged during the study period. During iron fortification, SIC rapidly increased and decreased after iron administration. The peak SIC 30 min after oral administration was 340.0 µg/dl for ferric pyrophosphate,
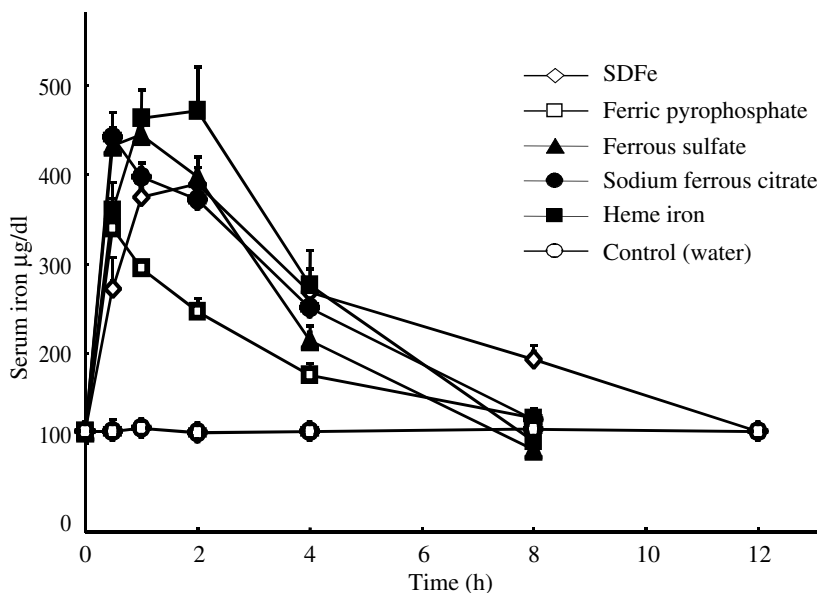
FIGURE 10.10   Serum iron level in normal rats after oral administration of SDFe and other iron compounds (2 mg Fe/kg body weight).

441.2 μg/dl for sodium ferrous citrate and 444.4 μg/dl for ferrous sulfate 60 min after oral administration, following which SIC rapidly decreased in the control. In the case of SDFe and commercial heme iron, the Fe absorption peak of SIC was delayed compared with other iron sources, reaching 388.8 and 471.6 μg/dl 2 h after oral administration. High serum iron concentrations were even observed 8 h after oral administration of SDFe (Figure 10.10).

The average value of the area under the curve was 2839, 1573, 2108, 2001, 2294 and 909 μg/dl for SDFe, ferric pyrophosphate, sodium ferrous citrate, ferrous sulfate, commercial heme iron and the control, respectively (Figure 10.11). The physico-chemical form of inorganic iron for iron fortification includes ferrous iron and ferric iron. Studies carried out on iron absorption by the SIC method following oral administration of ferrous sulfuric, sodium ferrous citrate or ferric pyrophosphate showed similar serum iron curves (Setsuda and Sato 1981; Heinrich 1987; Ekenved et al. 1976) to those found in this study. By contrast, SIC following oral iron administration of SDFe showed a lag in peak time and sustained release of iron in the serum. This could be because the SDFe preparation is a nano-sized encapsulated iron formulation.

10.3.2 Iron Bioavailability from SDFe by HRE Method

Using the technique of Zhang et al. (1989), iron bioavailability from SDFe and other test iron compounds was determined by the hemoglobin regeneration efficiency (HRE) based on the hemoglobin repletion assay in anemic rats. To create iron-deficient anemic rats, 4-week-old rats were fed an iron-deficient diet for 5 weeks.
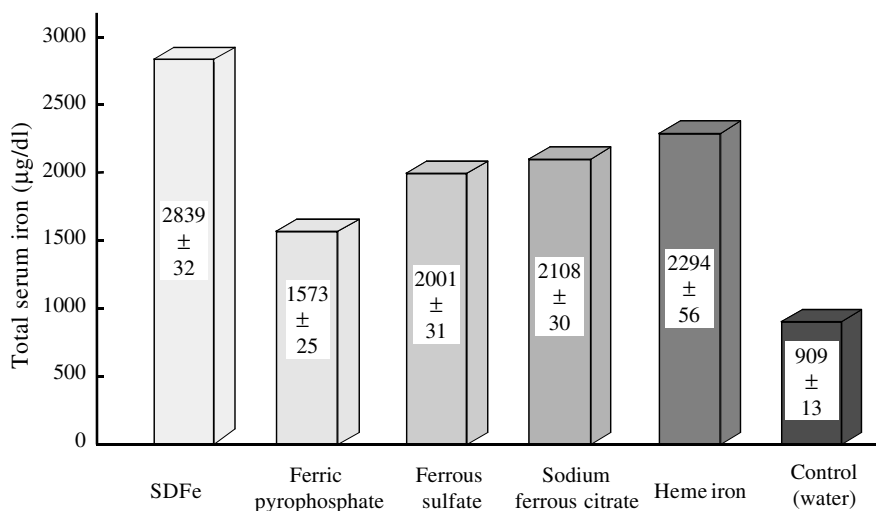
FIGURE 10.11   Incremental area under the curves following intake of iron (2 mg Fe/kg body weight).

The iron-deficient anemic rats were randomized into five groups, each with eight rats and approximately equal average body weight among the groups. Experimental diets were prepared by adding 3.5 mg Fe/kg iron to the iron-deficient diets using SDFe, ferric pyrophosphate, sodium ferrous citrate or ferrous sulfate. Experimental diets were fed *ad libitum* for 28 days. Control rats were fed a standard diet. Dietary intakes were recorded. After 0, 4, 7, 11, 14, 18, 21 and 28 days, body weights were measured and blood samples were taken from the tail to measure hemoglobin values. Diets were ashed at 550°C in the presence of 1 N HNO$_3$ for 24 h. Ash samples were dissolved with concentrated HCl and diluted with deionized water. Iron contents in the diets were determined by atomic absorption (Hitachi 180-30, Tokyo). Hemoglobin was determined by the cyanmethemoblobin method. The HRE value was calculated by the following method (Forbes et al. 1989; Zhang et al. 1989):

$$\text{Hb Fe (mg)} = \text{body wt (kg)} \times 0.075 \text{ L blood/body wt (kg)} \times$$
$$\text{Hb(g)/blood (L)} \times 3.35 \text{ mg Fe/Hb (g)}$$

$$\text{HRE} = ((\text{Hb Fe (mg)})_{\text{final}} - (\text{Hb Fe (mg)})_{\text{initial}})/\text{Fe consumed (mg)}$$

The relative biological value (RBV) was calculated by HRE of SDFe, ferric pyrophosphate or sodium ferrous citrate divided by the mean HRE of ferrous sulfate. After 2 weeks of iron fortification, the hemoglobin regeneration efficiencies were 55, 41, 53, and 53 for SDFe, feric pyrophosphate, sodium ferrous citrate and ferrous sulfate, respectively. The RBVs of iron sources were 1.05, 0.78 and 1.00 for SDFe, ferric pyrophosphate and sodium ferrous citrate, respectively. SDFe showed the highest value of HRE as well as RBV among the iron compounds tested (Figure 10.12).
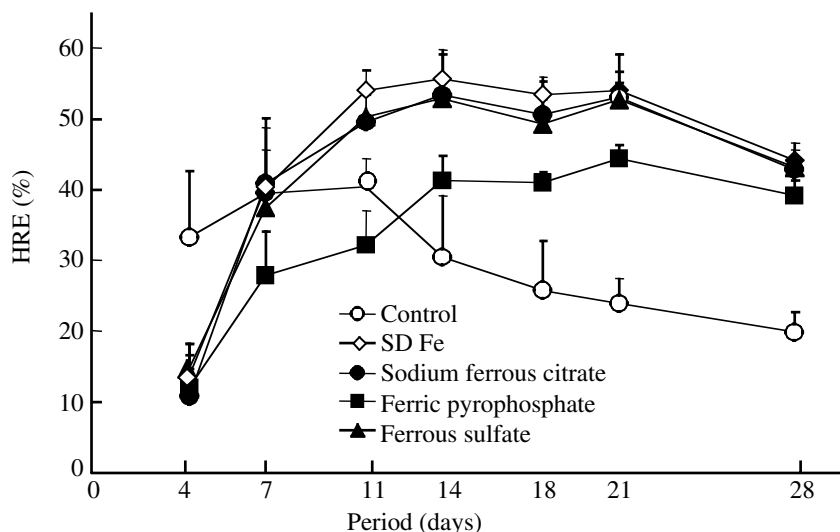
FIGURE 10.12   Hemoglobin regeneration efficiency (HRE) value in iron-deficient anemic and normal rats (dose: 3.5 mg Fe/100 g).

10.3.3 Iron Bioavailability by the Modified AOAC Method

Iron bioavailability from SDFe was determined by the AOAC method (Forbes et al. 1989). Iron-deficient anemic rats were prepared and randomized into five groups, each with eight rats and approximately equal average body weight among the groups. Experimental diets were prepared by adding 0, 6, 12, 18 or 24 mg Fe/kg to iron-deficient diets using SDFe, ferric pyrophosphate or ferrous sulfate, respectively. Experimental diets were fed *ad libitum* for 2 weeks. The bioavailability of each test iron source relative to ferrous sulfate was calculated by comparing the gain in hemoglobin with the iron level in the diet by the slope ratio procedure. The slope value of each test iron was 0.270, 0.145 and 0.259 for SDFe, feric pyrophosphate and ferrous sulfate, respectively, and RBV was 1.04 and 0.56 for SDFe and ferric pyrophosphate, respectively (Figure 10.13). Using the SIC, HRE and modified AOAC methods, SDFe showed high iron bioavailability compared with other iron sources.

Dietary constituents that solubilize iron may enhance absorption, whereas compounds that cause precipitation or molecular aggregation of iron decrease it (Conrad et al. 1993; Benjamin et al. 1967). The reason for the higher iron bioavailability of SDFe compared with ferric pyrophosphate may be due to a higher degree of solubility compared with ferric pyrophosphate and the mechanism of iron absorption due to the different particle size distributions of ferric pyrophosphate (Figure 10.2). Further studies are warranted to explain the mechanism of iron absorption of SDFe.
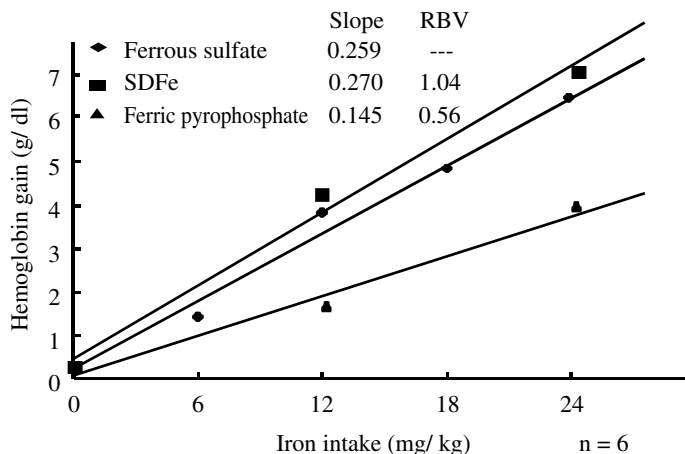
FIGURE 10.13   Relative biological value of SDFe and other test iron sources (modified AOAC method) in iron-deficient anemic rats.

10.4 BIOAVAILABILITY OF IRON FROM MILK CONTAINING SDFᴇ IN HUMANS

Hallberg et al. (1992) found that iron absorption from a meal was reduced due to the presence of calcium. To evaluate the effect of calcium on iron absorption, 13 young female students with hemoglobin levels less than 12 g/dl received 200 ml of milk containing SDFe (5 mg as iron) for 72 days. After the experiment, the hemoglobin and hematocrit levels were significantly higher than initial values (Figure 10.14). The study demonstrated that the iron in milk containing SDFe was bioavailable, even though the milk contained high concentrations of calcium.
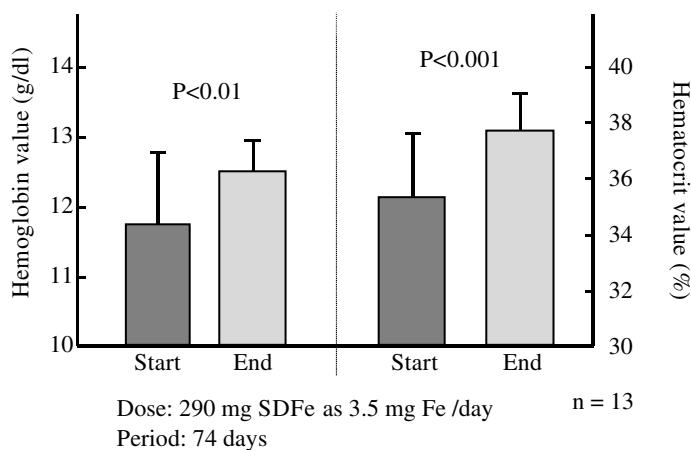
FIGURE 10.14   Efficacy of SDFe in young females.

10.5 GASTRIC TOLERANCE OF SDFᴇ AND OTHER TEST IRON COMPOUNDS

Often when iron is continuously consumed and/or high doses of iron are taken, side effects such as nausea, emesis, anorexia, abdominal pain, diarrhea or constipation occur (Hallberg et al. 1966b; World Health Organization 1992b). Thus, the effect of SDFe on gastric tolerance in rats was evaluated. A standard diet was fed to 8-week-old rats for 5 days. All rats were randomized into four groups of ten rats each so that mean body weight was approximately equal among the groups and they were fasted for 48 h prior to the experiment. Thereafter, SDFe, ferric pyrophosphate, ferrous sulfate, and sodium ferrous citrate dissolved in distilled water (30 mg Fe/kg body weight) were orally administered by gavage three times in the 24-h period. Approximately 5 h after the final iron administration, the stomach was removed and the extent of gastric ulcers induced by the iron compounds was evaluated using the method of Adami et al. (1964). Hemorrhagic suffusion or ulcers were observed in the sodium ferrous citrate and ferrous sulfate groups, but the SDFe group did not show any lesions or toxicity. The results showed that SDFe was tolerated well and did not have any harmful effect on the gastrointestinal system compared with the other iron compounds.

10.6 ACUTE TOXICITY STUDIES

In an acute toxicity study, male and female rats were treated orally by giving them a maximum of 635 mg Fe/kg in relation to the iron content in SDFe. No significant differences were observed between the control and the SDFe group in relation to general behavior, mortality, body weight, or food and water intake for 14 days. The LD_{50} of SDFe was found to be 635 mg Fe/kg body weight. The acceptable daily intake of iron phosphate is 70 mg/kg in humans, so the LD_{50} of SDFe is quite high.

SDFe was examined for mutagenic activity in two independent Ames tests using the histidine-requiring *Salmonella typhmurium* strains TA1535, TA1537, TA98 and TA100, the tryptophan-requiring *Escherichia coli* strain WP2 *uvrA*, and a liver fraction of Aroclor-induced rats for metabolic activation (S9-mix). SDFe was not toxic to any of the strains, as was demonstrated by the absence of a drastic decrease in the mean number of revertants. SDFe was not found to be mutagenic under the conditions employed in the study.

10.7 CONCLUSIONS

SDFe was found to have excellent iron absorption properties, iron bioavailability, safety and ease of use in food applications compared with other iron sources. By applying this unique technology, a "nutrition delivery system" (NDS) has been developed to fortify foods with minerals, vitamins and other nutrients. SDFe disperses insoluble iron in liquid formulations and produces no precipitation by the unique superdispersion technology. It also masks disagreeable flavors and the taste of iron without affecting the flavor of the final product. SDFe creates a milky-white solution, instead of the usual brownish color of most iron-fortified products and

maintains flavor- and taste-masking properties during baking and retort processes. SDFe showed excellent absorption properties and bioavailability compared with other iron sources. Because of its high stability, SDFe has been used for the fortification of milk, soft drinks, yogurt, yogurt drinks, ice cream, soups and dressings. It is a novel concept in iron fortification for dairy and other food product applications.

ACKNOWLEDGMENT

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11 European Perspective on Development of a Health Claim Dossier for a Functional Dairy Product

Gertjan Schaafsma and Jean Feord

CONTENTS

11.1 INTRODUCTION

The market for functional foods and dietary supplements is expanding rapidly: on the basis of a marketing survey, Rabo Bank International (2001) projected a world-wide 100% growth within 5 years. Questions about safety and efficacy of these

products must be answered. The novel food law in European Union (EU) member states (Regulation No 258/97) implies that the safety of novel foods must be assessed before their marketing.

Novel foods are defined as foods that hitherto have not been used for human consumption to a significant degree within the European community. No specific legislation exists in European countries for functional foods. As far as these foods are novel, their safety must be assessed before marketing. For health claims, no specific European legislation exists. Two basic rules, however, are in force: (1) consumers may not be misled and (2) medical claims for foods are not allowed. Internationally, and even among member states of the EU, detailed legislation with respect to health claims shows wide variations; thus, further harmonization of food legislation is required. The focus of this chapter is to discuss the evidence needed for health claim support, taking into account the general opinion that claims may not mislead consumers. Special attention is given to studies with human subjects because these are the final stage for obtaining scientific evidence for a particular health effect of a food or dietary supplement.

