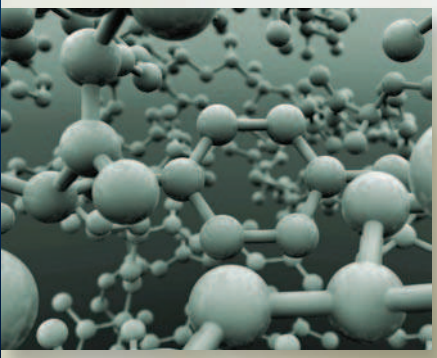
Bioactive Components in Milk and Dairy Products



YOUNG W. PARK EDITOR

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Bioactive Components in Milk and Dairy Products

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Foreword

An old saying declares that "times are changing." This book, *Bioactive Components in Milk and Dairy Products*, is a fine example of the truth in that old saying. For at least the last 100 years dairy science textbooks were about milk production from cows almost exclusively and about cow milk being the basic food for young and old. Now dairy science is joining the globalization of commerce worldwide and opening new focus on the long-neglected diversity of milk production from different mammals and on the complexity of milk composition as a source of a multitude of ingredients. These ingredients provide much more than basic nourishment to young and old; they also offer essential components with significant effects and benefits on body metabolism and body health.

This book provides an important contribution to the new globalized dairy science by detailing what is known of the milk of the other dairy animals whose milk contributes significantly to people's nutrition and health in countries other than the developed West: buffaloes, goats, sheep, camels, and horses. The milk of yaks and reindeer is not covered in this book, but it will be in the future because of the importance of these animals in certain regions of the world.

Bioactive Components in Milk and Dairy Products also presents a comprehensive discussion of the many components in milk of all species; these components have a role beyond the basic nutrients of protein, fat, sugar, and minerals. Chapters detail the chemistry and function of a long list of widely unknown factors that have been proven to have properties and activities beneficial to body health: bioactive milk proteins; β-lactoglobulin; α-lactalbumin; lactoferrin; immunoglobulins; lysozyme; lactoperoxidase; peptides from α_{s1}-, α_{s2}-, and β-; κ-caseins; glycomacropeptides; phosphopeptides; oligosaccharides; conjugated linoleic acid; polar lipids; gangliosides; sphingolipids; medium-chain triglycerides; trans fatty acids; milk minerals; growth factors; and approximately 16 hormones in milk, vitamins, and nucleotides. These proven beneficial effects include being antimicrobial, biostatic, antihypertensive, ACE inhibitive, antiadhesion, antidiabetic, anticholesterol, anticarcinogenic, immunomodulatory, anticariogenic, antiobesity, probiotic, and prebiotic. These factors and effects are discussed for milk and dairy products. Furthermore, regulatory and technological aspects of purification, analyses, and fortification into functional foods are presented.

Renowned world authorities researching the different dairy species and the bioactive components in their milk are the contributors in this book, which makes it especially valuable as a new reference source, for which the editor deserves much credit, and for which a wide distribution of this book is greatly deserved and highly recommended.

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Bioactive Components in Milk and Dairy Products

1
Overview of Bioactive Components in Milk and Dairy Products

Young W. Park

INTRODUCTION

Milk has been known as nature's most complete food. However, the traditional and contemporary view of the role of milk has been remarkably expanded beyond the horizon of nutritional subsistence of infants. Milk is more than a source of nutrients for any neonate of mammalian species, as well as for growth of children and nourishment of adult humans. Aside from nutritional values of milk, milkborne biologically active compounds such as casein and whey proteins have been found to be increasingly important for physiological and biochemical functions that have crucial impacts on human metabolism and health (Schanbacher et al. 1998; Korhonen and Pihlanto-Leppälä 2004; Gobbetti et al. 2007). Recent studies have shown that milk furnishes a broad range of biologically active compounds that guard neonates and adults against pathogens and illnesses, such as immunoglobulins, antibacterial peptides, antimicrobial proteins, oligosaccharides, and lipids, besides many other components at low concentrations (so-called "minor" components, but with considerable potential benefits). During the past decades, major progress has been made in the science, technology, and commercial applications of the multitude and complexity of bioactive components, particularly in bovine milk and colostrum. Cow milk has been the major source of milk and dairy products in developed countries, especially in the Western world, although more people drink the milk of goats than that of any other single species worldwide (Haenlein and Caccese 1984; Park 1990, 2006). Among the many valuable constituents in milk, the high levels of calcium play an important role in the development, strength, and density of bones in children and in the prevention of osteoporosis in elderly people. Calcium also has been shown to be beneficial in reducing cholesterol absorption, and in controlling body weight and blood pressure. Recent numerous research activities and advanced compositional identification of a large number of bioactive compounds in milk and dairy products have led to the discovery of specific biochemical, physiological, and nutritional functionalities and characteristics that have strong potential for beneficial effects on human health. Four major areas of bioactivity of milk components have been categorized: 1) gastrointestinal development, activity, and function; 2) infant development; 3) immunological development and function; and 4) microbial activity, including antibiotic and probiotic action (Gobbetti et al. 2007).

MILK AS A RICH SOURCE OF BIOACTIVE COMPONENTS

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 (Korhonen and Pihlanto-Leppälä 2004). Milk has been shown to contain an array of bioactivities, which extend the range of influence of mother over young beyond nutrition (Gobbetti et al. 2007). Peptides are in a latent or inactive state within protein molecules but can be

released during enzymatic digestion. Biologically active peptides released from caseins and whey proteins contain 3 to 20 amino acids per molecule (Korhonen and Pihlanto-Leppälä 2004). Researchers for the last decade have demonstrated that these bioactive peptides possess very important biological functionalities, including antimicrobial, antihypertensive, antioxidative, anticytotoxic, immunomodulatory, opioid, and mineral-carrying activities. A simple schematic representation of major bioactive functional compounds derived from milk is presented in Figure 1.1.

Most of the bioactivities of milk proteins are latent, being absent or incomplete in the original native protein, but full activities are manifested upon proteolytic digestion to release and activate encrypted bioactive peptides from the original protein (Clare and Swaisgood 2000; Gobbetti et al. 2002). Bioactive peptides (BPs) have been identified within the amino acid sequences of native milk proteins. They may be released by proteolysis during gastrointestinal transit or during food processing. Enzymes such as digestive, naturally occurring in milk, coagulants and microbial enzymes, especially those from adventitious or lactic acid starter bacteria, usually generate these bioactive compounds. BPs are released from milk proteins during milk fermentation and cheese maturation, which enriches the dairy products (Gobbetti et al. 2002). The major biologically active milk components and functions in milk precursors and components are summarized in Table 1.1.

Several milk-derived peptides have shown multifunctional properties, and specific peptide sequences have two or more distinct physiological activities. Certain regions in the primary structure of casein contain overlapping peptide sequences that exert different activities, as shown in Table 1.2. These fragments have been considered as strategic zones that are partially protected from further proteolytic

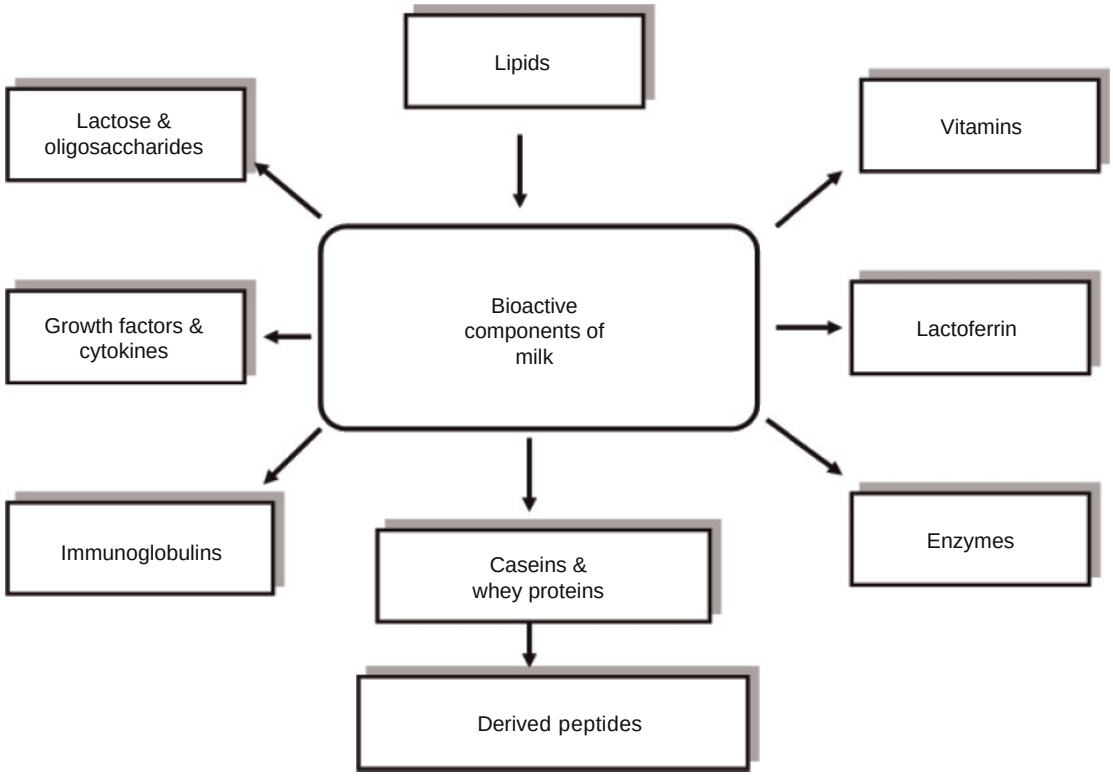

Figure 1.1. Schematic representation of major bioactive functional compounds derived from milk.

Table 1.1. Major biologically active milk components and their functions[1]

Milk Precursors or Components	Bioactive Compounds	Bioactivities Observed
α-, β-caseins	Casomorphins	Opioid agonist (Decrease gut mobility, gastric emptying rate; increase amino acids and electrolytes uptake)
α-, β-caseins	Casokinins	ACE inhibitory (Increase blood flow to intestinal epithelium)
α-, β-caseins	Phosphopeptides	Mineral binding (Ca binding; increase mineral absorption, i.e., Ca, P, Zn)
α-, β-caseins	Immunopeptides Casomorphins Casokinins	Immunomodulatory (Increase immune response and phagocytic activity)
α_{s1}-casein	Isracidin	Antimicrobial
α_{s2}-casein	Casocidin	Antimicrobial
κ-casein	Casoxins	Opioid antagonist
κ-casein	Casoplatelins	Antithrombotic
κ-casein	κ-casein glyco-macropeptide	Probiotic (Growth of bifidobacteria in GI tract)
α-lactalbumin (α-La) β-lactoglobulin(β-La)	Lactorphins	Opioid agonist
Serum albumin	Serorphin	Opioid agonist
α-La, β-La and Serum albumin	Lactokinins	ACE inhibitory
Immunoglobulins	IgG, IgA	Immunomodulatory (Passive immunity)
Lactoferrin	Lactoferrin	Immunomodulatory (Increase natural killer cell activity, humoral immune response, thymocyte trafficking immunological development, and interleukins-6; decrease tumor necrosis factor-α) Antimicrobial (Increase bacteriostatic inhibition of Fe-dependent bacteria; decrease viral attachment to and infections of cells) Probiotic activity (Increase growth of Bifidobacteria in GI tract)
Lactoferrin	Lactoferroxins	Opioid antagonist
Oligosaccharides	Oligosaccharides	Probiotic (Increase growth of bifidobacteria in GI tract)
Glycolipids	Glycolipids	Antimicrobial (Decrease bacterial and viral attachment to intestinal epithelial cells)
Oligosaccharides	Oligosaccharides	
Prolactin	Prolactin	Immunomodulatory (Increase lymphocyte and thymocyte trafficking, and immune development)
Cytokines	Interleukins-1,2,6, & 10 Tumor necrosis factor-α Interferon-γ Transforming growth Factors-α, β; leukotriene B_4 Prostaglandin E_2, Fn	Immunomodulatory (Lympocyte trafficking, immune development)
Growth factors	IGF-1, TGF-α, EGF, TGF-β	Organ development and functions
Parathromone-P	PTHrP	Increase Ca^{+2} metabolism and uptake

[1]Adapted from Schanbacher et al. (1998), Meisel (1998), and Clare and Swaisgood (2000).

Table 1.2. Examples of physiologically active milk peptides derived from milk[1]

Peptide Sequence[2]	Name	AA[3] Segment	Physiological Classification	Release Protease	Reference
FFVAP	α_{s1}-Casokinin-5	α_{s1}-CN (f23–27)	ACE inhibitor	Proline endopeptidase	Maruyama et al. (1985)
AVPYPQR	β-Casokinin-7	β-CN (f177–183)	ACE inhibitor	Trypsin	Maruyama et al. (1985)
YGLF	α-Lactorphin	α-LA (f50–53)	ACE inhibitor and opioid agonist	Synthetic peptide	Mullally et al. (1996)
ALPMHIR	β-Lactorphin	β-LG (f142–148)	ACE inhibitor	Trypsin	Mullally et al. (1997)
KVLPVPQ	Antihypertensive Peptide	β-CN (f169–174)	Antihypertensive peptide	Lactobacillus CP790 protease, and synthetic peptide	Maeno et al. (1996)
MAIPPKKNQDK	Casoplatelin	κ-CN (f106–116)	Antithrombotic	Trypsin and synthetic peptide	Jollès et al. (1986)
KDQDK	Thrombin	κ-CN glycol-inhibitory peptide macropeptide (f112–116)	Antithrombotic	Trypsin	Qian et al. (1995b)
KRDS	Thrombin inhibitory peptide	Lactotransferrin (f39–42)	Antithrombotic	Pepsin	Qian et al. (1995a)

Peptide Sequence[2]	Name	AA[3] Segment	Physiological Classification	Release Protease	Reference
QMEAES*IS*S*S*EEIVPNS*VEQK	Caseinophospho-peptide	α_{s1}-CN (f59–79)	Calcium binding and transport	Trypsin	Schlimme and Meisel (1995)
LLY	Immunopeptide	β-CN (f191–193)	Immunostimulatory (+)	Synthetic	Migliore-Samour et al. (1989)
FKCRRWQWRMKKLGAPSITCVRRAF	Lactoferricin B	Lactoferrin (f17–41)	Immunomodulatory (+) and antimicrobial	Pepsin	Bellamy et al. (1992)& Miyauchi et al. (1998)
YQQPVLGPVR	β-Casokinin-10	β-CN (f193–202)	Immunomodulatory (+/–) and ACE inhibitor	Synthetic	Meisel and Schlimme (1994)
RYLGYLE	α-Casein exorphin	α_{s1}-CN (f90–96)	Opioid agonist	Pepsin	Loukas et al. (1983)
YGFQNA	Serorphin	BSA (f399–404)	Opioid agonist	Pepsin	Tani et al. (1994)
YLLF.NH_2	β-Lactorphin amide	β-LG (f102–105)	Opioid agonist = ACE inhibitor	Synthetic or trypsin	Mullally et al. (1996)
YIPIQYVLSR	Casoxin C	κ-CN (f25–34)	Opioid antagonist	Trypsin	Chiba et al. (1989)
[YVPF PPF]	Casoxin D	α_{s1}-CN (f158–164)	Opioid antagonist	Pepsin-chymotrypsin	Yoshikawa et al. (1994)
YLGSGY-OCH_3	Lactoferroxin A	Lactoferrin (f318–323)	Opioid antagonist	Pepsin	Yamamoto et al. (1994)

[1]Adapted from Clare and Swaisgood (2000).
[2]The one-letter amino acid codes were used; S* = Phosphoserine.
[3]Amino acid.

breakdown (Fiat et al. 1993). Various peptide fragments have different physiological activities. Peptides containing different amino acid sequences can exhibit the same or different bioactive functionalities. The specific bioreactions associated with each physiological class have been characterized, and recent research data have been classified according to their physiological functionality. Some examples of BPs are compiled as shown in Table 1.2 (Clare and Swaisgood 2000).

BIOACTIVE COMPOUNDS IN FOODS AND FUNCTIONAL FOODS

In recent years, functional foods and bioactive components in foods have drawn a lot of attention and interest of food scientists, nutritionists, health professionals, and general consumers. A functional food may be similar in appearance to a conventional food, is consumed as a part of normal diet but has various physiological benefits, and can reduce the risk of chronic diseases beyond basic nutritional functions. The volume of market sales for functional foods has grown steadily. The global functional foods market continues to be a dynamic and growing segment of the food industry (Marketresearch.com 2008). Rapid growth is predicted to continue for the next year, but taper off by 2010, when functional foods are expected to represent 5% of the total global food market. The current global functional foods market is estimated to be US$7–63 billion, depending on sources and definitions (Marketresearch.com 2008), and is expected to grow to US$167 billion by 2010. The global growth rate for functional foods will likely achieve an average of 14% annually through 2010. After 2010, the functional foods market size is expected to comprise approximately 5% of total food expenditures in the developed world (Marketresearch.com 2008). Mintel International Group Ltd. (2008) reported that U.S. sales in the dairy segment of functional (bioactive) foods increased by more than 33% over the review period of 2005–07, and its share of the total market grew from 71–74% with a value of nearly US$2 billion, to nearly 75% of the total market (Table 1.3). In the bars and snacks segment, sales of functional foods more than doubled from 2005 to 2007 to a value of US$197 million. However, the segment still remains quite small, accounting for just 7% of the market in 2007. Functional cereal sales gains were modest as 6% from 2005 to 2007 with a value of US$434 million, making up 16% of market share. Bakery's share of the functional food sales stood at just 2%, which was a 29% decrease for the same period, suggesting that functional foods for the bakery segment may be the one that represents the most untapped potential (Table 1.3).

The consumption volumes of functional foods, especially for those manufactured using dairy bioactive compounds, are likely to increase in the developed countries. The U.S. sales of functional foods were forecasted to increase by 46% from 2007 to 2012 at current prices and by 28% at inflation-adjusted prices, following strong performance from 2002 to 2007 (Table 1.4). The number of new functional products drastically increased between 2002 and 2007. Product proliferation is a boon to this market because it promotes familiarity with the functional concept. However, the Mintel reports (2008) predicted that proliferation would lead to market saturation and that future growth in the spe-

Table 1.3. Sale volumes of functional foods in the U.S. during 2005 and 2007

	2005		2007		Change 2005–07
	$million	%	$million	%	%
Dairy and margarine	1,459	71	1,959	74	34
Cereal	410	20	434	16	6
Bars and snacks	92	5	197	7	113
Bakery	79	4	56	2	–29
Total	**2,041**	**100**	**2,646**	**100**	**30**

Data may not equal totals due to rounding.

Source: Mintel/based on Information Resources, Inc. InfoScan® Reviews Information.

Table 1.4. Total U.S. sales and forecast of functional foods at current prices, 2002–2012

Year	$million	% Change	Index (2002 = 100)	Index (2007 = 100)
2002	1,620	—	100	61
2003	1,666	2.8	103	63
2004	1,776	6.6	110	67
2005	2,041	14.9	126	77
2006	2,385	16.9	147	90
2007	2,646	10.9	163	100
2008 (fore)	2,879	8.8	178	109
2009 (fore)	3,117	8.3	192	118
2010 (fore)	3,359	7.8	207	127
2011 (fore)	3,611	7.5	223	136
2012 (fore)	3,874	7.3	239	146

Source: Mintel/based on Information Resources, Inc. InfoScan® Reviews Information; Mintel forecasts inflation-adjusted growth of 28% during 2007–12.

cifically defined market may slow down as manufacturers and marketers favor more general promotional methods (Table 1.4).

Globally, digestive health claims are leading functional claims for foods. A recent report also showed that functional foods and beverages providing digestive health benefits are growing, both in the traditional categories where the claim emerged for "yogurt and dairy-based beverages" and in unique and new categories, such as prepared meals and snack mixes (Prepared Foods 2008). In coming years, functional foods and beverages are expected to grow continuously. This trend stems from an ever-growing number of products capitalizing on natural ingredients that provide digestive health benefits. When it comes to digestive health, the key to business successes for manufacturers is to identify creative positioning strategies and launch new product introductions that would make a clear difference (Prepared Foods 2008). Table 1.5 shows the top 10 U.S. digestive health subcategories by number of new product introductions up to May 2008.

Table 1.5. Top U.S. digestive health subcategories (by number of new product introductions)

Category	2008*	2007	2006	2005
Spoonable yogurt	42	28	24	0
Cheese	7	15	1	0
Dairy-based frozen products	7	1	0	0
Snack/Cereal/ Energy bars	5	9	2	0
Soy yogurt	5	0	0	0
Juice	4	2	0	1
Prepared meals	4	0	0	0
Hot cereals	3	2	0	0
Drinking yogurts/ liquid cultured milk	2	6	1	1
Cold cereals	2	3	1	0
Snack mixes	2	0	0	0

Source: Mintel GNPD (*through May 5, 2008); Prepared Foods (2008).

The term *bioactive components* refers to compounds either naturally existing in food or ones formed and/or formulated during food processing that may have physiological and biochemical functions when consumed by humans. Food scientists have been exploiting bioactive components of milk and dairy products for application in functional foods and for potential pharmaceutical use. Milk is often considered as a functional food since it contains varieties of different bioactive components. Because of its chemical composition and structural properties, milk is also a good vehicle to formulate

functional foods. Although bioactive compounds in milk and dairy products have been extensively studied during the last couple of decades, especially in human and bovine milk and some dairy products, there are very few publications on this topic available for other dairy animal species that provide valuable nourishment, especially in developing countries in Asia and Africa.

WHY IS THIS BOOK UNIQUE?

So far only a limited number of publications have been available about the biochemistry and technology of bioactive and nutraceutical compounds in bovine and human milk, and the milk of other mammalian species has not received much research attention in this regard. This book is therefore unique in also covering extensively bioactive components in milk and dairy products of goats, sheep, buffalo, camels, and horses by internationally renowned scientists who are in the forefront of research in functional components of milk and dairy products and their chemistry and technology. The bovine milk chapter starts the discussion in order to present the updated reference scientific information and research data in the field of bioactive components and functional food ingredients with respect to those in other dairy species. This book benefits readers around the world, including students, scientists, and health-conscious consumers who are looking for scientific information on bioactive compounds and therapeutic substances in milk and dairy products from different dairy animal species as referenced to cow milk. This book presents the best available current knowledge and reports on the up-to-date information written and forwarded by world authorities and experts in bioactive and nutraceutical components in milk and processed milk products of different dairy species.

This volume is uniquely different from other published works because it not only contains rich compilations of a variety of research data and literature on milk of different mammalian species, but because it also extensively delineates bioactive compounds in the various important manufactured dairy products. Other integral aspects of functionality of bioactive compounds are also included in this book, such as potential for improving human health with these components in milk and dairy products. The introductory section describes the overview of bioactive components in milk and dairy products and the general concept of bioactive compounds and functional foods derived from milk and dairy products. Section I covers the bioactive components in milk of the different major dairy species, which makes this book a special reference source of detailed information not otherwise available. This work therefore would be valuable to readers who seek special scientific information and data for their unique locations, environments, traditions, and availabilities of their own dairy species. Considering this rapidly emerging and fascinating scientific area in human health and nutrition, this work is a special reference source because of its unique and significant contributions to the field. Section II looks closely at the bioactive components in manufactured dairy products, such as caseins, caseinates, cheeses, yogurt products, koumiss and kefir, whey products, probiotics, and prebiotics. Section III touches on other related issues in bioactive compounds in dairy products, such as regulatory issues and functional health claims on bioactive compounds, new technologies for isolation, and analysis of bioactive compounds. This section also delineates several aspects of potential for improving human health, including immunomodulation by dairy ingredients, calcium bioavailability of milk and dairy products, and iron fortification of dairy products.

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Section I
Bioactive Components in Milk

2
Bioactive Components in Bovine Milk

Hannu J. Korhonen

INTRODUCTION

Functional foods have emerged as a new approach to improve human nutrition and health in an environment where lifestyle diseases and aging populations are considered a threat to the well-being of the society. The advent of this new food category has been facilitated by increasing scientific knowledge about the metabolic and genomic effects of diet and specific dietary components on human health. Accordingly, opportunities have arisen to formulate food products that deliver specific health benefits, in addition to their basic nutritional value.

Bovine milk and colostrum are considered the most important sources of natural bioactive components. Over the last 2 decades major advances have taken place with regard to the science, technology, and commercial applications of bioactive components present naturally in bovine milk and colostrum.

Bioactive components comprise specific proteins, peptides, lipids, and carbohydrates. Chromatographic and membrane separation techniques have been developed to fractionate and purify many of these components on an industrial scale from colostrum, milk, and cheese whey (Smithers et al. 1996; McIntosh et al. 1998; Korhonen 2002; Kulozik et al. 2003; Pouliot et al. 2006; Korhonen and Pihlanto 2007a). Fractionation and marketing of bioactive milk ingredients has emerged as a new lucrative sector for dairy industries and specialized bioindustries. At present many of these components are being exploited for both dairy and nondairy food formulations and even pharmaceuticals (Korhonen et al. 1998; Shah 2000; German et al. 2002; Playne et al. 2003; Rowan et al. 2005; Krissansen 2007). The dairy industry has achieved a leading role in the development of functional foods and has already commercialized products that boost, for example, the immune system; reduce elevated blood pressure; combat gastrointestinal infections; help control body weight; and prevent osteoporosis (FitzGerald et al. 2004; Zemel 2004; Cashman 2006; Korhonen and Marnila 2006; Hartmann and Meisel 2007). There also is increasing evidence that many milk-derived components are effective in reducing the risk of metabolic syndrome, which may lead to various chronic diseases, such as cardiovascular disease and diabetes (Mensink 2006; Pfeuffer and Schrezenmeir 2006a; Scholz-Ahrens and Schrezenmeir 2006). Beyond essential nutrients milk seems thus capable of delivering many health benefits to humans of all ages by provision of specific bioactive components. Figure 2.1 gives an overview of these components and their potential applications for promotion of human health.

This chapter reviews the current knowledge about technological and biological properties of milk- and colostrum-derived major bioactive components and their exploitation for human health. A particular emphasis has been given to bioactive proteins, peptides, and lipids, which have been the subjects of intensive research in recent years.

BIOACTIVE PROTEINS AND PEPTIDES

The high nutritional value of milk proteins is widely recognized, and in many countries dairy products

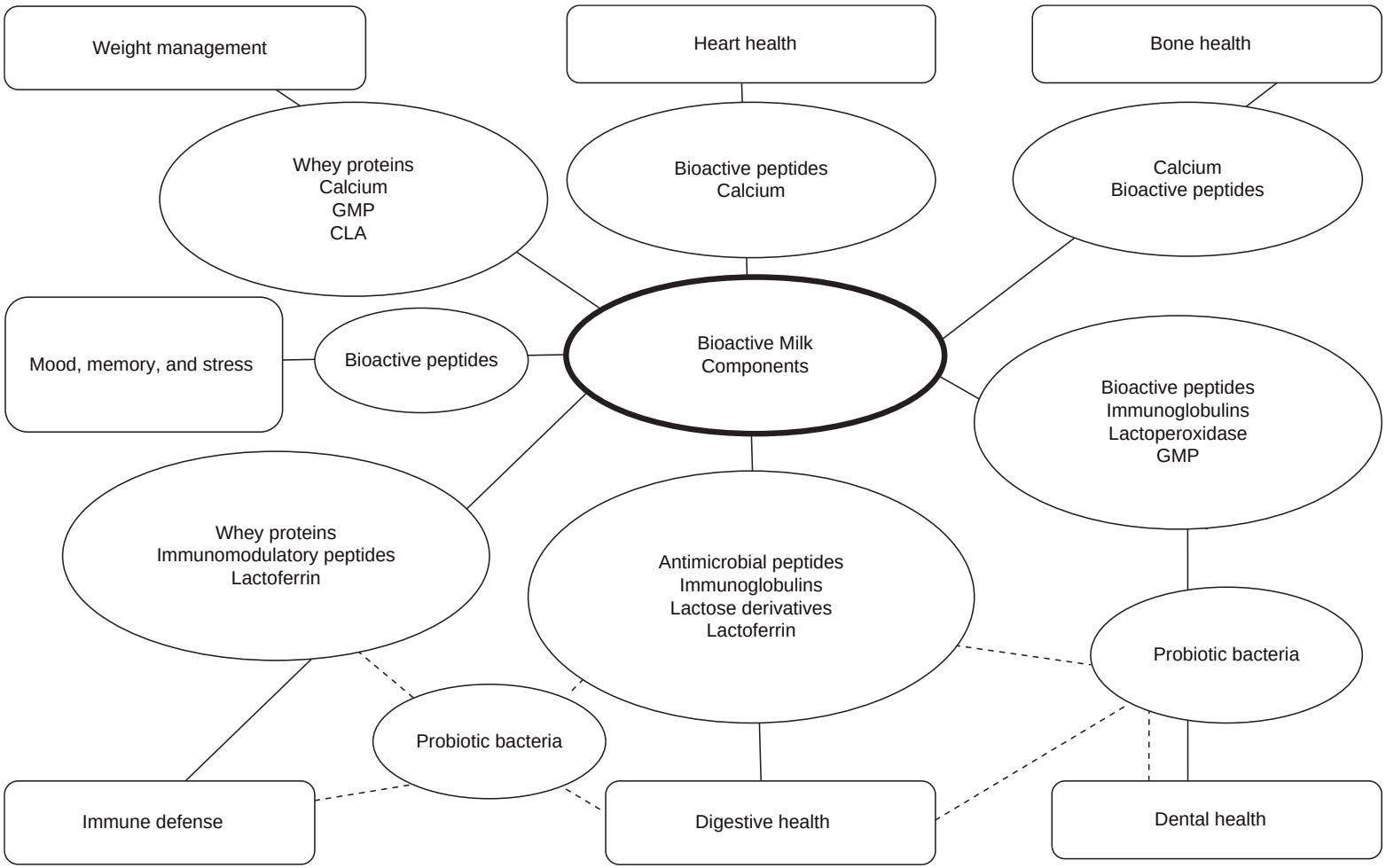

Figure 2.1. Bioactive milk components and their potential applications for health promotion.

contribute significantly to daily protein intake. The multiple functional properties of major milk proteins are now largely characterized (Mulvihill and Ennis 2003). Increasing scientific and commercial interest has been focused on the biological properties of milk proteins. Intact protein molecules of both major milk protein groups, caseins and whey proteins, exert distinct physiological functions in vivo (Shah 2000; Steijns 2001; Walzem et al. 2002; Clare et al. 2003; Floris et al. 2003; Pihlanto and Korhonen 2003; Madureira et al. 2007; Zimecki and Kruzel 2007). Table 2.1 lists the major bioactive proteins found in bovine colostrum and milk and their concentrations, molecular weights, and suggested biological functions. Many of the bioactive whey proteins, notably immunoglobulins, lactoferrin and growth factors, occur in colostrum in much greater concentrations than in milk, thus reflecting their importance to the health of the newborn calf (Korhonen 1977; Pakkanen and Aalto 1997; Scammel 2001). Furthermore, milk proteins possess additional physiological functions due to the numerous bioactive peptides that are encrypted within intact proteins. Their functions are discussed in the section "Production and Functionality of Bioactive Peptides" later in this chapter.

Fractionation and Isolation of Bioactive Milk Proteins

Increasing knowledge of the biological properties of individual milk-derived proteins and their fractions has prompted the need to develop technologies for obtaining these components in a purified or enriched form. Normal bovine milk contains about 3.5% of protein, of which casein constitutes about 80% and whey proteins 20%. Bovine casein is further divided into α-, β- and κ-casein. Various chromatographic and membrane separation techniques have been developed for fractionation of β-casein in view of its potential use, e.g., in infant formulas (Korhonen and Pihlanto 2007a). However, industrial manufacture of casein fractions for dietary purposes has not progressed to any significant extent, so far. Whole casein is known to have a good nutritive value owing to valuable amino acids, calcium, phosphate and several trace elements. Individual casein fractions have been proven to be biologically active and also a good source of different bioactive peptides (see review by Silva and Malcata 2005).

The whey fraction of milk contains a great variety of proteins that differ from each other in their chemical structure, functional properties and biological functions. These characteristics have been used to separate individual proteins, but the purity has proven a critical factor when evaluating the biological activity of a purified component. Membrane separation processes, such as ultrafiltration (UF), reverse osmosis (RO) and diafiltration (DF), are now industrially applied in the manufacture of ordinary whey powder and whey protein concentrates (WPCs) with a protein content of 30–80%. Gel filtration and ion-exchange chromatography techniques can be employed in the manufacture of whey protein isolates (WPI) with a protein content of 90–95% (Kelly and McDonagh 2000; Etzel 2004). The chemical composition and functionality of whey protein preparations are largely affected by the method used in the process. Their biological properties are also affected and are difficult to standardize due to the complex nature of the bioactivities exerted by different proteins (Korhonen 2002; Foegeding et al. 2002). Therefore there is growing interest in developing specific techniques for isolation of pure whey protein components. Industrial or semi-industrial scale processing techniques are already available for fractionation and isolation of major native milk proteins, e.g., alpha-lactalbumin (α-La), beta-lactoglobulin (β-Lg), immunoglobulins (Ig), lactoferrin (LF) and glycomacropeptide (GMP). Current and potential technologies have been reviewed in recent articles (Korhonen 2002; Kulozik et al. 2003; Chatterton et al. 2006; Korhonen and Pihlanto 2007a). Recently Konrad and Kleinschmidt (2008) described a novel method for isolation of native α-La from sweet whey using membrane filtration and treatment of the permeate with trypsin. After a second UF and diafiltration of the hydrolysate, the calculated overall recoveries were up to 15% of α-La with a purity of 90–95%. In another recent method β-Lg was isolated from bovine whey using differential precipitation with ammonium sulphate followed by cation-exchange chromatography (Lozano et al. 2008). The overall yield of purified β-Lg was 14.3% and the purity was higher than 95%. The progress of proteomics has facilitated the use of proteomic techniques, for example, two-dimensional gel electrophoresis, in characterization of whey proteins (O'Donnell et al. 2004; Lindmark-Måsson et al. 2005). Using this technique, Fong et al. (2008)

Table 2.1. Major bioactive protein components of bovine colostrum and milk: Concentration, molecular weight, and potential biological functions*

Protein	Concentration (g/L)		Molecular Weight	Biological Activity
	Colostrum	Milk	Daltons	
Caseins (α_{s1}, α_{s2}, β, and κ)	26	28	14.000–22.000	Ion carrier (Ca, PO_4, Fe, Zn, Cu), precursor for bioactive peptides immunomodulatory, anticarcinogenic
β-lactoglobulin	8.0	3.3	18.400	Vitamin carrier, potential antioxidant, precursor for bioactive peptides, fatty acid binding
α-lactalbumin	3.0	1.2	14.200	Effector of lactose synthesis in mammary gland, calcium carrier, immunomodulatory, precursor for bioactive peptides, potentially anticarcinogenic
Immunoglobulins	20–150	0.5–1.0	150.000–1000.000	Specific immune protection through antibodies and complement system, potential precursor for bioactive peptides
Glycomacro-peptide	2.5	1.2	8.000	Antimicrobial, antithrombotic, prebiotic, gastric hormone regulator
Lactoferrin	1.5	0.1	80.000	Antimicrobial, antioxidative, anticarcinogenic, anti-inflammatory, iron transport, cell growth regulation, precursor for bioactive peptides, immunomodulatory, stimulation of osteoblast proliferation
Lactoperoxidase	0.02	0.03	78.000	Antimicrobial, synergistic effects with immunoglobulins, lactoferrin, and lysozyme
Lysozyme	0.0004	0.0004	14.000	Antimicrobial, synergistic effects with immunoglobulins, lactoferrin, and lactoperoxidase
Serum albumin	1.3	0.3	66.300	Precursor for bioactive peptides
Milk basic protein	N.A	N.A	10.000–17.000	Stimulation of osteoblast proliferation and suppression of bone resorption
Growth factors	50 µg–40 mg/L	<1 µg–2 mg/L	6.400–30.000	Stimulation of cell growth, intestinal cell protection and repair, regulation of immune system

*Compiled from Pihlanto and Korhonen (2003) and Korhonen and Pihlanto (2007b); N.A. = not announced.

identified a large number of minor whey proteins after first fractionating bovine whey by semicoupled anion and cation exchange chromatography. Such proteomic display appears useful in the future design of strategies for purification of selected milk proteins or their fractions containing targeted bioactivities.

Biological Functions and Applications of Major Milk Proteins

Proteins contained in colostrum and milk are known to exert a wide range of nutritional, functional, and biological activities (Walzem et al. 2002; Pihlanto and Korhonen 2003; Marshall 2004; Tripathi and Vashishtha 2006; Yalcin 2006; Zimecki and Kruzel 2007). Apart from being a balanced source of valuable amino acids, milk proteins contribute to the structure and sensory properties of various dairy products. A brief overview is given about the established and potential physiological functions of main milk proteins with special emphasis on whey proteins and bioactive peptides.

Whole casein and electrophoretic casein fractions have been shown to exhibit different biological activities, such as immunomodulation (Bennett et al. 2005; Gauthier et al. 2006b) and provision of a variety of bioactive peptides, including antihypertensive (Lóopez-Fandiño et al. 2006), antimicrobial (López-Expósito and Recio 2006; Pan et al. 2006), antioxidative (Pihlanto 2006) and opioidlike (Meisel and FitzGerald 2000). These recent findings suggest that casein hydrolysates provide a potential source of highly functional ingredients for different food applications. Examples of such products are the fermented milk products Calpis® and Evolus®, which are based on hypotensive tripeptides Val-Pro-Pro and Ile-Pro-Pro derived from both β-casein and κ-casein. Owing to emerging health properties and documented clinical implications, the bovine milk whey proteins have attained increasing commercial interest (Krissansen 2007; Madureira et al. 2007). The total whey protein complex and several individual proteins have been implicated in a number of physiologically beneficial effects, such as

1. Improvement of physical performance, recovery after exercise, and prevention of muscular atrophy (Ha and Zemel 2003)
2. Satiety and weight management (Schaafsma 2006a,b); Luhovyy et al. 2007)
3. Cardiovascular health (Yamamoto et al. 2003; FitzGerald et al. 2004; Murray and FitzGerald 2007)
4. Anticancer effects (Parodi 1998; Bounous 2000; Gill and Cross 2000)
5. Wound care and repair (Smithers 2004)
6. Management of microbial infections and mucosal inflammation (Playford et al. 2000; Korhonen et al. 2000; Korhonen and Marnila 2006)
7. Hypoallergenic infant nutrition (Crittenden and Bennett 2005)
8. Healthy aging (Smilowitz et al. 2005)

Although many of these effects remain still putative, a few have been substantiated in independent studies. In the following discussion, the health-promoting potential of major whey proteins and examples of their commercial applications will be described briefly.

Immunoglobulins

Immunoglobulins (Ig) carry the biological function of antibodies and are present in colostrum of all lactating species to provide passive immunity against invading pathogens. Igs are divided into different classes on the basis of their physicochemical structures and biological activities. The major classes in bovine and human lacteal secretions are IgG, IgM, and IgA. The basic structure of all Igs is similar and is composed of two identical light chains and two identical heavy chains. These four chains are joined with disulfide bonds. The complete basic Ig molecule displays a Y-shaped structure and has a molecular weight of about 160 kilodaltons. Igs account for up to 70–80% of the total protein content in colostrum, whereas in milk they account for only 1–2% of total protein (Korhonen et al. 2000). The importance of colostral Igs to the newborn calf in protection against microbial infections is well documented (Butler 1994). Colostral Ig preparations designed for farm animals are commercially available, and colostrum-based products have found a growing worldwide market as dietary supplements for humans (Scammel 2001; Tripathi and Vashishtha 2006; Struff and Sprotte 2007; Wheeler et al. 2007). Igs link various parts of the cellular and humoral immune system. They are able to prevent the adhesion of microbes, inhibit bacterial metabolism, agglutinate

bacteria, augment phagocytosis of bacteria, kill bacteria through activation of complement-mediated bacteriolytic reactions, and neutralize toxins and viruses.

The concentration of specific antibodies against pathogenic microorganisms can be raised in colostrum and milk by immunizing cows with vaccines made of pathogens or their antigens (Korhonen et al. 2000). Advances in bioseparation techniques have made it possible to fractionate and enrich these antibodies and formulate so-called immune milk preparations (Mehra et al. 2006). The concept of "immune milk" dates back to the 1950s when Petersen and Campbell first suggested that orally administered bovine colostrum could provide passive immune protection for humans (Campbell and Petersen 1963). Since the 1980s, a great number of studies have demonstrated that such immune milk preparations can be effective in prevention of human and animal diseases caused by different pathogenic microbes, e.g., rotavirus, *Escherichia coli, Candida albicans, Clostridium difficile, Shigella flexneri, Streptococcus mutans, Cryptosporidium parvum,* and *Helicobacter pylori*. The therapeutic efficacy of these preparations has, however, proven to be quite limited (Weiner et al. 1999; Korhonen et al. 2000; Korhonen and Marnila 2006). A few commercial immune milk products are on the market in some countries, but the unclear regulatory status of these products in many countries has emerged as a constraint for global commercialization (Hoerr and Bostwick 2002; Mehra et al. 2006). In view of the globally increasing problem of antibiotic-resistant strains causing endemic hospital infections, the development of appropriate immune milk products to combat these infections appears as a highly interesting challenge for future research.

α-Lactalbumin

α-La is the predominant whey protein in human milk and accounts for about 20% of the proteins in bovine whey. α-La is fully synthesized in the mammary gland where it acts as coenzyme for biosynthesis of lactose. The health benefits of α-La have long been obscured, but recent research suggests that this protein can provide beneficial effects through a) the intact whole molecule, b) peptides of the partly hydrolyzed protein, and c) amino acids of the fully digested protein (Chatterton et al. 2006). α-La is a good source of the essential amino acids tryptophan and cystein, which are precursors of serotonin and glutathion, respectively. It has been speculated that the oral administration of α-La could improve the ability to cope with stress. A human clinical study with a group of stress-vulnerable subjects showed that an α-La–enriched diet affected favorably biomarkers related to stress relief and reduced depressive mood (Markus et al. 2000). In a later study the same researchers (Markus et al. 2002) observed that α-La improved cognitive functions in stress-vulnerable subjects by increased brain tryptophan and serotonin activity. In another clinical study (Scrutton et al. 2007) it was shown that daily administration of 40 g of α-La to healthy women increased plasma tryptophan levels and its ratio to neutral amino acids, but no changes in emotional processing was observed. Furthermore, there is significant evidence from animal model studies that α-La can provide protective effect against induced gastric mucosal injury. The protection was comparable to that of a typical antiulcer drug (Matsumoto et al. 2001; Ushida et al. 2003). Bovine α-La hydrolysates and specific peptides derived from these hydrolysates have been associated with many biological activities, for example, antihypertensive, antimicrobial, anticarcinogenic, immunomodulatory, opioid, and prebiotic (Pihlanto and Korhonen 2003; Chatterton et al. 2006).

Based on its high degree of amino acid homology to human α-La, bovine α-La and its hydrolysates are well suited as an ingredient for infant formulae. A few α-La enriched formulae have already been launched on the market.

β-Lactoglobulin

β-Lg is the major whey protein in bovine milk and accounts for about 50% of the proteins in whey, but it is not found in human milk. β-Lg poses a variety of functional and nutritional characteristics that have made this protein a multifunctional ingredient material for many food and biochemical applications. Furthermore, β-Lg has been proven an excellent source of peptides with a wide range of bioactivities, such as antihypertensive, antimicrobial, antioxidative, anticarcinogenic, immunomodulatory, opioid, hypocholesterolemic, and other metabolic effects (Pihlanto and Korhonen 2003; Chatterton et al. 2006). Of particular interest is the antihypertensive

peptide β-lactosin B [Ala-Leu-Pro-Met; f(142–145)], which has shown significant antihypertensive activity when administered orally to SHR rats (Murakami et al. 2004) and a tryptic peptide [Ile-Ile-Ala-Glu-Lys; f(71–75)], which has exerted hypocholesterolemic activity in rat model studies (Nagaoka et al. 2001). Also an opioid peptide β-lactorphin [Tyr-Leu-Leu-Phe; f(102–105)] has been shown to improve arterial functions in SHR rats (Sipola et al. 2002). β-Lg–derived peptides carry a lot of biological potential, but further in vivo studies are essential to validate the physiological effects. This view is supported by a recent study of Roufik et al. (2006), who showed that a β-Lg–derived ACE-inhibitory peptide [ALPMHIR; f(142–148)] was rapidly degraded upon in vitro incubation with human serum, and after oral ingestion it could not be detected in sera of human subjects.

Lactoferrin

LF is an iron-binding glycoprotein found in colostrum, milk, and other body secretions and cells of most mammalian species. LF is considered to be an important host defense molecule and is known to confer many biological activities, such as antimicrobial, antioxidative, antiinflammatory, anticancer, and immune regulatory properties (Lönnerdal 2003; Wakabayashi et al. 2006; Pan et al. 2007; Zimecki and Kruzel 2007). Furthermore, several antimicrobial peptides, such as lactoferricin B f(18–36) and lactoferrampin f(268–284) can be cleaved from LF by the action of digestive enzyme pepsin. LF is considered to play an important role in the body's innate defense system against microbial infections and degenerative processes induced, e.g., by free oxygen radicals. The biological properties of LF have been the subject of scientific research since its discovery in the early 1960s. Initially, the role was confined largely to antimicrobial activity alone, but now the multifunctionality of LF has been well recognized. The antimicrobial activity of LF and its derivatives has been attributed mainly to three mechanisms: a) iron-binding from the medium leading to inhibition of bacterial growth; b) direct binding of LF to the microbial membrane, especially to lipopolysaccharide in Gram-negative bacteria, causing fatal structural damages to outer membranes and inhibition of viral replication; and c) prevention of microbial attachment to epithelial cells or enterocytes. As reviewed by Pan et al. (2007) the bactericidal effect of LF can be augmented by the action of lysozyme or antibodies. LF can also increase susceptibility of bacteria to certain antibiotics, such as vancomycin, penicillin, and cephalosporins. The in vitro antimicrobial activity of LF and the derivatives has been demonstrated against a wide range of pathogenic microbes, including enteropathogenic *E. coli; Clostridium perfringens; Candida albicans; Haemophilus influenzae; Helicobacter pylori; Listeria monocytogenes; Pseudomonas aeruginosa; Salmonella typhimurium; S. enteriditis; Staphylococcus aureus; Streptoccccus mutans; Vibrio cholerae;* and hepatitis C, G, and B virus; HIV-1; cytomegalovirus; poliovirus; rotavirus; and herpes simplex virus (Farnaud and Evans 2003; Pan et al. 2007). The antitumor activity of LF has been studied intensively over the last decade and many mechanisms have been suggested, e.g., iron-chelation–related antioxidative property and immunoregulatory and antiinflammatory functions. In in vitro experiments LF has been shown to regulate both cellular and humoral immune systems by 1) stimulation of proliferation of lymphocytes; 2) activation of macrophages, monocytes, natural killer cells, and neutrophils; 3) induction of cytokine and nitric oxide production; and4) stimulation of intestinal and peripheral antibody response (Pan et al. 2007).

During the past 2 decades it has become evident in many animal and human studies that oral administration of LF can exert several beneficial effects on the health of humans and animals. These studies have been compiled and reviewed in several excellent articles (Teraguchi et al. 2004; Wakabayashi et al. 2006; Pan et al. 2007; Zimecki and Kruzel 2007). Animal studies with mice or rats have demonstrated that orally administered LF and related compounds suppressed the overgrowth and translocation of certain intestinal bacteria, such as *E. coli, Streptococcus,* and *Clostridium* strains but did not affect intestinal bifidobacteria. Also, oral administration of LF and lactoferricin reduced the infection rate of *H. pylori, Toxoplasma gondii,* candidiasis, and tinea pedis as well as prevented clinical symptoms of influenza virus infection. Further animal studies have demonstrated that orally ingested LF and related compounds improved nutritional status by reducing iron-deficient anemia and drug-induced intestinal inflammation, colitis, arthritis, and decreased mortality caused by endotoxin shock.

Also, LF increased weight gain in preweaning calves. A recent study (Cornish et al. 2004) has showed that oral LF administration to mice regulated the bone cell activity and increased bone formation. In addition animal studies have shown beneficial effects of LF ingestion on inhibition of carcinogen-induced tumors in the colon, esophagus, lung, tongue, bladder, and liver. Clinical studies in infants with bovine LF preparations have demonstrated that oral administration increased the number of bifidobacteria in fecal flora and the serum ferritin level, while the ratios of *Enterobacteriaceae, Streptococcus,* and *Clostridium* decreased. A recent clinical study (King et al. 2007) has shown that LF supplementation to healthy infants for 12 months was associated with fewer lower respiratory tract illnesses and higher hematocrits as compared to the control group, which received regular infant formula. Other human studies have shown that LF increased the eradication rate of *H. pylori* gastritis when administered in connection with triple therapy. Also, LF ingestion decreased the incidence of bacteremia and severity of infection in neutropenic patients. In other human studies LF was shown to alleviate symptoms of hepatitis C virus infection and reduce small intestine permeability in drug-induced intestinal injury. LF ingestion has also been shown to promote the cure of tinea pedis. Recent human studies reviewed by Zimecki and Kruzel (2007) suggest that bovine LF administration could be beneficial in stress-related neurodegenerative disorders and treatment of certain cancer types. For the reasons above bovine LF has attracted increasing commercial interest and many products containing added LF have already been launched on the market in Asian countries, in particular. LF is produced industrially by many companies worldwide and it is expected that its use as an ingredient in functional foods and pharmaceutical preparations will increase drastically in the near future (Tamura 2004). Current commercial applications of LF include, e.g., yogurt products marketed in Japan and Taiwan and baby foods and infant formulas marketed in South Korea, Japan, and China. In addition LF has been applied in different dietary supplements that combine, for example, LF and bovine colostrum or probiotic bacteria. Due to potential synergistic actions LF has been incorporated together with lysozyme and lactoperoxidase into human oral health care products, such as toothpastes, mouth rinses, moisturizing gels, and chewing gums.

Lactoperoxidase

LP is a glycoprotein that occurs naturally in colostrum, milk, and many other human and animal secretions. LP represents the most abundant enzyme in milk and can be recovered in substantial quantities from whey using chromatographic techniques (Kussendrager and Hooijdonk 2000). LP catalyzes peroxidation of thiocyanate anion and some halides in the presence of hydrogen peroxide source to generate short-lived oxidation products of SCN-, primarily hypothiocyanate (OSCN-), which kill or inhibit the growth of many species of microorganisms. The hypothiocyanate anion causes oxidation of sulphydryl (SH) groups of microbial enzymes and other membrane proteins leading to intermediary inhibition of growth or killing of susceptible microorganisms. The complete antimicrobial LP/SCN/H202 system was originally characterized in milk by Reiter and Oram (1967). Today this system is considered to be an important part of the natural host defense system in mammals (Boots and Floris 2006). A recent finding is the presence of LP in human airway epithelia where it is likely to actively combat respiratory infections caused by microbial invaders (Gerson et al. 2000). In in vitro studies the LP system has been shown to be active against a wide range of microorganisms, including bacteria, viruses, fungi, molds, and protozoa (Seifu et al. 2005). The LP system is known to be bactericidal against Gram-negative pathogenic and spoilage bacteria, such as *E.coli, Salmonella* spp., *Pseudomonas* spp., and *Campylobacter* spp. On the other hand, the system is bacteriostatic against many Gram-positive bacteria, such as *Listeria* spp., *Staphylococcus* spp., and *Streptococcus* spp. This mechanism also is inhibitory to *Candida* spp. and the protozoan *Plasmodium falciparium,* and it has been shown to inactivate in vitro the HIV type 1 and polio virus (Seifu et al. 2005). In view of the broad antimicrobial spectrum against a variety of spoilage and pathogenic microorganisms the LP system has been investigated extensively for many potential applications. The LP system is considered to provide a natural method for preserving raw milk because milk contains naturally all necessary components to make the system func-

tional. The natural concentrations of thiocyanate and H2O2 can, however, be critical in milk, and the system usually requires activation by addition of a source of these components. The effectiveness of the activated LP system has been demonstrated in many pilot studies and field trials worldwide (Reiter and Härnulv 1984; Anon. 2005; Seifu et al. 2005). Since 1991, the LP system has been approved by the Codex Alimentarius Committee for preservation of raw milk under specified conditions. The method is now being utilized in practice in a number of countries, where facilities for raw milk cooling are not available or are inadequate. The LP system has also found other applications, for example, in dental health care products and animal feeds. A potential new target is gastritis caused by *H. pylori* because this pathogen is killed in vitro by the LP system (Shin et al. 2002). More applications have been envisaged for preservation of different products, for example, meat, fish, vegetables, fruits, and flowers (Boots and Floris 2006).

Glycomacropeptide

GMP is a C-terminal glycopeptide f(106–169) released from the κ-casein molecule at ^{105}Phe-^{106}Met by the action of chymosin. GMP is hydrophilic and remains in the whey fraction in the cheese manufacturing process, whereas the remaining part of κ-casein, termed as para-κ-casein precipitates into the cheese curd. GMP has a molecular weight of about 8,000 Daltons, and it contains a significant (50–60% of total GMP) carbohydrate (glycoside) fraction, which is composed of galactose, *N*-acetyl-galactosamine and *N*-neuraminic acid. The nonglycosylated form of GMP is often termed *caseinomacropeptide* or *CMP*. GMP is the most abundant protein/peptide in whey proteins produced from cheese whey, amounting to 20–25% of the proteins (Abd El-Salam et al. 1996; Thomä-Worringer et al. 2006). The biological activities of GMP have received much attention in recent years. Extensive research has shown that GMP inactivates in vitro microbial toxins of *E.coli* and *V. cholerae*, inhibits in vitro adhesion of cariogenic *Str. mutans* and *Str. sobrinus* bacteria and influenza virus, modulates immune system responses, promotes growth of bifidobacteria, suppresses gastric hormone activities, and regulates blood circulation through antihypertensive and antithrombotic activity (Brody 2000; Manso and López-Fandiño 2004). Recently, Rhoades et al. (2005) have demonstrated that GMP inhibited effectively in vitro adhesion of pathogenic (VTEC and EPEC) *E.coli* strains to human HT29 colon carcinoma cells, whereas probiotic *Lactobacillus* strains were inhibited to a lesser extent. In another study (Brück et al. 2003) oral GMP administration was shown to reduce *E. coli*–induced diarrhea in infant rhesus monkeys.

Owing to its glycoprotein nature GMP has interesting nutritional and physicochemical properties. GMP is rich in branched-chain amino acids and low in methionin, which makes it a useful ingredient in diets for patients suffering from hepatic diseases. GMP has no phenylalanin in its amino acid composition and this fact makes it suitable for nutrition in case of phenylketonuria. On the other hand, animal model studies have suggested that the high sialic acid content of GMP may deliver beneficial effects for brain development and improvement of learning ability (Wang et al. 2001). The potential role of GMP in regulation of intestinal functions has been investigated in a number of studies (Brody 2000; Manso and López-Fandiño 2004). GMP has been reported to inhibit gastric secretions and slow down stomach motility. It has also been suggested that GMP stimulates the release of cholecystokinin (CKK), the satiety hormone involved in controlling food intake and digestion in the duodenum of animals and humans (Yvon et al. 1994). Another interesting finding is that GMP can be absorbed intact and partially digested into the blood circulation of adult humans after milk or yogurt ingestion (Chabance et al. 1998). In recent years commercial products containing GMP or CMP have been launched on the market for the purpose of appetite control and weight management. The clinical efficacy of such products, however, remains to be established. A study conducted with human adults for a short time period revealed that CMP had no effect on food energy intake or subjective indicators of satiety (Gustafson et al. 2001). On the other hand, GMP may have a beneficial role in modulation of gut microflora, because GMP has been shown to promote the growth of bifidobacteria, probably due to its carbohydrate content (Manso and López-Fandiño 2004). Pure GMP can be recovered in large quantities from cheese whey by chromatographic or ultrafiltration techniques. The biological efficacy of

this component in humans still remains to be established in clinical studies. On the other hand, the multiple physicochemical properties of GMP make it a potential ingredient for many food applications (Thomä-Worringer et al. 2006).

Production and Functionality of Bioactive Peptides

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts and Weiler 2003). The activity of peptides is based on their inherent amino acid composition and sequence. The size of active sequences may vary from 2–20 amino acid residues, and many peptides are known to reveal multifunctional properties. Milk proteins are considered the most important source of bioactive peptides. Over the last decade a great number of peptide sequences with different bioactivities have been identified in various milk proteins. The best characterized sequences include antihypertensive, antithrombotic, antimicrobial, antioxidative, immunomodulatory, and opioid peptides (Korhonen and Pihlanto 2003). These peptides have been found in enzymatic protein hydrolysates and fermented dairy products, but they can also be released during gastrointestinal digestion of proteins, as reviewed in many articles (Meisel 1998; Clare and Swaisgood 2000; FitzGerald and Meisel 2003; Kilara and Panyam 2003; Pihlanto and Korhonen 2003; Meisel 2005; Korhonen and Pihlanto 2006, 2007b; Gobbetti et al. 2007; Hartmann and Meisel 2007). Milk-derived bioactive peptides may exert a number of physiological effects in vivo on the gastrointestinal, cardiovascular, endocrine, immune, central nervous, and other body systems, as shown in Figure 2.2.

Bioactive peptides are inactive within the sequence of the parent protein molecule and can be released from precursor proteins in the following ways: 1) enzymatic hydrolysis by digestive enzymes, 2) fermentation of milk with proteolytic starter cultures, and 3) proteolysis by enzymes derived from microorganisms or plants. In many studies a combination of 1 and 2 or 1 and 3, respectively, has proven effective in generating biofunctional peptides (Korhonen and Pihlanto 2007a). Examples of bioactive peptides produced by the treatments above are described below. A great number of studies have demonstrated that hydrolysis of milk proteins by digestive enzymes can produce biologically active peptides (Korhonen and Pihlanto 2006). The most prominent enzymes are pepsin, trypsin, and chymotrypsin, which have

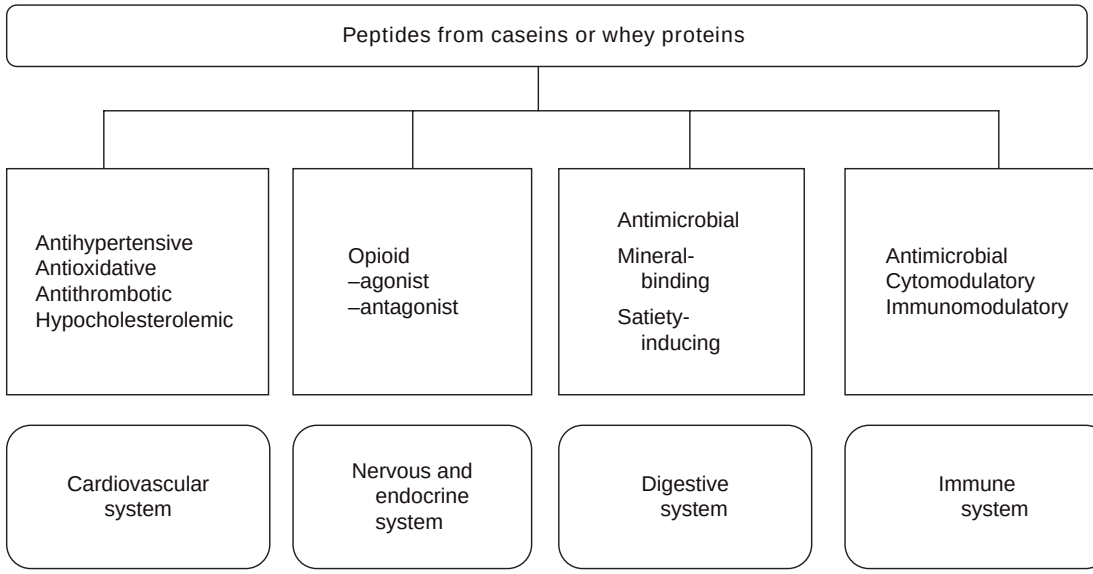

Figure 2.2. Physiological functionality of milk-derived bioactive peptides.

been shown to liberate a great number of antihypertensive peptides, calcium-binding phosphopeptides (CPPs), antibacterial peptides, immunomodulatory peptides, and opioid peptides both from different casein (α-,β- and κ-casein) and whey protein (α-La, β-Lg, and GMP) fractions (FitzGerald et al. 2004; Meisel and FitzGerald 2003; Yamamoto et al. 2003; Gobbetti et al. 2004; 2007). Blood pressure–reducing peptides, which inhibit the angiotensin converting enzyme I (ACE), are the most studied ones (López-Fandiño et al. 2006; Murray and FitzGerald 2007). Casein hydrolysates have in some studies (Otte et al. 2007) produced higher ACE-inhibitory activity than whey protein hydrolysates, but also whey peptides, e.g., Ala-Leu-Pro-Met-His-Ile-Arg (ALPMHIR) or lactokinin from tryptic digest of β-Lg, have been identified with strong antihypertensive activity (Mullally et al. 1997; Maes et al. 2004; Ferreira et al. 2007). Other proteolytic enzymes, such as alcalase, thermolysin, subtilisin, and successive treatment with pepsin and trypsin in order to simulate gastrointestinal digestion also have been employed to release various bioactive peptides, including CCPs (McDonagh and FitzGerald 1998), ACE-inhibitory (Pihlanto-Leppälä et al. 2000; de Costa et al. 2007; Roufik et al. 2007), antibacterial (López-Expósito and Recio 2006; López-Expósito et al. 2006), antioxidative (Pihlanto 2006), immunomodulatory (Gauthier et al. 2006a), and opioid-like (Pihlanto 2001). Mizuno et al. (2004) measured the ACE-inhibitory activity of casein hydrolysates upon treatment with nine different commercially available proteolytic enzymes. Among these enzymes, a protease isolated from *Aspergillus oryzae* showed the highest ACE-inhibitory activity in vitro per peptide.

Many of the industrially utilized lactic acid bacteria (LAB)–based starter cultures are highly proteolytic and the release of different bioactive peptides from milk proteins through microbial proteolysis is now well documented (Matar et al. 2003; Fitzgerald and Murray 2006; Gobbetti et al. 2007). Many studies have demonstrated that *Lactobacillus helveticus* strains are capable of releasing antihypertensive peptides, the best known of which are ACE-inhibitory tripeptides Val-Pro-Pro and Ile-Pro-Pro. The hypotensive capacity of these peptides has been demonstrated in many rat-model and human studies (Nakamura et al. 1995; Hata et al. 1996; Sipola et al. 2002; Seppo et al. 2003; Jauhiainen et al. 2005). Also yogurt bacteria, cheese starter bacteria and commercial probiotic bacteria have been demonstrated to produce different bioactive peptides in milk during fermentation (Gómez-Ruiz et al. 2002; Fuglsang et al. 2003; Gobbetti et al. 2004; Donkor et al. 2007). In a recent study (Virtanen et al. 2006) it was demonstrated that fermentation of milk with single industrial dairy cultures generated antioxidant activity in the whey fraction. The activity correlated positively with the degree of proteolysis suggesting that peptides were responsible for the antioxidative property. In another study (Chen et al. 2007) fermentation of milk with a commercial starter culture mixture of five LAB strains followed by hydrolysis with a microbial protease increased ACE-inhibitory activity of the hydrolysate and two strong ACE-inhibitory tripeptides (Gly-Thr-Trp) and (Gly-Val-Trp) were identified. The hypotensive effect of the hydrolysate containing these peptides was demonstrated in an animal model study using spontaneously hypertensive rats (SHR). Quirós et al. (2007) identified several novel ACE-inhibitory peptides in milk fermented with *Enterococcus faecalis* strains isolated from raw milk. Two β-casein–derived peptides f(133–138) and f(58–76) showed distinct antihypertensive activity when administered orally to SHR rats. Donkor et al. (2007) studied the proteolytic activity of several dairy LAB cultures and probiotic strains (*Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Lactobacillus casei*) as determinant of growth and in vitro ACE-inhibitory activity in milk fermented with these single cultures. All the cultures released ACE-inhibitory peptides during growth with a *B. longum* strain and the probiotic *L. acidophilus* strain showing the strongest ACE-inhibitory activity. Kilpi et al. (2007) studied the impact of general aminopeptidase (PepN) and X-prolyl dipeptidyl aminopeptidase (PepX) activity of the *L. helveticus* CNRZ32 strain on the ACE-inhibitory activity in fermented milk by taking advantage of peptidase-negative derivatives of the same strain. Increased ACE-inhibitory activity was attained in milk fermented by the peptidase-deficient mutants. The results suggested that both peptidases are involved in the release or degradation of ACE-inhibitory peptides during the fermentation process. This type of gene engineering approach could enable future production of tailor-made bioactive peptides using genetically modified LAB strains.

A number of in vitro studies have demonstrated that fermentation of milk with starter cultures or enzymes derived from such cultures prior to or after treatment with digestive enzymes can enhance the release and alter the profile of bioactive peptides produced. It can be speculated that such events may happen also under in vivo conditions in the gastrointestinal tract. Sütas et al. (1996) found that successive hydrolysis of casein fractions with pepsin and trypsin, respectively, produced both immunostimulatory and immunosuppressive peptides as measured in vitro with human blood lymphocytes. When the caseins were hydrolyzed by enzymes isolated from a probiotic strain of *Lactobacillus GG* var. *casei* prior to pepsin-trypsin treatment, the hydrolysate became primarily immunosuppressive, suggesting that the bacterial proteinases had modified the immunomodulatory profile of caseins. These results imply that it may be possible to reduce allergenic properties of milk proteins by a double enzymatic treatment as described above. Pihlanto-Leppälä et al. (1998) studied the potential formation of ACE-inhibitory peptides from cheese whey and caseins during fermentation with various commercial dairy starters used in the manufacture of yogurt, ropy milk, and sour milk. No ACE-inhibitory activity was observed in these hydrolysates. However, additional successive digestion of the hydrolysates with pepsin and trypsin resulted in the release of several strong ACE-inhibitory peptides derived primarily from α_{s1}-casein and β-casein. In a recent study Lorenzen and Meisel (2005) demonstrated that trypsin treatment of yogurt milk prior to fermentation with yogurt cultures resulted in a release of phosphopeptide-rich fractions. In particular, the release of the CPP sequences β-CN(1–25)-4P and α_{s1}–CN(43–79)-7P during trypsin treatment was pronounced, whereas the proteolysis caused by peptidases of the yogurt cultures was not significant.

Bioactive Peptides in Dairy Products and Novel Applications

A great variety of naturally formed bioactive peptides have been found in fermented traditional dairy products, such as yogurt, sour milk, dahi (an Indian fermented milk), quark, and different types of cheese (FitzGerald and Murray 2006). The occurrence, specific activity, and amount of bioactive peptides in fermented dairy products depend on many factors, such as type of starters used, type of product, time of fermentation, and storage conditions (Korhonen and Pihlanto 2006; Ardö et al. 2007). Ong et al. (2007) have studied the release of ACE-inhibitory peptides in cheddar cheese made with starter lactococci and commercial probiotic lactobacilli. The results demonstrated that ACE inhibition in cheese was dependent on proteolysis to a certain extent. The addition of probiotic strains increased the ACE-inhibitory activity in cheese during ripening at 4 °C.

Table 2.2 gives examples of bioactive peptides found in different fermented dairy products. It is noteworthy that in these products peptides with different bioactivities, e.g., calcium-binding, antihypertensive, antioxidative, immunomodulatory, and antimicrobial, can be found at the same time. The formation of peptides can be regulated to some extent by starter cultures used, but the stability of desired peptides during storage seems difficult to control (Ryhänen et al. 2001; Bütikofer et al. 2007). The potential health benefits attributable to bioactive peptides formed in traditional dairy products warrant extensive research. In a recent study, Ashar and Chand (2004) demonstrated that administration of dahi reduced blood pressure in middle-aged hypertensive male subjects. Two fermented milk products are on the market, and they have been developed with the aim to provide clinically effective amounts of antihypertensive peptides, that can be consumed on a daily basis as part of a regular diet. These commercial products are the Japanese fermented milk product Calpis or Ameal S and the Finnish fermented milk product Evolus; both contain the same ACE-inhibitory peptides Ile-Pro-Pro and Val-Pro-Pro. The blood pressure–reducing effects of both products have been established in many animal-model and human studies (FitzGerald et al. 2004; Hartmann and Meisel 2007; Korhonen and Pihlanto 2007b).

An increasing number of ingredients containing specific bioactive peptides derived from milk protein hydrolysates have been launched on the market within the past few years or are currently under development. Such peptides possess, e.g., anticariogenic, antihypertensive, mineral-binding, and stress-relieving properties. Examples of these commercial ingredients and their applications are listed in Table 2.3.

Table 2.2. Bioactive peptides identified in fermented dairy products*

Product	Examples of Identified Bioactive Peptides	Bioactivity	Reference
Cheese Type			
Cheddar	α_{s1}- and β-casein fragments	Several phosphopeptides	Singh et al. (1997)
Italian varieties: Mozzarella, Crescenza, Italico, Gorgonzola	β-cn f(58–72)	ACE inhibitory	Smacchi and Gobbetti (1998)
Gouda	α_{s1}-cn f(1–9) β-cn f(60–68)	ACE inhibitory	Saito et al. (2000)
Festivo	α_{s1}-cn f(1–9), f(1–7), f(1–6)	ACE inhibitory	Ryhänen et al. (2001)
Emmental	α_{s1}- and β-casein fragments	Immunostimulatory, several phosphopeptides, antimicrobial	Gagnaire et al. (2001)
Manchego (sheep milk)	Sheep α_{s1}-, α_{s2}- and β-casein fragments	ACE inhibitory	Gómez-Ruiz et al. (2002)
44 samples of hard, semihard and soft cheese samples	Val-Pro-Pro, Ile-Pro-Pro	ACE inhibitory	Bütikofer et al. (2007)
Cheddar	α_{s1}-cn f(1–6), f(1–7), f(1–9), f(24–32), f(102–110), β-cn f(47–52), f(193–209)	ACE inhibitory	Ong et al. (2007)
Fermented Milk			
Sour milk	β-cn f(74–76), f(84–86) κ-cn f(108–111)	Antihypertensive	Nakamura et al. (1995)
Dahi	Ser-Lys-Val-Tyr-Pro	ACE inhibitory	Ashar and Chand (2004)
Yogurt (sheep milk)	Active peptides not identified	ACE inhibitory	Chobert et al. (2005)
Kefir (goat milk)	PYVRYL, LVYPFTGPIPN**	ACE inhibitory	Quirós et al. (2005)
Fermented milk (probiotic and dairy strains)	Active peptides not identified	ACE inhibitory	Donkor et al. (2007)

Abbreviations: α_{s1}-cn = α_{s1}-casein, β-cn = β-casein, κ-cn = κ-casein.

*Modified from Korhonen and Pihlanto (2006).

**One-letter amino acid code.

BIOACTIVE LIPIDS

Bovine milk fat is composed of more than 400 fatty acids with different chemical composition. Most of these fatty acids are esterified to the glycerol molecule backbone and appear in milk fat mostly as triacylglycerols (Jensen 2002). Conjugated linoleic acid (CLA) refers to a collection of positional and geometrical isomers of *cis*-9, *cis*-12-octadecadienoic acid (C18:2) with a conjugated double bond system. The major CLA isomer in milk fat is 9-*cis*, 11-*trans*, also called *rumenic acid* (Collomb et al. 2006). CLA is formed partially by bioconversion of polyunsaturated fatty acids in the rumen by anaerobic bacteria, e.g., *Butyrovibrio fibrisolvens*, and primarily endogenously by Δ9-desaturation of vaccenic acid in the mammary gland of lactating cows (Griinari et al. 2000). Milk fat is the richest natural source of CLA, and contents ranging from 2 to 53.7 mg/g fat have been recorded in different studies (Collomb et al.

Table 2.3. Commercial dairy products and ingredients based on bioactive peptides*

Brand Name	Type of Product	Bioactive Peptides/Protein Hydrolysates	Health/Function Claims	Manufacturer
Calpis/Ameal S	Sour milk	Val-Pro-Pro, Ile-Pro-Pro, derived from β-casein and κ-casein	Blood pressure reduction	Calpis Co., Japan
Evolus	Fermented milk	Val-Pro-Pro, Ile-Pro-Pro, derived from β-casein and κ-casein	Blood pressure reduction	Valio Oy, Finland
BioZate	Hydrolyzed whey protein isolate	β-lactoglobulin fragments	Blood pressure reduction	Davisco, USA
BioPURE-GMP	Whey protein isolate	κ-casein f(106–169) (Glycomacropeptide)	Anticariogenic, antimicrobial, antithrombotic	Davisco, USA
ProDiet F200/Lactium	Flavored milk drink, confectionery, capsules	α_{s1}-casein f(91–100) (Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg)	Stress symptoms relief	Ingredia, France
Festivo	Fermented low-fat hard cheese	α_{s1}-casein f(1–9) α_{s1}-casein f(1–6) α_{s1}-casein f(1–7)	Antihypertensive	MTT Agrifood Research Finland
Cysteine Peptide	Ingredient	Milk protein–derived peptide	Aids sleep	DMV International, the Netherlands
C12 Peption	Ingredient	Casein-derived dodeca-peptide FFVAPFPEVFGK**	Blood pressure reduction	DMV International, the Netherlands
Capolac	Ingredient	Caseinophosphopeptide	Helps mineral absorption	Arla Foods Ingredients, Sweden
PeptoPro	Flavored drink	Casein-derived di- and tripeptides	Improves athletic performance and muscle recovery after exercise	DSM Food Specialties, the Netherlands
Vivinal Alpha	Ingredient	α-lactalbumin rich whey protein hydrolysate	Aids relaxation and sleep	Borculo Domo Ingredients (BDI), the Netherlands
Praventin	Ingredient	Lactoferrin-enriched whey protein hydrolysate	Helps reduce symptoms of skin infections	DMV International, the Netherlands

*Modified from Korhonen and Pihlanto (2006).

**One-letter amino acid code.

2006). The wide range of CLA values can be attributed to various factors such as feeding regime, forage preservation, geographical regions, and breed of cows as well as goats and sheep. A number of studies have confirmed that pasture feeding can increase significantly CLA concentrations as compared to indoor feeding. Also the grass composition of pasture seems to affect the CLA level because it was found to be almost 2–3 times higher in the milk of cows grazed in the alpine region as compared to milk from cows grazed in the lowlands (Collomb et al. 2001). Many experimental studies have demonstrated that feeding cows with meals containing plant oils and/or marine oils can effectively increase the CLA content. The majority of these studies suggest that concentrates rich in linoleic acid (rapeseed, soybean, sunflower) have a better impact than other polyunsaturated plant oils (peanut, linseed), whereas the dietary fish oils and their combination with plant oils appear even more effective than plant oils alone (Shingfield et al. 2006).

The effects of manufacturing conditions on the content of CLA in milk and dairy products have been studied by many authors. As reviewed by Collomb et al. (2006), inconsistent results have been reported on the impact of heat treatment on the CLA content in milk. CLA-enriched milk has been shown to produce softer butter than butter from ordinary milk. In cheese manufacture, processing seems to have only minor effects on the CLA content of final products (Chamba et al. 2006; Bisig et al. 2007). Properties of cheeses manufactured from CLA-enriched milk have been shown to differ from control cheeses. In cheddar and Edam types the texture of cheese made from CLA-enriched milk was found to be softer than in control cheese but no major differences were noticed in the organoleptic properties (Avramis et al. 2003; Ryhänen et al. 2005). In most studies the CLA content has been found to be higher in organic milk and organic dairy products in comparison to conventionally produced milk and products (Bisig et al. 2007). Possibilities for increasing the CLA content of dairy products with microbial cultures have been investigated in many studies (see reviews by Sieber et al. 2004; Bisig et al. 2007). Many dairy starter cultures, such as propionibacteria, lactobacilli, and bifidobacteria have been found to be able to convert linoleic acid into CLA in culture media. However, inconsistent results have been obtained about CLA production by these cultures in yogurt and cheese. Because this approach seems to have limited success, Bisig et al. (2007) have suggested as a technological solution the production of CLA in a special culture medium and addition of the isolated CLA into dairy products.

During the past 20 years a large number of animal model studies have demonstrated multiple health benefits for dietary CLA. These positive effects include anticarcinogenic, antiatherogenic, antidiabetic, antiobesity, and enhancement of immune system (Parodi 1999; Pariza et al. 2001; Belury 2002; Collomb et al. 2006; Pfeuffer and Schrezenmeir 2006b). The effects seem to be mediated primarily by two CLA isomers: *cis*-9, *trans*-11 and *trans*-10, *cis*-12, but the impact may differ depending on the isomer. For example, Park et al. (1999) have shown that the latter isomer is responsible for inducing reduction of body fat in mice, whereas the former isomer enhances growth in young animal models (Pariza et al. 2001). In some other cases these isomers may act together to produce a particular effect. In milk fat the *cis*-9, *trans*-11 isomer amounts to 75–90% of total CLA. The average total daily dietary intake of CLA is estimated to range between 95 and 440 mg and differs from country to country. Optimal dietary intake remains to be established, but it has been proposed that a daily intake of 3.0 to 3.5 grams of CLA is required to provide anticarcinogenic response in humans (Collomb et al. 2006). Strong evidence from animal trials supports an influence of CLA intake on lowering of body weight and fat mass and increase in lean body mass. Human studies carried out so far do not support, however, any weight loss–inducing effect of CLA, but suggest a lowering effect on body fat associated with an increase in lean body mass (Larsen et al. 2003).

As for the antidiabetic effect of CLA, contradictory results have been reported in animal and human studies. The antiatherogenic effect of CLA has also been a controversial issue. In rodents and humans, dietary CLA supplementation has produced inconsistent results, as far as the reduction of serum cholesterol and triglyceride levels are concerned (Terpstra 2004). A great number of in vitro experiments and animal trials have been conducted on anticarcinogenic effects of synthetic CLA and rumenic acid–enriched milk fat (Ip et al. 2003; Parodi 2004; Lee and Lee 2005). A majority of these

studies support the role for dietary CLA in protection against various types of cancer, e.g., breast, prostate, and colon cancer, but the underlying mechanisms warrant further studies. Very few relevant human experimental data are available as yet. An epidemiological study (Aro et al. 2000) has suggested an inverse association between dietary and serum CLA and risk of breast cancer in postmenopausal women. Another recent cohort study suggested that the high intake of CLA containing dairy foods may reduce the risk of colorectal cancer (Larsson et al. 2005).

Another biologically interesting lipid group in milk fat is the polar lipids, which are mainly located in the milk fat globule membrane (MFGM). It is a highly complex biological structure that surrounds the fat globule stabilizing it in the continuous aqueous phase of milk and preventing it from enzymatic degradation by lipases (Mather 2000; Spitsberg 2005). The membrane consists of about 60% proteins and 40% lipids that are mainly composed of triglycerides, cholesterol, phospholipids, and sphingolipids. The polar lipid content of raw milk is reported to range between 9.4 and 35.5 mg per 100 g of milk. The major phospholipid fractions are phosphatidylethanolamine and phosphatidylcholine followed by smaller amounts of phosphatidylserine and phosphatidylinositol. The major sphingolipid fraction is sphingomyelin with smaller portions of ceramides and gangliosides (Jensen 2002). In processing milk into different dairy products, the polar lipids are preferentially enriched in the aqueous phases like skimmed milk, buttermilk and butter serum.

The polar lipids in milk are gaining increasing interest due to their nutritional and technological properties. These compounds are secondary messengers involved in transmembrane signal transduction and regulation, growth, proliferation, differentiation, and apoptosis of cells. They also play a role in neuronal signaling and are linked to age-related diseases, blood coagulation, immunity, and inflammatory responses (Pettus et al. 2004). In particular, sphingolipids and their derivatives are considered highly bioactive components possessing anticancer, cholesterol-lowering, and antibacterial activities (Rombaut and Dewettinck 2006). Furthermore, butyric acid and butyrate have been shown to inhibit development of colon and mammary tumors (Parodi 2003). These promising results from cell culture and animal-model studies warrant further confirmation and human clinical studies but suggest that sphingolipid-rich foods or supplements could be beneficial in the prevention of breast and colon cancers and bowel-related diseases.