There is no generally accepted definition for functional foods. In the opinion of the authors, the general characteristics of these foods (or drinks) are that they have been made on the basis of knowledge of nutrition and health and that they offer specific physiological benefits to the consumer beyond the basic nutritional value of existing (traditional) foods. Consumption of functional foods offers the opportunity to ingest a healthy balanced diet with additional benefits, and the concept challenges the food industry to make existing foods better and to market new innovative foods. Functional foods thus offer the consumer health benefits and the food industry new possibilities to market so called "added-value" products. According to this concept, functional foods can be designed for the general population as well as for special target groups such as sports people, infants and patients.

Two dimensions in the concept of functional foods can be recognized: traditional nutritional value and bioactivity beyond traditional nutritional value (Figure 11.1). The first dimension may include well-designed convenience foods like meal-replacing products (such as cereal breakfast bars and shakes and drinks developed in conjunction with weight-loss programs) with a well-balanced nutritional composition and foods fortified with nutrients. The second dimension refers to the supplementation or fortification of foods with bioactive components such as prebiotics, probiotics, phytochemicals, bioactive peptides, long-chain polyunsaturated fatty acids and herbals (e.g., fortified cereals, yogurts and dairy drinks, cholesterol-lowering spreads). Nutrition claims are often attached to products of the first category (dimension 1), whereas health claims are attached particularly to foods of the latter category (dimension 2). In the scientific community this latter category of foods is considered to be functional (Diplock et al. 1999). Fortification of foods with nutrients (vitamins, minerals, trace elements, essential fatty acids and amino acids) can serve both dimensions, depending on the concentrations used. When nutrient concentrations in foods are such that a daily portion of these foods contributes significantly to daily recommended intakes, they can be considered as
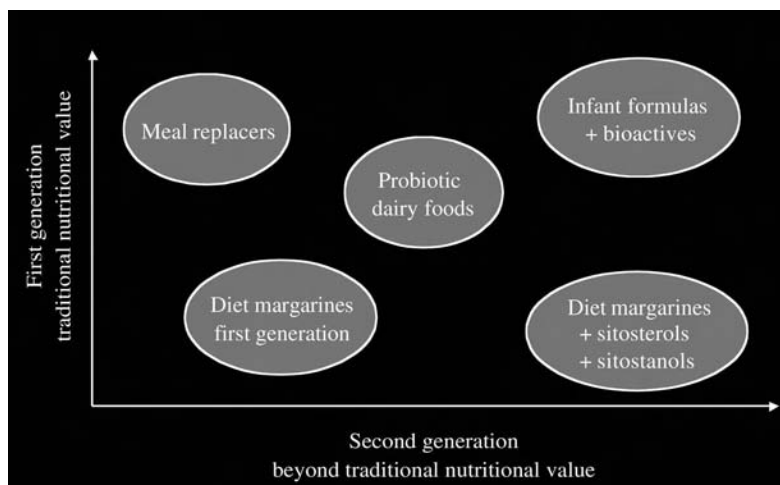
FIGURE 11.1 First and second generations of functional foods.

offering traditional nutritional value. Nutrient concentrations in excess of traditional recommendations could serve the second dimension of functional foods.

11.2 DIETARY SUPPLEMENTS, NEUTRICEUTICALS, AND NUTRACEUTICALS

According to the Dietary Supplement Health and Education Act (1994) in the U.S., dietary supplements are products intended to supplement the diet to enhance health. These supplements appear on the market as pills, capsules, powders, or drinks. They are also called nutriceuticals or nutraceuticals. The legal status of dietary supplements may vary among countries. In most European countries dietary supplements fall under food legislation. Only some EU countries — for example, Germany and Denmark — tend to view dietary supplements as pharmaceuticals; Finland views them distinctly from food, although not as pharmaceuticals. In some other countries, these products are considered legally as a distinct category of foods; for example, in the U.S. they fall under the Dietary Supplement Health and Education Act (1994). The authors consider nutriceuticals to be dietary supplements containing essential nutrients or mixtures thereof (vitamins, minerals, trace elements) and nutraceuticals to be dietary supplements containing other types of bioactive substances. This discrimination between nutri- and nutraceuticals is quite relevant because bioactivity for essential nutrients is not in doubt, whereas this activity still needs to be demonstrated for many nutraceuticals.

Special attention in this chapter is given to claims, the evidence needed for claim substantiation and the experiments with human volunteers that are generally considered the final stage of claim substantiation. A general model layout is described for the preparation of a dossier containing the information needed by regulatory

bodies that must evaluate the health effect of a food, food ingredient or dietary supplement. A health effect is the basis of a health claim.

11.3 CLAIMS

According to the Codex Alimentarius, a claim is defined as "any representation, which states, suggests or implies that a food has certain characteristics relating to its origin, nutritional properties, nature, production, processing, composition, or any other quality." With respect to nutritional properties of functional foods and dietary supplements, two main types of claims are recognized (Directorate General Health and Consumer Protection of the European Commission 2001): nutrition claims and health claims. In this section, these claims will be discussed and the data needed to substantiate the health effects on which the claims are based will be indicated.

The basic rules dominating the application of nutrition and health claims for foods and dietary supplements are:

- Consumers have the basic right to be informed about characteristics of their foods.
- Claims should not mislead consumers.
- Medical claims are not allowed for foods.
- Claims should not be in conflict with generally accepted guidelines for a healthy balanced diet.

With respect to the second point, the important question to be answered is "How much evidence is at least required to avoid accusations of misleading the consumer or quackery?" Here the right balance should be found between the available evidence and the content of the claim. Also, claims should not suggest a health benefit when scientific evidence for such a benefit does not exist. As mentioned earlier, two main types of claims for foods can be identified with respect to functional effects: nutrition and health claims.

11.3.1 NUTRITION CLAIMS

Nutrition claims refer to information about the composition of a food beyond general nutrient content information. Nutrition claims can be easily evaluated by food analysis and can be positive ("rich in calcium," "good source of fiber") or negative ("low in fat," "no sugar added," "free of cholesterol").

Positive nutrition claims often refer to essential nutrients, whereas negative nutrition claims refer to compounds that are considered to lead to undesirable side effects when consumed in excessive amounts. As a rule of thumb, a daily portion of a food for which a nutrition claim is made should provide at least 15 to 25% of the daily recommended intake of the nutrient involved. In the case of negative claims, it makes no sense to use these claims for foods that normally do not contain the "negative" substance. Thus the claim "free of cholesterol" for a cucumber is nonsense, as is the claim "no sugar added" for foods that normally do not contain sugar, like plain yogurt.

A positive nutrition claim that refers to an ingredient not considered to be an essential nutrient and for which no scientific evidence exists for a beneficial health effect in the consumer should, in the authors' opinions, be considered misleading to consumers. However, from a legislative point of view, this is not punishable. An example of such a misleading nutrition claim is that for taurine in so-called "energy" drinks. The application of taurine in energy drinks suggests a positive (performance-enhancing) effect, whereas scientific evidence for such an effect is lacking. On the other hand, nutrition claims for non-nutrients are acceptable when they refer to substances for which scientific evidence of health effects is available, provided the amount of the substance in a daily portion of the product is at least 25% of the effective dose. Even when the scientific evidence for such substances is not yet completely conclusive, the nutrition claim may be considered acceptable and not misleading to consumers, taking into account the right of consumers to be informed.

Nutrition claims referring to specific nutrients may be extended by providing additional information about the physiological function of the nutrients in question (e.g., "this product is rich in calcium and an adequate intake of calcium is required for bone development"). Such a "nutrient function claim" is very close to a health claim (see the next section). The information can be checked easily and is acceptable when formulated in a way that is not in conflict with the principles of a healthy balanced diet. Nutrition claims may also refer to products that have been designed to have a composition consistent with dietary guidelines or that are targeted to have a specific function in the total diet, such as meal-replacing foods.

11.3.2 HEALTH CLAIMS

A health claim is a message about a positive effect on health of a specific ingredient, dietary supplement or food. At the lowest impact level, a health claim may refer to improvement of the nutritional status of the consumer and is similar to a nutrient function claim. Such health claims are often obvious and do not need specific scientific substantiation. Two other types of health claims can be identified: reduced risk claims and functional claims (Diplock et al. 1999). Reduced risk claims, also called health claim type A, are used for products that can help to reduce the risk of chronic nutrition-related diseases. Functional claims (health claim type B) are used to communicate the positive effect a product may have on a body function. Relevant chronic nutrition-related diseases and body functions are listed in Table 11.1.

One problem in Europe with type A claims is that they will be considered by the authorities as medical claims, which are not allowed for foods. At the moment, type A health claims are formulated in such a way that the name of the disease involved is not directly communicated. Instead, the reduction of a validate biomarker for the disease in question (like serum LDL cholesterol or blood pressure for cardiovascular disease risk) by the consumption of a particular functional food is mentioned.

The majority of the scientific community agrees that health claims (types A and B) for functional foods should have a sound scientific basis — for example, as formulated by the Food and Drug Administration in the U.S. (Nutrition Labeling and Education Act 1990):

TABLE 11.1
Examples of Health Claim Areas[a] for Functional
Foods and Dietary Supplements

Diseases (health claims type A)	Body functions (health claims type B)
Cardiovascular disease	Natural defense
Osteoporosis	Gut function (stool habits)
Obesity	Mood
Diabetes type II	Physical performance
Allergy	Skin protection
Cancer	Oral health
Arthritis	Brain function

[a] Prevention of nutrition-related diseases and enhancement of body functions.

> … based on the totality of publicly available scientific evidence (including evidence from well-designed studies conducted in a manner which is consistent with generally recognized scientific procedures and principles), there is significant scientific agreement among experts qualified by scientific training and experience to evaluate such claims, the claim is supported by such evidence …

It is also recognized in the Western world that the scientific evidence should include results of at least two independent studies in human volunteers. (Results should be reproducible.) All relevant information should be compiled in a dossier that should be submitted by an independent expert panel (preferably before marketing of the functional food) to the institution that organizes the evaluation. Such a system (code of practice) exists in various European countries, e.g., Sweden (Swedish Nutrition Foundation 1997), the United Kingdom (Joint Health Claims Initiative 1997), and The Netherlands (Voedingscentrum 1998). These systems have no regulatory status, but it is expected that they will be followed in the future by legislation of health claims at a European level.

In other major markets, health claims are handled in a variety of ways. Japan probably has the most well-developed system for recognition of health benefits in functional foods under the FOSHU (foods of specific health use) scheme. Foods are approved by the Japanese Minister of Health and Welfare for FOSHU status based on whether the ingredients used in the foods are proven to have a beneficial effect on health. These foods are identified by the FOSHU logo. As of June 2003, 369 FOSHU containing ingredients that have benefits, including digestive health, cardiovascular health, mineral balance, blood sugar control and dental health foods, have been approved in Japan.

In other countries, schemes similar to FOSHU have not yet been embraced and systems are developing with the common element of handling claims on a

case-by-case-basis. In the U.S., three types of health claims are recognized by the Food and Drug Administration (FDA). The first is the NLEA (Nutrition Labeling and Education Act 1990)–authorized health claim, pertaining to substance–disease relationships, e.g., "diets high in calcium may reduce the risk of osteoporosis," which may be made for foods and dietary supplements. These FDA-authorized claims are based on significant scientific agreement of the nutrient–disease relationship established though extensive available scientific literature. The second type is a health claim based on an authoritative statement, which can be made on foods only. The authoritative statement must be made by a scientific body belonging to the U.S. government or the National Academy of Sciences. The third type is a qualified health claim that can be made on dietary supplements only. A qualified health claim is permitted if more evidence is available for the claim than against it. However, the claim is not endorsed by the validity of claims made under the NLEA. Details on these claims and further details relating to claims in the U.S. can be found on the FDA website (http://www.fda.gov/).

In Canada, Health Canada has drafted proposals for product-specific authorization of health claims. In permitting companies to apply for the authorization of claims specific to their food product, this goes beyond the already published regulation of authorized generic structure/function claims. Further information on the proposals in Canada can be found on the Health Canada website (http://www.hc-sc.gc.ca/food-aliment/e_index.html). The Australian and New Zealand Food Authority (ANZFA) has proposed a management framework for health, nutrition and related claims about food. This framework is still under development, but intends to introduce a co-regulatory scheme for government and industry to develop a code of practice for authorization and control of health claims. Results of the latest developments can be found at http://www.anzfa.govt.nz/.

Dietary supplements, although falling under the food law in many European countries, should be considered as a separate category of foods, for example, as in the U.S. Like all foods, dietary supplements should be safe under normal conditions of use and consumers should not be misled by their nutrition labeling or by nutrition or health claims connected to them. Taking into account the large market for dietary supplements, the fact that many companies acting in this market are relatively small, and the considerable cost of studies with human volunteers, one may ask whether it is feasible to perform such studies for all dietary supplements. For this separate category of foods, one could consider installing a special code of practice offering dietary supplement companies the possibility to have the health effects they claim for their supplements evaluated. A simple scoring system could be worked out to identify the strength of the scientific evidence for the health effect involved (no evidence < weak evidence < circumstantial evidence < substantial evidence< scientifically proven). This scoring system could assist the consumer in his decision to buy or not to buy the product. A dossier should be prepared for evaluation for submission under such a code of practice and the applicant could then receive a certificate that could be printed on the supplement label. This system would encourage industry to protect their product claims from "me-too" companies who copy their claims.

11.3.3 GENERAL PRINCIPLES FOR MAKING A HEALTH CLAIM

According to the Confederation of the Food and Drink Industries in the European Union (CIAA 2000), communication of health claims should be truthful and not misleading to consumers, and must not exaggerate or deceive directly or indirectly. The company responsible for placing a product on the market is also responsible for justifying any health claim on the basis of sound scientific evidence. Substantiation must be available to authorities on request. Moreover, the substantiation should be valid until the end of shelf life. The appropriate amount of intake to obtain the desired effect should be indicated; health claims should be justified in the context of the entire diet and must be applicable to the amount of food normally consumed.

11.3.4 MEDICAL CLAIMS

In Europe, any statement that a food, dietary supplement or ingredient can or will prevent, treat or cure a disease is a medical claim. Although forbidden, medical claims for foods can be true. This implies that the right of consumers to be informed about characteristics of their foods is in conflict with the prohibition of medical claims for foods — an unsatisfactory situation, which should be eliminated by proper legislation. As long as such legislation is lacking, type A health claims are forbidden unless formulated so that they are not considered to be medical claims. However, taking into account the disparity of opinions within the European community, harmonization of type A health claims will take time due to their complexity. An overview of the different types of claims is shown in Figure 11.2.
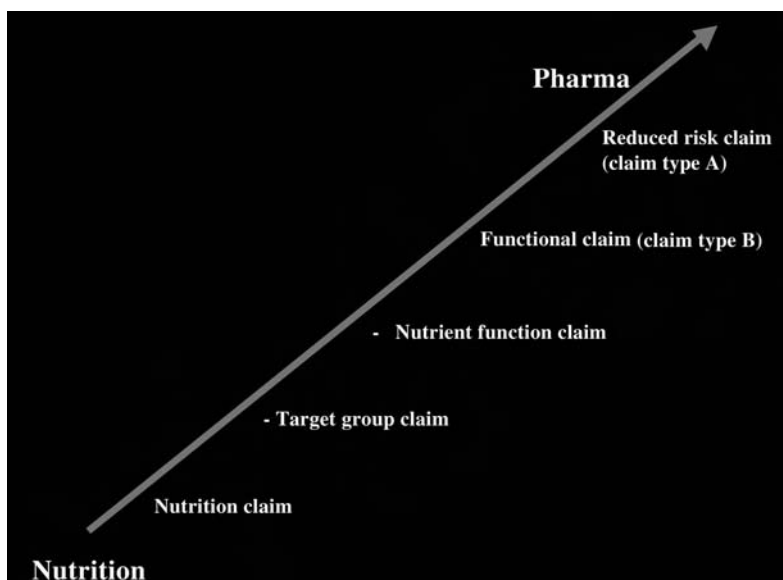
FIGURE 11.2 Functional food claims.

11.4 DOSSIER DEVELOPMENT

The preparation of a dossier refers to substantiation of health claims as described earlier and, specifically, to health claim types A and B. It should be stressed that such a dossier is distinct from the dossier needed for safety assessment of the food or food ingredient, for example, under the Novel Food Regulation in Europe. Future legislation on health claims will, however, benefit from the European novel food regulation when it comes to guidelines for dossier preparation. Therefore, the reader is referred to the EU Commission recommendation 97/618/EC concerning guidelines for the preparation of a dossier to be submitted to the regulatory bodies involved in the safety evaluation of novel foods. A similar dossier should be prepared for the evaluation of the efficacy of functional foods and dietary supplements. Codes of practice for health claims in Europe, like those of the CIAA (2000) and the Dutch Nutrition Center (Voedingscentrum 1998), provide useful information about issues that should be addressed, although the information required to substantiate a health claim within any of the existing or proposed schemes is similar. The dossier should be a report of the applicant's evaluation of efficacy and should contain the following information:

- Name and description of the company that markets the product and submits the application
- Detailed bioactive ingredient specifications
- Detailed description of applications in foods, supplements
- Description of target group
- Anticipated use by target group
- Description of health effect in target group
- Dose–effect relationship in target group
- Bioavailability of active ingredient in the food matrix
- Bioactivity: review of *in vitro*, *in situ*, animal, and epidemiological studies, and experimental studies in human subjects relevant to the described health effect in the target group. It should also provide a:
  - Description and evaluation of potential side effects (weighing benefits vs. risks)
  - Demonstration that consumption (in recommended levels) is not in conflict with a healthy diet
  - Discussion and conclusion
  - List of references

11.5 HUMAN STUDIES

Experimental studies in humans are often the final and decisive step in the assessment of efficacy and claim substantiation of functional foods or dietary supplements. According to the International Conference on Harmonization (ICH) of technical requirements for registration of pharmaceuticals for human use (ICH 1998), studies with human volunteers should be in compliance with good clinical practice (GCP) when the clinical data are to be submitted to regulatory bodies. It is recommended that the guidelines also be applied to other clinical investigations that may have an

impact on the safety and well-being of human subjects. Human volunteer studies on the efficacy and safety testing of functional foods, novel foods and dietary supplements belong to these "other clinical investigations," although the word "clinical" merely refers to studies in hospitals, whereas human volunteer studies can also be performed at other locations. TNO Nutrition and Food Research decided in 1995 that all its nutrition studies with human volunteers would be performed in compliance with GCP. In view of the novel food regulation of 1997 and the anticipated regulation of health claims for functional foods in the near future, this was a rational decision. Although TNO conducts all studies with human subjects according to GCP, it is not yet stipulated in the guidelines for health claim submissions.

11.6 GUIDELINES FOR GOOD CLINICAL PRACTICE (GCP)

GCP is an international ethical and scientific quality standard applicable to designing, conducting, recording, reporting and auditing trials that involve human subjects as participants. Compliance with this standard provides public assurance that the rights, safety and well-being of trial subjects are protected consistent with principles that have their origin in the Declaration of Helsinki, and that the clinical trial data are credible. GCP guidelines describe in detail how studies with human volunteers should be performed to be in compliance with its principles. Essential elements of GCP are the protocol, independent ethics committee, informed consent, inspections, standard operating procedures, quality assurance and quality control, privacy protection, and medical handling. Principles of GCP are:

- Before trial initiation, risks and inconveniences that can be foreseen should be weighed against benefits for the subjects and society. Only when the anticipated benefits justify the risks should a trial be initiated.
- Rights, safety and well-being of subjects prevail over the interests of science and society.
- Available information on a test product should be adequate to support the proposed trial.
- Trials should be scientifically sound and described in a clear and detailed protocol.
- A trial should be conducted in compliance with the protocol and not before the protocol has received approval by the Institutional Review Board/Independent Ethics Committee.
- Medical care given to subjects and medical decisions made about subjects should always be the responsibility of a qualified physician (or qualified dentist).
- Each individual involved in conducting a trial should be qualified by education, training and experience to perform his or her respective tasks.
- Freely given informed consent should be obtained from every subject prior to trial participation.

- All trial information should be recorded, handled, and stored in a way that allows its accurate reporting, interpretation and verification.
- The confidentiality of records that could identify subjects should be protected so that the privacy and confidentiality rules are respected in accordance with the applicable regulatory requirements.
- Test products (functional foods, novel foods and dietary supplements) should be manufactured, handled, and stored in accordance with applicable good manufacturing practice. They should be used in accordance with the approved protocol.
- Systems with procedures that ensure the quality of every aspect of the trial should be implemented.
- Human volunteer intervention studies should be scientifically sound. This means that all GCP criteria should be fulfilled. Specific issues are designs, biomarkers to be selected, statistical power in relation to the number of volunteers in the study, selection of volunteers and measurement of compliance.

Any design should include a proper control group, thus allowing the measurement of true effects. The control treatment should be selected carefully so that the difference in response between test and control treatment is attributable purely to the variable of interest. A control group is also required to account for so-called "time of measurement effects." These effects may occur during the study as a consequence of changes in variables not under strict control that influence the dynamic and/or kinetic parameters of the study independently of the variables investigated. Volunteers included in the study should be distributed randomly over the control and treatment groups. It may be necessary to use matching procedures so that the different groups are comparable with respect to all particular variables that could influence the response to treatment (for example, body weight, age, body mass index, gender, blood lipid levels).

It is also highly desirable that the volunteers and investigators are unaware of which treatments are given to the subjects; this is called "double blinding." When the investigator is unaware of the treatment, differences in treatment of subjects and bias in the investigator's interpretation of data are avoided. Blinding of the subjects means that psychological effects in response to treatment and treatment-dependent changes in behavior are prevented. Thus, ideally, the study should be double blind, placebo controlled, and randomized.

Basically, well-accepted study designs are (1) the parallel design and (particularly for nutrition science) (2) the cross-over design (Motulsky 1995; Snedecor and Cochran 1989). In the parallel design, test and control groups enter the study at the same time and are exposed to the same procedures during the intervention. Subjects remain on their treatment during intervention. In the cross-over design, on the other hand, different treatments are given to the same subjects in a balanced order. This is done in such a way that each subject receives each treatment. The advantage of the cross-over design for nutrition studies is that each subject can serve as his own control so that relatively fewer subjects are needed than in a parallel study; also, the

cross-over study is less sensitive to the lack of blinding. In fact, in nutrition studies it is not always possible to perform truly double-blind studies.

11.6.1 BIOMARKERS

Selection of the right biomarkers for the study is very critical. In fact, two different types of biomarkers can be identified: biomarkers of exposure and biomarkers of effect (efficacy). In nutrition studies related to functional foods or dietary supplements, interest is particularly in biomarkers of efficacy (and safety). For biomarkers of efficacy and safety, there are so-called endpoint biomarkers (e.g., a disease or a body function) and surrogate biomarkers, which provide relevant information about disease risk or body function. Examples of validated surrogate biomarkers are bone density (for osteoporosis risk), blood pressure (for cardiovascular disease risk) and LDL cholesterol (for coronary heart disease risk). It is a challenge for nutrition science to develop and validate new biomarkers that can be used to study the beneficial effects of functional foods and dietary supplements. In fact, the current range of validated biomarkers is insufficient to cover the main areas of interest. This is especially the case for the assessment of gastrointestinal health, including the effects on the intestinal flora. The availability of validated biomarkers is absolutely required for providing the scientific basis of health claims for functional foods and dietary supplements.

11.6.2 STATISTICAL POWER

Before any study is started, the number of volunteers to be included in the study should be computed. A sufficient number of volunteers is required to avoid type I and type II statistical errors. A type I statistical error means that a nonexistent effect is identified as a significant effect (acceptance of the alternative hypothesis when the null hypothesis is valid). A type II statistical error means that a significant (real) effect is not detected (rejection of the alternative hypothesis when this hypothesis is valid). Insufficient statistical power of a study is an important ethical issue due to the burden on subjects, in addition to the waste of money and resources. The number of volunteers required is thus dependent on the desired power of the study; it can be computed on the basis of the selected study design and the inter- and intraindividual variability of the main biomarker of efficacy. This variability can be derived from existing data. If this is not the case, a pilot study should be performed to acquire this knowledge.

11.6.3 SELECTION OF VOLUNTEERS

Volunteers should be prepared to comply with the protocol; the selected volunteers should represent the target population. For example, in calcium bioavailability studies (performed in view of osteoporosis prevention) peak bone mass development and reduction of age-related bone loss are relevant. For the first issue, the target group is adolescents, whereas the target group for the second issue may well be postmenopausal women. For studies on enhancement of natural defense, the particular target group will be elderly people who may display a reduced immune

competence or colonization resistance against pathogenic bacteria. For studies on reduction of cardiovascular disease risk, the target population may consist of middle-aged people with elevated blood lipid levels or hypertension. Inclusion and exclusion criteria should be defined in detail before selection.

11.6.4 COMPLIANCE

Measurements of compliance should be part of the protocol. Lack of compliance decreases the power of the study and can lead to type II statistical errors. A useful method is to add a marker of compliance and its measurement in body fluids to the test products (and placebo).

11.7 FUNCTIONAL DAIRY PRODUCTS

Traditionally, milk and dairy products have been considered products with a high nutritional value because of their content of high-quality proteins and the large variety of bioavailable nutrients. Dairy products therefore offer an excellent matrix for functional foods. Important examples are fermented dairy products with prebiotics, probiotics and peptides that inhibit the conversion of angiotensin I (ACE-inhibiting peptides) in the blood vessel wall. Also, milk with added nutrients for special target groups (including infant formulas and preparations) has gained popularity. Milk and whey are also used as sources of bioactive ingredients and dietary supplements. Examples here are milk proteins (caseins and whey proteins), bioactive proteins (e.g., lactoferrin, lactoperoxidase, growth factors and bioactive peptides [ACE-I-inhibiting peptides, casomorphins, immune-enhancing peptides, casein glycomacro peptide, tryptophan and cystein peptides], and lipid fractions [phospholipids and conjugated linoleic acid isomers (CLAs)]). The reader is referred to Schaafsma and Steijns (2000) for a detailed review of dairy ingredients as a source of functional foods. Taken together, it is not surprising that dairy products have a dominant position in the functional food market. Thus, it is remarkable that, to the authors' knowledge, no efficacy dossiers for functional dairy products have been submitted to EU regulatory bodies for evaluation. For the success of these products in the long term, such dossiers should be prepared and submitted.

11.8 DISCUSSION AND CONCLUSIONS

The long-term success of functional foods and dietary supplements depends on the reliability of nutrition claims and, particularly, health claims connected to these products. Health claims (type A and B) should have a sound scientific basis and the underlying health effects should be evaluated by expert panels. Therefore, applicants should make dossiers with the substantiation of health effects available.

At the moment, codes of practice for health effects of functional foods do not directly address dietary supplements, but the general feeling is that a code of practice for dietary supplements would be applicable too. However, it is realized that these products are considered a separate category of foods for which it is not always feasible to perform expensive studies with human volunteers to demonstrate efficacy.

Therefore, an evaluation system based on structure–function relationships and a scoring system for the strength of the evidence of efficacy may be developed for dietary supplements. Claims should be formulated in conformity with the strength of the scientific evidence. Also, preparation and submission of an efficacy dossier is necessary for such a system. Further experience, to be built up in Europe with the codes of practice, can be used in a later stage for EU legislation of functional foods and health claims.

For type A or B health claims connected to functional foods or dietary supplements, studies with human volunteers should be the final and required step in providing proof of efficacy. Similarly to the requirement for pharmaceutical studies, nutrition studies should fulfill the GCP criteria. Consumer organizations, nutrition information councils and health professionals should assist consumers in developing a critical attitude toward nutrition and health claims for functional foods and dietary supplements. Such an attitude will serve as a watch dog on the food industry, particularly those companies looking for short-term financial success without having an eye for consumer interest.

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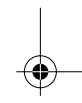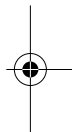
12 Communicating the Science behind the Health Benefits of Dairy Products: The American Experience

Allison Madell, Densie Webb, and Gregory D. Miller

CONTENTS

12.1 INTRODUCTION

Official recommendations, including the U.S. Department of Agriculture's (USDA) Food Guide Pyramid and the USDA/Department of Health and Human Services'

(DHHS) Dietary Guidelines for Americans, recognize milk and milk products as important components of a healthy diet. The food guide pyramid recommends two to three servings a day from the milk, yogurt, and cheese group. The dietary guidelines for Americans also recommend that children and adults aim for two or three servings from this group each day (USDA/DHHS 2000). In 1999, the American Academy of Pediatrics issued a new policy statement, Calcium Requirements of Infants, Children, and Adolescents, in which it stated (American Academy of Pediatrics 1999),

> Recent studies and dietary recommendations have emphasized the importance of adequate calcium nutriture in children, especially those undergoing the rapid growth and bone mineralization associated with pubertal development. The current dietary intake of calcium by children and adolescents, however, is well below the recommended optimal levels. The available data support recent recommendations for calcium intakes of 1200 to 1500 mg/day beginning during the preteen years and continuing through adolescence.

The consensus is that calcium is an essential mineral lacking in many diets and that dairy products are the best source of dietary calcium. This chapter briefly reviews the role of calcium in health and discusses the importance of dairy products as a source of calcium in the American diet. It also explores ways in which the media, policy makers and researchers can work together to counteract negative messages disseminated by activist groups and effectively communicate the health benefits of dairy foods to consumers.

12.2   DAIRY FOODS AND OSTEOPOROSIS

Osteoporosis affects an estimated 10 million Americans, with an additional 18 million at increased risk due to low bone mass; of those, 80% are women. Millions more have low bone density (National Institutes of Health Osteoporosis and Related Bone Diseases National Resource Center 1999). Calcium plays a critical role in preventing and treating osteoporosis, especially after menopause. The importance of calcium intake was confirmed at the National Institutes of Health Consensus Development Conference on Osteoporosis Prevention, Diagnosis, and Therapy, 2000, in which an expert panel determined that "calcium is the specific nutrient most important for attaining peak bone mass and for preventing and treating osteoporosis" (National Institutes of Health 2000). An adequate intake of calcium and vitamin D is considered the mainstay of osteoporosis prevention and treatment, yet recent government dietary surveys show that calcium intakes in the U.S. typically are below the current recommended intake of 1000 to 1300 mg daily (Institute of Medicine (IOM) 1997; USDA 1994–1996).

Accumulating scientific evidence indicates that a sufficient intake of calcium throughout life helps protect against osteoporosis by achieving genetically programmed peak bone mass by about 30 to 35 years of age or earlier and reducing postmenopausal and age-related bone loss (National Institutes of Health 1994; American Medical Association (AMA) Council on Scientific Affairs 1997; IOM

1997; Heaney 2000). Variations in calcium status early in life may account for a 5 to 10% difference in peak bone mass, which in turn contributes to more than a 50% difference in rates of hip fractures later in life (Matkovic 1996). The bulk of the evidence supports the hypothesis that consuming an adequate intake of calcium protects bone.

Moreover, dairy foods, which provide 73% of the calcium available in the diet, are considered the preferred source of calcium (Gerrior and Bente 1997). Increasing calcium intake from dairy products has been found to benefit bone health in pre-menopausal women (New et al. 1997; Baran et al. 1990). An expert panel on optimal calcium intake convened by the National Institutes of Health has recognized milk and other dairy foods as important sources of calcium for Americans, as has the AMA. Increasing dietary intake of calcium and vitamin D by consuming more servings of dairy foods is a safe and effective way to benefit bone health.

12.3   DAIRY FOODS AND HYPERTENSION

Since the early 1980s, a considerable body of evidence has accumulated from investigations in experimental animals, epidemiological studies, and clinical inter-vention trials in humans to support a beneficial role for calcium or calcium-rich foods, such as milk and other dairy foods, in blood pressure control (Reusser and McCarron 1994; Sacks et al. 2001; Miller et al. 2000a, b). Research has shown that consuming adequate calcium, potassium and magnesium lowers blood pressure. Dairy foods are the best food source for providing all three nutrients simultaneously in meaningful amounts.

Experimental animal studies, epidemiological investigations, and clinical trials in humans also indicate that an adequate potassium intake may protect against hyperten-sion (Reusser and McCarron 1994; McCarron et al. 1984). Overall findings indicate that dietary potassium (e.g., in foods such as fruits, vegetables, and dairy foods) may have a major role as a nonpharmacological agent in the control of high blood pressure (Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure 1997). Not only may potassium protect against hypertension and its associated vascular diseases (including stroke), but it also may reduce the need for antihypertensive medication (Reusser and McCarron 1994). Because of their potassium content, dairy foods may be especially beneficial for people with hypertension and those at risk for hypertension. In October, 2000, the Food and Drug Administration (FDA) approved a health claim for potassium and stroke, citing an already existing body of research showing a connection between the two (U.S. FDA 2000). The health claim may state that "diets containing foods that are good sources of potassium and low in sodium may reduce the risk of high blood pressure and stroke." Recent findings from the CARDIA (coronary artery risk development in young adults) study revealed that drinking at least four glasses of low-fat milk a day might decrease the risk of heart attack (American Heart Association, 2001).

Although more remains to be learned about the relationship between calcium and blood pressure, most investigators recommend that individuals consume cur-rently recommended intakes of this mineral to treat or reduce risk of hypertension (Joint National Committee on Prevention, Detection, Evaluation, and Treatment of

High Blood Pressure 1997; National High Blood Pressure Education Program Working Group 1993; McCarron et al. 1991). Calcium supplements, however, are not advised to reduce blood pressure. Rather, food sources of calcium such as dairy foods are recommended because, in addition to calcium they provide other nutrients, such as potassium and magnesium, that may protect against hypertension.

12.4   DAIRY FOODS AND COLON CANCER

Recently it has begun to be appreciated that several components in dairy foods may protect against colon cancer (Parodi 1997): specifically, calcium and vitamin D, bacterial cultures (e.g., *Lactobacillus acidophilus*), a class of fatty acids known as conjugated dienoic derivatives of linoleic acid (CLA), sphingolipids, butyric acid and milk proteins.