GROWTH FACTORS

The presence of factors with growth-promoting or growth-inhibitory activity for different cell types was first demonstrated in human colostrum and milk during the 1980s and later in bovine colostrum, milk, and whey (Pakkanen and Aalto 1997; Gauthier et al. 2006a; Pouliot and Gauthier 2006). At present the following growth factors have been identified in bovine mammary secretions: BTC (beta cellulin), EGF (epidermal growth factor), FGF1 and FGF2 (fibroblast growth factor), IGF-I and IGF-II (insulin-like growth factor), TGF-β1 and TGF-β2 (transforming growth factor)and PDGF (platelet-derived growth factor). The concentrations of all known growth factors are highest in colostrum during the first hours after calving and decrease substantially thereafter. The most abundant growth factors in bovine milk are EGF (2–155 ng/mL), IGF-I (2–101 ng/mL), IGF-II (2–107 ng/mL), and TGF-β2 (13–71 ng/mL), whereas the concentrations of the other known growth factors remain below 4 ng/mL (Pouliot and Gauthier 2006). Basically, the growth factors are polypeptides and their molecular masses range between 6,000 and 30,000 Daltons with amino acid residues varying from 53 (EFG) to about 425 (TGF-β2), respectively. It is noteworthy that the growth factors present in milk seem to withstand pasteurization and even UHT (ultrahigh temperature) heat treatment of milk relatively well (Gauthier et al. 2006a).

The main biological functions of milk growth factors have been reviewed recently (Gauthier et al. 2006a; Pouliot and Gauthier 2006; Tripathi and Vashishtha 2006). In essence EGF and BTC stimulate the proliferation of epidermal, epithelial, and embryonic cells. Furthermore, they inhibit the secretion of gastric acid and promote wound healing and bone resorption. The TGF-β family plays an important role in the development of the embryo, tissue repair, formation of bone and cartilage, and regulation of the immune system. Both forms of TGF-β are known to stimulate proliferation of connective tissue cells and inhibit proliferation of lymphocytes

and epithelial cells. Both forms of IGF stimulate proliferation of many cell types and regulate some metabolic functions, e.g., glucose uptake and synthesis of glycogen (Pouliot and Gauthier 2006).

Contradictory results are reported from studies concerning the stability of growth factors in the gastrointestinal tract. Many animal-model studies have, however, shown that EGF, IGF-I, and both TGF forms can provoke various local effects on the gastrointestinal tract and can be absorbed intact or partially from intestine into blood circulation. These results are supported by findings that the presence of proteins and protease inhibitors in milk can protect EFG against gastric and intestinal proteolytic degradation (Gauthier et al. 2006b). At present there are very few human studies conducted about the potential physiological effects of oral administration of bovine growth factors. Mero et al. (2002) have demonstrated that oral administration of a dietary colostrum-based food supplement increased serum IGF-I in male athletes during a short-term endurance and speed training. According to Gauthier et al. (2006a) increasing evidence supports the view that orally administered growth factors would retain their biological activity and exhibit in the body a variety of local and systemic functions. In recent years several health-related applications of growth factors based on bovine milk or colostrum have been proposed but just a few have been commercialized until now. The main health targets of those product concepts have been skin disorders, gut health, and bone health (Donnet-Hughes et al. 2000). Playford et al. (2000) suggested that colostrum-based products containing active growth factors could be applied to prevent the side effects of nonsteroid anti-inflammatory drugs (NSAIDs) and symptoms of arthritis. To this end specific methods for extraction of certain growth factors from acid casein or cheese whey have been developed. An acid casein extract rich in TGF-β2 has been tested successfully in children suffering from Crohn's disease. Another growth factor extract from cheese whey has shown promising results in animal models and humans in treatment of oral mucositis and wound healing, for example, leg ulcers (Smithers 2004; Pouliot and Gauthier 2006). Other potential applications could be treatment of psoriasis, induction of oral tolerance in newborn children against allergies, and cytoprotection against intestinal damage caused by chemotherapy.

OTHER BIOACTIVE COMPOUNDS

In addition to the major bioactive components described above, bovine mammary secretions are known to contain a great number of other indigenous minor bioactive components with distinct characteristics. These include hormones, cytokines, oligosaccharides, nucleotides, and specific proteins. A few of the best characterized of these factors are described briefly below.

A large number of hormones of either steroidic or peptidic origin are found in bovine colostrum and milk (Grosvenor et al. 1993; Jouan et al. 2006). These molecules belong to the following main categories: gonadal hormones (estrogens, progesterone, androgens), adrenal (glucocorticoids), pituitary (prolactin, growth hormone), and hypothalamic hormones (gonadotropin-releasing hormone, luteinizing-hormone–releasing hormone, thyrotropin-releasing hormone), and somatostatin. Other hormones of interest are bombesin, calcitonin, insulin, melatonin, and parathyroid hormone. In general the hormones occur in very small concentrations (picograms or nanograms per millilitre) and the highest quantities are usually found in colostrum, declining thereafter drastically at the onset of the main lactation period. For example, the concentration of prolactin found in colostrum varies between 500 and 800 ng/mL compared to 6–8 ng/mL in milk, as cited by Jouan et al. (2006). The biological significance of hormones occurring in mammary secretions is not fully elucidated, but in general they are considered important both in the regulation of specific functions of the mammary gland and in the growth of the newborn, including development and maturation of its gastrointestinal and immune systems. Hormones in colostrum could also temporarily regulate the activity of some endocrine glands until the newborn's hormonal system reaches maturity (Bernt and Walker 1999). There is some evidence from animal-model and human studies that oral ingestion of melatonin-enriched milk improves sleep and diurnal activity (Valtonen et al. 2005).

Nucleotides, nucleosides, and nucleobases belong to the nonprotein-nitrogen fraction of milk. These components are suggested to be acting as pleiotrophic factors in the development of brain functions (Schlimme et al. 2000). Because of the important role of nucleotides in infant nutrition, some infant and follow-up formulae have been supplemented

with specific ribonucleotide salts. Also nucleotides could have significance as exogenous anticarcinogens in the control of intestinal tumor development (Michaelidou and Steijns 2006).

Bovine colostrum contains relatively high concentrations of cytokines, such as IL-1 (interleukin), IL-6, TNF-α (tumor necrosis factor), IF-γ (interferon), and IL-1 receptor antagonist, but their levels decline markedly in mature milk (Kelly 2003). The biological role of these components and their potential applications have been reviewed recently (Struff and Sprotte 2007: Wheeler et al. 2007).

Among the minor whey proteins many biologically active specific molecules and complexes, such as proteose-peptones, serum albumin, osteopontin, and the enzymes lysozyme and xanthine oxidase, have been characterized (Mather 2000; Fox and Kelly 2006). Another interesting protein complex isolated from whey is milk basic protein (MBP), which consists of several low-molecular compounds, such as kininogen, cystatin, and HMG-like protein. In cell culture and animal-model studies BMP has been shown to stimulate bone formation and simultaneously suppress bone resorption (Kawakami 2005). The bone-strenghtening effects of BMP have been confirmed in human trials and the product has been evaluated for safety and commercialized in Japan.

The milk fat globule membrane contains several lipid and protein fractions, e.g., butyrophilin, CD36, mucin, and fatty acid binding protein that have shown specific biological functions. These components have been reviewed recently by Spitsberg (2005) and Fong et al. (2007).

Colostrinin™ (CLN) is a proline-rich polypeptide complex that was originally isolated from ovine colostrum. Colostrinin has been identified and isolated also from bovine colostrum in the form of a peptide complex with a molecular mass around 14,000 Daltons (Sokolowska et al. 2008). In in vitro and animal studies, ovine colostrinin has been shown to exert immunomodulatory properties. A recent clinical study (Billikiewicz and Gaus 2004) suggests that colostrinin is beneficial in the treatment of mild or moderate Alzheimer's disease. The mechanism of action remains, however, to be elucidated and the efficacy confirmed in further clinical studies.

Human milk contains significant concentrations (5–10 g/L) of complex oligosaccharides, which are considered to exert different health benefits to the newborn (Kunz et al. 2000; Kunz and Rudloff 2006). They may contribute to the growth of beneficial flora in the intestine, stimulation of the immune system and defense against microbial infections. Similar oligosaccharides and glycoconjugates occur in bovine milk and colostrum but in much smaller concentrations (Gopal and Gill 2000; Martin et al. 2001). The low concentration and complex nature of these compounds has hindered their characterization and utilization in health care and food products. Recent advances in analytical techniques have, however, enabled a more detailed structural and functional analysis of bovine milk–derived oligosaccharides. Membrane separation-based processes have been developed for the recovery of sialyloligosaccharides from cheese whey. Such ingredients could have potential, for example, as a prebiotic component in functional foods and infant formulae (Mehra and Kelly 2006).

CONCLUSIONS

The current global interest in developing health-promoting functional foods provides a timely opportunity to tap the myriad of innate bioactive milk components for inclusion in such formulations. Industrial or semi-industrial scale processing techniques are available for fractionation and isolation of major proteins from colostrum and milk, and a few whey-derived native proteins, peptides, growth factors, and lipid fractions are already manufactured commercially. They present an excellent source of well-studied natural ingredients for different applications in functional foods. It can be envisaged that in the near future several breakthrough products based on these ingredients will be launched on worldwide markets. They could be targeted to infants, the elderly, and immune-compromised people as well as to improve performance and prevent diet-related chronic diseases. Moreover, there is a need to study and exploit the health potential of other minor components occurring naturally in colostrum and milk. In future studies more emphasis should be given also to the health-promoting potential hidden in the vast range of traditional dairy products consumed worldwide and produced from other mammals besides cows, such as goats, sheep, camels, mares, yaks, etc.

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3
Bioactive Components in Goat Milk

Young W. Park

INTRODUCTION

As science advanced and people became more conscious about their health and health-related foods, a new field of research emerged dealing with bioactive or biogenic compounds, which includes bioactive peptides in foods. Bioactive peptides (BPs) were described earlier by Mellander (1950); he elucidated the effects of casein (CN)-derived phosphorylated peptides in the vitamin D–independent bone calcification of rachitic infants (Gobbetti et al. 2007). During the last 2 decades, food proteins have gained increasing value due to the rapidly expanding knowledge about physiologically and functionally active peptides released from a parent protein source (Korhonen and Pihlanto 2007).

BPs have been defined as specific protein fragments that have a positive impact on body function or condition and may ultimately influence health (Kitts and Weiler 2003). BPs can be delivered to the consumers in conventional foods, dietary supplements, functional foods, or medical foods. BPs are inactive within the sequence of the parent protein and can be released in three ways: 1) through hydrolysis by digestive enzymes, 2) through hydrolysis of proteins by proteolytic microorganisms, and 3) through the action of proteolytic enzymes derived from microorganisms or plants (Korhonen and Pihlanto 2007). In 1991, the claim of BPs was defined as the concept of Food for Specified Health Use (FOSHU) in Japan, and in 1993 the U.S. introduced the concept of BPs as health claims for foods having the properties of reducing diseases (Gobbetti et al. 2007).

Milk from various mammals contains a heterogeneous mixture of secretory components with a wide variety of chemical and functional activity. This diversified composition and functionality is exemplified by the protein component, which represents a myriad of serum and glandular-derived species differing in molecular size, concentration, and functionality (Regester et al. 1997). The study of these bioactive components in human or other species' milk has been difficult because of their biochemical complexities, the small concentrations of certain very potent agents in milk, the need to develop special methods to quantify certain factors due to their particular forms in milk, the compartmentalization of some of the agents, and the dynamic effects of length of lactation and other maternal factors upon the concentrations or functions of the components of the systems (Goldman and Goldblum 1995).

Caprine milk has been recommended as an ideal substitute for bovine milk, especially for those who suffer from cow milk allergy (CMA) (Rosenblum and Rosenblum 1952; Walker 1965; Van der Horst 1976; Taitz and Armitage 1984; Park 1994; Park and Haenlein 2006). Caprine milk has played an important role in the nutritional and economic well-being of humanity by providing daily essential proteins and minerals, such as calcium and phosphate, to the people of developing countries where cow milk is not readily available (Haenlein and Caccese 1984; Park and Chukwu 1988). Compared to cow or human milk, goat milk reportedly possesses unique biologically active properties, such as high digestibility, distinct alkalinity, high buffering capacity, and certain therapeutic values in medicine and

human nutrition (Gamble et al. 1939; Walker 1965; Devendra and Burns 1970; Haenlein and Caccese 1984; Park and Chukwu 1988; Park 1991, 1994; Park and Haenlein 2006).

Although numerous studies have been conducted and reported recently on BPs and/or bioactive components in human and bovine milk and/or their products, the research on other species' milk, including goats, has not been explored sufficiently. This chapter presents the current knowledge on bioactive components of caprine milk with respect to various forms of naturally occurring bioactive compounds, their physiological, nutritional, and biochemical functionalities for human health, and potential applications and production in functional foods.

BIOACTIVE PROPERTIES OF GOAT MILK: ITS HYPOALLERGENIC, NUTRITIONAL, AND THERAPEUTIC SIGNIFICANCE

Dietary proteins of animal or plant foods can provide rich sources of biologically active peptides. Once bioactive peptides are liberated by digestion or proteolysis, they may impart in the body different physiological effects on the gastrointestinal, cardiovascular, endocrine, immune, and nervous systems (Korhonen and Pihlanto 2007). However, the original macromolecular proteins such as cow milk caseins and whey proteins can cause allergic responses to certain individuals. Goat milk, on the other hand, has been known for its hypoallergenic and therapeutic properties in human nutrition and health, suggesting that caprine milk may possess certain bioactive and metabolically active components that may be unique to this species' milk.

CMA is a frequent disease in infants, although its etiologic mechanisms are not clearly defined (Heyman and Desjeux 1992; Park 1994; Park and Haenlein 2006). Caseins as well as beta-lactoglobulin (MW 36,000) which is the major whey protein in cow milk, not found in human breast milk, are mostly responsible for cow milk allergy (Heyman et al. 1990; Park 1994). It has been suggested that increased gastrointestinal absorption of antigens followed by adverse local immune reactions may contribute to a major etiological factor in development of food allergies like CMA (Walker 1987). Infants afflicted with CMA were associated with an inflammatory response in the *lamina propia* of the intestinal membrane by prolonged exposure to cow milk. Such inflammatory response also can occur by a constant increase in macromolecular permeability and electrogenic activity of the epithelial layer, even in the absence of milk antigen (Robertson et al. 1982; Heyman et al. 1988). The clinical symptoms of CMA are transient, since all disease parameters return to normal after several months on a cow milk–free diet (Heyman et al. 1990).

Goat milk has been recommended as the cow milk substitute for infants and allergic patients who suffer from allergies to cow milk or other food sources (Rosenblum and Rosenblum 1952; Walker 1965; Van der Horst 1976; Taitz and Armitage 1984; Park 1994; Haenlein 2004). There has been much documented and anecdotal evidence for the potential of goat milk as an effective natural, hypoallergenic, and bioactive dairy food source for human nutrition and health.

Hypoallergenic Properties of Goat Milk

Considering the bioactive components in milk, the hypoallergenic properties of goat milk are of great importance to human health and medicine. This premise has been of continuous keen interest to goat milk producers and consumers, especially in recent years in developed countries (Park and Haenlein 2006). In a recent study, treatment with goat milk resolved significant numbers of cases of children who had cow milk allergy problems; and in another allergy case study, 49 of 55 treated children benefited from the treatment with goat milk (Bevilacqua et al. 2000).

Various anecdotal literature has shown that goat milk has been used for hypoallergenic infant food or milk substitute in infants allergic to cow milk and in those patients suffering from various allergies such as eczema, asthma, chronic catarrh, migraine, colitis, hay fever, stomach ulcer, epigastric distress, and abdominal pain due to allergenicity of cow milk protein (Walker 1965; Wahn and Ganster 1982; Taitz and Armitage 1984; Park 1994; Haenlein 2004).

Soothill (1987) reported that children who were reactive or allergic to bovine milk but not to goat milk also reacted to bovine milk cheese but not to

goat milk cheese. In another study, administration and feeding of goat milk also improved gastrointestinal allergy in certain infants (Rosenblum and Rosenblum 1952). In an extensive feeding trial, Walker (1965) showed that only 1 in 100 infants who were allergic to cow milk did not thrive well on goat milk. Of 1,682 patients with allergic migraine, 1,460 were due to food, 98 due to inhalants, 98 due to endogenous (bacterial) substances, and 25 due to drugs (including tobacco). Among the 1,460 patients with food allergy, 92% were due to cow milk or dairy products; 35% wheat; 25% fish; 18% egg; 10% tomato; and 9% chocolate. Some patients were allergic to more than one food. In another experiment, approximately 40% of allergic patients sensitive to cow milk proteins were able to tolerate goat milk proteins (Brenneman 1978). These patients may be sensitive to cow lactalbumin, which is species specific. Other milk proteins, such as β-lactoglobulin, are also shown to be responsible for cow milk allergy (Zeman 1982; Heyman and Desjeux 1992).

Many scientists have recommended evaporated goat milk or goat milk powder for infant formula (McLaughlan, et al. 1981; Juntunen and Ali-Yrkko 1983; Taitz and Armitage 1984; Coveney and Darnton-Hill 1985), because heat applied to manufacturing processes reduces allergic reactions (Perlman 1977). Heat denaturation alters basic protein structure by decreasing its allergenicity (Macy et al. 1953), and high heat treatment removes sensitizing capacity of milk (McLaughlan, et al. 1981). Because goat milk has relatively low α_{s1}-casein content, it is logical that children with high sensitivity to α_{s1}-casein of cow milk should tolerate goat milk quite well (Chandan et al. 1992; Juàrez and Ramos 1986).

Perlman (1977) observed that lactalbumin from goat milk showed a different skin reaction in comparison with its bovine milk counterpart and that there was a variation of skin test reaction to allergenic fractions of bovine milk and goat milk (Table 3.1). The data indicate that some proteins of bovine milk gave higher incidences of positive skin test reactions than goat milk. Podleski (1992) reported that inconsistency in cross-allergenicity among milk of different species may be qualitative and quantitative. A few reports using gel electrophoretic precipitation analysis also showed that there was a certain immunological cross-reactivity between cow and goat milk proteins (Saperstein 1960, 1974; Parkash and Jenness 1968; McClenathan and Walker 1982).

There is a wide variety of genetic polymorphisms of the different caseins and whey proteins (Grosclaude 1995), which adds to the complexity of the CMA situation and the difficulty of determining which protein is mainly responsible for an allergic reaction. However, Bevilacqua et al. (2000) have shown that this genetic protein diversity may actually help identify which protein is the allergen, if genetic polymorphisms of milk proteins are specifically used for clinical tests. In their study, guinea pigs had allergic reactions to goat milk with α_{s1}-casein, similar to cow milk, which only has this protein polymorph, and which may explain the commonly found cross-immune reaction between cow milk and some goat milk (Park and Haenlein 2006). However, guinea pigs fed goat milk without this polymorph but instead with α_{s2}-casein showed an allergic reaction in only 40%, indicating that goat milk lacking in α_{s1}-casein is less allergenic than other goat milk. Compared to cow milk, goat milk contains much less or nondetectable amounts of α_{s1}-

Table 3.1. Variations in skin test reactions to fractions of cow milk and goat milk[1]

Patients	Fractions of Cow Milk			Bovine Plasma Albumin	Goat Milk Albumin
	α-lactalbumin	β-lactalbumin	Casein		
GF	++++	+	—	—	—
VWW	++	+	—	—	—
DK[1]	+	++++[2]	+	—	—
VDB	++++	+++	+++	Not done	+++

[1]Perlman (1977).

[2]Beta-lactalbumin heated to 100 °C still gave ++ reaction, but after heating to 120 °C for 20 min all skin test reactions disappeared.

casein (Jenness 1980; Chandan et al. 1992; Remeuf 1993; Park 1994 2006).

In French clinical studies over 20 years with cow milk allergy patients, Sabbah et al. (1997) concluded that substitution with goat milk was followed by "undeniable" improvements. In other French extensive clinical studies with CMA children, the treatment with goat milk produced positive results in 93% of the children and was recommended as a valuable aid in child nutrition because goat milk had less allergenicity and better digestibility compared to cow milk (Fabre 1997; Grzesiak 1997; Reinert and Fabre 1997).

Nutritional and Therapeutic Properties of Goat Milk

Goat milk also exhibits significant nutritional and therapeutic functions in abnormal or disease conditions of human nutrition and health, due mainly to some of its biologically active compounds. Reports have shown that therapeutic and nutritional advantages of goat milk over cow milk come not from its protein or mineral differences, but from the lipids, more specifically the fatty acids within the lipids (Babayan 1981; Haenlein 1992; Park 1994; Park and Haenlein 2006). Goat milk fat contains significantly greater contents of short- and medium-chain length fatty acids (C4:0–C12:0) than the cow counterpart (Babayan 1981; Juàrez and Ramos 1986; Chandan et al. 1992; Haenlein 1992 2004; Park 1994; Park and Haenlein 2006).

Goat milk has smaller fat globule size compared to cow and other species' milk. Comparative average diameters of fat globule for goat, cow, buffalo and sheep milk were reported as 3.49, 4.55, 5.92, and 3.30 μm, respectively (Fahmi et al. 1956; Juàrez and Ramos 1986). The smaller fat globule size of goat milk would have better digestibility compared to cow milk counterparts (Haenlein and Caccese 1984; Stark 1988; Chandan et al. 1992). The short- and medium-chain fatty acids (MCT) in goat milk have been shown to possess several bioactive functionalities in digestion and metabolism of lipids as well as treatment of lipid malabsorption syndromes in a variety of patients (Park 1994; Haenlein 1992 2004; Park and Haenlein 2006). These properties of short- and MCT in goat milk are further delineated in the bioactive lipid section of this chapter.

Goat milk proteins are also believed to be more readily digestible, and their amino acids absorbed more efficiently than those of cow milk. Caprine milk forms a softer, more friable curd when acidified, which may be related to lower contents of α_{s1}-casein in the milk (Jenness 1980; Haenlein and Caccese 1984; Chandan et al. 1992). It may be logical that smaller, more friable curds of goat milk would be attacked more rapidly by stomach proteases, giving better digestibility (Jenness 1980).

Caprine milk also has better buffering capacity than bovine milk, which is good for the treatment of ulcers (Devendra and Burns 1970; Haenlein and Caccese 1984; Park 1991, 1992). In a comparative study of buffering capacity (BC) using caprine milk, bovine milk, and commercial bovine milk infant formulae, Park (1991) reported that Nubian goat milk had the highest BC among all tested milk and that the major buffering entities of milk were influenced by species and breeds within species (Table 3.2). Due to the compositional differences, milk of Nubian goat breed showed a higher BC compared with the milk of Alpine breed, Holstein cows, and Jersey cows. Nubian goat milk had highest levels of total N, protein, nonprotein N (NPN) and phosphate (P_2O_5) among the four breeds of goat and cow milk. Regardless of breed, goat milk contained significantly higher nonprotein N than cow milk (Park 1991). The BC is influenced by proteins, primarily casein and phosphate components in milk (Watson 1931). Soy-based infant formulae contained less total N and NPN compared with natural goat and cow milk, and BC of the formulae were also lower than those of natural milk (Fig. 3.1). The higher BC of Nubian goat milk compared to cow milk would be important in human nutrition.

Mack (1953) conducted a nutrition trial involving 38 children (20 girls and 18 boys) aged 6 to 13 years by feeding one-half of them 0.946 liter of goat milk and the other half 0.946 liter of cow milk daily for 5 months. The study revealed that children in the goat milk group surpassed those on cow milk in weight gain, statue, skeletal mineralization, bone density, blood plasma vitamin A, calcium, thiamine, riboflavin, niacin, and hemoglobin concentrations. Statistical differences were minimal for blood hemoglobin and various other biochemical and structural measurements between the two groups. In another

Table 3.2. Concentration of total N, NPN, and phosphate in natural goat and cow milk and soy-based infant formulas[1]

		Total N		NPN		P_2O_5	
Milk Group	n[2]	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
Goat Milk							
Alpine	25	.390[c]	.032	.048[b]	.008	.166[a]	.020
Nubian	25	.556[a]	.013	.061[a]	.013	.212[a]	.015
Cow Milk							
Holstein	25	.392[c]	.058	.033[c]	.002	.173[a]	.022
Jersey	25	.505[b]	.043	.038[c]	.004	.211[a]	.118
Formula Milk							
Brand A	5	.227[d]	.026	.020[d]	.003	.211[a]	.008
Brand B	5	.259[d]	.016	.019[d]	.003	.192[a]	.053

[a,b,c,d]Means with different superscripts within a same column are significantly different ($P < .01$).

[1]Expressed in grams per 100 mL.

[2]Number of determinations per mean value.

Adapted from Park (1991).

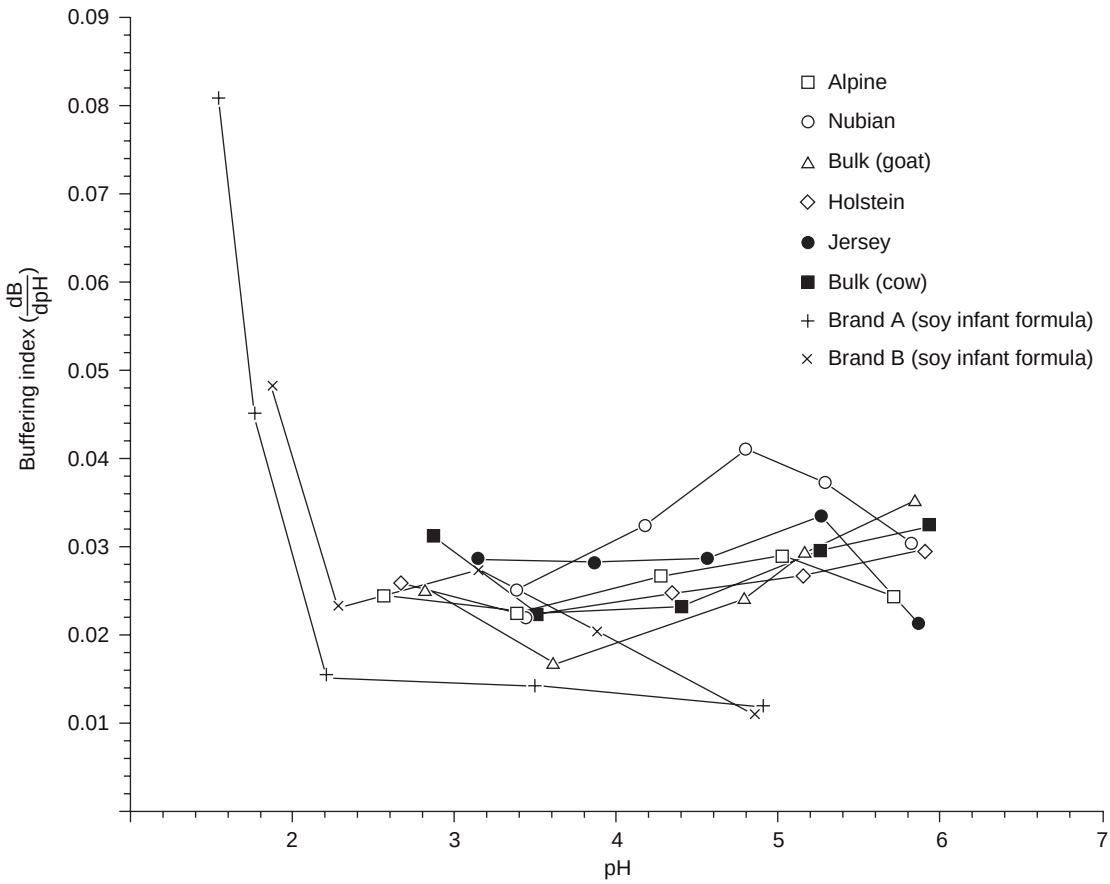

Figure 3.1. Buffering capacities of natural goat and cow milk compared with those of soy-based infant formula. Number of observations for Alpine, Nubian, Holstein, Jersey, and brands A and B formula milk were 25, 25, 25, 5, and 5, respectively. (Adapted from Park 1991).

study of a feeding trial of anemic rats, goat milk also showed a greater iron bioavailability than cow milk (Park et al. 1986), indicating that the iron compounds in goat milk, such as lactoferrin, may be more bioactive than those in cow milk.

In recent Spanish studies, Barrionuevo et al. (2002) removed 50% of distal small intestine of rats by resection, simulating the pathological condition of malabsorption syndrome, and found that the feeding of goat milk instead of cow milk as part of the diet resulted in significantly higher digestibility and absorption of iron and copper, thereby preventing anemia. In a separate trial, they also found that the utilization of fat and weight gain was improved with goat milk in the diet, compared to cow milk, and levels of cholesterol were reduced, while triglyceride, HDL, GOT, and GPT values remained normal (Alferez et al. 2001). It was concluded that the consumption of goat milk reduces total cholesterol levels and the LDL fraction because of the higher presence of medium-chain triglycerides (MCT) (36% in goat milk vs. 21% in cow milk), which decreases the synthesis of endogenous cholesterol. In an Algerian study, Hachelaf et al. (1993) also found that 64 infants with malabsorption syndromes, who had the substitution of cow milk with goat milk, resulted in significantly higher rates of intestinal fat absorption. These study results indicate that MCT in caprine milk may be considered as a bioactive compound.

In a study in Madagascar, Razafindrakoto et al. (1993) fed either cow or goat milk, in addition to the regular diet, to the 30 hospitalized undernourished children between 1 and 5 years of age. Malnutrition is apparently frequent among children in Madagascar and cow milk is not affordable or available in sufficient quantities, while goat milk was cheaper to produce and more readily available. The children on goat milk outgained the cow milk children in body weight by 9% daily ($8.53\,g/kg/day \pm 1.37$ vs. 7.82 ± 1.93) over the 2-week trial period and fat absorption tended to be better in the goat milk children. Thus goat milk was again recommended as a "useful alternative to cow milk for rehabilitating undernourished children." Considering the results of these nutritional studies, caprine milk apparently has certain growth factors and bioactive components, which may not be equally available in bovine milk.

FUNCTIONALITIES OF BIOACTIVE PEPTIDES IN MILK AND DAIRY PRODUCTS

Physiologically and functionally active peptides are produced from several food proteins during gastrointestinal digestion and fermentation of food materials with lactic acid bacteria (Korhonen and Pihlanto 2007). Once bioactive peptides are liberated, they exhibit various physiological effects in the body, such as gastrointestinal, cardiovascular, endocrine, immune, and nervous systems. These functionalities of the peptides include antimicrobial, antihypertensive, antithrombotic, antioxidative, and immunomodulatory activities (FitzGerald and Meisel 2003; Korhonen and Pihlanto 2003). Many milk protein–derived peptides exhibit more than one functional role, including peptides from the sequence 60–70 of β-casein, which has immunostimulatory, opioid, and ACE-inhibitory activities (Korhonen and Pihlanto 2007) (Fig. 3.2). The bioactive peptides derived from a variety of dietary proteins have been reviewed by many researchers (Clare et al. 2003; FitzGerald and Meisel 2003; Pellegrini 2003; Pihlanto and Korhonen 2003; Li et al. 2004).

Antihypertensive Peptides

Angiotensin is one of two polypeptide hormones and a powerful vasoconstrictor that functions in the body by controlling arterial blood pressure. The angiotensin-I converting enzyme (ACE, peptidyl-peptide hydrolases; EC 3.4.15.1) has been known as a multifunctional ectoenzyme that is located in different tissues, such as plasma, lung, kidney, heart, skeletal muscle, pancreas, arteries, and brain, and plays a key physiological role in regulating peripheral blood pressure, as well as in the renin-angiotensin, kallikrein-kinin, and immune systems (Gobbetti et al 2007; Korhonen and Pihlanto 2007). The ACE causes increase in blood pressure by converting angiotensin-I to the potent vasoconstrictor, angiotensin-II, and by degrading bradykinin, a vasodilatory peptide, and enkephalins (Petrillo and Ondetti 1982).

The antihypertensive or ACE-inhibitory peptides have been isolated from the enzymatic digest of various food proteins, and they are recently the most greatly investigated group of bioactive peptides

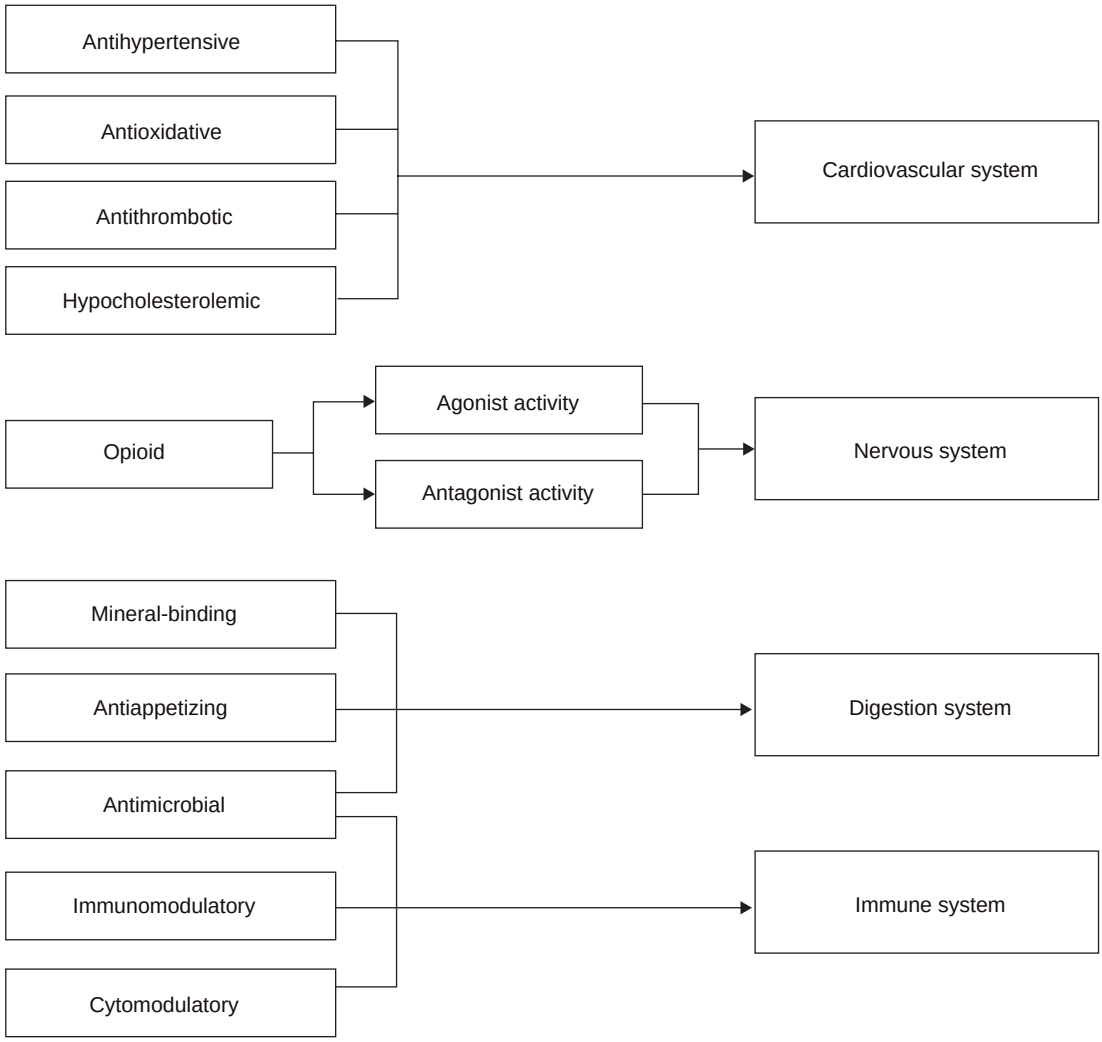

Figure 3.2. Physiological functionality of food-derived bioactive peptides (Korhonen and Pihlanto 2007).

(Korhonen and Pihlanto 2007). Exogenous ACE inhibitors having an antihypertensive effect in vivo were first discovered in snake venom (Ondetti et al. 1977). As shown in Table 3.1, several ACE-inhibitory peptides were identified by in vitro enzymatic digestion of milk proteins or chemical synthesis of peptide analogs (Gobbetti et al. 2002, 2004). The ACE-inhibitors derived from milk proteins account for different fragments of casein, named *casokinins* (Meisel and Schlimme 1994), or whey proteins, named *lactokinins* (FitzGerald and Meisel 2000).

Many recent in vivo and in vitro studies have shown the antihypertensive effect of CN-derived peptides contained in dairy products (FitzGerald and Meisel 2000; Gobbetti et al. 2004, 2007). ACE-inhibitory peptides were purified from Calpis, which is a Japanese soft drink manufactured from skim milk fermented by *Lactobacillus helveticus* and *S. cerevisiae* (Nakamura et al. 1995). Milk inoculated with *Lb. helveticus* released Val-Pro-Pro and Ile-Pro-Pro peptides from α_{s1}- and β-CN (Yamamoto et al. 1994). In a placebo-controlled study, Hata

et al. (1996) observed that the blood pressure of hypertensive patients significantly decreased after 4 and 8 weeks of daily ingestion of 95 mL sour milk, which contained the two tripeptides, and that resulted in ingested dosage of ACE-inhibitory peptides of 1.2 to 1.6 mg/day.

The presence of ACE-inhibitory peptides of low molecular mass was found in several ripened cheeses (Meisel et al. 1997). They further observed that the ACE-inhibitory activity increased as proteolysis developed, while the ACE-inhibitory effect decreased when the cheese maturation exceeded a certain level during proteolysis. Four novel ACE-inhibitory peptides were identified and purified from the hydrolysates of caprine β-Lg that was digested with thermolysin: f46–53, f58–61, f103–105, and f122–125 (Gobbetti et al. 2007). Very highly active ACE-inhibitory peptides were found, which were corresponding to casokinins such as α_{s1}-CN f23–27 and f1–9, β-CN f60–68 and f177–183, and α_{s2}-CN f174–181 and f174–179, having IC_{50} values lower than 20 μmol/L (Saito et al. 2000; Meisel 2001).

Antioxidative Peptides

Antioxidative peptides can be released from caseins, soybean, and gelatin in hydrolysis by proteolytic enzymes (Korhonen and Pihlanto 2003a). Researchers (Suetsuna et al. 2000; Rival et al. 2001a,b) have shown that peptides derived from α_s-casein have free radical–scavenging activity and inhibit enzymatic and nonenzymatic lipid peroxidation.

Bounous and Gold (1991) reported that low-temperature–processed whey protein contains high levels of specific dipeptides (glutamylcysteine), which can promote the synthesis of glutathione, an important antioxidant involved with cellular protection and repair processes.

Antithrombotic Peptides

Caseinomacropeptide (CMP) is a peptide split from κ-casein at the time of milk coagulation by rennin. This CMP is reported to have peptide sequences, which inhibit the aggregation of blood platelets and the binding of the human fibrinogen γ-chain to platelet surface fibrinogen receptors (Fiat et al. 1993). There are two reported antithrombotic peptides derived from human and bovine κ-caseinoglycopeptides, which were identified in the plasma of 5-day-old newborns after breast-feeding and ingestions of cow milk–based formula (Chabance et al. 1998).

The milk clotting mechanism through interaction of κ-CN with chymosin is comparable to the blood clotting process through interaction of fibrinogen with thrombin. Clare and Swaisgood (2000) reported that the C-terminal dodecapeptide of human fibrinogen γ-chain (residues 400–411) and the undecapeptide (residues 106–116) from bovine κ-CN are structurally and functionally quite similar. Casoplatelin, the peptide derived from κ-CN, affected platelet function and inhibited both the aggregation of ADP-activated platelets and the binding of human fibrinogen γ-chain to its receptor region on the platelet's surface (Jollès et al. 1986). Sheep CN-derived κ-caseinoglycopeptide (106–171) decreased thrombin- and collagen-induced platelet aggregation in a dose-dependent manner (Qian et al. 1995).

Hypocholesterolemic Peptides

The serum cholesterol-lowering activity is dependent on the degree of fecal steroid excretion (Nagata et al. 1982). Cholesterol is rendered soluble in bile salt-mixed micelles and then absorbed (Wilson and Rudel 1994). This fact prompted a hypothesis that a peptide with high bile acid-binding capacity could inhibit the reabsorption of bile acid in the ileum and decrease the blood cholesterol level (Iwami at al. 1986).

In a recent study, a novel hypocholesterolemic peptide (Ile-Ile-Ala-Glu-Lys) was identified from the tryptic hydrolysate of β-lactoglobulin (Nagaoka et al. 2001). This peptide was shown to suppress cholesterol absorption by Caco-2 cells in vitro and elicit hypocholesterolemic activity in vivo in rats after oral administration of the peptide solution. Four bioactive peptides were identified in the hydrolysate, which corresponded to β-lactoglobulin f9–14, f41–60, f71–75, and f142–146. The micellar solubility of cholesterol in the presence of β-lactoglobulin tryptic hydrolysate was markedly low (Nagaoka et al. 2001). However, the mechanism of the hypocholesterolemic effect by these peptides has not been delineated (Korhonen and Pihlanto 2007).

Opioid Peptides

Opioid peptides are opioid receptor ligands with agonistic or antagonistic activities. The peptides with

opioid activity have been found in milk protein and wheat gluten hydrolysates (Teschemacher 2003). Opioids are defined as peptides (i.e., enkephalins) that have an affinity for an opiate receptor and opiate-like effects, inhibited by naloxone (Gobbetti et al. 2007). Bioactive peptides derived from milk proteins may function as regulatory substances, defined exorphins, which have pharmacological properties similar to enkephalins (Meisel et al. 1989; Meisel and Schlimme 1990; Schanbacher et al. 1998).

β-casomorphins, which are β-casein opioid peptides, have been detected in the duodena chime of minipigs and in the human small intestine after in vivo digestion of casein or milk (Meisel 1998; Meisel and FitzGerald 2000). The α_{s1}-casein-exorphin (α_{s1}-CN f90–96), β-casomorphins-7 and -5 (β-CN f60–66 and f60–64, respectively), and lactorphins (α-lactalbumin f50–53 and β-lactoglobulin f102–105) act as opioid agonists, whereas casoxins (i.e., κ-CN f35–42, f58–61, and f25–34) act as opioid antagonists (Meisel and FitzGerald 2000; Gobbetti et al. 2007).

Among endogenous and exogenous opioid peptides, the common structural feature is the presence of a Tyr residue at the amino terminal end (except for α_{s1}-CN-exorphin, casoxin 6, and lactoferroxin B and C) and of another aromatic residue, Phe or Tyr, in the third or fourth position (Gobbetti et al. 2007). Chang et al. (1981) reported that the negative potential, localized in the vicinity of the phenolic hydroxyl group of Tyr, appeared to be essential for opioid activity, and removal of the Tyr residue results in a total absence of activity. Mierke et al. (1990) observed that the Pro residue in the second position is also crucial to maintaining the proper orientation of the Tyr and Phe side chains.

The pepsin/trypsin hydrolysis of *Lactobacillus* GG fermented UHT milk released several opioid peptides derived from α_{s1}- and β-CN, and α-lactalbumin (Rokka et al. 1997). Proteolysis of α-lactalbumin with pepsin produced directly α-lactorphin, while digestion of β-Lg with pepsin and then trypsin yielded β-lactorphin (Gobbetti et al. 2007).

The in vivo liberation of β-casomorphins from β-CN was observed in the small intestine of adult humans after the intake of cow milk (Svedberg et al. 1985). β-Casomorphins were found in the analogous position of the natural proteins in cow, sheep, water buffalo, and human β-CN (Meisel and Schlimme 1996).

Mineral-Binding Peptides

Mineral-binding phosphopeptides or caseinophosphopeptides (CPPs) function as carriers for different minerals by forming soluble organophosphate salts, especially Ca^{2+} (Meisel and Olieman 1998). The α_{s1}-, α_{s2}-, and β-CN of cow milk contain phosphorylated regions, which can be released by digestive enzymes. In this situation, specific CPPs can form soluble organophosphate salts and lead to enhanced Ca absorption by limiting the precipitation of Ca in the distal ileum (Korhonen and Pihlanto 2007).

Most CPPs contain a common motif, such as a sequence of three phosphoseryl followed by two glutamic acid residues (Gobbetti et al. 2007). The negatively charged side chains, particularly the phosphate groups, of these amino acids represent the binding sites for minerals (Gobbetti et al. 2007). Berrocal et al. (1989) reported that dephosphorylated peptides do not bind minerals, while chemical phosphorylation of α_{s1}- and β-CN increased the binding capacity and the stability of these proteins in the presence of Ca^{2+} (Yoshikawa et al. 1981). The Ca^{2+} binding constants of CPPs are in the order of 10^2–10^3/mol, and about 1 mol of CPP can bind 40 mol of Ca^{2+} (Sato et al. 1983; Schlimme and Meisel 1995).

Enzymatic (pancreatic endoproteinases, especially trypsin) digestion of casein can generate CPPs. FitzGerald (1998) reported that other enzyme combinations, such as chymotrypsin, pancreatin, papain, pepsin, thermolysin, and pronase, have been used for in vitro CPP production. CPPs increase Ca^{2+} and Zn^{2+} absorption from a rice-based infant gruel in human adults by about 30%, while there was no effect when CPPs were ingested in either high- or low-phytate whole-grain cereal meals (Hansen 1995).

Antiappetizing Peptides

The total whey protein in the diet has been linked to a lowering of LDL cholesterol and to heightened release of an appetite-suppressing hormone, cholecystokinin (Zhang and Beynen 1993). The bioactivity for total whey protein may reside with combinations of active whey protein fractions or amino acid sequences. This physiological role of total whey protein suggests a great potential for processed whey products in developing new and

lucrative health food markets as functional food ingredients (Regester et al. 1997).

Antimicrobial Peptides

Peptides having antimicrobial activities have been purified from several bovine milk protein hydrolysates, edible plants, fish, and eggs (Clare et al. 2003; Floris et al. 2003; Pellegrini 2003; Gobbetti et al. 2004). The total antibacterial effect in milk is greater than the sum of the individual contributions of immunoglobulin and nonimmunoglobulin (lactoferrin, lactoperoxidase, and lysozyme) defense proteins or peptides (Gobbetti et al. 2007). This may be attributable to the synergistic activity of naturally occurring proteins and peptides, in addition to peptides generated from inactive protein precursors (Clare and Swaisgood 2000). Antimicrobial peptides in mammals are found both at the epithelial surfaces and within granule phagocytic cells, and they are an important component of innate defenses because they are able to modulate inflammatory responses in addition to killing microorganisms (Devine and Hancock 2002).

The most studied antimicrobial peptides are the lactoferricins, derived from bovine and human lactoferrin (Kitts and Weiler 2003; Wakabayashi et al. 2003). Lactoferricins exhibit antimicrobial activity against various Gram-positive and -negative bacteria, yeasts, and filamentous fungi (Korhonen and Pihlanto 2007). The antibacterial activity of these peptides is partly attributed to the disruption of normal membrane permeability. Although the antimicrobial mechanisms and physiological importance exerted by other food-derived peptides have not been well postulated, most antibacterial peptide activities can be broadly defined as membrane-lytic functions, where these peptides tend to assemble to form channels with specificity for prokaryotic cell membranes (Gobbetti et al. 2007).

Lactenin was perhaps the first antibacterial factor derived from milk that has been treated with rennet (Jones and Simms 1930). Casecidins are a group of basic, glycosylated, and high-molecular-weight (about 5 kDa) polypeptides, which reportedly had bactericidal properties against lactobacilli and also against various pathogenic bacteria such as *Staphylococcus aureus*. Isracidin is another antibacterial peptide derived from α_{s1}-CN treated with chymosin. This peptide corresponding to the N-terminal fragment of this protein (f1–23) was isolated by Hill et al. (1974). Isracidin was shown to have an inhibitory effect on the in vitro growth of lactobacilli and other Gram-positive bacteria, only at relatively high concentrations (0.1–1 mg/mL). This peptide, however, showed a strong protective effect in vivo against *S. aureus*, *Streptococcus pyogenes* and *Listeria monocytogenes* at very low doses of 10 μg/mouse prior to bacterial challenge.

Immunomodulatory Peptides

Protein hydrolysates and peptides derived from milk caseins and major whey proteins have immunomodulatory effects (exert immune cell functions), such as lymphocyte proliferation, antibody synthesis, and cytokine regulation (Gill et al 2000). During fermentation of milk by lactic acid bacteria, casein peptides are produced.

These peptides have been shown to modulate the proliferation of human lymphocytes, to downregulate the production of certain cytokines, and to stimulate the phagocytic activities of macrophages (Korhonen and Pihlanto 2003a,b,c; 2007; Matar et al 2003). Because of immune cell functions, these peptides have been of special interest to food researchers and the food processing industry.

Milk-derived immunomodulatory peptides include α_{s1}-CN f194–199 (α_{s1}-immunocasokinin) and β-CN f193–202, f63–68, and f191–193 (immunopeptides), which are synthesized by hydrolysis with pepsin-chymosin. Kayser and Meisel (1996) showed that β-casomorphin-7 and β-CN immunopeptides suppressed the proliferation of human peripheral blood lymphocytes at low concentrations ($<10^{-7}$ mol/L) but stimulated it at higher concentration. Several peptides derived from β-CN enhanced the IgG production in mouse spleen cell cultures (Gobbetti et al. 2007). The proliferation of human colonic lamina propria lymphocytes was inhibited by β-casomorphin-7, where the antiproliferative effect of micromolar concentrations was reversed by the opiate receptor antagonist naloxone (Elitsur and Luk 1991). It was also reported that glutamine-containing peptides may substitute for the free amino acid glutamine, which is required for lymphocyte proliferation and utilized at a high rate by immunocompetent cells (Calder 1994).

Cytomodulatory Peptides

There is increased evidence that milk-derived peptides act as specific signals that may trigger viability of cancer cells (Gobbetti et al. 2007). McDonald et al. (1994) found that bacterial hydrolysis of casein using commercial yogurt starter cultures can yield bioactive peptides that affect colon cell Caco-2 kinetics in vitro. Roy et al. (1999) also found that bovine skimmed milk digested with cell-free extract of the yeast *Saccharomyces cerevisiae* had antiproliferative activity towards leukemia cells. Purified peptides which are equivalent to sequences of casein, also showed the modulation of cell viability such as proliferation and apoptosis in different human cell culture models (Hartmann et al 2000).

Caseinophosphopeptides (CPPs) have also been reported to exert cytomodulatory effects. Cytomodulatory peptides derived from casein fractions inhibit cancer cell growth or stimulate the activity of immunocompetent cells and neonatal intestinal cells (Meisel and FitzGerald 2003). Peptides from a lyophilized extract of Gouda cheese inhibited proliferation of leukemia cells. Cancer cell lines were more reactive to peptide-induced apoptotic stimulation than nonmalignant cells (Gobbetti et al. 2007).

BIOACTIVE PEPTIDES AND PROTEINS IN CAPRINE MILK

ACE-Inhibitory Peptides Derived from Caprine Milk Caseins

Casein (CN) constitutes approximately 80% of the total milk protein fraction. Recent research in vitro and on animal models suggests that peptides derived from CN are not only nutrients but also a source of low-molecular-weight peptides having various biological activities. These peptides are generated and become active after digestion by proteolytic enzymes or during the fermentation and maturation processes of cheese and yogurt (Korhonen and Pihlanto 2007). Antihypertensive and immuno-stimulating peptides can be generated from caprine β-CN as well as bovine β-CN (Geerlings et al. 2006; Silva et al. 2006).

A number of peptide fragments and sequences of bioactive peptides derived from caprine milk and its cheese proteins on the basis of different bioactivity are listed in Table 3.3. Although bioactive peptides from goat milk have not been studied to the level of those from bovine milk, at least two main functional bioactivities of caprine milk and its cheese products, such as ACE-inhibitory and antimicrobial active compounds, are listed in Table 3.3. For casein fractions, α-, β-, and κ-CN can be sources of bioactive components of goat milk proteins.

ACE-inhibitory peptides have been recently produced by hydrolysis of goat milk caseins (Lee et al. 2005). The peptic hydrolysate from goat casein was found to be the most active and several ACE-inhibitory peptides have been isolated from the hydrolysate (Table 3.3). Minervini et al. (2003) hydrolyzed sodium caseinates prepared from cow, sheep, goat, pig, buffalo and human milk by a partially purified proteinase of *Lb. helveticus* PR4. They found caprine β-CN f58–65 and α_{s2}-CN f182–187 among the produced peptides were also ACE-inhibitory peptides (Table 3.3).

Geerlings et al. (2006) have shown clear graphical demonstrations on the ACE-inhibitory effect of goat protein hydrolysate (GP-hyd) diet in spontaneously hypertensive rats (SHR). The systolic blood pressure (SBP) of the GP (goat protein) control group showed a gradual increase from weaning that reached maximal values at 10 weeks of life (Fig. 3.3). On the other hand, a long-term GP-hyd diet partly prevented the increase in SBP in SHR, and this effect reached statistical significance after 4 weeks of treatment (Fig. 3.3). The researchers isolated three new inhibitory peptides for ACE such as TGPIPN, SLPQ, and SQPK as shown in Table 3.3. The inhibitory concentration 50% (IC_{50}) values of individual peptides were 316, 330, and 354 μmol/L, respectively.

The same authors also measured the ACE activities in protein extracts from different tissues. The SHR fed the GP-hyd diet or the captopril diet for 12 weeks showed significantly lower ACE activities of the heart, aorta, and kidney compared with rats fed the GP-control diet (Fig. 3.4). The SHR were fed for 12 weeks the GP-hyd enriched diet of a hydrolysate containing those isolated peptides (estimated intake of TGPIPN 230 mg/kg/day), and showed approximately 15 mmHg lower systolic blood pressure than animals fed the control diet. No significant differences in tissue ACE activity were found between GP-hyd and captopril groups (Fig. 3.4).

Table 3.3. Peptide fragment and sequence of bioactive peptides derived from goat milk and its cheese proteins

Peptide Fragment	Sequence	References
ACE Inhibitory Peptides		
Caprine α_{s1}-CN f(143–146)	AYFY	Lee et al. (2005)
Caprine α_{s2}-CN f(4–8)	HPIKH	Minervini et al. (2003)
Caprine α_{s2}-CN f(174–179)	KFAWPQ	Quirós et al. (2005)
Caprine and ovine α_{s2}-CN f(203–208)	PYVRYL	Quirós et al. (2005) López–Expósito and Recio (2006)
Caprine β-CN f(58–65)	LVYPFPGP	Minervini et al. (2003)
Caprine β-CN f(78–83)	TGPIPN	Greerlings et al. (2006)
Caprine β-CN f(84–87)	SLPQ	Greerlings et al. (2006)
Caprine β-CN f(181–184)	SQPK	Greerlings et al. (2006)
Caprine and ovine β-CN f(47–51)	DKIHP	Gómez-Ruiz et al. (2005, 2006)
Caprine κ-CN f(59–61)	PYY	Lee et al. (2005)
Caprine β-Lg f(46–53)	LKPTPEGD	Hernández-Ledesma et al. (2002)
Caprine β-Lg f(58–61)	LQKW	Hernández-Ledesma et al. (2002)
Caprine β-Lg f(103–105)	LLF	Hernández-Ledesma et al. (2002)
Caprine β-Lg f(122–125)	LVRT	Hernández-Ledesma et al. (2002)
Antimicrobial/Antibacterial Peptides		
Caprine α_{s1}-CN f(24–30) (cheese)	VVAPFPE	Rizzello et al. (2005)
Caprine β-CN f(60–68) (cheese)	YPFTGPIPN	Rizzello et al. (2005)
Caprine β-CN f(183–187) (cheese)	MPIQA	Rizzello et al. (2005)
Caprine and ovine LF f(17–41)	ATKCFQWQRNM-RKVRGPPVSCIKRD	Vorland et al. (1998)
Caprine and ovine LF f(14–42)	QPEATKCFQWQRN-MRKVRGPPVSCIKRDS	Recio and Visser (2000)

Silva et al. (2006) obtained ACE-inhibitory and antioxidant active peptides in water-soluble extracts from raw and sterilized ovine and caprine cheeselike systems coagulated with enzymes of the plant *Cynara cardunculus*. They found that peptides Tyr-Gln-Glu-Pro, Val-Pro-Lys-Val-Lys, and Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-* from β-CN, as well as Arg-Pro-Lys and Arg-Pro-Lys-His-Pro-Ile-Lys-His-* from α_{s1}-CN exhibited ACE-inhibitory activity. They also found the only peptides released upon cleavage of the peptide bond Leu190-Tyr191 of caprine or ovine β-CN, and corresponding to the β-CN sequence Tyr-Gln-Glu-Pro-*, possessed antioxidant activity.

Many peptides can be released during fermentation of kefir manufacture by proteolytic enzymes of lactic acid bacteria. Sixteen peptides in a commercial caprine kefir were identified using HPLC coupled to tandem mass spectrometry (Quirós et al. 2005). Two of these peptides having sequences PYVRYL [α_{s2}-CN f(203–208)] and LVYPFPGP [β-CN f(58–65)] revealed potent ACE-inhibitory activity with IC_{50} values of 2.4 and 27.9 μM, respectively (Table 3.3). Recio et al. (2005) observed that the first of these peptides also had potent antihypertensive activity in spontaneously hypertensive rats. These results demonstrated the impact of digestion on the formation of new ACE-inhibitory peptides (Quirós et al. 2005).

In a study on diverse technologic processes with one Spanish goat milk cheese and Cabrales, Idiazabal, Roncal, Manchego, and Mahon sheep milk cheeses, Gómez-Ruiz et al. (2005, 2006) found their ACE-inhibitory activity was virtually concentrated in the 1 kDa permeate. Most of these peptides were derived from α_{s2}- and β-CN, and the peptide DKIHP [β-CN f(47-51)] was identified in all cheeses with the exception of Mahon cheese. This peptide showed the greatest inhibitory activity with an IC_{50} value of 113.1 μM.

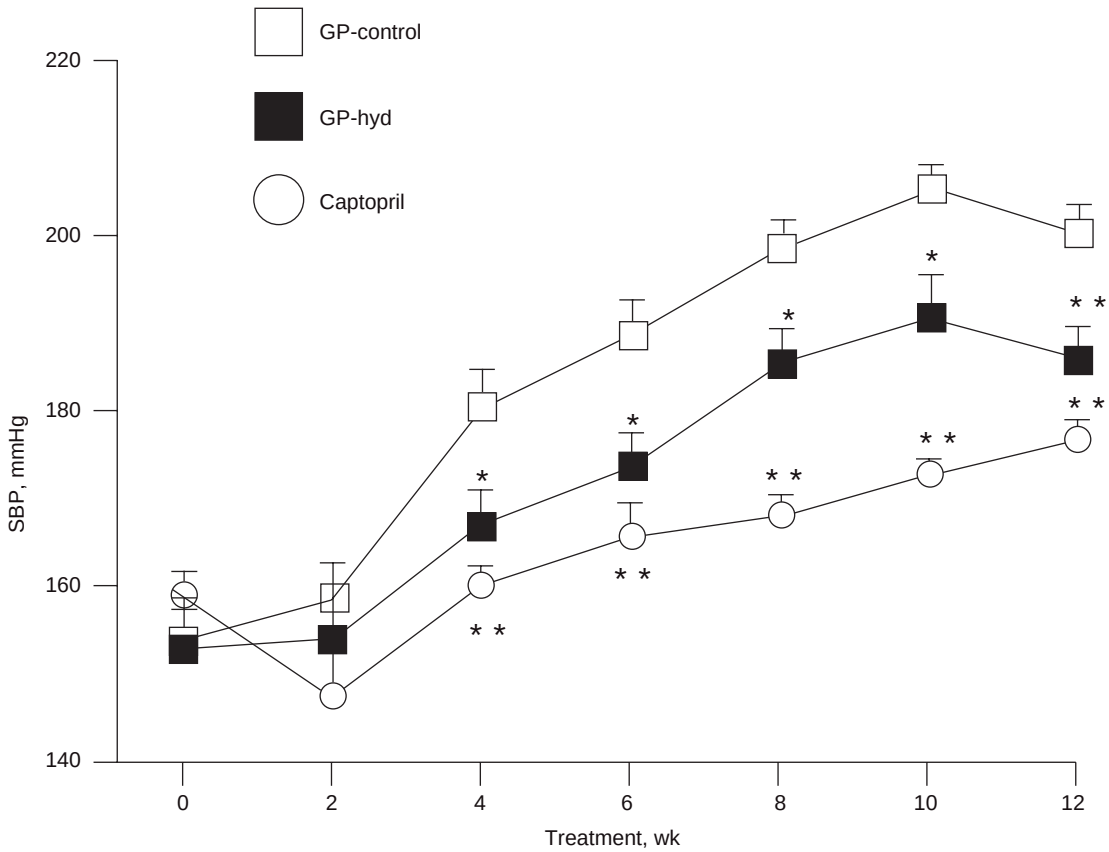

Figure 3.3. Systolic blood pressure (SBP) measured by tail-cuff plethysmography in spontaneously hypertensive rats (SHR) fed a diet containing goat protein (GP-control), an ad libitum diet containing goat protein hydrolysate (GP-hyd) diet, and a diet containing goat protein and captopril for 12 weeks.
*$P < 0.05$ and **$P < 0.01$. (Adapted from Geerlings et al. 2006).

ACE-Inhibitory Peptides Derived from Caprine Milk Whey

The ACE-inhibitory activity of hydrolysates of whey protein, mainly β-lactoglobulin from goat and sheep milk, has been reported. Hernández-Ledesma et al. (2002) observed that higher ACE-inhibitory activities of caprine and ovine β-Lg hydrolysates were obtained with enzymes of microbial origin than those hydrolysates prepared with digestive enzymes. They purified and identified four new ACE-inhibitory peptides from the hydrolysate of caprine β-Lg prepared with termolisin (Table 3.3). These peptides were identified as β-Lg fragments f(46–53), f(58–61), f(103–105), and f(122–125), and their IC_{50} values ranged from 34.7 to 2470 μM. These authors also delineated an interesting peptide LLF, which is included within the sequence of opioid peptide β-lactorphin (YLLF), and it is considered a "strategic zone" partially protected from digestive breakdown (Hernández-Ledesma et al. 2002).

Evaluating the ACE-inhibitory activities of bovine, caprine, and ovine κ-CMP and their tryptic hydrolysates, Manso and López-Fandiño (2003) showed that these κ-CMP have moderate ACE-inhibitory activity that increased considerably after digestion under simulated gastrointestinal conditions. They produced active peptides from CMP via proteolysis with trypsin, which were identified as MAIPPK and MAIPPKK peptides, corresponding to

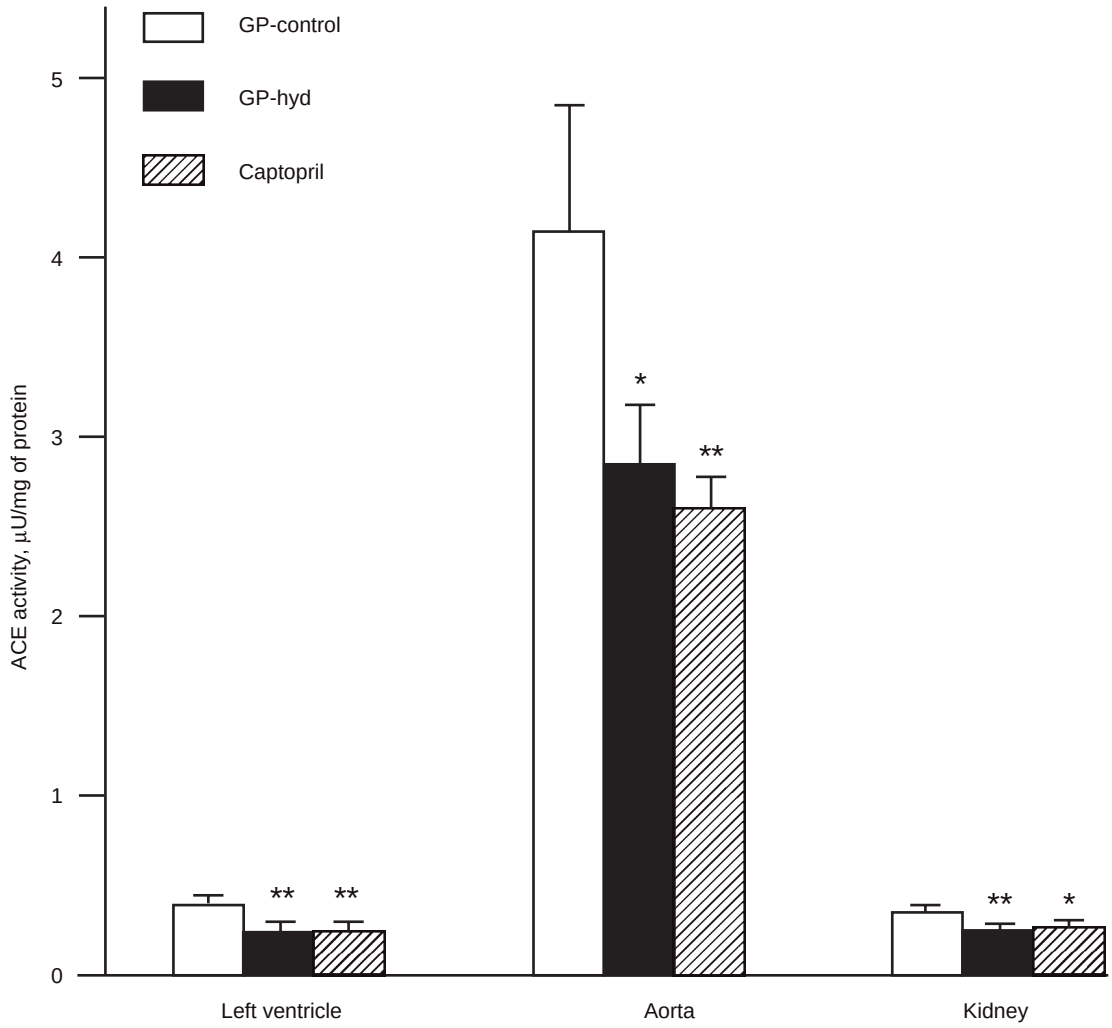

Figure 3.4. Angiotensin-converting enzyme (ACE) activity in different tissues from spontaneously hypertensive rats (SHR) after different treatments. GP-control = a diet containing goat protein; GP-hyd = an ad libitum diet containing goat protein hydrolysate; captopril = a diet containing goat protein and captopril. *$P < 0.05$ and **$P < 0.01$ vs. control group. (Adapted from Geerlings et al. 2006).

κ-CN fragments f(106–111) and f(106–112), respectively (Table 3.3). The authors observed that these peptides had a moderate activity, while their digestion under simulated gastrointestinal conditions allowed the release of potent ACE-inhibitory peptide IPP (IC_{50} value of 5 µM). These outcomes could enhance κ-CMP as multifunctional active ingredients, broadening the potential uses of rennet whey from various sources (Park et al. 2007).

Antimicrobial Peptides Derived from Caprine Caseins

Milk proteins can act as antimicrobial peptide precursors, which would promote the organism's natural defenses against invading pathogens. Consequently, food proteins may be considered as components of nutritional immunity (Pellegrini 2003). The total antibacterial effect in milk is generally expected to

be greater than the sum of the individual contributions of immunoglobulin and nonimmunoglobulin defense proteins, such as lactoferrin (LF), lactoperoxidase, lysozyme, and peptides (Park et al. 2007). This condition may be attributable to the synergistic effect of naturally occurring proteins and peptides in addition to peptides generated from inactive protein precursors (Gobbetti et al. 2004).

Goat milk caseins can be a source of antimicrobial peptides. López-Expósito and Recio (2006) recently identified four antibacterial peptides from a pepsin hydrolysate of ovine α_{s2}-CN that correspond to α_{s2}-CN fragments f(165–170), f(165–181), f(184–208) and f(203–208). The fragments f(165–181) and f(184–208) are homologous to those previously identified in the bovine protein (Recio and Visser 1999). The fragment f(165–181) was shown to have the strongest activity against all bacteria tested. Recio et al. (2005) showed that the peptide corresponding to ovine α_{s2}-CN f(203–208) is a good example of a multifunctional peptide because it exhibited not only antimicrobial activity, but also potent antihypertensive and antioxidant activity. The activities of these peptides can also be extended to caprine proteins because the amino-acid sequence of these peptides is the same in both caprine and ovine proteins (Park et al. 2007).

Antimicrobial Peptides Derived from Caprine Milk Whey

Lactoferrin (LF) is the major iron-binding whey protein in milk of many species including humans, mares, and goats (Renner et al. 1989). LF concentration in the mammary gland increases markedly during clinical infection. Peptides derived from LF have antibacterial activities that have drawn much attention during the last decade (Park et al. 2007). Tomia et al. (1991) first demonstrated that the enzymatic release of antibacterial peptides has more potent activity than the precursor LF. Shortly afterward, Bellamy et al. (1992) purified and identified the antibacterial domains of bovine LF f(17–41) and human LF f(1–47) as bovine and human lactoferricin (LFcin), respectively.

In caprine and ovine milk LF studies, Vorland et al. (1998) performed a chemical synthesis of fragment f(17–41) of caprine LF, which exhibited antibacterial activity that had a lesser extent than the bovine counterpart. Antibacterial hydrolysates were produced from the hydrolysis of caprine and ovine LF by pepsin. These hydrolyzed peptides were homologous to LFcin, corresponding to fragment f(14–42), which was identified in the caprine LF hydrolysate. The authors showed that caprine LFcin has lower antibacterial activity than bovine LFcin against *Escherichia coli* but comparable activity against *Micrococcus flavus* (Table 3.3). Recio and Visser (2000) also hydrolyzed ovine LF by the action of pepsin and observed ovine LF hydrolysate activity from the corresponding region to the LFcin within the sequence of LF.

Antithrombotic Peptides Derived from Caprine Milk Proteins

Coronary heart diseases, such as blood clotting thrombosis, are among the leading causes of mortality of adult humans in industrialized countries (Park et al. 2007). In this regard, antithrombotic agents, including antithrombotic peptides derived from milk proteins, may be very important for their application in human health. In blood coagulation, fibrinogen plays an important role, particularly because it binds to specific glycoprotein receptors located on the surface of the platelets, which allows them to clump.

Qian et al. (1995) found two very active sequences that have inhibitory activity of human platelet aggregation induced by thrombin and collagen after hydrolysis of ovine κ-CMP with trypsin. Furthermore, Manso et al. (2002) observed that bovine, ovine, and caprine κ-CMP and their hydrolysates with trypsin acted as inhibitors of human platelet aggregation. Jollès et al. (1986) showed that bovine κ-CN–derived peptide, casoplatelin, affected platelet function and inhibited both the aggregation of ADP-activated platelets and the binding of human fibrinogen γ-chain to its receptor region on the platelet's surface. In addition, a smaller κ-CN fragment f(106–110), casopiastrin, was synthesized by trypsin hydrolysis, and this peptide exhibited platelet aggregation but did not affect fibrinogen binding to the platelet receptor (Jollès et al. 1986; Mazoyer et al. 1992). The caprine κ-CN peptides in this regard have not been reported but are expected to have similar effects.

Other Bioactive Peptides Derived from Caprine Milk

Although more studies are required to demonstrate potential bioactive compounds for opioid, mineral-binding, antioxidant, and anticarcinogenic activities as functional foods in human nutrition, it can be predicted that the peptides reported as bioactive agents and released from bovine proteins are also found within goat and sheep proteins because of the great homology among the sequences of bovine, ovine, and caprine milk proteins (Park et al. 2007).

Recent reports (Clare and Swaisgood 2000; Mader et al. 2005) indicated that a number of peptides with opioid activity isolated from hydrolysates of bovine milk proteins can modulate social behavior, increase analgesic behavior, prolong gastrointestinal transient time by inhibiting intestinal peristalsis motility, exert antisecretory action, modulate amino acid transport, proliferate apoptosis in different carcinoma cell lines, and stimulate endocrine responses such as the secretion of insulin and somatostatin.

Another study suggested that casein phosphopeptides (CPP) can form soluble organophosphate salts and may function as carriers for different minerals, especially calcium in the intestine (Sato et al. 1986). Moreover, calcium-binding CPP can have anticariogenic effects by inhibiting caries lesions through recalcification of the dental enamel, along with competition of dental plaque-forming bacteria for calcium (Reynolds 1987). Peptides derived from caseins and whey proteins released by enzymatic hydrolysis (Suetsuna et al. 2000; Rival et al. 2001a,b; Hernández-Ledesma et al. 2005) and by milk fermentation (Kudoh et al. 2001) have shown to be of great interest because of their potent antioxidant activities.

BIOACTIVE LIPID COMPONENTS IN GOAT MILK

There are several lipid components that have bioactive functions, such as short- and medium-chain fatty acids (MCT), phospholipids, cholesterol, gangliosides, and glycolipids, etc. Milk lipids consist of 98–99% triglycerides, which are located in the fat globule. The remaining 1–2% are minor lipid components, including diglycerides 0.3–1.6%, monoglycerides 0.002–0.1%, phospholipids 0.2–1.0%, cerebrosides 0.01–0.07%, sterols 0.2–0.4%, and free fatty acids 0.1–0.4% (Renner et al. 1989).

As described in the section on therapeutic significance of goat milk earlier in this chapter, caprine milk has smaller fat globule size than cow milk, which is advantageous for better digestibility and a more efficient lipid metabolism compared with cow milk fat (Park 1994; Haenlein 1992, 2004; Park and Haenlein 2006). The short- and medium-chain fatty acids in goat milk exhibit several bioactivities in digestion, lipid metabolism, and treatment of lipid malabsorption syndromes. The important bioactive lipid components in goat milk are further delineated in the following sections.

Short- and Medium-Chain Fatty Acids in Goat Milk

Because the high level of short- chain and medium-chain triglycerides (MCT) in goat milk is species specific, it has been suggested that goat milk fat may have at least three significant contributions to human nutrition: 1) goat milk fat may be more rapidly digested than cow milk fat because lipase attacks ester linkages of short- or medium-chain fatty acids more easily than those of longer chains (Jenness 1980; Chandan et al. 1992; Park 1994; Haenlein 1992, 2004); 2) these fatty acids provide energy in growing children by their unique metabolic abilities and also exhibit beneficial effects on cholesterol metabolism, such as hypocholesterolemic action on tissues and blood via inhibition of cholesterol deposition and dissolution of cholesterol in gallstones (Greenberger and Skillman 1969; Kalser 1971; Tantibhedhyangkul and Hashim 1975; Haenlein 1992; Park amd Haenlein 2006); and 3) they also have been used for treatment of various cases of malabsorption patients suffering from steatorrhea, chyluria, hyperlipoproteinemia, intestinal resection, coronary bypass, childhood epilepsy, premature infant feeding, cystic fibrosis, and gallstones (Greenberger and Skillman 1969; Tantibhedhyangkul and Hashim 1975; Haenlein 1992, 2004; Park 1994). In addition, goat milk fat products such as goat butter, ghee, and related products with higher contents of MCT than fluid goat milk have not been studied in relation to physiological well-being of human subjects (Park and Haenlein 2006).

Manipulation of goat feeding regimes toward higher contents of beneficial unsaturated fatty acids

in goat milk fat by feeding special feed supplements such as protected fats can be used to produce "tailor-made functional foods" and to even further improve the nutritional value of goat milk (Sanz Sampelayo et al. 2002). Goats fed a high level of pasture forage had higher milk fat contents of C4:0, C6:0, C18:0, C18:1, C18:3, C20:0, *iso-*, *ante-iso-*, and odd fatty acids, but lower values of C10:0, C12:0, C14:0, C16:0, and C18:2, than those fed the low levels of forage (LeDoux et al. 2002). These dietary manipulations can lead to increased bioactive compounds in goat milk.

Phospholipids

Phospholipids are essential components of cell membranes in human, animal, and plant tissues. They are involved in the function of cell membranes, and they have the ability to interact with metabolites, ions, hormones, antibodies, and other cells (Weihrauch and Son 1983). Phospholipids are polar lipids that make up approximately 1.6% of the total lipids. Of the polar lipid fraction, glycolipids make up 16% in goat milk as compared to 6% in cow milk (Morrison et al. 1965). Quantitative analysis of the phospholipid fraction of bound lipids of goat milk revealed that it had 35.4% phosphatidyl ethanolamine (PE), 3.2% phosphatidyl serine (PS), 4.0% phosphatidyl inositol (PI), 28.2% phosphatidyl choline (PC; lecithin), and 29.2% sphingomyelin (SP). Species differences in phospholipid fractions appear to be insignificant, although goat milk contains slightly higher PE, SP, and PS than cow milk (Table 3.4). Human milk has more PS, PC, and SP than goat milk.

Table 3.4. Distribution of phospholipid subclasses in goat, cow, and human milks

	Percent of Total Phospholipids		
Phospholipid Fraction	Goat Milk	Cow Milk	Human Milk
Phosphatidyl ethanolamine	35.4	35	32
Phosphatidyl choline	28.2	30	29
Sphingomyelin	29.2	24	29
Phosphatidyl inositol	4.0	5	5
Phosphatidyl serine	3.2	2	4

Data from Cerbulis et al. (1982) and Renner et al. (1989).

Concerning bioactive functions, phospholipids contribute to a rapid absorption of fat because they form a membrane around the fat globules, which keep them finely dispersed. Phopholipids help transport fat from the liver through their lipotropic activity. Imaizumi et al. (1983), in their rat experiment, observed that a decrease in serum cholesterol occurred in dietary phosphatidyl ethanolamine, but not in dietary lecithin. They observed the distribution pattern of phospholipid subclasses in the liver and noted that the fatty acid compositions of hepatic and plasma phospholipids were altered by PC (lecithin).

Galli et al. (1985) found that more than 90% of PC (lecithin) is absorbed by the intestinal mucosa and incorporated into chylomicrons, and then taken up by the high-density lipoprotein (HDL) fraction. HDL levels were increased, even though the levels of plasma total cholesterol and triglycerides did not appear to be modified by PC. The same authors also discovered that oral PC administration reduced the platelet lipid and cholesterol content in healthy volunteer human subjects.

Phospholipids are also major constituents of the brain, nerve tissue, heart muscle, liver, and sperm (Renner et al. 1989). The effect of ingested lecithin on improved learning and memory in animals and humans has drawn the attention of researchers, although the phospholipids may not be considered as the essential nutrient because the body itself can synthesize PC (Weihrauch and Son 1983). Although these reports are based on bovine milk data, bioactivities of phospholipids in caprine milk are expected to be similar or greater.

Conjugated Linoleic Acid

Conjugated linoleic acid (CLA) has received much attention of nutritionists, food consumers, and researchers in recent years, because of its several beneficial and bioactive functions on human health, including anticarcinogenic, antiatherogenic, immune-stimulating, growth-promoting, and body fat–reducing activities, etc. (Pariza et al. 1996; Lawless et al. 1998; Dhiman et al. 1999; Park 2006). The generic name of *CLA* is a collective term embracing all positional and geometric isomers of linoleic acid (C18:2) that contain conjugated unsaturated double bonds (Dhiman et al. 1999; Park et al. 2007). The most biologically active isomer of

CLA is *cis*-9, *trans*-11-octadecadienoic acid, which accounts for more than 82% of the total CLA isomers in dairy products (Dhiman et al. 1999; Park 2006).