Furthermore, new clinical findings in humans indicate that increasing intake of low-fat dairy foods may reduce the risk of colon cancer (Holt et al. 1998). Numerous epidemiological, experimental animal, *in vitro*, and clinical studies in humans have investigated the protective effect of calcium, vitamin D and dairy foods against colon cancer (Newmark and Lipkin 1992; Wargovich et al. 1991; Van der Meer et al. 1998). Although many epidemiological studies support an inverse association between calcium, vitamin D and dairy food intake and colon cancer, it is difficult to draw conclusions from these studies (Miller et al. 2000a). Dietary recommendations to reduce cancer emphasize a reduction in total fat intake, especially from high-fat foods. Although some consumer organizations have interpreted this to mean that milk and other dairy products should be reduced or eliminated from the diet, care should be taken to ensure that recommended intakes of dairy foods are not jeopardized because of the many chemopreventive components in dairy foods. All individuals, and particularly those at high risk for colon cancer, should consume the recommended number of servings from the milk group every day.

The relationship between cultured dairy products and colon cancer is another area under investigation. A recent study in France found that people who regularly consumed yogurt had a lower risk of developing large colorectal adenomas compared with those who did not (Boutron et al. 1996). Two population-based case-controlled studies have found that, as yogurt and fermented milk consumption goes up, the incidence of colon cancer goes down (Young and Wolf 1998; Peters et al. 1992). In two prospective studies, a similar trend was found between yogurt consumption and colorectal polyps, intermediates in the development of colon cancer (Kampman et al. 1994). In addition, when elderly subjects with atrophic gastritis (chronic inflammation of the stomach) consumed yogurt, it decreased levels of the procarcinogenic fecal enzymes, nitroreductase and azoreductase (Pedrosa et al. 1995). Another group of adults taking *L. casei* experienced an 80% reduction in beta-glucuronidase activity, a potentially cell-damaging enzyme, over a 4-week period (Goldin et al. 1992). The evidence that consumption of milk and dairy products fermented with lactic acid bacteria reduces the risk of cancer is promising and deserves further investigation.

12.5   DAIRY FOODS AND OTHER HEALTH CONNECTIONS

Some studies have suggested that getting adequate calcium my help alleviate symptoms of premenstrual syndrome (PMS). It has been suggested that disturbances in calcium regulation may underlie the pathophysiologic characteristics of premenstrual syndrome and that increasing calcium intake may be an effective therapeutic approach (Thys-Jacobs et al. 1998; Thys-Jacobs 2000). Although most research has been conducted with calcium supplements, dietary calcium has been found to have a positive effect on PMS symptoms as well (Penland and Johnson 1993).

At least one study has found that a diet high in low-fat dairy products such as milk and yogurts may help control body fat (Zemel et al. 2000). Animal models demonstrate that a high-calcium diet exerts a corresponding 51% inhibition of adipocyte fatty acid synthase expression and activity and stimulation of lipolysis by 3.4- to 5.2-fold. Thus, increasing dietary calcium suppresses adipocyte intracellular calcium and modulates energy metabolism and thus reduces obesity risk. The researchers also examined the U.S. National Health and Nutrition Examination Survey data. After controlling for energy intake, relative risk of being in the highest quartile of body fat was set to 1.00 for the lowest quartile of calcium intake and reduced to 0.75, 0.40, and 0.16 for the second, third, and fourth quartiles, respectively, of calcium intake for women. A similar inverse relationship was also noted in men. In a separate paper, five clinical studies of calcium intake, designed with a primary skeletal endpoint, were reevaluated to explore associations between calcium intake and body weight (Davies et al. 2000). The researchers found significant negative association between calcium intake and weight.

Milk and dairy products are among the best sources of dietary calcium. Unfortunately, people often cut down on dairy foods when they are trying to lose weight. This latest research suggests that including plenty of low-fat dairy products within a calorie-controlled diet can help optimize fat loss. All of the research demonstrating a positive connection between regular consumption of dairy foods and good health and disease prevention needs to be communicated to consumers in a positive way that overrides negative messages about dairy foods disseminated via some media outlets and consumer activist groups.

12.6   WHY CARE ABOUT COMMUNICATING YOUR RESEARCH?

Why get involved in communicating and interpreting your research for consumers? If you do not do it, someone else will and may not get it right. A good health reporter will check with both sides of an issue before writing a story. The "other side," however, is often consumer activist groups, many of whom have developed sophisticated and efficient public relations efforts that deliver their political agendas as scientific truths, sometimes taking science out of context to further their message. Health professionals must be in the front lines of the communications effort because, in the media world, it is a race to see who will get the news first, not necessarily

who will get it right. The nutrition community is its own best spokesperson. Experts who are readily available to the media are making a major contribution to helping them get the story right. Major media outlets have writers or reporters that specialize in health and nutrition stories and some even have MDs or RDs; however, the vast majority of the smaller media outlets do not. These are generalist reporters trying to make sense of a complex story and present their readers with practical take-home messages.

A wide variety of nutrition messages is picked up by the media, who are increasingly involved in disseminating scientific information to the public. The high degree of public interest in nutrition information in recent years presents both opportunity and jeopardy (Goldberg 1997). Public interest in new scientific findings affords nutrition professionals the opportunity to improve dietary behaviors by providing information that can facilitate adoption of healthful diets. However, consumer perceptions that the message about diet and health is constantly changing, or that dietary recommendations are conflicting, sometimes undermine the scientific community's ability to positively influence eating behavior and, ultimately, health.

The media are the most popular vehicle through which consumers receive nutrition information and are powerful in influencing food selection and health behaviors. A recent survey by the American Dietetic Association found that television was cited as the leading source of nutrition information by 48% of respondents (American Dietetic Association 2000). Magazines ranked a close second, with 47% citing them as their main source of nutrition information; newspapers placed a distant third (18% of respondents ranked it as their most important source). Unfortunately, media messages surrounding nutrition may often be inconsistent or confusing and not help the public make positive changes in health behaviors. To the scientist, it often appears that the media get the story wrong; to the public, it appears that the scientists are always changing their minds (Goldberg 1997).

Many nutrition messages are in front of the consumer, but even more messengers are trying to put their own unique spin on the story. Newspapers, newsletters, Web sites, books and television stations are all on the lookout for the latest nutrition news. Twenty years ago nutrition research was considered an esoteric area, garnering little public interest. Only when major government public health announcements were made or concerns aired, such as the connection between cholesterol and heart disease or the concern over saccharin and cancer, did the public become aware of such topics. Today, however, virtually every nutrition study, no matter how small or narrow its focus, is fodder for the news. Studies in major medical journals such as *The New England Journal of Medicine*, *Lancet* and the *Journal of the American Medical Association* routinely make the front page of *The New York Times* and *The Washington Post* and are delivered throughout the country via newswires like AP and Reuters.

It comes as no surprise, then, that anecdotal evidence of the public's becoming skeptical about nutrition messages exists (Goldberg 1992). Given the public's growing skepticism and the continued increase in media coverage of breaking nutrition news, practical and easily understandable information on the relationships between diet and health must be a major component of public health efforts to promote healthy dietary patterns (Patterson et al. 2001).

Although consumers appear to understand the broader message that diet has an impact on health (American Dietetic Association 1995), apparently it is not being translated into positive changes in behavior, as demonstrated by the epidemic of obesity and related illnesses (National Institutes of Health 1998) and the low intake of critical nutrients such as calcium (IOM 1997). Consumers fail to understand how to eat healthy diets for many reasons. Their understanding of healthy eating behaviors, including the message to incorporate more dairy foods into their diets, is prerequisite to the following considerations:

- **Messages about diet and health often change over time as a result of increased scientific knowledge.** Although the public may view these ordinary, even expected shifts as scientific indecision, researchers view them as "another day at the office." A prime example is the margarine vs. butter issue. For years, health professionals recommended that everyone switch from butter to margarine in an effort to lower Americans' intake of saturated fat and cholesterol. Then researchers discovered that the *trans* fatty acids found in many margarines could also be detrimental to blood cholesterol levels. The dietary pendulum began to swing the other way as new stories began hinting that butter may not be so bad after all. Most experts, however, tried to give a middle-of-the-road message that butter is acceptable in moderation as part of an overall balanced diet and that margarines and oils low in *trans* fatty acids were a better choice. Consumer confusion resulted.
  - A longer running, but similar evolution of expert advice involves sodium intake. More than 20 years ago, health professionals began recommending that everyone cut back on sodium intake to reduce the risk of high blood pressure. In the interim, researchers have come to realize that not everyone is susceptible to the blood pressure–raising effects of sodium and that other factors in the diet, such as calcium, potassium and magnesium, may mitigate the effects of sodium (Jackson 1991; Alderman et al. 1998; Appel et al. 1997). However, the message to lower sodium stuck in the consumer's mind and altering that message to reflect current knowledge has been an uphill battle.
  - Another long-accepted nutrition message has been to cut back on dietary fat; in fact, avoiding dietary fat has become synonymous with the concept of eating healthily in America (Taubes 1997). Eating low-fat foods is so entrenched in dietary habits that the creation and marketing of reduced-fat food products has become a formidable force in the food industry, creating more than 15,000 reduced-fat foods (Taubes 1997). However, even this message has been questioned as to its worth and whether it needs altering because it may have originated from ill-informed good intentions (Taubes 1997). Whether the low-fat message will stand the test of time remains to be seen, but it may be easier to change the scientific stance than the public's perception.

- **Diet and health messages have become increasingly complex**, particularly as new scientific knowledge has led to varying dietary recommendations for prevention of different diseases. Although the variety of disease-specific dietary prescriptions seems understandable and reasonable to health professionals, to consumers these messages may appear to be incomprehensible contradictions in advice. For example, in years past the message regarding cholesterol was relatively simple: dietary cholesterol raises blood cholesterol, which increases risk of heart disease. Today, however, health professionals and researchers in the area know that the issue is far more complex — not only total blood cholesterol levels determine heart disease risk, but LDL, HDL and triglyceride levels also play an important role in determining risk. Most recently, new blood markers for risk, such as C-reactive protein and homocysteine, which appear to be influenced by diet, have been identified as important parts of the risk equation.
  - Alcohol is a classic example of consumer confusion over conflicting dietary recommendations. A growing body of evidence suggests that moderate drinking may offer some protection against heart disease. However, it has also been found that even moderate drinking may increase a woman's risk of breast cancer. That leaves older women perplexed over whether moderate drinking is acceptable: it could lower their risk of heart disease, but may be a poor choice because it could raise their risk of developing breast cancer. Consumers do not need to wait to see their doctors to learn about these complex issues and make decisions. They can simply pick up a newspaper or magazine or turn on the evening news.

- **There are many sources of health messages** including government agencies, professional health-related organizations, the food industry, and special-interest consumer groups, each of which can have markedly different agendas that result in conflicting messages (Goldberg 1992; Patterson et al. 2001). Consumer confusion, then, stems not only from the complexity of the messages, but also from the unique perspectives of the various groups and organizations developing them. The average consumer does not expect and cannot be expected to understand these agendas and biases (Goldberg 1992). A consumer interested in finding out about osteoporosis, for example, is understandably confused by mixed messages from what he views as equally credible sources. The National Osteoporosis Foundation and the National Institutes of Health urge Americans to get more calcium in their diets for good bone health. The USDA and the National Dairy Council recommend that people consume more low-fat dairy products to get calcium. These groups recommend increased calcium consumption through calcium-rich foods such as dairy products, but some animal activist groups insist that consuming more of these products actually exacerbates the osteoporosis problem and that if Americans ate less protein, their calcium requirements would be lessened.

- Rather than developing a coherent picture of what constitutes a health-ful diet, much of the public has a collection of fragmented facts and a misguided notion that foods can be clearly classified as "good" or "bad" (Anon. 1990). Who is the consumer to believe? It is likely to be the most vocal group that most frequently makes the news, gets the largest audience and presents the most compelling message. Well-informed health professionals and researchers that are readily available sources for reporters increase the likelihood that the health message presented to consumers will promote understanding, rather than increase confusion.

In the past, the biggest obstacle to overcome in delivering a health message about dairy products was dietary fat. The message health professionals delivered was a relatively straightforward one: substitute reduced-fat or fat-free dairy products for full-fat varieties and decrease fat, while still getting the calcium, protein and B vitamins found in dairy products. If full-fat dairy foods were chosen, other ways to reduce fat in the diet must be found. However, in recent years, the message has become much more complex, as issues such as the purported links between consumption of dairy products and diseases such as diabetes, obesity and osteoporosis are raised by extremely vocal consumer advocacy groups that use limited or incorrect data. Only well-informed and well-spoken researchers and health professionals can begin to undo some of the damage done by the growing and increasingly visible advocacy and animal rights groups.

12.7 THE POWER OF THE MEDIA

The old saw in journalism, "if it bleeds, it leads," is a harsh but realistic truth. A study confirming the role of dairy calcium in preventing osteoporosis is not likely to make front page news, but a story suggesting that milk consumption has been linked to prostate cancer is a real attention grabber. Many times such scientific observations come from preliminary findings, yet are described in the media as fact. This can be a problem when research findings are communicated prematurely. For example, preliminary research data or results from pilot studies that were once the exclusive domain of professional meetings are now distributed via press releases to journalists. In recent years, it has also become common practice for medical journals such as *The Lancet*, *Journal of the American Medical Association* and the *New England Journal of Medicine* to issue press releases regularly to the media on newsworthy studies. The result is often that the media become privy to the information before researchers in the field have even received the latest issue of the medical journal. This creates a situation in which researchers may be called on to comment on a study that they have not read yet. Often an expert voice is needed to bring home the ultimate message that the research is far from conclusive (Goldberg 1997).

Probably the most unregulated media outlet right now for health and nutrition information is the Internet. Though many Web sites are invaluable sources of reliable information for consumers and health professionals alike, countless more draw in

their audience by making baseless accusations about the food supply, such as "processed foods provide no nutrients" and "milk causes cancer." Some Web sites are hosted by consumer activist groups with a clear agenda that often targets specific branches of the food industry, such as the beef, egg or dairy industries. They are often quite effective at communicating their messages to consumers and the media, even though the messages are not grounded in good science. The media, understandably looking for the next controversial story, more often than not take the bait.

12.8   OVERCOMING MYTHS AND MIXED MESSAGES

Animal activist groups have not yet made an impact on the science battle, but the scientific community still has cause for concern because public opinion can influence the agenda for research funding. Dairy opponents today are better organized and mobilized worldwide than ever before. Their sophistication in manipulating the media and consumer perception continues to rise, just as their appeal to emotionalism and sensationalism continues to fuel rumors and consumer unrest. A case in point is activist groups such as the Norfolk, Virginia–based People for the Ethical Treatment of Animals (PETA) and Washington, D.C.–based Physicians Committee for Responsible Medicine (PCRM) that fuel consumer fears about consuming dairy products. Looking to further their own agendas, groups such as these present the media with research findings that are often misreported, oversimplified or not put into the context of the total available research. They work from an emotional and ideological, rather than a scientific, perspective and can be quite persuasive. A cause to which they have obviously given top priority is a systematic antimilk campaign that furthers their own animals rights (PETA) and vegan (PCRM) agendas.

PCRM, for example, has interpreted observational research to make claims via press releases and on the Internet that unnecessarily scare consumers and steer them away from milk. One of PCRM's claims is that calcium does not reduce fracture rates in older women, but actually increases osteoporosis-related fracture risk. Rather than look at the totality of evidence, they are basing their claims against milk on a few observational studies, such as the Harvard Nurses' Health Study (Feskanich et al. 1997), while ignoring a whole body of stronger evidence to the contrary. Based on very limited epidemiological data, both groups inaccurately claim that increased intake of calcium from dairy products is associated with a higher fracture risk.

When the Calcium Summit was held in Washington, D.C., in June 1999, PCRM (http://www.pcrm.org/) issued a press release blasting the event as a "pseudoscientific dairy promotion." PCRM went on to criticize the summit for not being a government-sponsored event, even though, ironically, the organization has been highly critical of almost all government-issued dietary recommendations. In 2000, PCRM even went so far as to file a petition against the USDA and the DHHS calling for the withdrawal and redrafting of the U.S. Dietary Guidelines 2000. The group's president, Neal Barnard, MD, stated in a press release that "it's clear that a meat-and dairy-based diet is killing us." In a commentary issued on the dietary guidelines, PCRM went further than anyone could have anticipated and accused the guidelines of being "racist." This accusation was based on the fact that the guidelines encourage

consumption of dairy products, although more than 90% of Asian Americans, 70% of Native Americans and African Americans, and 50% of Hispanic Americans are believed to suffer from some degree of lactose intolerance. PCRM further claimed that lactose intolerance is nature's warning against "doing dairy" and is the "dietary equivalent of yanking a hand back from a hot stove — a protective reaction." Such antidairy rhetoric will be the lone voice if health professionals do not make their voices heard.

Sometimes these consumer activist groups cross the line, e.g., when PETA posted prominent billboards in Wisconsin with New York City Mayor Rudy Giuliani sporting a milk mustache and posing the question, "Got prostate cancer?" parodying the popular "Got Milk?" ads. (Mayor Giuliani was diagnosed with prostate cancer in 2000.) The billboards were later removed after a pubic outcry over the organization's insensitivity and poor judgment, proving that counteractions will be heard. Although PETA's primary agenda is animal rights, not nutrition, they are against the housing and milking of cows and promote vegetarianism as the only healthful eating alternative.