CLA is considered an important bioactive component in goat milk. Generally milk fat contains not only the greatest level of CLA, but also the highest content of vaccenic acid (physiological precursor of CLA). It was reported that average total CLA content appeared to decrease in the following order: ewe > cow > goat milk fat—1.08, 1.01, and 0.65%, respectively (Jahreis et al. 1999). However, the stage of lactation, season, and feeding conditions are not known in this report. Dairy goats fed on only pasture can produce higher CLA content in goat milk as shown in a cow milk study (Dhiman et al. 1999). Milk CLA concentration in different ruminant species varied with the season mainly due to variations in feeding factors (Chilliard and Ferlay 2004). Ewe milk had the greatest seasonal differences in CLA content, showing 1.28% in summer and 0.54% at the end of the winter period.

It is also possible to increase CLA content of goat milk by dietary manipulation and supplementation. Mir et al. (1999) showed that feeding canola oil at 2 and 4% of grain intake to Alpine milking goats increased CLA in milk by 88 and 210%, respectively, compared to the nontreated control group. Although the CLA content in dairy products is affected by many factors, animal feeding strategies especially with seed/oil supplemented diets rich in PUFA, have shown high effectiveness in CLA increase for goat, sheep, and cow milk (Stanton et al. 2003; Chilliard and Ferlay 2004). Enhancing CLA content by dietary changes also results in milk fat containing a lower proportion of saturated FA and greater amounts of monounsaturated FA (i.e., vaccenic acid) and PUFA (Park et al. 2007).

Cholesterol

Sterols are a minor fraction of total lipids in milk, the main sterol being cholesterol (300 mg/100 g fat, equivalent to 10 mg/100 mL bovine milk) (Park et al. 2007). Cholesterol is located mainly in the fat globule membrane where it represents 0.4–3.5% of the membrane lipids (Renner 1989). Milk cholesterol exists mostly as free cholesterol (85–90%), while a minor portion occurs as esterified form, usually combined with long-chain fatty acids (Renner et al. 1989).

Mean cholesterol concentrations of goat, cow, and human milk were reported as 11, 14, and 14 mg/100 g milk, respectively (Posati and Orr 1976), indicating that goat milk contains less cholesterol than other milk, whereas goat milk generally contains higher total fat than cow milk. The low level of cholesterol in goat milk may be important to human nutrition, since cholesterol is implicated with coronary heart disease. In this regard, cholesterol may be considered a bioactive compound in milk. Cholesterol in goat milk is usually in the range of 10–20 mg/100 mL milk (Jenness 1980).

Fatty acid composition of cholesterol esters reveals that goat milk cholesterol esters have greater palmitic and oleic acid fractions than cow counterparts (Jenness 1980; Juàrez and Ramos 1986). Cholesterol esters of cow milk fat represent about one-tenth of the sterol content in cow milk. On the average, 66% of the free and 42% of the esterified cholesterol are associated with goat milk fat globules (Keenan and Patton 1970). The level of unsaponifiable matter in goat milk is 24 mg/100 mL or 46 mg/100 g fat, which is comparable to that in cow milk. Cholesterol content was significantly varied among different breeds, and most cholesterol in goat milk was in free state, with only a small fraction in the ester form, 52 mg/100 g fat (Arora et al. 1976).

Cholesterol is mainly synthesized in the liver from acetic acid via acetyl coenzyme A. It has essential functions in the body as a structural component of cellular and subcellular membranes, plasma lipoproteins, and nerve cells (Innis 1985; Renner et al. 1989). Cholesterol is a metabolic precursor of bile acids and steroid hormones including vitamin D, and it is required for the metabolic systems involved in DNA synthesis and cell division, and also plays an integral role in lipid transport (Innis 1985). The body synthesizes 1–4 g cholesterol daily, and the total amount of cholesterol in the human body is 100–150 g, while 10–12 g is constantly present in blood (Renner et al. 1989).

A higher amount of cholesterol is synthesized in the body itself than is taken up with diet, implying that there is no significant correlation between cholesterol intake and the level of blood cholesterol. Since the body has a compensatory regulation system in blood cholesterol level, the endogenous synthesis is decreased by an increased

intake of dietary cholesterol, as well as by the amount of biliary cholesterol excretion (Innis 1985; McNamara 1985). There is a higher hepatic efflux of cholesterol in low-density lipoproteins (LDL) or its precursors after cholesterol consumption (Beynen et al. 1986).

Other Minor Lipids in Milk

Minor lipids in milk may include gangliosides, glycolipids, glycosphingolipids, and cerebrosides, etc., which can be considered as bioactive components. Although these compounds exist ubiquitously in mammalian tissues, studies on these minor lipids are available for bovine and human milk, but those for goat milk are almost nonexistent.

The functions of these minor lipids are important in cell-to-cell interaction and cell differentiation, proliferation, and immune recognition, as well as in receptor functions in relation to protein hormones, interferon, fibronectin, and bacterial toxins (Renner et al. 1989). Ceramide glucoside and ceramide dihexoside were shown to be the major neutral glycosphingolipids and gangliosides in the bovine milk fat globule membrane, and ceramide galactoside and ceramide dihexoside were identified in pooled human milk (Takamizawa et al. 1986). Digestibility of milk for the neonate can be affected by the presence of ceramide glucoside or ceramide galactoside at the surface of the fat globules. In addition, these minor milk compounds contain long-chain fatty acids (C22–C24), which are required for the synthesis of glycosphingolipids in the constitution of the nervous system (Bouhours et al. 1984). Laegreid and Kolsto Otnaess (1985) extracted a ganglioside from human milk that inhibits *Escherichia coli* heat-labile enterotoxin and cholera toxin.

Another minor lipid, alkylglycerol, occurs as nonesterified or esterified with fatty acids and/or phospholipids in milk (Ahrne et al. 1983). The range of total amounts of neutral alkylglycerols is 0.1–0.2 mg/g of milk fat with higher amounts in colostrum as shown in Table 3.5. Studies using crude mixtures of alkylglylcerols reportedly have shown several therapeutic functions, including tuberculostatic and antiinflammatory effects (Renner et al. 1989). Alkylglycerol is a highly potent substance at nanomolar concentrations and is identified as a platelet-activating factor (Ahrne et al. 1983; Bjorck 1985).

Table 3.5. Alkylglycerol in colostrum and milk of different mammals

	Alkylglycerol Content (mg/g fat)	
Mammal	Colostrum	Milk
Cow	1.5	0.1
Sheep	1.0	0.2
Goat	2.0	1.0
Pig	3.6	1.4
Human	2.0	1.0

Bjorck (1985).

GROWTH FACTORS AND RELATED COMPONENTS IN GOAT MILK

Growth Factors in Goat Milk

Goat milk is a source of another physiologically active compound, growth factor (Wu and Elsasser 1995). Human milk contains physiological levels of growth factor, while bovine milk has much lower growth factor activity (Grosvenor et al. 1992; Wu and Elsasser 1995). Colostrum of most mammals usually contains rich levels of growth factors and others bioactive compounds, whereas the levels of these molecules decrease rapidly during the first 3 days of lactation (Brown and Blakeley 1984; Denhard et al. 2000). Epidermal growth factor (EGF) was identified using antibody neutralization assays (Carpenter 1980). Several other growth factors have been identified in human milk, including IGF-I (Grosvenor et al. 1992), hapatocyte growth factor (HGF; Yamada et al. 1998), and vascular endothelial growth factor (Nishimura et al. 2002).

Growth factors in milk have been shown to be involved in the development of the neonatal gastrointestinal tract (Koldovsky 1996). EGF enhances the establishment of the epithelial barrier in the gastrointestinal tract (Puccio and Lehy 1988), and protects the gastroduodenal mucosa by activating ornithine decarboxylase in polyamine synthesis (Konturek et al. 1991). Growth factors can withstand the harsh conditions of gastric acid exposure and can be absorbed through the GI tract to act on other tissues. Playford et al. (2000) suggested that milk-derived growth factors may be useful as an orally

Table 3.6. Growth factor activity[1] in goat milk from different breeds

	Breed				
	Nubian	Saanen	Alpine	Toggenburg	LaMancha
No. Observ.	10	10	10	9	7
Mean[2]	1,085	692	616	590	620
SD	270	96	73	60	80

[1]Units of growth factor activity in milk.

[2]The Nubian average was statistically higher ($P < 0.01$) than the other averages.

There were no differences among any other samples. Mean = average units of growth factor activity for each breed. SD = standard deviation.

Adapted from Wu et al. (2006).

administered treatment for a wide variety of gastrointestinal disorders.

Goat milk has a much higher level of growth factor activity than that of cow milk (Wu and Elsasser 1995), whereby goat milk may be a feasible nutraceutical for gastrointestinal disorders (Wu et al. 2006). The presence of EGF in caprine milk was observed using a human EGF (hEGF) polyclonal antibody (Denhard et al. (2000). Caprine milk also has been shown to possess the ability to reduce heat-induced gastrointestinal hyperpermeability (Prosser et al. 2004).

Wu et al. (2006) found that Nubian goat milk contained the highest growth factor activity among the five breeds of dairy goats tested (Table 3.6). In an earlier study, Park (1991) showed that Nubian milk contained higher ($P < 0.05$) total protein and nonprotein nitrogen contents than the Alpine breed, which might be associated with the highest growth factor activity observed in Nubian milk by Wu et al. (2006). The authors also found that the milk of pregnant does had a significantly higher growth factor activity than those of postpartum goats (Table 3.7). Growth factor activity decreased during the first 8 weeks of lactation, fluctuated thereafter, and then increased dramatically after natural mating. Growth factor activity during weeks 1 through 8, was inversely correlated with milk yield and week of lactation, while no correlation was found during weeks 9 through 29. Growth factor activity in the milk after natural mating of goats correlated significantly with somatic cell count and conductivity, but was inversely correlated with milk yield.

Wu et al. (2007), using dialysis and ultrafiltration with 50, 30, and 3 kDa cutoff membranes, characterized that more than 90% growth factor activity was present in the >50 kDa fraction, in contrast to the 6 kDa molecular weight of EGF (human epidermal growth factor). The growth factor activity of goat milk was around pH 6.3 fraction, which differed from the pI 4.6 of EGF. They concluded that the major growth factor of the molecular fraction of goat milk was different from that of human milk.

Table 3.7. Growth factor activity in goat milk from pre- and postpartum Nubian does

Doe Number	Prepartum[1]	Postpartum[1]
1	1,310	795
2	6,610	792
3	8,380	702
4	27,000	2,760
Mean[2]	13,800	1,260
SD	8,010	864

[1]Units of growth factor activity in milk.

[2]Mean = Mean growth factor activity for all four does. The means were significantly different at $P < 0.05$.

Adapted from Wu et al. (2006).

Other Growth-Promoting Factors in Milk

The β-casein–derived peptides that stimulate DNA synthesis in mouse fibroblasts have been identified in tryptic hydrolysates of casein (Yamauchi 1992). These peptides offer the biotechnology industry a potential source for production of cell growth promotants.

Lactulose is produced from lactose during heat processing of milk. It is a disaccharide of galactose and fructose, which was originally recognized as a

bifidus growth promoter, and it has been utilized for medicinal products and infant milk formulae. Morinaga Milk Industry in Japan uses lactulose in a lactic acid bacteria drink, powdered milk, and ice cream (Regester et al. 1997).

Growth factors in whey proteins are highly potent hormonelike polypeptides derived from blood plasma that play a critical role in regulation and differentiation of a variety of cells. Many growth factors in both human and bovine milk have been identified (Donovan and Odle 1994), such as insulin-like growth factor, epidermal growth factor, platelet-derived growth factor, and transforming growth factor. Even though making up less than 0.1% of the total milk protein, these growth-promoting compounds in milk have specific biological actions in the regulation and/or stimulation of cell growth and repair. Isolation procedure produced a whey protein fraction, which can promote significant growth stimulation in different cell lines, providing in some cases superior growth performance to fetal bovine serum (FBS). A colostrum-based product has been available as an FBS substitute for supporting the growth of mammalian cells in culture (Regester et al. 1997).

Somatotropin is a growth hormone secreted in milk, which has an enormous impact on the dairy industry. This hormone can be produced biotechnologically and daily injections of this compound can increase milk yield up to 40%. Bovine milk contains approximately 5 mg/mL, and its concentration in milk did not change by a single injection of up to 100 mg somatotropin (Peel and Bauman 1987). Administration of any exogenous somatotropin may be degraded by lysozymal enzymes and other proteolytic enzymes in the blood and at the target organs.

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).

In contrast to bovine milk, human milk contains a growth-promoting activity for *Lactobacillus bifidus* var. *Pennsylvanicus* (Gyorgy et al. 1974). This activity appears to be responsible for the predominance of *Lactobacillus* in the bacterial flora of large intestines of breast-fed infants. These bacteria are capable of producing acetic acid, which helps in suppressing the multiplication of enteropathogens. Caprine milk has yet to be studied in this premise. The bifidus growth-factor activity is attributable to N-containing oligosaccharides (Gyorgy et al. 1974) and glycoproteins and glycopeptides (Bezkorovainy et al. 1979). The oligosaccharide moiety of those molecules may possess the bifidus growth-promoter activity associated with caseins (Bezkorovainy and Topouzian 1981).

Dietary nucleotides have been postulated as growth factors for the neonate and have been implicated in the superior iron absorption from human milk (Janas and Picciano 1982). The effect of dietary nucleotides also has been positively ascribed to the lipoprotein metabolism during the neonatal period in relation to an increase of high-density lipoproteins (HDL) and a decrease of very low-density lipoproteins (VLDL) (Sanchez-Pozo et al. 1986).

A small polypeptide mitogen was identified as an epidermal growth factor producing significant biological effects in mammals, particularly in fetuses and infants (Renner et al. 1989). Bovine milk contains 324 mg/mL, which is almost the same as the level found in human milk when expressed as the proportion of protein (Yagi et al. 1986). Alpha-liponic acid, an organic acid, is located in the fat globule membrane and can also be considered a growth factor (Renner 1983).

MINOR BIOACTIVE COMPONENTS IN GOAT MILK

Minor Proteins

Immunoglobulins

Immunoglobulins are glycoproteins secreted by plasma cells in milk of mammals that function as antibodies in the immune response by binding to specific antigens, whereby they are considered as bioactive compounds in milk. Immunoglobulins are important for the immunity of the newborn young. Goat milk has similar ranges of immunoglobulins to those of cow and sheep milk and colostrums (Table 3.8).

The relative proportions of immunoglobulins in total milk protein are about 2% (1–4%) and in whey protein about 12% (8–19%) (Ng-Kwai-Hang and Kroeker 1984). In the colostrums of cow milk, IgG is the predominating form as approximately 80–90% of total immunoglobulins, while the proportion of

Table 3.8. Some of the minor protein contents in goat, cow, and human milk

Proteins	Goat	Cow	Human
Lactoferrin (μg/mL)	20–200	20–200	<2000
Transferrin (μg/mL)	20–200	20–200	50<
Prolactin (μg/mL)	44	50	40–160
Folate-binding protein (μg/mL)	12	8	—
Immunoglobulin:			
IgA (milk: μg/mL)	30–80	140	1000
IgA (colostrum:mg/mL)	0.9–2.4	3.9	17.35
IgM (milk: μg/mL)	10–40	50	100
IgM (colostrum:mg/mL)	1.6–5.2	4.2	1.59
IgG (milk: μg/mL)	100–400	590	40
IgG (colostrum:mg/mL)	50–60	47.6	0.43
Nonprotein N (%)	0.4	0.2	0.5

Adapted from Remeuf and Lenoir (1986), Renner et al. (1989), and Park (2006).

IgM is about 7% and IgA 5%. IgG_1 accounts for 80–90% of IgG, and IgG_2 for 10–20% (Guidry and Miller 1986). The levels of all isotypes of immunoglobulins diminish rapidly postpartum, whereas IgG predominates also in mature milk, as shown in Figure 3.5 (Guidry and Miller 1986; Renner et al. 1989).

In ruminant species such as goats, sheep, and cows, the newborn receives immunoglobulins through colostrum and milk, since the placental structure of ruminants does not permit the transfer of immunoglobulins from the mother to the fetus. In contrast, IgG in humans passes through the placenta and the passive immunity in the newborn is gained by the intrauterine transfer of antibodies (Renner et al. 1989). The immunoglobulins, mainly IgA, are not broken down by the digestive enzymes.

Lactoferrin

Serum transferrin is named transferrin in contrast to lactoferrin as the component in milk. Lactoferrin and transferrin are different in the pH-dependence of their iron-binding capacity; lactoferrin retains iron even at pH 3; transferrin loses it at pH 4.5 (Nemet and Simonovits 1985). Transferrins constitute a family of homologous glycoproteins present in all vertebrate species (Renner et al. 1989). Transferrins consist of a single polypeptide chain with one or two attached carbohydrate groups, and they have a molecular weight of nearly 80,000. With two metal binding sites, they can bind a ferric iron (Fe^{3+}) to each site together with a bicarbonate ion. Iron is delivered from transferrin to cells through mediation by binding of transferrin-Fe^{3+} complexes to specific cellular receptors (Metz-Boutigue et al. 1984; Renner et al. 1989).

Human milk has approximately 10 times higher lactoferrin contents than goat and cow milk, where lactoferrin is the major iron-binding protein in human milk (Table 3.8). On the other hand, transferrin contents of goat and cow milk are much higher than in human milk (Table 3.8). Milk from goats, cows, guinea pigs, mice, and sows contains nearly the same amount of lactoferrin and transferrin; transferrin is predominant in milk from rats and rabbits (Fransson et al. 1983; Table 3.8).

Lactoferrin has several biological functions, including microbial activity. It does not exert a bacteriostatic effect in normal milk, whereas the growth of *Escherichia coli* and other bacteria has been reduced in the milk of cows having acute mastitis (Rainard 1987). The native state of lactoferrin is only partly saturated with iron (8–30%), which has a physiological importance, since it can chelate iron and inhibit bacteria by depriving them of iron, which is essential for growth (Renner et al. 1989). Lacto-

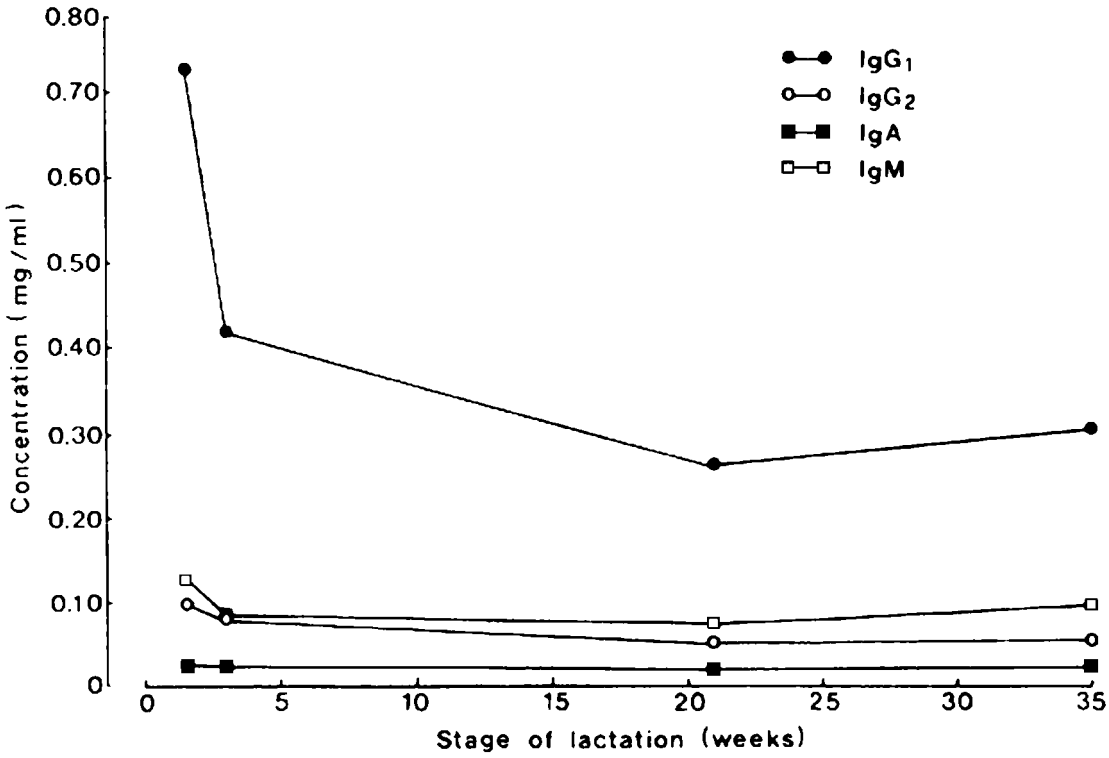

Figure 3.5. Immunoglobulin concentrations in cow milk at different stages of lactation (adapted from Guidry and Miller 1986).

ferrin can retain iron at very low pH (up to 2.2), so that it should pass through the acid gastric fluid unharmed (Reiter 1985a). Lactoferrin also chelates free iron continuously in the intestinal tract, when free iron becomes available during digestion from iron bound by fat and casein (Reiter 1985b). Lactoferrin has a growth-promoting effect on lymphocytes via transportation of free iron to the cell surface. The effect was also suggested on macrophages, granulocytes and neutrophilic leukocytes (Renner et al. 1989).

Free Amino Acids

Free amino acids (FAA) mainly consist of the nonessential amino acids glutamic acid, glycine, aspartic acid, and alanine with small quantities of other amino acids as FAA (Renner et al. 1989). FAA represent 10–20% of nonprotein nitrogen in milk, which is 5–8 mg/100 mL. Ornithine is found in some colostrum as an intermediate metabolic product, which may be an indicator for the destruction of body protein.

Carnitine plays an important role in facilitating the transport of fatty acids, particularly long-chain fatty acids, into the mitochondrial matrix for oxidation, in initiation of ketogenesis and in the maintenance of thermogenesis (Baltzell et al. 1987). Carnitine is a critical nutrient for the human neonate, since its endogenous synthesis from lysine may be lower than that in adults. Carnitine content of cow milk is higher than in human milk: 160–270 vs. 30–80 nmol/mL (Sandor et al. 1982).

Taurine is a sulfur-containing free amino acid, and occurs in a wide variety of mammalian tissues (Erbersdobler et al. 1984). Taurine functions in the formation of the bile salts, which facilitate the digestion and absorption of lipids, while it is not utilized either for protein synthesis or as a source of energy (Erbersdobler 1983).

Organic Acids

Literature on organic acid content of goat milk has been very scarce. However, Park et al. (2006) studying organic acid profiles of goat milk plain soft (PS) and Monterey Jack (MJ) cheeses, reported that soft goat cheese contained formic 2.32, orotic 0.042, malic 1.13, lactic 10.04, acetic 2.86, citric 0.69, uric 0.017, propionic 0.71, pyruvic 0.00, butyric acid 1.07 mg/g of cheese, respectively. The goat milk PS and MJ cheeses contained 60.1 and 42.1% moisture, respectively.

Orotic acid is an intermediate compound in pyrimidine biosynthesis, and thereby it is a component of all cells. Approximately 80% of the nucleotides in cow milk exist as the form of orotic acid that is almost absent in human milk (Counotte 1983). High concentrations of orotate have been found in ruminant milk. Cow milk contains about 60 mg/L with a range between 10 and 120 mg/L (about 400–600 μmol/L), while goat and sheep milk have lower contents, about 120 and 30 μmol/L, respectively (Tiemeyer et al. 1984). Orotic acid is hypocholesterolemic in rats, but not in mice, hamsters, or guinea pigs.

Nucleic acids are found in milk as ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and nucleotides, and they are constituents of all cells. Cow milk and human milk have similar DNA contents—1.2 and 1.5 mg/100 mL, respectively. However, human milk contains higher RNA than cow milk (11.5 vs. 5.4 mg/100 mL) (Renner 1983). An increased intake of nucleic acids leads to the formation of uric acid, which may result in urinary calculi and gout.

Dietary nucleotides may be growth factors for the neonate and have been implicated in the superior iron absorption from human milk (Janas and Picciano 1982). Dietary nucleotides have a positive effect on lipoprotein metabolism during the neonatal period by increasing high-density lipoproteins (HDL) and decreasing very low-density lipoproteins (VLDL) (Sanchez-Pozo et al. 1986).

Pyruvic acid is an important organic acid that is a key intermediate in the intermediary metabolism of carbohydrates, amino acids, and citrate by many organisms. It is excreted into the milk during the catabolism of lactose and the oxidative deamination of alanine by microorganisms, and the initial content is 1 mg/L (Marshall et al. 1982). Pyruvate can be converted by microorganisms into a variety of end products, and it is not a fermentation end product, but it is a transitional substance in bacterial metabolism.

Citric acid constitutes approximately 90% of the organic acids in milk. Citrate is a part of the buffer system of milk, has an effect on the distribution of Ca in milk, contributes to the stability of the calcium caseinate complex, and is the starting material for flavor substances in cultured milk products. Citrate is a carboxylic acid, synthesized in the mammary gland from pyruvic acid (Renner 1983). The average concentration of citrate in milk is 1.7 g/L, ranging from 0.9 to 2.3 g/L (Renner 1983).

Neuraminic or sialic acid plays a role in the stability of the casein complex and also contributes to the inhibition of the growth of *coli* and *staphylococci* bacteria. Neuraminic acid in milk occurs in acetylated form as N-acetyl neuraminic acid, and the mean sialic acid content in milk is approximately 150 mg/L, ranging from 80–1000 mg/L (Renner et al. 1989).

Uric acid is considered to have antioxidative activity, and occurs in milk at about 200 μmol/L of protein and fat-free milk extract. It is a final product of the purine metabolism. α-Liponic acid is regarded as a growth factor and is located in the fat globule membrane (Renner 1983). Although organic acids in human and bovine milk have been studied to a great extent, limited research has been documented on those in goat milk.

BIOACTIVE CARBOHYDRATES IN GOAT MILK

Lactose

Lactose contents of goat, cow, and human milk are 4.1, 4.7, and 6.9 g/100 mL, respectively (Haenlein and Caccese 1984; Park 2006). The high content of lactose in human milk may explain, at least in part, the stimulation of the development of a *bifidus* flora, which is associated with a decrease in the luminal pH and an increased colonization resistance against pathogenic organisms in the human intestine (Schulze and Zunft 1991).

Lactose stimulates the vitamin D–independent component of the intestinal calcium transport system in laboratory animals such as rats, probably due to the decreased luminal pH and increased calcium

solubility by the fermentation of undigested lactose in the intestinal lumen (Schaafsma et al. 1988). Lactose may enhance calcium absorption in situations where calcium solubility or active vitamin D–dependent calcium absorption is limited (Schaafsma and Steijns 2000).

Lactose and bile salts are a few example agents that can augment vitamin D–independent ileal absorption of calcium through the paracellular pathway (Lee et al. 1991). The bulk of calcium absorption occurs in the ileum, which is a segment with a limited capacity of active calcium absorption and is relatively insensitive to the action of calcitriol (the active vitamin D metabolite) (Lee et al. 1991).

Researchers have demonstrated that lactose has a lower glycemic index than sucrose or glucose, indicating that lactose can be regarded as suitable in the diet of diabetics (Wolever et al. 1985). Lactose is also reportedly less cariogenic compared to other major sugars, including glucose, fructose, and maltose (Edgar 1993).

Lactose-Derived Compounds

Lactose-derived products are: lactulose, lactitol, lactobionic acid, and galacto-oligosaccharides. Lactulose is produced from lactose during heat processing of milk, and it is a disaccharide of galactose and fructose. Lactulose was first acknowledged as a bifidus growth promoter and has been used in medicinal products and in infant milk formulae (Regester et al. 1997). Lactulose content in heated milk ranges from 4 to 200 mg/100 mL (Andrews 1989).

Lactulose and lactitol are nondigestible, but both of these lactose-derived compounds serve as a source of soluble fiber and are widely used in the treatment of constipation and chronic hepatic encephalopathy, where they act in a similar way on the intestinal microflora (Camma et al. 1993; Blanc et al. 1992). A 10 g per day intake of lactulose increased calcium absorption in postmenopausal women (Van den Heuvel et al. 1999).

Lactobionic acid is not digested in the small intestine while it can be fermented by the intestinal flora. Lactobionic acid forms soluble complexes with minerals such as calcium, due to its prebiotic properties (Schasfsma and Steijns 2000). The galacto-oligosaccharides were shown to have prebiotic properties owing to their selective stimulation of bifidobacteria in the intestine, and also have the property of increasing the intestinal absorption of calcium (Chonan and Watanuki 1995).

Oligosaccharides

Milk contains minor forms of carbohydrates other than lactose, such as free and bound to lipids, proteins, or phosphate. Among these compounds, oligosaccharides may be the most important and they are a class of carbohydrates that comprise from 2 to 10 monosaccharide units. These carbohydrates contain galactose, fucose, N-acetylglucosamine and N-acetylneuraminic acid (NANA) in different proportions as well as a glucose residue (Cheetham and Dube 1983; Renner et al. 1989). More than 50 oligosaccharides have been identified from human milk, and more than 30 from bovine and caprine milk (Saito et al. 1984; Martĭnez-Férez et al. 2006). Oligosaccharides in milk belong to the group of bifidus factor, promoting the growth of *Lactobacillus bifidus* in the intestinal tract.

Goat milk has a unique difference in milk carbohydrate patterns compared to cow milk. Goat milk has been shown to have 10 times higher oligosaccharides than cow milk, which closely resembles human milk. This is of special interest to infant nutrition since goat milk oligosaccharides have functional effects on human nutrition. Human milk oligosaccharides are thought to be beneficial for the infant in relation to their prebiotic and antiinfective properties (Martĭnez-Férez et al. 2006).

Characterization and quantification of neutral and sialylated lactose-derived oligosaccharides in mature caprine milk has been recently conducted to compare those in ovine, bovine, and human milk (Martnez-Férez et al. 2006). It was found that a large amount and variety of acidic and neutral oligosaccharides were present in goat milk compared to cow and sheep milk. Furthermore, they identified 15 new oligosaccharide structures in caprine milk.

Lara-Villoslada et al. (2006) investigated the effect of goat milk oligosaccharides (GMO) on dextran sodium sulfate (DSS-) induced colitis using a rat model. They found that DDS induced a decrease in body weight that did not occur in rats fed the GMO, and they also observed that GMO rats exhibited less severe colonic lesions and a more favorable intestinal microbiota. This study demonstrated that GMO can reduce intestinal inflammation and

contribute to the recovery of damaged colonic mucosa.

BIOACTIVE MINERALS IN GOAT MILK

Milk as a dietary source of minerals plays an important role in human health. Many major and trace minerals are bioactive in physiology and metabolism of the human body. There is an important relationship between dietary minerals and the occurrence of specific diseases such as hypertension, osteoporosis, cancer, and cardiovascular disease.

Up to 1960, six major and eight trace elements were recognized as essential minerals for growth, metabolism, and development of pathology (Underwood 1977). The six macrominerals are sodium, potassium, calcium, phosphorus, magnesium, and chloride, and the eight trace minerals are iron, iodine, copper, manganese, zinc, cobalt, selenium, and chromium.

Concentrations of major and trace minerals of goat milk are compared with those of cow and human milk shown in Table 3.9. Goat milk has higher calcium, phosphorus, potassium, magnesium, and chlorine, and lower sodium and sulfur contents than cow milk (Table 3.9; Park and Chukwu 1988; Haenlein and Caccese 1984; Park 2006). The bioactivities and functionalities of some of the selected minerals are delineated in the following sections.

Table 3.9. Mineral and vitamin contents of goat milk as compared with those of cow and human milk

Constituents	Goat	Cow	Human
		Amount in 100 g	
Mineral			
Ca (mg)	134	122	33
P (mg)	121	119	43
Mg (mg)	16	12	4
K (mg)	181	152	55
Na (mg)	41	58	15
Cl (mg)	150	100	60
S (mg)	2.89	—	—
Fe (mg)	0.07	0.08	0.20
Cu (mg)	0.05	0.06	0.06
Mn (mg)	0.032	0.02	0.07
Zn (mg)	0.56	0.53	0.38
I (mg)	0.022	0.021	0.007
Se (μg)	1.33	0.96	1.52
Vitamin			
Vitamin A (I.U.)	185	126	190
Vitamin D (I.U.)	2.3	2.0	1.4
Thiamine (mg)	0.068	0.045	0.017
Riboflavin (mg)	0.21	0.16	0.02
Niacin (mg)	0.27	0.08	0.17
Pantothenic acid (mg)	0.31	0.32	0.20
Vitamin B_6 (mg)	0.046	0.042	0.011
Folic acid (μg)	1.0	5.0	5.5
Biotin (μg)	1.5	2.0	0.4
Vitamin B_{12} (μg)	0.065	0.357	0.03
Vitamin C (mg)	1.29	0.94	5.00

Data from Posati and Orr (1976), Park and Chukwu (1988), Jenness (1980), Haenlein and Caccese (1984), and Park (2006).

Major Minerals

Calcium

Calcium is an important bioactive nutrient involved in the growth, metabolism, and health of bone (Kanis 1993). Calcium as a bioactive mineral is demonstrated widely in a range of calcium-fortified foods, including modified milk and beverages. In Western diets, milk and dairy products provide approximately 70% of the recommended daily intake for calcium. In the U.S., the Food and Drug Administration has advised men and women over 50 years to increase their calcium intakes toward 1200 mg/day.

The role of calcium as a protective factor in the etiology of colon cancer has been well documented (Sorenson et al. 1988). Calcium is also believed to be associated with binding and removal of carcinogenic agents (bile salts, etc.) along the gastrointestinal tract (Regester et al. 1997). In a study with rats, the involvement of dietary calcium in resistance against infections of pathogenic bacteria has been shown (Bovee-Oudenhoven et al. 1997).

Calcium is important for development and maintenance of skeletal integrity and prevention of osteoporosis (Schaafsma et al. 1987). Hypertension is another disease that is related to a low calcium intake, and calcium supplementation reduced blood pressure in hypertensive patients (Grobbee and Hofman 1986). A study showed that people who ingest diets low in sodium and high in potassium, magnesium, and calcium do not have hypertension and cardiovascular disease (Morgan et al. 1986).

Phosphorus

Goat milk contains about 134 mg Ca and 121 mg P/100 g (Table 3.9; Park and Chukwu 1988). Human milk contains only one-fourth to one-sixth of these minerals. Phosphorus exerts several important bioactive metabolic functions in the body, including bone mineralization, energy metabolism (i.e., ATP and chemical energy), fat and carbohydrate metabolisms, body buffer system (acid-base balance and pH of the body), and formation and transport of nucleic acids and phospholipids across cell membranes for body cell functioning, etc.

In underdeveloped countries, where meat consumption is very limited, goat milk is an important daily food source of animal protein, phosphate, and calcium due to lack of availability of cow milk (Haenlein and Caccese 1984; Park 1991; 2006). Approximately 3 times higher P and Ca in goat milk compared to human milk would have significant bioactivities of these two minerals in human nutrition, physiology, and metabolism in underdeveloped and developing countries.

Trace Minerals

Unlike major minerals, concentrations of trace minerals are affected by diet, breed, individual goats, and stages of lactation (Park and Chukwu 1988). It has been reported that a large proportion of copper, zinc, and manganese is bound to milk casein. Iron and manganese are partly bound to lactoferrin, a bacteriostatic protein, which occurs in the whey protein fraction of milk (0.2 mg/mL) (Lönnerdal et al. 1981, 1983, 1985).

Iron contents of goat and cow milk are lower than in human milk (Table 3.9). Iron occurs in milk in combination with several proteins, such as lactoferrin, transferrin, and ferrilactin. Iron also occurs in blood as hemoglobin and transferrin in the plasma in the ratio of 1000:1, and a small quantity of ferritin is present in erythrocytes. Iron deficiency causes anemia, impaired growth, and lipid metabolism (Underwood 1977). In a comparative study of bioavailability of milk iron, Park et al. (1986) reported that goat milk had a greater hemoglobin regeneration efficiency and iron bioavailability than cow milk, which was fed to iron-deficient, anemic rats (Fig. 3.6). A Spanish study also showed that a goat milk diet produced a greater iron nutritive utilization in comparison with a cow milk diet (Lopez-Aliaga et al. 2000). The same research group later also reported that rats fed a goat milk diet had greater iron and copper apparent digestibility coefficient and bioavailability in different animal organs compared to those fed a cow milk diet, especially those animals with malabsorption syndrome (Barrionuevo et al. 2002).

Zinc content is greatest among trace minerals (Table 3.9), and Zn in goat and cow milk is greater than in human milk (Park and Chukwu 1989). Zinc deficiency would result in skin lesions, disturbed immune function, growth retardation, and impaired wound healing (Underwood 1977). In a feeding trial with rats, animals fed a goat milk diet exhibited a greater bioavailability of zinc and selenium

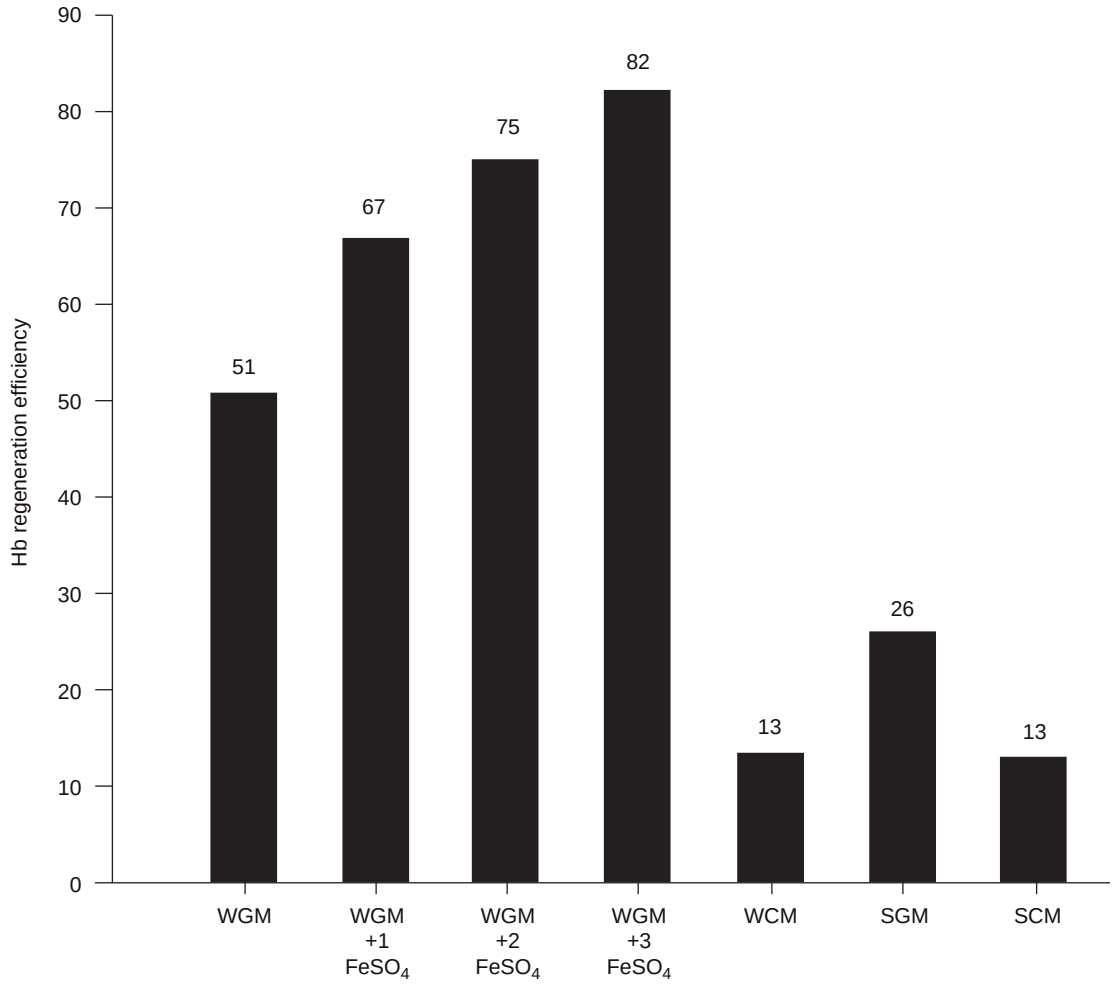

Figure 3.6. Hemoglobin regeneration efficiencies (HRE) of whole goat milk (WGM) diet; whole goat milk diet supplemented with 50, 100, or 200 ppm ferrous sulfate; whole cow milk (WCM) diet; skim goat milk diet; or skim cow milk diet fed to anemic growing rats for 10 days. HRE for WGM is greater than that for WCM ($P < .01$), and SGM is greater than the SCM group ($P < .05$). (Adapted from Park et al. 1986).

compared to those fed a cow milk diet (Alferez et al. 2003).

Goat and cow milk contain significantly greater levels of iodine than human milk, which may be important for human nutrition, since iodine and thyroid hormone are closely related to the metabolic rate of physiological body functions (Underwood 1977).

Goat and human milk contain higher concentrations of selenium than cow milk (Table 3.9). Less than 3% of the total selenium is associated with the lipid fraction of milk. The selenium-dependent enzyme, glutathione peroxidase, is higher in goat milk than in human and cow milk. Goat milk total peroxidase activity (associated with glutathione peroxidase) was 65% as opposed to 29% for human and 27% for cow milk (Debski et al. 1987). There are several other trace minerals that exhibit active bioactive functions in the body metabolisms, including Mo, Cr, Co, Mn, F, As, Sn, and V.

BIOACTIVE VITAMINS IN GOAT MILK

Vitamins are physiological, biochemical, and metabolical bioactive compounds occurring in milk. Vitamins are divided into two categories: water-soluble and fat-soluble vitamins. All known vitamins are contained in milk, and they have specific biological functions in the body. Cow milk is the rich source of human dietary requirements of vitamins, particularly riboflavin (B_2) and vitamin B_{12}.

Goat milk has higher amounts of vitamin A than cow milk. Goat milk supplies adequate amounts of vitamin A and niacin and excesses of thiamin, riboflavin, and pantothenate for a human infant (Table 3.9; Parkash and Jenness 1968; Ford et al. 1972). A human infant fed solely on goat milk is oversupplied with protein, Ca, P, vitamin A, thiamin, riboflavin, niacin, and pantothenate in relation to the FAO-WHO requirements (Jenness 1980).

Goat milk, however, has a significant drawback of deficiencies in bioactive vitamins, folic acid, and vitamin B_{12} as compared to cow milk (Collins 1962; Davidson and Townley 1977; Jenness 1980; Park et al. 1986). Cow milk has 5 times more folate and vitamin B_{12} than goat milk, where folate is necessary for the synthesis of hemoglobin (Collins 1962; Davidson and Townley 1977). Vitamin B_{12} deficiency has been reportedly implicated in "goat milk anemia," which is a megaloblastic anemia in infants (Parkash and Jenness 1968). However, the major cause of the anemia has been reportedly associated with folate deficiency in goat milk.

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4
Bioactive Components in Sheep Milk

Isidra Recio, Miguel Angel de la Fuente, Manuela Juárez, and Mercedes Ramos

INTRODUCTION

Although the production of sheep milk (about 8 million tonnes) is of marginal importance compared to cow milk in quantitative terms (2% of the total world supply), it is of major interest in Mediterranean and Middle Eastern countries, where climatic conditions are not favorable for cattle raising. The numbers of sheep do not fully reflect the amount of milk produced, since sheep are often used for other purposes, such as meat and wool. Although sheep milk is richer in nutrients than cow milk, it is rarely used as milk for drinking. In general, sheep milk is utilized essentially for cheese production but in some countries part of the milk is made into yogurt or whey cheeses (Haenlein and Wendorff 2006).

The nutritional importance of sheep milk is due to its composition (Table 4.1). Sheep milk generally contains higher total solids and major nutrient contents than goat and cow milk. As has been reported for bovine milk, composition of sheep milk varies with diet, breed, animals within breed, parity, season, feeding and management conditions, environmental conditions, locality, and stage of lactation (Haenlein 2001; Pulina et al. 2006). Ovine milk is an excellent source of high-quality protein, calcium, phosphorus, and lipids. There is a good balance between the protein, fat, and carbohydrate components, each being present in similar amounts. The supply of nutrients is high in relation to the calorie content of the food. The fat and protein ratio is higher than in cow milk and therefore cheese yield is also higher (approximately 15% for sheep milk compared with 10% for cow milk).

Information on nutritional characteristics of sheep milk is essential for successful development of dairy industries as well as for marketing their products. With the progress in the knowledge of the composition and role of milk components, it became apparent during recent years that some milk compounds possess biological properties beyond their nutritional significance and have an impact on body function or condition and ultimately on health. Major advances have occurred with regard to the science, technology, and applications of these bioactive components present naturally in milk. These raw materials have proven to be rich and unique sources of chemically defined components that can be isolated and utilized as ingredients for health-promoting functional foods or as nutraceuticals. As a result, there is a growing interest by the dairy industry to design and formulate products that incorporate specific bioactive components derived from different kinds of milk. With the research tools available now, the presence of many minor compounds with biological activity has been demonstrated in bovine milk, but less is known about ovine milk. The purpose of this chapter is to address the nutritional properties of sheep milk mainly on lipids and proteins, with emphasis on the different bioactive compounds present in these fractions.

LIPIDS

Lipids are one of the most important components of milk in terms of physical and sensory characteristics and in the nutritional properties that they confer to sheep dairy products. Lipids are present in the form

Table 4.1. Compositions of sheep and cow milk

	Sheep			Cow
Component	Average Content (%)	Range (%)	Average of Dry Matter (%)	Average Content (%)
Water	81.6	79.3–83.3		80.0
Lactose	4.6	4.1–5.0	25.0	3.5
Fat	7.1	5.1–8.7	38.5	3.9
Crude Protein (total nitrogen X 6.38)	5.7	4.8–6.6	31.1	3.2
Casein	4.4			2.6
Whey Proteins	1.0			0.6
Nonprotein nitrogen	0.1			0.1
Ash	0.9	0.7–1.1	4.9	0.8
Total solids	18.4	16.2–20.7		11.5
Nonfat solids	11.3			7.6

Source: Walstra and Jennes (1984) and Ramos and Juárez (2003).

of globules, which in ovine milk are characteristically abundant in sizes of less than 3.5 μm. Among ruminants, the average fat globule size is the smallest in sheep milk (Park et al. 2007). This is advantageous for digestibility and more efficient lipid metabolism compared with cow milk. Structure and composition of the globule membrane is similar to cow and goat milk fat, and it represents approximately 1% of total milk fat volume. Fat globules contain one hydrophobic lipid core, consisting mainly of triglycerides (TAG), surrounded by a membrane made mainly of phospholipids and glycoproteins. Among the health beneficial components of milk fat globule membrane are cholesterolemia-lowering factor; inhibitors of cancer cell growth; vitamin binders; xanthine oxidase as a bactericidal agent; butyrophilin as a possible suppression of multiple sclerosis; and phospholipids as agents against colon cancer, gastrointestinal pathogens, Alzheimer disease, depression, and stress (Spitsberg 2005). All of the above compel us to consider fat globule membrane as a potential nutraceutical.

Different fatty acids composed of fat globule core and membrane, address a potentially healthy profile, and have been detected in sheep milk (Scolozzi et al. 2006). The C14:0 and C16:0 were present in greater amounts in the core, while polyunsaturated (PUFA) omega-3, conjugated linoleic acid (CLA), and the precursors of the latter are more represented within the globule membrane. The unsaturated/saturated fatty acids ratio was lower in the fat globule core than in the membrane.

Triglycerides (TAG)

TAG constitute the biggest group of lipids (nearly 98%), including a large number of esterified fatty acids. TAG in sheep present a wide range of molecular weights when distributed according to the number of carbon atoms (taking into account the sum of the carbon atoms of the three acyl radicals) from C26 to C54 (Goudjil et al. 2003a; Fontecha et al. 2005). The TAG profile of sheep milk (Table 4.2) shows similarities to that reported for cow milk (Precht 1992). However, sheep have a higher percentage of medium-chain TAG (C26–C36) than cow milk and a lower proportion of long-chain TAG (C46–C54)(Goudjil et al. 2003a). Compared with TAG containing mainly long-chain fatty acids, medium-chain TAG comprising saturated fatty acids with 6–10 carbons have a lower melting point, smaller molecule size, are liquid at room temperature, and less energy dense. These distinct chemical and physical properties affect the way they are absorbed and metabolized. Their medical and nutritional values have been the subject of research, demonstrating real benefits in different diseases (Haenlein 2001). However the unique content of about 25% medium-chain TAG in total sheep milk fat and its possible quantitative modification through feeding has not

Table 4.2. Triglyceride compositions of sheep and cow milk fats

Triglyceride[a] (Total Acyl Carbon Number)	Sheep[b] (wt%)	Cow[b] (wt%)
C26	0.60–1.00	0.20–0.30
C28	1.30–2.50	0.40–0.80
C30	2.00–3.40	0.80–1.90
C32	3.10–5.00	1.80–3.20
C34	5.70–7.00	4.40–6.90
C36	9.20–10.30	9.10–12.40
C38	12.50–14.00	11.80–14.60
C40	11.00–12.50	9.50–12.10
C42	8.00–9.70	6.20–7.90
C44	7.30–8.60	5.40–7.80
C46	6.50–6.90	5.60–8.30
C48	6.10–7.80	6.90–10.70
C50	4.70–9.10	9.70–12.80
C52	5.00–9.40	7.20–12.60
C54	3.10–5.30	2.70–7.80
C56	<0.50	0.40–0.60

[a]Triglycerides are identified by the number of acyl carbon atoms per glyceride molecule.

[b]Range calculated on the results given by different authors.

Source: Ramos and Juárez (2003) and McGibbon and Taylor (2006).

been exploited commercially or deeply explored in research.

Saturated Fatty Acids

Most fatty acids, from acetic (C2:0) to arachidic acid (C20:0), contain an even number of carbon atoms. The five most important fatty acids in quantitative terms (C18:1, C16:0, C10:0, C14:0, and C18:0) account for >70% of total fatty acids (Table 4.3). Sheep milk does not substantially differ in butyric acid (C4:0) content but it contains more caproic (C6:0), caprylic (C8:0), and capric (C10:0) acids than cow milk (McGibbon and Taylor 2006). These fatty acids are associated with the characteristic flavor of cheeses and possess different biological properties.

Low concentrations of butyric acid can inhibit growth in a wide range of human cancer cell lines, including prostate (Williams et al. 2003). Animal studies have shown that dietary fibers, which liberate a constant and elevated supply of butyrate to the colon, are most effective for prevention of chemically induced colon tumors. Moreover, the level of butyric acid in the colonic lumen of patients with colorectal cancer and adenomas was found to be lower than that in healthy individuals (Parodi 2004). Furthermore, synergism between butyrate and other dietary components and common drugs in reducing cancer cell growth have also been shown. A summary of these related cell-growth–inhibiting effects induced by butyric acid is outlined by Parodi (2006).

Beneficial effects of caproic, caprylic, and capric acids have recently been reviewed (Marten et al. 2006). These authors reported the potential of these fatty acids to reduce body weight and body fat. Acids C6:0, C8:0, and C10:0 are particularly digestible because they are hydrolyzed preferentially from the TAG and are transferred directly from the intestine to the portal circulation without resynthesis of TAG. Thus, there is only a low tendency for adipose formation. Furthermore, these fatty acids are a preferred source of energy (β-oxidation). Given in moderate amounts in diets with moderate fat supply, they may actually reduce fasting lipid levels more than oils rich in mono- or polyunsaturated fatty acids (Marten et al. 2006). As several studies indicate, moderate doses are better than excessive loads. Further studies are necessary to examine which dose and dairy matrix offers the most benefits and whether in milk fat naturally occurring fatty acids at position sn-1 of a TAG molecule and TAG oils have the same effect.

Unsaturated Fatty Acids

Oleic acid (*cis*-9 C18:1), the second predominant fatty acid in sheep milk fat (Table 4.3), is regarded as an antiatherogenic agent. Human diets high in oleic acid are mostly reported to decrease the level of LDL cholesterol, whereas HDL cholesterol levels are not affected significantly (Molkentin 2000). The polyunsaturated fatty acids (PUFA) in sheep milk fat mainly comprise linoleic (*cis*-9 *cis*-12 C18:2) and α-linolenic (*cis*-9 *cis*-12, *cis*-15 C18:3), as well as smaller amounts of their positional and geometric isomers. Both are essential fatty acids, have many diverse functions in human metabolism and, overall, promote an antiatherogenic effect. Sheep milk is not a rich source of linoleic and α-linolenic acids (Table

Table 4.3. Mean values and minimum (Min) and maximum (Max) contents of sheep and cow milk fat main fatty acids (% in total fatty acid methyl esters)

	Sheep		Cow	
Fatty Acid	Mean	Min/Max	Mean	Min/Max
C4:0	3.5	3.1–3.9	3.9	3.1–4.4
C6:0	2.9	2.7–3.4	2.5	1.8–2.7
C8:0	2.6	2.1–3.3	1.5	1.0–1.7
C10:0	7.8	5.5–9.7	3.2	2.2–3.8
C12:0	4.4	3.5–4.9	3.6	2.6–4.2
C13:0	0.2	0.1–0.2	0.2	
C14:0	10.4	9.9–10.7	11.1	9.1–11.9
iso C15:0	0.3	0.3–0.4	0.4	
anteiso C15:0	0.5	0.3–0.6	0.4	
C15:0	1.0	0.9–1.1	1.2	0.9–1.4
iso C16:0	0.2	0.2–0.3	0.4	
C16:0	25.9	22.5–28.2	27.9	23.6–31.4
iso C17:0	0.5	0.4–0.6	0.5	
anteiso C17:0	0.3	0.3–0,4	0.5	
C17:0	0.6	0.6–0.7	0.6	
C18:0	9.6	8.5–11.0	12.2	10.4–14.6
C20:0	0.5	0.4–0.5	0.4	
C10:1	0.3	0.2–0.3	0.2	
C14:1	0.3	0.2–0.5	0.8	0.5–1.1
C16:1	1.0	0.7–1.3	1.5	1.4–2.0
C17:1	0.2	0.2–0.3	0.4	
cis C18:1	18.2	15.3–19.8	17.2	14.9–22.0
trans C18:1	2.9	2.5–3.2	3.9	
C18:2	2.3	1.9–2.5	1.4	1.2–1.7
C18:2 conjugated	0.7	0.6–1.0	1.1	0.8–1.5
C18:3	0.8	0.5–1.0	1.0	0.9–1.2

Source: Goudjil et al. (2004) and McGibbon and Taylor (2006).

4.3). Mean linoleic acid content accounts for 70–75% of the total C18:2, excluding conjugated in sheep milk, whereas the rest of the *trans* C18:2 isomer group represent slightly more than a quarter of this fraction and 0.5–0.9% of the total fatty acids (Goudjil et al. 2004). On average, α-linolenic acids rarely exceed 1% (Table 4.3). However other omega-3 PUFA are hardly found in sheep milk fat, except when animal diets are supplemented with marine oil source (Mozzon et al. 2002; Reynolds et al. 2006).

TRANS Fatty Acids (TFA)

TFA content in sheep milk fat ranges from 2.5 to 5% of total fatty acids, mainly depending on diet and season. Monoene TFAs are the most abundant in all species. In ruminants, sheep milk presents the highest quantities, after cow and, finally, goat milk fat. The pattern of *trans* C18:1 isomer distribution is, however, quantitatively identical in the three species (Precht et al. 2001). TFA in dairy fat are not seen as bioactive lipids in a positive sense. But since TFA have come under scrutiny due to their influence on lipid levels and on other risk factors for coronary heart disease (CHD), the question of whether all TFA are alike or whether TFA isomers from dairy fat may have metabolic properties distinct from those of other origins, hydrogenation reaction, for instance, has gained increasing relevance.

The main source of TFA consumed daily by humans is partially hydrogenated vegetable fats and oils, although these compounds also occur naturally in ruminant milk as a result of partial biohydrogena-

tion of PUFA caused by rumen microorganisms. There is considerable overlap of TFA isomers in fats of ruminant origin and partially hydrogenated vegetable oils, with many isomers in common. However, the isomer profile of hydrogenated vegetable fats is very different.

During hydrogenation of vegetable fats a wide range of *trans* monounsaturated fatty acids are principally formed (e.g., *trans*-9 C18:1, elaidic acid), while the main TFA in milk fat is *trans*-11 C18:1, vaccenic acid (VA) (IDF 2005). The importance of VA lies in its role as a precursor of the main isomer of CLA, rumenic acid (RA) (*cis*-9 *trans*-11 C18:2), physiologically the most relevant bioactive compound present in milk fat. This synthesis not only occurs in the bovine mammary gland (Griinari and Bauman 1999), but also in human tissues (Turpeinen et al. 2002; Mosley et al. 2006; Kuhnt et al. 2006). The proportion of VA in milk fat total monoene TFA in sheep is around 45–60% (Wolff 1995; Precht et al. 2001; Goudjil et al. 2004), whereas elaidic acid is present only in considerably smaller amounts (average 5% of the total TFA). Thus, in contrast to the majority of hydrogenated vegetable oils enriched in *trans*-10 and *trans*-9 C18:1, the consumption of dairy products represents a very low intake of these components.

Individual TFA isomers could have differing physiological effects. There is evidence of unfavorable effects of TFA from hydrogenated vegetable oils on LDL and other risk factors of atherosclerosis, whereas the predominant TFA in milk, VA, would not exert these detrimental effects (IDF 2005; Parodi 2006). Most studies reported that the positive association with risk of CHD could be explained entirely by the intake of TFA from hydrogenated vegetable oils. Pfeuffer and Schrezenmeir (2006) also compiled works addressing the effect of TFA intake on CHD. Several of the large studies, which established that intake of TFA increases CHD risk, showed a significant inverse association with intake of animal or dairy TFA, a nonsignificant inverse

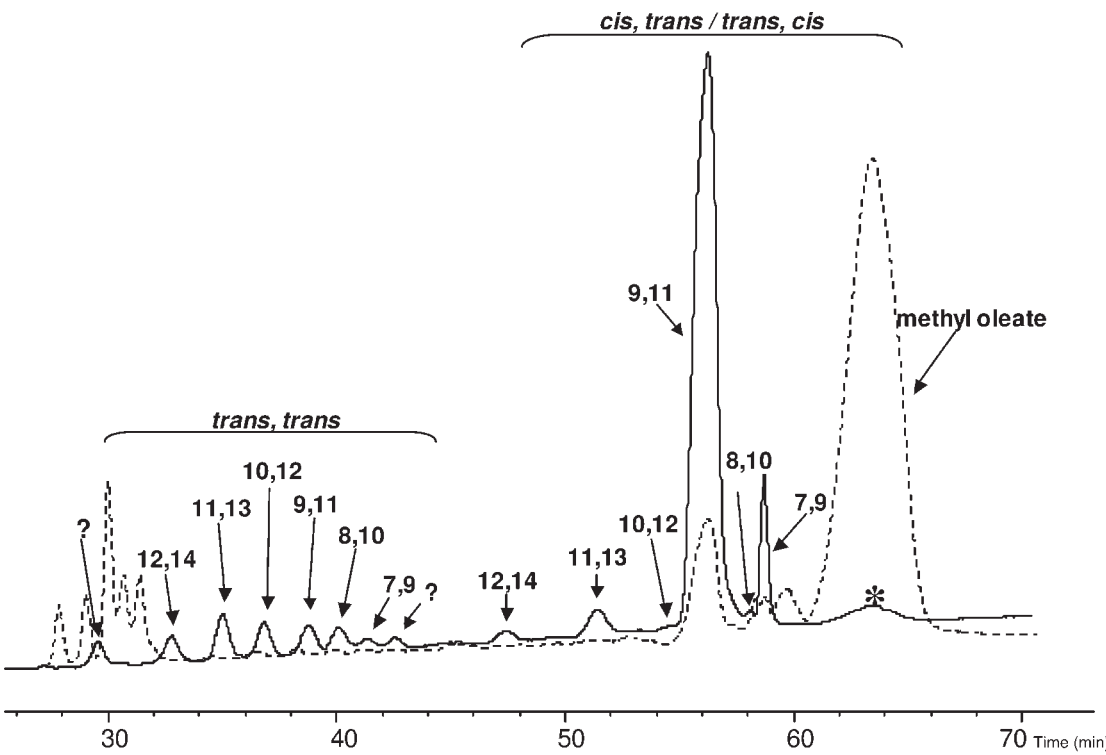

Figure 4.1. Silver ion-HPLC profile with three capillary columns in series of sheep milk fat fatty acid methyl esters using UV detector at 233 nm (solid line) and 205 nm (broken line). Asterisk represents methyl oleate. *Source:* Luna et al. (2005a) (used with permission).

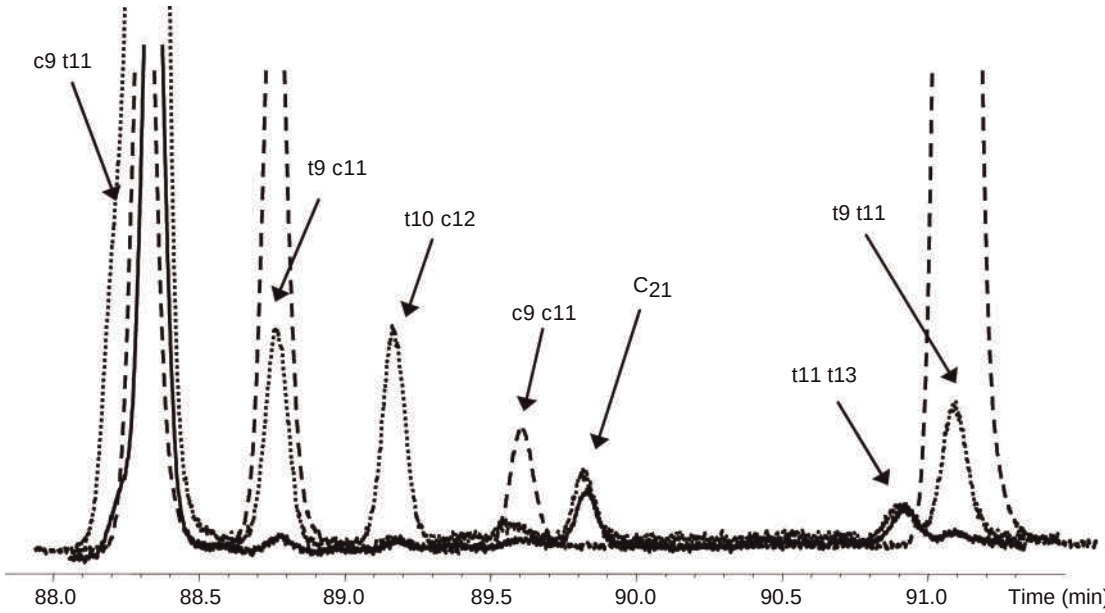

Figure 4.2. Partial chromatograms (conjugated linoleic acid area) by gas chromatography of fatty acid methyl esters of milk fat from sheep fed diets containing 0 (—) or 60 g of sunflower oil/kg dry matter (····) and an isomerized standard of *cis*-9 *trans*-11 C18:2 (---) containing the four geometrical isomers of 9–11 C18:2. *c* and *t* mean *cis* and *trans* configuration, respectively.

trend or at least no change with increasing intake of TFA from such sources (Pfeuffer and Schrezenmeir 2006).

Conjugated Linoleic Acid (CLA)

The generic name CLA is a collective term embracing all positional and geometric isomers of linoleic acid, which contain a conjugated double bond system. Data from in vitro studies and animal models have been used to suggest that the RA isomer is responsible for CLA anticarcinogenic and antiatherogenic properties, as well as a multiplicity of potentially beneficial effects on human health (Lee et al. 2005; Bhattacharya et al. 2006; Yurawecz et al. 2006).

Among ruminants, sheep milk fat could contain not only one of the highest levels of CLA, but also the major content of VA, its physiological precursor. In the first studies (Jahreis et al. 1999), total CLA mean content would seem to decrease in the following order: sheep > cow > goat milk fat, 1.2; 0.7, and 0.6% of total fatty acids, respectively. Other reports on sheep milk fat (Prandini et al. 2001; Barbosa et al. 2003) quantified the most prominent components assigned to CLA by GC. However, this main GC peak includes more than one component and minor CLA isomers masked by the RA peak. The combination of GC/MS of fatty acid methyl esters (FAME) and 4.4-dimethyloxazoline derivatives with silver-ion high-performance liquid chromatography (Ag^+-HPLC) of FAME helped to reveal the CLA isomer profile in sheep milk (Luna et al. 2005a). Table 4.4 shows the range of the relative composition of CLA isomers by Ag^+-HPLC. RA represents more than 75% of total CLA. *Trans*-7 *cis*-9 C18:2 is, from a quantitative point of view, the second CLA molecule (5–10% of total CLA), whereas minor amounts of other CLA isomers with different positional and geometrical configurations can also be found (Figs. 4.1, 4.2).

Trans-10 cis-12 C18:2 has lean body mass–enhancing properties (Belury 2002; Pariza 2004), and several studies in animals and humans have suggested that this isomer could be responsible for decreasing glucose levels and increased insulin

Table 4.4. Relative composition (% of total conjugated linoleic acid) determined by silver-ion high-performance liquid chromatography of conjugated linoleic acid (CLA) isomers in ewe milk (,*t/t*, corresponds to the sum of - plus -).

Isomer	Range (% Total CLA)
trans-12 *trans*-14	1.31–3.47
trans-11 *trans*-13	1.21–5.08
trans-10 *trans*-12	1.17–1.77
trans-9 *trans*-11	1.13–1.99
trans-8 *trans*-10	1.05–1.37
trans-7 *trans*-9	0.48–0.61
12–14 (*cis-trans* plus *trans-cis*)	0.52–1.83
11–13 (*cis-trans* plus *trans-cis*)	0.76–4.23
10–12 (*cis-trans* plus *trans-cis*)	0.28–0.41
9–11 (*cis-trans* plus *trans-cis*)	76.5–82.4
8–10 (*cis-trans* plus *trans-cis*)	0.11–0.71
7–9 (*cis-trans* plus *trans-cis*)	3.31–9.69

Source: Luna et al. (2005a).

resistance (Khanal 2004). However, the amount of this isomer in sheep milk fat is very low, less than 1% of total CLA (Table 4.4).

Information on the biological activity of other CLA minority isomers detected in sheep milk fat is very scant. *Cis*-9 *cis*-11 C18:2 has been shown to be a blocking agent of estrogen signalling in human breast cancer cells by using *in vitro* assays (Tanmahasamut et al. 2004). Other studies have reported the potent inhibitory effect of *trans*-9 *trans*-11 C18:2 on the growth of human colon cancer cells (Beppo et al. 2006) as well as antiproliferative and proapoptotic effects on bovine endothelial cells (Lai et al. 2005). However, in this field, further research is needed.

There is a great interest in increasing CLA content and changing the fatty acid profile in dairy products to provide value-added foods. Processing of sheep milk to cheese appears to have no effect on the final concentration of CLA, and the isomeric profile and its content are primarily dependent on the CLA level of the unprocessed milk (Luna et al. 2005b, 2007, 2008). On the other hand, no other factors, such as breed, parity, or days in milk can significantly affect CLA content in milk fat (Tsiplakou et al. 2006), which means that the dietary ones remain the sovereign factors explaining the highest proportion of CLA content variability in sheep milk fat.

The most important factor between the intrinsic and extrinsic variables to modulate milk fatty acid composition is the feed, in particular by adding lipid supplement to the diet as reviewed in cows and in sheep (Haenlein 2001; Bocquier and Caja 2001; Pulina et al. 2006). Changes in fatty acid profile of ovine milk fat should not substantially differ from the pattern previously described for cow milk. Milk CLA concentration in different ruminant species varied with the season mainly due to variations in feeding factors. The greatest seasonal differences were measured in sheep milk, 1.28% in summer and 0.54% at the end of the winter period (Jahreis et al. 1999). The effect of feeding fresh forages or Mediterranean pastures and season (related to changes in pasture quality) on the fatty acid composition of sheep milk, with special emphasis on the content of CLA and its precursors, has been reported (Addis et al. 2005; Cabiddu et al. 2005; Nudda et al. 2005).

In addition to enhancing CLA content, the dietary changes with vegetable seeds and oils also result in milk fat containing a lower proportion of saturated fatty acids and greater amounts of monounsaturated fatty acids (including VA) and PUFA (Antongiovanni et al. 2004; Luna et al. 2005b; Zhang et al. 2006). A more recent study confirmed the feasibility of an entire system approach for the production of CLA and omega-3 enriched sheep milk and cheese (Luna et al. 2008). The CLA and VA contents of milk and cheese from sheep receiving a ration rich in linolenic and α-linolenic acids were twofold higher than in milk from ewes fed with a control ration. Additionally, supplementation with flax seed and sunflower oil in the ration of sheep increased the C18:3 and C18:2 content in milk and reduced the concentration of the main saturated fatty acid (C16:0) of milk; other short-chain FA up to C10 increased significantly. Furthermore, consumer acceptability attributes of CLA-enriched cheese manufactured from sheep fed a lipid supplement were not different from those of cheeses manufactured from milk from animals fed with nonsupplemented diets (Luna et al. 2008).

It has also been reported in cows that marine oil is more effective than plant lipids for enhancing milk fat CLA content, and these responses can be further increased when fish oil is fed in combination with supplements rich in linoleic acid (Stanton et al.

2003). However, to date, data on CLA enhancement in sheep by adding fish oil supplements are very limited (Reynolds et al. 2006).

Other Minor Lipid Compounds

Along with TAG, sheep milk presents complex lipids (phospholipids) and different liposoluble compounds (sterols, β-carotene, vitamins) with biological activity.

Phospholipids are associated with the milk fat globule membrane and account for 0.2–1% of total milk lipids. Sphingomyelin and its metabolites, ceramide and sphingosine, are reported to have tumor-suppressing properties by influencing cell proliferation and are highly bioactive compounds with bacteriostatic and cholesterol-lowering properties (Parodi 2004, 2006). Further, some phospholipids exhibit antioxidative properties in dairy fat products with low water content (Molkentin 2000). However, to date, only very limited data are available on the phospholipid content in dairy products and the influence of processing and environmental variables on its concentration and relative distribution. The proportions of corresponding phospholipid classes in sheep milk are remarkably similar to other ruminants (McGibbon and Taylor 2006). Phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin are the most abundant, with smaller amounts of phosphatidylinositol and phosphatidylserine.

Sterols are a minor fraction of sheep milk total lipids, the main sterol being cholesterol (about 300 mg/100 g fat, equivalent to approximately 10 mg/100 mL milk). The sterol fraction of milk is of nutritional interest because high levels of cholesterol in plasma are associated with an increasing risk of cardiovascular disease. Cholesterol is also important for the resorption of fats and for its role as precursor in the synthesis of steroid hormones. Values reported for the cholesterol content of sheep milk vary considerably and are associated with breed and the use of different analytical techniques. Small amounts of other sterols implicated in cholesterol biosynthesis have also been found in ovine milk: lanosterol (5–15 mg per 100 g of fat) and, in even smaller proportions, dihydrolanosterol, desmosterol, and lathosterol (Goudjil et al. 2003b).

Other molecules present in the milk lipid fraction at low amounts have been claimed as bioactive components. Sheep milk fat for instance, contains a small amount of ether lipids (Hallgren et al. 1974). These compounds and their derivatives have a potent antitumor activity. It is believed that ether lipids are incorporated and accumulated in cell membranes and thereby influence biochemical and biophysical processes. There is also substantial epidemiological evidence for an association between diets rich in vitamin A and β-carotene and a decreased risk of cancer (Parodi 2006). Ovine milk contains virtually no β-carotene but supplies an adequate amount of vitamin A, which is higher than bovine milk (Park et al. 2007). Notwithstanding, little information about these topics is found in the literature. Thus, more research should be undertaken in this field to gain a better knowledge of the raw materials available and a deeper insight into the contribution of sheep dairy products in maintaining health.

PROTEINS AND THEIR PEPTIDES

The protein components of milk have multiple functions. They provide amino acids, which are necessary for growth and development. In addition, milk proteins and peptides have more specific physiological functions. The biological properties of milk proteins include antibacterial activity, such as immunoglobulins, lactoferrin, lactoperoxidase, lysozyme, hormones, and growth factors. Most studies are performed with proteins of bovine and human origin, but these activities can be extrapolated to milk from other origins.

Immnunoglobulins were among the first host protein defense systems described. There are five classes of immunoglobulins, but IgA predominates in colostrum and milk. Recent commercial and industrial applications have involved the targeted immunization of cows (Mehra et al. 2006). The iron-binding protein, lactoferrin, is widely considered to be one of the most important defense proteins present in milk fluids. Lactoferrin exhibits activity as an antimicrobial agent for host defense and as a physiological regulator with respect to both inflammatory and immune responses. Several reviews have recently been published in which the various physiological functions of proteins are addressed, as well as the important in vivo experimental results that promote their use in regulating mucosal immune responses (Wakabayashi et al. 2006). Lysozyme and lactoperoxidase are also important antimicrobial proteins found in mammalian milk and colostrum.

Lysozyme is mainly active against Gram-positive microorganisms, whereas Gram-negative microorganims containing catalase, such as pseudomonads, coliforms, salmonellae and shigellae, are killed by activated lactoperoxidase, provided that the hydrogen peroxide substrate is present in excess.

Hormones, growth factors, and analogs are also present in milk, and they could act as development and metabolic regulators. They are comprised of polypeptide hormones or growth factors such as prolactin and insulinlike growth factor-I. This group is constituted by various components with higher concentrations in milk than in blood, and they are supposed to play a physiological role for both mother and newborn. Several studies have particularly reported a role of these compounds in DNA synthesis, proliferation, differentiation, and metabolic effects (Pouliot and Gauthier 2006; Jouan et al. 2006).

Bioactive Peptides Derived from Sheep Milk Proteins

Enzymatic hydrolysis of milk proteins can release fragments able to exert specific biological activities, such as antihypertensive, antimicrobial, opioid, antioxidant, immunomodulatory, or mineral binding. Such protein fragments, known as bioactive peptides, are formed from the precursor inactive protein during gastrointestinal digestion and/or during food processing (Fitzgerard and Murray 2006). Due to their physiological and physicochemical versatility, milk peptides are regarded as highly prominent components for health-promoting foods or pharmaceutical applications. Research in the field of bioactive peptides has focused mainly on milk proteins of bovine origin and has been extensively reviewed (López-Fandiño et al. 2006, Korhonen and Pihlanto-Leppälä 2003; Gobbetti et al. 2004; Silva and Malcata 2005). However, during the last years, research has been extended to milk proteins from other mammals, including ovine and caprine species (Park et al. 2007). An overview of the major classes of bioactive peptides obtained from sheep milk is provided in the following sections.

Angiotensin-Converting Enzyme (ACE)–Inhibitory Peptides

Among the bioactive peptides known so far, those with ACE-inhibitory properties are receiving special attention due their potential beneficial effects in the treatment of hypertension. ACE is a multifunctional enzyme, located in different tissues, and is able to regulate several systems that affect blood pressure. It is responsible for generating vasopressor angiotensin II and inactivation of the vasodepressor bradykinin.

Currently, milk proteins are the main source of ACE-inhibitory peptides. Several techniques have been applied for releasing these peptides from native milk proteins: 1) hydrolysis with digestive enzymes of mammalian origin, 2) hydrolysis with enzymes of microbial and/or plant origin, 3) fermentation of milk with proteolytic starter cultures, 4) successive combination of hydrolysis with fermentation, and 5) chemical synthesis based on combinatorial library designs of peptides having similar structures to those known to inhibit ACE.

Most of the published reports on ACE-inhibitory and/or antihypertensive peptides are derived from bovine milk proteins. However, in recent years, sheep milk proteins have become an important source of ACE-inhibitory peptides (Table 4.5).

Studies about the ACE-inhibitory activity of β-lactoglobulin (β-Lg) hydrolysates from ovine and caprine milk with trypsine, chymotrypsin, proteinase K, and thermolysin enzymes have been carried out by Hernández-Ledesma et al. (2002). Higher activities were observed for caprine and ovine β-Lg hydrolysates obtained with enzymes of microbial origin than those prepared with digestive enzymes. Several peptide sequences with ACE-inhibitory activity were identified in a caprine β-Lg hydrolysate, but these domains are maintained in ovine β-Lg. Therefore, these peptides, especially the most active ones, LQKW and LLF, could be responsible for the activity found in the hydrolysates of ovine origin. In this work, lower IC_{50} values (protein concentration needed to inhibit original ACE activity by 50%), i.e., higher activity, was obtained for the hydrolysates prepared from sweet whey β-Lg than those achieved for the digests of β-Lg from acid whey. This higher activity was attributed to the presence of ACE-inhibitory peptides derived from caseinmacropeptide (CMP). According to these results, Manso and López-Fandiño (2003) found that undigested bovine, caprine, and ovine CMP exhibited moderate ACE-inhibitory activity, but it increased considerably after digestion under simulated gastrointestinal conditions. Several ACE-inhibitory peptides could be identified from CMPs

Table 4.5. Sequence of bioactive peptides derived from ovine milk proteins

Peptide Fragment	Sequence[a]	Biological Activity[b]	Produced By	References
β-Lg f(58–61)	LQKW	ACE-inhibitory (3.5 μM) Antihypertensive	Hydrolysis with thermolysin	Hernández-Ledesma et al. (2002) Hernández-Ledesma et al. (2007)
β-Lg f(103–105)	LLF	ACE-inhibitory (82.4 μM) Antihypertensive	Hydrolysis with thermolysin	Hernández-Ledesma et al. (2002) Hernández-Ledesma et al. (2007)
β-Lg f(142–148)	ALPMHIR	ACE-inhibitory	Tryptic hydrolysis	Chobert et al. (2005)
β-Lg f(1–8)	IIVTQTMK	ACE-inhibitory	Tryptic hydrolysis	Chobert et al. (2005)
κ-CN f(106–111)	MAIPPK	ACE-inhibitory	Tryptic hydrolysis	Manso and López-Fandiño (2003)
κ-CN f(106–112)	MAIPPKK	ACE-inhibitory	Tryptic hydrolysis	Manso and López-Fandiño (2003)
α_{s2}-CN f(205–208)	VRYL	ACE-inhibitory (24.1 μM)	Cheese ripening	Gómez-Ruiz et al. (2002)
α_{s1}-CN f(102–109)	KKYNVPQL	ACE-inhibitory (77.1 μM)	Cheese ripening	Gómez-Ruiz et al. (2002)
κ-CN f(108–110)	IPP	ACE-inhibitory (5 μM) Antihypertensive	Cheese ripening	Bütikofer et al. (2007)
β-CN f(84–86)	VPP	ACE-inhibitory (9 μM) Antihypertensive	Cheese ripening	Bütikofer et al. (2007)
β-CN f(95–99)	VPKVK	ACE-inhibitory (93.7 μM)	Cheeselike system	Silva et al. (2006)
α_{s1}-CN f(1–3)	RPK	ACE-inhibitory (36.7 μM)	Cheeselike system	Silva et al. (2006)
β-CN f(47–51)	DKIHP	ACE-inhibitory (113 μM)	Cheese ripening	Gómez-Ruiz et al. (2006)
α_{s1}-CN f(27–29) β-CN f(110–112)	PFP	ACE-inhibitory (144 μM)	Cheese ripening	Gómez-Ruiz et al. (2006)
β-CN f(114–121)	YPVEPFTE	Opioid Antihypertensive	Probiotic yoghurt	Papadimitriou et al. (2007) Lebrun et al. (1995)

α_{s2}-CN f(203–208)	PYVRYL	Antibacterial ACE-inhibitory (2.4 μM) Antioxidant Antihypertensive	Peptic hydrolysis	López-Expósito et al. (2006b) López-Expósito et al. (2007) Quirós et al. (2005) Recio et al. (2005)
α_{s2}-CN f(165–170)	LKKISQ	Antibacterial ACE-inhibitory (2.6 μM)	Peptic hydrolysis	López-Expósito et al. (2006b) López-Expósito et al. (2007)
κ-CN f(25–30)	YIPIQY	ACE-inhibitory (10 μM)	Hydrolysis with gastrointestinal enzymes	Gómez-Ruiz et al. (2007)
κ-CN f(12–17)	EKDERF	ACE-inhibitory (14.3 μM)	Hydrolysis with gastrointestinal enzymes	Gómez-Ruiz et al. (2007)
κ-CN f(22–24)	IAK	ACE-inhibitory (15.7 μM)	Hydrolysis with gastrointestinal enzymes	Gómez-Ruiz et al. (2007)
κ-CN f(56–60)	LPYPY	ACE-inhibitory (28.9 μM)	Hydrolysis with gastrointestinal enzymes	Gómez-Ruiz et al. (2007)
α_2-CN f(165–181)	LKKISQYYQKFAWPQYL	Antibacterial	Peptic hydrolysis	López-Expósito et al. (2006b)
α_{s2}-CN f(184–208)	VDQHQAMKPWT-QPKTKAIPYVRYL	Antibacterial	Peptic hydrolysis	López-Expósito et al. (2006b)
α_{s1}-CN f(10–21)	GLSPEVLNENLL	Antibacterial fraction	Cheese ripening	Rizzello et al. (2005)
α_{s1}-CN f(22–30)	RFVVAPFPE	Antibacterial fraction	Cheese ripening	Rizzello et al. (2005)
α_{s1}-CN f(24–31)	VVAPFPEV	Antibacterial fraction	Cheese ripening	Rizzello et al. (2005)
β-CN f(155–163)	RFVVAPFPE	Antibacterial fraction	Cheese ripening	Rizzello et al. (2005)
κ-CN f(112–116)	KDQDK	Antithrombotic	Tryptic hydrolysis	Qian et al. (1995a)
κ-CN f(98–105)	HPHPHLSF	Antioxidant	Hydrolysis with gastrointestinal enzymes	Gómez-Ruiz et al. (2008)
Colostrinin		Immunomodulatory		Zimecki (2008)

[a]Amino acids are designated with one letter code.