In addition, PETA has charged that milk causes acne and suggested in ads that beer ("Got Beer?") is a healthful alternative to milk. The beer ads were withdrawn after PETA received complaints from organizations like Mothers Against Drunk Driving. Yet, according to news reports, PETA considered the ad campaign a success simply because it attracted so much attention. In February 2001, the group reached out to middle schools by handing out "milk suckers" trading cards with stomach-turning characters such as "Pimply Patty," "Windy Wanda," "Chubby Charlie" and "Loogy Louie" — all suffering from ill-health effects that the group says are associated with milk. The backs of the cards clearly warn kids that dairy consumption can cause unpleasant side effects such as "gas, pimples, and a throat full of phlegm." These erroneous health claims have been countered by the American Council on Science and Health, the *Berkeley Wellness Letter*, *The New York Times* and others. Such alarming innuendo and increasingly aggressive scare tactics designed to decrease milk consumption can have an enormous negative impact on consumers' behaviors and, ultimately, their health.

Despite all the negative messages, proof exists that good science can prevail, most recently when the Dietary Guidelines 2000 continued to recommend that people get two to three servings of dairy foods each day (USDA/DHHS 2000). The American Heart Association included the recommendation to include fat-free and low-fat dairy products regularly as part of a heart-healthy diet and, more specifically, for maintaining a healthy blood pressure (Krauss et al. 2000).

Health professionals clearly must band together to disseminate positive messages to counter negative campaigns. Organizing a national network of spokespeople available to speak to media and give presentations to organizations is one proactive way that positive messages can be delivered. Many such networks already exist — join one or call on one to support you in your efforts. Whether you speak out verbally, write letters or articles, or lobby influential people and organizations, it is critical that the voice of sound science, reason and experience rise above these organizations' noise.

12.9   THE POWER OF SCIENCE

The process to disseminate positive messages regarding dairy products begins with communication between scientists and journalists. The power of the press to influence health behavior can be positive and scientists would be better served to view members of the media as allies rather than adversaries (Goldberg 1997). A call from a reporter should not be regarded as a nuisance to be avoided, but rather as an educational opportunity. Many journalists covering health and nutrition topics have little or no background in health sciences or nutrition and are looking for understandable explanations of the latest study, to address readers' questions, or simply to follow their editors' instructions to "cover the story." The rule of thumb for any researcher or health professional talking with a reporter is to keep it simple. Unless the reporter asks sophisticated questions that reveal an understanding of the subject matter, researchers should keep responses short and to the point and always discuss what the real-life implications might be. The subtleties of research findings and statistical manipulations may seem fascinating — even essential — to a researcher, but reporters live by deadlines and would rather get to the bottom line as quickly as possible.

12.10   KNOW YOUR RIGHTS

It is also important to know the type of publication for which the reporter writes. A magazine, book or newsletter should be able to offer enough time to request a prepublication copy to approve quotes and check for errors. For a newspaper or wire service, however, the turnaround is rapid and reviewing a story for approval may not be possible. Be realistic about the power to veto copy before it runs. Veto power ultimately lies with the editor, not the writer. If a mistake is made, make it a point to write, call, e-mail or fax the editor and writer and point out the error, without making accusations of carelessness. Although some damage may have already been done, chances are it will not happen again — at least not on that specific topic with that particular writer. The more the lines of communication are kept open between scientists and reporters, the more likely reporters are to get the story right and to feel comfortable in seeking an expert's assistance and insights in the future.

12.11   SPREAD THE GOOD WORD

Some research findings, on the other hand, naturally lend themselves to the delivery of positive messages. News reports on research with the DASH diet (dietary approaches to stop hypertension) (Appel et al. 1997) are good examples of accurate, positive messages provided from research findings. Its success has in part been due to the simple message communicated: a diet high in fruits, vegetables, and low-fat dairy foods substantially reduces the risk of hypertension. The message has been delivered via consumer magazines, nutrition newsletters, newspapers and television reports and has been received by a broad consumer audience. In addition, the dietary principles of the DASH diet have been incorporated into the American Heart Association's official dietary guidelines (Krauss et al. 2000). Another simple message,

backed by years of research and now well accepted by consumers, is the link between calcium and osteoporosis prevention. Messages from the National Institutes of Health, the National Osteoporosis Foundation and popular media outlets have firmly established in consumers' minds the fact that calcium can help prevent osteoporosis. This demonstrates that scientists and scientific organizations can have an impact on consumers.

12.12   MAKE YOUR VOICE HEARD

The ability of nutrition researchers and health professionals to have an impact on nutrition policymakers may be the most untapped source of influence within the nutrition community. Prior to most public policy announcements regarding nutrition, there is a period for open comments. For example, prior to the National Institutes of Health issuing its 1994 and 2000 Consensus Statements on Osteoporosis, scientists had opportunities to participate in developing the consensus by attending the meetings and speaking out. Experts are also invited to write opinions on the topic to the conference panel that will be reviewed by the panel when drafting the consensus statements. These consensus reports can affect policy decisions and determine how research dollars will be spent. By providing information and insight in their area of expertise, researchers and health professionals can affect policy and, ultimately, consumer behavior. The same is true of the Dietary Guidelines, which are revised every 5 years and are next scheduled to be revised in 2005. As a part of developing new dietary guidelines, the Dietary Guidelines Advisory Panel accepts written and oral comments regarding any changes that may be made. The same has been true of the updated Dietary Reference Intakes for nutrients. In reviewing the science, the National Academy of Sciences provided researchers the opportunity to present information and provide comments in their areas of expertise.

In addition, opportunities for review papers and commentaries are always offered in scientific journals such as *Nutrition Reviews*, *Journal of the American Dietetic Association*, *American Journal of Clinical Nutrition*, *Journal of the American College of Nutrition* and others; these are seen by health professionals, researchers and reporters alike. Making your voice heard through publication affects consumers because review papers are often covered in the media, and also may affect policymakers because comprehensive review papers are often considered when making changes in current nutrition policy or considering a new application for fast-track approval of labeling health claims.

12.13   SHARE YOUR KNOWLEDGE

Lobbying is the most direct legal means for organized interests to gain access to lawmakers and regulators (Sims 1998). It is part of the political process for involved parties to send lobbyists to Washington, D.C., to speak out for their interests. The Institute of Food Technologists regularly sponsors a congressional fellow who spends a year in Washington as a staff person to learn how things get done on Capitol Hill. Nutrition organizations would do well to emulate that practice.

Researchers may be asked to serve on advisory boards for industry and advocacy groups. If the goal of the company or group is an admirable one, serving on an advisory board increases visibility and allows the opportunity to deliver a message of healthful eating to a wider audience. With cooperation between scientists and the media, the public can be motivated to change unhealthy behaviors and adopt a healthy diet that includes dairy products on a daily basis.

For good or bad, politics, science and the media are inextricably intertwined. Oftentimes it is difficult to know who is following whom: is the media influencing consumers, who in turn drive politics, which drives scientific research funds? Or is politics driving science, which is driving the media and influencing consumers? Regardless of who is in the lead position, the influence each one of these sectors has on the other cannot be ignored. To do so is to dismiss opportunities to have a positive impact on research funds, public health policy and consumer demands. Governmental action to improve the health of the public through changes in nutrition policy has been a part of the national health agenda for decades. Two of the most affecting government nutrition messages are the dual messages of limiting sodium and cholesterol. Both clearly show the power of the media and public policy to affect consumer attitudes and behaviors significantly.

- **Cholesterol** — although the consensus among experts is that elevated plasma cholesterol levels are related to increased risk for cardiovascular disease, the shift has been gradually away from the emphasis on dietary cholesterol alone as an important determinant of blood cholesterol levels. In the late 1970s, the American Heart Association recommended limiting total cholesterol intake to a maximum of 300 mg per day. Rather than being based on a specific study or studies, the 300-mg number was little more than a "best guess" of an appropriate limit; however, that number has not been seriously challenged in the 20+ years since (Callaway 1994). A pivotal moment in cholesterol awareness came from the now infamous *Time* magazine cover on March 26, 1984, which displayed two fried eggs with the cover headline "Cholesterol. And Now the Bad News." The connection among eggs, cholesterol and heart disease was forever imprinted on the American consciousness. The dietary cholesterol message and the 300-mg/day maximum have been reinforced again and again through public health education programs such as the National Cholesterol Education Program launched in 1985 and through most major media outlets, such as women's magazines, health and fitness magazines, *The New York Times*, *The Washington Post* and *USA Today*.
  - More recently, several studies have found little or no connection among dietary cholesterol (several studies looked specifically at egg consumption), blood cholesterol levels and heart disease (Ginsberg et al. 1994, 1995; Schnohr et al. 1994). As a result, the volume on the cholesterol message, or at least on the antiegg message, was turned down a notch. In 1999, a highly publicized article published in the *Journal of the American Medical Association* (April 21) found no connection between egg consumption and heart disease risk (Hu et al. 1999). Although the

American Heart Association has not altered its official advice regarding egg consumption, the organization's most recent dietary guidelines highlight the findings of the JAMA study, citing it as a challenge to current recommendations (Krauss et al. 2000). However, despite the best efforts of the National Egg Board, it would seem that Americans still equate eggs with high cholesterol and heart disease.

- The message that high blood levels of cholesterol can cause heart disease was loud and clear to consumers. The problem developed when new research indicated that dietary cholesterol was not a major factor in determining blood cholesterol levels for most individuals. Refining or even reversing the original message has not been easy. As with many other evolving nutrition issues, consumers viewed the new findings about eggs, cholesterol and heart disease as a flip-flop, rather than a refinement of an old message.

- **Sodium** — policy makers exerted just as great as, if not greater than, an influence on the public's perception of the importance of sodium intake on hypertension. In the early 1980s, the FDA began its Sodium Initiative, a nationwide effort to educate the public about the dangers of eating too much salt. The campaign was successful in increasing consumer concern for dietary sodium beyond what science now demonstrates to be the scope and nature of the problem (Callaway 1994). Official recommendations still urge Americans to limit sodium intake to no more than 2400 mg a day, just as they did more than 20 years ago; however, a heated debate has ensued among researchers as to the importance of the entire population limiting dietary sodium to control blood pressure. Although no consensus has yet been reached about who might be sodium sensitive, the consumer still operates under the assumption of widespread public health messages that sodium equals high blood pressure. If research deems it necessary and a scientific consensus can be reached, undoing or refining the original message will not be an easy task.

The cholesterol and sodium sagas clearly show that policy makers can have an impact on consumer dietary habits. Government and other groups that provide dietary recommendations must make recommendations while reminding consumers of the evolving science. They need to be ready to change recommendations as the science changes and not get locked into "old" science.

12.14 WHEN EVERYTHING COMES TOGETHER: A GAME PLAN

For nutrition researchers and health professionals to deliver their nutrition education messages effectively, they will need some education of their own — education on working with the media and how to have an impact on public policy, both of which, in turn, affect consumer behavior. Just such a symposium was held at the 2001 meeting of the American Society for Nutrition Sciences/American Society of Clinical

Nutrition. Presentations were given on communicating research, managing the media, using the Internet as a communications tool and the interrelations among public health policy, research and communications. More meetings and symposia like this need to be held to educate researchers and health professionals on how to deal with the rapidly changing and increasingly intersecting landscape of health research and communications. Educational programs designed to increase consumption of dairy products are likely to be most successful when many different groups support them. Coordinated efforts involving the public and private sectors are essential. Development of such a plan requires patience, political skills, and good will on the part of public officials and others involved in the political process (Sims 1998). Such programs may require the involvement of federal, state and local governments, private and voluntary groups and public comment on draft documents (Sims 1998).

Organizations that should be engaged as health allies or dealt with defensively as adversaries in any nutrition education or promotion program, including one involving dairy products, include:

- **Professional associations**, such as the American Dietetic Association or American College of Nutrition, may provide a platform at their annual convention for symposia, presentations and exhibits.
- **Consumer advocacy groups** may be allies, such as the National Osteoporosis Foundation, whose goal is to advance osteoporosis as one of the top national health care agenda items, or adversaries, such as PCRM or PETA, whose goal is to eliminate dairy products from the American diet. The bottom line is to build allies but be prepared to respond to adversaries.
- **Coalitions** comprising industry groups or consumer advocacy groups that have banded together as a result of common goals also become important in the balance of power. Both can have a major impact on the amount and quality of information delivered to consumers.
- **Public–private partnerships** are also increasingly common and create even more opportunities for researchers and health professionals to communicate the health benefits of dairy foods. A successful public–private partnership is that of the National Cancer Institute and Produce for Better Health in the 5-A-Day Campaign to increase Americans' fruit and vegetable consumption. Another, the Dietary Guidelines Alliance formed in 1995, comprises 13 food, health and consumer and government organizations to develop easy-to-understand, action-oriented messages regarding the U.S. Dietary Guidelines.

Researchers and health professionals need to come together to address animal advocacy groups' claims that are not grounded in science. A willingness to discuss specific questions about claims, cite research and explain why they may or may not be true is necessary to communicate the story effectively. The following list contains important considerations for experts trying to communicate their positive messages to consumers and the media.

12.14.1 Checklist for Communicating

- **Think globally, act locally.** Speak out at any available opportunity, whether it is a church or community gathering or giving a talk at a school or adult education classes. The antimilk organizations are quite vocal in their opinions, effectively reaching out to adults and children alike. Researchers and health professionals would do well to emulate their enthusiasm for their cause.
- **Be available.** If you want reporters to get the facts straight about your research or your area of expertise, you must be readily available via phone, fax or e-mail to answer questions and also for follow-up questions; ask the writer, editor or fact-checker to obtain your approval for any quotes.
- **Keep your message simple.** The more complex the message, the more difficult it is for reporters to get it right. Their mandate is to make it short, make it relevant and give it a headline the reader cannot resist. If you do not provide the "sound bites" and practical information, they will go to someone else who will.
- **Put it in context.** It is important to explain not only the findings of a particular study, but also how it falls into the big picture of a person's diet.
- **Be patient.** What may seem too basic to mention may be just the foundation a reporter needs to make sense of it. For example, it may be necessary to explain or reiterate that the findings of a population study do not demonstrate a cause–effect relationship, but only show an association that may or may not be meaningful.
- **Ask for a copy of an article, once it is in print.** If it is to be published in a magazine or a book, ask to approve the copy before it goes into print. For newspapers, which have tight deadlines and quick turnarounds, ask that your quote be read back, faxed or e-mailed for your approval.
- **Limit use of scientific jargon.** Some reporters have science or health backgrounds, but many do not. If you sense the reporter on the phone has a limited science background, always try to explain terms before using them.
- **Paint a picture.** To explain complex mechanisms or interrelationships, use vivid analogies whenever you can. Instead of talking about receptor sites, refer to them as lock-and-key mechanisms, or instead of talking about compounds that compete for absorption, refer to them as playing a game of musical chairs, in which the one that moves the fastest gets a place and a compound is ultimately left without a chair.
- **Resist the temptation to overinterpret findings from any one study, even if it is your own.** Be sure to put research into perspective, explaining how it fits into the big picture.
- **Know the difference in reporters' goals.** Some reporters are looking for detailed background information, while others have done the background work and are simply looking for a pithy quote to spice up the story.
- **Know your opponent.** Educate yourself as to what the other side is saying. Check out their Web sites and publications and be prepared with a rejoinder for each false or alarmist statement made.

12.15 SUMMARY: A COMMITMENT TO COMMUNICATE

The issues affecting the nutrition community today are among the most significant and difficult to manage; most are not well understood by consumers or media and many are misguided as a result. Misjudgment of impact on the consumer can evoke fear and change their minds and behavior for the worse. Aggressive and thoughtful management of these issues is paramount. Science, politics, and the media must be in sync with consumers' views about food and food values.

As part of the commitment to communicate effectively, health professionals must be willing to initiate and participate in symposia, press conferences and media roundtables and be willing to share their own experiences and insights in helping the media make sense of the latest research. Nutrition messages about the importance of dairy foods in the diet must not fall on deaf ears; seasoned professionals must be daring enough to lead the way. The American Dietetic Association, in its position paper on nutrition education for the public, recognizes that research provides credible, scientifically supported information that contributes to the success of nutrition education (Krauss et al. 2000). However, researchers and their research findings can contribute to nutrition education only if the researchers are an active and vocal part of the nutrition education process, which includes policy makers, health professionals and the media. You have the power to communicate — changing consumer minds and behaviors for the better — but no one will hear you unless you speak out.

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13 Biotechnology of Food Cultures for the Nutritional Enhancement of Milk and Dairy Products

Eoin Barrett, Catherine Stanton, Gerald Fitzgerald, and R. Paul Ross

CONTENTS

13.1   INTRODUCTION

Milk contains many health-promoting constituents, including immunoglobulins, bio-active fatty acids and peptides (often encrypted in casein and whey protein); many of these have been associated with health benefits following consumption. Studies

have shown that consumption of milk and dairy products may have positive health consequences such as decreased vulnerability to colon cancer and heart disease (Wells 2001). This healthy image of milk has resulted in dramatic growth in the diversification of dairy products in recent years and in huge increases in the varieties of products such as dairy desserts, flavored milk drinks, cheeses and yogurts. Indeed, milk can be considered a very suitable starting point for the innovative development of foods for health, or functional foods. Functional foods are already a well-developed concept in Japan, where the term was first introduced in 1984 (Arai 1996); the term refers to processed foods that are nutritious as well as an aid to bodily functions (Hasler 1998). Interestingly, markets for functional foods in Europe are predicted to increase dramatically in the next 5 years (Stanton et al. 2001). Indeed, in 2000, $100 billion of the global market was attributed to functional food use (Weststrate et al. 2002). For these reasons, milk components such as lactoferrin, β-lactoglobulin and conjugated linoleic acid are examined for specific health purposes on an on-going basis.