[b]ACE = angiotensin-converting enzyme. For peptides with ACE-inhibitory activity, the IC_{50} value its indicated between brackets when reported.

via proteolysis with trypsin, peptides MAIPPK and MAIPPKK, corresponding to κ-CN f(106–111) and f(106–112), respectively (Table 4.5). These peptides showed moderate activity, but their digestion under simulated gastrointestinal conditions allowed the release of a potent antihypertensive peptide IPP (IC_{50} value of 5 μM). These findings might help promote further exploitation of CMP as multifunctional active ingredients, broadening the potential uses of rennet whey from various sources.

Chobert et al. (2005) have investigated the ACE-inhibitory activity of ovine β-Lg hydrolyzed with trypsin and yogurt from ovine milk with different starters. A higher susceptibility was found for β-Lg variant B to tryptic hydrolysis than for variant A, as previously observed for pepsin (El-Zahar et al. 2005). In addition, several peptides from this tryptic hydrolysate were identified by tandem mass spectrometry. Interestingly, the ACE-inhibitory activity after fermentation of ovine milk was higher than that obtained after tryptic hydrolysis of ovine β-Lg, showing that hydrolysis of caseins with enzymes of bacterial origin constitute a more efficient substrate and procedure for the formation of ACE-inhibitory peptides.

Caseins are an important source of peptides with ACE-inhibitory activity after enzymatic hydrolysis and/or milk fermentation. Because ovine milk is mainly used for cheese-making, the formation of bioactive peptides during cheese ripening is of special interest. Cheeses constitute an important source of peptides, due to the diversity of the proteolytic systems involved in cheese ripening and to the different intensity of proteolysis during ripening depending on the cheese type. However, because identification of biologically active peptides in complex matrices such as cheese is a challenging task, there are only a few papers related to the ACE-inhibitory activity of peptides liberated from casein during ovine cheese ripening.

Several ACE-inhibitory peptides have been isolated from extracts of Italian cheeses (Gobbetti et al. 2004) and from a Spanish Manchego cheese prepared by inoculating ovine milk with *Lactococcus lactis* subsp. *lactis* and *Leuconostoc mesenteroides* (Gómez-Ruiz et al. 2002). In this work 22 peptides from α_{s1}-, α_{s2}-, and β-casein were sequenced by tandem mass spectrometry comprised in several active chromatographic fractions. Two of these peptides, VRYL and KKYNVPQL, exhibited considerable ACE-inhibitory activity, with IC_{50} values of 24.1 μM and 77.1 μM, respectively (Gómez-Ruiz et al. 2004a). In addition, peptide VRYL was only partly hydrolyzed after simulated gastrointestinal digestion retaining the ACE-inhibitory activity, and this peptide was found to be a true inhibitor of the enzyme showing a competitive inhibition pattern. The formation of this and other active peptide sequences during Manchego cheese ripening could be followed by HPLC coupled *on line* to a tandem mass spectrometer, and it was found that the use of selected bacterial strains favored the formation of ACE-inhibitory peptides in addition to other biologically active sequences (Gómez-Ruiz et al. 2004b). This analytical technique, HPLC-MS, has also been successfully employed for the quantitative determination of two well-known antihypertensive tripeptides, IPP and VPP, in semihard and soft cheeses. It was found that in various traditional cheese samples these two peptides were present at concentrations able to produce a physiological effect on blood pressure. However, the amount of these peptides in the cheeses of ovine origin, Roquefort and Manchego, was moderate (below 50 mg/kg) (Bütikofer et al. 2007). The presence of other ACE-inhibitory peptides has been investigated in different Spanish cheeses (Cabrales, Idiazábal, Roncal, Manchego, Mahón, and a goat milk cheese), elaborated with milk of different species and by using diverse technological processes. The ACE-inhibitory activity of these cheeses was essentially concentrated in the 1 kDa-permeate, showing that the activity was mainly due to small peptides. The major peptides contained in each cheese type were identified by HPLC-MS, most of them being derived from α_{s1}-casein and β-casein (Gómez-Ruiz et al. 2006). Peptide DKIHP, corresponding to β-CN f(47–51), which was found in all cheeses except in Mahon cheese, showed different ACE-inhibitory activity depending on conformation of the C-terminal proline residue. The change of *trans*-proline to *cis*-proline produced a decrease in activity probably due to the loss of interactions with the ACE (Gómez-Ruiz et al. 2004c). This study revealed the importance of performing structural studies of peptides before testing their ACE-inhibitory activity. More recently, other ACE-inhibitory peptides have been identified in ovine and caprine cheeselike systems prepared with proteases from *Cynara cardunculus*. Two of the identified sequences in the ovine cheeselike

systems, VPKVK and RPK, showed potent ACE-inhibitory activities (Silva et al. 2006).

The presence of ACE-inhibitory peptides has also been investigated in other food matrices, such as ovine yogurt and hydrolysates of ovine milk proteins. Several previously described active sequences have been found in sheep milk yogurt produced with a yogurt culture enriched with a probiotic strain (Papadimitriou et al. 2007). A peptide derived from β-casein, YPVEPFTE, with well-established ACE-inhibitory and opiatelike activity was identified in this probiotic yogurt. Novel ACE-inhibitory sequences have also been found in a peptic α_{s2}-casein hydrolysate with pepsin (López-Expósito et al. 2007), and in a hydrolysate of ovine κ-casein prepared with digestive enzymes (Gómez-Ruiz et al. 2007). Some of these novel sequences exhibited IC_{50} values as low as 2.4 and 2.6 μM (Table 4.5).

Antimicrobial Peptides

Bioactive proteins and peptides derived from milk have been reported to provide a nonimmune disease defense and control of microbial infections (McCann et al. 2006). It is generally accepted that the total antibacterial effect in milk is greater than the sum of the individual contributions of immunoglobulin and nonimmunoglobulin defense proteins such as lactoferrin (LF), lactoperoxidase, lysozyme, and peptides. This may be due to the synergistic activity of naturally occurring proteins and peptides, in addition to peptides generated from inactive protein precursors (Gobbetti et al. 2004). It has been proved that milk proteins can also act as antimicrobial-peptide precursors, and in this way, might enhance the organism's natural defenses against invading pathogens. Consequently, food proteins can be considered as components of nutritional immunity (Pellegrini 2003).

Peptides derived from LF are the antibacterial peptides from milk proteins that have attracted more attention during the last decade. The first report that demonstrated the enzymatic release of antibacterial peptides with more potent activity than the precursor LF dates from 1991 (Tomita et al. 1991). Shortly afterward, the antibacterial domains of bovine LF f(17–41) and human LF f(1–47), called respectively bovine and human lactoferricin (LFcin), were purified and identified (Bellamy et al. 1992). These peptides showed a potent antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria (Wakabayashi et al. 2003). Hydrolysis of caprine and ovine LF by pepsin resulted in antibacterial hydrolysates, and a homologous peptide to LFcin, corresponding to fragment f(14–42), was identified in caprine LF hydrolysate. The region corresponding to the LFcin within the sequence of ovine LF was hydrolyzed by the action of pepsin, and hence, the activity observed in the ovine LF hydrolysate could be caused by other LF fragments (Recio and Visser 2000). In addition to these studies, El-Zahar et al. (2004) obtained a peptic hydrolysate of ovine α-lactalbumin (α-La) and β-Lg that inhibited the growth of *Escherichia coli* HB101, *Bacillus subtilis* Cip5262, and *Staphylococcus aureus* 9973 in a dose-dependent manner, but responsible peptides were not identified.

Caseins are also a source of antimicrobial peptides in the same manner as whey proteins (for a recent review see López-Expósito and Recio 2006a, 2008). In a preliminary study, an ovine β-casein hydrolysate with pepsin, trypsin, and chymotrypsin showed inhibition of bioluminescent production by *Escherichia coli* JM103, but the peptides responsible for this activity have not been identified (Gómez-Ruiz et al. 2005). Recently, four antibacterial peptides were identified from a pepsin hydrolysate of ovine α_{s2}-casein (López-Expósito et al. 2006b). The peptides corresponded to α_{s2}-casein fragments f(165–170), f(165–181), f(184–208), and f(203–208); the fragments f(165–181) and f(184–208) are homologous to those previously identified in the bovine protein (Recio and Visser 1999) (Table 4.5). These peptides showed a strong activity against Gram-negative bacteria. Of them, the fragment f(165–181) was the most active against all bacteria tested. The peptide corresponding to ovine α_{s2}-casein f(203–208), with sequence PYVRYL, is a good example of multifunctional peptides because it exhibited not only antimicrobial activity, but also potent antihypertensive and antioxidant activity (Recio et al. 2005).

Antimicrobial activity has also been found in the water-soluble extract of several Italian cheese varieties, some of them manufactured from ovine milk. Most of the extracts exhibited a large inhibitory spectrum against Gram-positive and Gram-negative microorganisms, including potentially pathogenic bacteria of clinical interest. Some peptide sequences were identified in these extracts and some of them

corresponded or showed high homology with previously described antimicrobial peptides (Table 4.5) (Rizzello et al. 2005).

Other Biological Activities of Peptides from Ovine Proteins

It has been reported that milk proteins of ovine origin are a source of peptides with other biological activities. For instance, κ-caseinomacropeptide is one of the main components of whey and it is obtained as a by-product in cheese-making. The κ-CMPs from several animal species have been reported as good sources of antithrombotic peptides. Qian and co-workers (1995a) found two very active sequences with inhibitory activity of human platelet aggregation induced by thrombin and collagen after hydrolyzing ovine κ-CMP with trypsin (Table 4.5). Furthermore, bovine, ovine, and caprine κ-CMPs and their hydrolysates with trypsin were found to be inhibitors of human platelet aggregation (Manso et al. 2002). In this work, the hydrolysate obtained from ovine κ-CMP showed the strongest effect, but the peptides responsible for this activity were not identified. Similarly, a chromatographic fraction from a peptic hydrolysate of ovine lactoferrin has also shown inhibitory activity on platelet aggregation (Qian et al. 1995b).

Several studies have centered their interest on the identification of peptides derived from caseins and whey proteins of bovine origin with potent antioxidant activity acting by different mechanisms. These peptides are released by enzymatic hydrolysis and milk fermentation. More recently, the potential role of different ovine casein fractions and their hydrolysates to exert antioxidant activity has also been studied (Gómez-Ruiz et al. 2008). Of special interest was the identification of a κ-casein fragment, HPHPHLSF, which resulted as a potent inhibitor of linoleic acid oxidation with an activity similar to that obtained with the synthetic antioxidant BHT.

A proline-rich peptide, called *colostrinin*, which was originally found as a fraction accompanying ovine immunoglobulins, was found to promote T-cell maturation. This peptide promoted procognitive functions in experimental animal models, indicating prevention of pathological processes in the central nervous system. In humans, the therapeutic benefit of colostrinin has been demonstrated in Alzheimer's disease patients by delaying progress of the disease (Zimecki 2008).

Therefore, although research on bioactive peptides has mainly been focused on bovine milk proteins, paying less attention to milk from other origins, such as ovine milk, these findings justify further studies. In addition, given the high homology among the sequences of bovine, ovine, and caprine milk proteins, it would be predictable that the peptides reported as bioactive agents and released from bovine proteins were also within sheep and goat proteins.

OLIGOSACCHARIDES

Lactose is the major carbohydrate in ovine milk, which is composed of glucose and galactose bonded by a β 1–4 glycosidic linkage. The lactose content in sheep milk is similar to bovine milk, while the fat and protein contents are considerably higher (Ramos and Juárez 2003). Lactose is a valuable nutrient because it favors intestinal absorption of calcium, magnesium, and phosphorous, and the utilization of vitamin C. Carbohydrates other than lactose, such as glycopeptides, glycoproteins, and oligosaccharides, are also found in ovine milk. Milk oligosaccharides are thought to be beneficial for the human milk–fed infant with regard to their prebiotic and antiinfective properties. Recent studies suggest that human milk oligosaccharides have potential to modulate the gut flora, to affect different gastrointestinal activities, and to influence inflammatory processes (Kunz and Rudloff 2006). Milk from other mammals also contains oligosaccharides, but they are found in lower amounts than in human milk. Recently, it was found that the amount of oligosaccharides in caprine milk was in the range of 250 to 300 mg/L (Martĭnez-Férez et al. 2006). This represents 4–5 times the amount of oligosaccharides in bovine milk. The amount of oligosaccharides in ovine milk is in the range of 20 to 30 mg/L; however, their content, as in other mammalian milk, is considerably higher in colostrum. The chemical structures of oligosaccharides from ovine colostrum have been described by Urashima and co-workers (1989). Three neutral milk oligosaccharides, isomers of galactosyllactose Gal (α1–3) Gal (β1–4) Glc, Gal (β1–3) Gal (β1–4) Glc, and Gal (β1–6) Gal (β1–4) Glc have been identified. Three acid milk oligosaccharides from the ovine colostrums have been isolated and identified by ^{1}H-NMR (Nakamura et al. 1998). These acid milk oligosaccharides contained sialic acid. *Sialic acid* is a general name for N-acetylneuraminic acid (Neu5Ac) and

N-glycolylneuraminic acid (Neu5Gc). The sialyl oligosaccharides described are Neu5Ac (α2–3) Gal (β1–4) Glc, Neu5Gc (α2–3) Gal (β1–4) Glc, and Neu5Gc (α2–6) Gal (β1–4) Glc. More recently, Martĭnez-Férez and co-workers (2006) also identified oligosaccharides with similar structures in sheep milk. These components could be of interest because it has been suggested that sialic acid present in milk oligosacharides promotes the development of the infant's brain (Nakamura and Urashima 2004); it also has been shown that sialic acid-containing oligosacharides reduce the adhesion of leukocytes to endothelial cells, an indication for an immune-regulatory effect of certain human milk oligosacharides (Kunz and Rudloff 2008).

MINERALS

In recent years, interest in the nutritional significance of milk minerals and trace elements has markedly increased. There are about 20 minerals that are considered nutritionally essential for humans (Na, K, Cl, Ca, Mg, P, Fe, Cu, Zn, Mn, Se, I, Cr, Co, Mb, F, As, Ni, Si, and B). Milk and dairy products can make an important contribution to the daily intake of some of them, especially Ca and P. Detailed descriptions of the biochemical role of these essential minerals and trace elements as well as of the nutritional significance of milk as a source of these micronutrients have been profusely discussed (Renner et al. 1989; Flynn and Cashman 1997; Cashman 2002a,b) and are not discussed in this chapter.

The minerals in sheep milk have not been as extensively studied as in bovine milk, even though they may be of nutritional and health interest. Sheep milk has around 0.9% total minerals or ash, compared to 0.7% in cow milk (Juárez and Ramos 1986). The most abundant elements are Ca, P, K, Na, and Mg; Zn, Fe, Cu, and Mn are the trace elements. Representative values for the average mineral contents of milk are presented in Table 4.6. The levels of Ca, P, Mg, Zn, Fe, and Cu are higher in sheep than in cow milk; the opposite appears to be the case for K and Na. The contents of macrominerals and trace elements in other sheep dairy products have been presented elsewhere (Martĭn-Hernández et al. 1992; Coni et al. 1999). In general, mineral contents of sheep milk seem to vary much more than those of cow milk. The mineral content of sheep milk is not constant but is influenced by a number of factors such as stage of lactation, nutritional status of the animal, and environmental and genetic factors due to feeding differences and seasonal variations (Polychroniadou and Vafopoulou 1985; Rincón et al. 1994).

The chemical form in which a macromineral and trace element is found in milk is important because it may influence intestinal absorption and *utilization* (the process of transport, cellular assimilation, and conversion into a biologically active form) and thus bioavailability. The salt balance in sheep milk is interesting as a contribution to the knowledge of nutritional characteristics of these types of milk, and to the retention of these elements in the curd during cheese-making. Because sheep milk is mainly used in cheese-making and most of the soluble elements are lost in the whey during manufacture, the knowledge of element distribution would allow evaluation of the influence of milk composition on the mineral content in cheese.

Na, K, and Cl in milk are almost entirely soluble and fully available in the whey. Ca, Mg, and P in sheep milk are associated in different proportions to the colloidal suspension of casein micelles. Due to these bindings, these minerals are partly retained in the curd during cheese-making. In samples from different herds on farms in Spain the percentages of Ca, Mg, and P in the soluble phase of sheep milk were 21, 56, 35%, respectively, within the ranges of variation reported in other countries (Polychroniadou and Vafopoulou 1986; Pellegrini et al. 1994). Although the proportions of P and Mg linked to casein were, in general terms, higher than in cow milk, the most striking aspect of these findings is the distribution of Ca. Percentage of Ca in the soluble phase is lower than in milk from other ruminants, and thereby higher levels of this element could potentially be incorporated to the curds.

Data on sheep milk about the distribution of trace elements are scarce. It appears that a large proportion (up to 90%) of Zn and Mn are found in the micellar fraction (Kiely et al. 1992; Shen et al. 1995; De la Fuente et al. 1997); this is presumably casein, as occurs in cow milk, the principal Zn-binding ligand in this species. The distribution of Fe and Cu differed more. Along with Cu, Fe is the microelement found most abundantly in the soluble phase. This fraction contains 29% and 33% of total Fe and Cu, respectively (De la Fuente et al. 1997). Additionally, of all elements considered here, Fe is probably the one that was bound in the highest proportion

to the lipid fraction. Se availability in sheep milk appears to be significantly less than in human, bovine, and caprine milk (Shen et al. 1996).

The content of Ca and P in cheese is higher than that in milk, 4–5 times higher in fresh cheeses, 7–8 times higher in semihard cheese, and 10 times higher in hard cheeses. The bioavailability of Ca in cheese is comparable to that in milk, and is not affected by the ripening process (Ramos and Juárez 2003).

VITAMINS

Bibliographical data on the vitamin content of sheep milk are given in Table 4.6. Most of the known vitamins are contained in ovine milk and for some of them this foodstuff is a rich source. Daily riboflavin (B_2) requirements, for instance, are completely covered by drinking just 2 cups of sheep milk without eating anything else (Haenlein 2001). Because drinking sheep milk is not widespread, it is likely that 2 cups of sheep milk yogurt would meet those daily requirements, or the milk equivalent in 90 g of sheep cheese. From the literature (Juárez and Ramos 1986; Park et al. 2007) the conclusion is that sheep milk is richer than cow milk in most of the vitamins. However, documented research data on vitamins of sheep milk are too sparse to offer a definitive picture.

Table 4.6. Mean value and range for vitamins (per 100 g) and minerals (per liter) of sheep milk

	Mean Value	Range
Vitamin		
Vitamin A (μg)	50.00	
Thiamin (B_1) (μg)	48.00	28.00–70.00
Riboflavin (B_2) (mg)	0.23	0.16–0.30
Nicotinamide (mg)	0.45	0.40–0.50
Pantothenic acid (mg)	0.35	
Biotin (μg)	9.00	
Niacin (mg)	0.42	
Vitamin B_6 (mg)	0.06	
Vitamin B_{12} (μg)	0.51	0.30–0.71
Vitamin C (mg)	4.25	3.00–6.00
Folic Acid (μg)	5.60	
Vitamin D (μg)	0.18	
Vitamin E (μg)	120.00	
Mineral		
Ca (g)	1.98	1.80–2.39
Mg (g)	0.18	0.10–0.22
Na (g)	0.50	0.27–0.81
K (g)	1.20	0.96–1.96
P (g)	1.30	1.17–1.70
Fe (mg)	0.76	0.34–1.40
Cu (mg)	0.07	0.04–0.14
Zn (mg)	7.50	4.70–12.00
Cl (g)	1.50	0.04–0.14
S (g)	0.29	
Mn (μg)	7.00	
I (mg)	0.02	
Se (μg)	1.00	

Source: Ramos and Juárez (2003) and Park et al. (2007).

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5
Bioactive Components in Buffalo Milk

A. J. Pandya and George F. W. Haenlein

INTRODUCTION

Milk is a complete food for newborn mammals. It is the sole food during the early stages of rapid development. Milk also contains high levels of the critically important immunoglobulins and other essential physiologically active compounds for warding off infection in the newborn and in adults. Milk proteins are currently the main source of a range of biologically active peptides. Concentrates of these peptides are potential health-enhancing nutraceuticals for dietary and pharmaceutical applications, for instance, in the treatment of diarrhea, hypertension, thrombosis, dental diseases, mineral malabsorption, and immunodeficiency. Minor whey proteins, such as lactoferrin, lactoperoxidase, lysozyme, and immunoglobulins are considered antimicrobial proteins. Milk also contains natural bioactive substances, including oligosaccharides, hormones, growth factors, mucins, gangliosides, endogenous peptides, milk lipids like conjugated linoleic acids, medium-chain triglycerides, *trans* fatty acids, polar lipids, minerals, trace elements, vitamins, and nucleotides (Chatterton et al. 2006).

Increased awareness of diet-health relationships has brought about a new trend in nutrition science in many countries, where more attention is given to the health effects of individual foods and the role of diet in the prevention and treatment of diseases, as well as improving body functions. New achievements in analytical techniques and technology in the dairy industry offer opportunities to isolate and concentrate or modify food components with biological activity so that their dietary application as supplements, nutraceuticals, or medically beneficial foods has become possible (Meisel 1997; Aimutis 2004).

Dairy buffalo, which presently number about 166 million head, dominate the dairy industry in parts of the world and contribute a major share to the world's milk supply and dairy products (Pandya and Khan 2006). India has the highest number of dairy buffalo, about 96 million head, and the greatest buffalo milk production and consumption. India is also the home for some of the best breeds of dairy buffalo in the world. Possession of dairy buffalo and their numbers in an Indian household present a socioeconomic status for the farmer. Buffalo milk production increased about 59, 39, 64, and 155% in India, Pakistan, China and Italy, respectively, in recent years. Buffalo milk production contributes about 66, 25, 4, and 0.2% of all milk produced in India, Pakistan, China and Italy, respectively.

Information on bioactive components in buffalo milk is very sparse because this kind of research is still in its infancy and not urgent in Western countries, where cows dominate the dairy industry. However, the results on components in cow milk may be used with some caution. They are discussed briefly in this chapter only to provide continuity and possible extrapolation to its close relative, the buffalo (Calderone et al. 1996; Berger et al. 2005).

BIOACTIVE MILK PROTEINS

The proteins in milk are divided principally into caseins (CN) and whey proteins. The caseins consist

mainly of α_{s1}-, α_{s2}-, β- and κ-CN molecules, which have several genetic polymorphisms and posttranslational modifications with phosphorylation and/or glycosylation. The major whey proteins are β-lactoglobulin (β-Lg) and α-lactalbumin (α-La), which also have genetic variants. In addition the whey fraction contains significant amounts of immunoglobulins and blood serum albumin (BSA), and minor but important proteins, lactoferrin and lactoperoxidase, which are available commercially. Due to the presence of the endopeptidase enzyme plasmin in milk, β-CN can be degraded into *N*- and *C*-terminal peptides with different properties. Buffalo milk and colostrum, like that from cows, also contain minor bioactive components such as peptides, hormones, and growth factors (Howarth et al. 1996), which had beneficial intestinal effects in rat trials.

β-Lactoglobulin

Buffalo milk has slightly higher concentrations of β-Lg than cow milk, and it is also the major whey protein, while human milk contains no β-Lg (Table 5.1). β-Lg contains essential and branched chain amino acids, and a retinol-binding protein, which has the potential to modulate lymphatic responses. β-Lg can yield antibacterial peptides, which are released after proteolytic digestion with trypsin and are active against Gram-positive bacteria (Sahai 1996; Pellegrini et al. 2001).

The molecular weight of buffalo β-Lg was estimated to be 38.5 kDa on the basis of sedimentation coefficients, and it is somewhat higher than that obtained from amino acid composition. It is a small, soluble, and globular protein. The molecular mass of cow milk β-Lg is in comparison about 18 kDa at

Table 5.1. Average concentrations and biological functions of major proteins in milk of buffalo, cow, and human (Pandya and Khan 2006)

Protein	Concentration (g/L^{-1})			Function
	Buffalo	Cow	Human	
Total caseins	37.84	26.0	2.7	Ion carrier (Ca, PO_4, Fe, Zn, Cu), precursors of bioactive peptides
α-Casein	16.6–20.8	13.0		
β-Casein	12.6–15.8	9.3		
κ-Casein	4.3–5.4	3.3		
Total whey proteins		6.3	5.5	
β–Lactoglobulin	3.9	3.2	-	Retinol carrier, binding fatty acids, possible antioxidant
α–Lactalbumin	1.4	1.2	1.9	Lactose-synthesis in mammary gland, Ca carrier, immunomodulation, anticarcinogenic
Immunoglobulins (A, M, and G)	10.66	0.7	1.3	Immune protection
Serum albumin	0.29	0.4	0.4	
Lactoferrin	0.32	0.1–0.5	1.5–2.0	Antimicrobial, antioxidative, immunomodulation, iron absorption anticarcinogenic
Lactoperoxidase	5.2–9.8[†]	0.03		Antimicrobial
Lysozyme	0.000152	0.0004	0.1	Antimicrobial, synergistic effect with immunoglobulins and lactoferrin
Miscellaneous	NA	0.8	1.1	
Proteose peptone	3.305	1.2	NA	Not characterized
Glycomacropeptide	NA	1.2	NA	Antiviral, bifidogenic

[†]Units/mL^{-1}.

1	11	20	28	45	50	52	53	56	59	64	78	80	84	87	108	116	126	130	148	150	158	162
I	**D**	**Y**	**D**	**E**	**P**	**G**	**D**	**I**	**Q**	**G**	**I**	**A**	**I**	**L**	**E**	**A**	**P**	**D**	**R**	**S**	**E**	**V**

Example of amino acid sequences for β-Lg in milk of *Bubalus arnee* and *Bubalus bubalis*.

a pH < 3; at a pH between 3 and 7, bovine β-Lg exists in solution as dimer with an effective molecular mass of about 36 kDa. Buffalo β-Lg exists as a dimer at pH 5.2, but changes to a monomer at a pH below 3.5 or above 6.5. At low temperature nearing 0° and at pH 3.5 the molecule exists as a tetramer (Malik and Bhatia 1977).

The amino acid composition of buffalo β-Lg is almost identical to that of cow β-Lg (Sahai 1996). Both are also identical in terms of electophoretic mobility, sedimentation, and titration behavior. However, buffalo β-Lg does not seem to have genetic polymorphisms. Amino acid sequences have been identified for β-Lg in milk of *Bubalus arnee* and *Bubalus bubalis* (Sawyer 2003). See sample above.

Buffalo β-Lg is very close to the sequence of cow β-Lg B variant (except in *1*, *162* L, I, respectively). β-Lg and its peptide fragments have a variety of useful nutritional and functional bioactivity characteristics, which have made this protein of considerable interest in the formulation of modern foods and beverages.

Health Benefits

Angiotensin-converting enzyme (ACE) plays a major role in the regulation of blood pressure and thereby hypertension. Various peptides derived from proteolytic digestion of β-Lg have been shown to have inhibitory activity against ACE in cow milk studies (Mullally et al. 1997a,b). Proteolytic digestion of bovine β-Lg by trypsin yields four peptide fragments (f15–20, f25–40, f78–83, and f92–100) with bactericidal activity against Gram-positive bacteria (Pellegrini et al. 2001). Modulation of the peptides via targeted amino acid substitution expanded the bactericidal activity to the Gram-negative *E. coli* and *Bordetella bronchiseptica.*

Several studies have reported that β-Lg, chemically modified with 3-hydroxyphthalic anhydride to form 3-hydroxyphthaloyl-β-Lg, can be effective in inhibiting HIV-1, HIV-2, simian immunodeficiency virus, herpes simplex virus types 1 and 2, and *Chlamydia trachomatis* infection in vitro (Superti et al. 1997; Oevermann et al. 2003). A tryptic peptide from β-Lg (f71–75; Ile-Ile-Ala-Glu-Lys) has been shown to have hypocholesterolemic activity in rat trials, and β-lactotensin, a neurotensin agonist derived from β-Lg (f146–149), had hypocholesterolemic activity in mice (Yamauchi et al. 2003).

Whey proteins, including β-Lg, have been shown to provide protection against development of cancer in animal models when delivered orally, which may be related to their sulphur amino acid content that appears to bind mutagenic heterocyclic amines with carcinogenic properties (Yoshida et al. 1991). In processing, β-Lg has excellent heat set gelation, water binding, and texturization characteristics for commercial production of various foods and as an alternative to egg albumin (Chatterton et al. 2006).

α-Lactalbumin

α-Lactalbumin is the other main protein in milk. Calderone et al. (1996) studied its amino acid sequence and showed one difference at position 17 between buffalo (Asp) and bovine (Gly) α-La. The crystal structure of buffalo α-La contains another difference at position 27 (Ile). The overall features of the structures are similar to those reported for baboon and human α-La (Figs. 5.1, 5.2). In human and baboon α-La structures the polypeptide has a distorted helical conformation, whereas in buffalo α-La structure it forms a flexible loop. The conformation of the flexible loop has particular interest in understanding α-La functions. Since the amino acid sequence of buffalo milk α-La is almost identical to the bovine sequence, the substantial amount of functional bovine data can be used. Based on mutagenesis data on bovine α-La, amino acid substitutions at position 106, 107, and 110 have considerable effect on α-La's ability to bind GT (Calderone et al. 1996).

Buffalo and cow milk α-La have the same crystalline form and similar nitrogen, tyrosine, and tryptophan contents. The molecular weight of buffalo α-La is 16,200 Da, which is close to the molecular weight of cow α-La (Malik et al. 1988). In the absence of calcium, this protein is very unstable (T_m of 43 °C). Therefore, binding of calcium is of utmost importance for maintaining the structure of this protein.

α-La shares a high homology with lysozyme (LZ) with respect to amino acid sequences and protein

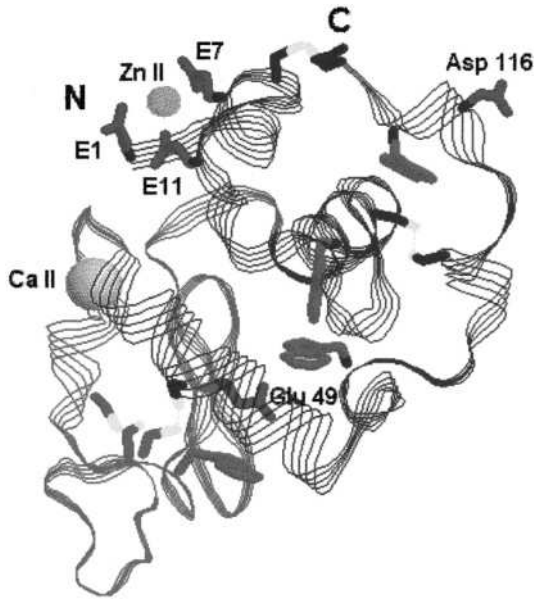

Figure 5.1. X-ray α-La structure derived from native buffalo milk (courtesy of Dr. Acharya) and recombinant bovine protein (taken from Brookhaven Protein Data Bank), prepared in conjunction with Serge E. Permyakov from the Institute for Biological Instrumentation, Pushchino, Russia, and Dr. Charles Brooks, Department of Veterinary Biosciences, Ohio State University, Columbus, OH, U.S. α-domain is shown in blue; β-domain is in green. Trp residues are shown in blue and S-S bridges are in yellow. The residues, which take part in coordination of Zn^{2+} ions, are shown in red (Permyakov and Berliner 2000).

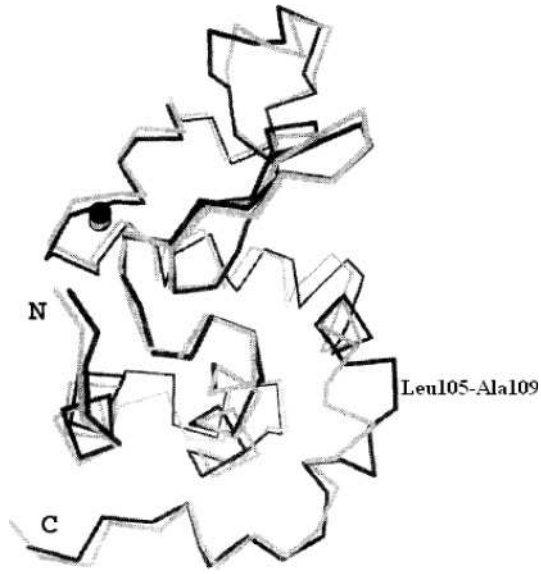

Figure 5.2. A view of the C^{α} backbone of human α-La (grey) and buffalo α-La (black) after least squares superimposition. A portion of the molecule, which differs in the two structures (residues 105–109), known to possess conformational flexibility (helix in human α-La; coil or loop in buffalo α-La, present study) is highlighted. The Ca^{2+} ion is shown as a large sphere. The figure was produced by MOLSCRIPT (Calderone et al. 1996).

and gene structures (McKenzie and White 1986), but both differ strongly in biological functions. LZ possesses bactericidal properties, while the primary function of α-La is related to the synthesis of lactose. α-La hydrolyzed with pepsin and trypsin inhibits the metabolic activity of *E. coli*.

Heat-treatment alters the disulphide bond patterns within proteins, causes intermolecular cross-linking, and alters bioactivity of peptides. Intermolecular disulphide bonds between α-La and β-Lg, involving Cys61 (part of the antibacterial peptide) and Cys111 (Livney et al. 2003) or α-La and BSA have been reported (Havea et al. 2001).

Health Benefits

Health benefits of α-La for human consumption derive from 1) the intact, whole protein, 2) peptides of the partly hydrolyzed protein, or 3) the amino acids of the fully digested protein. α-La is a particularly good source of the essential amino acids Trp and Cys, precursors of serotonin and glutathione, respectively. The peptide with the sequence of the amino acids Tyr-Gly-Gly-Phe (f50–53), released from α-La by pepsin treatment, has structural similarities to the opioid peptide human leuenkephalin, termed α-*lactorphin*, and had opioid effects in mouse studies. The same peptide also inhibits ACE with an IC_{50} value of 733 μM (Mullally et al. 1997a,b). Similar ACE-inhibition effects were reported for other α-La dipeptides Try-Gly (f18–19 and f50–51), Leu-Phe (f52–53) with IC_{50} values of 1523 and 349 μM, respectively, and tripeptide Try-Gly-Leu (f50–52) with similar IC_{50} values (409 μM) (Pihlanto-Leppälä et al. 2000).

A variant of human α-La has been discovered recently in mice studies to have anticancerogenic properties by entering tumor cells and inducing apoptosis in cell nuclei. α-La has been shown to

have antimicrobial activities, particularly against *Streptococcus pneumoniae* and *Haemophilus influenzae*. Trypsin treatment of α-La releases two antibacterial peptides Glu-Gln-Leu-Thr-Lys f(1–5), and Gly-Tyr-Gly-Gly-Val-Ser-Leu-Pro-Glu-Trp-Val-Cys-Thr-Thr-Phe f(17–31) disulphide-bonded to Ala-Leu-Cys-Ser-Glu-Lys f(109–114); chymotrypsin results in another antibacterial peptide, Cys-Lys-Asp-Asp-Gln-Asn-Pro-His-Ile-Ser-Cys-Asp-Lys-Phe f(61–68) disulphide bound to f(75–80). These peptides were mostly active against Gram-positive bacteria. Pepsin- or trypsin-released peptides from α-La inhibited the growth of *E. coli* JM103 at a peptide concentration of 25 mg/mL^{-1} (Pihlanto-Leppälä et al. 2000; Gauthier and Pouliot 2003).

Peptides from hydrolyzed α-La have growth-promoting effects on *Bifidobacterium longum* ATCC 15707 and can be prebiotic food supplements. α-La was observed to improve cognitive performances in stressed individuals by increased brain tryptophan and serotonin activity (Markus et al. 2002). Clinical trials suggest that α-La could be used to improve sleep in adults (Matsumoto et al. 2001; Minet-Ringuet et al. 2004; Kelleher et al. 2003).

Purified α-La is very useful in infant formula manufacturing because of its structural similarity to human α-La (Clustal 2006); however, due to cost reasons demineralized whey with higher levels of β-Lg is often used, making the formula less similar to human milk (Raiha et al. 1986a,b; Heine et al. 1996; Sarwar and Botting 1999; Bruck et al. 2003a,b).

Minor Bioactive Proteins from Milk

Lactoferrin

Lactoferrin (LF) is an iron-binding glycoprotein of the transferrin family, which was first fractionated as an unknown "red fraction" from cow milk by Sørensen and Sørensen (1939). LFs are single-chain polypeptides of about 80,000 Da, containing 1–4 glycans, depending on the species. Bovine and human LF consist of 689 and 691 amino acids, respectively. Their sequence identity is 69%. LFs have also been identified in buffalo, pig, horse, goat, and mouse milk. LF is isolated commercially from milk, which contains between 20 to 200 mg/mL^{-1}. LF can also be found in tears, synovial fluids, saliva, and seminal fluid, with concentrations from 2 mg/mL^{-1} to 10 mg/ mL^{-1}). Blood plasma LF is derived from neutrophils, which degranulate and synthesize lactoferrin during inflammation (Abe et al. 1991; Britigan et al. 1994).

LF exhibits a range of biological activities including antimicrobial, antiviral, antioxidant, and immunomodulation effects on cell growth, binding, and inhibition of bioactive compounds lipopolysaccharides and glycosaminoglycans (Baveye et al. 1999; Chierici 2001). The in vitro activity of LF includes transcriptional activation of several genes. Pepsin hydrolysate of LF has more potent antimicrobial activity than the native protein (Tomita et al. 1991).

The purified active peptide from LF hydrolysate was named *Lactoferricin* (Bellamy et al. 1992). The three-dimensional conformations of LF of various species have been studied in detail. The three-dimensional structures of bovine and human LF are very similar, but not entirely superimposable. In the natural state, bovine LF is only partly saturated with iron (15–20%) and has a salmon-pink color, the intensity of which depends on the degree of iron saturation. Iron-depleted LF with less than 5% iron saturation is called *apo-lactoferrin*, whereas iron-saturated LF is referred to as *holo-lactoferrin*. The LF found in breast milk is apo-lactoferrin. The average LF content in buffalo milk is 0.32 mg/mL^{-1}, which is higher than in cow milk (0.05 mg/mL^{-1}) (Bhatia and Valsa 1994). In buffalo colostrum (0–12 h) the LF content is high, about 4.8 mg/mL^{-1}, but decreases on the first day to about 1.2 mg/mL^{-1}, similar to trends in cow milk but differing in magnitude between breeds (Abd El-Gawad et al. 1996; Mahfouz et al. 1997).

The molecular weight of buffalo LF is 73,700–74,000 Da and its metal binding sites and other properties are similar to cow LF. The four most abundant amino acids of buffalo milk LF are Ly, Glu, Asp, Leu; in cow LF they are Glu, Asp, Leu and Al. The carbohydrate moiety of buffalo LF contains 2.2–3.2 g mannose, 1.7–1.9 g N-acetylglucosamine, 0.4 g sialic acid and 0.2 g fucose per 100 g LF and is similar to that of milk LF of Friesian and Brown-Swiss cattle (Mahfouz et al. 1997).

Health Benefits

The oral administration of LF exerts various beneficial antiinfection health benefits in infants, adult

animals, and humans (Tomita et al. 2002; Wakabayashi et al. 2003; Wakabayashi 2006). Over 60% of administered bovine LF survived passage through the adult human stomach and entered the small intestine intact (Kuwata et al. 1998, 2001; Troost et al. 2001). The cationic N-terminus of bovine LF is of special interest because of its reported antibacterial activity (Bellamy et al. 1992*)*. Orally administered LF enhanced interleukin (IL)-18 production in the intestinal epithelial cells and increased the numbers of $CD4^+$ cells, $CD8^+$ cells, and natural killer (NK) cells in the intestinal mucosa (Kuhara et al. 2000; Wang et al. 2000).

LF in tears, saliva, and seminal fluids, as well as in milk, suggests that it has a role in the defense against invading pathogens. Its broad antimicrobial spectrum includes Gram-positive and Gram-negative bacteria, yeasts, and fungi, with added antiviral activity against cytomegalovirus, herpes, influenza, HIV, rotavirus, and hepatitis C) (Kawasaki et al. 1993; Teraguchi et al. 1994, 1995; Superti et al. 1997). Ingestion of bovine LF for 8 weeks decreased serum hepatitis C virus (HCV)-RNA levels in chronic hepatitis C patients with low viral loads (Tanaka et al. 1999). In adults, a 7-day bovine LF treatment aided in the eradication of *Helicobacter pylori* gastric infection (Di Mario et al. 2003). Several animal studies have reported that LF can inhibit the development and progression of colonic tumors in rats (Sekine et al. 1997a,b), besides having chemopreventive effects on cancers in the esophagus, lung, tongue, bladder, and liver.

Antimold activity of buffalo and cow LF was studied against four test mold strains (*Aspergillus niger, Rhizopus oryzae, Penicillium roquefortii,* and *P. camemberti*). The lowest effective inhibitory concentration of buffalo and cow LF was in the range of 25–75 and 25–125 µg/mL, respectively (Shilpa et al. 2005). The antifungal activity of buffalo LF against five yeast strains (*Kluveromyces marxianus* NCDC39, *Saccaromyces cerviae* NCDC 47, *Rhodotorula glutinis* NCDC *51,* and *Candida guillermondi* NCDC44) was also studied. The lowest effective inhibitory concentration of buffalo LF was in the range of 25–250 $\mu g/mL^{-1}$. Among all yeast cultures *Rhodotorula glutinis* NCDC 51 was most sensitive to buffalo LF, *Asperigillus niger* NCDC 267 was the most sensitive mold against buffalo and cow LF, and mold sporulation was inhibited.

In infants, increases of *Bifidobacterium* in the fecal flora were obtained by supplementing infant formula with bovine LF at $1\,mg/mL^{-1}$ (Kawaguchi et al. 1989a,b; Roberts et al. 1992; Chierici et al. 1992; Chierici 2001), which suggests that feeding LF-enriched formula can lead to a *Bifidobacterium*-dominant intestinal flora in infants. In cats, bovine LF is used for the treatment of intractable stomatitis (Sato et al. 1996). In mice, bovine LF induced mucosal and systemic immune responses (Debbabi et al. 1998; Wang et al. 2000). Current commercial applications of bovine LF include infant formulae, nutritional iron supplements and drinks, fermented milk, chewing gums, immune-enhancing nutraceuticals, cosmetic formulae, and pet care supplements.

Bovine LF as a food ingredient is thought to be safe because there is a long dietary history of its use. People who live on dairy products must have ingested LF for a long time, because raw milk and natural cheese contain $0.1–0.4\,mg/mL^{-1}$ and about $3\,mg^{-1}$ of LF, respectively. LF did not show any toxicity upon single-dose oral ingestion or 4-week or 13-week oral repeated-dose ingestion at a maximum dose of $2\,mg/kg^{-1}\,day^{-1}$ in rats (Yamauchi et al. 2000). A human clinical study in chronic hepatitis C patients showed that high oral doses, up to $7.2\,g/body^{-1}/day^{-1}$, were well tolerated (Okada et al. 2002). Use of bovine LF as a nutritional supplement is considered to be Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration. Since LF is denatured by heat treatment, pasteurized milk is not a suitable source for LF purification (Law and Reiter 1977; Yoshida et al. 2000; Sanchez et al. 1992). Heat stability of LF is affected by conditions of pH, salts, and whey proteins (Kussendrager 1994). LF is used to supplement foods such as infant formula, supplemental tablets, yogurt, drinks and sports foods, and skin and oral care products (Table 5.2) (Tomita et al. 2002; Wakabayashi et al. 2003; 2006)).

Immunoglobulins

The function of immunoglobulins (Igs) in milk and colostrum (about $50\,gL^{-1}$ in the early bovine colostrum) is to protect the neonatal calf and the mammary gland against pathogens (Elfstrand et al. 2002; Lilius and Marnila 2001). The Ig content decreases sharply after the first 2 days of lactation. Commercially, Ig

Table 5.2. Commercial lactoferrin applications available in Japan (Wakabayashi 2006)

Category	Product	Brand Name	Expected Effect
Food	Infant formula	Hagukumi, Chilmil Ayuni, Non-Lact, E-Akachan, GP-P, New-NA-20 (Morinaga)	Antiinfection, improvement of orogastrointestinal microflora, immunomodulation, antiinflammation, antioxidation
	Supplemental tablet	Lactoferrin Plus, Lactoferrin Original Type (Live well), Acito Lactoferrin (Asahi), Lactoferrin (DHC)	
	Yogurt	Lactoferrin 200 Yogurt, Onakani-Haitatsu Yogurt, Ikiikigenki-Nomu Yogurt (Morinaga), Bifine M (Yakult)	
	Skim milk	Ca Lactoferrin skim milk (Morinaga), Tetsu Lactoferrin Plus (Snow Brand)	
	Drink	Lactoferrin Plus (Morinaga)	
	Pet food	Lactoferrin 200, Lactonin	
Skin care (cosmetics)	Lotion, cream, face wash	Milk protein (DHC), Miss Yoko essential lotion/white cream/essence (Yoko)	Hygiene, moistening, antioxidation
Oral care	Mouthwash, mouth gel, toothpaste chewing gum	Biotene Oral Balance/mouthwash/toothpaste (Laclede) Hamigaki Gum (Kanebo)	Hygiene, moistening

products are used effectively in human and animal health care (Table 5.3) (Loimaranta et al. 1997 and 1999; Korhonen et al. 2000; Marnila and Korhonen 2002; Casswall et al. 2002; Marnila et al. 2003; Kelly 2003; Tawfeek et al. 2003; Brinkworth and Buckley 2003; Earnest et al. 2005). Igs in buffalo colostrum, milk, and blood were investigated by Mahran et al. (1997). Three classes of Igs have been identified: IgG, IgM, and IgA. The IgG was present in two subclasses: IgG1 and IgG2. Colostrum Igs contained higher values of all essential amino acids except Leu and Lys and more nonessential amino acids than the corresponding serum. Goel and Kakker (1997) reported from colostrum samples of three buffalo immediately postpartum IgG1 31.6, IgG2 2.0, IgM 33.6, and IgA 0.4 mg/mL, declining 7-fold by 24 hours, and more than 30-fold by 84 hours postpartum (Table 5.4). Nawar (1999) studied optimal ammonium sulfate concentrations (35, 40 and 45%) for fractionation of buffalo serum and colostrum in order to obtain pure Igs. Fractionation of blood serum and colostral whey was similar and optimal recovery of Igs was obtained by 2 times precipitation with 40% saturated ammonium sulfate.

Periodic changes of the chemical composition and amino acid content, and effects of heat treatment and soft cheese making on the buffalo Ig contents were also studied (Mahran et al. 1997). Commercial pasteurization results in incomplete denaturation of IgG1 and IgG2 in buffalo milk. The milk permeate from UF treatment had free Igs because this treatment rejects all milk proteins in the retentate; consequently the resultant UF soft cheese contained high values of IgG1 and IgG2. El-Loly et al. (2007) studied denaturation of Igs in buffalo milk and reported that IgG and IgM were incompletely denatured upon heating up to 88 °C for 15 minutes,

Table 5.3. Commercial colostrum and immune milk products (Pakkanen and Aalto 1997; Scammell 2001)

Product	Company	Claimed Health Benefits
Intact™	Numico RA (Australia)	Immune-enhancing, athletic performance
Gastrogard-R™	Northfield Laboratories (Australia)	Prevents diarrhea caused by rotavirus in infants and children <4 years
PRO-IMMUNE 99 Proventra™ natural immune components	GalaGen Inc. (U.S.)	Prevents scours caused by *E. coli* in calves; boosts immunity and enhances body's natural resistance
Lactimmunoglobulin Biotest	Biotest Pharm GmbH (Germany)	Product for humans, treatment of diarrhea in AIDS patients
ColostrumGold™ liquid Colostrumune™ powder	Sterling Technology, Inc. (U.S.)	Immune system booster
First Defence^{R}	Immucell (U.S.)	Reduces mortality and morbidity from scours caused by *E. coli* K99 rand coronavirus in calves

Table 5.4. Immunoglobulin concentrations in the first milking colostrum and milk of buffalo and cows (Marnila and Korhonen 2002; Elfstrand et al. 2002; Goel and Kakker 1997)

		Concentration (g/L^{-1})			
		Milk		Colostrum	
Immunoglobulin Class	Molecular Mass (kDa)	Cow	Buffalo	Cow	Buffalo
IgG1	146–163	0.3–0.6	0.36–1.15	15–180	27.72–34.08
IgG2	146–154	0.06–0 12	0.10–0.19	1–3	1.91–2.03
IgG total		0.15–0.8	0.46–1.34	20–200	29.75–36.03
IgA	385–430	0.05–0 I	0.01–0.03	1–6	0.18–0.57
IgM	900	0.04–0.1	0.04	3–9	0.47–0.57

whereas IgA was completely denatured at any temperature between 63–88 °C. This means that IgA is the most heat sensitive of the Igs. The rate of denaturation of buffalo milk Igs was lower at 63 °C compared to 88 °C. Elagamy (2000) found that the whole activity of IgG in buffalo or cow milk was lost at 75 °C/30 minutes versus 69% loss of camel IgG.

Lysozyme

Lysozyme is an important antimicrobial agent in buffalo milk, which kills bacteria by cleaving the β-1,4-glycosidic bond between N-acetylglucosamine residues of the peptidoglycan in the bacterial cell wall (Priyadarshini and Kansal 2002a,b). Together with LF, lysozyme (LZ) is one of the most extensively studied antibacterial milk proteins. LZ is a major component of the whey fraction in human milk ($0.4\,g/L^{-1}$) although its concentration in bovine milk is several orders of magnitude lower ($0.13\,mg/L^{-1}$) (Chandan et al. 1965). Milk LZ has demonstrated antibacterial activity against Gram-positive and some Gram-negative bacteria that are completely resistant to egg-white LZ. Specific activity of buffalo milk LZ is 10 times that of bovine milk LZ (White et al. 1988), 5 times that of camel milk (Duhiman 1988), and 3 times that of mare's milk (Bell et al. 1981). Milk and egg LZ are similar to those of human milk LZ (Parry et al. 1969). Mean LZ activity in buffalo milk was found to be $60 \pm 3.9 \times 10^{-3}$ units/mL, which is double the value observed in bovine milk ($29.1 \pm 1.5 \times 10^{-3}$

units/mL) (Priyadarshini and Kansal 2002a,b), but Nieuwenhove et al. (2004b) had reported 424 ± 349 U/mL^{-1} for Murrah buffalo milk LZ activity.

Buffalo colostrum showed LZ activity 5 times that of mature milk (Priyadarshini and Kansal 2002b). Human and buffalo milk LZ possess greater positive charges than egg-white LZ and are about 3 times more active. LZ activity in buffalo milk was not influenced by parity and stage of lactation; however, it increased during extreme weather conditions in winter and summer. LZ in buffalo and cow milk exhibited maximum activity at pH 7.4. Buffalo milk LZ was fully stable (El-Dakhakhny 1995; Priyadarshini and Kansal 2002b), whereas cow milk LZ was partly inactivated by pasteurization. Nieuwenhove et al. (2004b) found that LZ in Murrah buffalo milk was completely inactivated by low (65 °C, 30 min) and high pasteurization (72 °C, 15 s). LZ in buffalo milk was more stable than in cow milk during storage. A 10- to 50-fold increase in milk LZ activity was observed in mastitic cows. An assay of LZ activity in milk can be used to diagnose mastitis in cattle, but not in buffalo. Some buffalo exhibited thousandfold greater LZ activity and moderately raised somatic cell counts in milk, but there was no sign of mastitis (Priyadarshini and Kansal 2002b). Elagamy (2000) reported that loss of activity of LZ at 85 °C/30 minutes was 56, 74, and 82%, respectively, in camel, cow, and buffalo milk.

Table 5.5. Antibacterial activity of lysozyme from buffalo milk (BMLZ) and egg white (EWLZ) (Priyadarshini and Kansal 2002a)

	Determination of Inhibition, mm	
Bacteria	BMLZ	EWLZ
Micrococcus luteus	16.0	19.5
Bacillus subtilis	13.5	15.50
Bacillus cereus	0	0
Lactococcus lactis ssp. Lactis	13.5	15.5
Enterococcus faecalis	10.0	0
Lactobacillus delbruckii ssp. Bulgaricus	0	0
Staphylococcus aureus	0	12.5
Escherichia coli	0	0
Proteus vulgaris	0	0
Pseudomonas aeruginosa	0	0
Salmonella typhi	0	0

Priyadarshini and Kansal (2002a) found the molecular weight of buffalo milk LZ to be 16 kDa compared with 14.3 kDa for standard egg-white LZ. Milk LZ from different species is antigenically different. Egg-white LZ showed no cross-reactivity with anti–buffalo milk LZ. Antibacterial activity of LZ from buffalo milk and egg white was compared by Priyadarshini and Kansal (2002a) (Table 5.5). The favorable ionic environment of buffalo milk and the high specific activity of LZ can play important roles in preventing growth of some Gram-positive microorganisms. The *N*-terminal sequence of 23 amino acid residues of buffalo milk LZ shows high homology with bovine milk LZ (56%), followed by human milk LZ (48%), egg-white LZ (35%), and mare's milk LZ (30%) (Table 5.6). Lys at position 1, Cys at 6, Ala at 9, Asp at 18, and Gly at positions 16 and 22 are conserved in all five LZ, while positions 3 and 20 have aromatic amino acids (Phe or Tyr) and positions 1 and 5 have basic amino acids (Lys or Arg) in all five LZ. Important variations in buffalo milk LZ are Arg at position 4; Ile at 13; Asn at 7; and Ala at positions 8, 17, and 19, which differ from the other four LZ (Priyadarshini and Kansal 2002a,b). El-Dakhakhny (1995) investigated effects of heat treatments of 60–90 °C for 5–30 minutes on LZ activity in buffalo milk. Concentration of LZ was higher in buffalo milk (29 μg/100 mL^{-1}) than in cow milk (21 μg/100 mL^{-1}). LZ activity increased with 4% sodium chloride, while antibiotics at concentrations of up to 50 mg/L^{-1} had negligible effects.

Lactoperoxidase

Lactoperoxidase (LP) is an enzyme present in milk of different species in varying concentrations and has antimicrobial properties. LP is also present and active in other secretory fluids of the body (Clare et al. 2003). Buffalo milk LP has been studied extensively by Kumar (1994), Kumar et al. (1995), and Kumar and Bhatia (1998, 1999).

Ozdemir et al. (2002) purified water buffalo LP (WBLP) with Amberlite CG 50 H+ resin, CM Sephadex C-50 ion-exchange chromatography, and Sephadex G-100 gel filtration chromatography from skim milk. They reported Km value at optimum pH and optimum temperature for the WBLP was 0.82 mM; Vmax value was 13.7 μmol/mL^{-1}/min^{-1}. Km value at optimum pH and 25 °C for the WBLP

Table 5.6. N-terminal sequence of buffalo milk lysozyme in comparison with lysozyme from milk of cows, human, equine, and egg white (Priyadarshini and Kansal 2002a)

Buffalo milk	lys	Ile	Tyr	Arg	Arg	Cys	Asn	Ala	Ala	Arg	Thr	Leu	Ile	Lys	Ile	Gly	Ala	Asp	Ala	Tyr	Gly	Gly	Val
Bovine milk	lys	lys	Phe	Gln	Arg	Cys	Glu	Leu	Ala	Arg	Thr	Leu	Lys	Lys	Leu	Gly	Leu	Asp	Gly	Tyr	Arg	Gly	Val
Human milk	lys	Val	Phe	Glu	Arg	Cys	Glu	Leu	Ala	Arg	Thr	Leu	Lys	Arg	Leu	Gly	Met	Asp	Gly	Tyr	Arg	Gly	Ile
Equine milk	lys	Val	Phe	Ser	Lys	Cys	Glu	Leu	Ala	His	Lys	Leu	Lys	Ala	Gln	Gly	Met	Asp	Gly	Phe	Gly	Gly	Try
Egg white	lys	Val	Phe	Gly	Arg	Cys	Glu	Leu	Ala	Ala	Ala	Met	Lys	Arg	His	Gly	Leu	Asp	Asn	Tyr	Arg	Gly	Try

was 0.77 mM; Vmax value was 4.83 μmol/mL^{-1}/in^{-1}. The purified WBLP had high antibacterial activity in a thiocynate-H_2O_2 medium against pathogenic bacteria, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginose*, *Shigella sonnei*, *Staphylococcus saphrophyticus*, *Staphylococcus epidermidis*, and *Shigella dysenteriae*, and compared well with antibiotics, tetracycline, penicillin, and netilmicine. The LP activity in buffalo milk has been reported to be 24% higher than in cow milk (Kumar and Bhatia 1994). The important biological function is the bactericidal effect against Gram-negative and -positive bacteria in the presence of hydrogen peroxide and SCN or halogens (DeWit and van Hooydonk 1996). LP has been used to extend the shelf life of milk and milk products (Chakraborty et al. 1986; Earneshaw et al. 1989; Denis and Ramlet 1989). LP activity and thiocyanate level in Murrah buffalo milk were reported by Nieuwenhove et al. (2004b) as 2.49 ± 0.86 U/mL^{-1} and 8.64 ± 2.08 ppm. The biocidal activity of LP results from the products of the chemical reaction it catalyzes. The primary reaction product hypothiocyanite is known to react with the thiol groups of various proteins that are important for the viability of pathogens, thereby inactivating crucial enzyme and protein systems (Kumar 1994; Shin et al. 2001). The thermal stability of LP in permeates and in buffer systems has been shown to be less than that in whey or in bovine milk (Hernandez et al. 1990). The denaturation of LP in buffalo milk is highly sensitive around 80 °C, resulting in complete loss of LP activity in 30 seconds (Kumar and Bhatia 1998). During the pasteurization process, LP is not inactivated, suggesting its stability as a preservative.

Kumar and Bhatia (1999) observed that LP is more stable in whey prepared from buffalo milk, with the relative order of thermostability being in neutralized acid whey > rennet whey > skim milk. They showed Arrhenius energy for buffalo skim milk, rennet whey and neutralized acid whey were 710 kJ/mol^{-1}, 1022 kJ/mol^{-1}, and 1398 kJ/mol^{-1}, respectively. Buffalo LP was pH sensitive undergoing denaturation at low pH, while relatively stable in the range of pH 5–10 (Kumar 1994). Sato et al. (1996) reported greater heat stability of LP toward acidic pH in cow milk. Kumar and Bhatia (1999) reported that at 72 °C buffalo milk LP alone in acetate buffer (0.1 M, pH 6.0) was completely inactivated at zero time, while the presence of salts induced thermal protection to LP structure. The relative effect was in the order of KCl > NaCl > $CaCl_2$ > $MgCl_2$, but sulphates of Na, K, and Mg had no thermoprotective effects. Nieuwenhove et al. (2004b) found 16% inactivated LP by low pasteurization (65 °C, 30 minutes) and 80% by high pasteurization (72 °C, 15 seconds); SCN content was unmodified by any heat treatment. They concluded that LP was the most abundant enzyme in buffalo milk, whereas LZ had a very low activity. Because of its broad biocidal and biostatic activity LP has found many commercial applications, especially targeting oral pathogen (Tenovuo 2002).

BIOACTIVE MILK PEPTIDES

Milk contains many protein components from which bioactive peptides can be generated in vivo through gastrointestinal processes (Clare and Swaisgood 2000). They can exert many direct antimicrobial effects (Isaacs et al. 1990; Epand and Vogel 1999; Clare et al. 2003; Morrow et al. 2005; Isaacs 2005). Caseins as well as whey proteins are sources of antimicrobial peptides. The first discovery about antimicrobial properties of milk was made by Jones and Simms (1930).

Antimicrobial activities of buffalo casein-derived peptides were studied by Aziz et al. (2004) and Bajaj et al. (2005). Buffalo casein was separated from pooled milk at pH 4.6 using HCl, subjected to hydrolysis by chymosin at pH 6.4 (E:S:1:17000), incubated at 30 °C for 30 minutes. The enzyme was inactivated by raising the temperature to 80 °C for 10 minutes, and hydrolyzed casein was precipitated with 2% TCA followed by 12% TCA. The precipitates were dissolved by raising pH to 7.2, dialyzed and freeze-dried. The buffalo casein peptide fraction resulted in antimicrobial activity against *Escherichia coli* NCDC134, *B. cereus*, and *Kluveromyces.* At 1000 μg/mL^{-1} concentration of peptide, a 50% reduction of viable cell count of *Escherichia coli* was observed; in *B. cereus* the reduction of total count was from 140 × 103 to 40 × 103 as concentration increased to 1000 μg/mL^{-1}. There was also a strong effect of peptides against yeast with 50% reduction at 250 μg/mL^{-1} concentration.

Aziz et al. (2004) prepared casein from buffalo milk and hydrolyzed it at 37 °C for 6 hours and pH 7.0, using pancreatin and trypsin. The casein hydrolysate contained peptides from 20,000 kd to

116,000 kd, the whey hydrolysate contained peptides from 20,000 to 45,000 kd, and it was concluded that the two protein hydrolysates can be used for therapeutic purposes in dairy foods. Recently, McCann et al. (2005) have isolated and identified cationic peptides from bovine α_{s1}-casein, which had an MIC (minimum inhibitory concentration) of 125 μg/mL against the Gram-positive bacteria *B. subtilis* and *Listeria innocua*; against Gram-negative bacteria, f(99–109) presented activity against *Salmonella typhimurium* (MIC 125 μgm/mL), *E. coli* (MIC 250 μg/mL), *Sal. enteritidis* (MIC *125* μg/mL), and *Citrobacter freundii* (MIC 500 μg/mL). Isracidin was the first peptide with antimicrobial properties identified in a sequence of bovine α_{s1}-casein (Hill et al. 1974). It was obtained by chymosin digestion of bovine casein and corresponded to the N-terminal fragment, α_{s1}-casein (f1–23). Isracidin was found to inhibit the in vitro growth of *lactobacilli*, and of a variety of Gram-positive bacteria, but only at high concentrations. Casocidin-I is the first described antimicrobial peptide derived from α_{s2}-casein (Zucht et al. 1995; Recio and Visser 1999; Bargeman et al. 2002; Bradshaw 2003). Kappacin is an antimicrobial peptide derived from κ-casein (Malkoski et al. 2001) with antimicrobial activities against *Sty. mutans*, *E. coli* and *Porphyromonas gingivalis*. Kappacin and isracidin have been detected in the water-soluble extracts of different Italian cheese varieties (Rizzello et al. 2005), which demonstrated that antimicrobially active peptide sequences can be generated by milk fermentation. β-casein peptides also have proved to exert biological activities related to host protection (Coste et al. 1992; Hata et al. 1998; Otani et al. 2001). Glycomacropeptide (GMP) is a casein macropeptide present in whey at 10–15%, due to the action of chymosin on casein during the cheese-making process (Delfour et al. 1965).

El-Shibiny et al. (2001) studied the percentage and composition of GMP released from buffalo, cow, goat, and sheep caseins by the action of Mucor [Rhizomucor] miehei protease, Mucor pusillus protease, calf rennet, recombinant chymosin, and rennilase. The total GMP contents released from the different caseins using these enzymes were nearly the same, but the released GMP had variable sialic acid and total carbohydrate contents. Also, the amino acid composition of GMP from different species caseins varied slightly depending on the enzyme used. The results suggest the heterogeneity of the released GMP of caseins from different species. The biological activities of GMP have received much attention in recent years (Abd EI-Salam et al. 1996; Brody 2000; Dziuba and Minkiewicz 1996; Manso and López-Fandiño 2004). In cows with mastitis, isracidin obtained a success rate of over 80% in the treatment of chronic streptococcal infection (Lahov and Regelson 1996). A tryptic casein hydrolysate for treatment and prophylaxis of newborn calf colibacillosis (Biziulevicius et al. 2003) had a 93% therapeutic and 94% prophylactic efficacy. Caseinophosphopeptides (CPPs) added to toothpaste may prevent enamel demineralization and exert an anticariogenic effect (Tirelli et al. 1997). Casein-derived phosphopeptides form organophosphate salts with trace elements such as Fe, Mn, Cu, and Se, which function as carriers and are used in the treatment of rickets (Kitts and Yuan 1992; Meisel and Schlimme 1990). Two commercial products, a casein hydrolysate containing the peptide FFVAP-FEVFGK (α_{s1}-casein) f(23–34) (Casein DP, Kanebo, Ltd, Japan, and Cl2 peptide, DMV, The Netherlands) and a whey protein hydrosylate (BioZate, Davisco, U.S.) were claimed to lower blood pressure in humans (FitzGerald et al. 2004). See Figure 5.3.

Sodium caseinates prepared from buffalo, bovine, goat, sheep, pig, and human milk were hydrolyzed by a partially purified proteinase of *Lactobacillus helveticus* PR4 and had a peptide concentration of 0.515 (goat), 0.693 (human), 0.710 (sheep), 0.966 (pig), 1.238 (buffalo), and 1.812 mg/mL (cow). Various ACE-inhibitory peptides were found in the hydrolysates: the cow α_{S1}-casein (α_{S1}-CN) 24–47 fragment f(24–47), f(169–193), and β-CN f(58–76); buffalo β-CN f(58–66); sheep α_{S1}-CN f(1–6) and α_{S2}-CN f(182–185) and f(186–188); goat β-CN f(58–65) and α_{S2}-CN f(182–187); and a mixture of three tripeptides originating from human β-CN (Azuma et al. 1984; Minervini et al. 2003). Before fractionation by RP-FPLC, the above sodium caseinate hydrolysates showed ACE-inhibitory activities of 2 to 43%. The RP-FPLC peptide profiles of the sodium caseinate hydrolysates differed according to the milk species. Hydrolysates of cow and goat caseinates were rich in peptides in the hydrophobic and hydrophilic zones, respectively, of the acetonitrile gradient, while the buffalo and other hydrolysates showed rather similar profiles. Hydrolysates of sodium caseinate prepared from buffalo, sheep,

101 … – ... – ... – … – … – Met – Ala – Ile – Pro – Pro – Lys – Lys – Asn –Gln – Asp – Lys – Thr – Glu – Ile – Pro

Ile (Var. B)

121 Thr – Ile – Asn – Thr – Ile – Ala – Ser – Gly – Glu – Pro – Thr – Ser – Thr – Pro – – Glu – Ala – Val – Glu –

Thr (Var. A)

Ala (Var. B)

141 Ser – Thr – Val – Ala – Thr – Leu – Glu – – Ser – Pro – Glu – Val – Ile – Glu – Ser – Pro – Pro – Glu – Ile – Asn–

Thr (Var. A)

161 Thr – Val – Gln – Val – Thr – Ser – Thr – Ala – Val

Figure 5.3. Primary structure of bovine caseinomacropeptide (CMP) variants A and B (Thomä-Worringer et al. 2006).

and pig milk contained fractions, which had ACE inhibition of Ca. 70% and which was lower than for human, cow and goat hydrolysates (Table 5.7). A peptide contained within the sequence 58–76 of β-CN, f(58–66), was found in the fraction with most ACE-inhibition purified from buffalo sodium caseinate hydrolysate. When only part of the above peptide (β-CN f(58–66)) was present, i.e., fractions 3 and 40 of the buffalo and goat sodium caseinate hydrolysates, the IC_{50} was slightly higher (Walstra and Jenness 1984; Minervini et al. 2003; Gobetti et al. 2004).

BIOACTIVE MILK CARBOHYDRATES

Complex oligosaccharides constitute a large portion of the total solids of human milk (Gopal and Gill 2000). Human milk oligosaccharides (HMOs) perform biological functions that are closely related to their structural conformation. They contribute to the growth of beneficial intestinal flora in the colon, postnatal stimulation of the immune system, and provide defense against bacterial and viral infections by acting as competitive inhibitors for binding sites on the intestinal epithelial surface (Kunz et al. 2000).

Compared with human milk and colostrum, the levels of oligosaccharides in milk of domestic mammalian animals (cows, sheep, and goats) are much lower (Urashima et al. 1997; Martinez-Ferez et al. 2006). The low concentration of oligosaccharides in bovine milk and colostrum has stalled their utilization as biologically active ingredients in the health-care and food sector. This has necessitated research toward development of methods and processes for large-scale separation and enrichment of bovine milk oligosaccharides, as well as for expression of HMO in human milk substitutes. Much interest has focused on the potential of milk oligosaccharide in infant nutrition.

Human milk contains 5–10 g/L^{-1} of lactose-derived oligosaccharides, the third largest component of human milk (Kunz and Rudloff 2002). Oligosaccharides are strictly defined as carbohydrates, which contain between 3 and 10 monosaccharides covalently linked through glycosidic bonds. HMO monomers are D-glucose, D-galactose, N-acetylglucosamine, L-fucose, and N-acetyl neuraminic acid. These oligosaccharides carry lactose at their reducing end with a few exceptions. Further structural variations occur due to the enzymatic activity of several fucosyltransferases and sialyltransferases.

Milk oligosaccharides are divided broadly into neutral and acidic classes (Gopal and Gill 2000). Neutral oligosaccharides do not contain any charged monosaccharide residues, while acidic oligosaccharides contain one or more residues of sialic acid, that are negatively charged. About 150 and 200 neutral and acidic oligosaccharides, respectively, have been isolated from human and domestic farm animal milk and colostrum, and their chemical structures

Table 5.7. Sequences and corresponding casein fragments of peptides in crude fractions from sodium caseinate hydrolysates produced by a partially purified proteinase of *L v* PR4 from milk of cows, sheep, goats, buffalo, and humans (Minervini et al. 2003)

Milk Source or Fraction	Sequence[a]	Casein Fragment	Calculated Mass[b]	Expected Mass[b]
Bovine				
16	LVYPFPGPIPNSLPQNIPP	β-CN f58–76	2,100.28	2,100.13
	FVAPFPEVFGKEKVNELSKDIGSE	α_{S1}-CN f24–47	2,194.35	2,194.14
	LGTQYTDAPSFSDIPNPIGSENSEK	α_{S1}-CN f169–193	2,122.27	2,121.93
17	LVYPFPGPIPNSLPQNIPP	β-CN f58–76	2,100.28	2,100.13
	FVAPFPEVFGKEKVNELSKD IGSE	α_{S1}-CN f24–47	2,194.35	2,194.14
18	LVYPFPGPIPNSLPQNIPP	β-CN f58–76	2,100.28	2,100.13
Sheep				
4	RPKHPI	α_{S1}-CN f1–6	746.9	746.46
	RPKH	α_{S1}-CN f1–4	537.32	537.35
	HPIKH	α_{S1}-CN f4–8	631.37	631.38
6	TVDQ	α_{S2}-CN f182–185	599.42	599.35
	HQK	α_{S2}-CN f186–188	411.46	411.22
Goat				
3	LVYPFPGP	β-CN f58–65	888.47	888.96
4	TVDQHQ	α_{S2}-CN f182–187	727.33	727.24
Buffalo				
4	LVYPFPGPI	β-CN f58–66	1,002.14	1,001.56
Human				
4	QPQ	β-CN f44–46	371.26	371.3
	VPQ	β-CN f77–79 or f137–139 or 155–157	342.26	342.22
	IPQ	β-CN f141–143 or f163–165 or β-CN f74–76	356.36	356.39
19	QELLLNPTHQYPVTQPLAPVHNPI SV[c]	β-CN f184–210	3,132.8	3,133.39

[a]Single-letter amino acid code is used.
[b]Monoisotopic masses are reported.
[c]The only peptide that had antibacterial activity. All the other peptides were ACE inhibitors.

determined (Boehm and Stahl 2003; Nakamura and Urashima 2004). Oligosaccharide distributions in human milk, and colostrums and milk of domestic animals are listed in Table 5.8. Oligosaccharide distributions in human milk and colostrums and milk of domestic animals have also been studied by Mehra and Kelly (2006), but no data were reported for buffalo milk HMO total concentration decreases during lactation, with contents in early lactation being 5–10 times higher than in late lactation (Kunz et al. 2001).

Besides much lower contents of oligosaccharides cow, sheep, goat, and horse milk (Urashima et al. 1997) than in human milk, there are also structural differences. The major oligosaccharides in human milk, fucosylated oligosaccharides (containing L-fucose), could not be detected in cow, sheep, goat, and horse milk (Finke 2000). While 3′- and 6′-sialylactose and 3′- and 6′-galactosyl-lactose are common to human and bovine milk, the structures of other sialyloligosaccharides are different in the two types of milk (Boehm and Stahl 2003). Furthermore, bovine colostrum contains sialyloligosaccharides with two types of sialic acid, Neu5Ac and Neu5Gc, and human milk or colostrum contains only Neu5Ac (Urashima et al. 2001). In bovine

Table 5.8. Oligosaccharides in milk and colostrums of humans, cows, goats, sheep, and buffalo (Mehra and Kelly 2006)

Oligosaccharide	Human Milk (g/L^{-1})	Cow Milk (g/L^{-1})	Cow Colostrum (g/L^{-1})	Goat Milk/ Colostrum (g/L^{-1})	Sheep Milk/ Colostrum (g/L^{-1})	Buffalo Milk/ Colostrum ($g\pm L^{-1}$)
Lactose	55–70	40–50	40–50	43–48	41–49	~52
Neutral Oligosaccharides						
Lacto-*N*-tetraose	0.5–1.5	Trace	—	—	—	—
Lacto-*N*-fucopentaose I	1.2–1.7	—	—	—	—	—
Lacto-*N*-fucopentaose II	0.3–1.0	—	—	—	—	—
Lacto-*N*-fucopentaose III	0.01–0.2	—	—	—	—	—
Lacto-*N*-difucohexaose I	0.1–0.2	—	—	—	—	—
Lacto-*N*-novopentaose			b	—	—	—
N-acetylgalactosaminyl glucose			b	—	—	—
N-acetylgalactosyl-lactose			b	—	—	
α-3'-galactosyl-lactose			b	0.03–0.05	b	
β-3'-galactosyl-lactose			b		b	
6'-galactosyl-lactose			b	b	b	
N-acetyl-lactoseamine			b	—	—	
N-acetylglucosaminyl-lactose				0.02–0.04		
Acidic Oligosaccharides						
NeuAc(α2-6)lactose	0.3–0.5	0.03–0.06 (combined)	0.019	0.05–0.07	—	
NeuAc(α2-3)lactose	0.1–0.3		0.095	0.03–0.05	b	
N-glycolylneuraminyl-lactose				0.04–0.06		
NeuAc-lacto-*N*-tetraose a	0.03–0.2	Trace	—	—	—	
NeuAc-lacto-*N*-tetraose c	0.1–0.6	Trace	—	—	—	
NeuAc2-lacto-N-tetraose	0.2–0.6	Trace	—	—	—	
6-Sialyl-lactosamine			0.047	b	—	
3-Sialyl galactosyl-lactose			Trace (3 μmol/ L^{-1})	—	—	
Disialyl-lactose			0.028	0.001–0.005	—	b
Sialyl-lactose-1-phosphate			Trace (3 μmol/ L^{-1})	—	—	—
Sialyl-lactose-6-phosphate			Trace (1 μmol/ L^{-1})	—	—	—
3-Glucolylneuraminyl-lactose			Trace (2 μmol L^{-1})	—	—	—
6-Glucolylneuraminyl-lactose			b	b	b	
GlcNAcβ(1–3)Galf3(1–4)	—	—	—	—	—	b
GlcNacβ(1–3)Galf3 (1–4)Glc						

b = too low.

milk, the concentration of oligosaccharides decreases during lactation, except in late lactation, when the level of sialylated oligosaccharides increases (Martin et al. 2001).

A processed oligosaccharide mixture of buffalo milk induced significant stimulation of antibody, delayed-type hypersensitivity response to sheep red blood cells in BALB/c mice and also stimulated nonspecific immune response of the animals in terms of macrophage migration index. Saksena et al. (1999) isolated a novel pentasaccharide from buffalo milk oligosaccharides containing a fraction with immunostimulant activity. The results of structural analyses, i.e., proton nuclear magnetic resonance, fast atom bombardment mass spectrometry, chemical transformations and degradations, are consistent with the following structure: GlcNAc β(1–>3)Gal β(1–>4)GlcNAc β(1–>3)Gal β(1–>4)Glc.

Methods for Oligosaccharide Production

The technology for the development of oligosaccharide-enriched ingredients from any milk is still in its beginning and research is fragmentary. Approaches include 1) production of HMO by fermentation of genetically engineered bacteria, 2) concentration and fractionation technologies such as membrane filtration, and 3) expression of HMO in transgenic animals. Sialyloligosaccharide in situ production from waste streams of cheese processing and from other dairy sources using α-(2,3)-trans-sialidase enzyme have been described (Pelletier et al. 2004). Disialyllactose from buffalo colostrum has been studied by Aparna and Salimath (1995). Another process has been reported for the isolation of goat milk oligosaccharide fractions using membrane filtration technology (Martinez-Ferez et al. 2006). A two-stage tangential ultrafiltration-nanofiltration of goat milk, using 50 and 1 kDa molecular mass cutoff membranes, respectively, was employed. A virtually lactose and salt-free product was obtained containing more than 80% of the original oligosaccharide content.

Sarney et al. (2000) demonstrated a scalable approach to the recovery of biologically active oligosaccharides from human milk using a combination of enzymatic hydrolysis of lactose and nanofiltration. Recovered oligosaccharides by this method were shown to inhibit binding of intimin, an adhesion molecule of enteropathogenic *Escherichia coli*, to epithelial cells in vitro. Nakano (1998) produced sialyllactose-rich preparations by desalting (electrodialysis and/or ion-exchange dialysis), evaporation, and drying bovine UF permeate. A sialic acid-containing glycolipid called *ganglioside GM3* (monosialoganglioside) was also prepared from bovine buttemilk by ultrafiltration, organic solvent separation of the glycolipid fraction from the phospholipid fraction, and desialization of GD3 (disialoganglioside) present in the glycolipid fraction.