Apart from these milk components, the health attributes associated with fermented and probiotic milks and dairy products are currently receiving intense attention. For millennia, milk has been preserved by fermentation through the action of lactic acid bacteria (LAB), which convert lactose to lactic and other organic acids, thereby lowering the pH and consequently inhibiting the growth of pathogenic and spoilage bacteria. Moreover, these LAB produce a range of secondary metabolites that can influence product flavor, aroma compounds and texture as well as produce antimicrobial peptides (bacteriocins). These bacteria also possess a diverse complement of proteases and peptidases that aid in the digestion of milk proteins. In addition, many lactobacilli and bifidobacteria are increasingly exploited in probiotic dairy products such as cheese, yogurt and milk drinks, about whose human health-promoting activities the clinical evidence is accumulating. Over the last 20 years, many of the LAB have been genetically manipulated, opening up new possibilities for engineering novel bioactivities into these strains. A landmark in LAB research was reached in 1999 with determination of the complete genome sequence for *Lactococcus lactis* IL1403 (Bolotin et al. 2001). At present, complete and/or partial sequences are available for many LAB, including *L. plantarum, L. johnsonii, L. acidophilus* and *B. longum* (Klaenhammer et al. 2002); the U.S. Department of Energy joint genome initiative has compiled draft microbial genomes of a number of LAB (http://www.jgi.doe.gov/JGI_microbial/html/index.html).

The purpose of this review is to bring together some of these exciting developments in dairy-related biotechnology and demonstrate the huge potential for product innovation in this sector, with particular emphasis on functional foods. The review will concentrate on examples in which bioactivities of LAB can be exploited or accentuated to improve the quality or nutritional value of milk and dairy products. These examples will initially include nutritional metabolites such as bioactive peptides and vitamins that are naturally produced by LAB during "normal" fermentation processes. In addition, examples will be given in which new properties may be superimposed on strains through metabolic engineering strategies, yielding so-called second generation LAB.

13.2 LACTIC CULTURES AS CELL FACTORIES FOR METABOLITE PRODUCTION

LAB have a relatively simple homo- or heterofermentative metabolism. These heme-negative bacteria lack a terminal electron transport chain and when grown in milk generally rely on lactose as their main carbohydrate source. Dairy LAB include members of the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus*. LAB fermentation yields primarily lactic acid, which plays a vital function in safeguarding food products against spoilage through reduction of pH. Furthermore, the products of LAB metabolism beneficially affect the texture and flavor of fermented foods. For example, the viscosity and texture of fermented dairy products can be greatly enhanced by the production of polysaccharides by LAB (Ruas-Madiedo et al. 2002), while compounds such as diacetyl, ethanol, acetaldehyde and alanine play roles in flavor development in fermented foods. In addition, many LAB produce compounds of human nutritional value as regular endproducts of their metabolism, including some B vitamins.

Evidence is also accumulating that many strains of LAB and bifidobacteria produce other metabolites that promote human health. These biogenic compounds include health-promoting components derived from microbial activity not involving intestinal activity (Mitsuoka 2000), such as bioactive peptides generated from milk proteins as a result of their proteinase and peptidase complement and the production of CLA from linoleic acid by strains of lactobacilli and bifidobacteria. Table 13.1 lists nutritional metabolites and the strains that produce them, as well as whether these strains have been genetically modified to overproduce these metabolites.

13.3 PRODUCTION OF BIOACTIVE PEPTIDES BY LAB

Bioactive peptides have been identified within the amino acid sequences of milk proteins and may be released through the action of digestive enzymes in the intestine, added proteases or lactic cultures. Such peptides potentially have a wealth of health-related activities, including antimicrobial, opiate, antithrombotic, antihypertensive and mineral-utilizing properties (for review, see Meisel 1997). One of the best studied examples of bioactive peptides is the angiotensin-converting enzyme (ACE)-inhibitor. ACE is involved in influencing blood pressure and acts on angiotensin 1 to produce angiotensin 11, which increases blood pressure by causing a strong vasconstricting action (Skeggs et al. 1956).

Examples of fermented dairy products containing peptides that exhibit strong anti-ACE activity (Yamamoto et al. 1999; Gobbetti et al. 2000; Fuglsang et al. 2002) abound. A number of LAB strains, including *Lactobacillus* GG, *Lactobacillus helviticus* CP790, *Lactobacillus helveticus* CPN4, *Lactobacillus delbrueckii* ssp. *bulgaricus* SS1 and *Lactococcus lactis* ssp. *cremoris* FT4 have been used in the generation of anti-ACE peptides during the fermentation of milk (Maeno et al. 1996; Rokka et al. 1997; Yamamoto et al. 1999; Gobbetti et al. 2000; Fuglsang et al. 2002).

One such product is the Japanese soft drink Calpis™ that is fermented by *Lactobacillus helveticus* and *Saccharomyces cerevisae* and contains two known ACE-inhibitory peptides (Val-Pro-Pro and Ile-Pro-Pro). It has been shown that

TABLE 13.1
Nutrition Metabolites Produced by a Selection of Bacteria

Species	Nutrient/Metabolite	GMO[a]	Reference
Bifidobacterium breve	CLA[b]	–	Coakley et al., 2003
Bifidobacterium dentium	CLA	–	Coakley et al., 2003
Bifidobacterium longum	Folate	–	Lin and Young, 2000
Corynebacterium glutamicum	Lysine	+	de Graaf et al., 2001
Lactobacillus acidophilus	CLA	–	Ogawa et al., 2001
Lactobacillus bulgaricus	Folate	–	Rao et al., 1984
Lactobacillus delbrueckii ssp. bulgaricus	ACE[c] inhibitor	–	Gobbetti et al., 2000
Lactobacillus GG	ACE inhibitor	–	Rokka et al., 1997
Lactobacillus helveticus	ACE inhibitor	–	Maeno et al., 1996; Yamamoto et al., 1999; Fuglsang et al., 2002
Lactobacillus plantarum	Mannitol	+	Ferain et al., 1996
Lactobacillus plantarum	CLA	–	Kishino et al., 2002
Lactococcus lactis	Mannitol	+	Neves et al., 2000
Lactococcus lactis	Alanine	+	Hols et al., 1999
Lactococcus lactis	EPS[d]	+	Stingele et al., 1999
Lactococcus lactis ssp. *cremoris*	Menaquinone	–	Morishita et al., 1999
Lactococcus lactis ssp. *lactis*	Menaquinone	–	Morishita et al., 1999
Lactococcus lactis ssp. *cremoris*	ACE inhibitor	–	Gobbetti et al., 2000
Leuconostoc mesenteroides	Mannitol	–	Vandamme et al., 1987
Leuconostoc pseudomesenteroides	Mannitol	–	Grobben et al., 2001
Propionibacteria freundenreichii ssp. *freundenreichii*	CLA	–	Jiang et al., 1998
Propionibacteria freundenreichii ssp. *shermanii*	CLA	–	Jiang et al., 1998
Propionibacteria shermani	Folate	–	Bullerman and Berry, 1966b; Marwaha et al., 1983
Saccharomyces cerevisae	ACE inhibitor	–	Nakamura et al., 1995
Streptococcus thermophilus	Folate	–	Rao et al., 1984
Xanthomonas campestris	Xanthan gum	+	Fu and Tesng, 1990

[a] GMO: genetically modified organism.

[b] CLA: conjugated linoleic acid.

[c] ACE: angiotensin converting enzyme.

[d] EPS: exopolysaccharide.

administering this sour milk containing antihypertensive peptides significantly decreases the systolic blood pressure in spontaneously hypertensive rats 4 to 8 h after consumption (Nakamura et al. 1995). The beneficial effects of Calpis sour milk consumption on the blood pressure of elderly hypertensive patients, most of whom were receiving antihypertensive medication, has been demonstrated (Hata et al. 1996). Consumption of Calpis resulted in a decline in systolic and diastolic

blood pressure without any of the side effects observed when patients are treated with traditional medical ACE inhibitors. In addition, long-term consumption of Calpis and a similar fermented milk product containing a larger concentration of the peptides Val-Pro-Pro and Ile-Pro-Pro reduced hypertension development in spontaneously hypertensive rats (Sipola et al. 2002). Indeed, the greater the intake of these tripeptides the lower the resulting blood pressure is. This example serves to demonstrate the potential effect that biogenic peptides can have on human health.

13.4 PRODUCTION OF CONJUGATED LINOLEIC ACID BY LAB AND BIFIDOBACTERIA

Conjugated linoleic acid (CLA) refers to a mixture of positional and geometrical isomers of linoleic acid. A number of these isomers have attracted considerable attention recently due to their potentially beneficial effects, including anticarcinogenic and anti-inflammatory activities and inhibition of the onset of diabetes (for reviews, see Bassaganya-Riera et al. 2002; Belury 2002). Fats derived from ruminant animals are among the major dietary sources of CLA, of which, c9, t11 C18:2 is the major isomer (Chin et al. 1992; O'Shea et al. 2000). This isomer was first reported as an intermediate formed by the microbial biohydrogenation of linoleic acid in the rumen (Bartlet and Chapman 1961) by the action of the linoleic acid isomerase enzyme of certain rumen microorganisms such as *Butyrivibrio fibrisolvens* (Kepler et al. 1966). It has been shown more recently that food-grade strains, including a number of LAB, can also produce CLA. For example, Jiang et al. (1998) reported that the dairy starters *Propionibacteria freundenreichii* ssp. *freundenreichii* and *shermani* were capable of producing CLA, with the isomers c9, t11 and t9, c11 found to represent 70 to 90% of the total CLA formed. Further studies involving propionibacteria have been aimed at optimizing a method for the increased production of CLA (Rainio et al. 2001). Tested bacterial cultures tolerated concentrations of up to 1 mg/ml linoleic acid in whey permeate-based media and conversion rates of up to 80% were recorded from 600 μg/ml linoleic acid. Slightly higher yields were obtained with higher concentrations (1 mg/ml) of linoleic acid but yields as a percentage of the initial linoleic acid (57%) decreased due to its inhibitory effect (Rainio et al. 2001).

A number of studies have reported that the levels of CLA are higher in fermented dairy products than in unprocessed milk (Ha et al. 1989; Aneja and Murthi 1990). Six strains of LAB were shown to produce CLA from linoleic acid in milk medium (Lin et al. 1999), with *Lactobacillus acidophilus* generating the highest yield of CLA from 1 mg/ml linoleic acid after 24 h. It was suggested that protective components in milk prevented CLA oxidation and protected against the inhibitory effect of CLA toward cultures (Lin et al. 1999). Furthermore, the pathway for CLA production from linoleic acid in *Lactobacillus acidophilus* AKU 1137 has recently been reported (Ogawa et al. 2001). The production of CLA by *Lactobacillus acidophilus* AKU 1137 involves the production of hydroxy fatty acids, 10-hydroxy-*trans*-12-octadecanoic acid and 10-hydroxy-*cis*-12-octadecanoic acid from linoleic acid and their

subsequent decrease with increased formation of CLA. In a further study, 250 strains from various genera were screened for the ability to convert linoleic acid to CLA (Kishino et al. 2002). Considerable amounts of CLA (greater than 0.07 mg/ml reaction mixture) were formed by strains from the genera *Enterococcus*, *Pediococcus*, *Propionibacteria* and *Lactobacillus*. *L. plantarum* AKU1009a produced the greatest amount of CLA, with washed cells (33% wet cell w/v) producing 40 mg/ml of CLA from 12% w/v linoleic acid after 108 h (33% molar yield) (Kishino et al. 2002). However, by reducing the linoleic acid concentration to 2.6% and washed cell concentration to 23% wet w/v, CLA molar yields of 80% were obtained after 96 h. *L. plantarum* AKU1009a was found to produce similar quantities of CLA under aerobic and anaerobic conditions, suggesting a lack of oxidative linoleic acid degradation activity.

It has also been reported that most CLA production was associated with or in the cells as opposed to in the supernatant (Kishino et al. 2002). Interestingly, CLA production has also been demonstrated by a number of human-derived bifidobacteria, with substantial variation among species (Coakley et al. 2003). Of the nine bifidobacterial strains found to be capable of converting linoleic acid to CLA, *B. breve* proved most efficient, converting 65% of linoleic acid to *c*9, *t*11 CLA when grown in 0.55 mg/ml linoleic acid (Coakley et al. 2003) (Figure 13.1). In contrast to *L. plantarum* production of CLA (Kishino et al. 2002), bifidobacterially produced CLA was predominantly detected in the medium supernatant under anaerobic conditions (Coakley et al. 2003). Thus, although CLA production is not a general feature of LAB and bifidobacteria, strains are now available that can efficiently convert exogenous linoleic acid to the health-promoting fatty acid CLA. Such strains have potential for increasing the CLA content of dairy foods during fermentation. It is important to emphasize, however, that this activity would need to be accompanied by lipase activity to release the substrate from its triglyceride forms because the strains require linoleic acid in the free fatty acid form for isomerization to CLA.

13.5 VITAMIN PRODUCTION BY LAB AND BIFIDOBACTERIA

13.5.1 FOLATE PRODUCTION

Folate is a term used to describe salts of folic acid (pteroylmonoglutamic acid) (Briggs and Calloway 1979), which are vital components of the human diet and involved in the biosynthesis of nucleotides and cofactors in many metabolic reactions. Because mammalian cells cannot manufacture folates (Sarma and Duttagupta 1995), folate-synthesizing LAB can be used to augment the folate content in fermented dairy products (Lin and Young 2000). Folates have been shown to safeguard against some forms of cancer (Ames 1999) and a link has been established between folate supplementation and a reduced risk of neural tube defects during pregnancy (Collins 1994). Importantly, increased ingestion of folic acid has been shown to reduce substantially plasma homocysteine, a risk factor for the development of coronary heart disease (Morrison et al. 1996).
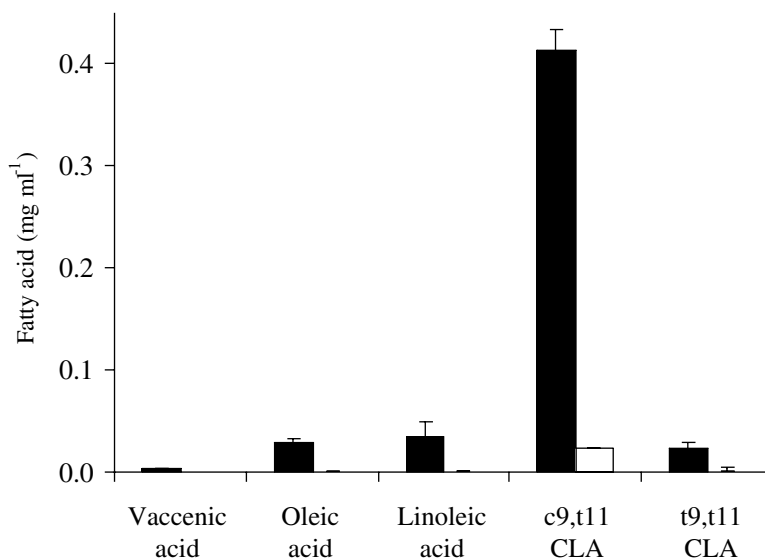
FIGURE 13.1 Fatty acid profile of *B. breve* supernatant (■) and pellet (□) following growth in cys-MRS plus 0.55 mg/ml linoleic acid. (Source: Coakley, M. et al., *J. Appl. Microbiol.,* 94, 138–145, 2003.)

Folic acid is formed by the linkage of three components, pteridine, para-aminobenzoic acid and one or more glutamic acid residues, with poly γ glutamyl tails differing in length from 1 to 10 glutamic acid residues (Briggs and Calloway 1979; Hamm-Alvarez et al. 1989). The term monoglutamyl folate refers to molecules with a single glutamyl tail, whereas polyglutamyl folate possesses at least two glutamyl residues, with increased occurrence of the monoglutamyl derivative increasing bioavailability of folate (Sybesma et al. 2002). Two conjugated forms of folic acid occur in food with three or seven γ-glutamic acid residues per molecule (Briggs and Calloway 1979).

Recent reports suggest that milk contains between 20 and 50 µg/l folate (Hugenholtz et al. 2002), while fermented milk is reported to contain even higher amounts (Alm 1980), with up to 142 µg/l detected in yogurt (Smid et al. 2001). Some starter cultures have been shown to synthesize folate and secrete it into the growth medium during fermentation in milk. Folate levels produced in reconstituted skim milk were found to be far higher than in complex media (MRS, M17), following the growth of a number of LAB (Lin and Young 2000). These included two *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* strains.

Lysis of yogurt bacterial cells occurs during gastric transit, as has been demonstrated by an alleviation of lactose intolerance due to the release of microbial β-galactosidase (Lin et al. 1991); therefore, it is reasonable to assume that folate would be available to the body whether it is naturally released from bacterial cells during growth or following cell lysis (Lin and Young 2000). It has also been reported that *Bifidobacterium longum* B6 can produce up to 100 ng/ml of folate (Lin and

Young 2000). Thus, consumption of 200 ml of milk fermented with *Bifidobacterium longum* B_6 would supply 10% of the recommended daily allowance for a child 4 to 8 years of age, according to the U.S. Food and Nutrition Board, Institute of Medicine, National Academy of Sciences.