Pending the development of commercially available large-scale preparations of oligosaccharides from bovine milk, oligosaccharides of structures simpler than HMOs are being increasingly used in food products to mimic health benefits of HMOs. Fructans and galactooligosaccharides represent oligosaccharides of nonmilk origin that have been studied for food applications. Galactooligosaccharides (GOS) are produced from lactose by enzymatic transgalactosylation using β-galactosidases (Tanaka and Matsumoto 1998) and consist of a chain of 3–8 units of galactose, usually with a glucose molecule at the reducing end. Enzymatic synthesis leads to the production of heterogeneous mixtures of GOS structures with varying chain length and linkages. These have been shown to have beneficial prebiotic effects similar to HMOs (Boehm and Stahl 2003).

Processes for large-scale production of human milk oligosaccharides by fermentation of genetically engineered bacteria have been developed. Two fucosyltransferase genes of *Helicobacter pylori* were engineered into *E. coli* cells to express fucosyltransferases for production of LeX oligosaccharides (Dumon et al. 2004). GlcNAcβ(1–3)Galβ(1–4)Glc, lacto-N-neotetraose, lacto-N-neohexaose, and sialyllactose have been shown to be produced by metabolically engineered *E. coli* (Priem et al. 2002).

Health-Promoting Aspects of Milk Oligosaccharides

Since the 1950s, oligosaccharides from human milk have been thought to be growth-promoting factors for the so-called *bifidus* flora in the gut of breastfed infants. However, these carbohydrates may have a more specific effect on the colonization of the gut by acting as soluble analogs to epithelial receptors for specific microorganisms, and thus preventing their adhesion to the intestinal wall. Since the pattern

of milk oligosaccharides depends on the blood group and secretor status of the donors, chemical structures vary individually, and their potential antiadhesive and antiinfective properties may be different (Kunz et al. 2000).

Sialic acid present in oligosaccharides, glycolipids, and glycoproteins in milk is considered to play an important role in the expression and development of brain and central nervous system functions in infants. As some acidic bovine milk oligosaccharides are structurally similar to those found in human milk, it is likely that they would also have similar biological functions and great potential in functional foods and infant formula. Recent studies suggest that HMOs resist digestion, get partially absorbed, and remain in the circulation long enough and in high-enough concentrations to exert systemic effects (Engfer et al. 2000; Gnoth et al. 2000). It has been shown that sialic acid containing oligosaccharides reduce the adhesion of leukocytes to endothelial cells, an indication for an immune regulatory effect of certain HMOs (Bode et al. 2004). A *bifidus* predominance of the intestinal flora of breastfed infants was reported long ago by Moro (1900), who already concluded that human milk contains a growth factor for these microorganisms. By using a "Bifidum mutant" (*Bifidobacterium bifidum* subsp. *Pennsylvanicum*, *B. bifidum* subsp. *Penn.*) György (1953) referred to a mixture of oligosaccharides containing *N*-acetylglucosamine (GlcNAc), which he called *gynolactose*, to be the *bifidus* factor. In many in vitro studies it has been demonstrated that GlcNAc containing oligosaccharides are able to enhance the growth of *B. bifidum* subsp. *Penn.*, whereas other *N*-containing sugars showed less growth-promoting activity. In addition to oligosaccharides there are several glycoconjugated fractions, i.e., glycoproteins or glycolipids, which may also have a bifidogenic effect.

Because human milk is still the gold standard for the production of infant formula, new products on the market include those containing prebiotics (PBOs) shown to be suitable to increase the number of bifidobacteria in an infant's gut. No data have been published so far, however, regarding the growth inhibition of pathogenic microorganisms. In HMOs, structures are present that may prevent the adhesion of certain microorganisms to epithelial cells by acting as soluble receptor analogs due to specific monosaccharide composition (Kunz and Rudloff 2006). A decisive pathophysiological factor for many infectious diseases such as diarrhea is the ability of microbial pathogens to adhere to the mucosal surface and their subsequent spreading, colonization, and invasion in the gut (e.g., *Escherichia coli*, *Helicobacter jejuni*, *Shigella* strains, *Vibrio cholerae*, and *Salmonella* species) (Beachey 1981; Ofek and Sharon 1990). Bacterial adhesion is often a receptor-mediated interaction between structures on the bacterial surface and complementary ligands on the mucosal surface of the host (Karlsson 1995). Human milk, with its high amount and large variety of oligosaccharides, might prevent the intestinal attachment of microorganisms by acting as soluble analogs competing with epithelial receptors for bacterial binding or binding of other pathogens.

The concept of PBO has received much attention during recent years. They are considered to influence the microbial composition of the human colon with potential health benefits. Most of the effects seem to be associated with prebiotic functions, i.e., being substrates for lactobacilli and bifidobacteria, thus stimulating their growth. PBOs are defined as the following: "Nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve host health" (Gibson and Roberfroid 1995). In general, PBOs are nondigestible oligosaccharides or disaccharides. For supplementation of infant formula galactosylated and/or fructosylated oligosaccharides (GOS and FOS) are often used. They may be derived from plants or produced by technological means, e.g., transgalactosylation. Such components are not present in human milk. The main monosaccharides in PBOs are galactose, glucose, fructose, xylose, and arabinose (Tungland and Meyer 2002). Besides glucose and galactose these monosaccharides are not present in human milk.

FOS, like inulin or oligofructose, are well-characterized components. GOS can be produced from lactose through specific processes. They consist of a number of β1–6 linked galactosyl residues bound to a terminal glucose unit via an α1–4-linkage (Tungland and Meyer 2002). In human milk, small oligosaccharides are fucosylated or sialylated, but solely galactosylated components do not occur. The linkage between monosaccharides is important for biological effects. It was shown that the addition of GOS and FOS to an infant formula may increase the

Table 5.9. Milk oligosaccharides as soluble ligands for potential pathogens (Kunz and Rudloff 2006)

Pathogen	Target Tissue	Milk Oligosaccharides
E. coli	Urinary tract	Man(α1–3)[Man(α1–6)]Man-
H. influenzae	Respiratory tract	$NeuAc(\alpha 2–3)_{0,1}$ Gal(β1–4)GlcNAc(β1–4)GlcNAc-
H. pylory	Stomach	NeuAc(αα2–3)Gal(β1–4)Glc(NAc)
		Fuc(α1–2)Gal(β1–3)[Fuc(α1–4)]Gal
S. pneumoniae	Respiratory tract	$NeuAc(\alpha 2–3)_{0,1}$ Gal(β1–4)GlcNAc(β1–4)GlcNAc

number of *bifidobacteria.* Milk oligosaccharides as potential ligands against some pathogens are given in Table 5.9.

Currently, there is only a limited amount of quantitative data on oligosaccharides in buffalo milk (Sahai 1996). The main difference between human and bovine milk is the predominance of neutral oligosaccharides in human milk, whereas the oligosaccharide fraction in mature bovine milk mainly consists of acidic components, most notably 3′ sialyl-lactose. As animal milk from the first days of lactation also contains a relatively high amount of neutral components, colostrum may be suitable for isolating larger amounts of individual oligosaccharides to be potentially added to infant formula. Certain structural prerequisites are necessary for milk oligosaccharides to be effective in different in vitro systems.

BIOACTIVE MILK LIPIDS

It is well known that buffalo milk contains higher amounts of fat than cow milk, almost twice the amount (Sahai 1996). Physiology of animal, stage of lactation, season, feed, breed, time, and sequence of milking, etc., are some of the factors affecting the fat content of buffalo milk. Bovine milk lipids (BML) contain a number of bioactive substances with interesting properties, mainly in the class of fatty acids, which also apply to the lipids in buffalo milk. Besides *trans* fatty acids (TFA), conjugated linoleic acids (CLA) are of particular interest. Apart from ruminant meat products, the main source of CLA in food are BML. Although TFA as well as saturated fatty acids are thought to be positively correlated with human atherosclerosis and coronary heart disease, CLA on the other hand are considered antiatherogenic. Further, CLA are reported to reduce adipose fat and to have anticarcinogenic properties.

Table 5.10. Average profile of major long-chain fatty acids in milk of Murrah buffalo (Nieuwenhove et al. 2004a)

Fatty Acid	Concentration (mg/g^{-1} of FAME)
C14:0	68.01
C14:1	5.04
C15:0	5.83
C16:0	319.41
C16:1	10.24
C17:0	4.39
C18:0	111.49
C18:1 *trans*-11	39.51
C18:1 *cis*-9	271.56
C18:2	16.46
C18:3	5.04
CLA*	4.83

*All conjugated linoleic acid isomer.

The varying CLA and TFA contents of lipids from milk and dairy products are correlated with one another. Anticarcinogenic effects are ascribed to butyric acid as well as to some phospholipids and other lipids present in BML. Moreover, the essential fatty acids 18:2n-6 and 18:3n-3 found in BML are involved in a variety of biochemical processes and functions in human metabolism. The total content of bioactive substances in BML is approximately 75%. Table 5.10 lists the profile of long-chain fatty acids in Murrah buffalo milk (Nieuwenhove et al. 2004a).

Conjugated Linoleic Acids in Milk Fat

Ha et al. (1987) identified CLA as a new anticarcinogen. The term *CLA* includes a collection of positional and geometrical isomers of octadecadienoic

acid, with conjugated double bonds ranging from 6,8 to 12,14. For every positional isomer, four geometric pairs of isomers are possible (i.e., *cis, trans*; *trans, cis*; *cis, cis*; and *trans, trans*). The term CLA therefore includes a total of 28 positional and geometrical isomers.

In cow milk fat, CLA levels may range from 2 to 37 mg/g^{-1} fat (Parodi 1999; Stanton et al. 2003), but recently *cis*-9, *trans*-11 CLA contents of 54 mg/g^{-1} (Shingfield et al. 2006) and 52 mg/g^{-1} of total FA (Bell et al. 2006) were reported. This large range in CLA contents can be attributed to a number of factors, especially diet. High values often occur with the feeding of fresh pasture and other forages (Dhiman et al. 1999; Chilliard et al. 2000; 2001; Collomb et al. 2001; 2002; Stanton et al., 2003; Lock and Bauman 2004).

In buffalo milk the high fat and CLA contents, besides increased yield, are especially valuable characteristics for dairy products producers and consumers. Nieuwenhove et al. (2004a) reported that the Murrah buffalo milk CLA average content was 4.83 mg/g^{-1} of FAME (fatty acid methyl ester) and a range between 3.2–6.5 mg/g^{-1} of FAME. The mean concentration of vaccenic acid (C18:1 *trans*-11) in buffalo milk fat was 39.51 mg/g^{-1} of FAME (range 24.3–56.7 mg/g^{-1} of FAME) (Table 5.10). They found a positive correlation (r = 0.90) between CLA and VA contents in the milk fat.

Secchiari et al. (2005) showed that when Italian buffalo are fed fresh forage, the percentages of medium-chain fatty acids and saturated fatty acids significantly decreased in milk fat, while those of monounsaturated fatty acids and polyunsaturated fatty acids increased. The CLA average content of milk was significantly enhanced by the inclusion of fresh forage in the TMR (total mixed ration) diet in a similar way to those reported in similar dairy cattle research. However, much higher CLA levels in milk were found when suitable TMR included safflower or fish oil (Lynch et al. 2005). Breed (Lawless et al. 1999; Kelsey et al. 2003), lactation number, age (Stanton et al. 1997), or individual animals can also influence CLA levels (Kelly et al. 1998; MacGibbon et al. 2001; Peterson et al. 2002).

Tissue Δ^9-desaturase activity and the viability of certain rumen microflora responsible for isomerization and biohydrogenation may be contributing factors (Bauman et al. 2003; Parodi 2003; Lock et al. 2005). Seasonal effects on milk CLA content have also been reported; the trend is that content is greatest when fresh pasture is plentiful, and it decreases throughout the growing season (Riel 1963; Banni et al. 1996; Jahreis et al. 1997; Lock and Garnsworthy 2003).

Health Benefits

The average total CLA human intake is estimated between 95 and 440 mg per day and differs by country. The different CLA isomers do not exhibit the same biological effects (Martin and Valeille 2002). Above all, the isomers *cis*-9, *trans*-11 and *trans*-10, *cis*-12 are currently of greatest interest. In milk fat *cis*-9, *trans*-11 CLA amounts to 75–90% of total CLA, whereas *trans-l0, cis*-12 CLA constitutes a minor isomer (Albers et al. 2003).

Evidence from animal trials has shown an influence of CLA on body composition, i.e., lowering of body weight and fat mass, and a relative increase in lean body mass (Roche et al. 2001). Results from human trials indicate a body fat lowering effect of CLA associated with an increase in lean body mass (Martin and Valeille 2002; Larsen et al. 2003). CLA supplementation for 24 months to healthy, overweight humans decreased body fat mass mainly during the first 6 months (Terpstra 2004; Gaullier et al. 2005). Studies in animals and humans have found an antidiabetic effect of CLA and suggested the *trans*-10, *cis*-12 isomer to be responsible for decreasing glucose levels and increasing insulin sensitivity (Khanal 2004), but other studies have not found similar results (Moloney et al. 2004; Wang and Jones 2004).

In rodents, dietary CLA supplementation lowered serum cholesterol and triacylglycerol concentrations (Terpstra 2004; Lock et al. 2005) and led to a reduction in the severity of cholesterol-induced atherosclerotic lesions in the aorta (Kritchevsky et al. 2000, 2002). With the exception of one study, results from human studies investigating CLA influence on body composition and blood lipids showed no significant effects (Terpstra 2004). However, Tricon et al. (2004b) compared the effects of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers on blood lipids and suggested that *cis*-9, *trans*-11 is the isomer with positive effects. Moloney et al. (2004) found significantly lower plasma fibrinogen concentrations after CLA supplementation compared with controls in humans with type 2 diabetes mellitus.

In vitro experiments and animal trials have been done regarding CLA inhibition of carcinogenesis (Belury 2002; Banni et al. 2003; Ip et al. 2003; Parodi 2004), but no human data are available so far. Epidemiologically there seems to be a negative connection between CLA and the incidence of breast cancer in humans (Aro et al. 2000; Voorrips et al. 2002). The results of a cohort study suggest that high intakes of high-fat dairy foods and CLA may reduce the risk of colorectal cancer (Larsson et al. 2005). Effects of CLA may differ according to CLA isomer, type, and site of the organ and stage of carcinogenesis (Lee and Lee 2005). Rat studies revealed a contribution of VA to the anticarcinogenic effects due to its desaturation to *cis*-9, *trans*-11 CLA (Ip et al. 1999; Corl et al. 2003; Lock et al. 2004).

CLA may also modulate the immune system and prevent immune-induced wasting in animals. In humans a beneficial effect in certain types of allergic or inflammatory responses was proposed due to CLA-induced increases of IgA and IgM and a decrease of IgE (O'Shea et al. 2004). Another study found raised protective antibody levels after hepatitis B vaccination in healthy men given a CLA concentrate compared with the control group (Albers et al. 2003). A study by Tricon et al. (2004a) found *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers to decrease mitogen-induced T lymphocyte activation in a dose-dependent manner in healthy humans, with both isomers showing similar impacts.

Processing Effects

A significant decrease in CLA content was documented due to HTST pasteurization (Herzallah et al. 2005), while a study by Nieuwenhove et al. (2004a) revealed that conventional pasteurization had no significant effects on CLA contents in buffalo milk (Table 5.11).

Fat supplementation of ruminant animal feeding rations modifies fatty acid (FA) contents in milk and can cause softer butter (Baer et al. 2001; Ramaswamy et al. 2001; Gonzalez et al. 2003). The churning time of cream with a modified FA composition may also be longer than normal. This may be due to the higher content of unsaturated fat and smaller fat globule size in modified milk (Avramis et al. 2003). No significant differences in the flavor of CLA-enriched butter have been documented (Baer et al. 2001; Ramaswamy et al. 2001). The storage stability of butter from modified milk seems to be good. The free FA and peroxidase values have been reported to remain within the expected ranges (Baer et al. 2001; Ryhänen et al. 2005).

Processing of milk to cheese appears to have no effect on the final content of CLA in cheeses. Its content is primarily dependent on the CLA level of the unprocessed milk (mozzarella, Dhiman et al. 1999; Edam, Ryhänen et al. 2005; Emmental, Gnädig et al. 2004; Gouda, cheddar, Shantha et al. 1995; Swedish Greve, Herrgardsost, Jiang et al. 1997; processed cheese, Luna et al. 2005a). However, Avramis et al. (2003) found that cheddar cheese made from milk fed supplemental fish oil ripened faster after the first 3 months of ripening and developed a more desirable texture and cheddar flavor. Cheeses from CLA-enriched milk seemed to be softer than normal. Jones et al. (2005) manufactured cheeses from milk obtained from cows fed fish oil. The experimental cheeses had CLA contents over 7 times higher and were significantly softer than the controls (Ryhänen et al. 2001; 2005). Luna et al. (2005a,b) reported that organoleptic characteristics of cheeses made from CLA-enriched milk from ewes fed linseed supplements did not differ from control cheeses. The total content and the isomer profile of CLA also did not change during ripening. In another study, cheddar cheese from cows grazing on pasture had CLA contents 3 times higher than cheeses manufactured from milk of cows fed conserved forage and grain. Taste panel evaluations showed no differences in the sensory characteristics

Table 5.11. CLA and VA content in raw buffalo milk after different treatments (Nieuwenhove et al. 2004a)

FAME	Control	Refrigeration	Homogenization	Pasteurization
CLA	4.83 ± 0.92	4.51 ± 1.31	4.63 ± 1.22	4.63 ± m1.09
VA	39.51 ± 9.53	38.65 ± 8.71	36.41 ± 10.23	36.13 ± 9.37
P		>0.05	>0.05	>0.05

between treatments, and the consumer acceptability of CLA cheese was similar to products with a low level of CLA (Khanal et al. 2005).

Possibilities to increase the CLA content of dairy products with microbial cultures have been studied (Sieber et al. 2004). Dairy starter bacteria strains, which are able to convert linoleic acid to CLA in vitro have been identified, such as *propionibacteria* (Jiang et al. 1998), lactic acid bacteria (Lin et al. 1999; Kim and Liu 2002), and *bifidobacteria* (Coakley et al. 2003; Oh et al. 2003; Song et al. 2005). The conversion has been suggested to result from the action of the isomerase enzyme (Lin et al. 2002, 2003; Lin 2006;). A potential approach to raising the CLA content in dairy products is the microbial conversion of free linoleic acid to CLA. Studies by Lin et al. (2003) showed that the production of CLA in set yogurt prepared with mixed cultures comprising *Lactobacillus acidophilus* and yogurt bacteria was significantly enhanced by the addition of linoleic acid (0.1%). Recently, Lin et al. (2005) reported that *Lb. delbrueckii* ssp. *bulgaricus* immobilized with polyacrylamide at pH 7 was effective in promoting CLA formation. Another method to produce CLA-enriched milk fat was published by Romero et al. (2000), who utilized carbon dioxide extraction to enhance CLA concentration in one of the fractions of milk fat.

Polar Lipids

Polar lipids are the main constituents of natural membranes, occurring in all living organisms in different polar lipid species and concentrations (Keenan et al. 1983). They are rich in sphingolipids, highly bioactive compounds that have profound effects on cell metabolism and regulation. Brain and bone marrow are rich in polar lipids, but since some of these sources are involved in diseases like bovine spongiform encephalitis, scrapie, and Creutzfeld-Jacob, it makes milk products an interesting alternative as a source of polar lipids. Phospholipids and sphingolipids of the polar lipids group are amphiphilic molecules with a hydrophobic tail and a hydrophilic head. The glycerophospholipids consist of a glycerol backbone on which two fatty acids are esterified on positions *sn*-l and *sn*-2. These fatty acids are more unsaturated than the triglyceride fractions of milk. On the third hydroxyl, a phosphate residue with different organic groups (choline, serine, ethanolamine, etc.) may be linked. Generally, the fatty acid chain on the *sn*-1 position is more saturated compared with that at the *sn*-2 position. Lysophospholipids contain only one acyl group, predominantly situated at the *sn*-l position. The head group remains similar. The characteristic structural unit of sphingolipids is the sphingoid base, a long-chain (12–22 carbon atoms) aliphatic amine containing two or three hydroxyl groups. Sphingosine (d 18: 1), is the most prevalent sphingoid base in mammalian sphingolipids, containing 18 carbon atoms, two hydroxyl groups, and one double bond. Monoglycosylceramides, like glucosylceramide or galactosylceramide, are often denoted as cerebrosides, while tri- and tetraglycosylceramides with a terminal galactosamine residue are denoted as globosides.

The polar lipids in milk are mainly situated in the milk fat globule membrane (MFGM). This is a highly complex biological membrane that surrounds the fat globule, thereby stabilizing it in the continuous phase of the milk and preventing it from enzymatic degradation by lipases (Danthine et al. 2000). The membrane consists roughly of 60% protein and 40% of lipids (Fox and McSweeney 1998; Keenan et al. 1988). The lipids of the MFGM are triacylglycerides, cholesterol, phospholipids, and sphingolipids in varying proportions. The lipids are like the proteins, asymmetrically arranged. The choline-containing phospholipids, phosphatidylcholine (PC) and sphingomyelin (SM), and the glycolipids, cerebrosides, and gangliosides are largely located on the outside, while phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) are concentrated on the inner surface of the membrane (Deeth 1997). Gangliosides are highly complex oligoglycosylceramides, containing one or more sialic acid groups in addition to glucose, galactose, and galactosamine. (Newburg and Chaturvedi 1992; Vesper et al. 1999; Pfeuffer and Schrezenmeir 2001; Christie 2003; Vanhoutte et al. 2004; Yang et al. 2004). After milk secretion and milking, compositional and structural changes in the MFGM occur and membrane material is shed into the skim milk phase. Factors like temperature, age, bacteriological quality, stage of lactation, and season influence these changes (Evers 2004).

The polar lipid content of raw milk is between 9 and 36 mg/100 g^{-1}. The major milk phospholipids are PE (20–42%, w/w), PC (19–37%, w/w), PS (2–10%, w/w), and PI (1–12%, w/w). The major milk

sphingolipids are glucosylceramide (GluCer) (2–5%, w/w), lactosylceramide (LacCer) (3–7%, w/w), and SM (18–34%, w/w) (Weihrauch and Son 1983; Zeisel et al. 1986; Souci et al. 2000; Fagan and Wijesundera 2004; Avalli and Contarini 2005; Rombaut et al. 2005). Newburg and Chaturvedi (1992) reported glycosphingolipid composition of bovine milk to be 0.7 mg GluCer and 1.7 mg LacCer 100 g^{-1} raw milk (using a conversion factor of 713 and 960 g/mol^{-1}, respectively). The polar lipid contents of various dairy products are listed in Table 5.12 (Rombaut et al. 2006). Variations can be due to fractionation of polar and neutral lipids on processing. Mechanical treatments like heating (Kim and Jimenez-Flores 1995; Lee and Sherbon 2002; Ye et al. 2002), homogenization (CanoRuiz and Richter 1997), aeration, and agitation (Evers 2004) can seriously enhance MFGM release into the serum phase of milk. Upon destabilization of the fat globule, like in churning, the membrane fraction is recovered in the buttermilk (Rombaut et al. 2006). Variations in the polar lipid content of raw milk can be ascribed to environmental factors such as breed of the animal, stage of lactation, season of the year, age, feeding of the cow and treatment of the milk (Christie et al. 1987; Keenan et al. 1988; Bitman and Wood 1990; Puente et al. 1996).

Gangliosides

Gangliosides (GS) are a different class of sphingolipids, which contain one to several sialic acid moieties and include species that differ from each other mainly by the nature of their ceramide moieties. The

Table 5.12. Polar lipid contents of different dairy products (Rombaut et al. 2006)

Sample	Polar Lipids ($mg/100\,g^{-1}$ Product)	Polar Lipids ($g/100\,g^{-1}$ Dry Matter)	Polar Lipids ($g/100\,g^{-1}$ Total Lipids)	Sphingolipids ($mg/100\,g^{-1}$ Product)
Butter	181	0.22	0.22	65
Butter	230	0.26	0.27	71
Buttermilk	91	1.15	21.85	19
Buttermilk (acid)	160	2.03	33.05	31
Buttermilk (reconstituted)	130	1.44	21.66	
Buttermilk Quarg	310	1.86	29.06	74
Buttermilk whey	100	1.84	23.66	19
Buttermilk whey (rennet)	104	1.55	—	
Butter serum[a,b]	660		14.8	—
Butter serum[b]	1250	11.54	48.39	379
Cheddar	153	0.25	0.47	39
Cottage cheese	376	—	5.30	139
Cream	190	0.4	0.45	49
Cream (centrifuged)	95.76		0.53	19.54
Cream (natural)	189.20		0.86	54.11
Quarg	32	0.25	24.66	10
Skimmed milk	20	0.28	19.06	6
Skimmed milk powder		—		14.4[c]
Swiss cheese				8.42[c]
Whey (cheddar)	18	0.26	5.32	5
Whey (Emmenthal)[a]	22	0.33	45.21	3
Whole milk[a]	14.48			4.89
Yogurt				2.81[c]

[a]Conversion factor of 1 g/mL^{-1} was used.
[b]The aqueous phase of butter.
[c]A conversion factor of 751 g/mol^{-1} was used.

backbone in a GS is a hydrophobic acylsphingosine (that is, ceramide), to which are attached hydrophilic oligosaccharide units containing sialic acid. The amino group in sphingosine species is acylated with a fatty acid, in which the chain length and unsaturation degree are the main variables (Laegreid et al. 1986). Gangliosides are abundant in the plasma membrane of nerve cells and other mammalian cells, interact with a variety of biologically active factors, and may play a role in signal transmission and cell-to-cell communication. Different gangliosides are present in bovine milk, some in trace amounts. The ganglioside content of bovine milk, of which monosialoganglioside 3 (GM3) and disialoganglioside 3 (GD3) are the predominant ones, varies between 0.1 and 1.1 mg/100 mL^{-1} (Rueda et al. 1998; Pan and Izumi 2000; Jensen 2002).

Buffalo milk is reported to comprise gangliosides that are not contained in bovine milk, such as gangliosides that belong to the GM1-class. Furthermore, buffalo milk is found to contain unknown gangliosides, denoted as ganglioside "F" and "L" (Berger et al. 2005). Buffalo milk gangliosides, surprisingly, are found in fractions of isolation procedures that were so far not considered to comprise gangliosides. Milk and milk serum from buffalo, as derived from mozzarella cheese production, contain specific gangliosides in the same amounts as human milk, which makes them suitable for humanization of infant formula. A side product of Italian production of buffalo mozzarella and ricotta, which is decaseinated milk serum, is that they contain high levels of GS, especially after simple delactosing procedures.

Health Benefits

Sphingolipids are biologically highly active through their metabolites: ceramide, sphingosine, and sphingosine phosphate (U.S. Patent 2005). These compounds are secondary messengers involved in transmembrane signal transduction and regulation, growth, proliferation, differentiation, and apoptosis of cells. They play a role in neuronal signaling and are linked to age-related diseases, blood coagulation, immunity, and inflammatory responses (Cinque et al. 2003; Kester and Kolesnick 2003; Colombaioni and Garcia-Gil 2004; Deguchi et al. 2004; Pettus at al. 2004; Radin 2004). All organs appear to be capable of de novo sphingolipid biosynthesis, and there is no evidence that consumption of dietary sphingolipids is required for growth under normal conditions (Vesper et al. 1999). Upon digestion, sphingolipids undergo sequential cleavage to ceramide and sphingosine in the regions of the small intestine and colon, and these are subsequently absorbed by intestinal cells and degraded to fatty acids or reincorporated into sphingolipids (Schmelz et al. 1994). However, not all of the ingested sphingolipids are absorbed. As such, these biologically active compounds might exert an effect on colon cancer cells, in which normal growth is arrested and apoptosis delayed. In tests on mice in which tumorgenesis was chemically induced by a chemical agent, or caused by an inherited genetic defect, sphingolipids were found to inhibit both the early and the late stages of colon carcinogenesis, even at concentrations between 0.025 and 0.1 g/100 g^{-1} of the diet. Furthermore, there was a significant shift in tumor type, from the malignant adenocarcinoma to the more benign adenomas (Schmelz et al. 1996; 2000; Schmelz 2004; Symolon et al. 2004). The effects of sphingolipids were found to be chemopreventive as well as chemotherapeutic on tumors in mice (Lemonnier et al. 2003). The concentrations of sphingolipids used in these tests (0.025–0.5% of the diet) were close to the estimated consumption in the United States (Vesper et al. 1999). Hertervig et al. (1997) found a decrease of sphingomyelinase activity in human colon adenomas and carcinomas of 50% and 75%, respectively. Similar findings were reported for patients with chronic colitis, who had an increased risk of developing colorectal cancer (Sjoqvist et al. 2002). These studies demonstrate the importance of sphingolipid-rich foods or supplements in the prevention of colon cancer and bowel-related diseases.

Sphingolipids are also involved in the intestinal uptake of cholesterol. In rat experiments, supplementation with 0.1%, 0.5%, and 5% milk SM to the feed resulted in a 20%, 54%, and 86% reduction of cholesterol absorption, respectively (Eckhardt et al. 2002). This decrease was found to be higher for SM from milk than from other sources (Noh and Koo 2003, 2004). There was a mutual effect on absorption because 38% of sphingolipid metabolites were recovered in the feces in the presence of cholesterol, while only 16% were found in the absence of cholesterol. These effects were attributed to the fact that SM, which shows a high affinity for cholesterol, decreases its micellar solubilization, thereby

decreasing the cholesterol monomers for uptake by enterocytes. A moderate daily intake of SM can lower cholesterol absorption in humans, reduce serum LDL (low-density lipoprotein), elevate HDL (high-density lipoprotein), and may inhibit colon carcinogenesis (Noh and Koo 2004). Many bacteria and viruses use glycosphingolipids to bind to cells (Karlsson 1989). It is plausible that food sphingolipids can compete for and act as cellular binding sites. This can cause a shift in the bacterial population of the colon. Rueda et al. (1998) reported that newborn infants, given an infant formula supplemented with gangliosides, had significantly fewer *Escherichia coli* and more *bifidobacteria* in feces than the control group. Sprong et al. (2001) also noted in vitro bactericidal effects of sphingolipid products on pathogenic bacteria. Italian buffalo milk–derived GM1 (gangliosides) species bound cholera toxin, and GM3 species from the same source bound rotavirus particles. Moreover, lipophilic GS isolated from Italian buffalo milk had anti-inflammatory effects. Positive effects were found in clinical trials with patients suffering from Alzheimer's disease, however, at elevated doses of 200 mg/day^{-1} (Pepeu et al. 1996; McDaniel et al. 2003). More positive health effects were reported by Kidd (2002), Blusztajn (1998), and Spitsberg (2005). Because of their origin and amphiphilic nature, dairy polar lipids, as well as the MFGM proteins, have good emulsifying capacities. MFGM isolates can be used as an emulsifier or fat replacer in products like mayonnaise, margarine, recombined butter, instant milk powder, cosmetics and pharmaceuticals (Correding and Dalgleish 1997; Roesch et al. 2004).

Medium-Chain Triglycerides

The term medium-chain triacylglycerides (MCT) refers to mixed triacylglycerides of saturated fatty acids with a chain length of 6–10 carbons, i.e., hexanoic acid (C6:0, common name *caproic acid*), octanoic acid (C8:0, common name *caprylic acid*), and decanoic acid (C10:0, common name *capric acid*). Sometimes, dodecanoic acid (C12:0, common name *lauric acid*) is included. In the 1950s, MCTs were introduced as a special energy source within a variety of clinical nutrition settings, including pancreatic insufficiency, fat malabsorption, impaired lymphatic chylomicron transport, severe hyperchylomicronemia, and total parenteral nutrition. MCTs are also being used in preterm infant formulae. Since 1994, the use of MCTs in food products is generally recognized as safe (GRAS status) by the U.S. Food and Drug Administration (Traul et al. 2000).

In buffalo milk C6:0–C10:0 may average 4% of all fatty acids, and 2% for C12:0, while bovine milk may have 4–12% and 2–5%, respectively, varying with genetics, stage of lactation, and feeding regimens (Sahai 1996; Jensen 2002). Compared with triglycerides containing mainly saturated long-chain fatty acids, MCTs have a lower melting point, have smaller molecule size, are liquid at room temperature, and are less energy dense (8.4 versus 9.2 $kcal/g^{-1}$). These distinct chemical and physical properties affect the way MCFAs are absorbed and metabolized. Intraluminal hydrolysis of MCTs is faster and more efficient than hydrolysis of long-chain triglycerides (LCT). Likewise, absorption of MCFAs is faster and more efficient than that of long-chain fatty acids (LCFAs). MCFAs stimulate cholecystokinin secretion, bile phospholipid and cholesterol secretion less than LCFAs.

Health Benefits

In patients with pancreatic insufficiency, steatorrhea was significantly lower during a 5-day intake of a diet supplemented with MCT oil compared with a diet supplemented with LCTs (butter fat) (Caliari et al. 1996). In contrast to other dietary fats, the majority of absorbed MCFAs are transported via the portal vein directly to the liver to enter the energy metabolism, whereas LCFAs are incorporated into chylomicron triglycerides and reach the systemic circulation via the lymph system to adipose tissues (Bach and Babayan 1982). This gives MCT a distinctly more beneficial role in human nutrition and health as compared to LCFA and is the reason for their clinical application in many disease conditions of infants and adults (Babayan 1981).

Trans Fatty Acids

Trans fatty acids (TFAs) in food attracted attention due to their potential adverse effects on human health. In hydrogenated vegetable oils the TFA content varies widely and may account for up to 60% of all fatty acids, whereas the TFA content of

beef and dairy lipids only accounts for up to 5% of the total fatty acids (Stender and Dyerberg 2003).

In dairy fat, vaccenic acid (VA, short name *t*11–18:1 or 18:1*t*, *n*-7) accounts on average for 48% of all *trans*-18:1 isomers, but may be much higher depending on feeding conditions (Kraft et al. 2003). There is no *trans*-18:3 isomer and only traces of *trans*-16:1. In their study Wolff et al. (2000) found, that *t*9–18:1, elaidic acid (EA), is the predominant *trans*-18:1 isomer in PHVO (partially hydrogenated vegetable oils), with a wide range of 15–46% (mean, 28%). The *t*10–18:1 isomer ranked second (mean, 21%), and VA represented, on average, 13%. Both animal and hydrogenated vegetable fats contain also *trans* isomers of linoleic acid (e.g., *t*9, *t*12–18:2, linolelaidic acid) to a varying degree. *Trans*-18:3 isomers are found in some PHVOs and may also be formed during deodorization of oils rich in linolenic acid (18:3, *n*-3). Different TFAs, depending on chain length or position of double bond(s), differ in their effect on lipoprotein cholesterol levels in humans (Almendingen et al. 1995; Vermunt et al. 2001; Parodi 2004). Consumption of dairy products (milk, yogurt, and butter) prepared from milk of rapeseed cake–fed cows, as compared with control products, decreased LDL, increased HDL, and thus reduced the ratio of LDL/HDL cholesterol significantly in human subjects (Seidel et al. 2005). However, in this study the enriched milk fat contained also less saturated and more mono- and polyunsaturated fatty acids. Part of dietary VA is converted into *c*9, *t*11-CLA in humans, on average 19% (Turpeinen et al. 2002). Therefore this CLA isomer needs to be taken into account when assessing health aspects of VA. In animal models, *c*9, *t*11-*CLA* showed antiinflammatory (Changhua et al. 2005), antiatherosclerotic (Kritchevsky et al. 2004), and anticarcinogenic (Ip et al. 2002) properties. There was an anticarcinogenic effect of VA in rats, which seemed to be due to the conversion of VA to c9, t11-CLA (Banni et al. 2001; Corl et al. 2003; Lock et al. 2004). Also, *c*9, *t*11-CLA had different effects on lipid metabolism from *t*10, *c*12-CLA and did not induce insulin resistance in mice (Roche et al. 2002). *c*9, *t*11-CLA also did not impair fatty acid metabolism and insulin sensitivity in human preadipocytes (Brown and McIntosh 2003). In man, a *c*9, *t*11-CLA–rich preparation–as compared with *t*10, *c*12-CLA–reduced glucose and triglyceride levels and improved the LDL/HDL cholesterol ratio (Tricon et al. 2004a,b; Risérus et al. 2004; Pfeuffer and Schrezenmeir 2006).

MILK MINERALS

Many of the nutrients and food components in the human diet can potentially have a positive or negative impact on bone health. These dietary factors range from inorganic minerals (including "milk minerals") through vitamins to macronutrients, such as protein and fatty acids (Cashman 2004). Formulation of dietary strategies for prevention of osteoporosis requires a thorough knowledge of the impact of these dietary factors on bone, individually and in combination. The mineral content of milk is not constant but is influenced by a number of factors such as stage of lactation, nutritional and health status of the animal, and environmental and genetic factors. Reported values in the literature for the concentration of many minerals and trace elements show a wide variation due to these factors (Anderson 1992).

Buffalo milk has been found to contain more minerals than cow milk (Table 5.13). Contents of macrominerals and selected trace elements in dairy products have been published by Cashman (2002a,b). The chemical form in which a macromineral and

Table 5.13. Average concentrations of major minerals and trace elements in buffalo and cow milk (Sahai 1996)

Mineral/Trace Element	Concentration (mg/100 mL^{-1})	
	Buffalo Milk	Cow Milk
Calcium	183.9	123
Magnesium	19.02	12
Sodium	44.75	58
Potassium	101.6	141
Phosphate	88.74	95
Citrate	177.6	160
Chloride	63.82	119
Boron	0.052–0.145	0.027
Cobalt	0.00069–0.00161	0.0006
Copper	0.007–0.021	0.013
Iron	0.042–0.152	0.045
Manganese	0.0382–0.0658	0.022
Sulphur	15.700–31.400	30
Zinc	0.147–0.728	0.390

trace element is found in milk or in other foods and supplements is important, because it will influence the degree of intestinal absorption and utilization, transport, cellular assimilation, and conversion into biologically active forms, and thus bioavailability.

Calcium

Of the 20 essential minerals, calcium (Ca) is surely the "milk mineral" that most people would associate with bone health. It is present in milk in relatively high levels such that 200 mL milk (a typical serving size) provides about 22% of the current U.S. RDA (1000 mg/day^{-1} for 19–50-year-olds; Institute of Medicine 1997). Buffalo milk contains about 180 mg/100 mL. Milk and yogurt contribute about 35% to the mean daily intake of Ca in Irish adults (Hannon et al. 2001). The role of Ca in supporting normal growth and development of the skeleton as well as its maintenance during later life is well established (Cashman 2002c; European Commission 1998). Intervention and cross-sectional studies have reported positive effects of Ca on bone mass, mineral content, and density in children, adolescents, adults, and the elderly (Institute of Medicine 1997; Cashman and Flynn 1999; Shea et al. 2004; Xu et al. 2004). Bonjour et al. (2001) suggested that some differences exist in the pharmacodynamic properties of Ca salts in the metabolism of growing bone, and they proposed that Ca phosphate, as present in milk, might have additional anabolic properties not shared by other Ca salts.

Besides the amount of Ca in the diet, the absorption of dietary Ca from foods is also a critical factor in determining the availability of Ca for bone development and maintenance (Cashman 2002c). Although Ca bioavailability from milk and dairy products is about 30%, this is higher than that from plant-based foods and supplements (Weaver et al. 1991). Priyaranjan et al. (2005) observed that the absorption and retention of Ca was much higher for the organic than for the inorganic salts of Ca in fortified buffalo milk (Table 5.14). A number of individual milk components, such as lactose, lactulose, casein phosphopeptides, and vitamin D are enhancers of calcium absorption (Scholz-Ahrens and Schrezenmeir 2000). Recent evidence from tissue culture and animal studies suggests that dairy fatty acids and CLA can aid in Ca absorption (Kelly et al. 2003; Jewell et al. 2005) and reduce the rate of bone resorption in ovariectomized rats (Kelly and Cashman 2004).

Phosphorus

The current recommendation of dietary phosphorus (P) intake by adults is about 700 mg/day^{-1} for 19–50-year-olds (Institute of Medicine 1997). Although P is an essential nutrient, there is concern that excessive amounts may be detrimental to bone, especially when accompanied by low Ca consumption. A rise in dietary P intake increases serum P concentration, producing a transient fall in serum-ionized Ca and resulting in elevated parathyroid hormone secretion and potential bone resorption (Katsumata et al. 2005; Huttunen et al. 2006). Milk contains significant levels of P (200 mL milk can provide about 25% of the current U.S. RDA) (Hannon et al. 2001). The ratio of P:Ca in milk is approximately 0.8:1.

Magnesium

The level of magnesium (Mg) in milk is such that 200 mL can provide about 12% of the U.S. RDA

Table 5.14. Bioavailability of calcium from fortified buffalo milk (Priyaranjan et al. 2005)

		Absorption		Retention	
Sample	Intake (mg)	mg	%	mg	%
Buffalo Milk	193.89 ± 13.26	97.33 ± 1.94	50.19	94.06 ± 1.47	44.48
Fortified Buffalo Milk					
Calcium chloride	255.88 ± 0.89	117.67 ± 1.97	45.98	114.07 ± 2.34	44.58
Calcium lactate	230.24 ± 18.26	123.92 ± 3.11	53.82	121.70 ± 3.12	52.86
Calcium gluconate	208.84 ± 17.60	145.62 ± 4.14	69.73	141.46 ± 3.64	67.74

Data are represented as means ± SEM (n = 5).

(310–320 and 400–420 mg/day^{-1} for 19–50-year-old women and men, respectively; Institute of Medicine 1997). Mg deficiency is a possible risk factor for osteoporosis in humans (Rude 1998; Tucker et al. 1999). Mg supplementation (6 months at 750 mg/day^{-1} followed by 250 mg/day^{-1} for 18 months) increased radial bone mass in 31 osteoporotic women after 1 year (Stendig-Lindberg et al. 1993).

Sodium

Increasing sodium chloride (NaCl) intake increases urinary calcium excretion. A number of studies suggest that increasing Na intake (within the range of 50–300 mmol/day^{-1}) can increase bone resorption in postmenopausal women, even when Ca intake is adequate, due to maladaption of Ca absorption to Na-induced calciuria (Doyle and Cashman 2004; Harrington and Cashman 2003). Two hundred mL of milk can contribute about 7% of the new U.S. Adequate Intake value for Na (1500 mg/day^{-1} for l8–50-year-olds; Institute of Medicine 2004).

Potassium

There has been increasing interest in the potential beneficial effects of potassium (K) on bone. Alkaline salts of K (e.g., potassium bicarbonate) have been shown to significantly reduce urinary Ca excretion in healthy adults (Morris et al. 1999), even in the setting of a high sodium intake. Alkaline salts of K are both natriuretic and chloruretic and as such can reduce the extracellular volume expansion that occurs with increased salt intake (Sellmeyer et al. 2002). In addition, these salts reduce endogenous acid production and increase blood pH and plasma bicarbonate concentration (Sebastian et al. 1994). Milk (200 mL) can contribute about 6% of the current U.S. adequate intake value for K (4700 mg/day^{-1} for 19–50-year-olds; Institute of Medicine 2004).

Zinc

Although zinc (Zn) is a trace element and is present in low levels in comparison with the macrominerals. 200 mL milk can contribute about 8% U.S. RDA (8 and 11 mg/day^{-1} for 18–50-year-old women and men, respectively; Institute of Medicine 2001). Zn plays an important role in nucleic acid synthesis, transcription, and translation as a cofactor for some of the enzymes involved, and will therefore participate in a broad range of metabolic activities in bone. Alkaline phosphatase (E.C. 3.1.3.1.), which is required for bone calcification, and collagenase (E.C. 3.4.24.3), which is required for bone resorption and remodeling (Swann et al. 1981), are Zn metalloenzymes (International Union of Biochemistry in Enzyme Nomenclature 1978). The skeleton is a major body store of Zn and in humans approximately 30% of total body Zn is found in bone (Moser-Veillon 1995), probably bound to hydroxyapatite (Sauer and Wuthier 1990). Some researchers have found an elevated urinary zinc excretion in osteoporotic women (Herzberg et al. 1990; Szathmari et al. 1993; Relea et al. 1995). Since urinary zinc excretion is almost uninfluenced by variation in diet, urinary zinc excretion may be used as a marker of changes in bone metabolism (Herzberg et al. 1996).

In conclusion, milk and milk products can make an important contribution to the daily intake of essential minerals. Milk-extracted Ca phosphate has a more favorable long-term effect on growing bone than that from other forms of supplemental Ca (Nielsen and Milne 2004). Milk also contains a number of nonmineral bioactive ingredients, proteins, peptides, and CLA that may augment the effect of milk minerals on bone growth and density; much more research is needed, especially concerning buffalo milk.

METABOLIC SYNDROME

Several epidemiological studies have indicated that the consumption of dairy products, especially low-fat products, was inversely associated with body mass index (BMI), blood pressure, plasma lipids, insulin resistance, and type-2 diabetes, but the literature is not unanimous. In a population-based study with >3,000 adults, being overweight was less common in individuals who consumed high quantities of milk products (Pereira et al. 2002). It was concluded that the relationship between dairy or Ca intake and obesity or components of the metabolic syndrome appears to be influenced by gender (Mennen et al. 2000; Loos et al. 2004), age (Dixon et al. 2005), ethnicity and degree of obesity (Loos et al. 2004), and absence of hypercholesterolemia (Dixon et al. 2005). Children who avoided milk consumption not only had lower bone mineral density

and more frequent bone fractures (Black et al. 2002), but also showed a higher prevalence for being overweight (Barba et al. 2005). In a randomized, controlled study of 32 obese young adults, a significant reduction of body fat, trunk fat, and waist circumference was found after 24 weeks on an energy restricted diet containing 400–500 mg Ca (Zemel et al. 2004). This effect was significantly more pronounced when the diet was supplemented with 800 mg Ca from calcium carbonate, and even more significant when the Ca was derived from dairy products.

In the study by Pereira et al. (2002), impaired glucose homeostasis, (fasting insulin >20 $\mu U/mL^{-1}$), hypertension (blood pressure >130/85 mm) and dyslipidemia (tHDL-cholesterol >35 mgd/L^{-1} or triglyceride >200 mgd/L^{-1}) were less common in individuals who consumed high quantities of milk products. In the QUEBEC family study, LDL-cholesterol and ratio of total/HDL-cholesterol were negatively correlated with Ca intakes (Jacqmain et al. 2003). In a clinical trial of 459 adults the effects of a "DASH" diet, which is rich in fruit, vegetables, and low-fat milk products, reduced systolic and diastolic blood pressure more effectively than the fruit and vegetable diet alone (Appel et al. 1997). In a double blind, placebo-controlled intervention study of young female adults, a decline was observed in systolic but not in diastolic blood pressure during a 6-week intervention with normal milk, but the decline was not found with mineral-poor milk (Van Beresteijn et al. 1990).

Dietary Ca may facilitate the loss of body weight and body fat by forming soaps with fatty acids in the gut and thereby lowering the amount of absorbed fat, as seen in human subjects and rats (Denke et al. 1993; Welberg et al., 1994; Papakonstantinou et al. 2003; Jacobsen et al. 2005). A diet with high Ca content caused a reduction of the Ca content of basal adipocytes in transgenic mice, independent of the Ca source (Shi et al. 2001). This was not the case on energy restriction alone. Furthermore, fatty acid synthase (FAS) activity was reduced in a high Ca diet. Lipolysis, as indicated by glycerol release, was stimulated after high dietary Ca intake, especially if Ca was derived from dairy products.

MILK GROWTH FACTORS

Bovine milk and colostrum contains growth factors, hormones and cytokines, which are involved in cell proliferation and differentiation (Gauthier et al. 2006). They are synthesized in the mammary gland, and their concentration is highest in colostrums, gradually decreasing during lactation. Rogers et al. (1996) and Belford et al. (1997) have isolated from whey a cationic-exchange fraction containing growth factors and have demonstrated its stimulating effect on the proliferation of a number of cell lines. Milk-derived growth factors are used in health products for the treatment of skin disorders and gastrointestinal diseases.

The more abundant growth factors in bovine milk and colostrum are insulinlike growth factor (IGF)-I, transforming growth factor (TGF)-$\beta 2$, members of the epidermal growth factor (EGF) family, and basic fibroblast growth factors (bFGF) and (FGF)-2 (Grosvenor et al. 1993; Pakkanen and Aalto 1997), all at levels between 5 and 1,000 ng/mL^{-1}. The concentrations in colostrum are generally higher than in milk. Quantitatively, the relative concentrations of growth factors in milk are IGF-I > TGFβ2 > EGF $\approx$ IGF-II > bFGF (Belford et al. 1997).

The function of members of the EGF family is to stimulate the proliferation of epidermal, epithelial, and embryonic cells. They also inhibit the secretion of gastric acid and promote wound healing and bone resorption (Gauthier et al. 2006). The TGF-β family plays an important role in embryogenesis, tissue repair, formation of bone and cartilage, and the control of the immune system. IGF-I stimulates cellular growth, differentiation, glucose uptake, and synthesis of glycogen. FGF-2 also stimulates proliferation, migration, differentiation of endothelial cells, fibroblasts, epithelial cells, angiogenesis, the synthesis of collagen, fibronectin, and hematopoiesis.

Casein micelles are at the upper limit of MW and could be removed from skim milk without affecting the fractionation of growth factors. Average MW of growth factors is between 6,400 g/mol^{-1} (EGF) and 30,000 g/mol^{-1} (PDGF). However, these MW values do not take into account the occurrence of binding proteins such as the latent TGF-β-binding proteins, and the IGF-binding proteins (IGFPB) (Farrell et al. 2004; Gauthier et al. 2006). The pI values of milk growth factors are between 6.5 (IGF-II) and 9.6 (bFGF and PDGF), except for EGF at 4.8. The neutral-to-alkaline pI values for growth factors are their important characteristics with regards to their extraction from milk. Growth factors can be

separated from whey proteins, which have a pI around 4.8–5.1. Hossner and Yemm (2000) achieved the separation of IGF-I and IGF-II on a 30,000 g/mol^{-1} UF membrane by performing DF at pH 8.0, which allowed the passage of IGF-II to the permeate due to its pI of 7.5.

Milk growth factors have been used to develop therapeutic compositions for wound healing (Ballard et al. 1999; Rayner et al. 2000) and for the treatment of gastrointestinal disorders (Johnson and Playford 1998a,b; Playford et al. 2000). An acid casein extract rich in TGF-β2 for an oral polymeric diet has been produced by Nestle, named CT3211 or Modulen (Francis et al. 1995), and has been effective as treatment for children with active Crohn's disease (Fell et al. 1999; 2000). This extract also improved pathological conditions of inflammatory bowel diseases (Oz et al. 2004; Lionetti et al. (2005).

HORMONES IN MILK AND MILK PRODUCTS

Yaida (1929) and Ratsimamanga et al. (1956) were the first authors to report hormones in bovine milk, essentially steroidic hormones of gonadal and adrenal origins. Since these early findings, a large number of studies, mostly published in the late 1970s and 1980s, have been devoted to the finding and dosage of hormones in cow milk. However, the scientific literature on their concentrations in milk has not progressed, while physiological studies have been more concerned with blood concentrations. Hormones found in milk can regulate, at least temporarily, the activity of endocrine glands until the newborn's hormonal system reaches maturity (Bernt and Walker 1999) (Table 5.15).

Table 5.15. Summary of the main hormones detected in bovine milk (Jouan et al. 2006)

Hormone	Ranges Reported in Bovine Milk
Gonadal Hormones	
Estrogens	5–l0 pg/mL^{-1}
Progesterone	2–20 ng/mL^{-1}
Androgens	0–50 pg/mL^{-1}
Adrenal Gland Hormones	
Glucocorticoids (cortisosterone, cortisol)	0–50 ng/mL^{-1}
5a-androstene-3,17-dione	3 ng/mL^{-1}
Pituitary Hormones	
Prolactin	5–200 ng/mL^{-1}
Growth hormone (GH)	0–1 ng/mL^{-1}
Hypothalamic Hormones	
Gonadotropin-releasing hormone (GRH)	0.5–3.0 ng/mL^{-1}
Luteinizing hormone-releasing hormone (LHRH)	0.5–3.0 ng/mL^{-1}
Thyrotropin-releasing hormone (TRH)	0–0.5 ng/mL^{-1}
Somatostatin	10–30 ng/mL^{-1}
Other Hormones	
Parathyroid hormone-related protein (PH-rP)	40–100 ng/mL^{-1}
Insulin	5–40 ng/mL^{-1}
Calcitonin	700 ng/mL^{-1}
Bombesin (gastrin-releasing peptide)	0.25–450 ng/mL^{-1}
Erythropoietin	n/a[a]
Melatonin	5–25 pg/mL^{-1}

[a]Occurrence in milk suspected but no analytical data available.

Gonadal Hormones

Estrogens

A number of techniques have been used to quantify estrogen content in milk, including colorimetry, spectrofluorometry, gaschromatography, and high-pressure chromatography. The most reliable data are obtained with radioimmunoassay (RIA). Wolford and Argoudelis (1979) determined 1% estradiol (E2), estrone (E1), and estriol (E3) concentrations in milk and in a number of dairy products. Concentrations of E2 were 10–14 pg/mL^{-1} in raw milk and 5–9 pg/mL^{-1} in skim milk; contents of E1 were 6–8 pg/mL^{-1} in raw milk and 9–20 pg/mL^{-1} in skim milk. Approximately 65% of E2 and 80% of E1 can be found in the milk fat fraction. In whey, 48% of E2 and 53% of E1 are bound to proteins. It is likely that these hormones are associated with bovine serum albumin, transported into the mammary gland by plasma serum albumin.

Progesterone

This hormone (Pg) was absent from colostrum. Its presence has been reported in milk approximately

15 days after parturition. Between days 15 and 57 of lactation, Pg concentrations in milk varied in a cyclic manner between 1 and 6 ng/mL^{-1}, were higher in evening milk than in the morning, were higher in cream than in skim milk (Darling et al. 1974), and were higher in milk than in blood plasma (Heap et al. 1973). According to Ginther et al. (1976), whole milk, skim milk, and cream contain 11 ± 6, 4 ± 4, and 59 ± 5 ng/mL^{-1} of Pg, respectively; butter had 133 ± 5 ng/g^{-1}, whole milk powder 98 ng/g^{-1}, and skim milk powder 17 ng/g^{-1} (Hoffmann et al. 1975).

Androgens

Very few studies have been published on androgen content of milk. Testosterone in milk has been determined by RIA after ether extraction and purification on a silica gel. The data varied from nondetectable to 50 pg/mL^{-1} in milk at estrus and l50 pg/mL^{-1} during the luteal phase. The ratio between free and conjugated testosterone was 1:1 (Hoffmann and Rattenberger 1977).

Adrenal Gland Hormones

The occurrence of corticosteroids in cow milk was demonstrated for the first time by Ratsimamanga et al. (1956). According to Gwazdauskas et al. (1977), glucocorticoid concentrations in milk vary between 0.7 and 1.4 ng/mL^{-1}. There is no difference between whole and skim milk. Milk cortisol represents 10–23% and corticosterone 60–90% of their plasma homologs (Tucker and Schwalm 1977). During lactation, glucocorticoid concentrations in milk decrease gradually and significantly. They go from 0.59 ± 0.11 ng/mL^{-1} during the first 2 months, to 0.28 ± 0.04 ng/mL^{-1} between 5–7 months, and 0.25 ± 0.02 ng/mL^{-1} between 9 and 11 months of lactation. This decrease is related to changes in cortisol concentrations, while corticosterone concentrations slightly increase during the same period. Contrary to estrogens, glucocorticoids are not concentrated in cream. Although 71–89% of the milk estrogens will be found in the cream fraction, it contains only 12–15% of the corticosteroids. In cow milk, corticosteroids are equally distributed between caseins and whey protein fractions.

Pituitary Hormones

Prolactin

Prolactin has been detected first in cow milk using RIA with a double antibody (Malven and McMurtry 1974). Contents varied from 5 to 200 ng/mL^{-1} with average value at 50 ng/mL^{-1}. The bioactivity of milk prolactin has been demonstrated in vitro on explants of mammary glands from pseudopregnant rabbits (Gala et al. 1980). Prolactin concentrations appear to fluctuate seasonally and are higher in summer than in winter. Storage of milk at 4 °C does not affect prolactin concentrations, whereas 59% is lost upon storage at −15 °C. Part of the prolactin content of milk is associated with milk fat globules, since a centrifugal separation of fat also removes 60% of the initial prolactin content of milk (Malven and McMurtry 1974). The prolactin content of colostrum is much higher than that of milk, varying between 500 and 800 ng/mL^{-1} (Kacsoh et al. 1991). Prolactin in milk probably originates from blood plasma. Its biological functions are not clearly established. It could stimulate lactation by a direct action on secretory cells. In the newborn, the permeability of the gastrointestinal tract allows the passage of prolactin from the intestine to the blood. In the adult, prolactin is probably hydrolyzed in the intestine.

Growth Hormone

The growth hormone (GH) or somatotropin in milk was first detected using RIA (Torkelson 1987) at concentrations lower than 1 ng/mL^{-1}. It was also found that GH increased the concentration of insulin-like growth factor-1 (IGF-1) in epithelial cells of the mammary gland of lactating cows (Glimm et al. 1988). In light of extensive use of injections of commercial bovine somatotropin to dairy cattle in recent decades to obtain significant increases in milk yield, it seems to be very important to focus more research on the presence, biological activities and fate of milk GH in the gastrointestinal tract of humans.

Hypothalamic Hormones

Gonadotropin-Releasing Hormones

Gonadotropin-releasing hormone (GnRH) was detected and quantified in cow milk by Baram et al.

1977). Concentrations of GnRH in milk vary between 0.5 and 3 ng/mL^{-1}. GnRH extracted from milk has similar biological activities as the hypothalamic hormone, since it induces the release of LH and FSH from rat pituitary glands incubated in vitro. GnRH content in milk is 5–6 times greater than in blood plasma, but is likely coming from an active transport by the mammary gland. In the newborn, GnRH is absorbed by the intestine in an active form. It may be involved in the masculinization of the male hypothalamus by stimulating androgens secretion that would act on the brain. Gonadotropin-releasing hormone-associated peptide (GAP) was also found in bovine colostrum, using RIA (Zhang et al. 1990). This 56-amino acid peptide has a sequence identical to the C-terminal end of GnRH and could be the precursor of GnRH. GAP concentration in defatted colostrum is 1.5 ± 0.1 pmol/g^{-1}.

Luteinizing Hormone-Releasing Hormone

Luteinizing hormone-releasing hormone (LH-RH) content in milk and colostrum has been determined by RIA, after methanol-acid extraction and HPLC (Amarant et al. 1982). In colostrum, LH-RH concentration is 11.8 ± 0.7 ng/mL^{-1}, whereas in milk it varies between 0.5 and 3 ng/mL^{-1}. Milk or colostrum contents of LH-RH exceed that of blood plasma. LH-RH from milk is biologically active. It induces the liberation of LH from rat pituitary glands incubated in vitro. LH-RH is absorbed in intact and active form by the newborn's intestine. Milk could be considered as a source of LH-RH for the newborn stimulating the secretion of pituitary gonadotropins.

Thyrotropin-Releasing Hormone

Thyrotropin-releasing hormone (TRH) has been measured in milk and colostrum by RIA (Amarant et al. 1982), using a procedure similar to that of LH-RH. TRH concentrations in colostrum and milk are 0.16 ± 0.03 and 0.05 ng/mL^{-1}, respectively. Both colostrum and milk show higher concentrations of TRH than blood plasma. TRH is absorbed in intact and active form by the newborn's intestine. The intestinal hydrolysis of TRH is low during the first 3 weeks after birth. It is possible that TRH modulates TSH secretion in the newborn.

Somatostatin

The occurrence of somatostatin in cow milk has been demonstrated by enzyme immunoassay (EIA) on defatted, decaseinated milk (Takeyama et al. 1990). Somatostatin concentrations in milk vary between 10 and 30 pmol/L^{-1}. It does not seem to be affected by parturition.

Other Hormones

Parathyroid Hormone-Related Protein

Parathyroid hormone-related protein (PTH-rP) is present in cow milk at about 96 ± 34 ng/mL^{-1} (Budayr et al. 1989; Ratcliffe et al. 1990) with no difference between fresh and pasteurized milk. Two biologically active molecular forms (27 and 21 kDa) were detected. The breed of cow seems to have an influence on PTH-rP content; milk from Jersey cows contained 52 ± 5 ng/mL^{-1}, whereas that from Friesians had 41 ± 5 ng/mL^{-1} (Law et al. 1991). PTH-rP content was 89 ± 9 ng/mL^{-1} in low-fat milk, and 118 ± 19 ng/mL^{-1} in skim milk (Budayr et al. 1989; Goff et al. 1991). The physiological functions of PTH-rP have not been clearly established.

Insulin

Insulin content in colostrum is between 0.67 and 5.0 nM, which is a hundredfold higher than the concentration in blood plasma (Ballard et al. 1982). According to Malven (1977), insulin concentrations in milk varied from 37 ± 14 ng/mL^{-1} during the prepartum period to 6 ± 0.6 ng/mL^{-1} after parturition.

Calcitonins

Calcitonin concentrations in human milk have been estimated at 700 ng/mL^{-1} using RIA by Koldovsky (1989). It inhibits the liberation of prolactin.

Bombesin

Bombesin (gastrin-releasing peptide) is a 14 amino acid peptide that is known to influence the gastric hormone secretions following ingestion (Lazarus et al. 1986). Satiety, blood sugar concentrations, gut acidity, and concentrations of some gastrointestinal hormones are known to be influenced by bombesin.

Bombesin has been found in human milk, cows' milk, milk powder, and whey (Lazarus et al. 1986). Concentrations in human, bovine, and porcine milk range from 0.25 to 450 ng/mL^{-1} (Koldovsky 1989).

Erythropoietin

Human milk is known to contain erythropoietin (Grosvenor et al. 1993). It has been suggested that erythropoietin is being transferred from the mother to the offspring by the milk and that it might be able to stimulate erythropoiesis in the offspring (Grosvenor et al. 1993). No analytical data are available for cow or buffalo milk.

Melatonin

Melatonin is a hormone synthesized by the pineal gland in a diurnal pattern reflecting photoperiodicity. Melatonin has been found in human, bovine, and goat milk (Eriksson et al. 1998; Valtonen et al. 2003) at a low concentration (5–25 pg/mL^{-1}). Its concentration in milk shows the diurnal maximal concentration at midnight and minimal concentration at noon, which parallels that of serum. It has been suggested that nighttime milk could serve as a source of melatonin to improve sleep and diurnal activity in elderly people (Valtonen et al. 2005).

Updated data on hormone levels in milk and milk products is needed, especially in light of impressive changes in the genetic background and performance levels of dairy cattle and dairy buffalo in the last decades, as well as in animal feeding, husbandry regimes, and new processes that have emerged in the dairy industry.

VITAMINS

Extensive research in the last decade has suggested that subtle deficiencies in B vitamins may be risk factors for vascular and neurological diseases and cancers (Brachet et al. 2004). A combined deficiency of folate and vitamin B_{12} is associated with neuropsychiatric disorders among the elderly, development of dementia, and Alzheimer's disease (Seshadri et al. 2002). Furthermore, folate, B_6, and B_{12} are factors known to influence homocysteine metabolism. Since an elevated level of plasma homocysteine is considered to be a risk factor for developing cardiovascular disease, the leading cause of mortality in most Western countries, increase in folate intake would be beneficial (Arkbage 2003; Graham and O'Allaghan 2000). Apart from the prevention of cardiovascular diseases, folates have come into focus for their protective role against birth defects—for example, neural tube defects (Berry et al. 1999; Forssen et al. 2000; Molloy 2002). Also, there is growing evidence that a low folate status is linked to an increased cancer risk, particularly colon cancer (Giovannucci et al. 1998; Rampersaud et al. 2002).

Thus, having a proven beneficial effect on human health, B vitamins are included in the list of nutraceuticals (Hugenholtz and Smid 2002; Levy 1998). Dairy products represent the most important application of lactic acid bacteria (LAB) to increase production levels of B vitamins at an industrial scale (Kleerebezem and Hugenholtz 2003). Novel dairy foods, enriched through fermentation using multivitamin-producing starters, can compensate for the B vitamin-deficiencies that are common even in highly developed countries (Sybesma et al. 2004).

Riboflavin deficiency usually occurs in combination with deficiencies of the other water-soluble vitamins. It is often considered as a problem of malnutrition in the developing world, but subclinical deficiency exists in developed countries, especially among the elderly, individuals with eating disorders, alcoholics, and patients with certain diseases (Hugenholtz et al. 2002).

Raw buffalo milk contained more riboflavin, B_6, and folic acid and less thiamin than raw cow milk. Heat treatment of the milk caused the loss of 7–37% of thiamin, 8–35% of B_6, 8–45% of folic acid, and 0.4–4% of riboflavin. Losses of all vitamins were higher in cow milk than in buffalo milk. Losses were lower for pasteurization than by microwave or conventional boiling and in-bottle sterilization (Sharma and Darshan 1998).

Natural fortification of foods, through fermentation, has major advantages over food fortification by the addition of chemically synthesized vitamins. It enables the use of naturally occurring molecules in physiological doses and in an environment most suited for optimum biological activity. This is of importance, especially for dairy products, since some bioactive molecules do not work in isolation, but require their specific binding proteins or other milk proteins for biological activity or for use as chaperones during intestinal transit (Stover and Garza 2002).

NUCLEOTIDES

Nucleotides, nucleosides, and nucleobases belong to the nonprotein-nitrogen (NPN) fraction of milk. The species-specific pattern of these minor constituents in milk from different mammals is unique and confirms their specific physiological role in early life. Nucleosides and nucleobases are the acting components of dietary and supplemented nucleic acid-related compounds in the gut (Schlimme et al. 2000). Due to the bio- and trophochemical properties of dietary nucleotides, the European Commission has allowed the use of supplementation with specific ribonucleotide salts in the manufacture of infant and follow-up formula.

Recent findings on effector properties in human cell model systems imply that modified nucleosides may inhibit cell proliferation and activate apoptosis. Food-derived inducers of apoptosis may be of significance as exogenous anticarcinogens in the control of malignant cell proliferation where the intestinal tract could be the primary target site for a possible selective apoptotic stimulant against malignant cells (Yamamoto et al. 1997; Schlimme et al. 2000; Grimble and Westwood 2001; Sanchez-Pozo and Gil 2002).

CONCLUSION

Milk is a most important source of essential nutrients and valuable bioactive components of great interest to proper nutrition and health of humans, young and adult, as it has also been during evolution for the newborns of buffalo and other mammals. Research into the identification of milk bioactive components and their functions has progressed tremendously in recent years for bovine milk, while this kind of research is just in its beginning for the milk of other dairy animals and humans. Nevertheless, much similarity between components and their functions in bovine and buffalo milk has been demonstrated, which allows the extrapolation from bovine to buffalo milk of many components for their potential nutraceutical applications, at least with some caution.

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6
Bioactive Components in Camel Milk

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INTRODUCTION

Milk is the sole fluid for mammals' neonates because it provides the complete nutritional requirements of each corresponding species. It also contains components that provide critical nutritive elements, immunological protection, and biologically active substances to both neonates and adults. Milk contains factors that have anticariogenic properties such as calcium, phosphate, casein, and lipids (Reynolds and del Rio 1984). As do other biological secretions—saliva, tears, bronchial, nasal, and pancreatic fluids—milk contains minor protective proteins. These are antibodies (immunoglobulins) and non-antibody components, i.e., complements, lysozyme, lactoferrin, lactoperoxidase, xanthine oxidase, and leukocytes. The antibodies are directed against specific antigens; the non-antibody protective proteins augment and complement the antibody mechanisms. The concentration of protective proteins varies according to species and needs of the offspring, and depends on such factors as maturity at birth, rate of growth, digestive system, and environment. What determines the variation in the concentration of the non-antibody protective proteins is not known. Human milk is rich in lactoferrin and lysozyme, while in bovine milk lactoperoxidase and xanthine oxidase are the main protective proteins (Reiter 1985). Camel milk is characterized by higher contents of immunoglobulins, lysozyme, and lactoferrin (El-Agamy and Nawar 2000). In addition to these protective proteins, milk contains caseins, α-lactalbumin (α-La), β-lactoglobulin (β-Lg), proteose-peptone fractions (heat-stable, acid-soluble phosphoglycoproteins), serum albumin, and other minor peptides.

In addition to native protective proteins, other bioactive peptides may be generated from milk proteins through gastrointestinal digestion or during processing via specific enzyme-mediated proteolysis. The resulting active peptides are of particular interest in food science and nutrition because they have been shown to play physiological roles, including opioidlike features, immunostimulating and antihypertensive activities, and the ability to enhance calcium absorption. The main objective of this chapter is to review the unique biological properties of camel milk bioactive components.

CAMEL MILK COMPOSITION AND NUTRITIVE VALUE

Normal camel milk has a very white color and is foamy (El-Agamy 1983). The taste of camel milk is usually sweet, when camels are fed on green fodder, but sometimes salty, due to feeding on certain shrubs and herbs in the arid regions (El-Agamy 1983, 1994a; Indra and Erdenebaatar 1994). All reported data (El-Agamy 2006) show that camel milk seems to be similar to cow milk and not to human milk (Table 6.1). Casein content of camel and cow milk is quite similar; however, the whey protein fraction is higher in camel milk. The ratio of whey protein to casein in camel milk is higher than in cow but lower than in human milk proteins (Table 6.1). This may explain why the coagulum of camel milk is softer than that of cow milk (El-Agamy 1983).

Table 6.1. Relative composition of camel and cow milk in relation to human milk 100% (El-Agamy 2006)

Constituents	Camel	Cow
Total solids	109	108
Fat	141	129
Protein	163	164
Casein	338	354
Whey proteins	74	58
Lactose	72	74
Ash	293	281
Minerals		
Ca	446	424
Mg	303	300
P	624	679
K	249	243
Na	433	446
Zn	98	140
Fe	380	100
Cu	390	75
Vitamins		
Vitamin C	149	77
Thiamin	322	421
Riboflavin	169	888
Niacin	260	37
Pantothenic acid	40	168
Vitamin B_6	473	455
Folacin	80	100
Vitamin B_{12}	400	1000
Vitamin A	54	48
Vitamin E	13	26
Energy (KCal)	107	113
Cholesterol	—	100
Essential amino acid		
Arginine	127	111
Histidine	99	107
Lysine	70	82
Threonine	67	87
Valine	78	93
Phenylalanine	122	108
Methionine	178	144
Leucine	100	98
Isoleucine	126	132
Tryptophan	240	280

Lactose content is slightly higher in camel milk than in cow milk. The ash content in camel milk is similar to that of cow milk. Chloride content of camel milk is higher than that of milk of other species. This may be due to the effect of feeding as well as the types of fodder grazed by camels (El-Agamy 1983; Yagil 1987). The freezing point of camel milk is between –0.57 °C and –0.61 °C (Wangoh 1997). It is lower than that of cow milk (–0.51 to –0.56 °C). The higher salt or lactose content in camel milk may contribute to this result (El-Agamy 1983). The viscosity of Egyptian camel milk was estimated at 2.2 cPas (Hassan et al. 1987), 2.35 cPas (El-Agamy 1983), which is higher than cow (1.7 cPas) and goat (2.12 cPas), lower than sheep (2.48 cPas), and similar to buffalo (2.2 cPas) (Mehaia 1974). The state of fat, i.e., diameter, and ratio of fat to casein can affect the smoothness of the formed curd. The ratio of fat to casein seems to be comparatively higher in camel milk than in cow milk.

Whole camel milk has a maximum buffer index between 0.060 and 0.062 at pH 5.20, while the minimum range is between 0.011 and 0.012 at pH 7.70 to 7.90 (El-Agamy 1983). The corresponding values for cow, buffalo, sheep, and goat milk were 0.034, 0.043, 0.049, and 0.042 at pH 5.20 for their maximum buffer indexes, respectively; their minimum buffer indexes were 0.006 (pH 8.40); 0.007 (pH 8.65), 0.007 (pH 8.45), and 0.006 (pH 8.50), respectively (Mehaia 1974). Other studies revealed that skim camel milk has a maximum buffering capacity at pH 4.95 versus pH 5.65 for skim cow milk (Al-Saleh and Hammad 1992). The differences in buffer capacity of camel milk and that of other species reflect the compositional variations in protein and salt constituents involved in buffering systems.

The concentrations of all major minerals Ca, Mg, P, Na, and K in camel milk seem to be similar to those of cow milk. The concentration of citrate in camel milk (128 mg/100 mL) (Moslah 1994) is lower than in cow milk (160 mg/100 mL). The low level of citrate in camel milk may be its excellent advantage in medicinal properties since lactoferrin (one of the antimicrobial factors) activity is enhanced with low levels of citrate. Camel milk is rich in Zn, Fe, Cu, and Mn and richer in Cu and Fe than cow milk as 0.7–3.7 mg/L (Fe), 2.8–4.4 mg/L (Zn), 0.11–1.5 mg/L (Cu), and 0.2–1.9 mg/L (Mn) (El-Agamy 1983; Gnan and Sheriha 1986; Al-Saleh and Hammad 1992; Gorban and Izzeldin 1997). In cow milk the corresponding values are 0.3–0.8 mg/L, 3.5–5.5 mg/L, 0.1–0.2 mg/L, and 0.04–0.20 mg/L, respectively (Sawaya et al. 1984). The ratio of Ca : P is 1.5 for camel milk versus 1.29 and 2.1 for cow and human

milk, respectively. This ratio is important, since if the cow milk-based formula used for feeding infants contains high phosphate, this may lead to hyperphosphatemia and low serum calcium (Kappeler 1998). Camel milk has a calorific value of 665 KCal/L versus 701 KCal/L for cow milk.

All reported data reveal that camel milk proteins have a satisfactory balance of essential amino acids quality or exceeding the FAO/WHO/UNU requirements (1985) for each amino acid, and have adequate nutritive values for human diets.

In amino acid composition, several differences exist between human and cow milk, which can present problems in feeding cow milk-based formulae to certain infants. Human milk has a high cystine : methionine ratio and some taurine (Sturman et al. 1970). Cow milk has a lower cystine : methionine ratio and essentially no taurine. The human infant's liver and brain have only low levels of cystathionase, the enzyme that converts methionine to cystine (the fetus and preterm infant are completely lacking this enzyme). Cysteine is important for central nervous system development (Sturman et al. 1970). Taurine is made from cystine and is needed for brain and retinal development and function, and the conjugation of bile salts (Sturman et al. 1970; Jelliffe and Jelliffe 1978). The ratio of cystine: methionine is lower in camel milk (0.38) than in cow (0.5) and human (0.6) milk due to the high content of methionine in camel milk proteins. Another amino acid problem in human milk versus cow milk or its formula, is the concentration of phenylalanine and tyrosine, since infants have limited ability to metabolize these amino acids, which can build up and cause phenylalanine ketone urea (PKU babies) (Jelliffe and Jelliffe 1978). Human milk has low levels of both phenylalanine and tyrosine (Jelliffe and Jelliffe 1978). The ratios of phenylalanine to tyrosine are 0.7, 2.7, and 2.5 for human, cow, and camel milk, respectively (El-Agamy 2006).

Camel milk can meet at least as well or better, significant portions of the daily nutrient requirements of humans. A typical serving size of milk is 1 cup with 245 mL content, which is used as a standard in the U.S. for comparison of nutrient intake (Posati and Orr 1976). The minimum daily requirements of 2,300 or 2,200 KCaL for an adult man or woman (Podrabsky 1992) can be met by 14 cups of camel milk. For meeting the needs of proteins, 8 cups of camel milk are sufficient. Because human requirements in the heat of arid lands are based less on calories and more on protein and especially liquid, relatively small amounts of camel milk supply man's needs. For minerals, the minimum daily requirements of calcium or phosphorus (800 mg) can be easily met by 2.5 and 4 cups for Ca and P, respectively. Only 7 cups of camel milk are sufficient to meet for the needs of vitamin C (60 mg). The same is true for meeting the needs of most essential amino acids.

CAMEL MILK PROTEIN STRUCTURE AND FUNCTION

Caseins

The protein fraction of cow milk consists of about 80% of caseins. Four different gene products are designated as α_{s1}-, α_{s2}-, β, and κ-caseins, which together form micellar structures of 20 nm to 500 nm by noncovalent aggregation (Swaisgood 1992). Casein is a phosphoprotein, which precipitates from raw skim milk upon acidification to pH 4.6 at 20 °C.

Size Distribution of Casein Micelles

The casein micelle determines the colloidal stability of the polydisperse system in milk. The dimension and composition of casein micelles are of great importance for the coagulation process. Coagulation time varies with micelle size and reaches an optimum with small and medium-size micelles, which have higher κ-casein contents than the larger micelles (Ekstrand 1980). Smaller micelles give firmer curd than larger micelles at the same casein concentration (Grandison 1986). Published data on the state of the casein micelle structure in camel milk are very scarce. In these studies, different techniques were applied; therefore, the results are contradictory to some extent, but all of them showed that camel milk casein micelle is different from that of cow milk. A study used electron microscopy after solidifying milk with agar. The casein micelle ranged in size from 25 to more than 400 nm, where no clear identification of smaller micelles was specified (Gouda et al. 1984).

Freeze-fractured samples of camel milk were examined by electron microscopy (Farah and Ruegg 1989), which showed that the distribution of casein

micelles is significantly broader than in cow and human milk, with a greater number of large particles. The particles in the lowest-size class with diameters smaller than 40 nm comprise about 80% of the total number of particles but represent only 4–8% of the mass or volume of casein. The volume distribution curve of casein micelles in camel milk is broad and shows a maximum between 260 and 300 nm versus 100–140 nm for cow milk casein. In another study (El-Agamy 1983), the diameter of casein micelle was estimated at 956 Angstrom (Å) (905–1031 Å) versus 823 Å, 801 Å, 716 Å, and 662 Å for buffalo, goat, sheep, and cow milk casein, respectively. This indicates that casein micelles of camel milk are bigger in diameter than those of other species.

Casein Fractionation

Whole camel milk caseins were separated on alkaline native polyacrylamide gel electrophoresis (Fig. 6.1) and compared with those of cow and human milk. Camel milk casein fractions were slower in migration than those of cow and human milk. This feature reveals the differences among the three types of milk caseins in both types and density of the charges (El-Agamy et al. 2006).

Molecular weights of camel α_{s1}-CN and β-CN were estimated at 33 and 29.5 kDa, respectively (El-Agamy et al. 1997). In another study camel milk casein was also fractionated on ion exchange chromatography and fractions were identified by polyacrylamide gel electrophoresis (Larsson-Raznikiewicz, M. and Mohamed, M.A. 1986). Four casein fractions were identified as α_{s1}-, α_{s2}-, β-, and κ-CN. Their corresponding molecular masses were 31, 25, and 27 kDa for α_{s1}-, α_{s2}-, and β-casein, respectively. The study showed also that α_{s1}- and β-casein were dominants, whereas α_{s2}-CN appeared as a diffuse band on the gel. While κ-CN band was absent from the gel, it was isolated by ion exchange and identified by amino acid sequence as homologous to cow milk κ-CN. Furthermore, camel milk α_{s1}- and β-CN were phosphorylated to about the same extent as in cow milk, while α_{s2}-CN was more heavily phosphorylated than that of cow milk casein (Kappeler 1998).

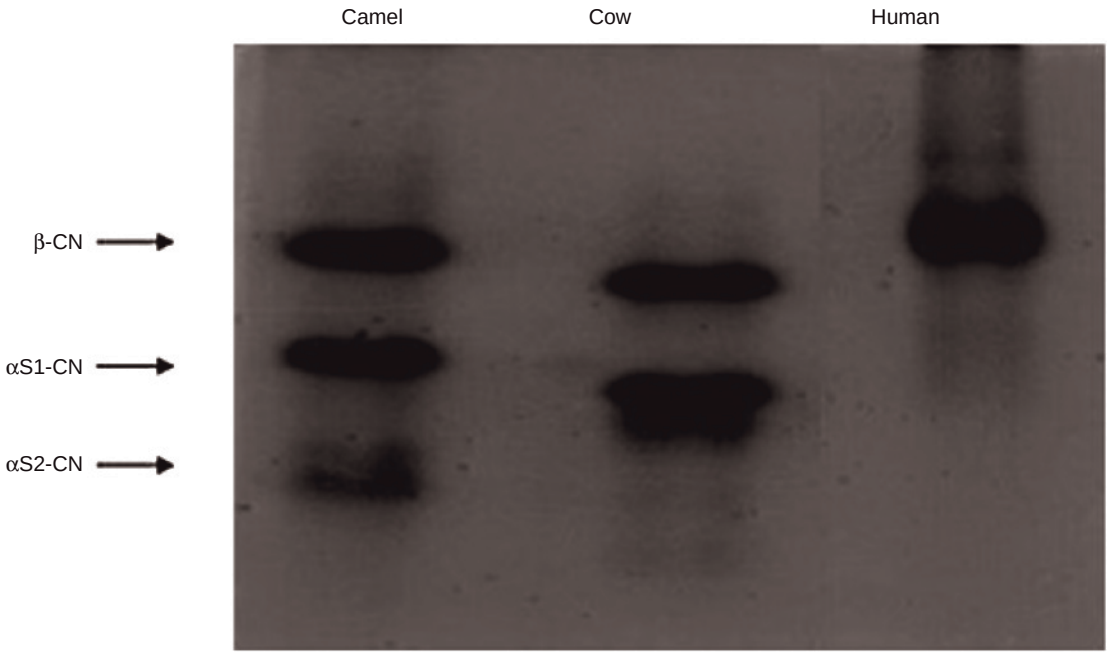

Figure 6.1. Alkaline native-PAGE of acid camel, cow, and human milk caseins. Anode is toward bottom of photo (El-Agamy et al. 2006).

The amino acid compositions of camel milk caseins are similar to cow milk casein fractions (Eigel et al. 1984). Camel milk acid-casein was fractionated on reversed-phase HPLC chromatography (Kappeler 1998). Four fractions were well identified as α_{s1}-CN, α_{s2}-CN, β-CN, and κ-CN in camel and cow milk as shown in Table 6.2. It was found that the ratio of β-CN to κ-CN is lower in camel milk casein than in cow milk. This low ratio affects some of the processing characteristics, heat treatment, and enzymatic coagulation of casein micelles in camel milk. The same study revealed that the pH values of isoelectric points (p^I) of camel and bovine milk caseins were similar.

Primary Structure

Camel α_{s1}-CN and β-CN, similarly to bovine caseins, are devoid of cysteine residues, and α_{s2}-CN and κ-CN both contained only two cysteines. The proline content in camel caseins is slightly higher than in cow caseins, with 9.2% in α_{s1}-CN, 4.5% in α_{s2}-CN, 17.1% in β-CN, and 13.6% in κ -CN, compared to 8.5%, 4.8%, 16.7%, and 11.8% in cow caseins, respectively. This higher proline content in camel caseins may lead to destabilization of secondary structures in a more pronounced manner than it does in cow milk caseins (Kappeler 1998).

Secondary Structure

Limited pronounced structural differences were found between camel and cow milk caseins when sequence comparison was made. Although α_{s1}-CN of camel and cow milk had a low percentage similarity in primary structure, similarities in the secondary structure (a series of α-helical regions followed by a C-terminus with little defined secondary structure) predominated. In camel milk α_{s1}-CN hydrophilicity of the N-terminal end was slightly more pronounced. Similarly to cow milk, camel α_{s2}-CN was the most hydrophilic among the four caseins and had a high potential for secondary structures, mainly α-helices. The two cysteine residues also occurred at about position 40 (Kappeler 1998).

For camel κ-CN secondary structure, it was found that it is similar to that of cow milk κ-CN, with an N-terminal α-helix containing one cys followed by β-pleated sheets and a second cys. Both cys residues are at the positions similar to those in bovine milk κ-CN.

It is well known that in bovine κ-CN, the site of cleavage by chymosin is Phe^{105}-Met^{106}, leaving a macropeptide of 6.707 kDa, 64 amino acids in length with a *p*I of the unmodified peptide at pH 3.87, and the amino acid sequence from His^{98} to Lys^{112} is involved in binding and cleavage of bovine κ-CN by chymosin (Visser et al. 1987). In camel milk κ-CN, the site of cleavage by chymosin was found to be Phe^{97}-Ile^{98} leaving a macropeptide of 6.774 kDa, 65 amino acids in length with a *p*I of the unmodified peptide at pH 4.13 (Kappeler 1998). As shown below, all protein residues are conserved in camel milk κ-CN and the bovine residue Leu^{103} was replaced by Pro^{95}.

Table 6.2. Physicochemical characteristics of camel and cow milk caseins (Eigel et al. 1984; Kappeler 1998)

Species	Casein Fraction	Molecular Mass (kDa)	*p*I	Relative Amount in Total Casein	Amino Acid Residues
Camel	α_{s1}-CNA	24.755	4.41	22.0%	207
	α_{s1}-CNB	24.668	4.40		
Cow	α_{s1}-CNB	22.975	4.26	38.0%	199
Camel	α_{s2}-CN	21.993	4.58	9.5%	178
Cow	α_{s2}-CNA	24.348	4.78	10.0%	207
Camel	β-CN	24.900	4.66	65.0%	217
Cow	β-CNA_2	23.583	4.49	39.0%	209
Camel	κ-CN	22.294	4.11	3.5%	162
		22.987			
Cow	κ-CNA	18.974	3.97	13.0%	169

Camel: *Arg^{90} – Pro – Arg – Pro – Arg – Pro – Ser – Phe^{97} – Ile^{98} –Ala – Ile – Pro – Pro – Lys – Lys^{104}*

Cow: *His^{98} – Pro – His – Pro – His – Leu – Ser – Phe^{105} – Met^{106} – Ala – Ile – Pro – Pro – Lys – Lys^{112}*

This additional proline residue is suggested to help the stabilization of the conformation of κ-CN in the active site cleft by camel chymosin and different to the conformation by cow milk κ-CN in the cleft of bovine chymosin. Moreover, histidine residues in the sequence His^{98} to His^{102} of cow milk κ-CN are replaced by more basic arginine residues in camel milk κ-CN. This leads to the fact that camel milk κ-CN backbone does not need to be bound as tightly to chymosin as it was shown for cow milk κ-CN (Plowman and Creamer 1995).

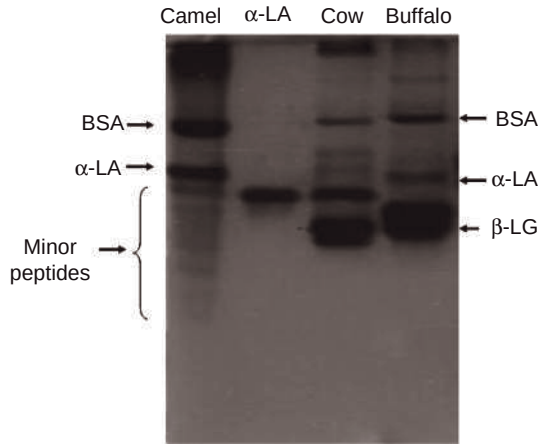

Figure 6.2. Polyacrylamide gel electrophoresis (alkaline native- PAGE), 12.5% T of camel milk whey proteins compared with those of other species. Anode is toward bottom of photo (El-Agamy et al. 1997).

Whey Proteins

It is well known that the major whey proteins of bovine milk are β-LG with 55% of total whey protein, α-LA with 20.25%, and BSA with 6.6%. Other minor whey proteins as immunoglobulins and proteose peptone were also well characterized (Butler 1983). Camel milk whey proteins (CMWPs) were isolated and well characterized by chromatographic, electrophoretic, and immunochemical analyses (Beg et al. 1985, 1986a, 1987; Conti et al. 1985; Farah 1986; El-Agamy et al. 1997, 1998a; Kappeler 1998).

CMWPs were fractionated on polyacrylamide gel electrophoresis using alkaline native-PAGE technique and compared with those of cow and buffalo milk (El-Agamy et al. 1997) (Fig. 6.2). Electrophoretic patterns of CMWPs showed a different electrophoretic behavior than those of other species. Camel milk α-LA is slower but BSA is faster in migration than cow and buffalo milk proteins. Similar to human milk, no distinguished band belonging to β-LG was detected in camel milk. This was confirmed by the molecular study (Kappeler 1998). Two different isoforms of camel α-LA were detected (Conti et al. 1985; El-Agamy et al. 1997). Camel milk proteins were characterized by the presence of several minor peptides being lower in molecular weights compared to milk of other species. These peptides may play an important role in the therapeutic value of camel milk (El-Agamy 1983, 2000a; El-Agamy et al. 1997). Kappeler et al. (2003) reported that concentration of α-La in camel milk (3.5 g/L) is closer to that of human milk (3.4 g/L), compared to bovine milk (1.26 g/L). El-Hatmi et al. (2007) recently reported that serum albumin is the major whey protein present in camel milk with an average concentration of 10.8 g/L.

In another study (Beg et al. 1985) the primary structure of camel α-LA was determined by analysis of the intact protein, and of CNBr fragments and enzymatic peptides from the carboxymethylated protein chain. Results showed that camel α-LA has 123 residues and a molecular mass of 14.6 kDa. The amino acid sequence is homologous to other α-LAs, but also exhibits extensive differences: 39 residues differ in relation to the bovine protein and only 35 residues are like other known α-LAs. The molecular mass of CMWPs was estimated at 67, 15, and 13.2 kDa for BSA, α-LA variants A, and B, respectively, versus 66.2 kDa for BSA and 14.4 kDa for α-LA of cow milk (El-Agamy et al. 1997).

Two different unknown proteins were isolated from camel whey having molecular masses of 14 and 15 kDa. Protein of 14 kDa is rich in cysteine/half-cystine; while that of 15 kDa has no cysteine. No obvious structural similarities were noted between these proteins and other known milk proteins (Beg et al. 1984, 1986a, 1987).

Recently, acid whey prepared from camel milk was separated by HPLC analysis. Three peaks were identified by N-terminal sequencing as whey acidic protein, α-LA and lactophorin (Kappeler 1998). Their ratios were 86.6, 11.5, and 1.9% for α-LA, lactophorin, and whey acidic protein, respectively. SDS-PAGE of fractionated proteins showed that BSA and other proteins coeluted with α-LA as minor fractions. Table 6.3 summarizes the physicochemical characteristics of camel milk whey proteins as compared with those of cow milk (Eigel et al. 1984; Hernandez et al. 1990; De Wit and Van Hooydonk 1996; Kappeler 1998). Camel milk lactophorin is a major protein in whey, whereas bovine lactophorin is a minor protein in whey. Camel milk lactophorin was not isolated from proteose peptone component$_3$ (PP_3) as it was in bovine milk protein. Bovine PP_3 consisted of several proteins of which lactophorin was just the main fraction. The protein had 60.4% amino acid sequence identity to a proteose peptone component 3 protein from bovine whey and 30.3% identity to the glycosylation dependent cell adhesion molecule 1 in mice. The N terminal heterogeneity of the protein was a result of alternative mRNA splicing. About 75% of the protein was expressed as a long variant A with 137 amino acid residues and a molecular mass of 15.7 kDa. About 25% was a short variant B with 122 amino acid residues and a molecular mass of 13.8 kDa. Both proteins are probably threefold phosphorylated. In contrast to the related proteins, no glycosylation was found in camel lactophorin. Because of this difference, specific interaction with carbohydrate binding proteins, as reported for the murine protein, can be excluded, and a function of the protein other than cell recognition or rotaviral inhibition is proposed. Pronounced similarities existed between the primary and secondary structures of bovine and camel proteins (Kappeler et al. 1999b). Meanwhile, the percent sequence similarity of camel lactophorin to bovine and caprine lactophorin is much higher than to the rat and murine one (Kappeler 1998).

The concentration of lactophorin in camel milk was found to be about 3 times higher than the concentration of the bovine homologue in bovine milk. This could be of higher potential benefit in milk processing, since lactophorin is an inhibitor of lipase (Glass et al. 1967).

It was reported that whey acidic protein, generally described as a major constituent of rodent milk, and peptidoglycan recognition protein, an intracellular protein binding to Gram-positive bacteria and presently not known to be a milk constituent, were detected in major amounts in camel whey, both on the cDNA and protein level (Kappeler et al. 2003).

It was found that camel milk whey has an acidic protein (12.5 kDa) possessing a potential protease inhibitor (Beg et al. 1986b). Depending on these findings it is suggested that the higher level of natural preserving agents may bring about longer storage or shelf life of raw camel milk as compared with raw cow milk (El-Agamy 1983; Farah 1986).

BIOACTIVE NATIVE PROTEINS IN CAMEL MILK

Immunoglobulins

Structure and Function

Immunoglobulins are known as antibodies, which are found in human or animal body fluids or blood serum due to the immune response from exposure

Table 6.3. Physicochemical characteristics of camel and cow milk whey proteins

Species	Protein	Molecular Mass (kDa)	*p*I	Amino Acid Residues	(mg/L) in Milk	Similarity to Corresponding Cow Milk Proteins
Camel	α-LA	14.430	4.87	123	>5,000	88.5%
Cow	α-LA	14.186	4.65	123	600–1700	
Camel	Lactophorin A	15.442	5.10	137	954	83.6%
Cow	Lactophorin	15.304	6.03	135	300	
Camel	Whey acidic protein	12.564	4.70	117	157	—
Cow	β-LG B	18.281	4.66	162	<4000	

Eigel et al. (1984); Hernandez et al. (1990); De Wit and Van Hooydonk (1996); Kappeler (1998).

to certain antigens, virus, bacteria, etc.; they are polypeptide chains having high molecular weights. Immunoglobulins are classified into five classes: immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA), immunoglobulin D (IgD), and immunoglobulin E (IgE).

The immunoglobulin molecule is composed of four polypeptide chains, two light chains (lambda or kappa) and two heavy chains (alpha, gamma, mu, and delta or epsilon). The type of heavy chain determines the immunoglobulin isotype, IgA (alpha), IgG (gamma), IgM (mu), IgD (delta), and IgE (epsilon). Immunoglobulin classes differ in amino acid composition and sequence as well as molecular weight. IgA is dominant in blood serum, and secretory IgA (sIgA) is dominant in milk. Three classes as IgG, IgA, and IgM are recognized in camel milk (El-Agamy 1989). IgG class is found to have three different subclasses: IgG_1, IgG_2 and IgG_3 (El-Agamy 1989; Hamers-Casterman et al. 1993).

The molecular weights of heavy and light chains of camel immunoglobulins are shown in Table 6.4. Camel immunoglobulins have molecular weights different from those of cow, sheep, goat, mare, buffalo, and human (El-Agamy 2006). Molecular masses of camel IgM heavy and light chains were estimated at 80 and 27 kDa, versus 75 and 22.5 for bovine IgM fragments (El-Agamy 1989). Camel IgA heavy and light chains have 55.5 and 22.5 kDa (El-Agamy 1989), versus 61 and 24 for heavy and light chains of bovine IgA (Butler 1983). Camel milk IgG subclasses were purified and their molecular masses determined (Hamers-Casterman et al. 1993) as 50, 46, and 43 kDa for IgG_1, IgG_2, and IgG_3 heavy chain, respectively. Only IgG_1 has a light chain of 30 kDa; however, IgG_2 and IgG_3 lack the light chain completely in their structure. Although these isotypes are devoid of light chains, they have an extensive antigen-binding repertoire. Camel heavy chain IgGs lack CH1, which in one IgG class might be structurally replaced by an extended hinge, and heavy chain IgGs are a feature of old and new world camelides (Hamers-Casterman et al. 1993). It has been reported that camel IgG2 and IgG3 act as true competitive inhibitors by penetrating the active sites of some enzymes (Lauwereys et al. 1998). The reactivity of camel blood serum or milk IgG to Protein A (El-Agamy 1989; Hamers-Casterman et al. 1993) or Protein G (Hamers-Casterman et al. 1993) was recognized. Secretory IgA and IgM have no affinity to protein A (El-Agamy 1989; Hamers-Casterman et al. 1993).

Table 6.4. Molecular mass (kDa) of immunoglobulins of different species

	Camel[a]		Cow[b]		Buffalo[c]	
Immunoglobulins	H	L	H	L	H	L
IgG(whole molecule)	60	29	55	26	56	28
IgM	80	27	75	22.5	66	33
IgA	55.5	22.5	61	24	58	30
*FSC	78		74		68	

H and L: heavy and light chains, respectively.
*FSC: free secretory component.
[a]El-Agamy (1989).
[b]Butler (1983).
[c]El-Agamy and Nawar (1997).

Content in Colostrum and Normal Milk

The concentration of immunoglobulins in milk varies depending on some factors as stage of lactation, health status of animal, and species. The study of El-Agamy and Nawar (2000) showed that camel milk contains the highest level of total IgG (1.64 mg/mL) versus 0.67, 0.63, 0.70, 0.55, and 0.86 for cow, buffalo, goat, sheep, and human milk, respectively.

The concentrations of IgG subclasses in camel colostrum and normal milk were determined (El-Agamy 1994b). The concentrations of IgG_1 and IgG_2 were high on the first day and declined in the following days (Table 6.5). IgG_1 represented 91.6% of total IgG on the first day, whereas IgG_2 represented only 8.4%. The high level of IgG_1 indicated the dominance of this type of immunoglobulin in camel colostrum, similar to that of bovine colostrum (Butler 1983, Korhonen 1977).

Total immunoglobulins in camel colostrum were determined by SDS-PAGE followed by densitometric analysis as 2.52% and 1.88% of total protein after 2 hours and 24 hours postparturition, respectively (Zhang et al. 2005). In another study (El-Hatmi et al. 2007), the concentration of total IgG in camel colostrum was 101.8 g/L and declined to 47.2 g/L after 24 hours of parturition. IgG_1 concentration represented 42.6% of total IgG in the same samples.

IgG was estimated in camel milk from Kazakhstan, where two species of camels (*Camelus*

Table 6.5. The average concentration of immunoglobulin subclasses in camel colostrum and normal milk

Days Postpartum	IgG1 (mg/mL)		IgG2 (mg/mL)	
	Range	Mean	Range	Mean
1	(25.6–84.3)	53.80	(1.8–6.0)	4.94
2	(18.7–70.9)	36.67	(1.3–5.0)	3.68
3	(13.1–40.1)	23.69	(0.9–2.8)	1.83
4	(7.0–27.2)	14.23	(0.5–1.9)	0.95
5	(4.8–17.4)	08.55	(0.3–1.2)	0.62
6	(4.3–12.0)	07.15	(0.3–0.9)	0.49
7	(4.1–10.1)	06.02	(0.3–0.7)	0.40
14	(1.0–02.3)	01.35	(0.1–0.2)	0.11

Adapted from El-Agamy (1994b).

bactrianus, *Camelus dromedarius*) and their hybrids cohabit. The concentration of IgG was determined according to three variation factors: region, season, and species. The mean values for IgG in raw camel milk was 0.718 ± 0.330 mg/mL. The seasonal effect was the only significant variation factor observed, with the highest values in the winter for IgG. The IgG concentration varied from 132 to 4.75 mg/mL through the first week after parturition (Konuspayeva et al. 2007).

Lactoferrin

Structure and Function

Lactoferrin, also named *lactotransferrin*, is a glycoprotein. It belongs to the family of transferrins, together with blood serotransferrin (siderophilin), egg white ovotransferrin (conalbumin), melanotransferrin of malignant melanomas, the porcine inhibitor of carbonic anhydrase and other proteins. The common property of this protein family is the binding of two metal cations, preferably (Fe^{3+}), at structurally closely related binding sites. Most lactoferrins are needed for storage or transport of iron. Lactoferrin was discussed to serve for iron scavenging in body secretions (Brock 1997). It is found in milk, different other body secretions, and neutrophil leukocytes (Masson 1970). Camel milk lactoferrin was found to contain 6.2% carbohydrates in colostral milk and 5.6% in milk collected 15 to 30 days postpartum (Mahfouz et al. 1997). The content of N-acetylglucosamine in camel milk lactoferrin was markedly higher than in other ruminants' milk lactoferrins (3.35% in colostral camel milk compared to about 1.75% in other colostral ruminants' milk). The carbohydrate content of camel lactoferrin from end-lactational milk is 6.2–6.8% of total protein mass (Kappeler 1998). Lactoferrin of colostral camel milk has a low iron saturation of 9% similar to lactoferrin of bovine colostral milk. In milk taken 15 to 30 days after parturition, camel lactoferrin was nearly completely iron saturated. Similar results were found for bovine lactoferrin from the milk of the same lactational stage (Mahfouz et al. 1997).

Lactoferrin was purified from camel milk, and its molecular weight was determined as 79.5 kDa (El-Agamy et al. 1996) and 75.3 kDa (Kappeler 1998). The corresponding molecular weights of cow, buffalo, and human lactoferrins were determined as 80 or 89 (Spik et al. 1994; Yoshida and Ye-Xiuyun 1991), 78.5 (Nawar 2001), and 82 kDa (Spik et al. 1994), respectively.

PCR amplification products of a full-length cDNA clone of camel lactoferrin were sequenced (Kappeler et al. 1999a). The study showed that the clone is 2336 bp long and contains a 5′-untranslated region of 21 bp and a 3′-untranslated region of 191 bp. The mature lactoferrin was 689 amino acids residues long, and the unmodified peptide had an isoelectric point of 8.14. Camel lactoferrin shares 91.6% sequence similarity with bovine or human lactoferrin and 91.3% with porcine lactoferrin. The high similarity in primary structures among lactoferrins of the three species indicates small variations in functional aspects of them.

Several studies showed that lactoferrin concentration in camel milk varies widely in normal milk from 0.02 to 7.28 mg/mL (El-Agamy 1994b; El-Agamy et al. 1996; Abd El-Gawad et al. 1996; Kappeler et al. 1999a; Zhang et al. 2005; El-Hatmi et al. 2007). This variation is mainly due to differences in lactation period, feeding regimen, number of analyzed samples, breeds, and methods of analysis. Comparative study on lactoferrin content in camel, cow, buffalo, sheep, goat, donkey, mare, and human normal milk was done (El-Agamy and Nawar 2000). The study showed that lactoferrin concentration varied considerably. The highest level was in human milk (1.7 mg/mL), while donkey milk had the lowest content (0.07 mg/mL). Camel milk contained a higher ($P \leq 0.01$) level (0.22 mg/mL) of

lactoferrin compared with other species except human milk. Lactoferrin in camel milk represents 2.44, 2.59, 2.20, 1.75, 3.33, and 2.27 times the lactoferrin in cow, buffalo, goat, sheep, donkey, and mare milk, respectively. Other studies showed that the concentration of lactoferrin in bovine milk ranged from 0.02–0.35 mg/mL (Korhonen 1977) and 0.10–0.50 mg/mL (Persson 1992).

Colostral camel milk was reported to have high lactoferrin content of 5.1 mg/mL on the second day after parturition compared to about 0.5 mg/mL in bovine colostral milk. After 30 days of parturition, the lactoferrin level in camel milk declined to 0.34 mg/mL, while in bovine milk it was 0.06 mg/mL (Abd El-Gawad et al. 1996). In another study a camel milk sample taken at the end of the lactation period, 360 days after parturition, contained 0.22 mg/mL (Kappeler 1998). Changes in lactoferrin in camel colostrum and normal milk revealed that the concentration of lactoferrin was highest in the first day and then decreased with milking progress (El-Agamy 1994b), which was similar to a pattern found in bovine milk (Korhonen 1997). The study of El-Hatmi et al. (2007) showed that the maximum level of lactoferrin (2.3 g/L) was observed at 48 hours after parturition.

Lactoferrin was estimated in camel milk from Kazakhstan, where two species of camels (*Camelus bactrianus*, *Camelus dromedarius*) and their hybrids cohabit. Concentrations of lactoferrin were determined according to season and species. The lactoferrin in raw camel milk was 0.229 mg/mL. The seasonal effect was the only significant variation factor observed, with the highest values in the spring. The lactoferrin concentration in the first week after parturition milk ranged from 1.422 to 0.586 mg/mL (Konuspayeva et al. 2007). An immunochemical study (El-Agamy et al. 1996) on camel lactoferrin showed no antigenic relationship with bovine lactoferrin when anticamel lactoferrin was used in immunodiffusion analysis.

Indigenous Enzymes

Lysozyme (EC 3.2.1.17)

Lysozyme cleaves beta (1–4) glycosidic bonds between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan, the constituent of bacterial cell walls. There are two types of lysozymes: those found in hen egg white and known as chick-type (c) lysozyme and those found in goose egg-whites or goose-type (g) lysozyme (Arnheim et al. 1973). Lysozymes c and g differ in their amino acids sequence and molecular weights. Lysozyme g is heat-labile and contains half as much cystine and tryptophan residues as lysozyme c (Arnheim et al. 1973). Lysozyme is found also in secretions of milk, tears, nasal secretions, urine, etc. Lysozymes in human, goat, mare, and camel milk are considered type c lysozymes; however, it is unclear whether bovine milk lysozyme is a type c or g lysozyme. Lysozyme was purified from camel milk (Duhaiman 1988; El-Agamy et al. 1996). The molecular mass of camel lysozyme was estimated at 14.4 (El-Agamy et al. 1996) and 15 kDa (Duhaiman 1988) versus 15 kDa for either human (Parry et al. 1969) or goat lysozyme (Jolles and Jolles 1984) and 18 kDa for cow lysozyme (Eitenmiller et al. 1971).

Immunological study (El-Agamy et al. 1996) on camel milk lysozyme showed that there are no antigenic similarities between camel and bovine milk lysozyme, suggesting different structures. The concentration of lysozyme in mammalian milk varies widely from 13 µg/100 mL in buffalo milk (El-Agamy et al. 1998b) to 79 mg/100 mL in mare milk (Jauregui-Adell 1975). Camel milk contained 228, 288, and 500 µg/100 mL of lysozyme (Barbour et al. 1984; Duhaiman 1988; El-Agamy et al. 1998b). In cow milk, the corresponding values were reported as 7 (Vakil et al. 1969), 13 (Korhonen 1977), and 37 µg/100 mL (El-Agamy et al. 1996). The variations in the reported values are mainly due to the effect of lactation stage.

A comparative study (El-Agamy and Nawar 2000) of lysozyme concentration in milk of different species showed that camel milk had a considerably higher concentration of lysozyme than cow, buffalo, sheep, and goat milk. However, lysozyme in camel milk was lower than those in human, donkey, and mare milk. The equivalent concentration of lysozyme in camel milk was 11, 18, 10, and 8 times that of cow, buffalo, sheep, and goat milk, respectively.

Lysozyme concentration in milk varies according to some factors such as lactation period and health status of the animal. It increases in precolostrum, colostrum, and udder infection (Carlsson et al. 1989; Persson 1992). The changes in lysozyme in daily samples of camel colostrum and normal milk are

shown in Table 6.6. Camel colostrum contained higher concentrations of lysozyme than normal milk. Camel milk lysozyme was highest on day 2 after parturition and then declined, and on day 5 there was a slight increase followed by a decrease. The trend was different in cow milk lysozyme. The concentrations of lysozyme in camel milk were found to decrease rapidly within the first months of lactation (Barbour et al. 1984).

Only one study has been carried out on the effect of heat treatment on protective proteins as immunoglobulins (IgG_S), lysozyme, and lactoferrin (El-Agamy 2000a). In this study camel, cow, and buffalo skim milk samples were heated at 65, 75, 85, and 100 °C for 30 minutes. The results showed that heating of the three kinds of milk at 65 °C for 30 minutes had no significant effect on lysozymes and lactoferrins; however, a significant loss in IgG_S activity was detected. The whole activity of IgG in both cow and buffalo milk was lost at 75 °C for 30 minutes versus 68.7% in loss activity of camel IgG. The entire activity of lactoferrins was lost at 85 °C for 30 minutes in the three kinds of milk; however, at this level of temperature, the activity losses of lysozymes were 56, 74, and 81.7% for camel, cow, and buffalo milk, respectively. Generally it was concluded that protective proteins of camel milk are significantly ($p \leq 0.01$) more heat-resistant than cow and buffalo milk proteins. Among the protective proteins, the order of heat resistance found was lysozyme > lactoferrin > IgG_S.

Table 6.6. Changes in lysozyme in camel and cow colostrum and normal milk (El-Agamy 1994b; Korhonen 1977)

Days Postpartum	Lysozyme (mg/L)			
	Camel Milk		Cow Milk	
	Range	Mean	Range	Mean
1	0.43–1.97	1.03	0.14–0.70	0.40
2	0.69–2.02	1.28	0.20–0.70	0.55
3	0.54–1.98	1.06	0.57–0.78	0.65
4	0.52–1.92	0.90	0.45–0.80	0.66
5	0.57–1.96	0.93	0.45–0.85	0.69
6	0.54–1.93	0.92	0.55–0.80	0.64
7	0.46–1.88	0.87	0.56–0.90	0.72
14	0.42–1.35	0.73	0.07–0.60	0.37

Lactoperoxidase (EC 1.11.1.7)

Lactoperoxidase is found in milk, tears, and saliva. It contributes to the nonimmune host defense system, exerting bactericidal activity mainly on Gram-negative bacteria. It is supposed that the main function in milk is the protection of the udder from microbial infections (Ueda et al. 1997). Lactoperoxidase is resistant to proteolytic digestion and acidic pH. Lactoperoxidase activity is maintained at a high level throughout lactation; however, human lactoperoxidase is present only in colostrum and becomes undetectable within one week after parturition (Ueda et al. 1997). Lactoperoxidase was first isolated, crystallized, and characterized by Theorell and Akesson (1943). Lactoperoxidase has been demonstrated in milk from many species: e.g., cow, goat, guinea pig, murine, human, and camel milk (Polis and Shmukler 1953; Gothefors and Marklund 1975; El-Agamy 1989; Stephens et al. 1979).

Bovine milk is rich in lactoperoxidase (30 mg/L) (Polis and Shmukler 1953). Lactoperoxidase is a glycoprotein and contains one heme group (Theorell and Pedersen 1944). The iron content is 0.0680–0.0709% and the carbohydrate content 9.9–10.2% (Carlstrom 1969).

Camel milk lactoperoxidase is a monomeric protein, which has 79.3% sequence similarity to human myeloperoxidase, and 79.2% sequence similarity to human eosinophil peroxidase. Both myeloperoxidase and eosinophil peroxidases are dimeric proteins (Kappeler 1998). Lactoperoxidase was purified from camel (El-Agamy et al. 1996) and bovine milk (Yoshida and Ye-Xiuyun 1991), and their molecular weights were estimated at 78 and 88 kDa, respectively.

PCR amplification products of a camel lactoperoxidase cDNA clone were sequenced (Kappeler 1998). The study showed that the clone was 2,636 bp long, and contained a 3′-untranslated region of 497 bp and a 5′-untranslated region of 4 bp. The molecular weight of camel lactoperoxidase is 69.46 kDa versus 69.57 kDa for bovine lactoperoxidase. The isoelectric point of camel lactoperoxidase is 8.63, whereas it is at pH 7.90 in bovine lactoperoxidase (Dull et al. 1990). Camel lactoperoxidase shares 94.9% sequence similarity with bovine lactoperoxidase and 94.1% with human salivary peroxidase. Immunochemical study on camel milk lactoperoxidase showed that there is a cross-

reactivity, i.e., antigenic similarities, with bovine milk lactoperoxidase when antiserum to camel milk lactoperoxidase was used in the test (El-Agamy et al. 1996).

Mode of Action

Antimicrobial activity of lactoperoxidase is performed by a so-called lactoperoxidase system (LPS), in which hydrogen peroxide (H_2O_2) is reduced and a halide, e.g., iodide (I^-) or bromide (Br^-), or a pseudohalide, e.g., thiocyanate (SCN^-), is subsequently oxidized to hypothiocyanate (OSCN) as shown in the following equation:

$$H_2O_2 + SCN^- \rightarrow OSCN^- + H_2O$$

Natural substrates of lactoperoxidase in milk are thiocyanate and iodide, of which milk contains trace amounts. Thiocyanate is provided by consumption of plants of the family Cruciferae as cabbage contains up to 5 g/kg thioglucosides, which are readily converted into SCN^- by enzymatic hydrolysis (Bibi 1989).

Cow milk contains 1–15 mg/L of SCN^- (De Wit and Van Hooydonk 1996). Concentration of H_2O_2 is very low in normal milk and it can be generated by oxidation of xanthine by xanthine oxidase or supplied by catalase-negative bacteria such as lactobacilli, lactococci, or streptococci, which naturally occur in milk (Reiter 1985). The bacterial action of the LP system is due to the effect of reaction products of thiocyanate oxidation, $OSCN^-$ and HOSCN, which are able to oxidize free SH-groups of the cytoplasmic membrane of Gram-negative bacteria. The structural damage on bacterial cell membranes results in diffusion of potassium ions, amino acids, and polypeptides out of the cell; meanwhile, the uptake of glucose and other metabolic substrates is inhibited. The action of the LP system on Gram-positive bacteria such as streptococci is different, because Gram-positive bacteria are protected from the action due to their rigid cell wall (Reiter 1985).

The lactoperoxidase system could be used as an alternative method for the preservation of raw camel milk, which is produced under high ambient temperature and low hygienic conditions when a cooling process is not found.

Other Native Peptides

A novel protein is involved in the primary immune response of vertebrates and invertebrates on Gram-positive bacteria and other invading organisms, such as nematodes (Yoshida et al. 1996; Kang et al. 1998). Inactivation of pathogens probably occurs by binding to peptidoglycan structures in bacterial cell walls, thus the name *peptidoglycan recognition protein (PGRP)*. It was isolated from camel whey by heparin-sepharose chromatography and probably serves the same function of specific pathogen inhibition in camel milk (Kappeler 1998). It is found in higher amounts in camel milk than concentrations of other protective proteins as lactoferrin, lysozyme, or lactoperoxidase (Kappeler 1998). It has 19.11 kDa and its N-terminal sequence of the reverse phase purified protein was determined as the following: Arg-Glu-Asp-Pro-Pro-Ala-Cys-Gly-Ser-Ile.

A full-length cDNA clone of 700 bp corresponding to the N-terminal sequence was found (Kappeler 1998). The isoelectric point of camel PGRP was at pH 8.73 and higher than those of human and murine, which were pH 7.94 and 7.49, respectively (Kappeler 1998). The mature PGRP had 91.2% similarity with human protein, 87.9% with murine. Camel PGRP protein is rich in arginine but poor in lysine, although the *p*I is highly basic. Its concentration in camel milk was determined as 370 mg/L (Kappeler 1998). In contrast, PGRP was isolated in major amounts from end-lactation milk. This indicated constant expression of the protein in camel milk in the course of lactation.

A protein rich in proline (25% of total protein) was isolated from camel milk by reverse-phase high-performance liquid chromatography. The N-terminal amino acid sequence showed that it is a fragment of β-casein, derived from a nontryptic type of cleavage of the parent protein and homologous to the c-terminal region of β-caseins from other species. The protein structure showed it contains no less than four regions characterized by alternating proline residues. This structure is one of the properties typical of peptides with opioid activity that have previously been characterized from proteose-peptone fractions of bovine β-casein (Beg et al. 1986b).

It has been reported that camel milk has more free amino acids and peptides than does bovine milk (Mehaia and Al-Kanhal 1992). The nonprotein-

bound amino acids in camel milk are easily digested by microorganisms and, therefore, camel milk has a higher metabolic activity when used in a starter culture preparation. The importance of free amino acids and peptides for the growth of bifidobacteria has been studied by Cheng and Nagasawa (1984). It was presumed that free amino acids could be utilized during the early stage of incubation and that peptides became available during the prolonged incubation of *Bifidobacterium* cultures. The study of Abu-Taraboush et al. (1998) evaluated the growth, viability, and proteolytic activity of four species of bifidobacteria in whole camel milk, and comparison was made with whole cow milk. The growth rate of *Bifidobacterium longum* was higher in camel milk than in bovine milk, while it was higher for *Bifidobacterium angulatum* in bovine milk than in camel milk. *Bifidobacterium bifidum* and *Bifidobacterium breve* showed the same trend as *B. angulatum* 16 h postinoculation. All species except *B. longum* showed higher proteolytic activity in fermented camel milk than in bovine milk.

ANTIMICROBIAL PROPERTIES OF CAMEL MILK BIOACTIVE PROTEINS

Antiviral Activity

Rotaviruses are the most frequent cause of nonbacterial gastroenteritis in infants or calves in most parts of the world. In Egypt, the Bedouins use camel milk to treat diarrhea (personal observation). Camel milk immunoglobulin (IgG) and secretory immunoglobulin A (sIgA) were purified and their neutralization activity against bovine (El-Agamy et al. 1992) or human (El-Agamy 2000b) rotavirus was studied. Individual camel (*Camelus dromedarius*) colostrum and normal milk samples were tested for the presence of antibodies to rota- and coronaviruses. All samples were negative for anticoronavirus antibodies; while some of colostrum and milk samples had specific antibodies to rotavirus. The antirotavirus activity, i.e., antibody titer in colostrum, was strong due to IgG, while sIgA in normal milk was high (Fig. 6.3A and B). This indicates that raw camel milk is considered a strong viral inhibitor to human rotavirus. Meanwhile, the high titer of sIgA against rotavirus reflects that she-camel mammary glands are able to synthesize a high concentration of such type of immunoglobulin as a defense factor. These findings may explain the reason for use of camel milk as a remedy to treat diarrhea by camel herdsmen (El-Agamy 1983).

The antiviral properties of freshly prepared or conserved Shubat, a national drink of fermented camel milk in Kazakhstan, were studied (Chuvakova et al. 2000). The results showed that Shubat is characterized by virucidal and virus-inhibiting properties against ortho- and paramyxoviruses, and these properties were not affected by shelf life. The antiviral activity of Shubat is suggested to be due to the presence of sialic conjugates and metabolic products of lactic acid bacteria and yeasts.

Antibacterial Activity

Lysozyme

The inhibitory effects of camel milk lysozyme in 200 individual milk samples on pathogenic bacteria were examined (Barbour et al. 1984). Results showed that percentages of inhibition were 7.5, 4.0, 2.0, and 1.0% for *Clostridium perfringens*, *Staphylococcus aureus*, *Shigella dysenteriae*, and *Salmonella typhimurium*, respectively; however, none of the whole samples inhibited *Bacillus cereus* or *Escherichia coli*.

The inhibition effect of camel milk lysozyme was also studied comparing with egg white lysozyme and bovine milk lysozyme against some strains of bacteria (El-Agamy 1989). Results revealed that camel milk lysozyme had a higher lysis value toward *Salmonella typhimurium* compared with other lysozymes. The results obtained by disc assay technique indicated that the clearance zone values were 22.2, 20.2, and 0.0 mm for camel, egg white, and bovine milk lysozyme, respectively. Camel milk lysozyme had no effect on *Lactococcus lactis* subsp. *cremoris*; however, the strain was highly affected by bovine milk lysozyme. All lysozymes were ineffective toward *Escherichia coli* and *Staphylococcus aureus*.

It has been found that lysozyme increases the antibacterial activity of lactoferrin. Commercial preparations of lysozyme are an interesting alternative to nitrate as an antisporulating agent, preventing the growth of *Clostridium tyrobutiricum* in cheese (Stepaniak 2004).

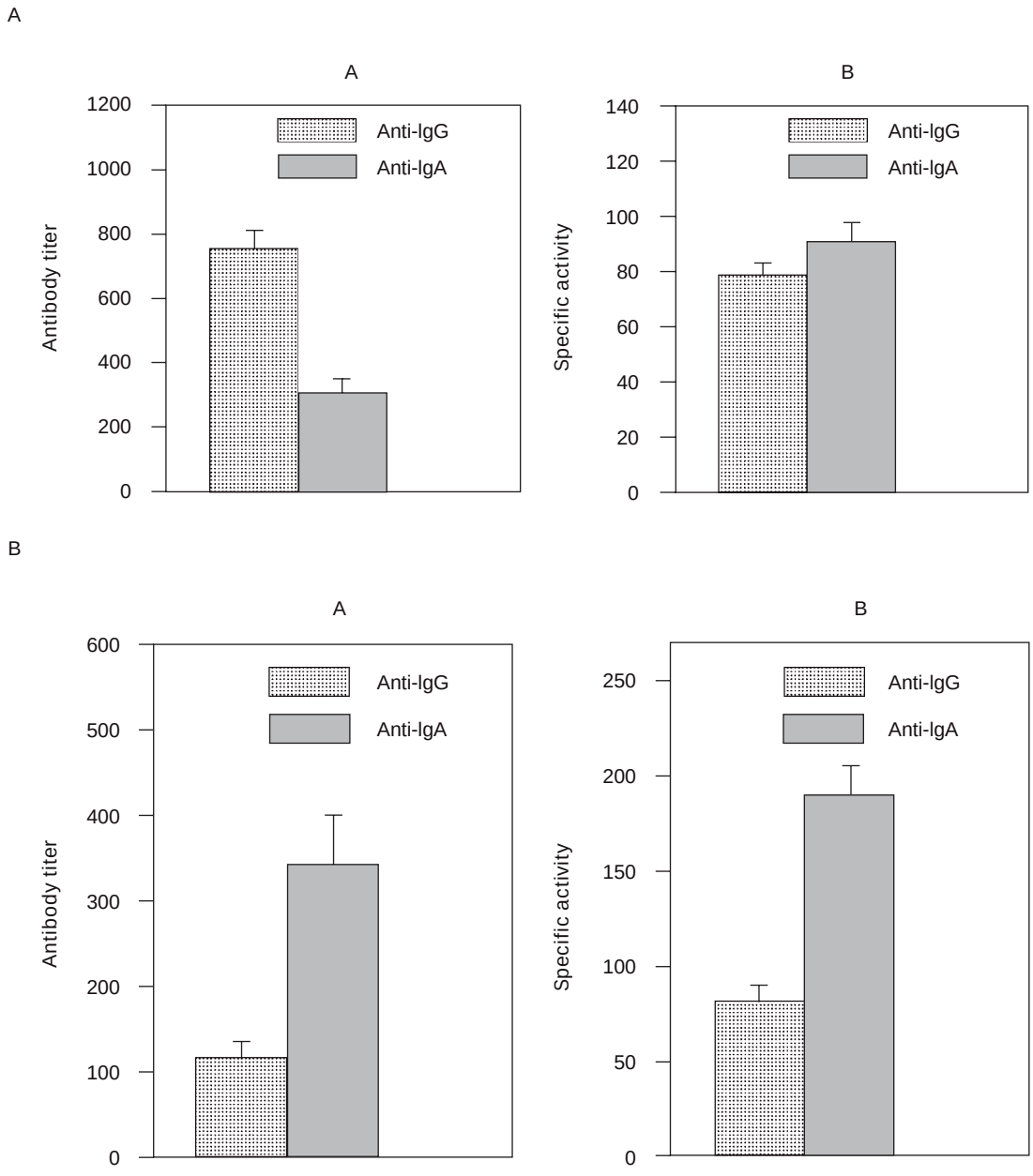

Figure 6.3. A. Antirotavirus activity in camel-colostral whey expressed as antibody titer (A) or specific activity (B) (El-Agamy 2000b). B. Antirotavirus activity in camel normal-milk whey expressed as antibody titer (A) or specific activity (B) (El-Agamy 2000b).

Lactoferrin

The inhibition effect of lactoferrin depends mainly on iron requirements of microorganisms. For example, *E. coli* is much more sensitive than lactic acid bacteria (Reiter 1985). The inhibition effect of camel and bovine milk lactoferrins against some strains of bacteria was studied (El-Agamy 1989). Both types of lactoferrins were effective against *Salmonella typhimurium*, and the clearance inhibition zones were 18.2 and 17.4 mm for camel and bovine milk lactoferrins, respectively. Neither camel nor bovine milk lactoferrin had a lysis effect toward *E. coli* and *Staphylococcus aureus*.

Taking into account that the inhibition rate of camel lactoferrin against such microorganisms was detected in synthetic media, this effect is probably different when liquid media such as milk are used, because it was reported that citrate ions can counteract the bacteriostatic activity of lactoferrin, i.e., compete for iron, unless the bicarbonate concentration is high (Reiter 1985). Therefore, it can be expected that lactoferrin activity in camel milk will be higher due to the lower concentration of citrate ions (El-Agamy 1983). It can be assumed that the inhibition effect of lactoferrin in camel milk, when ingested by the nomads in the desert, is due to two main factors: 1) the low content of citrate in camel milk and 2) the high bicarbonate concentration in the intestinal fluid, where bicarbonate is the main buffer (Reiter 1985). These two factors will provide the proper conditions for lactoferrin to bind iron and inhibit sensitive microorganisms such as *E. coli*.

The Lactoperoxidase System (LPS)

Camel milk lactoperoxidase was purified and its inhibition activity against lactic acid bacteria and some strains of pathogenic bacteria was studied (El-Agamy et al. 1992). The LP system had a bacteriostatic effect toward both *Lactococcus lactis* and *Staphylococcus aureus*; however, it was bactericidal for *E. coli* 0157:H7 and *Salmonella typhimurium*. The destructive effect of camel LP system on the cell walls of these bacterial strains was recorded (Fig. 6.4).

Medicinal Properties of Camel Milk

Camel milk in raw state and its fermented products are used as therapeutic agents to treat stomach ulcers, liver disorders, diarrhea, constipation, and wounds, as well as to enhance female ovaries for ovulation (personal observations). Camel milk is also given to children suffering from biliary atresia and postpartum respiratory insufficiency and kept alive until a liver transplant could be performed and lungs are developed (Yagil 1987). Fermented camel milk Shubat is used as a therapy for treating tuberculosis in different countries, India (Mal et al. 2000), Libya (Alwan and Tarhuni 2000), and Kazakhstan (Puzyrevskaya et al. 2000). Treatment of chronic diseases of the gastrointestinal tract using camel milk and Shubat was also reported (Djangabilov et al. 2000). Camel milk is used for chronic hepatitis, spleen inflammation (personal observations). Similar remarks were recorded in the former USSR (Sharmanov et al. 1978). It was reported that patients suffering from chronic hepatitis have improved liver functions after drinking camel milk. Early reports showed that camel milk is successfully used for stabilization of juvenile diabetes (Yagil 1987). It was found that one of the camel milk proteins has many characteristics similar to insulin (Beg et al. 1986a) and it does not form coagulum in an acidic environment (Wangoh 1993). This lack of coagulum formation allows the camel milk to pass rapidly through the stomach together with the specific insulinlike protein and remains available for absorption in the intestine. Breitling (2002) suggested that camel milk is having antidiabetic activity possibly because of insulinlike activity, regulatory and immunomodulatory functions on β cells.

Other reports showed that camel milk supplementation reduces the insulin requirement in type 1 diabetic patients (Agrawal et al. 2003). Moreover, it has been found that after 1 year of treatment with camel milk as an adjunct to insulin therapy, it was found that camel milk improves long-term glycemic control and reduction in doses of insulin in patients with type 1 diabetes (Agrawal et al. 2005).

Recently in India, Agrawal et al. (2007) observed a low prevalence of diabetes in a community consuming camel milk habitually. The prevalence of diabetes in a community (n = 501) was 0% versus 5.5% in a community not consuming camel milk (n = 529).The study concluded that the consumption of camel milk was statistically highly significant as a protective factor for diabetes. In Africa, Egypt, Sudan, Kenya, and Somalia, there is a common belief among the herdsmen of camels, especially

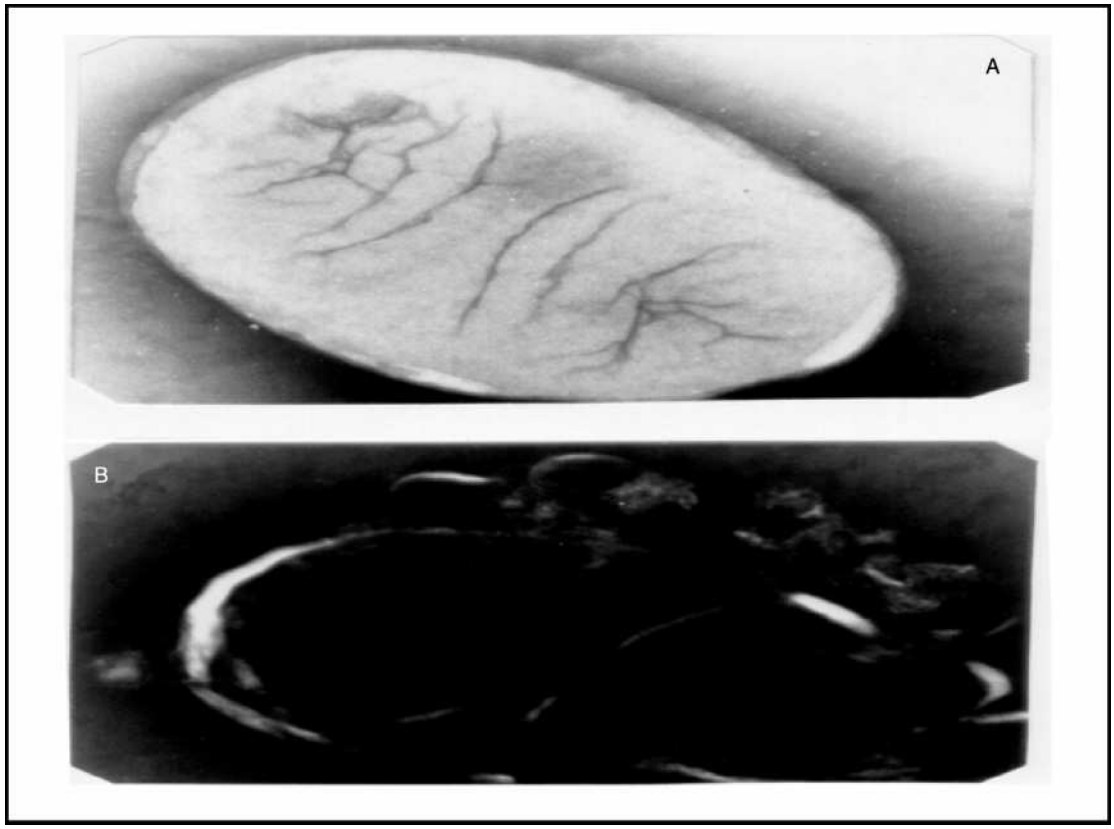

Figure 6.4. Electron micrographs showing the effect of lactoperoxidase system (LPS) on *E. coli* 0157:H7. The damage in outer membrane after exposure shown by arrow. (A) Intact cell. (B) PS–treated cell (El-Agamy 1989).

those grazing on herbs, that if a man drinks camel milk he becomes strong, swift, and virile (personal observations).

HYPOALLERGENIC AND THERAPEUTIC VALUE OF CAMEL MILK PROTEINS

Human milk is the most fit food for human infants, but when breast-feeding is not available, cow milk or infant formula, which is mainly based on cow milk, is usually used as a substitute for human milk. This substitution can lead to nutritional and immunological problems, such as allergy to cow milk proteins. This allergy results from an abnormal immunological response to one or more of milk proteins (El-Agamy 2007).

Cow Milk Allergy

The word *allergy* means an altered or abnormal reaction. Such a reaction may occur when there is contact between a foreign protein—an allergen—and body tissues that are sensitive to it. The allergy may reach the tissues by direct contact with the skin or mucous membranes or through the bloodstream after absorption. Allergic reactions have been classified into two types:

1. In the immediate reaction type, allergic manifestations occur within hours of the patient coming in contact with the allergen and often within seconds or minutes; in this form of allergy, skin tests are nearly always positive.
2. In the delayed reaction type, in which manifestations may not appear for many hours or even

for 2 or 3 days; in this type, skin tests are usually negative.

Cow milk allergy (CMA) is clinically an abnormal immunological reaction to cow milk proteins, which may be due to the interaction between one or more milk proteins and one or more immune mechanisms, resulting in immediate IgE-mediated reactions. On the other side, reactions not involving the immune system are defined as cow milk protein intolerance. CMA occurs in some infants after ingestion of an amount of cow milk. CMA is generally more serious in early infancy (Hill and Hosking 1996).

Milk Protein Cross-Reactivity

Cross-reactivity between milk allergens from different mammalian species and humans occurs when they share part of their amino acid sequence or when they have a similar capacity to bind specific antibodies due to their molecular structures. The cross-reactivity between milk proteins from different animal species has been studied (Prieels et al. 1975; El-Agamy et al. 1997; Carroccio et al. 1999; Restani et al. 2002; El-Agamy et al. 2006). Restani et al. (1999) showed that IgEs from sera of children allergic to cow milk are capable of recognizing most parts of milk proteins from European mammals: sheep, goat, and buffalo. Weak cross-reactivity was observed with milk proteins from mares and donkeys, but none with camel milk. IgEs from a child allergic to sheep milk did not recognize any proteins of camel milk.

The immunological relationship between human milk proteins and their counterparts of camel, cow, buffalo, goat, sheep, donkey, and mare milk was studied (El-Agamy et al. 1997). Human milk caseins had relationships with donkey and mare milk proteins; relations were weak with goat and camel milk and had no relations to cow, buffalo, and sheep milk proteins. Meanwhile, when antiserum to human milk whey proteins was applied in immunodiffusion analysis, two precipitin lines were detected between human and donkey milk proteins versus only one precipitin line with proteins of other species. It indicates that the antigenic similarities are stronger between donkey and human milk whey proteins than that of milk of other species.

El-Agamy et al. (2006) studied the immunological cross-reactivity between camel milk proteins and their counterparts in cow milk, using prepared antisera to camel milk proteins as well as sera from some children allergic to cow milk using immunoelectrophoretic, immunoblotting (Western blot) and ELISA techniques. Immunoelectrophoretic (Fig. 6.5) and immunoblotting analyses showed the absence of immunological cross-reactivity between camel and cow milk proteins when specific antisera to camel milk proteins were applied.

When sera from some allergic children to cow milk were tested for the specificity of immunoglobulin E (IgE) to camel milk proteins using ELISA technique (Fig. 6.6), the same results as of immunoelectrophoresis were obtained. The study concluded that the absence of immunological similarity between camel and cow milk proteins can be considered an important criterion from the nutritional and clinical points of view, since camel milk may be suggested as a new protein source for nutrition of cow milk allergic children and can be used as such or in a modified form.

Human Milk Alternatives

Human milk composition is different from that of other mammalian milk in both ratios and structure of milk constituents. The protein content in human milk is lower than in milk of ruminant dairy animals—cows, buffalo, yak, camel, goat, sheep, reindeer—but it is closer to that of donkey and mare milk (El-Agamy et al. 1997). The ratio of casein within total protein is lower in human milk, because whey proteins (soluble proteins) are higher than in cow, buffalo, and sheep milk, whereas they are at similar levels in donkey and mare milk (El-Agamy et al. 1997). This condition gives human milk the special property of forming a soft curd during digestion in the infant's gut, although goat milk is also known for this uniquely different property. The softness of the curd is due to the lower ratio of soluble calcium. This condition may explain why, in many parts of the world, mare and donkey milk as well as goat and camel milk are used as human milk substitutes for bottle-fed infants (El-Agamy 1983; Zhao 1994; El-Agamy et al. 1997). On the contrary, both cow and buffalo milk give a hard curd, which is preferred in cheese making. Dilution of bovine milk with water before using it in baby feeding must be practiced for safe nutrition, especially for very young babies. On the other hand, human milk

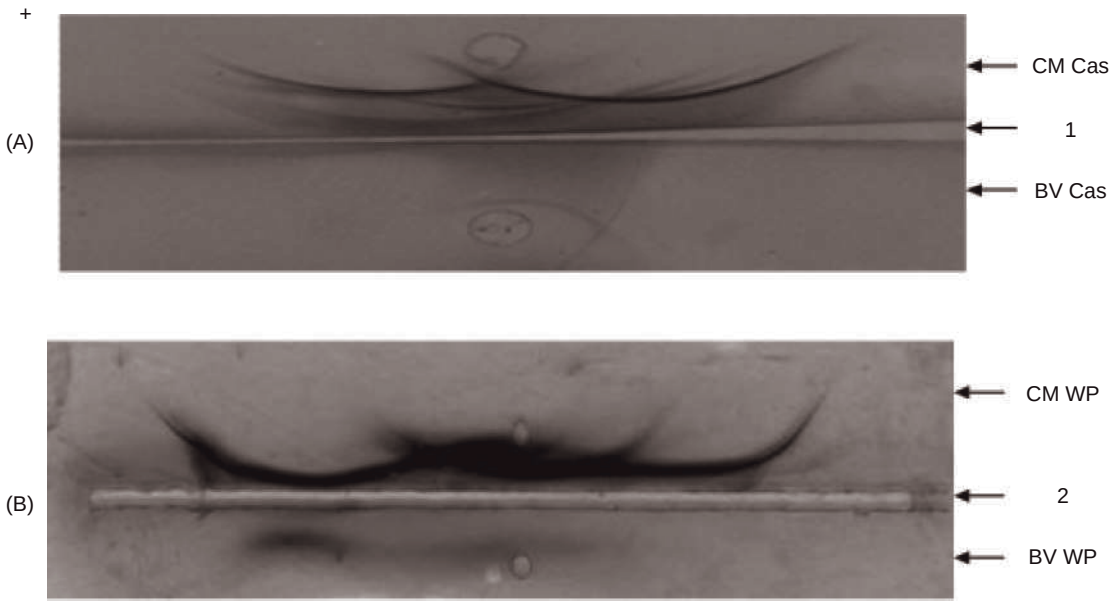

Figure 6.5. Immunoelectrophoretic analysis of camel and cow milk proteins. (A) CM Cas: camel milk casein; BV Cas: bovine casein; 1: rabbit antiserum to camel milk casein. (B) CM WP: camel milk whey proteins; BV WP: bovine milk whey proteins; 2: rabbit antiserum to camel milk whey proteins (El-Agamy et al. 2006).

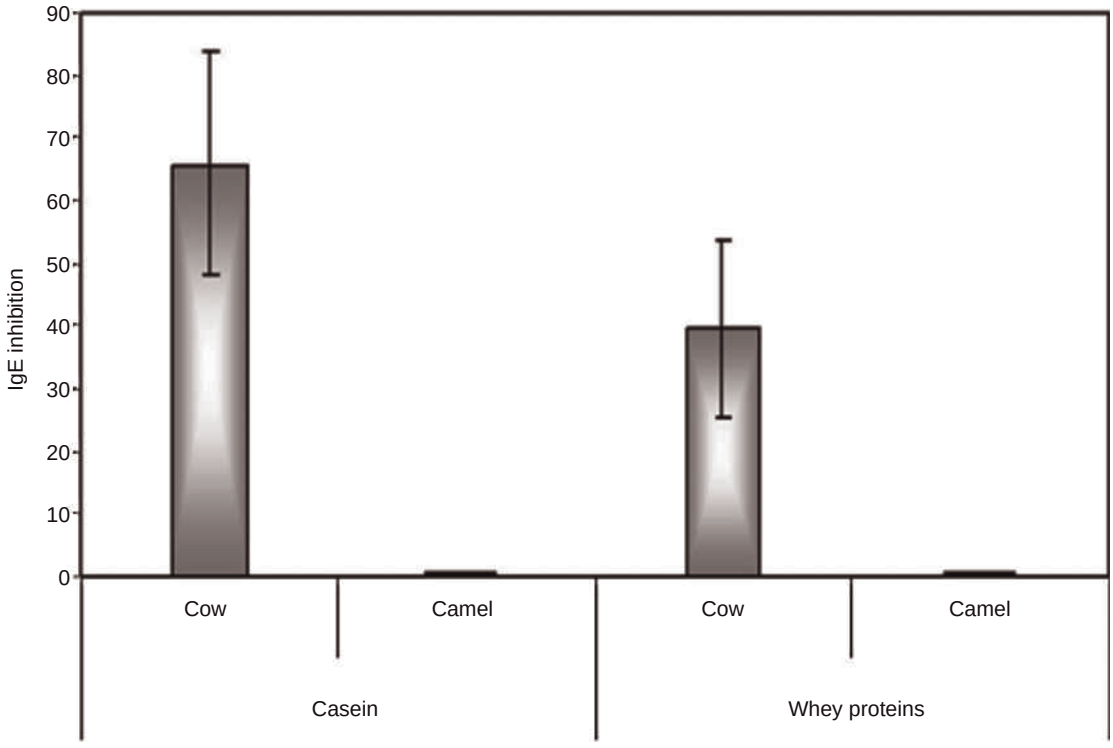

Figure 6.6. IgE-ELISA inhibition of cow and camel milk proteins (El-Agamy et al. 2006).

proteins are different in their composition and structure from those of milk of other species. Taylor (1986) reported that the major whey proteins of bovine milk are β-Lg with 55% of total whey proteins, α-La with 20%, and BSA with 7%.

These proteins differ in their types and ratios between goat, sheep, cow, camel, human, buffalo, mare, and donkey milk (El-Agamy et al. 1997). Human milk is free of β-Lg (Kappeler 1998), one of the major allergens in cow milk, similar to camel milk, which also has no β-Lg (El-Agamy and Nawar 2000). On the contrary, β-Lg is a major whey protein in cow, buffalo, sheep, goat, mare, and donkey milk (El-Agamy et al. 1997).

Several studies have evaluated the clinical use of milk from different animals such as goat (Cant et al. 1985; Park 1994; Alvarez and Lombardero 2002; Muraro et al. 2002; Restani et al. 2002), camel (El-Agamy et al. 2006), sheep (Dean et al. 1993; Restani et al. 2002), and mare and donkey (El-Agamy et al. 1997; Carroccio et al. 2000; Muraro et al. 2002). The available data in the literature show contradictory results concerning the use of animal milk as alternatives to human milk. Some studies revealed that goat (Cant et al. 1985; Coveney and Darnton-Hill 1985; Razafindrakoto et al. 1994; Bevilacqua et al. 2001), mare, donkey (El-Agamy et al. 1997, Carroccio et al. 2000), and camel milk (El-Agamy et al. 2006) can be considered as proper alternatives to human milk due to hypoallergenic properties of their proteins. On the other side, other studies showed that milk of goat (Jelert 1984; Cant et al. 1985; Wuthrich and Johansson 1995; Spuergin et al. 1997; Orlando and Breton-Bouveyron 2000; Alvarez and Lombardero 2002; Muraro et al. 2002; Restani et al. 2002; Pessler and Nejeat 2004), sheep (Wuthrich and Johansson 1995; Spuergin et al. 1997; Alvarez and Lombardero 2002; Restani et al. 2002), and buffalo (Restani et al. 2002) cannot be useful in all cases as alternatives to human milk, because they can be as allergic as cow milk, which also has been documented for soy milk in some cases. The study of Infante et al. (2003) with goat milk revealed that only 25% of 12 patients with CMA benefited and showed adequate immediate and late oral tolerance and negative results in immunological tests with RAST, specific IgE, SPT, and challenge tests, but other studies have found higher cure rates (Haenlein 2004).

In Egypt, camel milk is used by nomads, after dilution with water, to feed their infants (El-Agamy 1983). Similar behavior is found in China (Zhao 1994) and Mongolia (Indra and Erdenebaatar 1994). This traditional feeding regimen may be explained by the following: 1) camel milk is free of β-LG like human milk and 2) the ratio of whey protein to casein in camel milk is high, which results in soft curd and therefore digestibility is easier.

BIOACTIVE LIPID COMPONENTS

Milk fat is secreted in the form of globules surrounded by a membrane, i.e., the milk fat globule membrane, which maintains the integrity of the globules and renders them compatible with their aqueous environment. The fat globules consist almost entirely of triacylglycerols, while the membranes contain mostly the complex lipids.

Fat Globule Size and Digestibility

The bulk of the fat in milk exists in the form of small spherical globules of varying sizes. The size distribution of fat globules in milk of different species is markedly different. The average size of fat globules of camel, cow, buffalo, sheep, and goat milk have been reported (El-Agamy 2006). The highest diameter of fat globules is in buffalo milk, whereas the lowest diameter is in camel milk. Generally, camel, sheep, and goat milk fat globules are smaller in size compared with those of buffalo and cow milk. The smallest size of camel milk fat globules may explain the high softness of coagulum of camel milk and therefore better digestibility (El-Agamy 1983).

Lipid Composition

Milk lipids serve nutritionally as an energy source, act as a solvent for the fat-soluble vitamins, and supply essential fatty acids. In milk of all species studied to date, triacylglycerols are by far the major lipid class of milk fat, accounting for 97–98% of the total lipids in most species. The triacylglycerols, which contain a great variety of fatty acids, are accompanied by small amounts of diacylglycerols and monoacylglycerols, cholesterols, free fatty acids, and phospholipids (Jensen 2002).

Fatty Acid Composition

The major components of fats are the acids in the case of milk fat. The fatty acids account for over

85% and the glycerol for approximately 12.5% of weight. Glycerol is a nonvarying component of all fats, whereas the fatty acids represent a significant variable. Consequently the physicochemical properties of a particular fat depend primarily on its component acids. The fatty acids in milk are derived from two sources, the plasma lipids and synthesis in the mammary gland. Those fatty acids of the plasma may come from the diet, but also include fatty acids released from body tissues. Generally the fatty acid composition of the milk of a given species represents the balance between the contributions of fatty acids of diet and those synthesized in the mammary gland. It was found that milk fat contains *trans*-11 18:1 (Vaccenic acid), *cis*-9, *trans*-11 18:2 (Rumenic acid) or conjugated linoleic acid (CLA), *trans*-9 and *trans*-11 18:2 CLA that have been shown to elicit antimutagenic properties and alter lipoprotein metabolism (Parodi 1999, 2001; Bauman et al. 2005; Collomb et al. 2006).

The composition of fatty acids in camel, sheep, goat, and cow milk fat are listed in Table 6.7. Taking into account the influence of some factors such as diet and stage of lactation and genetic variations in the fatty acids composition in the milk of each species, data from different sources for each milk were collected. Within these limitations the general pattern of camel milk fatty acids indicates that their short-fatty acids C_4–C_{14}, are presented in very small amounts in milk fat compared with other species, but that the concentrations of $C_{16:0}$–$C_{20:0}$ are relatively high. It was reported that the higher the consumption of saturated fatty acids the higher the risk for cardiovascular disease, and it may also be related to lowered insulin secretion (Shingfield et al. 2008).

Table 6.7. Mean values of some relationships between fatty acids of camel, sheep, goat, and cow milk fat (Posati and Orr 1976; Farah et al. 1989; Ahmed1990; Bernard et al. 2005; El-Agamy 2006; Shingfield et al. 2008)

Relationships	Camel	Sheep	Goat	Cow
SFA%	62.41	74.56	73.70	70.08
USFA%	37.80	25.44	26.30	29.81
USFA/SFA	00.61	00.34	00.36	00.43
PUFA/USFA	00.11	00.14	00.10	00.11
SCFA(C_4–C_{14})	14.80	41.30	33.40	27.72
LCFA(C_{16}–C_{20})	85.20	58.70	66.60	72.18

SFA: saturated fatty acids; USFA: unsaturated fatty acids; PUFA: polyunsaturated fatty acids; SCFA: short-chain fatty acids; LCFA: long-chain fatty acids.

Zhang et al. (2005) found that in camel milk fat the predominant saturated fatty acids were C14:0, C16:0, and C18:0, whereas the main polyunsaturated fatty acid was C18:1, regardless of the stage of lactation. Many studies (Rao et al. 1970; Farah 1986; Abu-Lehia 1989; Farah et al. 1989; Ahmed 1990) showed that $C_{16:1}$ is present in greater proportions in camel milk fat than in the milk fat of other species. Many earlier studies showed that saturated fatty acids (C12:0, C14:0 and C16:0) are known to raise total and low-density lipoprotein (LDL) cholesterol (Katan et al. 1995; Temme et al. 1996), while stearic acid (C18:0) has been shown to be essentially neutral (Bonanome and Grundy 1988). Other in vivo studies on human subjects showed that 16:0 has no effect on plasma total or LDL cholesterol in hypercholesterolemic (Clandinin et al. 1999) or normocholesterolemic subjects, when the intake of $18:2_{(n-6)}$ exceeds 5.0% of dietary energy and cholesterol is less than 400 mg/day (Ng et al. 1992; Sundram et al. 1995; Clandinin et al. 2000; French et al. 2002).

On the other hand, supplying 0.6 or 1.2% of C14:0 as a source of energy in healthy men's diets resulted in significant decreases in plasma triacylglyceride and increases in HDL-cholesterol concentrations (Dabadie et al. 2005). Such findings may highlight the challenge of the nutritional state of camel milk fat for the prevention of chronic disease.

Camel milk fat is characterized by the higher proportion of unsaturated fatty acids compared with other species. This may be the main reason for the waxy texture of camel milk fat (Zhang et al. 2005). Sheep and goat milk fats contain relatively high concentrations of short-chain length fatty acids compared with those of camel and cow milk. Appreciable amounts of the essential fatty acid, linoleic acid—$18:2_{(n-6)}$—are found in camel milk fat. Compared with other species camel milk fat is characterized by the higher iodine value; acid value; refractive index; melting point; and lower Reicher Meissl, Polenske, and saponification values. This reflects its higher content of long-chain fatty acids (C_{14}–C_{18})

and lower content of short-chain fatty acids (C_4–C_{12}).

Trans Fatty Acids

Trans fatty acids are defined as all unsaturated fatty acids that contain nonconjugated one or more double bonds in a transconfiguration. Several studies indicated that there is a strong relation between trans fatty acids high intakes and increased rate of coronary heart disease compared with saturated fatty acids (Willett et al. 1993; Kromhout et al. 1995; Ascherio et al. 1999a,b; Mensink et al. 2003). Recently, it has been found that trans fatty acids are responsible for proinflammation, which is an independent risk factor for many aspects of chronic disease (Mozaffarian et al. 2006). *Trans*-11 18:1 is the major isomer in milk fat (Goudjil et al. 2004). Its profile in human milk fat is less distinct compared with cow and goat milk fat (Ledoux et al. 2002).

Branch-Chain Fatty Acids

It has been reported that milk fat contains 56 isomers of branch-chain fatty acids (BCFA) with chain lengths varying from 4 to 26 carbon atoms (Ha and Lindsay 1990; Jensen 2002). The major branch-chain fatty acids in milk fat can be classified into one of three classes: even-chain iso acids, odd-chain iso acids, and odd-chain anteiso (Vlaeminck et al. 2006). Milk fat also contains relatively minor amounts of *w*-alicyclic fatty acids with or without a substitution for double bonds or hydroxylation (Brechany and Christie 1992, 1994). In ruminant milk fat, 15:0 anteiso and 17:0 anteiso are typically the most abundant branch-chain fatty acids in milk. Their concentrations were determined as 462 and 501 mg/100 g fatty acids, respectively (Ha and Lindsay 1990; Vlaeminck et al. 2006). Several studies have shown that many BCFA possess anticarcinogenic properties. In vitro studies showed that BCFA are effective in inhibiting the fatty acid synthesis of human breast cancer cells (Wongtangtintharn et al. 2004). No data are available on BCFA of camel milk fat yet.

Phospholipids

Although phospholipid components comprise 0.2–1.0% of total lipids in cow milk fat (Rombaut et al. 2005), they are an important fraction of milk lipids. They are found mainly in the milk fat globule membrane and in other membranous material of the skim milk phase. The phospholipid content in camel, buffalo, and goat milk was reported as 4.77 mg/g fat (Ahmed 1990), 3.8–4.0 mg/g fat (Hofi et al. 1973), and 8–10 mg/g fat (Jenness 1980), respectively.

The composition of the phospholipids in camel, buffalo, and cow milk is shown in Table 6.8. Phosphatidyl choline (PC), phosphatidyl-ethanolamine (PE), and sphingomyelin (SP) are the majority in each case; phosphatidylserine (PS), phosphatidyl-inositol (PI), and lysophospholipids (LP) are also present, and there are marked similarities in the relative proportions of each of the phospholipids among the three species. It was found that the total concentration of phosphocholine-containing components is fairly constant (52–60%), presumably because these perform the same structural function in each species (Morrison 1968a). Camel milk PE is unusual in that it contains 15% plasmalogen, whereas the largest amount reported in other phospholipids is 4% plasmalogen in bovine milk phosphatidylcholine (Morrison et al. 1965).

The phospholipid fatty acids of camel, cow, buffalo, sheep, donkey, pig, and human milk were studied (Morrison 1968b). Phospholipid fatty acids of camel milk are not entirely characteristic of those of the ruminant herbivores. The ruminant herbivores have branched-chain fatty acids with more than two double bonds. Their sphingomyelin contains a high proportion of tricosanoic acid ($C_{23:0}$) but little

Table 6.8. Phospholipids composition in milk from camel, buffalo, and cow (Morrison 1968a)

	Amount (mol % of the Total Phospholipids)					
Species	PC	PE	PS	PI	SP	LP*
Camel	24.0	35.9	4.9	5.9	28.3	1.0
Buffalo	27.8	29.6	3.9	4.2	32.1	2.4
Cow	34.5	31.8	3.1	4.7	25.2	0.8

*Mainly lysophosphatidyl choline but also lysophosphatidyl ethanolamine.

PC: phosphatidyl choline; PE: phosphatidyl ethanolamine; PS: phosphatidyl serine; PI: phosphatidyl inositol; SP: sphingomyelin; LP: lysophospholipids.

nervonic acid (C_{24}:1_{n-9}). Nervonic acid plays a part in the biosynthesis of nerve cell myelin and is found in sphingolipids of white matter in the human brain. In diseases involving demyelination, such as adrenoleukodystrophy and multiple sclerosis (MS), there is a marked reduction of nervonic acid levels in sphingolipids (Sargent et al. 1994).

Camel milk phospholipid fatty acids have high amounts of linoleic acid (18:3_{n-3}) and long-chain polyunsaturated acids. Its sphingomyelin contains a higher proportion of nervonic acid and a lower proportion of tricosanoic acid than that of the other ruminant herbivores.

Sterols

Cholesterol is the major sterol component of most milk. It forms at least 95% of the total sterols, but small amounts of other sterols have been found. In ruminant milk, beta-sitosterol, lanosterol, dihydrolanosterol, delta-4-cholesten-3-one, delta-3, 5-cholestadiene-7-one, and 7-dehydrocholesterol have been isolated and adequately characterized. Cholesterol is needed by the infant in challenging the development of cholesterol metabolizing enzymes and it contributes to synthesis of nerve tissue and bile salts. Cholesterol in cow milk is quite similar to that of human milk (140 mg/L) (Jelliffe and Jelliffe 1978). Early data on cholesterol in camel milk showed that it is the major sterol in camel milk fat (Farag and Kebary 1992).

Gorban and Izzeldin (1999) found that total cholesterol in camel milk fat was 313.2 mg/L versus 256.3 mg/L for cow milk fat. The cholesteryl ester of total cholesterol in colostrum was 10–18% versus 26–39% for normal milk. Meanwhile, saturated and unsaturated fatty acids represented 52% and 48% of cholesteryl esters in normal milk fat, respectively.

Milk Fat Globule Membrane Components

The fat globules of milk are each surrounded by a thin protective layer, usually called a *milk fat globule membrane (MFGM)*. MFGM consists of the typical bilayer membrane as the outer coat and the monolayer of proteins and polar lipid that covers the triacylglycerol core. The very existence of the fat globules depends on their membranes. Study of the membrane is very important for many practical problems. All interactions between fat and plasma must take place through the membrane. The total area is considerable and the membrane contains many highly reactive materials and enzymes; hence it can react in many ways. Also, the physical stability of the fat globules depends largely on the properties of the membrane. In spite of the relatively small quantities of the MFGM in milk, it plays an indispensable role in determining the properties of milk-fat-rich dairy products. These properties are related to the composition of MFGM. As shown in Table 6.9, phospholipid compositions of the MFGM of camel, cow, and buffalo milk are similar because phosphatidylserine and phosphatidylinositol are the minor components. But other major components, phosphatidylcholine, phosphatidylethanolamine, differ among the three species (Sharma and Ray 1982; Ahmed 1990; Jensen et al. 1991).

The milk fat globule membrane is comprised of three major phospholipid species—sphingomyelin, phosphatidyl choline, and phosphatidyl ethanolamine—with sphingomyelin accounting for between 18–20% of total phospholipids in milk (Avalli and Contarini 2005). The bioactivity of phospholipids, including sphingolipids, revealed that sphingomyelin reduces the number of colon tumors and inhibits the proliferation of colon carcinoma cell lines (Berra et al. 2002; Schmelz 2003;

Table 6.9. Phospholipids composition of the fat globule membrane (FGM) of milk of different species

	Amount (mol % of the Total Phospholipids)					
Species	PC	PE	PS	PI	SP	Reference
Camel	23.00	35.50	4.60	5.50	28.00	Ahmed (1990
Buffalo	27.90	29.42	12.91	4.97	21.39	Sharma and Ray (1982)
Cow	33.60	22.30	2.30	2.00	35.30	Jensen et al. (1991)

Spitsberg 2005). Meanwhile, it has also been shown to reduce cholesterol absorption in rats (Noh and Koo 2004). Ceramide and sphingosine are biologically active metabolites of sphingomyelin known to be important in transmembrane signal transduction and cell regulation, causing the arrest of cell growth and the induction of cell differentiation and apoptosis (Parodi 2001).

MFGM Proteins

MFGM proteins represent only 1–4% of total milk protein content; nevertheless, the MFGM consists of a complex system of integral and peripheral proteins, enzymes, and lipids. Despite their low classical nutritional value, MFGM proteins have been reported to play an important role in various cellular processes and defense mechanisms in the newborn (Cavaletto et al. 2008).

Mather (2000) reported that bovine MFGM proteins were identified by electrophoretic and immunochemical techniques to seven major proteins (Fig. 6.7). Their functions are listed in Table 6.10. Recently, the proteome of bovine MFGM profile was identified using peptide mass fingerprinting (PMF) via mass spectrometry (MS), or peptide sequencing via tandem MS (MS/MS) analysis (Fong et al. 2007; Reinhardt and Lippolis 2006). The composition of MFGM was 69–73% lipid and 22–24% protein. Among the identified minor proteins were the following: apolipoprotein A and E, lactoperoxidase, polymeric immunoglobulin receptor, a heat shock protein (71 kDa), clusterin, and peptidylprolyl isomerase.

MFGM was fractionated by electrophoresis, and digested gel slices were subjected to multidimensional liquid chromatographic methods combined with MS (LC MS/MS) for protein identification. Of the 120 proteins identified, 71% were membrane-associated, but 29% were cytoplasmic proteins (Reinhardt and Lippolis 2006). Smolenski et al. (2007) studied the proteome of bovine MFGM in

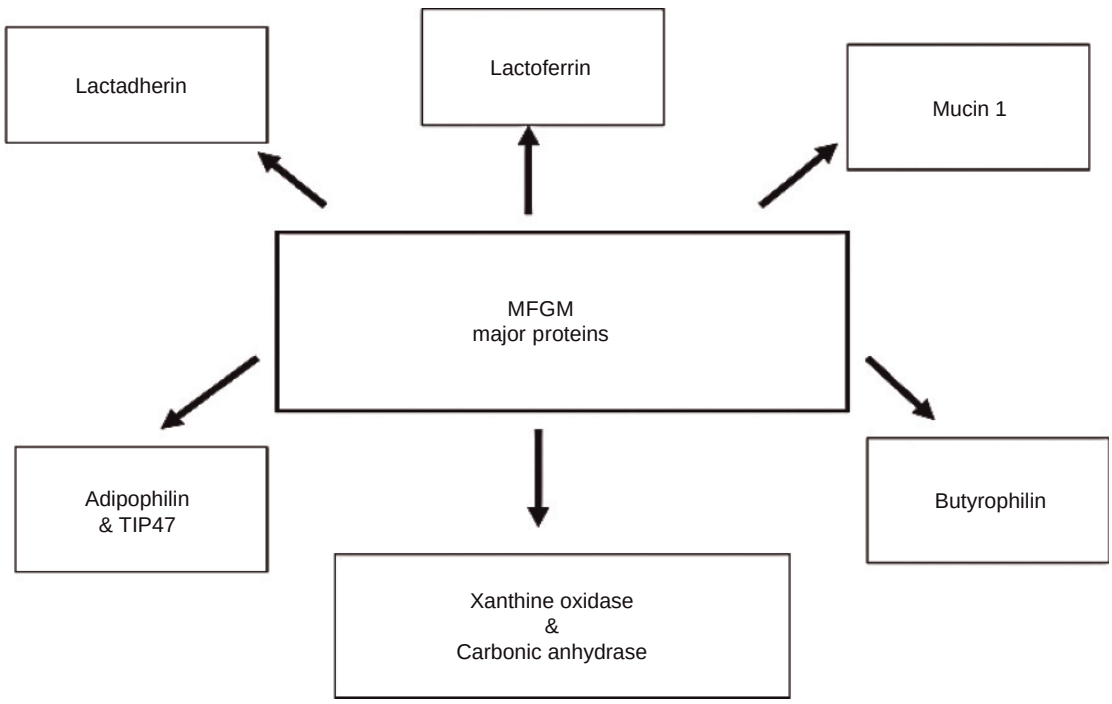

Figure 6.7. A schematic representation of different classes of major proteins of bovine milk fat globulin membrane (MFGM) (Mather 2000).