13.5.2 VITAMIN B_{12} PRODUCTION

Vitamin B_{12} is an important cofactor for the metabolism of fatty acids, amino acids, carbohydrates and nucleic acids (Quesada-Chanto et al. 1994); this vitamin can be produced by bacteria such as propionibacteria (Bullerman and Berry 1966a, b). The metabolic pathway involved in the production of vitamin B_{12} has been elucidated and involves an intricate pathway of at least 25 steps (Hugenholtz et al. 2002). Optimal production of this vitamin by *Propionibacterium shermani* ATCC 13673 was observed in 10% w/v cheese whey supplemented with 1.5% w/v yeast extract (Berry and Bullerman 1966; Bullerman and Berry 1966a, b). The production of B_{12} was observed after 80 to100% of cell growth was reached, while the addition of 10 ppm of the vitamin B_{12} precursor, 5, 6-dimethylbenzimidazole, along with 5 ppm cobalt during the aerobic stage of production, led to increased yields of 10 to15 mg/l (Berry and Bullerman 1966; Bullerman and Berry 1966a, b).

Propionibacterium shermani 566 was also shown to produce 5.12 µg/ml of vitamin B_{12} in 4% w/v whey lactose (carbon mix of 3.6% w/v whey lactose and 0.4% w/v glucose) with minor supplements, including 5 mg/l iron, 5 g/l yeast extract, 5 mg/l cobalt, and 0.5% w/v ammonium phosphate (Marwaha et al. 1983). The biological oxygen demand of whey was reduced by 90%, thereby reducing the associated pollution concerns of whey (Marwaha et al. 1983). A recently described patent for the production of vitamin B_{12} by *Propionibacterium freundreichii* CBS 929.97 on glucose observed yields of up to 200 mg vitamin B_{12}/kg mesh by controlling the aerobic and anaerobic phases and the transfer of cells from one phase to another in a semicontinuous production process (Hunik 2002). The use of improved fermentation techniques such as those described by Hunik (2002) may also lead to increases in vitamin B_{12} yields in whey.

13.5.3 VITAMIN K PRODUCTION

Vitamin K is an essential cofactor involved in the post-translational modification of glutamic acid residues in certain proteins such as blood clotting and proteins involved in tissue calcification (Price et al. 1976; Olson 1984); these proteins have also been detected in kidney tissue and artherosclerotic plaque (Suttie 1985). Two forms of vitamin K exist: phylloquinone (vitamin K_1), which occurs in green plants, and menaquinone (MK; vitamin K_2, numbered depending on the number of prenyl units on the attached side chain); in addition to occurring in animals, they are produced by intestinal bacteria (Briggs and Calloway 1979; Conly and Stein 1992). The term "vitamin K" describes 2-methyl-1,4-naphthoquinone and all derivatives exhibiting phyloquinone activity (Nomenclature policy 1990).

Among a range of LAB screened from the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Bifidobacterium*, *Streptococcus* and *Enterococcus*, five strains yielded

more than 250 nmol quinones/g lyophilized cells (Morishita et al. 1999). These included *Lactococcus lactis* ssp. *cremoris* YIT 2007, *Lactococcus lactis* ssp. *cremoris* YIT 2011, *Lactococcus lactis* ssp. *cremoris* YIT 2012, *Lactococcus lactis* ssp. *lactis* YIT 2027 and *Leuconostoc lactis* YIT 3001, which produced MK varying in length from MK-7 to MK-10. The ability to produce MK was common to all strains in soymilk medium, but only YIT2011 and YIT2012 grew and produced MK in milk-based medium with forms of MK ranging from MK-7 to MK-10 (Morishita et al. 1999). Such strains may be useful as dairy starter cultures to enrich the vitamin K content of fermented foods.

13.6 MANNITOL OVERPRODUCTION BY LAB

Mannitol is a nontoxic, nonhydroscopic sweetener estimated to be about half as sweet as sucrose (Dwivedi 1978; Debord et al. 1987). Humans are incapable of metabolizing mannitol entirely and therefore do not suffer from hyperglycemia following consumption (Griffin and Lynch 1972). Mannitol is a six-carbon naturally occurring sugar alcohol or polyol and is the most abundant polyol in nature (Wisselink et al. 2002). Many LAB can synthesize mannitol by employing one of two pathways for its production; the mode of synthesis depends on the pathway used for hexose fermentation; LAB generally use heterofermentative or homolactic fermentation for hexose catabolism.

Heterofermentative bacteria ferment hexose via the 6-phosphogluconate/phosphoketolase pathway (Kandler and Weiss 1986) to produce equimolar quantities of lactate, carbon dioxide and ethanol (Axelsson 1993), whereas homofermentative bacteria convert >90% of carbohydrates to lactic acid (Garrigues et al. 1997). The best mannitol-producing LAB employ heterofermentative metabolism; bacteria such as *Leuconostoc mesenteroides* ATCC 12291 are well known for their ability to produce mannitol when grown on sucrose (Vandamme et al. 1987). In heterofermentative bacteria, NAD^+ regeneration is achieved anaerobically using an electron acceptor; if fructose is reduced to regenerate NAD^+, mannitol is produced.

Mannitol production was also observed during growth in fructose by a mannitol-producing variant of *Leuconostoc pseudomesenteroides* DSM20193 (Grobben et al. 2001). In contrast to the parent strain, which did not produce mannitol on fructose, the variant did not exhibit diauxic growth patterns when grown in a medium with fructose and glucose as carbon sources. Both carbon sources were utilized simultaneously, with glucose fermented while fructose was converted to mannitol at a rate of 0.95 mol mannitol/mol fructose (Grobben et al. 2001). Mannitol dehydrogenase, which reduces fructose to mannitol, is essential for mannitol production in heterofermentative bacteria (Grobben et al. 2001).

Mannitol production occurs in homolactic fermentation under atypical conditions in which the Embden–Meyerhof–Parnas pathway is not completed as usual (Wisselink et al. 2002). Low mannitol yields were obtained when mannitol 1-phosphate dehydrogenase converted fructose 6-phosphate to mannitol 1-phosphate (Ferain et al. 1996). In homofermentative *Lactobacillus plantarum* (Ferain et al. 1996) and *Lactococcus lactis* (Neves et al. 2000), the inactivation of *LDH* leads to mannitol production due to NAD^+ renewal**.**

13.7 SECOND GENERATION LAB FOR PRODUCTION OF NUTRITIONAL METABOLITES

As stated earlier, phenomenal progress has been achieved in the last 30 years with respect to genetic manipulation of food bacteria. Such advances have led to the development of sophisticated genetic tools for engineering these bacteria. A good example of this is the nisin controlled expression (NICE) system, which allows overproduction of heterologous proteins in many LAB (Kleerebezen et al. 1997; Eichenbaum et al. 1998). This system is based on nisin, an antimicrobial peptide produced by *Lactococcus lactis,* which also acts as an extracellular signal that controls its own biosynthesis (Kuipers et al. 1995). In this case a membrane-bound sensor, histidine kinase (*nis K*), and a cytoplasmic response protein (*nis R*) are responsible for signal transduction. The interaction between extracellular nisin and membrane-bound *nis K* results in *nis K* being phosphorylated, followed by the transfer of a phosphate to *nis R.* This results in the expression of the *nis R* protein, which is responsible for initiation of transcription at the *nis A* promoter (Kuipers et al. 1995). Induction of the NICE system occurs at nisin concentrations far below those that cause growth inhibition (Kleerebezen et al. 1997), allowing expression of selected genes without inhibiting cells.

The NICE system has been introduced into *Lactococcus, Lactobacillus, Leuconostoc* and *Streptococcus* (Kleerebezen et al. 1997; Eichenbaum et al. 1998) and has been used for the overproduction of diacetyl, alanine and exopolysaccharides (EPS) in *Lactococcus lactis,* by overexpressing the *nox* gene *of Streptococcus mutans*, the *alaD* gene of *B. sphaericus* and *EPSD* gene of *Lactococcus lactis,* respectively (Hols et al. 1999; van Kranenburg et al. 1999a; Hugenholtz et al. 2000). Thus, using genetic tools such as the NICE system, it is now possible to engineer many LAB species to overproduce novel metabolites or specific metabolites over and above what they produce naturally. In a number of examples, such an approach has led to modified food-grade bacteria that overproduce certain nutritional metabolites, including amino acids, polysaccharides and folate.

13.7.1 AMINO ACID PRODUCTION

L-alanine is used in the food industry as a food sweetener and in pharmaceutical applications as a useful pre- and postoperative nutrition therapy (Leuchtenberger 1996). L-alanine is produced by *Lactococcus lactis* by the enzyme alanine dehydrogenase that uses NADH as a cofactor, whereby pyruvate is reduced to create L-alanine in the presence of ammonia and NAD^+ is regenerated. Researchers at the NIZO and Wageningen Center for Food Research recently overexpressed the alanine dehydrogenase (*alaD*) enzyme of *Bacillus sphaericus* in *Lactococcus lactis* (Hols et al. 1999) under the control of *nisA* promoter. Under optimal nisin induction levels, the alanine dehydrogenase enzyme totalled 30 to 40% soluble protein. The introduction of this system into resting wild-type *Lactococcus lactis* resulted in 35% of carbon being redirected to alanine production in the presence of surplus ammonia, which is necessary for the conversion of pyruvate to alanine by alanine dehydrogenase. Overexpression of L-*AlaD* in lactate dehydrogenase-lacking cells at a controlled pH

of 7.5, resulted in improved rerouting of pyruvate to alanine (140 mM, 75%) (Hols et al. 1999). Furthermore, subsequent optimization of the ammonia content, 150 mM $(NH_4^+)_2SO_4$, resulted in 200 mM of alanine being formed from 100 mM glucose or 99.5% alanine being formed from glucose. On the other hand, disruption of the alanine racemase gene of *Lactococcus lactis* resulted in strains producing solely L-alanine with D-alanine added as a growth supplement (Hols et al. 1999).

Another approach to the design of dairy strains that overproduce amino acids is to take an existing overproducing bacterium such as *Corynebacterium glutamicum* and confer on it the ability to ferment lactose. In this respect, *Corynebacterium glutamicum* is widely used for the industrial production of the essential amino acid lysine but is lactose negative. It is reported that production of the world's 420,000 t of lysine by fermentation is almost exclusively due to *Corynebacterium glutamicum* (de Graaf et al. 2001). A number of metabolic engineering strategies have been developed for optimizing amino acid yields from this strain (de Graaf et al. 2001). Detailed analysis of metabolic fluxes, rerouting of metabolic pathways, transformation techniques and optimization of fermentation conditions have resulted in increased amino acid yields (de Graaf et al. 2001; Kircher and Pfefferle 2001).

An example of a strategy adopted was to create *Corynebacterium glutamicum* strains harboring the *Escherchia coli* lactose operon that were capable of utilizing lactose, thereby making whey available as a potentially inexpensive substrate for amino acid production (Brabetz et al. 1991). The influence of the heterologous enzyme system in *Corynebacterium glutamicum* was examined. The effects on strain performance of the lactose permease protein and the influence of promoters transcribing the genes at two different levels were examined. Because the transformed strain carrying the lactose operon was more actively expressed, it grew at the same rate and to the same extent on 0.2% (w/v) lactose and 0.1% (w/v) glucose. Lactose and galactose measurements during growth demonstrated the utilization of lactose, while levels of galactose in the media increased to approximately 0.09% (w/v). As well as experiencing improved growth, the galactose moiety of lactose did not appear to be toxic to the cells because growth on 0.2% (w/v) lactose was similar to that of 0.1% (w/v) glucose.

Despite similar levels of β-galactosidase expression, large differences in the growth between strains possessing and those lacking the permease gene were observed in minimal media, suggesting that the permease protein was vital for lactose metabolism. There was no difference in growth of the parent *Corynebacterium glutamicum* strain and the strain expressing the lactose operon in Lauria Bertani media (Brabetz et al. 1991). This would suggest that the lactose permease did not restrain the growth in a rich medium that would be expected to occur following severe membrane damage (Padan et al. 1983; Brabetz et al. 1991). In addition, β-galactosidase was not excreted into the culture medium, to an extent that would have a significant impact on growth of the strain, by hydrolyzing lactose and leaving glucose available for consumption (Brabetz et al. 1991).

Further studies were performed on *Corynebacterium glutamicum* R163 (pECL1x/pECL3x) examining the expression of lactose permease as a functional

carrier protein (Brabetz et al. 1993). The permease protein must be inserted correctly into the membrane of the host; this was confirmed in the study of Brabetz et al. (1993). Furthermore, cells lacking lactose permease hydrolyzed the β-galactosidase substrate o-nitro-phenyl-galactoside (ONPG), which, when cleaved, resulted in the development of a measurable yellow color at about 15% of the extent found in those with lactose permease. The hydrolysis of ONPG was inhibited by addition of n-eth-ylmaleimide, a permease inhibitor that irreversibly binds to lactose permease; mea-suring the uptake and internalization of methyl-B-D-thiodigalactoside indicated the active transport process. Clearly the lactose permease was integrated and functioned correctly in *Corynebacterium glutamicum.*

In a related study, we have recently cloned the lactose operon from *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 and the galactose genes from *Lactococcus lactis* ssp. *cremoris* MG 1363 into a lysine overproducing *Corynebacterium glutamicum* (data unpublished). The resulting strain produced more than 2 g/l lysine when grown in whey-based media in comparison to the control, which did not produce any lysine.

13.8 EXOPOLYSACCHARIDE PRODUCTION

Exopolysaccharides (EPSs) are excreted outside the cell, do not remain attached to cell walls (Sutherland 1977) and play a valuable role in rheology and texture of fermented foods. Furthermore, some polysaccharides produced by LAB have been shown to exert prebiotic (Gibson and Roberfroid 1995), immunostimulatory (Hosono et al. 1997), antitumoral (Kitazawa et al. 1991) and cholesterol-lowering (Nakajima et al. 1992) effects. Scandinavian ropy fermented milk products, a term for fermented milks produced by slime producing mesophilic lactococci, have been shown to possess a number of EPS-producing LAB (Macura and Townsley, 1984). LAB can generate EPSs of two types: homopolysaccharides (e.g., dextran, glucan) containing a single type of monosaccharide and heteropolysaccharides containing repeating units of polysaccharides and also noncarbohydrate units (Ruas-Madiedo et al. 2002). The assembly of monosaccharides occurs outside the cell; these are incorporated into homopolysaccharides, which are synthesized by excreted enzymes (Jolly et al. 2002). On the other hand, repeating units of heteropolysaccharides are synthesized within the cell and subsequently relocated across the membrane by glycosyltrans-ferases and assembled extracellularly (De Vuyst et al. 2001; Ruas-Madiedo et al. 2002).

A large number of LAB have been shown to generate EPS and the biosynthesis pathways, structure, and functionality of many have been described (for reviews, see De Vuyst and Degeest 1999; Laws et al. 2001; Ruas-Madiedo et al. 2002). In particular, the EPS gene cluster of *Lactococcus lactis* NIZO B40 has been charac-terized in detail (van Kranenburg et al. 1999a). These EPS genes have been shown to be plasmid encoded and, therefore, may be transferred from one *Lactococcus* strain to another through the process of conjugation.

Heterologous overexpression of this EPS has been explored in lactococci (van Kranenburg et al. 1999b). The production of heterologous EPS has also been

explored with the transfer of the EPS gene cluster from *Streptococcus thermophilus* Sfi6 into *Lactococcus lactis* MG1363, a strain that does not produce EPS. This resulted in the creation of an altered EPS structure compared with the EPS of *S. thermophilus*, with a branching α-D-Gal*p* absent and a main chain α-D-*N*-acetyl-galactosamine with α-D-galactose (Stingele et al. 1999). However, more recently the EPS gene cluster of *Streptococcus thermophilus* Sfi39 was transformed into *Lactococcus lactis* MG 1363 with an identical EPS formed (Germond et al. 2001). In spite of these results, EPS production in LAB is not economically viable when compared with yields obtained from conventional methods (Becker et al. 1998).

A further example of metabolic engineering to produce metabolites on whey involves the construction of a lactose utilizing *Xanthomonas campestris* for the production of xanthan gum. Xanthan gum is a high molecular weight exopolysaccharide with a cellulose backbone that has applications in the food, cosmetic and oil industries and can be used in a number of applications such as stabilizing, viscosifying, emulsifying, thickening and suspending agents. Although *X. campestris* is not generally regarded as safe (GRAS), xanthan's rheological properties and cheap production costs result in its approved use in the food industry (De Vuyst and Degeest 1999). The xanthan-producing strain, *X. campestris,* possesses a low level of β-galactosidase; however, the bacterium cannot use lactose as an efficient carbon source, so attempts to use lactose-based substrates such as whey for gum production would be problematic unless a functional system utilizing lactose were introduced into and maintained in the bacterium. Expression of a heterologous β-galactosidase enabled *X. campestris* to produce xanthan gum in whey in amounts similar to that produced by cells grown in glucose with and without the constructed plasmid, pKMφLT, containing the *E. coli* lactose genes (Fu and Tseng 1990).