Table 6.10. Functions of bioactive major proteins of MFGM in milk

Protein	Function	References
Butyrophilin	Glycoprotein consists of two extracellular immunoglobulin-like domains. It has some receptorial function and modulates the encephalitogenic T cell response.	Cavaletto et al. (2002)
Carbonic Anhydrase	Essential factor in the normal growth and development of the gastrointestinal tract of the newborn.	Karhumaa et al. (2001); Quaranta et al. (2001)
Lactadherin	Promotes cell adhesion and is antiviral.	Quaranta et al. (2001)
Lactoferrin	Inhibits the classical pathway of complement activation and bacteriostatic action by competing with bacteria for iron.	Cavaletto et al. (2004); Smolenski et al. (2007)
Xanthine Oxidase	Involved in lipid globule secretion and defense protein and indispensable for milk fat secretion.	Mather (2000); Aoki (2006)
Mucin 1	Involved in protection against the attachment of fimbriated microorganisms.	Hamosh et al. (1999)
Adipophilin and TIP47	Structural role for lipid droplet packaging and storage. TIP47 involved in the trafficking of the mannose-6-phosphate receptor.	Fong et al. (2007); Fortunato et al. (2003); Sztalryd et al. (2006)

colostrum, mastitis, and normal milk at peak lactation: 95 distinct gene products were identified, comprising 53 proteins identified through direct LC-MS/MS and 57 through two-dimensional gel electrophoresis followed by MS. The study demonstrated that a significant fraction of minor proteins are involved in protection against infection.

ENDOCRINE FACTORS IN COLOSTRUM AND NORMAL MILK

Colostrum and normal milk contain hormones, growth factors, releasing factors, and cytokines (Blum 2006). Although their concentrations in milk are less than 1 mg/L, their activity needs only micro- or nanograms per liter to be achieved. These components have biological activity that influences both cell growth and immune function. These components are insulin, growth hormone (GH), prolactin (PRL), insulinlike growth factors (IGFs), IGF-binding proteins (IGFBPs) and glucagon (Gibson et al. 1998). Colostrum has a large amount of these bioactive proteins, peptides, and hormones to enable newborns in the first days. The concentration of these components are species differences. For example, IGFs are high in bovine colostrum (Blum and Hammon 2000), but components of the epidermal growth factor (EGF) family are higher in human colostrum than in bovine (Shing and Klagsbrun 1984).

Epidermal Growth Factors (EGF) are a family that shares a common structure and binds to the EGF receptor (EGFR) (Barnard et al. 1995). This family includes EGF, heparin-binding EGF-like growth factor, transforming growth factor-β (TGF- β1 and β2), amphiregulin, and betacellulin. It has been documented that EGF increases cell proliferation in vitro (Berseth 1987). The insulinlike growth factors (IGFs) are polypeptides with high sequence similarity to insulin. They are a part of a complex system consisting of ligands (IGF-1 and IGF-2) and their corresponding high-affinity cell-surface receptors (IGF-1R and IGF-2R), a family of six high-affinity

IGF-binding proteins (IGFBP 1–6), as well as associated IGFBP degrading enzymes. Based on studies on neonatal, it was found that IGFs can survive to a considerable extent in the small intestine (Xu et al. 2002). They can suppress the proteolytic degradation of lactase and its precursor, increase lactase synthesis, and reduce aminopeptidase activity (Burrin et al. 2001). Milk-derived TGF-β might be exploited in functional foods for the infant or during therapies for specific intestinal diseases or cancers. Active TGF-β molecules are highly conserved homodimeric proteins. The predominant form in bovine milk is TGF-β2. Biologically active TGF-β2 is a dimer of 224 amino acids (26 kDa) (Michaelidoua and Steijns 2006).

It was postulated that TGF-β2 in breast milk may modulate class II molecule expression on intestinal epithelial cells until the neonate is capable of handling the antigens to which it is exposed after weaning (Donnet-Hughes et al. 2000). Cell and animal studies have shown that growth factor preparations enriched in TGF-β2 may be responsible for protective effects on gut epithelial cells, due to the ability to arrest healthy gut cells in their growth cycle (Van Land et al 2002). Moreover, bovine colostrum contains cytokines such as interleukin-1b (IL-1b), IL-6, tumor necrosis factor (TNF-α), interferon-g(INF-g), and IL-1 receptor antagonist. Their levels fall markedly in mature milk (Hagiwara et al. 2000).

The amino acid sequence of isolated camel milk protein rich in half-cystine has been determined by peptide analyses (Beg et al. 1986a). The 117-residue protein has 16 half-cystine residues, which correspond to disulfide bridges and suggests a tight conformation of the molecule.

Comparisons of the structure with those of other proteins revealed that camel protein is clearly homologous with a previously reported rat whey phosphoprotein of possible importance for mammary gland growth regulation, and with a mouse protein of probable relationship to neurophysins. The camel, rat, and mouse proteins may represent species variants from a rapidly evolving gene. Residue identities in pairwise comparisons are 40% for the camel/rat proteins and 33% for the camel/mouse proteins, with 38 positions conserved in all three forms. Camel protein also reveals an internal repeat pattern similar to that for the other two proteins. The homology between the three milk whey proteins has wide implications for further relationships. Previously noticed similarities, involving either of the milk proteins, include limited similarities to casein phosphorylation sites for the camel protein, to neurophysins in repeat and half-cystine patterns for the mouse and rat proteins, and to an antiprotease for the rat protein. These similarities are reinforced by the camel protein structure and the recognition of the three whey proteins to be related. Finally, a few superficial similarities with the insulin family of peptides and with some other peptides of biological importance are recorded. Camel protein is suggested to be related with some binding proteins.

VITAMINS

Water-Soluble Vitamins

Extensive research in the last decade has suggested that deficiencies in B vitamins may be risk factors for vascular and neurological diseases and cancers (Brachet et al. 2004). There is growing evidence that a low folate status is linked to an increased cancer risk, particularly colon cancer (Rampersaud et al. 2002). It was reported that development of dementia and Alzheimer's disease among the elderly is due to a combined deficiency of folate and vitamin B12 (Seshadri et al. 2002). It was found that homocysteine metabolism is affected by folate, B6, and B12. An elevated level of plasma homocysteine is considered to be a risk factor for developing cardiovascular disease, the leading cause of mortality in most Western countries (Michaelidoua and Steijns 2006). Vitamins B_1, B_2, folic acid, and pantothenic acid are low in camel milk: its content of B_6 and B_{12} are quite similar to those of cow milk and higher than in human milk (Table 6.11). Camel milk is richer in niacin and vitamin C than cow milk. The high level of vitamin C in camel milk has been reported from several studies (El-Agamy 1983; Kon 1959; Knoess 1979; Farah et al. 1989, 1992; El-Agamy et al. 1998a; El-Agamy and Nawar 2000; Zhang et al. 2005). In comparison to the content of vitamin C in milk of other species, camel milk contained 52 mg/L versus 27, 22, 29, 16, 35, 49, and 61 mg/L for cow, buffalo, sheep, goat, human, donkey, and mares' milk, respectively (El-Agamy and Nawar 2000). It has been reported that camel milk contains 235–290 nmol/L of carnitin (vitamin B_T), which is 410 nmol/L more than cow milk (Alhomida 1996).

Table 6.11. Water-soluble vitamins in camel, cow, and human milk

Vitamin	Camel	Cow	Human
	mg/kg		
Thiamin (B_1)	0.33–0.60	0.28–0.90	0.14–0.16
Riboflavin (B_2)	0.42–0.80	1.22.0	0.36
Vitamin B_6	0.52	0.40–0.63	0.11
Vitamin B_{12}	0.002	0.002–0.007	0.0005
Niacin	4–6	0.5–0.8	1.47–1.78
Pantothenic acid	0.88	2.6–4.9	1.84–2.23
Folacin	0.004	0.01–0.10	0.052
Ascorbic acid	24–52	3–23	35–43

Adapted from Posati and Orr (1976); Ciba-Geigy (1977); Knoess (1977); Jelliffe and Jelliffe (1978); Sawaya et al. (1984); Farah et al. (1992).

Many milk lipid-soluble substances are bioactive, including vitamins and vitaminlike substances. Fat-soluble vitamins (A, D, E, and K) and carotenoids are known as highly lipophilic constituents in milk. They are obviously of great nutritional importance for the newborn. There are insufficient data available on contents of camel milk lipid-soluble vitamins. It is well known that in bovine milk, the content of fat-soluble vitamins is influenced by different factors as breed, parity, lactation period, production level, and health status (Baldi 2005). The contents of vitamin K and D are affected by the exposure of dairy animals to sunlight, but the type of forage affects the contents of vitamin E and A (McDowell 2006).

The compositions of fat-soluble vitamins in camel, cow and human milk are shown in Table (6.12). Vitamin A in camel milk ranged between 100 to 380 μg/L versus 170–380 and 540 μg/L for cow and human milk, respectively. The low level of vitamin A, compared with human milk, is a disadvantage in the composition of camel milk. A balanced diet with camel milk as basic foodstuff should consider this aspect. The problem of the low level of vitamin A results from the fact that green vegetables are a minor part of the diet in arid areas. It is well known that vitamin A is mainly present in milk in esterified form, and the mammary gland takes up retinol derived from the liver, esterifies it, and secretes it in milk (Tomlinson et al. 1974). Other sources of milk vitamin A are retinol esters derived from dietary β-carotene and dietary retinol. Milk also contains carotenoids, mainly β-carotene, which yield vitamin A. Vitamin A plays a central role in many essential biological processes including vision, growth and development, immunity, and reproduction (Debier and Larondelle 2005). Vitamin A can function as a scavenger of singlet oxygen and may also react with other reactive oxygen species (Baldi et al. 2006). Vitamin A has been suggested to play a role in the morphogenesis, differentiation, and proliferation of the mammary gland, probably via interactions with growth factors. Carotenoids are also important for immune function and fertility as well as inhibition of enzymes involved in carcinogenesis (Chew and Park 2004, Stahl and Sies 2005). Data on vitamin E in camel milk revealed that it has 530 μg/L versus 200–1300 and 2800 μg/L for cow and human milk, respectively (Jenness 1980; Sawaya et al. 1984; Belitz et al. 2004). Vitamin D contents in Bactrian camel milk samples on days 30 and 90 of lactation were 692 and 640 IU/L, respectively (Zhang et al. 2005), but no data are available on vitamin D in dromedary camel milk.

Table 6.12. Fat-soluble vitamins in camel, cow, and human milk

Milk	Vitamin A μg/L	Vitamin E μg/L	Vitamin D μg/L
Camel	100–380	530	—
Cow	170–380	200–1300	0.60–25
Human	540	2800	0.70

Adapted from Jenness (1980); Sawaya et al. (1984); Belitz et al. (2004); Zhang et al. (2005).

Vitamin E is also low in camel milk compared with cow and human milk. It has been documented that vitamin E is able to prevent the free radical-mediated tissue damage; therefore, it prevents or delays the inflammatory development (Baldi et al. 2006). It was found that tocotrienols, as a member of the vitamin E family, possess powerful neuroprotective, antioxidant, anticancer, and cholesterol-lowering properties (Sen et al. 2006). It has been reported that vitamin K may have protective actions against osteoporosis, atherosclerosis, and hepatocarcinoma (Kaneki et al. 2006).

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7
Bioactive Components in Mare Milk

Qinghai Sheng and Xinping Fang

INTRODUCTION

Horses can live in many diverse environments, where they have evolved in many different ways. The earliest ancestor of the modern horse (*Hyracotherium*) lived around 60 million years ago; its front feet had four toes, and its back feet had three (Futuyma 1986). Due to climatic changes, the vegetation changed as well, and thereby the body of the species continually evolved. Eventually, around 4 million years ago, Equus evolved by elongating its legs, altering its molars, and developing hooves. Modern horse feet have a single hoof (Edwards 1994).

Clutton-Brock (1987) once said that a tame animal differs from a wild one, in that it is dependent on man and will stay close to humans of its own free will. A variety of theories have been developed purporting to explain the origin of horse domestication throughout the whole 20th century. Horses were accompanied by well-preserved equipment, such as bridles, saddles, and harnessing in some of the south Siberian Iron Age tombs of Kurgans—Pazyryk, Bashadar, and Ak-Alakha (Park et al. 2006). Domestic horses and carriages appeared in the late Shang Dynasty in China, but archaeological findings of horses before that time are scarce (Levine 1987). The eneolithic (3600–2300 B.C.) Botai culture of the Eurasian Steppe region in northern Kazakhstan was heavily dependent on the horse for subsistence. No evidence exists that domestic crops were raised. Remote sensing using electrical resistivity and magnetic field gradient imaging revealed a large number of possible pit houses and post molds at the Krasnyi Yar site. Many of the post molds are found in near-circular and semicircular arrangements, suggesting horse corrals or stockades. Soil from a Copper Age site in northern Kazakhstan yielded new evidence for domesticated horses up to 5,600 years ago (Stiff et al. 2006). However, where, when, and for what purpose wild horses were domesticated by humans is still indistinct. There is no doubt that horses were caballine and that they were ridden or used for traction. A wild horse would be buried with a chariot. During the early stages of horse domestication, they were usually ridden without the use of a saddle or bridle (Levine 1987).

Now there are hundreds of different breeds of Equus all over the world. Many have been through the process of artificial selection by humans and have been bred to perform specific tasks, such as pull heavy loads, jump high obstacles, or gallop fast. Dairy horses are mainly located in the former USSR and in Mongolia. They are found in Kazakhstan, Kirghizia, Tadzhikistan, Uzbekistan, in some parts of Russia near Kazakhstan (Kalmukia, Bachkiria), and in Mongolia and its periphery (Buryatia in Siberia, Inner Mongolia in North China). Dairy horses are also found in Tibet and Xinjiang. To a lesser extent, horses have been used as dairy herds in Eastern Europe (Belarus, Ukraine) and central Europe, especially Hungary, Austria, Bulgaria and Germany (Doreau and Martin-Rosset 2002; Park et al. 2006).

If milking is accepted by mares, any horse breed can be developed into a milking heard. Thus, different breeds of dairy horses are used for mare milk production in the world. Several native Kazakh breeds, weighing 500–600 kg, are found being used for for milk production in the former USSR and

Mongolia (Park et al. 2006). In Mongolia, a number of crossed breeds are being used to produce milk (Doreau and Martin-Rosset 2002). Haflinger horses are the most important dairy breed, with an adult weight of 500 kg, and are famous for milk production capacity in European countries (Park et al. 2006). As an example, Haflinger mares have been machine milked 3 times per day near Heidelberg, Germany. These mares are milked for commercial fluid milk and yogurt production all year around where mare milk is being used in local therapy and for sale. Figures 7.1a and 1b show machine milking of dairy horses in Germany.

Besides being the most important nutritional resource for foals during the first month of life, mare milk is also one of the most important basic foodstuffs for some human populations in several regions of the world. From the early 20th century it was popular in Germany where it was delivered door to door, and it is also now back fashion in France, Belgium, Austria, and Holland in addition to Germany. Mare milk attracts health-conscious consumers because of the properties of high levels of vitamins and minerals, better digestibility, and lower fat content than cow milk (Chapman 2004).

CHEMICAL COMPOSITION

Different breeds of dairy horses are used for mare milk production in the world. Research results concerning mare milk composition are shown in Table 7.1. The gross composition of different breeds shows ranges (fat, 0.5–2.0; protein, 1.5–2.8; lactose, 5.8–7.0; and dry matter, 9.3–11.6 g/100 g milk) reported by Solaroli et al. (1993). When comparing it with human and cow milk, the characteristics of mare milk, such as high levels of polyunsaturated fatty acids and low nitrogen and cholesterol contents, suggest that it is of interest for use in human nutrition (Massimo et al. 2002).

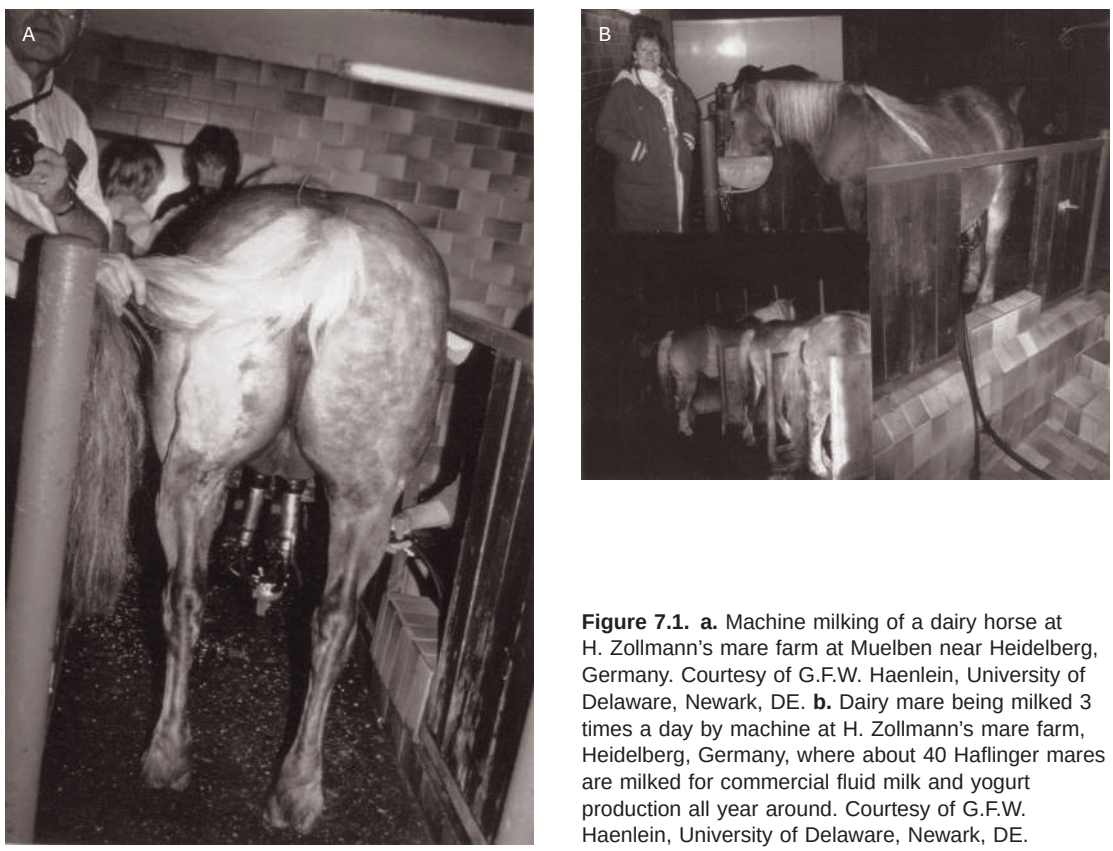

Figure 7.1. a. Machine milking of a dairy horse at H. Zollmann's mare farm at Muelben near Heidelberg, Germany. Courtesy of G.F.W. Haenlein, University of Delaware, Newark, DE. **b.** Dairy mare being milked 3 times a day by machine at H. Zollmann's mare farm, Heidelberg, Germany, where about 40 Haflinger mares are milked for commercial fluid milk and yogurt production all year around. Courtesy of G.F.W. Haenlein, University of Delaware, Newark, DE.

Table 7.1. Concentrations of some components in mare milk in different breeds (g/100 g of milk)

Breed	Days Postpartum	Fat	Protein	Lactose	Dry Matter
Quarter Horse[a]	61	0.27–0.39	1.45–1.69	—	9.63–9.92
Andalusian[b]	—	2.36–2.43	1.88–3.89	5.41–6.58	13.40–12.17
Standardbred[c]	—	0.31–2.35	1.77–4.30	6.08–7.44	9.74–13.55
Arabian[b]	—	1.89–2.01	2.35–3.33	5.57–6.13	11.76–12.10
Shetland pony[d]	125	1.8	1.9	—	9.8
Przewalski[e]	—	2.2	2.0	6.1	10.5

[a]Adapted from Kubiak et al. (1989).
[b]Adapted from Fuentes-Garcia et al. (1991).
[c]Adapted from Mariani et al. (1993).
[d]Adapted from Lukas et al. (1972).
[e]Adapted from Jenness and Sloan (1970).

Table 7.2. Casein distribution of mare milk in comparison to human and cow milk

	Mare	Human	Cow
Casein (g/kg^{-1})	10.7	3.7	25.1% of total casein
α_s-casein (%)	46.65 (40.2–59.0)	11.75 (11.1–12.5)	48.46[a] (48.3–48.5)
β-casein (%)	45.64 (40.1–51.4)	64.75 (62.5–66.7)	35.77 (35.8–37.9)
κ-casein (%)	(7.71)[b]	23.50 (22.2–25.0)	12.69[c] (12.7–13.8)
Micelles size (nm)	255	64	182

Mean value and, in parentheses, range values reported in literature.
References cited include Amit et al. (1997), Gobbi (1993), and György et al. 1954.
[a]38.46 α_{s1}-casein and 10.00 α_{s2}-casein.
[b]κ-casein and other fractions not characterized.
[c]100% was reached with γ-casein fraction (3.08%).
Adapted from Malacarne et al. (2002) and Park et al. (2006).

Mare milk is quite similar to human milk in composition of the major protein components, as shown in Table 7.2 (Malacarne et al. 2002; Park et al. 2006). Caseins of mare milk are mainly composed of equal amounts of β-casein and α_s-casein (Ochirkhuyag et al. 2000). Cow milk has a higher casein content than mare and human milk, where the former is defined as *caseineux* milk in French (Pagliarini. 1993; Solaroli et al. 1993; Csapo et al. 1995; Malacarne et al. 2002). Proteins of mare milk are made of 40–60% caseins, which are close to the proportion in human milk (40%) and much less than in the milk of other dairy species (Doreau and Martin-Rosset 2002).

Mare and human milk contain comparable levels of whey protein in toto and NPN content. Horse milk has approximately 10% nonprotein nitrogen (range; 8–15%), which is 2 times higher than in cow milk and half the amount of human milk (Doreau and Martin-Rosset 2002). The noncasein nitrogen is higher in mare milk with respect to both whey protein and NPN fractions (Marconi and Panfili 1998). The free amino acid fraction of mare milk is especially rich in serine and glutamic acid (Doreau and Martin-Rosset 2002).

Research has shown that whey protein represents about 40% of mare milk, containing 2–19% serum albumin, 25–50% α-lactalbumin, 28–60% β-lactoglobulin, and 4–21% immunoglobulin after the colostral stage (Doreau and Martin-Rosset 2002; Miranda et al. 2004). As in cow milk, mare milk contains β-casein and γ-casein, which represents 50% and less than 10% of total casein, respectively. Mare milk also contains α_{S1}- and α_{S2}-caseins as 40% of total caseins, and a small quantity of κ-casein. It is evident that mare milk is very similar to human milk with respect to the ratio between casein and whey proteins, which is important for digestibility. High casein content of cow milk causes the formation of a firm coagulum in the stomach, which requires 3–5 hours for digestion. Mare and human milk form a finer and softer precipitate, and the

evacuation time of the stomach is about 2 hours (Kalliala et al. 1951). It has a much lower thermal sensitivity than bovine milk, making mare milk less sensitive to thermal sanitation processes. From this point of view, considerable advantages have been found in using mare milk in infant feeding (Bonomi et al. 1994).

The fat content of mare milk is very low when compared to human and cow milk. It is poorer in triglycerides (78–80%) and richer in free fatty acids (9–10%) and phospholipids (5–19%) than cow and human milk (Doreau and Boulot 1989). According to the analysis of the triglyceride compositions of the milk fats of mares and other species, the milk fat of mare milk is largely of medium-chain fatty acids. It is different from the milk fat of humans, which has a high amount of long-chain fatty acids, and the milk fat of cows, which contains significant levels of short-chain fatty acids (Breckenridge and Kuksis 1967).

The higher lactose content and lower casein in mare milk, while compared with human and cow milk, also plays a major role in sensory characteristics, which are quite different from cow milk. Mare milk is more translucent and less white, has a sweet and at the same time somewhat harsh taste, and has an aromatic flavor (Massimo et al. 2002). Heger (1988) reported that mare milk has a typical coconut flavor. In some physical characteristics, such as density, osmotic pressure, and freezing point, mare milk is similar to cow milk, but it has lower electric conductivity, viscosity, and titrable acidity than cow milk. Additionally, the reaction of fresh mare milk is alkaline with very few exceptions when the milk is neutral (Vieth 1885).

The importance of minerals in mare milk to the nutritional demands by foals is sufficient and mare milk can provide the needed minerals even in the absence of other nonmilk food resources (Schryver et al. 1986a). The elements represent different functions. Sodium acts as an important cation in blood and extracellular fluid bathing cells and potassium is a monovalent cation significant to the maintenance of fluid integrity within the cell. Especially, bone development of the foals requires abundant calcium and phosphorus from the milk. Magnesium is also a part of the mineralized bone, although considerably less concentrated in the bone than are calcium and phosphorus (Martuzzi et al. 1997; Anderson 1991). Variations in major minerals of different breeds of dairy horses are shown in Table 7.3. In addition to the contents of iron in the milk of Italian saddle horses and copper in Bardigiano horse milk exceeding the ranges and the contents of magnesium and zinc in Arabian, and Malopolski milk showing a lower level, most element contents are in the ranges summarized by Doreau and Martin-Rosset (2002). Calcium content in Italian Saddle milk is similar to that in Bardigiano horse milk, but higher than in other breeds.

Vitamin contents of mare milk collecting at different postpartum days (Table 7.4) revealed that colostrum contains 2.6, 1.7, and 1.5 times higher vitamin A, D_3, and K_3 contents, respectively, com-

Table 7.3. Average macro and micro elements content of mare milk (mg/kg of milk).

Mare Breed	Days Postpartum	Ca	P	Mg	Na	K	Zn	Fe	Cu
Arabian and Malopolski[a]	—	—	394	29	—	—	0.89	1.46	0.25
Quarter Horse[b]	3–180	787	504	75	171	701			
Przewalski[c]	85–250	804	419	62	137	344	1.9	1.1	0.23
Thoroughbred[c]	6–120	811	566	53	140	410	1.9	0.27	0.25
Shetland pony[c]	6–120	857	418	77	127	250	1.7	—	0.37
Bardigiano[d]	5–35	1220	668	—	198	662	2.79	1.06	1.06
Italian saddle[d]	5–35	1155	678	—	167	573	2.95	1.47	0.73
Haflinger[e]	4–180	802	593	77	181	443	—	—	—

[a]Adapted from Kulisa (1986).
[b]Adapted from Anderson (1991).
[c]Adapted from Schryver et al. (1986b).
[d]Adapted from Martuzzi et al. (1998).
[e]Adapted from Summer et al. (2004).

Table 7.4. The vitamin contents of mare milk

VA[a] (mg/kg)	VD_3[a] (mg/kg)	VE[a] (mg/kg)	VK_3[a] (mg/kg)	VC[a,b] (mg/kg)	VB_{13}[d] (μg/mL)
0.88[1], 0.34[2]	0.0054[1], 0.0032[2]	1.342[1], 1.128[2]	0.043[1], 0.029[2]	23.8[1], 17.2[2], 17.2[3,*], 12.87[4]	2[6]
VB_1[b] (mgL)	VB_2[b] (mgL)	B_{12}[c] (μgL)	Nicotinic acid[b] (mgL)	Pantothenic acid[b] (mgL)	Folic acid[c] (μgL)
0.24[3], 0.39[4]	0.26[3], 0.11[4]	0.02[5]	0.72[3], 0.72[4]	2.77[4]	1.3[5]

*mgL.

[a]Adapted from Park et al. (2006).

[b]Adapted from Holmes et al. (1946).

[c]Adapted from Collins et al. (1951).

[d]Adapted from Larson and Hegarty (1979).

[1], [2], [3], [4], [5], and [6] show 0–0.5 postpartum days, 8–45 postpartum days, 1.5 postpartum month, 2–4 postpartum months, 10 postpartum days, and not identified postpartum day, respectively.

pared to the milk from 8–45 days of lactation. Vitamin B_1, B_2, and nicotinic acid contents collected at different lactation stages are 0.24 versus 0.39 mgL. 0.26 versus 0.11 mgL, and 0.72 versus 0.72 mgL, respectively. Vitamin B_{12} (0.02 μgL), B_{13} (2 μgL), pantothenic acid (2.77 mgL), and folic acid (1.3 μgL) also are reported. Vitamin C content is higher in colostrum (23.8 mg/kg), and then decreases to 17.2 mg/kg (Park et al. 2006; Holmes et al. 1946) and 12.87 mg/kg (Holmes et al. 1946) throughout lactation. Compared to human and cow milk, vitamin C content in mare milk is much richer, although no significant differences are observed in other vitamins. Moreover, vitamin C appears to be very resistant to oxidation, which is of great nutritional importance (Servetnik-Chalaya and Mal'tseva 1981; Solaroli et al. 1993).

BIOACTIVE COMPONENTS

In addition to providing the building blocks and energy necessary for growth, mare milk also has many specific functions, such as protection against infection and as metal carriers (Table 7.5). The potential role of these bioactive components in supplements for health promotion and disease prevention is important to the field of public health.

Protein

Whey protein distributions of mare milk compared to human and cow milk are shown in Table 7.6. Although whey protein is absent from human milk, mare and bovine milk contain significant amounts of β-lactoglobulin. In contrast to other dairy species, mare milk is rich in lysozyme and lactoferrin (Doreau and Martin-Rosset 2002). The lactoferrin content of mare milk is particularly high at 0.2–2 g/kg milk, which is 10 times higher than in cow milk and slightly lower than in human milk (Doreau and Martin-Rosset 2002). Detailed bioactivities of mare milk whey proteins are discussed below.

α-Lactalbumin

The presence of α-lactalbumin (La) from the milk of Australian horses and the colostrum of Persian Arab mares has been demonstrated and the primary structures were compared (Godovac-Zimmermann et al. 1987). Girardet et al. (2004) also reported the multiple forms of equine α-La. Approximately 1% of α-lactalbumin (α-La) is *N*-glycosylated, and two nonglycosylated α-La isoforms with similar molecular masses were found. They correspond to a nonenzymatic deamidation process. Deamidation induces a slight variation in secondary structure content, but no significant change in the tertiary structure of the equine α-La.

A characteristic property of La is a remarkable distortion on the surface disulfide between the 6-120 disulfide bond. It is easy to obtain a 3SS-La, which has only three disulfides and a nativelike structure with a retained function in lactose synthesis. Such abnormal disulfide also is found in equine α-lactalbumin (ELA), but is a typical α-lactalbumin. This structure may be used to explain why

Table 7.5. Specific bioactive functions of the components in mare milk

Component	Function
Protein[a1–a7]	
α-lactalbumin	Lactose synthesis; Ca carrier; cell lytic activity
β-Lactoglobulin	Retinol-binding activity
Immunoglobulins	Immune protection
Lactoferrin and lactotransferrin	Iron-chelating activity
Serum albumin	Lipid-binding activity
Fat[b1–b5]	
Sn-2 position palmitic acid	Benefiting assimilation
Linoleic and α-linolenic acids	Precursors of ω-3 and ω-6; allergy inflammation protection
Long-chain polyunsaturated fatty acids	Vasodilatory or vasoconstrictive effects; needed for brain and retinal function; precursors of eicosanoids
Phospholipids	Part of all living cells
CLA	Potential anticarcinogen
Carbohydrate[c1–c2]	
Galactose	Ensuring galactose levels
Oligosaccharides	Benefiting assimilation; bacteria infection inhibition; Bifidobacterium growth and/or metabolism stimulation; T-antigen
Enzyme[d1–d8]	
Lysozyme	Bacteriostatic and calcium-binding activities
Lipase	Fat lipolysis
Plasmin	β-casein hydrolyzation
Dehydrogenase	Lactic acid dehydrogenation
Aminotransferase	Influencing carbohydrate metabolism; mediating glutathione; amino acid decomposition and synthesization
Hormone and growth factor/protein[e1–e6]	
Insulin and insulinlike growth factor I	Promoting growth; favoring normal cell development; central nervous system protection;
Prolactin binding protein	Inhibitors or potentiators of prolactin-action
Parathyroid hormone-related protein	Modulating bone metabolism; participating in cell differentiation and proliferation
Triiodothyronine (T3) and 5′-monodeiodinases	Supporting lactogenesis; action within the intestinal tract
Progestogen	Diagnosing early pregnancy and postpartum estrous cycles
Leptin	Modulating appetite and energy consumption
Others[(f1–f7)]	
Bifidus factor	Activating *Lactobacillus bifidus* var. *Penn* growth
Carnitine	LCPUFA-binding activity
Lactadherin/epidermal growth factor	Antibacterial activity; promoting epidermal cell mitosis; inhibiting stomach acid secretion
Amyloid A	Neonatal intestine protection
Interleukin-1	Antiinflammatory activity
Lactic acid bacteria	Probiotics

[a1–a7]Adapted from Conti et al. (1984), Kaminogawa et al. (1984), Masson and Heremans (1971), McKenzie and White (1987), Pearson et al. (1984), Pérez et al. (1993), and Sugai et al. (1999), respectively.

[b1–b5]Adapted from Jahreis et al. (1999), Koletzko and Rodriguez-Palmero (1999), Massimo et al. (2002), Orlandi et al. (2006), and Winter et al. (1993), respectively.

[c1–c2]Adapted from Glöckner et al. (1976) and Newburg and Neubauer (1953), respectively.

[d1–d8]Adapted from Chilliard and Doreau (1985), De Oliveira et al. (2001), Egito et al. (2003), Hideaki et al. (1992), Nabukhotnyĭ et al. (1988), Neseni et al. (1958), Rieland et al. (1998), and Solaroli et al. (1993), respectively.

[e1–e6]Adapted from Cai and Huang (2000), Dore et al. (1997), Grosvenor et al. (1992), Hunt et al. (1978), Philbrick et al. (1996), and Slebodziński et al. (1998), respectively.

[f1–f7]Adapted from Bach (1982), Duggan et al. (2008), Francois (2006), György et al. (1954), Murray et al. (1992), Newburg et al. (1998), and Ying et al. (2004), respectively.

Table 7.6. Whey protein distributions of mare milk compared to human and cow milk

	Mare		Human[a]		Cow[b]	
True whey protein (g/kg^{-1})	8.3		7.6		5.7	
β-lactoglobulin (%)	30.75	(25.3–36.3)	Absent		20.10	(18.4–20.1)
α-lactalbumin (%)	28.55	(27.5–29.7)	42.37	(30.3–45.4)	53.59	(52.9–53.6)
Immunoglobulins(%)	19.77	(18.7–20.9)	18.15	(15.1–19.7)	11.73	(10.1–11.7)
Serum albumin(%)	4.45	(4.4–4.5)	7.56	(4.5–9.1)	6.20	(5.5–76.7)
Lactoferrin (%)	9.89		30.26		8.38	
Lysozyme (%)	6.59		1.66		Trace	

[a]Adapted from Lönnerdal (1985).
[b]Adapted from Solaroli et al. (1993).
Proteose-peptone fraction is not included in the table.

α-lactalbumin can interact with the enzyme *N*-acetyllactosaminide α-1,3-galactosyltransferase (EC 2.4.1.151), modifying its carbohydrate-binding properties in a way that promotes the transfer of galactose to glucose resulting in lactose synthesis (Kaminogawa et al. 1984; Sugai and Ikeguchi 1994; Godovac-Zimmermann et al. 1987).

The ability of α-lactalbumin to bind Ca^{2+} has recently been recognized. High resolution X-ray structure of α-lactalbumin reveals a Ca^{2+}-binding fold (Godovac-Zimmermann et al. 1987). Sugai et al. (1999) showed that the 6-120 disulfide stabilizes the tertiary structure in the holo and apo equine lactalbumin, and Ca^{2+} stabilizes it more markedly than the disulfide. Ca^{2+} or the 6-120 disulfide bond affects the stability of the tertiary structure of equine lactalbumin somewhat more markedly than for bovine lactalbumin.

Equine, human, bovine, and rat α-La represent trace cell lytic activity. Through analyzing the reaction kinetics of α-La with corresponding milk lysozymes, their lytic activities are not likely to result from trace lysozyme content. Thus, a weak cell lytic activity seems to be inherent to α-La (McKenzie and White 1987).

β-Lactoglobulin

β-lactoglobulin isolated from horse colostrum is heterogeneous and contains two components: β-lactoglobulin I and β-lactoglobulin II. Horse β-lactoglobulin II contains 166 amino acids, and it is unlike other β-lactoglobulins, which contain 162 amino acids. The additional four amino acids insert between positions 116 and 117 of other β-lactoglobulins so far sequenced, including horse β-lactoglobulin I. β-lactoglobulin II in mare milk shows structural homology with human retinol-binding protein. This may reveal similar biological functions and clues to the origin of milk proteins (Conti et al. 1984; Godovac-Zimmermann et al. 1985).

Immunoglobulin

With regard to equine colostral immunoglobulins, Genco et al. (1969) reported that the ratio of the three major serum immunoglobulins IgG, IgG(T), and IgM occur in the same ratio as they do in serum. However, Rouse and Ingram (1970) reported that IgG is the predominant immunoglobulin in colostrum of mare milk, and secretory IgA either does not occur in colostrum or makes only a minor contribution to the total immunoglobulin; in human milk, secretory IgA is the predominant immunoglobulin. The mean concentration of IgG in colostrum of the different breeds of horses also was examined. Arabian mares have higher IgG at the time of parturition than the average for Thoroughbreds. The average lapsed time of colostral IgG decreases to certain amounts for Arabian mares is significantly longer than for Thoroughbred mares. The higher content and longer duration of IgG in Arabian mare milk will show more immune function for the foals (Pearson et al. 1984).

Lactoferrin and Lactotransferrin

Sørensen and Sørensen (1939) first described a red protein fraction from bovine milk, which is later

shown to consist of two types of iron-binding components: a protein homologous to serum transferrin and a component absent in plasma. The latter was designated thereafter as *lactoferrin*. Masson and Heremans (1971) presented that mare milk shares this property. Lactotransferrin is similar to lactoferrin in metal-chelating properties, binding two atoms of iron and 2 molecules of bicarbonate involved in the reaction. However, they have different molecular weights and different physicochemical properties.

The preliminary crystallographic structure of mare lactoferrin was analyzed and several crystal forms were obtained (Sharma et al. 1996). Lactoferrin in colostrum/mare milk is an iron-binding glycoprotein with a molecular weight of 80 kDa. The protein has two iron-binding sites. It has two structural lobes, each housing one Fe^{3+} and the synergistic CO_3^{2-} ion. Mare lactotransferrin also has been purified and analyzed. Its molecular mass is 81 kDa (Jollès et al. 1984).

Serum Albumin

The ability to bind lipids of serum albumin and β-Lg of horse, human, pig, guinea pig, and sheep whey protein was examined by Pérez et al. 1993. Results showed that sheep β-Lg and serum albumin from all species have the ability to bind fatty acids in vitro. Albumin from horse contains approximately 2.9 mol fatty acids/mol protein. While β-Lg of mare milk does not share the ability of ruminant β-Lg to bind fatty acids, it has neither fatty acids physiologically bound nor the ability to bind them in vitro. In mare milk, albumin is the only whey protein detected with bound fatty acids as in human and guinea pig milk.

Fat

The triglyceride structure is a major factor influencing the action of lipase enzymes for fat absorption. In mare milk palmitic acid ($C_{16:0}$) is preferentially associated with the sn-2 position (Parodi 1982). It is similar to the $C_{16:0}$ in human milk, which is considered favorably by some authors for the assimilation of this fatty acid in children (Winter et al. 1993).

Mare milk seems to contain higher contents of linoleic (LA) and especially α-linolenic (ALA) polyunsaturated fatty acids, usually called essential fatty acids (EFA) and, respectively, precursors of ω-3 and ω-6, than cow milk. The prostaglandins (like PGI) with vasodilatory effects, tromboxans (like TXA) with vasoconstrictive effects, and docosahexaenoic acid (C22:6n-3, DHA) needed for brain and retinal development can derive from a-linolenic (C18:3n-3) and eicosapentaenoic (C20:5 n-3, EPA) acids. From linoleic acid (C18:2 n-6) derives many other prostaglandins (PGI2) and tromboxans with different influences on the circulatory system through the modulation of prostaglandin and leucotriene production. In addition to the inhibition of cellular activation and cytokine secretion, as well as the alteration of the composition and function of the epidermal lipids barrier, EFA also show the possibility to affect allergic inflammation. A deficit of n-6 EFA leads to inflammatory skin condition in both animals and humans (Uauy and Hoffman 2000). Male rats fed with EFA of mare milk show significant increase in the immunocompetent system and nonspecific resistance (Valiev et al. 1999). Grant et al. (1988) reported that linoleic acid in human milk is a precursor of prostaglandin E in the prevention of gastric ulcers. Moreover, some LC-PUFA are precursors of eicosanoids, which have a biological activity to modulate various cellular and tissue processes (Koletzko and Rodriguez-Palmero 1999).

Mare milk has higher levels of phospholipids than cow and human milk. Compared to human milk, phospholipids of mare milk are relatively richer in phosphatidylethanolamine (31% vs 20%) and in phosphatidylserine (16% vs 8%), but less rich in phosphatidylcholine (19% vs 28%) and phosphatidylinositol (trace vs 5%), while sphingomyelin is similar (34% mare vs 39% human). Being the components of the lipoprotein layers of the cell membrane, phospholipids consisting mainly of polyunsaturated fatty acids, are present in all living cells in particular of neural cells (Massimo et al. 2002).

Conjugated linoleic acids (CLA) refer to a class of positional and geometric isomers of linoleic acid. Research with cow milk indicates CLA as antioxidative and anticarcinogenic agents (Parodi 1999). The potential anticarcinogenic CLA, *cis*-9, *trans*-11 C18:2 also is determined in mare milk, despite the fact that mare milk is nearly CLA-free (mean value 0.09% of total fatty acids) (Jahreis et al. 1999).

Carbohydrate

Lactose is found in the milk of most mammals as the major source of carbohydrates, and mare milk has higher lactose than human and cow milk. Because lactose is found almost exclusively in milk, the presence of large amounts of lactose in conjunction with traces of other specific carbohydrates may favor colonization of the infant intestine by organisms more able to split lactose. This could result in a symbiosis in which favorable microflora are established that compete with and exclude many potential pathogens.

Galactose, once digested and absorbed by the infant, can be converted to glucose by epimerization and used for energy. Mare milk contains a significant amount of galactose in the form of lactose. The presence of galactose in milk could be related to some unique requirement common to all young, growing mammals. Most young mammals are undergoing a period of rapid brain development and myelination, which requires large amounts of galactosylceramide (galactosylcerebrosides) and other galactolipids. The liver is assumed to be capable of providing all of the galactose required for synthesis of galactolipids, but many adult liver functions are underdeveloped in the young. Milk galactose probably ensures galactose levels in the infant to be limited in galactosylceramide (galactocerebroside) production, which in turn limits optimal myelination and brain development. Milk galactose can play a unique role in providing the requirements of the rapidly developing infant brain (Newburg and Neubauer 1953).

In mare milk fat globules are coated with three layers: an internal protein layer, an intermediate layer consisting of a phospholipidic membrane, and an external layer consisting of high-molecular-weight glycoproteins. At the surface of glycoproteins there is a branched oligosaccharide structure, which is similar to human milk and is not found in cow milk. Branched glucosides of fat globules may slow down the movement of fat globules in the intestine, thus ensuring larger exposure to bile salts and lipases (Solaroli et al. 1993). Three neutral oligosaccharides—Galβ1-4GlcNAcβ1-3Galβ1-4Glc (HM-3a), Galβ1-4GlcNAcβ1-6Galβ1-4Glc (HM-3b), and Galβ1-4GlcNAcβ1-3[Galβ1-4GlcNAcβ1-6]Galβ1-4Glc (HM-5)—were obtained from horse colostrum by Urashima (1991). HM-3b and HM-5 have been found in human milk as lacto-*N*-neotetraose and lacto-*N*-neohexaose, respectively. HM-3b also has been isolated from goat milk. These oligosaccharides show significant inhibition to bacteria infection and stimulation to the growth and/or metabolic activity of bifidobacterium. One alkali-labile oligosaccharide was isolated from mare milk fat globule membranes. It is a tetrasaccharide containing the disaccharide (β-D-galactosyl (1-3)-N-acetyl-D-galactosamine) plus 2 molecules of sialic acid. Hemagglutination inhibition tests against human anti-T serum by desialylated glycoproteins show the presence of the T-antigen in mare milk fat globule membranes, and inhibition is proportional to the chemically detected amounts of disaccharides (Glöckner et al. 1976).

Enzymes

Lysozyme

Lysozyme in mare milk was identified by many methods (Jáuregui-Adell 1971; Jáuregui-Adell et al. 1972; Noppe et al. 1996). A minor *N*-glycosylated form of lysozyme was also found in equine milk (Girardet et al. 2004). The amino acid sequence of equine milk lysozyme has been elucidated. It consists of a single chain of 129 amino acid residues and has 14,647 kDa. Although equine milk lysozyme has the essential features of a c(chick)-type lysozyme, there is only 51% sequence homology with human milk lysozyme and 50% with domestic hen egg white lysozyme (McKenzie and Shaw 1985). Lysozyme in mare milk is thermostable in vitro. After a heat-treatment at 82 °C for 15 minutes, lysozyme in mare milk has 68% and in human milk only 13% residual activity (Jáuregui-Adell 1975).

The secondary and tertiary structures of equine lysozyme were very similar to those in bovine lactalbumin (Sugai and Ikeguchi 1994). Similarities in amino acid sequences, three-dimensional structures, and the exon-intron patterns of their genes have indicated that c-type lysozymes and alpha-lactalbumins are homologous proteins, i.e., descended by divergent evolution from a common ancestor (Bell et al. 1980; Acharya et al. 1994; Katsutoshi 2002). Like the alpha-lactalbumins, mare milk lysozymes all bind Ca^{2+}. The structure of calcium-binding equine

lysozyme was determined by means of molecular replacement (Hideaki et al. 1992; Tsuge et al. 1991; Zeng et al. 1990).

Equine lysozyme plays an important role in milk coagulation. It also can catalyze the hydrolysis of glycosidic bonds in the alternation β (1–4) linked copolymer of N-acetyl-D-glucosamine and N-acetyl-D-muramic acid, a constituent of bacterial cell walls. The antibacterial action of lysozyme is generally due to this lysis. The exact mechanism of its role in the defense of the infant gut from pathogens needs further development (De Oliveira et al. 2001; Solaroli et al. 1993).

Lipase

Neseni et al. (1958) first reported lipase activity in mare milk, which has lipase in very small amounts. The following research by Chilliard and Doreau (1985) reported that mare milk contains serum-dependent lipase activity. It is thermolabile and NaCl sensitive and has characteristics of lipoprotein lipase (LPL) (E.C. 3.1.1.34). Most long-chain and polyunsaturated fatty acids derive from blood triglycerides after hydrolysis by mammary LPL. Milk LPL might originate from mammary LPL. The significance of LPL activity in mare milk fat lipolysis and milk flavor during storage and processing needs further study.

Plasmin

Equine β-casein and to a lesser extent α_{s1}- and κ-caseins are good substrates for plasmin in solution. β-casein is readily hydrolyzed at the Lys^{47}–Ile^{48} bond to produce γ-like caseins of 23 kDa. These γ-like caseins are degraded by plasmin in solution subsequently to their formation. The presence of low amounts of endogenous plasmin in equine milk associated with equine caseinate may explain why γ-caseins are generated when a sodium caseinate solution is incubated at 37 °C and pH 8.0 for 24 hours or when caseinate is stored in a freeze-dried form at 7 °C for 2 years. The presence of PP5-like peptides of 20 kDa associated with caseins seems to arise from endogenous plasmin activity in equine milk (Egito et al. 2003), which could explain the significant presence of γ-like caseins. Humbert et al. (2005) showed that a large plasmin activity is in fresh mare milk. The mare milk stored at low or negative temperature has more soluble plasmin activity than at room temperature. They also reported that the protocols usually applied to bovine milk or equine blood plasminogens could not activate plasminogen in mare milk to plasmin.

Dehydrogenase

Lactic acid dehydrogenase (LDH) is an enzyme that helps produce energy. It is present in almost all of the tissues in the body. In the milk of breeding mares during the course of lactation, the enzyme activity of lactic dehydrogenase (LDH) was reported by Rieland et al. (1998). In mare milk, the LDH-activity is highest on the first day, shows a marked decrease on the third day, is followed by a slight decrease until the 20th day, and then remains at a constant level of about 80 UL.

Aminotransferase

The enzyme activities of γ-glutamyltransferase (γ-GT), glutamate-oxaloacetate transaminase (GOT) and glutamic-pyruvic transaminase (GPT), which were in the milk of breeding mares during the course of lactation, were reported by Rieland et al. (1998). The γ-GT-activity and the GPT-activity is highest on the third or first day, respectively, and decreases during lactation. The GOT-activity of the first day is higher than in mature milk. The γ-GT and GOT activity also is detected in cow colostrum and mature milk (Zanker et al. 2001). These enzymes of milk participate in amino acid decomposition and synthesization; influence carbohydrate metabolism, thus producing favorable effects on the adaptation processes in newborns; and mediate glutathione thus favoring the supply of ascorbic acid to bronchial cells (Corti et al. 2008; Nabukhotnyĭ et al. 1988).

Hormone and Hormone-Related Growth Factor/Protein

Insulin and Insulinlike Growth Factor I

In mare milk, concentrations of immunoreactive insulin (iI) and immunoreactive insulinlike growth factor I (iIGF-I) are high in first colostrum and then decrease drastically; the iI and iIGF-I changes in colostrum and milk occur in parallel. Colostrum iI and iIGF-I in mare milk behave similarly to those in

cow milk, because IGF-Is in mare and cow appear to be bound to proteins of similar molecular weight (Hess-Dudan et al. 1994). IGFs and insulin, in addition to their growth-promoting actions, are considered to play important roles in the development and maintenance of normal cell functions throughout life (Slebodziński et al. 1998). Direct binding in the central nervous system by insulin may relate to IGF (Dore et al. 1997).

Prolactin-Binding Proteins

The soluble prolactin-binding protein (PRL-BP) in mare milk is highly specific for the lactogenic hormones. This prolactin receptor belongs to the newly defined superfamily of cytokine receptors. The significance of PRL-BP in mare milk may be viewed as possible inhibitors or potentiators of hormone action by analogy with other polypeptide hormone-binding proteins, regulating growth and secretory functions of maternal mammary tissue and growth, development, and maturation of the neuroendocrine, reproductive, and immune systems in the suckling neonate (Amit et al. 1997; Grosvenor et al. 1992).

Parathyroid Hormone-Related Protein

The parathyroid hormone-related protein (PTHrP) in mare milk was reported by Care et al. (1997). Actually, PTHrP was first isolated from malignant cell tissues correlated with hypercalcemia. The following research showed that PTHrP can modulate bone metabolism and participate in cell differentiation and proliferation (Amizuka et al. 2002; Philbrick et al. 1996).

Triiodothyronine and 5′-monodeiodinases

The thyroid hormone triiodothyronine (T3), generated locally by 5′-monodeiodinase (5′-MD) in the mammary tissues in mare colostrum and milk, was measured by Slebodziński et al. (1998). Mare milk also shows the presence of propylthiouracil (PTU)-sensitive (type I) and PTU-insensitive (type II) 5′-monodeiodinases (5′-MD). The presence of 5′-MD of type II suggests that intramammary T3 generation may play a paracrine role supporting lactogenesis. Little amounts of colostral T3 are consumed daily by a suckling foal, and thus the T3 hormone action within the intestinal tract cannot be ruled out.

Progestogen

Progestogen in mare milk was examined (Allen and Porter 1987; Bailes and Holdsworth 1978; Gunther et al. 1980). Monitoring mare milk progestone can be used for evaluation of postpartum estrous cycles. It also shows useful aids in the diagnosis of early pregnancy (Borst et al. 1985; 1986; Hunt et al. 1978; Laitinen et al. 1981).

Leptin

Romagnoli et al. (2007) compared the leptin concentration in plasma and in mare milk during the interpartum period and showed that leptin concentration in the colostrum and milk has been significantly higher than in plasma samples at week 1 and between weeks 12 and 17. Leptin participates in body weight regulation. It can serve as an adiposity signal to inform the brain of the adipose tissue mass in a negative feedback loop regulating food intake and energy expenditure. Leptin also plays important roles in angiogenesis, immune function, fertility, and bone formation (Cai and Huang 2000).

Others

Bifidus Factor

The bifidus factor occurrence in mare milk and the milk of other species was reported by György et al. (1954). Mare milk is slightly richer in the growth factor with activity for growth of *Lactobacillus bifidus* var. *Penn* than the milk of ruminants, but it is still far below the level found in human milk.

Carnitine

When long-chain fatty acids are bound to acylcarnitine, the fatty acids can penetrate into the mitochondria to be oxidized. This oxidation is important during the neonatal period. It increases the role of lipids to meet the energy needs for the babies and maintain normal body temperature. Carnitine, especially acylcarnitine also is detectable in mare milk and the concentration of long-chain acylcarnitine in mare milk was under 1%, with similar functions (Bach 1982; Penn et al. 1987).

Lactadherin/Epidermal Growth Factor

Lactadherin, also known as milk fat globule epidermal growth factor 8 (MFG-E8), is expressed abundantly in lactating mammary glands in stage- and tissue-specific manners and has been believed to be secreted in association with milk fat globules. It has been shown that human milk lactadherin prevents symptoms in breast-fed infants infected with rotavirus by binding to the virus and inhibiting its replication. (Nakatani et al. 2006; Newburg et al. 1998). Bruner et al. (1948) found a lower level of antibacterial agglutinins in mare milk compared with colostrum. The epidermal growth factor (EGF)-like activity also was measured in mare colostrum and milk (Murray et al. 1992). The multiple physiological functions of EGFs from other sources, such as promoting epidermal cell mitosis and inhibiting stomach acid secretion, may also belong to the EGF-like activities in mare milk.

Amyloid A

Duggan et al. (2008) reported that Amyloid A3 (AA3) was found in equine colostrum and early milk at consistently higher concentrations than in peripartum maternal serum. There was no correlation between serum AA and colostrum AA3 concentrations at parturition. The production of this protein in the mammary gland is likely to be under a different stimulus than the production of serum AA and may have protective effects in the neonatal intestine.

Interleukin-1

Francois (2006) showed the antiinflammatory activity of an equine milk fraction selected from skim milk, lactoserum, and sodium caseinate. The bioactive component is claimed for having interleukin-1 (IL-1) to produce inhibiting activity.

Lactic Acid Bacteria

In mare milk, 191 lactic acid bacteria were isolated and they were classified into 10 groups. The isolates from mare milk consisted mainly of coccus: *Leuconostoc (Leuc.) mesenteroides, Leuc. pseudomesenteroides, Lc. garviae, Lc. Lactis* ssp. *lactis, Streptococcus (Sc.) parauberis* and *Enterococcus (Ec.) faecium*. The isolation rates were 45, 19, 7, 8, 16, and 6%, respectively. These lactic acid bacteria are useful as probiotics for humans and animals (Ying et al. 2004).

SPECIFIC UTILIZATION OF WHOLE MARE MILK

There are many bioactive components in mare milk, as mentioned above. Table 7.7 describes the specific utilizations of whole mare milk relating to the function of one bioactive component or the conjunct functions of different bioactive components.

The effect of mare milk consumption on functional elements of phagocytosis of human neutrophil granulocytes from healthy volunteers was researched by Ellinger et al. (2002). Results showed that drinking frozen mare milk modulated inflammation processes by decreasing chemotaxis and respiratory burst, which might be favorable for giving relief to diseases with recurrent inflammation. Heart failure, chronic hepatitis, tuberculosis, and peptic ulcer diseases are also treated by mare milk. Mirrakhimov

Table 7.7. Specific utilization of whole mare milk

Property	Utilization
Health[a1–a6]	Treating recurrent inflammation
	Treating series of cutaneous diseases
	Treating heart failure
	Treating chronic hepatitis; tuberculosis
	Treating peptic ulcer
	Treating hair loss
Nutrition[b1–b5]	Substitute for cow milk for the nutrition of cow milk allergic human infants
	Culturing equine blastocysts in vitro as part of the medium

[a1–a6]Adapted from Bliesener (2000), Ellinger et al. (2002), Mirrakhimov et al. (1986), Sharmanov et al. (1981), Sharmanov et al. (1982), and Zhangabylov and Salkhanov (1977), respectively.

[b1–b5]Adapted from Businco et al. (2000), Curadi et al. (2001), Lebedev and Lebedeva (1994), Muraro et al. (2002), and Robles et al. (2007), respectively.

et al. (1986) once used natural mare milk in the combined treatment of refractory heart failure. The therapeutic action of whole mare milk in clinical and laboratory findings was more remarkable in patients with chronic hepatitis (Sharmanov et al. 1982). After participating in the diet therapy with whole mare milk, peptic ulcer (in stomach or duodenum) patients had a complete wound healing (Sharmanov et al. 1981; Zhangabylov and Salkhanov 1977). Mare milk also shows pronounced antacid properties. Tuberculosis has often been treated with mare milk, which increases the number of erythrocytes and lymphocytes, and restores a normal erythrocyte sedimentation rate (Doreau and Martin-Rosset 2002). The effectiveness of Stutenmilch (mare milk) in the treatment and care of skin has been well known for a long time. Bliesener (2000) showed that when a composition of 50 weight percentage Stutenmilch, 25,000 I.E. retinol as acetate, and 150 I.E. α-tocopherolacetate were laid on the scalp, the diseased hair loss and further appearance of seborrhea-like excessive tallow gland isolation, shed, and scalp itching were no longer observed.

Gobbi (1993) reported pharmaceutical and dermocosmetic compositions containing equine colostrums, when applied on the skin, were particularly effective in the treatment of burns (caused by fire or sunlight), contact lesions (caused by jelly fishes or, generally, by vesicant agents), and cutaneous diseases. In the ratios specified above, the equine colostrum and milk may also be formulated into cosmetic compositions having antiburn, antiaging, and repairing activities. In northern Europe, mare milk is a treatment for acne sufferers and is becoming increasingly popular as an alternative treatment of various diseases such as psoriasis and atopic eczema (Fanta and Ebner 1998).

Cow milk allergy is a common disease of infancy and early childhood. If the baby is not breast-fed, a substitute for cow milk formula is necessary. The allergenicity of mare milk in children with cow milk allergy was researched. Twenty-five children with severe IgE-mediated cow milk allergy were studied. The results showed that all children showed strong positive skin test responses to cow milk and two children had positive skin test responses to mare milk. These data suggest that mare milk can be regarded as a good substitute for cow milk in most children with severe IgE-mediated cow milk allergy. Equidae milk might therefore be modified for use as a safe and adequate substitute for cow milk for the nutrition of cow milk–allergic human infants (Businco et al. 2000; Curadi et al. 2001; Muraro et al. 2002; Robles et al. 2007), but Gall et al. (1996) reported a case of allergic reaction to mare milk. Because mare milk is being increasingly used as "alternative medicine" for treatment of various ailments, more and more allergic reactions to this milk can be expected (Fanta and Ebner 1998).

In addition to the multiple uses for humans, mare milk has been used in a medium containing egg yolk, mare milk, and/or modified PBS for culturing equine blastocysts. Most of the blastocysts recovered nonsurgically. The percent content of mare milk and PBS was interchangeable in the experiment. After transfer of one cultured blastocyst per mare, two mares became pregnant and one lost the fetus at 9 months, while the other carried the fetus to term and foaled normally (Lebedev and Lebedeva 1994).

CONCLUSIONS

Mare milk has beneficial nutritive values and characteristics, such as high content of unsaturated fatty acids, vitamin C, and optimal Ca/P ratio. In addition, the aforementioned bioactive components—including enzymes, LC-PUFA, oligosaccharides, hormone and growth factor/protein, and carnitine—place mare milk as a high-quality natural product of great food interest for children, the elderly, and debilitated or convalescent consumers. This would be attributable not only to the composition of mare milk, which is close to that of human milk, but also to the fact that some countries have a long tradition of using mare milk as a substitute for human milk or as medicine. Since most people feel much better after drinking horse milk, it is possible that cow milk could be replaced by mare milk as a preferred beverage milk, if the latter is readily available.

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Section II
Bioactive Components in Manufactured Dairy Products

8
Bioactive Components in Caseins, Caseinates, and Cheeses

Ryozo Akuzawa, Takayuki Miura, and Hiroshi Kawakami

INTRODUCTION

Many milk-derived components have multifunctional properties. Caseins are currently the main source of a range of biologically active peptides. Peptides with the sequence of the parent protein can be released by enzymatic hydrolysis during gastrointestinal digestion or cheese ripening. Casein-derived peptides are regarded as highly prominent ingredients for health-promoting, functional foods of pharmaceutical preparations.

This chapter discusses the current knowledge on beneficial effects of casein-derived peptides and cheese on human health.

CASEIN AND ITS INDUSTRIAL PRODUCTION

Casein

Casein is composed of more than one protein (α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein), inorganic materials, and citric acid, which in the aggregate is called a *casein micelle* (Table 8.1).

For the casein micelle, some structural models have been proposed based on various studies (Walstra 1999; Holts 1992), none of which is entirely certain. However, the main points about the casein micelle structure are as follows: The casein micelle has a spherical shape, with smaller micelles having similar proportions to those of κ-casein (Fox 1992). It changes into a smaller casein micelle of 10–20 nm in diameter, if a chelating agent is added to the solution containing the casein micelle. The micelle precipitates when chymosin, which has substrate specificity on κ-casein (Phe105-Met106), is made to act on the casein (Fox 1992).

The primary structure of each casein has the following common property (Otani 2005): It has a phosphoserine residue and there is a relatively high proline content. The hydrophilic and hydrophobic amino acids are localized. The α_{s1}-casein, α_{s2}-casein, and β-casein, which have a phosphoserine concentration region, precipitate under calcium coexistence. They are called *calcium receptivity caseins*. On the other hand, κ-casein, which has only one phosphoserine, does not precipitate under the calcium coexistence (Waugh and von Hippel 1956). It is called *calcium non-receptivity casein*. The precipitation of the calcium receptivity caseins does not occur in milk, which is very high in calcium, because the surface of the casein micelle is covered with glycomacropeptides, which are abundantly hydrophilic (Swartz et al. 1991).

Casein has been considered a valuable amino acid supply source since ancient times because it is a protein with good digestibility. In the latter half of the 1970s, a variety of bioactive peptides has been isolated from a digestion of casein (Korhonen and Pihlanto et al. 2006) (Silva and Malcata 2005). The latency of the bioactive peptides in bovine casein and the casein as the ingredient that becomes the supply source of peptide are described in the following sections.

Acid Casein

Acid casein is produced from skim milk by direct acidification, usually with an acid solution, or by

Table 8.1. Composition of casein micelles in bovine milk

Component	Content (g/100 g Micelles)
Casein	93.3
α_{s1}-casein	35.6
α_{s2}-casein	9.9
β-casein (include γ-casein)	35.9
κ-casein	11.9
Mineral	6.24
Calcium	2.87
Magnesium	0.11
Sodium	0.11
Potassium	0.26
Inorganic phosphate(PO_4)	2.89
Citrate	0.4

Schmidt (1982).

fermentation with a *Lactococcus* culture to pH 4.6 (Fox and McSweeney 1998).

Direct acidification is achieved by using hydrochloric, phosphoric, or lactic acid, where hydrochloric acid is the most common method used worldwide for producing acid casein.

The fermentation method is performed by inoculation of milk with lactic acid-producing bacteria. *Lactococcus lactis* ssp. *lactis* or *cremoris*, commonly known as starters, convert some of the lactose in the milk to lactic acid during an incubation period of about 16–18 hours.

Rennet Casein

Rennet casein is precipitated from skim milk by the action of calf rennet or some other suitable milk-clotting enzyme. It is insoluble in water or alkali, but can be solubilized by treatment with polyphosphates.

A milk-clotting enzyme is added to coagulate the skimmed milk, usually at 30°C. The casein curd particles subsequently shrink to exclude whey. The curd is washed several times in warm water after whey is excluded from the curd by cooking. Rennet casein is calciumparacaseinate with a high content of colloidal calcium phosphate (Southward 2002).

Caseinate

Caseinates are produced by the neutralization of acid casein with alkali. All caseinates are substantially water soluble and are typically prepared as a solution of about 20% solids prior to spray drying. Roller-dried caseinates may be prepared from more concentrated solutions. Sodium caseinate is the most common product and is prepared by mixing a solution of sodium hydroxide and bicarbonate or carbonate with acid casein curd or dry acid casein suspended in water and then drying the resultant solution.

On a moisture-free basis, it has a slightly lower protein content than the acid casein from which it is made due to the sodium incorporated into it by the reaction of the casein with sodium hydroxide. Typical compositions of casein and sodium caseinate are shown in Table 8.2.

PRODUCTION OF BIOACTIVE PEPTIDES FROM CASEIN

Opioid Peptide

Opioid peptides are the peptides that play a key role in many physiological phenomena such as analgesia action, euphoria, stomachaches, and allaying anxiety.

In general, opioid peptides derived from caseins can be classified into two types based on their biofunctions. The α- and β-casein fragments produce agonist responses; those derived from κ-casein elicit antagonist effects. Such functions appear through the receptor located in the nervous, endocrine, and immune systems (Teschemacher et al. 1994). Therefore, this is an important structural motif that fits into the binding site of opioid receptors. The major exogenous opioid peptide fragments derived from β-casein are called β-*casomorphins* because of their morphinelike behavior (Clare and Swaisgood 2000) and have been characterized as μ-type receptors (Teschemacher et al. 1997). The first report in 1979 (Henschen et al. 1979) mentioned a β-casein-derived opioid, BCM-7, which can subsequently be metabolized to β-casomorphin 5 (BCM-5), and which has the strongest relative affinities to opioid receptors (Shah 2000). Opioid peptides derived from α-casein are called *exorphins*, which have been isolated from the peptic hydrolysates of α-casein fractions (Loukas et al. 1983) and characterized as δ-type receptors. In

Table 8.2. Compositional standards for casein products[a]

Composition	Acid Casein	Rennet Casein	Sodium Caseinate
	Amount/100 g		
Moisture (g)	12.0 max	12.0 max	8.0 max
Fat (g)	2.0 max	2.0 max	2.0 max
Protein (g) (nitrogen × 6.38) dry basis	90.0 min	84.0 min	88.0 min
Ash (g) (phosphorus fixed)	2.5 max	7.5 min	—
Lactose (g)	1.0 max	1.0 max	1.0 max
	Amount/1 kg		
Copper (mg)	5 max	—	5 max
Lead (mg)	5 max	—	5 max
Iron (mg)	20 max	—	20 max
	Amount/1 g		
Free acid (mL 0.1 N NaOH)	0.27 max	—	—
pH	—	—	8.0 max

[a]Codex Standard A-18 (1995).

Table 8.3. Opioid peptides derived from casein

	Casein Segment	Peptide Sequence	Enzyme	Peptide Name	Reference
Agonistic peptides	α_{s1} (90–95)	RYLGYL	Pepsin	Exorphin	Loukas et al. 1983
	α_{s1} (90–96)	YLGYLE			
	α_{s1} (91–95)	YLGYL			
	β (60–66)	YPFPGPI	Pepsin	β-Casomorphin-7	Henschen et al. 1979
	β (60–64)	YPFPG	Pepsin	β-Casomorphin-5	Henschen et al. 1979
	γ (114–121)	YPVEPFTE	Trypsin		Perpetuo et al. 2003
Antagonistic peptides	κ(35–42)	YPSYGLNY		Casoxin A	Meisel and FitzGerald 2000
	κ (58–61)	YPYY	Trypsin	Casoxin B	Meisel and FitzGerald 2000
	κ (25–34)	YIPIQYVLSR		Casoxin C	Meisel and FitzGerald 2000

general, their structures differ considerably from those of β-casomorphins. These fragments of α- and β-casein function as opioid agonists, whereas all κ-casein fragments called casoxins behave as opioid antagonists. Casoxins bind to κ-type receptors. Table 8.3 lists several opioid peptides based on their casein type and their biofunction.

Antihypertensive Peptides

Antihypertensive peptides have been well researched in various bioactive peptides and have been put to practical use as a functional food. The function of these peptides is to inhibit the angiotensin-converting enzyme (ACE). ACE (EC 3.4.15.1) is a

zinc-containing metalloenzyme associated with blood pressure adjustment.

Blood pressure is controlled by a number of different interacting biochemical pathways. Major blood pressure regulation has been associated with the renin-angiotensin system (RAS). RAS begins with the inactive precursor angiotensinogen, which is the only known precursor of angiotensin I as well as the only known substrate for rennin (EC 3.4.23.15). Renin acts on angiotensinogen and. as a result, releases angiotensin I, which is further converted into the active peptide hormone angiotensin II by the ACE (Fitzgerald et al. 2004).

Consequently, it increases blood pressure and aldersterone, thus further inactivating the depressor action of bradykinin. The inhibition of ACE is effective for the regulation of blood pressure. A great number of ACE-inhibitory peptides have been isolated from the enzymatic digest of various milk proteins. These casein-derived peptides, which are known as *casokinins*, exhibit sequences that have been found in α_{s1}-, β- and κ-casein.

These ACE-inhibitory peptides are generated with trypsin and pepsin; others are generated with the lactic acid bacterium or enzyme treatment and are included in dairy products such as cheeses or fermented milk. In vitro incubation of milk proteins with gastrointestinal proteinase preparations in pepsin, trypsin, and chymotrypsin activities results in the release of ACE-inhibitory peptides. Maruyama and Suzuki (1982) and Meisel and Schlimme (1994) reported that tryptic hydrolysates of casein correspond to f23–24 and f23–27 of α_{s1}-casein, as well as to f177–183 and f193–202 of β-bovine casein. Recently, Perpetuo et al. (2003) showed that the new ACE-inhibition peptides Glu-Met-Pro-Phe-Pro-Lys (f108–113) and Tyr-Pro-Val-Glu-Pro-Phe-Thr-Glu (f114–121) were generated by tryptic hydrolysis of γ-casein.

The bacterial proteinases can also be used to release ACE-inhibitory peptides. Pihlanto-Leppälä et al. (1998) detected ACE-inhibitory peptides from casein protein using fermentation with lactic acid bacteria and then hydrolysis by digestive enzymes. These peptides were identified as being from α and β-casein. *Lactobacillus helveticus* strains were capable of releasing ACE-inhibitory peptides into fermented soft drinks, such as the two potent casokinins derived from β-casein, which correspond to Val-Pro-Pro (f84–86) and Ile-Pro-Pro (f74–76). These peptides were found in "Calpis", a skim milk fermented soft drink with *Lactobacillus helveticus* CP790 and *Saccharomyces cerevisiae*. In a placebo-controlled trial with mildly hypertensive subjects, a significant reduction in blood pressure was recorded after a daily ingestion for 4 weeks of 95 mL of sour milk containing two such peptides(Hata et al. 1996). Another recent study (Ashar and Chand 2004) showed that a new casokinin Ser-Lys-Val-Tyr-Pro (f) was found in "Dahi" fermented milk, which was made from bovine milk fermented by *Lb. derbrueckii* ssp. *bulgaricus*, *Str. thermophilus* and *Lc. lactis* ssp. *lactis* biovar. diacetylactis.

Many studies have shown that ACE-inhibitory peptides can also be produced during cheese-making (Meisel et al. 1997). Recently, Ryhänen et al. (2001) detected a low-fat cheese containing an ACE-inhibitory peptide. In general, it appears that such an ACE-inhibitory peptide increases during cheese ripening. However, long-term ripening promotes the degradation of ACE-inhibitory peptides. As referred to above, the ability of ACE in vitro is indicative of the potential of a given casokinin to act as a hypotensive agent in vivo. However, there are various regulators of blood pressure in vitro, such as the kallikrein-kinin system, the natural endopeptidase system, and the endothelin-converting enzyme system that have been shown to generate additional vasoregulatory peptides independent of ACE (Fitzgerald et al. 2004). Therefore, more detailed studies will be required to improve our understanding of the blood pressure–reducing mechanisms of casein-derived peptides (Table 8.4).

Antithrombotic Peptides

The antithrombotic peptides derived from κ-casein are also considered as one of the factors that affect the cardiovascular system. The clotting of blood and the clotting of milk are two physiologically important coagulation processes and have been proved to be similar.

The first step in blood clotting is the aggregation of platelets that form into a "mesh," which adheres to the wound site (Minors 2007). The secondary step is the interaction of fibrinogen with thrombin. Fibrinogen is cleaved by thrombin to form fibrin, which is polymerized and binds to the mesh (more exactly, to a specific binding site on the ADP-activated platelet surface) to form a clot (Minors 2007).

Table 8.4. Antihypertensive peptides derived from casein

Casein Segment	Peptide Sequence	Enzyme	Reference
α_{s1} (1–9)	RPKHPIKHQ	Protease in festivo cheese	Ryhänen et al. 2001
α_{s1} (23–24)	FF	Trypsin	Maruyama and Suzuki 1982
α_{s1} (23–27)	FFVAP	Trypsin	Maruyama and Suzuki 1982
α_{s1} (102–109)	KKYKVPQ	Protease in manchego	Gomez-Ruiz et al. 2002
α_{s1} (104–109)	YKVPQL helveticus CP790	Protease from lactobacillus	Maeno et al. 1996
α_{s1} (142–147)	LAYFYP and Trypsin	Starter + Pepsin	Pihlanto-Leppälä et al. 1998
α_{s1} (157–164)	DAYPSGAW and Trypsin	Starter + Pepsin	Pihlanto-Leppälä et al. 1998
α_{s1} (194–199)	TTMPLW	Trypsin	Maruyama and Suzuki 1982, Pihlanto-Leppälä et al. 1998
α_{s2} (174–179)	FALPQY	Trypsin	Tauzin et al. 2002
α_{s2} (174–181)	FALPQYLK	Trypsin	Tauzin et al. 2002
β (74–76)	IPP	Protease from *Lactobacillus helveticus*, *Saccharomyces cerevisiae*	Nakamura et al. 1995a,b
β (84–86)	VPP	Protease from *Lactobacillus helveticus*, *Saccharomyces cerevisiae*	Nakamura et al. 1995b
β (177–183)	AVPYPQR	Trypsin	Maruyama et al. 1985
β (198–202)	TKVIP	Trypsin	Maeno et al. 1996
β (193–198)	YQEPVL	Starter + Pepsin and Trypsin	Pihlanto-Leppälä et al. 1998
β (193–202)	YQEPVLGPVRGPFPI	Trypsin	Maruyama and Suzuki 1982
β (199–204)	GPVRGPFPIIV	Protease in manchego	Gomez-Ruiz et al. 2002
κ (108–110)	IPP	Protease from *Lactobacillus helveticus*, *Saccharomyces cerevisiae*	Nakamura et al. 1995a,b
γ (108–113)	EMPFPK	Trypsin	Perpetuo et al. 2003
γ (114–121)	YPVEPFTE	Trypsin	Perpetuo et al. 2003

The coagulation of milk depends on the interaction of κ-casein with rennin or chymosin. Thus, the enzymatic hydrolyses are necessary for these processes to unfold. Jollès et al. (1978) reported that the human fibrinogen γ-chain (f400–411) and the peptide fragments from bovine κ-casein (f106–116) are structurally and functionally quite similar. The κ-casein–derived peptides are known as *casoplatelins*.

Other casoplatelins (f106–110, f106–112, and f113–116) obtained from κ-casein are inhibitors of both the aggregation of ADP-activated platelets and the binding of the human fibrinogen γ-chain to its specific receptor region on the platelet surface. However, a fragment (f103–111) inhibiting platelet aggregation is unable to prevent fibrinogen from binding to ADP-treated platelets (Fiat et al. 1993).

Although the potential physiological effects of these antithrombotic peptides have not been determined, bovine and human κ-caseinoglycopeptides, two antithrombotic peptides derived from the corresponding κ-caseins, have been detected in physiologically active concentrations in the plasma of newborn infants after ingestion of a cow's-milk–based formula or human milk, respectively (Table 8.5). It is suggested that these two bioactive peptides are released from milk proteins during digestion.

Immunomodulatory Peptides

The immune system is involved in the human body's defense mechanism, which consists of stimulation of the immune system and inhibition of microbial activity by bioactive peptides.

Table 8.5. Antithrombotic peptides "casoplatelins" from casein

κ-Casein Segment	Peptide Sequences	Enzyme	Reference
103–111	LSFMAIPPK	Trypsin	Fiat et al. 1993
106–110	MAIPP	Trypsin	Fiat et al. 1993
106–116	MAIPPKKNQDDK	Trypsin	Schlimme and Meisel 1995
106–112	MAIPPKK	Trypsin	Schlimme and Meisel 1995
113–116	NQDK	Trypsin	Schlimme and Meisel 1995

Immunomodulating peptides have been found to stimulate the proliferation of human lymphocytes. Jollès et al. (1981) reported that the Val-Glu-Pro-ile-Pro-Lys fragments of trypsin-hydrolyzed human β-casein (f54–59) possess immunostimulating activity. Subsequently, other immunomodulating peptides were isolated, such as f63–68, f191–193, and f193–209 from bovine β-casein and f194–199, f1–23 from bovine α_{s1}-casein, by trypsin or chymosin hydrolysates (Migliore-Samour 1989). Those peptides derived from casein hydrolysates were shown to increase the phagocytotic activity of human and murine macrophages in vitro, and the immunostimulatory activity against *Klebsiella pneumoniae* was demonstrated in vivo (Migliore-Samour 1989). The small peptides from κ-casein increased the proliferation of human peripheral blood lymphocytes in vivo (Kayser and Meisel 1996). Caseinphosphopeptides (CPPs) released both in vitro and in vivo by gastrointestinal trypsin from α_{s1}-, α_{s2}-, or β-casein, also showed immunomodulatory activity. Hata et al. (1999) studied a commercially available CPP-III, consisting mainly of f1–32 from bovine α_{s2}-casein and f1–28 from β-casein. CPP-III is a stimulation lipopolysaccharide, phytohemagglutinin. Concavalin A enhances the proliferative response.

The antimicrobial activity of milk is mainly attributed to immunoglobulins, casein, lactoferrin, lactoperoxidase, and lysozyme (Table 8.6). Isolated antibacterial fragments have also been derived from α_{s1}-, α_{s2}-, or κ-casein (Lahov and Regelson 1996). Casocidin-I (f165–203) derived from α_{s2}-casein can inhibit the growth of *Escherichia coli* and *S. carnosus* (Zucht et al. 1995). Two other peptides were recently isolated from α_{s2}-casein (f183–207 and f164–179), depending on the target microorganism. The inhibitory concentrations of their peptides are different. These peptides exhibited inhibition against Gram-positive and -negative bacteria with minimal inhibitory concentrations ranging from 8–95 μmol/L (Recio and Visser et al. 1999).