13.9   FOLATE

Genetic manipulation of LAB strains can also lead to increased folate production in fermented dairy products. It has been reported that a gene cluster coding for the synthesis of folate is present in *Lactococcus lactis* (Sybesma et al. 2002). The *Lactococcus lactis* cluster consists of six genes: GTP cyclohydrolase (*gch*), folate synthase *(folC)*, dihydropteroate synthase *((dps)folP))*, dihydroneopterin aldolase *(dhna)*, 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine *(hppk),* and dihydrofolate reductase *(dhfr).* According to a recently described patent, the γ glutamyl hydrolase enzymes (catalyzing the hydrolysis of folypoly-γ-glutamates and antifolypoly-γ-glutamates by removing γ-linked polyglutamates and glutamates) (McGuire and Coward 1984) of rat and human origin were overexpressed in *Lactococcus lactis* under the control of the NICE system. In both cases, deconjugation of polyglutamyl to monoglutamyl folate was observed (Sybesma et al. 2002). Furthermore, overexpression of the GTP cyclohydrolase *(gch)* gene resulted in increased levels of intra- and extracellular folate when compared with the control (twice as much total folate). Extracellular folate was approximately 20-fold higher than the control and it was suggested that the higher monoglutamyl residues in this system were due to low *folC* activity (Sybesma et al. 2002).

13.10   CONCLUSIONS

As outlined in these examples, food cultures can naturally produce a range of nutritional metabolites with applications in dairy foods or can be engineered to do so. In this respect, the exploitation of metabolic engineering strategies for the production of nutritional metabolites using lactic cell factories promises to be a very exciting field of study toward the development of innovative dairy-based functional food ingredients and the improvement of consumer health. Moreover, as genome information from various food cultures rapidly accumulates, so will understanding of the metabolic diversity in these organisms and how best to exploit it.

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14 The Safety Evaluation of Functional Dairy Foods

John O'Brien[*]

CONTENTS

14.1 INTRODUCTION

The development of functional foods, and nutraceuticals, can be viewed as an extension of supplementation and fortification strategies. It also represents a complement to the removal of unhealthy ingredients and a trend toward the addition to or generation of healthy properties in products. Many definitions of functional foods have been proposed in the literature, including a European Concerted Action Project's recent suggestion (Diplock et al. 1999):

> A food can be regarded as "functional" if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease.

Diplock et al. (1999) indicate that "a functional food can be a natural food, a food to which a component has been added, or a food from which a component has been removed by technological or biotechnological means."

Whatever definition is used, the distinguishing feature of functional food products is the objective of designing biological activity into the product. One type of biological activity, i.e., functional efficacy, is sought while at the same time avoiding another — side effects and toxicity. This implies a degree of specificity that may go beyond conventional food product development and process optimization for

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[*] Opinions expressed herein are those of the author and do not necessarily represent the policy of the Danone Group.

organoleptic attributes and safety. Furthermore, the marketing of food products based on the health benefits of consumption places a high expectation among consumers in terms of efficacy and safety.

The preceding considerations may lead to problems of communication and a challenge for the methodology of safety evaluation. The goal is to ensure that a product is at least as safe as existing traditional products — i.e., to ensure relative safety. This endpoint is reflected in the safety evaluation process, which is based as far as possible on a starting point of the safety database for existing foods and ingredients. The amount of work necessary to ensure the safety of a new product depends on the adequacy of this existing database and the degree of "novelty" of the product. Whatever the source of the functional food or constituent (traditional or not), the golden rule of Paracelsus, the father of toxicology, is pertinent: "What is there that is not poison, all things are poison and nothing is without poison. Solely the dose determines that a thing is not a poison."

The safety of functional foods is governed by general food law; no specific legislation covers the safety evaluation of functional foods. If a functional food is also deemed to be novel, then safety evaluation may be covered by special legislation requiring premarket approval; for example, in Europe this would be covered by the EC Novel Foods Regulation (EC 258/97) that came into law in May 1997. This chapter will focus on the context of launching functional food products in Europe, but the same principles apply to the safety evaluation of such products regardless of local regulations.

What distinguishes all foods, including many functional foods, from food additives and contaminants, however, is the potentially high levels of exposure. Therefore, exposure estimation (dose) plays a critical and central role in ensuring safety in use of functional foods. Extracts or concentrates of food fractions or components may lead to increased exposure compared with traditional intakes due to increased intakes or increased bioavailability. The safety or efficacy of a food component in a food does not ensure the safety or efficacy of the extracted or purified material. For example, human epidemiology studies consistently showed a reduced risk of lung cancer associated with high intakes of carotenoids from fruits and vegetables. Similarly, high plasma β-carotene was associated with reduced lung cancer incidence. However, high doses of synthetic β-carotene led to an increased rate of lung cancer and cardiovascular disease in a chemoprevention trial involving smokers (EC Scientific Committee on Food 2000), provoking a World Health Organization warning.

Whole foods, including functional foods, require nutritional evaluation in addition to traditional toxicological, microbiological and other safety considerations. In fact, the term "wholesomeness" describes the evaluation of novel foods better than "safety"; wholesomeness encompasses many issues, including toxicology, nutrition, microbiology and even environmental effects. Some companies have retrained toxicologists as nutritionists — a reflection of the number of products in which nutritional safety or adequacy is as important as or even more important than toxicology per se.

14.2 FUNCTIONAL DAIRY PRODUCTS

A variety of functional ingredients have been identified as having potential for addition to dairy products: β-glucan, caffeine, glucosamine, green tea extract,

lactoferrin, lutein, magnesium, phytosterols, probiotics, S-adenosyl-L-methionine, soy protein, vitamin C and vitamin E (Pszczola 2001). Plant sterols derived from a number of botanical sources have been considered for use in dairy products for their cholesterol-lowering properties. Several of these ingredients have undergone previous safety evaluation and a detailed toxicological literature is available.

Conjugated linoleic acid (CLA) is a natural constituent of bovine milk and some other foods. Concentrations in milk may be increased by the diet of the animal and by some types of fermentation and other types of processing. Dietary CLA has attracted interest in recent years due to anticancer effects and effects on body weight and body composition. In relation to the latter effects, the functionality of CLA may find applications in animal feed and in human foods. Toxicologically, CLA belongs to a structurally heterogeneous class of substances known as peroxisome proliferators that interact with a group of receptors known as peroxisome proliferator-activated receptors (PPAR) (Moya Camarena et al. 1999). The isomers of CLA appear to exhibit different toxicology profiles, which may help to direct research toward optimizing the safety of CLA preparations for human use. However, further toxicological research is required.

The uses of bovine colostrum and immunoglobulin-containing whey protein concentrates from immunized cows have received attention recently as potential approaches to preventing infections due to specific intestinal pathogens in humans. The safety evaluation and efficacy of milk and milk products prepared in this way is still ongoing.

Lactoferrin has demonstrated potential as an antimicrobial agent. The lower lactoferrin content of bovine milk compared with human milk has been identified as an opportunity for functional dairy product development (Antonini et al. 1997). However, it is possible that the cost of safety studies is one of the barriers that have prevented the widespread application of natural antimicrobials such as lactoferrin (Gould 1996). In addition to conventional safety studies on toxicology, allergenicity and tolerance, antimicrobials would also require extensive efficacy testing using pathogen challenge testing *in vitro* and *in vivo* as appropriate to anticipated applications. This would include studies on potential effects on the normal gut microflora.

Perhaps the most important category of functional dairy products is based on probiotic activity. To date most of the organisms used in such probiotic products have been lactobacilli or bifidobacteria; several publications are available on the safety of organisms such as probiotics (Mogensen et al. 2002a, b; Boriello et al. 2003). Important prerequisites for the use of any organism as a probiotic include (O'Brien et al. 1999; Boriello et al. 2003):

- Detailed phenotypic and genetic characterization of the organism
- Deposition in an internationally recognized culture collection
- Absence of pathogenicity
- Sensitivity to clinically important antibiotics
- Absence of transferable antibiotic resistance elements

New strains not previously found in human foods may require detailed safety evaluation.

14.3   FRAMEWORK FOR SAFETY EVALUATION OF NEW PRODUCTS

The safety evaluation of functional foods should commence with a thorough characterization of the food or ingredient. The preliminary data required are summarized as follows:

- Characteristics of the material: the chemical composition, Latin and common names (where applicable), specification, contaminants, active ingredients, etc.
- Exposure: can present or potential exposure be quantified and can exposure from other sources be estimated (including exposure assessment using biomarkers)?
- Who is likely to be exposed? Are there vulnerable groups?
- Dietary source of the material: is it a traditional or a novel food?

The proposed use of the product is also important because it will determine who is likely to be exposed and possible use and abuse patterns. As mentioned in the introduction, the safety evaluation of functional foods falls under existing legislation that includes, in Europe, the novel foods regulation. Under the regulation a novel food is defined as (EC 258/97) "foods and food ingredients which have not hitherto been used for human consumption to a significant degree within the community."

Novel foods are then subclassified, according to source, as foods and food ingredients:

- Containing or consisting of genetically modified organisms
- Produced from, but not containing, genetically modified organisms
- With a new or intentionally modified primary molecular structure
- Consisting of or isolated from microorganisms, fungi or algae
- Consisting of or isolated from plants and food ingredients isolated from animals, except for foods and food ingredients obtained by traditional propagating and breeding and having a history of safe food use
- To which a production process not currently used has been applied in which that process gives rise to significant compositional or structural changes that affect nutritional value, metabolism, or level of undesirable substances

A slightly different classification was published by the EC Scientific Committee on Food (SCF) that gives a more detailed guide as to the safety data required (EC 618/97). These data are described in detail by the SCF and in the regulation. It is important that the test material used in toxicology studies have the same specifications as the food or ingredient intended for marketing. In addition, the level of testing should be appropriate to projected intakes. This means that, although a minimum dataset of a 90-day rat feeding study and an assessment of genotoxicity *in vitro* may be acceptable, if exposure is likely to be widespread and/or high, chronic toxicity/carcinogenicity and reproductive toxicity testing may be required. Because of

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the normal necessity of including high levels of test materials in the diets of animals, test protocols should always take the nutritional requirements of the test species into account. This implies that exposure projection and nutritional assessment are required prior to any toxicology studies. Nutritional testing is also required to ensure that

- Nutritional status is not jeopardized by unknowing substitution of dietary components of known nutritive value (on which dietary recommendations may be based) with inferior substitutes
- Nutrient intakes are not inadvertently distorted due to unusual levels of particular nutrients or the presence of antinutrients affecting the nutritional value and bioavailability of other dietary components

In turn, the extent of the nutritional database depends on whether:

- The food or ingredient is consumed in significant amounts
- The food or ingredient has a specific nutritional function
- The foreseeable use of the food or ingredient is likely to cause nutritional imbalance
- A novel process causes changes in nutritional composition not observed in conventional processes
- Toxicological testing in animals is necessary (because it is essential to have adequate data on nutritional quality in advance)

At the heart of the process of safety evaluation of novel foods is to determine whether the novel product or process has an equivalent among the traditional foods consumed in European Union countries. ILSI Europe has classified products based on such equivalence (Jonas et al. 1996):

- Class 1 — foods or food ingredients substantially equivalent to a traditional reference food
- Class 2 — foods or food ingredients sufficiently similar to a traditional reference food
- Class 3 — foods or food ingredients neither substantially equivalent nor sufficiently similar to a traditional reference food

Thus, products may be substantially equivalent (possibly requiring few new data to conduct a safety evaluation), partially equivalent (possessing sufficient similarity to use some historical data and identify gaps in the database) or classified as foods that are neither and therefore require generation of an original safety dataset.

Unfortunately, the term "substantial equivalence" has sometimes led to consumer concern and confusion. Analysis of equivalence is not a safety assessment in absolute terms, but permits comparison with the safety of existing foods. This approach avoids doing the same thing twice, thus avoiding the ethically questionable excessive use of animal experiments and encouraging a holistic approach to safety evaluation based on mechanistic insights, nutritional safety and original toxicology when necessary.

The process of establishing substantial equivalence should include lengthy and detailed analysis of a product; the technical approach will vary depending on whether one is dealing with animals, plants or microorganisms, or ingredients derived from them.

In the absence of substantial or partial equivalence, a general rule of thumb in terms of minimum toxicology requirements is a 90-day subchronic feeding study plus *in vitro* genotoxicity. However, chronic studies and an assessment of reproductive toxicity may also be required.

The overall safety assessment dataset for a novel product requires the following information:

- Instructions for use
- Evidence of previous human exposure
- Extent of use/intake
- Details of process and product specification
- Nutritional studies
- History of organism
- Characterization of derived strain
- Toxicological assessment
- Human studies
- Genetic modification procedure
- Effect of genetic modification on properties of parent organism
- Genetic stability of modified organism
- Site expression of novel gene product
- Transfer of genetic material
- Ability of microorganism to survive, colonize, and replicate in the human gut

The expected intake will determine the amount of safety data required. Nutritional studies and detailed characterization studies should precede toxicological assessment. This is necessary to avoid nutritional effects that might confuse toxicological interpretation.

In particular, nutritional testing is necessary to ensure that the nutritional status of consumers is not jeopardized by unknowing substitution for existing foods of known nutritional value and to ensure that nutrient intakes are not distorted due to unusual levels of certain nutrients or due to the presence of antinutrients. The extent of the nutritional database required depends very much on the same factors that influence the generation of the toxicology database. In practice the nutritional evaluation depends on a thorough examination of composition, effects of processing and storage, and the presence and activity of antinutrients. Animal studies can be used to determine metabolizable energy, protein quality and bioavailability of essential nutrients.

Human tolerance should be determined in confirmatory tests after a thorough safety evaluation and preferably before marketing as opposed to uncontrolled release followed by postmarketing surveillance for adverse reactions. These human studies should be conducted in addition to sensory evaluation and may take the form of

single-dose or repeat-dose short-term studies of digestibility and tolerance. They could also be conducted as part of allergenicity assessment (within ethical constraints), taking due account of observations or occupationally exposed individuals.

Postlaunch monitoring is mandated in the novel food regulation when deemed necessary in the prelaunch safety assessment. The National Poisons Information Service based at Guys Hospital recently published an analysis of inquiries received after consumers reported adverse reactions to food supplements, health foods and herbal products. Of these three categories, the group that gave most cause for concern were the herbal products, of which composition is variable and uncertain and mechanisms of action poorly understood.

The need for safety evaluation will undoubtedly have a marked bearing on the development and marketing of functional foods. Because of the potential need to conduct expensive and time-consuming nutrition and toxicology studies, the process of safety evaluation should start as early as possible and be concurrent with other tasks and activities. The time scale here describes the potential extremes; however, if a project to conduct a 90-day study might take the best part of year and cost a minimum of $140,000, the feasibility of a food product development project might well be determined by the preliminary safety evaluation.

If potential costs of toxicology studies are examined, it can be seen that the sums of money are quite small relative to the cost of developing a drug. However, the margins for most foods are too low to permit heavy investment in this area. Safety evaluation may well be the rate-limiting step in functional food development. If the cost of advanced toxicology protocols were added to this list, one could easily move above a million dollars for a toxicology package.

14.4 NUTRIENT-FORTIFIED PRODUCTS

Milk has been an important vehicle for nutrient fortification for many years. Because of the importance of milk as a source of dietary calcium, this applies especially to vitamin D fortification. Fortification of milk with vitamin D has been practiced in the U.S. since the 1930s and, although not mandatory, most fluid milk in the U.S. contains added vitamin D. Vitamin D fortification of milk is also practiced on the European continent but is not permittted in the U.K.

Several cases of hypervitaminosis D have been associated with overfortification of drinking milk in Boston (Jacobus et al. 1992; Blank et al. 1995). These cases arose due to a failure to apply good manufacturing practices to fortified products, leading to prolonged overaddition of vitamin D to products (up to 245,840 IU per liter compared with a target level of ~380 IU/l; i.e., 400 IU/quart). The problem was compounded by mislabeling of concentrate used for fortification. Several contemporaneous studies revealed significant variability in vitamin D levels in fortified products compared with label declarations (Tanner et al. 1988; Jacobus et al. 1992; Holick et al. 1992; Chen et al. 1993).

At an industrial level, fortification should be under the supervision of trained personnel, calibration of metering pumps should be checked regularly, and levels in products should be verified by analysis on a regular basis. Despite the duration of the overfortification (several years) and the number of people consuming the product

(33,000 customers), only 19 people developed hypervitaminosis D. Most of those affected were older than 60 years. In spite of these cases associated with a single dairy, milk is an ideal medium for vitamin D fortification provided that suitable quality management procedures are implemented. Correctly fortified milk has not been associated with hypervitaminosis D; nevertheless, accurate labeling of vitamin D–fortified products is imperative due to hypersensitivity considerations and con-traindications in some groups of patients (Vieth 1999). Similar considerations may apply to the labeling of all functional foods. Recently, a model for the safe addition of micronutrients to foods was proposed based on three categories of micronutrient added as a percentage of EC RDA per 100-kcal portion of food (Flynn et al. 2003).

14.5   CONCLUSIONS

The development of functional food products introduces the need to conduct safety studies of a type not frequently associated with the food industry. The nature of the safety dataset required depends directly on the predicted dietary exposure to the new product and the consumers exposed. Whatever the nature of functionality sought in a product, the product is still a food; consequently, the focus of safety evaluation is on novelty. In this context, novelty of use may also be an important consideration. As emphasized earlier, an integrated approach incorporating toxicology, nutrition, microbiology and characterization studies is necessary because the safety of a food component in a food does not assure the safety of the purified extracted material.

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