Other peptides derived from α_{s1}-casein (f1–23) (called *isracidin*) have demonstrated antibiotic activity in vivo, and it can be used against mastitis (Lahov and Regelson 1996).

Metal-Binding Peptides

Some casein-derived peptides are able to bind to metal ions, thus acting as biocarriers in vitro. It has been shown that caseinophosphopeptides (CPPs) play the role of metal carriers. CCPs have negatively charged side chains of those amino acids, which represent the binding sites for minerals (Meisel 1998).

CCPs have been shown to bind to metals such as Ca, Mg, Fe, Zn, Ba, Cr, Ni, Co, and Se. Limiting the precipitation of calcium in the distal ileum especially enhances calcium absorption. However, the calcium-binding ability is different for each CCP fraction from casein. According to Meisel (1998), that can be attributed to the influence of additional amino acids around the phosphorylated binding site. Moreover, the high concentration of negative charged amino acid of CPPs contributes to its resistance to further proteolysis. CPPs have also been found after in vitro and in vivo digestion of α_{s1}-, α_{s2}-casein and β-casein.

The metal binding ability of CCPs can have a positive impact on human health. It has been shown that they can induce anticariogenic effects by promoting the recalcification of tooth enamel. These characteristics have been incorporated into dental care products that are now commercially available (Adamson and Reynolds 1995; Reynolds 1997).

Table 8.6. Immunomodulating and antimicrobial peptide sequences in the primary structure of caseins

κ-Casein Segment	Peptide Sequences	Enzyme	Biological Activity	Reference
α_{s1} (1–23)	RPKHPIKHQGL PQEVLNENLLRF	Trypsin or Chymosin	Immunomodulating and antimicrobial	Minkiewicz et al. 2000
α_{s1} (194–199)	TTMPLW	Trypsin or Chymosin	Immunomodulating	Migliore-Samour et al. 1989
α_{s2} (1–32)	KNTMEHVSSSEESI ISQETYKQEKNMAINPSK	Trypsin	Immunomodulating	Hata et al. 1999
α_{s2} (164–179)	LKKISQRYQKFALPQY	Pepsin	Antimicrobial	Recio and Visser 1999
α_{s2} (165–203)	KKISQRYQKFALPQYLKT VYQHQKAMKPWIQPKTKVIPY	Trypsin or Synthetic	Antimicrobial	Zucht et al. 1995
α_{s2} (183–207)	VYQHQKAMKPWIQPKTKVI PYVRYL	Pepsin	Antimicrobial	Recio and Visser 1999
β (1–28)	RELEELNVPGEIVESLSSS EESITRINK	Trypsin	Immunomodulating	Hata et al. 1999
β (63–68)	PGPIPN	Trypsin or Chymosin	Immunomodulating	Migliore-Samour et al. 1989
β (191–193)	LLY	Trypsin or Chymosin	Immunomodulating	Migliore-Samour et al. 1989
β (193–209)	YQEPVLGPVRGPFPIIV	Trypsin or Chymosin	Immunomodulating and antimicrobial	Migliore-Samour et al. 1989

BIOACTIVE COMPONENTS IN CHEESES

Beneficial Effects of Cheese on Health

Among dairy products, cheese has been a part of dietary culture since the dawn of history and is produced using unique food processing procedures, such as fermentation and ripening. A wide variety of cheeses has been produced around the world to satisfy different taste and health requirements, depending on differences in the lactic acid bacteria used as the starter and manufacturing process. In recent years, cheese has been suggested to have a variety of health-promotive effects. Human intervention studies have demonstrated that cheese does not increase plasma cholesterol concentrations (Tholstrup et al. 2004; Biong et al. 2004; Nestel et al. 2005). Comparison of the effects of consumption of butter and cheese, prepared using an identical amount of milk fat, revealed that consumption of cheese was associated with lower serum cholesterol levels than the consumption of butter with an identical amount of fat (Biong et al. 2004). Regarding homeostasis, it was shown that women with the highest cheese consumption had beneficially lower concentration of plasminogen activator inhibitor 1 (Mennen et al. 1999). Recently, an animal experiment was conducted to examine the effects of Gouda-type cheese produced using *Lactobacillus helveticus* on some biological markers of the metabolic syndrome (Higurashi et al. 2007). Because *Lactobacillus helveticus* has higher protease activity than other starter microorganisms, they focused on some peptides from the cheese and also investigated the effects of the peptides on the production of adiponectin from primary cultures of rat abdominal adipose cells.

Effect of Cheese Consumption on Abdominal Adipose Accumulation and Serum Adiponectin Levels

In order to investigate the effect of ingestion of a Gouda-type cheese on abdominal adipose accumulation and the production of adiponectin, Higurashi et al. (2007) conducted an animal experiment in which the animals were divided into an experimental cheese diet group, fed a diet containing cheese, and a control group, fed a diet consisting of raw materials and nutritional ingredients containing casein and butter oil. Four weeks after study initiation, a significant difference in serum cholesterol in rats with cheese versus without cheese intake for 8 weeks was noted in both rat groups, and it was revealed that the rats with cheese intake had significantly lower serum cholesterol. Analysis of lipoproteins was performed in the serum samples collected at 8 weeks after the start of the experiment. The contents of triglyceride and cholesterol were measured in each of the four classes of lipoproteins, namely, chylomicrons, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). The amount of cholesterol contained in the VLDL was lower ($p < 0.01$) in the cheese diet group compared with the control group.

VLDL is a triglyceride-rich lipoprotein synthesized in the liver, and it is known to be increased by high-fat loading. VLDL is also a precursor of LDL, known as “bad” cholesterol. Therefore, this result suggested that cheese intake inhibits the synthesis of VLDL in the body. Furthermore, the lipoproteins were divided into 20 fractions based on their molecular diameter, and subclass analysis was performed. The amount of cholesterol in the VLDL was lower in the cheese diet group for all molecular radii of the lipoproteins. The serum level of LDL was lower in the cheese diet group in the molecular radius range of large, medium, and very small apolipoprotein. The serum HDL was lower in the cheese diet group for the molecular diameters of small and very small. The epidemiological investigation described in past articles found that the risk of developing cardiovascular disease had increased fifteenfold when large VLDL particles and small HDL particles were both increased (Freedman et al. 1998; Cheung et al. 1991). In the study reported by Higurashi et al. (2007), the amounts of both large VLDL particles and small HDL particles were controlled in the group with cheese intake, which suggested that the cheese intake might reduce the risk of developing cardiovascular disease. Moreover, the amount of adiponectin in blood at the 4th week and the 8th week was measured. The group with cheese intake maintained the amount of adiponectin, but the level had significantly decreased in the group without cheese intake.

The visceral fat tissues, the mesenteric, perinephric, peritesticular, and posterior abdominal wall

adipose tissues were removed separately and weighed, and the weight of the mesenteric adipose tissue, which is the major white adipose tissue, was significantly lower in the cheese diet group. Results of previous studies (Berg et al. 2002; Tsao et al. 2002) have revealed the role of abdominal adipose tissue as the largest endocrine tissue in the living body, and it is understood that maintenance of normal homeostasis of the abdominal adipose tissue is important for the prevention of metabolic syndrome and inhibition of the development of atherosclerosis. It has been suggested that excessive abdominal adipose accumulation is associated with disruption of regulation of the secretion of adipocytokines, which are secreted from the abdominal adipose tissue, resulting in the development of various clinical diseases (Matsuzawa et al. 2004). In particular, it is known that abdominal adipose accumulation is associated with lower serum levels of adiponectin, which is specifically secreted from adipose tissue and usually exists in the blood at high concentrations. Because adiponectin may have a physiological role in the prevention of diabetes mellitus, atherosclerosis, inflammation, hypertension, etc., it is considered very important to increase the concentration of adiponectin in blood or attenuate any decrease in its concentration in order to avoid the metabolic syndrome (Fruebis et al. 2001). The data confirmed that, when given a 20% higher caloric intake than an ordinary diet, the cheese intake reduced visceral fat and also maintained a good balance between serum cholesterol and adiponectin, suggesting that cheese intake might be an effective prophylaxis against the metabolic syndrome.

Lactic Acid Bacteria and Protease in Cheese

Lactic acid bacteria possess various types of protease. The characteristics and types of enzymes differ between the different species of bacteria. Therefore, a variety of peptides can be produced in cheese, depending on the types of starters used, or whether or not nonstarter bacteria exist (Fenelon et al. 2000). The main proteases that contribute to hydrolysis are coagulants (chymosin, pepsin, fungal acid protease, plant acid protease) used for cheesemaking and plasmin, which originate from raw milk. In addition, cathepsins originating from lysosome, peptidase originating in starters, di- and triaminopeptidase with carboxypeptidase, and proline-specific exopeptidase contribute in a small way. Various physical properties and flavors are produced as different types of cheese contain different ratios of each enzyme, and the reactivity of enzymes relies upon a wide range of manufacturing conditions and environmental factors. There have been many reports regarding the peptides found in cheese (Gouldsworthy et al. 1996; Muehlenkamp and Warthesen 1996; Ferranti et al. 1997; Meisel et al. 1997; Smacchi and Gobbetti 1998; Jarmolowska et al. 1999; Saito et al. 2000; Gomez-Ruiz et al. 2004; Upadhyay et al. 2004). Table 8.7 represents only the structures of peptides of which bioactivity has been suggested.

Antioxidant Peptide in Cheese

The presence of antioxidant activity was searched in 20 types of cheeses (Igoshi et al. 2008). Although milk protein before ripening had little antioxidant activity, it was revealed that the ripened cheeses such as Gouda, Parmesan, and Camembert, had antioxidant activities. Antioxidant activity was particularly high in the moldy cheeses. Then, they searched the origin of antioxidant activity in cheese and identified some antioxidant peptides.

The antioxidant peptides contained in the ripened cheeses are listed in Table 8.7. These peptides were decomposed into smaller peptides by digestive enzymes, and it was revealed that these decomposed substances also had antioxidant activity. In addition, it was confirmed that the activity of the cheese-based antioxidant peptides is relevant to tea catechin, a well-known antioxidant component, and furthermore, it had twice the activity of the marketed antioxidant peptide known as *carnosine*. The amino acid sequence of the peptide with high antioxidant activity separated from the water-soluble fraction of Gouda-type cheese produced using *Lactobacillus helveticus* as the starter corresponded to the sequence from the 4th to the 13th amino acids at the N-terminal end of α_{s1}-casein, and was a decapeptide, His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln (Higurashi et al. 2007). The decapeptide was broken down into two peptide fragments (His-Pro-Ile-Lys and His-Gln-Gly-Leu-Pro-Gln) by artificial digestive juices, and the antioxidant activity of these peptides was comparable to that of tea catechin in an equivalent molar ratio.

Table 8.7. Bioactive peptides in cheese

Peptide Sequence	Biological Activity	Casein Segment	Source Cheese	Reference
RPKHPIKHQGLPQ	Antihypertension	α_{s1}(1–13)	Gouda	Saito et al. 2000
HPIKHQGLPQ	Antioxidation	α_{s1} (4–13)	Gouda	Higurashi et al. 2007
DIGSESTEDQAMEDIKEME–AESISSSEEIVPNSVEEK	Anticariogenesis	α_{s1} (43–79)	Cheddar	Reynolds 1997
VPSERYL[a]	Antihypertension	α_{s1} (86–91)	Manchego	Gomez-Ruiz et al. 2004
KKYNVPQL	Antihypertension	α_{s1} (102–109)	Manchego	Gomez-Ruiz et al. 2004
LEIVPK	Antihypertension	α_{s1} (109–114)	Manchego	Gomez-Ruiz et al. 2004
IPY	Antihypertension	α_{s2} (202–204)	Manchego	Gomez-Ruiz et al. 2004
VRYL	Antihypertension	α_{s2} (205–208)	Manchego	Gomez-Ruiz et al. 2004
RELEELNVPGEIVESLSSS–EESITRINK[a]	Anticariogenesis	β (1–28)	Cheddar	Reynolds 1987
FQSEE	Antioxidation	β (33–37)	Blue	Igoshi et al. 2008
YPFPGPI	Opioid activity	β (60–66)	Cheddar, Swiss	Jarmolowska et al. 1999
			Brie, Limburger Blue	Muehlenkamp and Warthesen 1996
YPFPGPIPN	Antihypertension	β (60–68)	Gouda	Saito et al. 2000
MPFPKYPVQPF	Antihypertension	β (109–119)	Gouda	Saito et al. 2000
VRGPFP	Antihypertension	β (199–204)	Manchego	Gomez-Ruiz et al. 2004

[a]S represents phosphoserine.

Effect of Antioxidant Peptides on Adiponectin Production of Abdominal Adipose Cells

The effects of antioxidant peptides on the production of adiponectin from primary cultures of rat abdominal adipose cells were investigated (Higurashi et al. 2007). It was evaluated using VAC01, a kit for primary culture of rat abdominal adipose cells. When the antioxidant decapeptide was added to the culture medium in the concentration range of 10, 50, and 100 mM, the production of adiponectin was significantly increased as compared with that in the culture not treated with the decapeptide. The concentration of adiponectin was the highest when 50 mM of the peptide was added to the medium. Ford et al. (2003) reported that subjects with the metabolic syndrome have significantly lower serum concentrations of antioxidants, such as carotenoids, vitamin C, and vitamin E. Furukawa et al. (2004) demonstrated that exposure of visceral fat to oxidant stress affects the regulation of adipocytokine production, thereby increasing the risk of development of metabolic syndrome and atherosclerosis. The antioxidant peptide contained in the cheese potentially contributed, at least in part, to the effect of the dietary cheese consumption on the production of serum adiponectin and reduction of abdominal adipose accumulation.

Other Bioactive Components in Cheese

Peptides

There have been many reports regarding peptides found in cheese. Although ACE-inhibitory activity peptides (Meisel et al. 1997; Smacchi and Gobbetti 1998; Saito et al. 2000; Gomez-Ruiz et al. 2004), β-casomorphin (Muehlenkamp and Warthesen 1996; Jarmolowska et al. 1999), and calcium-binding phosphopeptides (Ferranti et al. 1997) were identified in cheese, the stability of these peptides under the conditions of cheese ripening was dependent on pH, salt, and type of enzymes present. Muehlenkamp and Warthesen (1996) suggested that β-casomorphin might be degraded under conditions common for cheddar cheese. On the other hand, Gomez-Ruiz et al. (2004) reported that the ACE-inhibitory activity of peptides was not severely affected by simulated gastrointestinal digestion.

Calcium

Cheese is rich in calcium with excellent bioavailability. In particular, hard-type cheeses are rich in calcium, due to the acidity of the curd after the whey has been removed. It has been known for a long time that the bioavailability of calcium in natural cheese and processed cheese is very high (Kansal and Chaudhary 1982). One of the reasons put forward has been that it is due to the stimulatory effect on calcium absorption by casein phosphopeptides (CPP), which are produced during ripening (Bouhallab and Bougle 2004). A study on the level of thyroid hormone (PTH) in blood after consumption of high-calcium foods has confirmed that, in terms of suppressing PTH production, cheese surpasses milk (Karkkainen et al. 1997).

In recent years, research has repeatedly shown that an insufficient intake of calcium, in particular, increases the risk of obesity, hyperlipidemia, and insulin-resistance syndrome (Parikh and Yanovski 2003; Teegarden 2003; Zemel 2004). A diet rich in dairy calcium intake enhanced weight reduction in type 2 diabetic patients. Such a diet could be tried in diabetic patients, especially those with difficulty adhering to other weight reduction diets (Shahar et al. 2007). Results from an intervention trial have demonstrated the effectiveness of fortifying calcium in the area of lipid balance, with the ingestion of calcium in the form of dairy foods in particular reportedly enhancing its effectiveness (Zemel et al. 2000; Jacqmain et al. 2003). In addition, intervention trials and epidemiological studies have demonstrated that the blood pressure of people with high dairy food consumption is lower than in those with a low intake of dairy foods (Ackley et al. 1983; Hajjar et al. 2003). Calcium and peptides with ACE-inhibitory activity have been suggested as potential working mechanisms (Smacchi and Gobbetti 1998; Saito et al. 2000). Furthermore, Kashket and DePaola (2002) reported that cheese rich in calcium and CPP had a pronounced anticaries effect by reducing demineralization and enhancing remineralization.

Conjugated Linoelic Acid (CLA)

Some 75% or more of CLA in cheese is *cis*9, *trans*11 CLA isomer, while trace levels of *trans*10, *cis*12 CLA isomer are also present (Yurawecz et al. 1998). Lin et al. (1995) measured the CLA content of 15 types of cheese (Table 8.8), and reported that the amount differed according to the type of cheese. The *cis*9, *trans*11 CLA content of Japanese and imported cheeses has been investigated by Takenoyama et al. (2001), who found that the CLA content of imported cheese was higher than in Japanese cheese. At the same time, Ha et al. (1989) have reported that CLA content increases when processed cheese is manufactured from natural cheese. Shantha et al. (1992) have investigated the relationship between manufacturing conditions and the CLA content by producing processed cheese using cheddar cheese as the main ingredient, and then adding whey protein concentrate (WPC) and its low molecular fraction. They found that the processing conditions and whey components do in fact influence CLA content.

There have also been some reports regarding the ability of cheese starters to produce CLA (Jiang et al. 1998; Lin 2000). Cheeses such as Blue, Brie, Edam, and Swiss contain a relatively high amount of CLA (Lin et al. 1995). Although the CLA content of cheese relies heavily on the amount of CLA in raw milk, there are also reports that the microorganisms in cheese play a role in CLA production (Lin 2000). In particular, lactic acid bacteria (Ogawa et al. 2001; Kishino et al. 2002) and propionic acid bacteria (Jiang et al. 1998) are known to produce CLA using oleic acid as a substrate. In addition, it has been reported that lactic acid bacteria and *Bifi-*

Table 8.8. Conjugated linoleic acid content of dairy products (adapted from Lin et al. 1995)

Dairy Products	Lipid Basis Amount[a]	Product Basis Amount[b]
Natural cheese		
Blue1	4.87 ± 0.10	147.5 ± 3.7
Blue2	7.96 ± 0.12	228.5 ± 4.1
Brie	4.75 ± 0.28	129.4 ± 31.4
Cheddar-medium	4.02 ± 0.29	140.0 ± 13.0
Cheddar-sharp	4.59 ± 0.30	161.2 ± 12.2
Cougar gold	3.72 ± 0.17	130.0 ± 8.6
Cream	4.30 ± 0.42	142.9 ± 14.2
Cottage	4.80 ± 0.30	19.6 ± 1.2
Edam	5.38 ± 0.90	141.7 ± 20.3
Monterey jack	4.80 ± 0.38	142.8 ± 15.0
Mozzarella	4.31 ± 0.21	91.4 ± 4.4
Parmesan	4.00 ± 0.53	89.9 ± 8.0
Swiss	5.45 ± 0.59	160.9 ± 17.2
Viking	3.59 ± 0.01	119.5 ± 0.7
Processed American	3.64 ± 0.15	91.1 ± 3.0
Whole milk	4.49 ± 0.64	14.2 ± 2.1
Fermented milk	3.82 ± 0.13	7.4 ± 0.1

[a]Milligram of CLA (*cis*-9, *trans*-11 isomer)/g of lipid.
[b]Milligram of CLA (*cis*-9, *trans*-11 isomer)/g of product (wet weight basis).
Values are means ± standard error.

dobacterium longum produce CLA when they are cultured on media containing linoleic acid (Alonso et al. 2003; Coakley et al. 2003).

Regarding the physiological effects, CLA has been found to play a role in body fat reduction, cancer inhibition, and immune modulation. McIntosh et al. (2006) reviewed some of the evidence regarding the potential contribution of omega-3 fatty acids and CLA in cheese fat to disease prevention. Roupas et al. (2006) proposed that fat derived from cheese might be preferable to tallow in its influence on some risk markers for cardiovascular disease. Belury et al. (1996) showed that the progress of phorbol ester-induced skin cancer in rats was suppressed by adding 1.0–1.5% of CLA to their feed. It has also been found that feeding rats with a diet containing 0.5% CLA reduces the number of early stage carcinogen-induced colonic carcinomas from developing (Liew et al. 1995). Ip et al. (1999) reported that feeding rats with a diet prepared with butter containing high CLA (0.8% CLA in feed) reduced mammary tumorigenesis by approximately 50% in comparison to a control group (0.1% CLA). Ritzenthaler et al. (2001) estimated CLA intake in adult males and females from a 3-day dietary record. The results were 212 ± 14 mg/day for males and 151 ± 14 mg/day for females. They reported that, judging from animal trial data, it is necessary for humans to ingest at least 500 mg of CLA per day for it to be effective in preventing cancer.

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9
Bioactive Components in Yogurt Products

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INTRODUCTION

Yogurt is defined as fermented milk obtained by lactic acid fermentation due to the presence of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salvarius* ssp. *thermophilus* in milk. Other lactic acid bacteria (LAB) species are now frequently added to give the final product unique characteristics. Strictly speaking, the microorganisms in the final product must be abundant and viable. Yogurt products come in a wide variety of flavors, forms, and textures and include lowfat, nonfat, light, Swiss or custard, and frozen yogurts. Yogurt might contain active cultures, be heat-treated, be in liquid or drinkable form, and have fruit at the bottom. Yogurt has been considered as a functional food. *Functional foods* are generally considered processed foods containing ingredients that aid specific body functions, in addition to being nutritious. The functionality of such foods is derived from their bioactive components. Bioactivity refers to the application of bioactive ingredients or nutraceuticals in foods like prebiotics, probiotics, flavonoids, phytosterols, phytostanols, bioactive peptides, and bioactive carbohydrates (Arvanitoyannis and van Houwelingen-Koukaliaroglou 2005). In fact, yogurt contains properties or components that can benefit human health (Parvez et al. 2006).

The history recording the beneficial properties of yogurt dates back many centuries. According to Persian tradition, Abraham owed his fecundity and longevity to the regular ingestion of yogurt; in the early 1500s, King Francis I of France was reportedly cured of a debilitating illness after eating yogurt made from goat's milk (van de Water 2003). At the beginning of the 20th century, scientific interest concerning the health benefits of yogurt was sparked by a Russian bacteriologist, Elie Metchnikoff. He attributed the good health and longevity of Bulgarians to the fact that they regularly ate large amounts of yogurt (van de Water 2003). Studies on the health effect of lactic acid bacteria continued throughout the century. Many studies have provided support to Metchnikoff's theory and confirmed that yogurt may indeed be beneficial to health (van de Water et al. 1999; Lourens-Hattingh and Viljoen 2001; Mercenier et al. 2003). Yogurt has been studied for its effects on cholesterol metabolism, immunologic effect, diarrhea, *Helicobacter pylori* eradication, antimutagenic activity, colon cancer, and antioxidant activity.

HEALTH ATTRIBUTES OF YOGURT

Several claims are available on proposed beneficial health effects of fermented dairy foods including yogurt. A few reviews have elaborated on this subject (Meydani and Ha 2000; Parvez et al. 2006). Table 9.1 summarizes some of the suggested health benefits associated with probiotic yogurt consumption. However, conflicting opinions still abound. Both bacterial and nonbacterial components in yogurt are thought to play a role through their effects on a host's immune system. The roles of yogurt in the control of some of these diseases are elaborated upon in this section.

Table 9.1. Suggested health benefits associated with probiotic yogurt consumption

Health Condition	Suggested Mechanism	References
Improved lactose tolerance	Predigestion of milk lactose by bacterial lactase to absorbable glucose and galactose; reduction of gut pH by lactic acid; probiotic modulation of colonic microbiota.	Alm 1982; Marteau et al. 1990; He et al. 2008
Protection against gastrointestinal infections	Limiting gut colonization of pathogenic microorganisms; influencing gut microflora population; alteration of toxin binding sites, making intestinal conditions less favorable for pathogenicity.	Sanders 1999; Gionchetti et al. 2000; Lourens-Hattingh and Viljoen 2001; Zubillaga et al. 2001
Relief of constipation	No suggested mechanism.	Bu et al. 2007
Cholesterol reduction	Active deconjugation of bile salts by the bacterial enzyme, bile salt hydrolase; possible binding of dietary cholesterol with bacterial cells in the gut; antioxidative effect.	Lourens-Hattingh and Viljoen 2001; Zubillaga et al. 2001; Hosono et al. 2002
Blood pressure–lowering effect	Inhibition of ACE* by ACE inhibitors. Inhibiting the production of the vasoconstrictor, angiotensin II and the degradation of the vasodilator, bradykinin.	Maeno et al. 1996; Yamamoto et al 1999; Nurminen et al. 2000; Seppo et al. 2003
Antiallergic effects	Immunostimulatory activities of oligonucleotide (OND) sequences derived from the DNA of different probiotic strains; stimulation of B lymphocytes, induction of TH1 cytokines and inhibition of IgE production by ONDs; prevention of antigen translocation into the bloodstream.	Zubillaga et al. 2001; Takahashi et al. 2006
Anticancer effects	Detoxification of ingested carcinogens, cellular uptake of mutagenic compounds, alteration of intestinal microflora environment, production of active compounds (e.g., butyrate), and inhibition of carcinogen-producing enzymes by other colonic microbes.	Lidbeck et al. 1992; Sanders 1999; Lourens-Hattingh and Viljoen 2001; Zubillaga et al. 2001; Isolauri 2004
Improving immunity	Enhancement of specific and nonspecific immune mechanisms against infections and tumor development.	Sanders 1999; Lourens-Hattingh and Viljoen 2001; Zubillaga et al. 2001
Relief of small bowel bacterial overgrowth	Interference with activities of bacterial overgrowth, limiting toxic metabolite production and presenting less favorable growth conditions	Lourens-Hattingh and Viljoen 2001; Zubillaga et al. 2001
Hepatic encephalopathy	Inhibiting growth of urease-producing flora	Sanders 1999

*ACE: angiotensin-converting enzyme.

Yogurt and the Immune System

The immune system functions to eliminate invading microorganisms and viruses, rid the host system of damaged tissue, and destroy neoplasm. Yogurt is known to have immunostimulatory effects, which are thought to be through its components but the exact mechanisms are not yet fully understood.

The major microorganisms in yogurt are Gram-positive bacteria and have cell wall components

such as peptidoglycan, polysaccharide, teichoic acid, and lipoproteins. All these components have been shown to have immunostimulatory properties (Takahashi et al. 1993; Akira et al. 2006). Other lactic acid bacterial metabolites such as exopolysaccharides can also influence the immune system (Meydani and Ha 2000). On entering the intestine, biologically active probiotic particles may activate both specific and nonspecific immune responses. There is evidence that ingestion of lactic acid bacteria exerts an immunomodulatory effect in the gastrointestinal system of both humans and animals (Meydani and Ha 2000). Antigen processing and the initial cellular events of the immune response in the intestine occur in the Peyer's patch and other gut-associated lymphoid tissue (GALT), where pathogenic microorganisms and other antigens encounter macrophages, dendritic cells, B lymphocytes, and T lymphocytes. When the mucosal immune response is induced, primed T and B cells migrate through the lymphatic system and then enter the peripheral blood circulation (Perdigón et al. 1999). Therefore, immunostimulatory effects of yogurt are not only observed in GALT but also in the other peripheral lymphoid tissues to enhance cytokine production, phagocytic activity, specific humoral immune response, T lymphocyte (CD4+ and CD8+) function, and NK cell activity (Meydani and Ha 2000). For example, ingestion of yogurts containing *Bifidobacterium* spp. and *L. acidophilus* increased significantly the percentages of T helper (CD4+) cells in the spleens of mice and also enhanced mucosal and systemic IgA response to Cholera toxic immunogen (Tejada-Simon et al. 1999; Pestka et al. 2001).

Other nonbacterial milk components (whey protein, calcium, vitamins, and trace elements) and components produced during the fermentation process (free amino acids and peptides) may also contribute to the immunostimulatory attributes of yogurt. Both animal and human studies have contributed enormously to the understanding of the immunostimulatory effects of probiotic yogurt (Meydani and Ha 2000; Cogan et al. 2007).

Yogurt and Gut Diseases

Gastrointestinal diseases result from a change in the gut microflora caused by invading pathogens. Before the appearance of symptoms, the invading pathogen must first establish itself in sufficient numbers in the gut. Yogurt consumption is known to modulate conditions such as acute viral and bacterial diarrhea, inflammatory bowel disease, traveler's diarrhea as well as antibiotic associated diarrhea, necrotizing enterocolitis, irritable bowel syndrome, and constipation through actions of containing probiotics and other compounds.

Diarrhea means frequent loose or liquid stools. Treatment of diarrhea by administering living lactic acid bacteria to restore a disturbed intestinal microflora has a long tradition (de Vrese and Marteau 2007). The primary pathogens for acute infectious diarrhea are rotavirus, *Shigella*, *Salmonella*, *Escherichia coli*, and *Clostridium difficile*, with rotavirus being the most common cause of severe acute diarrhea in children worldwide (Yan and Polk 2006). During acute diarrhea, there is a decrease of protective commensal microflora including lactobacilli and bifidobacteria, followed by overgrowth of urease-producing pathogenic bacteria. Exogenously administered *lactobacilli* may reverse such development and attenuate the clinical course of diarrhea. Clinical studies have shown that *Lactobacillus* effectively colonized the gastrointestinal tract after administration and significantly shortened the duration of acute rotavirus diarrhea (Shornikova et al. 1997). Antibiotic-associated diarrhea is most common among patients receiving antibiotic treatment induced by a disturbance of the intestinal microbial balance (Yan and Polk 2006). It has been demonstrated that yogurt supplementation could effectively decrease the incidence and duration of antibiotic-associated diarrhea. A randomized clinical trial of yogurt for the prevention of antibiotic-associated diarrhea in hospitalized patients receiving oral or intravenous antibiotics demonstrated that yogurt supplementation effectively decreased the incidence and duration of antibiotic-associated diarrhea (Beniwal et al. 2003). *L. acidophilus*– and *L. casei*–fermented milk were shown to have a preventive effect against antibiotic-associated diarrhea and to reduce *C. difficile*–associated incidence (Beausoleil et al. 2007).

Inflammatory bowel disease is an umbrella term used for Crohn's disease and ulcerative colitis. One characteristic common to both is diarrhea. This symptom can be particularly distressing to an individual with inflammatory bowel disease and is often manifested by fear, anxiety, and embarrassment (Savard and Sawatzky 2007). The intestinal micro-

flora plays an important role in the pathogenesis of inflammatory bowel disease (Noble et al. 2008). A number of microbial agents are implicated as initiating factors in the pathogenesis of inflammatory bowel disease. These include *Mycobacterium paratuberculosis*, measles virus, *Listeria monocytogenes*, and adherent *E. coli*. Therefore, alteration of the gut microflora through introduction of probiotic lactic acid bacteria could theoretically result in some clinical improvement. Furthermore, modulation of cytokine expression and stabilization of the mucosal barrier by probiotic lactic acid bacteria could promote disease resolution. Probiotics offer an alternative by altering the intestinal microflora and modulating the immune response without the risk of side effects associated with conventional therapy (Sheil et al. 2007). In addition, probiotic yogurts alter the physiological response by enhancing the intestinal microflora and the pathophysiological response, thereby decreasing the symptom of diarrhea of inflammatory bowel disease patients (Savard and Sawatzky 2007). Baroja et al. (2007) showed that the consumption of yogurt supplemented with *Lactobacillus* strains GR-1 and RC-14 by patients with inflammatory bowel disease promoted the formation of a desirable antiinflammatory environment, and the effect was associated with an increase in the proportion of putative CD4+ CD25+ T_{reg} cells ($CD4^+$ $CD25^{high}$) in peripheral blood.

Helicobacter pylori is a spiral Gram-negative microaerophilic pathogen that can colonize epithelial cells lining the antrum of the stomach and survive in the acidic environment. *H. pylori* causes chronic gastritis (Blaser 1990), peptic ulcer (Everhart 2000) and has been linked to development of gastric malignancies such as gastric mucosa–associated lymphoid tissue lymphomas and gastric adenocarcinoma (Horie et al. 2004; Wang et al. 2004). In comparison with the quadruple therapy (tetracycline, bismuth, metronidazole, and a proton-pump inhibitor), supplementation of yogurt to patients improved the efficacy of the quadruple therapy in eradicating residual *H. pylori* (Sheu et al. 2006).

Yogurt and Cancer

Cancer is generally caused by genetic mutations in cells. These mutations may be due to the effects of carcinogens, such as tobacco smoke, radiation, chemicals, or infectious agents, may be randomly acquired through errors in DNA replication, or are inherited. Since some carcinogens or cancer-causing chemicals can be ingested or generated by metabolic activities of gut microorganisms, yogurt and other probiotic cultures or preparations may modulate cancer development through detoxification of ingested carcinogens, cellular uptake of mutagenic compounds, alteration of intestinal microflora, and production of compounds (e.g., butyrate) that will participate in the process of apoptosis, have anticarcinogenic properties, and enhance immune response.

Yogurt consumption has been demonstrated to be associated with decreased risk of colon cancer. It was proposed about two decades ago that diet-induced gut microfloral alteration may retard the development of colon cancer (Hitchins and McDonough 1989). After ingestion of fermented milk or probiotics, Saikali et al. (2004) reported a shift of intermediate markers of colorectal cancer risk in human subjects from a high- to low-risk pattern. In a case–control study, Boutron et al. (1996) showed a significant inverse relationship between consumption of moderate amounts of yogurt and the risk of large colonic adenomas (benign tumors) in both women and men. Clinical studies also support a protective role of dairy foods and calcium intake on colon cancer. Increased intake of calcium from 600 to 1,500 mg/d from food sources, especially lowfat milk, was shown to reduce the risk of colon cancer (Holt et al. 2001). Yogurt consumption was also found to inhibit the development of a colorectal carcinoma induced by carcinogen 1,2 dimethylhydrazine (Perdigón et al. 2002). Further, oral administration of certain *L. bulgaricus* strain could prevent 1,2 dimethylhydrazine–induced DNA breaks in the colon in vivo (Wollowski et al. 1999).

The mechanism of the antimutagenic action of yogurt is not fully understood. The presence of large numbers of probiotic bacteria in the gut serves to decrease the populations and metabolic activities of harmful bacteria that may generate carcinogenic compounds. Some pathogenic bacteria may convert procarcinogens to carcinogens, and limiting their growth means a reduction in the amount of carcinogens in the intestine. Reducing the enzymes that promote the conversion of procarcinogens to carcinogens in the gut may be another possible mechanism responsible for the antitumor activity of yogurt.

Bacterial β-glucuronidase and nitroreductase have been considered as key enzymes for the metabolic activation of carcinogens in the colon lumen and suggested to play a role in human colon carcinogenesis. Several researchers have demonstrated that yogurt consumption may reduce the potentially harmful enzyme activities of β-glucuronidase and nitroreductase (Guerin-Danan et al. 1998). Yogurt bacteria can also reduce the concentration of the nitrogenous compound, nitrite, in the gut, thereby eliminating a key substrate in the formation of carcinogenic compounds (Dodds and Collins-Thompson 1984). Another concept in the possible reduction or delay of tumor development by probiotic bacteria is that they might bind to nitrogenous compounds in the gut, thereby reducing their absorption into circulation (Lidbeck et al. 1992; Isolauri 2004). The binding of the mutagens to the cell wall of lactic acid bacteria seems to be an important step in this process (Matsumoto and Benno 2004). Secondly, it is also possible that the metabolites of probiotics act as important antimutagenic factors. The released peptides can contribute to the mechanism for the antimutagenic activity of yogurt. It has been reported that milk proteins, such as caseins, α-lactalbumin, and β-lactoglobulin, are able to bind mutagenic heterocyclic amines at high percentages (Yoshida et al. 1991; Yoshida and Ye 1992). Further, inhibitory activity by caseins against nitroquinoline-1-oxide (known as a carcinogen) increased when caseins were hydrolyzed by pepsin (van Boekel et al. 1993). In addition, antimutagenic compounds were produced in milk during fermentation by *L. helveticus* and the release of peptides is one possible contributing mechanism (Matar et al. 1997). Exopolysaccharide produced by lactic acid bacteria is another possible contributing factor for the antimutagenic activity of yogurt. Polysaccharides produced by *Bifidobacterium longum* revealed high levels of antimutagenicity (Sreekumar and Hosono 1998). The lactic acid bacterial spermidine might also be another factor for the antimutagenic activity of yogurt. Spermidine is an antimutagen for some frameshift mutations, and the antimutagenicity of spermidine is probably due to binding to DNA, thereby preventing DNA alkylation, and reducing, in turn, the frequency of UV-induced mitotic gene conversion and reverse mutation. It was demonstrated that consumption of *Bifidobacterium*-containing yogurt increased gut spermidine level and was responsible for the reduction of mutagenicity in the gut of healthy adults (Matsumoto and Benno 2004). Third, the beneficial effects of lactic acid bacteria on cancer therapy have been associated with the ability to modulate immune parameters, including T cell, natural killer (NK) cell, and macrophage activity, which are important for hindering tumor development (Ouwehand et al. 1999). Activated macrophages, NK cells, and some T lymphocyte subpopulations (such as CD4+ TH1 cells) can secrete tumor necrosis factor (TNF), which can induce both apoptotic and necrotic forms of tumor-cell lysis (Laster et al. 1988; Fiers 1991).

Yogurt and Cardiovascular Diseases

Cardiovascular disease, defined as a range of diseases of the heart and circulatory system, is the most important cause of death in Western countries. A high level of serum total cholesterol is generally considered to be a risk factor for cardiovascular disease. Therefore, it is very important to decrease elevated serum cholesterol levels in order to prevent cardiovascular disease (Hasler 2002). The discovery by Mann and Spoerry (1974) that people who drank yogurt had very low values of blood serum cholesterol opened up a new area of study. A number of studies have been performed with experimental animals, and also humans, in order to elucidate the effect of yogurt on serum cholesterol (Akalin et al. 1997; Anderson and Gilliland 1999; Hepner et al. 1979). Although some contradictory results have been obtained (de Roos et al. 1998), the majority of results from these reports indicate that yogurt possesses hypocholesterolemic properties.

The mechanisms responsible for the hypocholesterolemic effects of yogurt are still under investigation. It has been proposed that the lactic acid bacteria incorporated in yogurt products may be responsible for the hypocholesterolemic effects. One proposed mechanism of the hypocholesterolemic activity of lactic acid bacteria is assimilation of cholesterol by the bacterial cells (Buck and Gilliland 1994). Removal or assimilation of cholesterol by intestinal organisms in the small intestine could reduce the amount of cholesterol available for absorption from the intestine, thus exerting some control on serum cholesterol levels (Pigeon et al. 2002). Alternatively, the hypocholesterolemic activity of lactic acid bacteria could be due to the suppression of bile acid resorption by deconjugation as a function of the

bacterial bile salt hydrolase activity (Xiao et al. 2003). The major metabolites of cholesterol in the body are bile acids; therefore, greater excretion of bile acids should, in principle, lead to a lower level of serum cholesterol (Ho et al. 2003). Deconjugated bile acids are excreted in the feces, whereas conjugated bile acids are recycled to the liver via the enterohepatic circulation (Gilliland 1990). In vitro experiments have demonstrated that some strains of *Lactobacillus* produced the enzyme bile-salt hydrolase and had the ability to deconjugate bile acids (Gilliland et al. 1985). Finally, exopolysaccharide produced by lactic acid bacteria is also another possible mechanism of the hypocholesterolemic activity of yogurt. It was demonstrated that exopolysaccharide-producing strains of *L. bulgaricus* bound significantly greater amounts of bile acids than was the case for the nonexopolysaccharide-producing strains (Pigeon et al. 2002). There is potential for these bacterial exopolysaccharides to interfere with the absorption of cholesterol and bile acids from the intestines by binding with and removing them from the body.

Milk components such as immunoglobulin, magnesium, riboflavin, and orotic acid have also been proposed as potential mediators of hypocholesterolemic effects of milk products (Buonopane et al. 1992; Earnest et al. 2005). Furthermore, some bacterial cultures have been shown to have proteolytic activity. For example, *L. bulgaricus* was shown to have a high proteolytic activity during milk fermentation, as indicated by elevated concentrations of peptides after milk fermentation. Thus, the concentrations of peptides are higher in yogurt than in milk. A number of milk-protein–derived peptides have been shown to be able to elicit a cholesterol-lowering effect. For example, a low molecular-weight peptide, α-lactotensin (His-Ile-Arg-Leu), derived from β-lactoglobulin, was found to reduce serum cholesterol in mice subsequent to oral administration (Yoshikawa et al. 2000). Another peptide (Ile-Ile-Ala-Glu-Lys) derived from β-lactoglobulin was also found to significantly influence serum cholesterol levels for rats, and exhibited a pronounced hypocholesterolemic effect (Nagaoka et al. 2001).

High blood pressure or hypertension is another risk factor for cardiovascular diseases such as coronary heart disease, peripheral arterial disease, and stroke. Angiotensin-converting enzyme (ACE)-inhibiting peptides are released during bacterial fermentation of milk proteins (Nakamura et al. 1995; Takano 1998), indicating that fermented milk products including yogurt may possess blood pressure–lowering effects.

Yogurt and Other Human Diseases

Allergy is characterized by an abnormal immunological reactivity in certain individuals to antigens. This response generates a wide variety of symptoms and clinical manifestations expressed in several affected organ systems such as skin, respiratory tract, and gastrointestinal tract. The epidemiological data and results of animal experiments suggest that a disorder of normal intestinal microflora is closely related to allergy development (Tanaka and Ishikawa 2004). Dietary studies have suggested that long-term consumption of yogurt can reduce some of the clinical symptoms of allergy in adults with atopic rhinitis or nasal allergies, and can lower serum levels of IgE (Trapp et al. 1993; van de Water et al. 1999). A polarized Th2 response is often observed in patients with allergy (Romagnani et al. 1994). T helper cells can be subdivided into Th1 and Th2, and the cytokines they produce are known as Th1-type cytokines and Th2-type cytokines, respectively. Th1-type cytokines tend to produce the proinflammatory responses responsible for killing intracellular parasites and for perpetuating autoimmune responses. The Th2-type cytokines include interleukins (IL) 4, 5, and 13, which are associated with the promotion of IgE and eosinophilic responses in atopy, and also IL-10, which has more of an antiinflammatory response. Allergy is regarded as a Th2 weighted imbalance of immune responses. Therefore, enhancing a Th1-type immune response is expected to be beneficial for treatment of allergic disease. It has been demonstrated that certain strains of *L. casei* possess the ability to stimulate macrophage secretion of IL-12, shift the cytokine production pattern from Th2 to Th1 predominance, and then suppress IgE production (Shida et al. 1998). In addition, a murine model of food allergy has also been used to demonstrate that intraperitoneal administration of *L. plantarum* can downregulate casein-specific IgE antibody levels in vivo, as well as the in vitro capacity of splenic lymphocytes to secrete IL-4 (Murosaki et al. 1998).

Numerous studies indicate that probiotic yogurt is well tolerated, compared to milk, by individuals

who are lactose intolerant. The beneficial effect is a consequence of the lactic acid bacteria in yogurt (Gilliland and Kim 1984; Marteau et al. 1990). The presence of microbial β-galactosidase derived from yogurt bacterial culture, like intestinal lactase, can break down lactose to easily absorbable component sugars, glucose and galactose, thus reducing the symptoms of lactose intolerance in lactase-deficient individuals. Another suggested mechanism is that lactic acid produced during the fermentation process may act as a preservative by reducing pH. It may also influence the physical properties of the casein curd by inducing a finer suspension, which in turn may promote digestibility. Yogurt's acidity and finer dispersion of protein in the stomach act to retard the emptying of the contents of the ileum into the colon. Slow movement may allow for a longer breakdown activity of lactases from gut or bacterial origin, consequently leading to a more efficient lactose digestion (reviewed by Buttriss 1997).

Despite the debate that exists regarding the role of exogenous calcium in the prevention of osteoporosis, yogurt remains a recommended source of calcium (Murray 1996; Weinsier and Krumdieck 2000). In addition, earlier studies with ingested yogurt containing *L. acidophilus* culture suggested its prophylactic use against candidal vaginitis and bacterial vaginosis (Hilton et al. 1992; Shalev et al. 1996).

Several reactive oxygen species, including the superoxide radical, hydroxyl radical, hydrogen peroxide, and the peroxide radical, are known to cause oxidative damage not only to food systems but also to living systems. Accumulating evidence suggests that reactive oxygen species and their subsequent modification of cellular macromolecules play a significant pathological role in human diseases such as cancer, atherosclerosis, hypertension, and arthritis (Frenkel 1992). Although the human body has an inherent antioxidative system (i.e., superoxide dismutase, glutathione peroxidase, and uric acid) to protect itself from damage caused by peroxidants, the system is not sufficiently effective to totally prevent such damage (Simic 1988). Hence, there is an increasing interest in finding natural antioxidants from food, because it is believed that they can protect the human body from the attack of free radicals and retard the progress of many chronic diseases, as well as retarding the lipid oxidative rancidity in foods (Pryor 1991). The antioxidative effect of lactic acid bacteria has been reported only recently. Certain strains of *L. acidophilus* and *B. longum* were able to inhibit linoleic acid peroxidation and showed the ability to scavenge α,α-diphenyl-β-picrylhydrazyl (DPPH) free radical (Lin and Yen 1999; Lin and Chang 2000). The mechanisms responsible for the antioxidative effects of lactic acid bacteria are still under investigation. It has been previously reported that proteins deriving from dairy products reveal some antioxidant potential (Wong and Kitts 2003). The specific amino-acid residue side-chain groups or the specific peptide structure of the peptides deriving from milk protein hydrolysates may be attributable to chelation of prooxidative metal ions and termination of the radical chain reactions, thus inhibited lipid oxidation (Pena-Ramos and Xiong 2001). In addition, some peptides deriving from the pepsinic hydrolysate of caseins were demonstrated possessing DPPH radical-scavenging activities (Suetsuna et al. 2000). It was observed that heat treatment could enhance the DPPH radical-scavenging activity of skim milk and the activity being further increased by fermentation with *L. casei*. The casein hydrolysate from the fermented milk might be one of the factors enhancing radical-scavenging activity (Nishino et al. 2000).

BIOACTIVE COMPONENTS IN YOGURT

Almost all milk components may contribute potential health benefits—including proteins, peptides, lipids, minor carbohydrates, minerals, and vitamins. In the manufacture of yogurt, live bacteria are added. Others such as bioactive peptides are generated during the fermentation process while others such as minerals are added to increase the nutritional quality or flavor of yogurt products. Probiotic microorganisms can exert their beneficial properties through two mechanisms: direct effects of the live microbial cells (probiotics) or indirect effects via metabolites of these cells. The most important metabolites in fermented milk may be peptides that are not present prior to fermentation. Tables 9.2 and 9.3 summarize the bioactive components found in yogurt.

Bacterial Components

Yogurt is increasingly seen as a safe and enjoyable means to deliver probiotics to the gut, in which case

Table 9.2. Examples of bioactive components in yogurt

Category	Examples
Probiotics	Main cultures: *Lactobacillus bulgaricus* and *Streptococcus thermophilus* Supplementary cultures: *L. acidophilus, L. casei*, *Bifidobacterium bifidum*, *B. longum*, *B. infantis*, *B. breve*
Prebiotics	Those being tested are β-glucans, inulin, pectin, gums and resistant starch, fiber
Bioactive peptides	See Table 9.3
Major milk proteins	α_{S1}-casein, α_{S2}-casein, β-casein, κ-casein, α-lactalbumin, β-lactoglobulin, proteose-peptone, glycomacropeptide
Minor proteins and naturally occurring bioactive peptides	Adrenocorticotropic hormone, calcitonin, plasmin enzymes (plasmin, catalase, gluthathion peroxidase, lactoperoxidase), growth factors, immunoglobulins, insulin, lactoferrin, lactoperoxidase, luteinizing hormone-releasing hormone, lysozyme, prolactin, relaxin, somatostatin, thyroid stimulating hormone, thyrotropin-releasing hormone, transferrin
Bioactive lipid	May become useful:conjugated linoleic acid
Vitamins	Vitamin D, vitamin B_{12}, thiamine, riboflavin, niacin, folate
Minerals	Calcium, phosphorus, magnesium, zinc

patients and healthy individuals alike will benefit from both the rich nutrients and probiotic content. Certain species, particularly lactic acid bacteria, are used in yogurt manufacture. Basically, for a product to be called *yogurt* in North America, it must be fermented with a symbiotic blend of *Streptococcus salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. These two species are commonly referred to as *yogurt bacteria*. These symbiotic bacteria do not however adequately survive gastric passage or colonize the gut, thus necessitating the addition of other LAB species in yogurt preparations: *L. acidophilus, L. casei*, *B. bifidum*, *B. logum*, *B. breve*, *B. infantis*, and *B. lactis*, and others (Guarner et al. 2005; Mater et al. 2005; Guyonnet et al. 2007; Cogan et al. 2007). These microorganisms are capable of partially resisting gastric and bile secretions in vitro and in vivo and can deliver enzymes and other substances into the intestines (Alvaro et al. 2007).

Lactic acid bacteria in yogurt products enhance resistance against intestinal pathogens mainly via antimicrobial mechanisms. These include competitive colonization and production of organic acids, such as lactic and acetic acids, bacteriocins, and other primary metabolites (Kailasapathy and Chin 2000; Lemberg et al. 2007). By competitive colonization, lactic acid bacteria inhibit the adhesion of gastrointestinal pathogens to the intestinal mucosa. Production of organic acids, such as lactic and acetic acids, by lactic acid bacteria lowers intestinal pH and thereby inhibits the growth of pathogens. These organic acids also increase peristalsis, thereby indirectly removing pathogens by accelerating their rate of transit through the intestine (Kailasapathy and Chin 2000). Through competitive colonization, lactic acid bacteria inhibit the adhesion of gastrointestinal pathogens to the intestinal mucosa. In addition, probiotic *Lactobacillus* strains have been shown to increase the secretion of IgA and certain antiinflammatory cytokines and to promote the gut immunological barrier in animal models, thus enhancing the host immune response. Millette et al. (2007) indicated that a probiotic milk culture of *L. acidophilus* and *L. casei* was capable of delaying the growth of the foodborne pathogens *Staphylococcus aureus*, *Enterococcus faecium*, and *faecalis* and *Listeria innocua*.

In order to provide health benefits, probiotic yogurt must contain a certain number of live bacteria at the time of manufacture. Even with such strict regulations in place, less than optimal numbers of bacterial counts have been found in commercial yogurt products and also a further decline after 3

Table 9.3. Summary of bioactive peptides derived from milk fermentation and/or processing according to their physiological classification[1]

Protein Precursor	Peptide Name	Physiological Classification
α_{S1}-casein	α_{S1}-Casecidin	Antimicrobial
	Isracidin	Antimicrobial
	α_{S1}-Casokinin-5	ACE[2] inhibitor
	Caseinophosphopeptide	Calcium binding and transport
	α_{S1}-casein exorphins	Opioid agonist
	Casoxin D	Opioid agonist
	α_{S1}-casein exorphins	Opioid agonists
α_{S2}-casein	Casocidin-1	Antimicrobial
	Caseinophosphopeptide	Mineral binding
β-casein	β-casokinin-7	ACE inhibitor
	β-casokinin-10	Immunomodulatory and ACE inhibitor
	Antihypertensive peptide	Antihypertensive
	Immunopeptide	Immunostimulatory
	β-casomorphin-4, -5, -6, -7 and -11	Opioid agonists
	Morphiceptin	Opioid agonist
	Caseinophosphopeptide	Mineral binding
κ-casein	κ-casecidin	Antimicrobial
	Casoplatelin	Antithrombotic
	Thrombin inhibitory peptide	Antithrombic
	Casoxin A	Opioid antagonist
	Casoxin B	Opioid antagonist
	Casoxin C	Opioid antagonist
	Caseinophosphopeptide	Mineral binding
α-lactalbumin	α-lactorphin	ACE inhibitor and opioid agonist
β-Lactoglobulin	β-Lactorphin	ACE inhibitor and opioid agonist
Lactoferrin	Lactoferricin A	Opioid agonist
	Lactoferricin B	Immunomodulatory and antimicrobial
Lactotransferrin	Thrombin inhibitory peptide	Antithrombic
Bovine serum albumin	Serorphin	Opioid agonist

[1]More detailed information is available in Clare and Swaisgood (2000), German et al. (2002), FitzGerald and Meisel (2003), FitzGerald and Murray (2006). Some of the peptides have not been specifically identified in yogurts but their presence is not excluded.

[2]ACE = Angiotensin-converting enzyme.

weeks of refrigeration at 4°C (Ibrahim and Carr 2006). It is generally assumed that probiotic microorganisms in a dairy product need to be viable in order to exert positive health effects. However, use of nonviable instead of viable microorganisms would be economically attractive because of longer shelf life and reduced requirements for refrigerated storage. Additionally, products that are fermented and then pasteurized could expand the potential use of probiotics to areas where strict handling conditions cannot be met in some developing countries (Ouwehand and Salminen 1998). There are some reports to indicate that consumption of cell-free whey from milk fermented with bifidobacteria was capable of modifying the human intestinal ecosystem. After consumption of the cell-free fermented whey for 7 days, fecal excretions of *Bacteroides fragilis*, *Clostridium perfringens*, and clostridial spores decreased, while counts of bifidobacteria increased (Romond et al. 1998).

Research has demonstrated that both the cell wall and cytoplasmic fractions of lactic acid bacteria were able to stimulate macrophages to produce significant amounts of tumor necrosis factor-alpha (TNF-α), IL-6, and nitric oxide (Tejada-Simon and Pestka 1999). Both heat-killed bacteria and highly purified lipoteichoic acid, a protoplast component in *Lactobacillus*, mediated NF-κB activation and TNF-α secretion in macrophage cells (Matsuguchi et al. 2003). NF-κB is an important mediator of various functions of macrophages, including the production of TNF-α, IL-1β, IL-6, and inducible nitric oxide synthase (Matsuguchi et al. 2003).

Prebiotics are nondigestible complex carbohydrates that selectively stimulate the growth and activity of bacteria in the colon and also beneficially affect the host (Gibson and Roberfroid 1995). Examples of probiotic ingredients being tested for yogurt production are β-glucans, inulin, pectin, gums, and resistant starch (Cummings et al. 2001; Vasiljevic et al. 2007).

Nonbacterial Components

The presence of lactic acid bacteria is thought to be essential for yogurt to exert immunostimulatory effects but components of nonbacterial origin, such as whey protein, short peptides, and conjugated linoleic acid (CLA), are believed to contribute to yogurt's beneficial effects as well. Besides the main proteins and naturally occurring bioactive peptides and minor proteins in milk, the activities of bacterial proteases during the fermentation process change the physicochemical state of milk proteins leading to the release of free amino acids and bioactive peptides (see Tables 9.2 and 9.3).

Lactic acid bacterial cells possess cell-envelope–located proteinases, which cause the degradation of caseins into oligopeptides. Because microbial proteases hydrolyze milk proteins more randomly than intestinal proteases, the fermentation process results in more significant amounts of free amino acids and bioactive peptides in yogurt than in milk. A large body of scientific research has established that the action of bacterial proteases on milk proteins during yogurt fermentation or during digestion by gut bacteria results in the release of bioactive peptides, exhibiting different bioactivities that may be beneficial to health (Clare and Swaisgood 2000; German et al. 2002; FitzGerald and Meisel 2003; FitzGerald and Murray 2006). The peptidic profile of milk proteins is significantly different after microbial fermentation, suggesting that microbial proteolysis can be a potential source of bioactive peptides (LeBlanc et al. 2002). These oligopeptides can be a direct source of bioactive peptides. Several casein-derived peptides may play a role in the modulation of the immune system. Fragments of β-casein have been shown to stimulate phagocytosis of sheep red blood cells by peritoneal macrophages, protect against infections, enhance the proliferation of human peripheral blood lymphocytes in vitro, and increase proliferation of murine peripheral blood lymphocytes in vivo. Lactoferricin, a peptide deriving from lactoferrin, has been shown to contain immunostimulating peptides, which can enhance the proliferation of spleen cells and can stimulate the phagocytic activity of human neutrophils (LeBlanc et al. 2002). Therefore, the immunoenhancing effects of yogurt may be attributable, in part, to peptides released from milk proteins during lactic acid bacterial fermentation.

Many lactic acid bacterial strains are capable of forming exopolysaccharides, which give a higher viscosity and a thicker texture to yogurt products. In addition, it was shown that some exopolysaccharides derived from certain lactic acid bacterial strains possess B cell mitogen activity, the capacity to induce cytokine production, and the capacity to modify some macrophage and splenocyte functions. Some lactic acid bacterial exopolysaccharides could induce a gut mucosal response for protective immunity, maintaining intestinal homeostasis, enhancing the IgA production at both the small and large intestine level, and influencing the systemic immunity through the cytokines released to the circulating blood (Vinderola et al. 2006). Thus, exopolysaccharide produced by lactic acid bacteria may also contribute to the immunoenhancing effect of yogurt.

Biochemical changes in milk fat occur during the fermentation process in yogurt production. Through the activity of bacterial lipase, large amounts of free fatty acids are released (Chandan and Shahani 1993). The process of biohydrogenation during fermentation results in higher CLA concentrations of yogurt than does the milk from which the yogurt was processed (Shantha et al. 1995). Since there is greater preference for lowfat and nonfat varieties of yogurt, the hydrolysis of milk lipids contributes little to the

composition of most yogurt products. However, research indicates that the yogurt composition of CLA can be manipulated/increased (Lin 2003; Xu et al. 2005; 2006) while CLA enriched yogurt has been shown to have effects on energy metabolism and adipose tissue gene expression in healthy subjects (Nazare et al. 2007).

PERSPECTIVES AND CONCLUDING REMARKS

Today, a large number of claims associate particular food ingredients and bioactive components with remedies for certain health conditions in humans. Although substantial evidence currently exists to support beneficial effects of yogurt consumption on human health, there are inconsistencies in reported results. The exact reasons for such inconsistencies are unknown. They may include different strains of LAB used in investigational procedures or may be due to the criteria used to assess health indices. To further complicate the matter, no strict regulations govern production of functional foods such as yogurt in most countries. False advertising about the benefit of yogurt products misleads the general public and can lead to potential lawsuits and mistrust by the public. Any health claims about yogurt or any other milk products have to be supported by solid scientific evidence and verification. Further, a health benefit claim based on research outcome from in vitro studies or laboratory animal subjects could be misleading if such claims are not independently validated in humans. Therefore, further research is required to substantiate benefits of those substances in intended end users and relationships with physiological conditions or health states validated. In particular, more human clinical studies are needed to prove efficacy in all cases. These studies have to be well designed and be of adequate duration.

With the large body of scientific research implicating different bioactive components such as peptides and CLA, and probiotics and prebiotics with modulatory effects for different health conditions, the possibilities of developing yogurt into nutraceuticals are enormous. The effects of addition of beta-glucan, CLA, folic acids, and some immune enhancers have been studied. Inclusion and augmentation of these beneficial components in yogurt preparations should be pursued. Yogurt products with confirmed or novel health claims can become important components of a healthy lifestyle and can greatly benefit public health.

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10
Bioactive Components in Kefir and Koumiss

Jia-ping Lv and Li-Min Wang

INTRODUCTION

The origin of fermented food goes back to the earliest stages of human history. Archaeological evidence has indicated that the process of fermentation in foods was discovered accidentally thousands of years ago. Fermented foods soon became popular because they may have longer storage time and improved nutritional values compared to their unfermented raw foods.

Milk and milk-derived products have constituted a significant part of the diet of western countries and some ethnic groups. Fermented dairy products from milk of various dairy animal species are perhaps the most common fermented foods worldwide. Kefir and koumiss are less well known than yogurt. However, these two products are popular and important for the people in certain regions of the world, and the research data on their nutritional and chemical composition indicate that they may contain various bioactive components that can provide humans with unique health benefits.

ORIGINS AND MANUFACTURE OF KEFIR

Outline of Kefir

Kefir is a viscous, slightly carbonated acid milk beverage that contains small quantities of alcohol and is believed to have its origins in the Caucasian mountains of Tibet and Mongolia (Jin 1999). It is also manufactured under a variety of names including *kephir, kiaphur, kefer, knapon, kepi*, and *kippi* (Zhou et al. 2003). Kefir can be made from the milk of different dairy species, but it is generally made from cow milk. The product is manufactured by standardizing milk to the desired fat and milk solids (typically 0.5–4% fat and 8%–11% milk solids), followed by pasteurization at 80–85 °C for 30 minutes. Traditionally, the heated milk is cooled to a temperature of 20–22 °C and then is fermented by adding kefir grains (a mass of proteins 1; polysaccharides; mesophilic, homofermentative lactic acid streptococci; lactobacilli; acetic acid bacteria and yeasts) to a quantity of milk (Hu and Huang 2006; Zhou et al. 2003). Kefir grains are irregularly shaped, gelatinous masses varying in size from 1 to 6 mm in diameter, and they resemble cauliflower florets in shape and color. Kefir grains exhibit an irregular surface and consist of a gel matrix in which yeasts and bacteria are imbedded and live symbiotically (Fig. 10.1).

Yeast is important in kefir fermentation (Irigoyen et al. 2005). Kefir grains usually contain lactose fermenting yeast (*Kluyveromyces lactis, Kluyveromyces marxianus, Torula kefir*), as well as nonlactose fermenting yeasts (*Saccharomyces cerevisiae*). The principal polysaccharide is a water-soluble substance known as *kefiran*, which is produced by multiple yeasts and bacteria in kefir grains (Keizo et al. 1990; Edward 2005). Grains are kept viable by transferring them daily into fresh milk and allowing them to grow for approximately 20 hours. Grains must be replicated in this way to retain their viability, since old and dried kefir grains have little or no

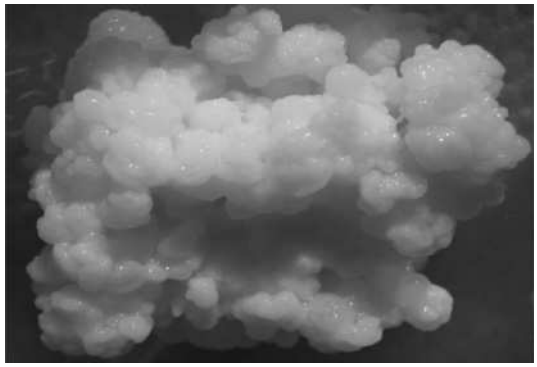

Figure 10.1. Kefir grains. Adapted from Liu hui (2004). Permission granted by the author.

ability to replicate (Edward 2005). Low temperature storage appears to be the best way to maintain kefir grains for long periods.

Due to the metabolic activity of the yeasts present in the product, kefir contains both ethanol and carbon dioxide. Furthermore, it is possible to achieve alcohol contents of not more than 1%, and lower levels are normally found in products made using the more defined starters. The alcohol is formed by the decomposition of lactose by kefir yeast. The sharp acid and yeasty flavor, together with the prickly sensation contributed by the carbon dioxide produced by the yeast flora can be considered as the typical kefir flavor (Motaghi et al. 1997). Kefir is more easily digested because a portion of the casein is transformed into a soluble segment and the rest forms very tiny coagulated flakes. The older a kefir is, the more hemialbumoses and peptones it contains.

Chemical Composition of Kefir

The chemical composition of kefir depends greatly on the type of milk that was fermented. However, during the fermentation, changes in composition of ingredients and nutrients have also been shown to occur. The major end products of the fermentation are lactic acid, acetaldehyde, acetoin, diacetyl, ethanol, and CO_2. Moreover, during the fermentation, vitamins B_1, B_{12}, soluble calcium, amino acids, folic acid, and vitamin K may increase in the kefir (Irigoyen et al. 2005). L (+) lactic acid is the highest concentration among organic acids after fermentation of kefir. Kefir contains 100% L(+) lactic acid. D(−) and L(+) lactic acids are different optical isomers of lactic acid. In general, L(+) lactic acid is beneficial to human beings. It may lower the pH in the stomach, promote the assimilation of protein and inhibit the growth of pathogenic microorganisms in the intestines. On the other hand, D(−) lactic acid cannot be assimilated due to the lack of its metabolic enzyme in the body. Therefore, if infants take large amounts of D(−) lactic acid one time, they will get acid hematic illness (Jin 1999).

Figure 10.2 presents the flow chart of kefir manufacture technology.

ORIGINS AND MANUFACTURE OF KOUMISS

Outline of Koumiss

Koumiss is a traditional drink of nomadic cattle breeders in Central Asia. It is a very popular fermented dairy product for the people of Mongolia, Kazakhstan, Kirgizstan, and some regions of Russia and Bulgaria (Svetla et al. 2005). It is a traditional beverage that is manufactured by Tartars and on the Steppes of the Kirgises. In the time of the Scythians, the antecedents of the Magyars, koumiss was a favorite drink.

Koumiss is usually made from mare milk by spontaneous fermentation of lactose to lactic acid and alcohol. Three types of koumiss exist—"strong," "moderate," and "light"—depending on the lactic acid content. Strong koumiss is generated by lactic acid bacteria (*Lactobacillus bulgaricus*, *Lactobacillus rhamnosus*) that acidify the milk to pH 3.3–3.6 and whose conversion ratio of lactose into lactic acid is about 80–90%. Moderate koumiss contains *Lactobacillus* bacteria (*L. acidophilus*, *L. plantarum*, *L. casei* and *L. fermentum*) with restricted acidification properties, that lower the pH to 3.9–4.5 at the end of the process, and the conversion ratio averages 50%. Light koumiss is a slightly acidified product (pH 4.5–5.0) and is formed by *Streptococcus thermophilus* and *Str. cremoris*. Moderate koumiss has the best fragrance and taste (Svetla et al. 2005).

Koumiss has been the subject of a limited number of studies. It is manufactured from fresh mare milk, incubating with yeast and lactic acid bacteria for a few hours, as shown in Figure 10.3. (Kücükcetin et al. 2003; Li et al. 2006). Lactobacilli play a major

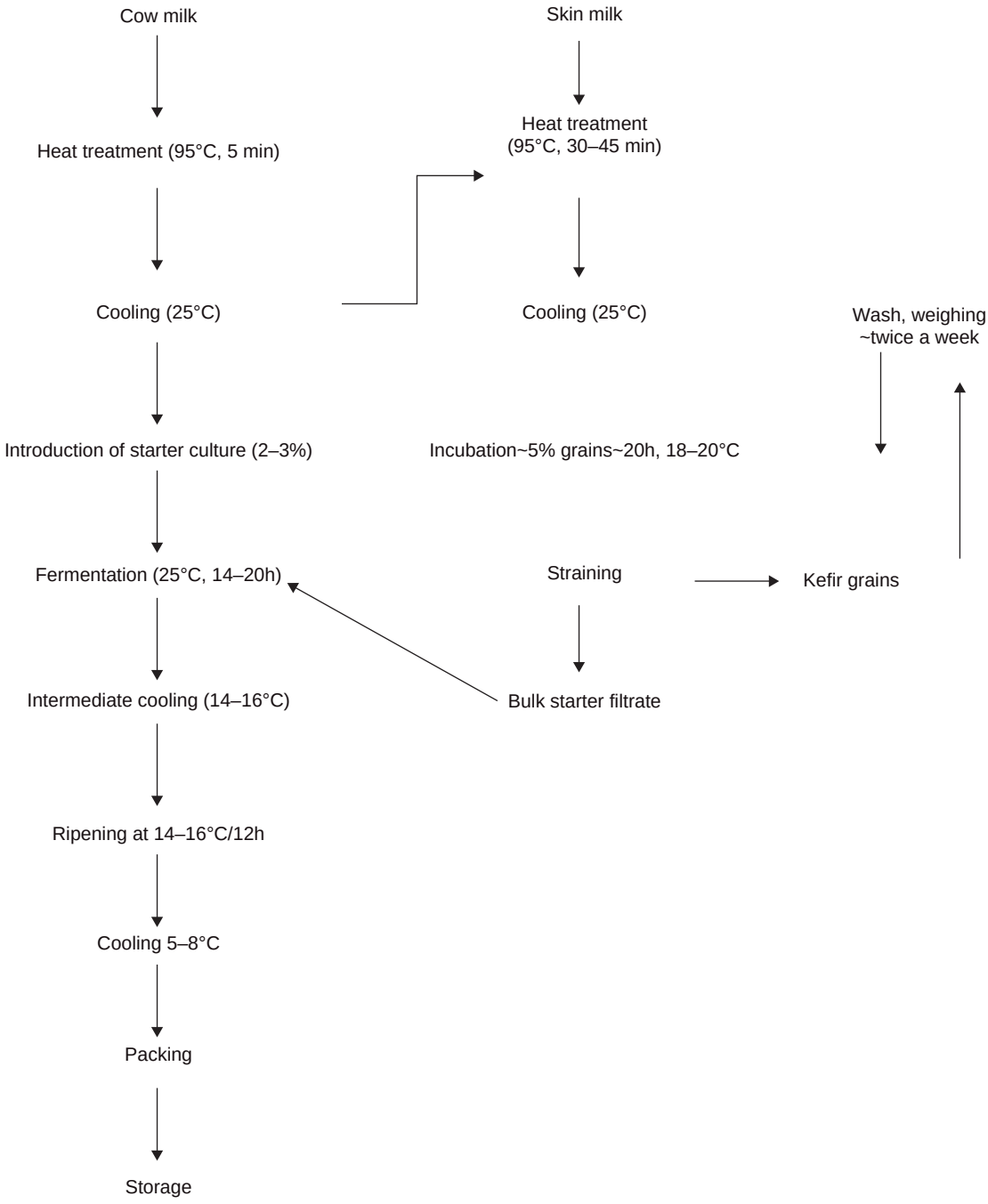

Figure 10.2. The flow chart of kefir manufacture technology.

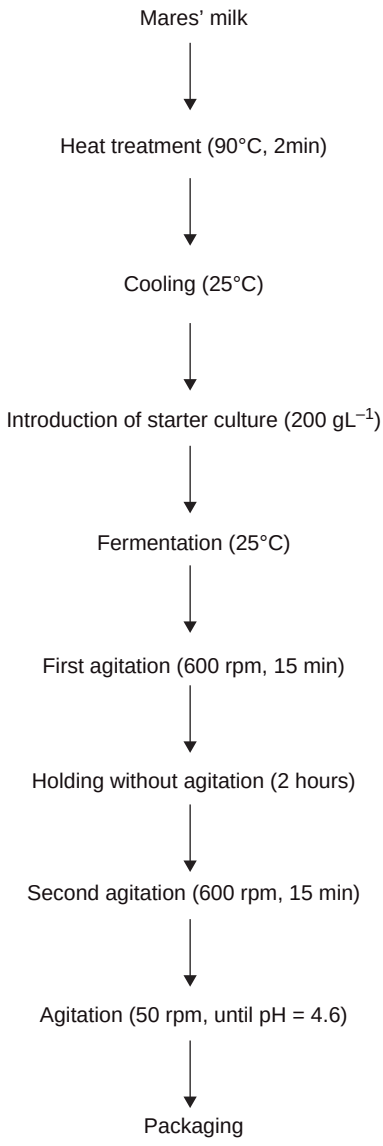

Figure 10.3. The flow chart of koumiss manufacture technology.

fermentative role affecting the aroma, texture, and acidity of the product as well as being of some benefit to human health.

Chemical Composition of Koumiss

The composition of koumiss depends mainly on the mare milk, and some bioactive components may be formed through fermentation of lactic acid bacteria and yeasts. Horses are single-stomach animals and have many physiological functions that are different from those of ruminants such as cows, goats, sheep, and camels. Mare milk contains a lower content of protein and higher lactose content than cow and sheep milk.

The protein content is 1.7–2.2% and depends on the milk used. Although mare milk protein content is lower than that in cow milk, the ratio of casein to whey protein is 1:1, which is close to human milk (Ha et al. 2003). Mare milk contains more essential fatty acids, especially linoleic and linolenic acid, than cow milk (Huo et al. 2002). Lactose is a major component in mare milk. Its concentration is 6.7% and may favor the fermentation of lactose. Lactose is decomposed into lactic acid, alcohol, and other small molecular substances by microorganisms, and the content of lactose in koumiss is about 1.4–4.4%. Mare milk contains calcium, phosphorus, magnesium, zinc, iron, copper, and manganese, which are beneficial to human beings. The ratio of calcium to phosphorus is 2:1, which is similar to human milk (Ha et al. 2003). Furthermore, vitamins A, C, E, B_1, B_2, B_{12}, pantothenic acid, and bacteriocin may increase in koumiss. Tables 10.1 and 10.2 list the chemical composition of koumiss.

Processing Technology of Koumiss

Because the cost of mare milk is a major restricting factor, the use of bovine milk to substitute for mare

Table 10.1. Nutritional values of koumiss

Ingredient	Fat (%)	Lactose (%)	Protein (%)	Alcohol (20 °C,%, v:v)	Amino acid (%)	Fatty acid (%)	Acidity (°T)	Vitamin C (mg/kg)
Quantity	1.75	2.80	2.00	2.20	1.77	1.65	110.00	78.40

Li et al. (2006).

Table 10.2. Some typical values of the major constituents of cow milk, mare milk koumiss, and cow milk kefir (%)

Ingredient	Cow Milk	Koumiss	Kefir
Protein	3.10	1.12	3.80
Fat	3.80	2.05	2.00
Lactose	4.60	2.20	2.00
Lactic acid	—	1.15	0.90
Alcohol	—	1.65	0.80
Water and salts	88.50	91.83	90.50

Adapted from Zhang and Cheng (1997).

milk for koumiss production has been of great interest to researchers. Because of the difference in composition between mare and bovine milk, it is necessary to modify bovine milk to make it suitable for the production of koumiss. Various methods have been used to modify bovine milk, such as decreasing fat content and adding water, lactose, and ultrafiltration retentate of bovine milk (Kücükcetin et al. 2003). However, the success of these approaches has been limited.

Koumiss is digestible and it has a somewhat sweet-sour taste. It is expensive and its curative properties are probably not better than those of kefir. It also contains higher alcohol content (1–3%) than kefir.

BIOACTIVE COMPONENTS IN KEFIR AND KOUMISS

The chemical composition of a foodstuff provides a useful indication of its potential nutritional value; the data shown in Table 10.2 indicate the main components of typical koumiss and kefir.

The area of functional foods has attracted a great deal of attention because it is now recognized that many foods contain bioactive ingredients, which may have health benefits or reduce the risk of getting certain diseases. One portion of functional foods is probiotic foods, in which there are several possible sources of bioactive ingredients: the microorganisms themselves (dead or alive), metabolites of the microorganisms formed during fermentation (including antibiotics or bacteriocins), or breakdown products of the food matrix, such as peptides and organic acids.

Koumiss and kefir have been known for their health benefits for centuries, especially in Eastern Europe and Inner Mongolia of China. The benefits of consuming kefir and koumiss are numerous. Kefir is reported to possess antibacterial, immunological, antitumoral, and hypocholesterolemic effects (Gabriel et al. 2006). For centuries, koumiss has been known as a wholesome beverage. The Mongols have developed the "koumiss therapeutics" method and koumiss medical centers have been set up in Russia, Mongolia, and Inner Mongolia. Favorable effects on the circulatory and nervous systems, blood-forming organs, kidney functions, endocrine glands, and immune system have been reported by many authors (Marcela et al. 2001; Ha et al. 2003; Li et al. 2006). Today koumiss is considered a functional food in China.

As the fermented mare milk product, koumiss has many nutritional and therapeutic properties, which are considered beneficial to elderly patients and infants. The composition of mare milk is similar to human milk, in particular regarding its low protein content and its low casein-to-whey protein ratio. In addition, several characteristics of mare milk, such as a high level of polyunsaturated fatty acids and low cholesterol content, seem to support the interest in increasingly using mare milk for human consumption.

Carbohydrates

Carbohydrates consist of "available carbohydrates" and "unavailable carbohydrates." Available carbohydrates are all carbohydrates that can be digested by the human body and hence can act as a source of energy for metabolism. In the case of natural koumiss and kefir, a number of mono- and disaccharides are present in trace amounts. Unavailable carbohydrates refer to the long-chain polysaccharides composed of regular arrangements of monosaccharide units, and the molecules cannot be hydrolyzed by digestive enzymes in the human body (Tamime and Robinson 1985). In koumiss and kefir, most available carbohydrates are produced by a variety of lactic acid bacteria, including *Lactobacillus*, *Streptococcus*, *Lactococcus* and *Leuconostoc* (Huo et al. 2002; Edward 2005).

Available carbohydrates include exopolysaccharides (EPS), which are extracellular, long-chained, branched, and high molecular mass polymers con-

taining α- and β-linkages that can be either homopolysaccharides or heteropolysaccharides (Zisu and Shah 2007). They are cell-surface carbohydrates and often loosely bound to the cell membrane. Kefiran contains D-glucose and D-galactose in a ratio of 1:1. Examples can be found in the literature, where the same bacterial strain produced different EPSs in different media. *Lactobacillus* sp. KPB-167B was found to produce capsular polysaccharide. Kefir grains grown in soy milk produce an EPS that has been shown to be primarily composed of D-glucose and D-galactose with a molecular weight of approximately 1.7×10^6 Da (Liu et al. 2002).

Clinical studies have shown that these EPSs stimulate the mammalian immune system by activating macrophages and subsequently increasing the T cell cascade, enhancing the secretion of cytokines like TNF-α, IFN-γ, IL-1β, etc., and potentiate the delayed-type hypersensitivity response associated with tumor growth suppression (Kodama et al. 2003; Edward 2005).

The effect of kefiran on the biological activity of *Bacillus cereus* strain B10502 extracellular factors was assessed by using cultured human enterocytes (Caco-2 cells) and human erythrocytes. In the presence of kefiran concentrations ranging from 300 to 1000 mg/L, the ability of *B. cereus* B10502 spent culture supernatants to detach and damage cultured human enterocytes was significantly abrogated. In addition, mitochondrial dehydrogenase activity was higher when kefiran was present during the cell toxicity assays. Protection was also demonstrated in hemolysis and apoptosis/necrosis assays. Scanning electron microscopy showed the protective effect of kefiran against structural cell damages produced by factors synthesized by *B. cereus* strain B10502 (Zeynep et al. 2005). The protective effect of kefiran depended on a strain of *B. cereus*. The findings demonstrate the ability of kefiran to antagonize key events of *B. cereus* B10502 virulence. This property, although strain-specific, gives new perspectives to the role of bacterial exopolysaccharides in functional foods (Micaela et al. 2008).

Lactic Acid

The ability to produce lactic acid is one of the most important characteristics of the starters. The amount of lactate influences the quality and variety of the final koumiss and kefir products. There is limited published information about the process of lactic acid fermentation in koumiss and kefir.

A proportion of the global human population is unable to digest lactose because of lack of lactase, and the free lactose may be used by other intestinal bacteria to produce a range of unpleasant symptoms, such as abdominal bloating, cramping, and diarrhea. This reaction to the ingestion of milk is usually referred to as *lactose intolerance*. Some lactic acid bacteria and yeasts in koumiss and kefir may be tolerant of the low acidity and are responsible for converting lactose to lactic acid with the help of its β-galactosidase.

The primary role of lactic acid bacteria in koumiss and kefir is to utilize lactose as a substrate and convert it into lactic acid during the fermentation. Lactose is taken up as the free sugar and split with β-galactosidase to glucose and galactose. Both glucose and galactose are metabolized simultaneously, via the glycolytic and D-tagatose 6-phosphate pathways, respectively. In addition, galactose can also be further metabolized by enzymes of the Leloir pathway. Probiotic fermented milk products have recently become widely used to promote a healthy gastrointestinal system. Acidophilus milk contains 1% lactic acid, but for therapeutic purposes those with 0.6–0.7% are usual. The consumption of koumiss and kefir may have similar effects and with improved sensory quality.

The amount of lactic acid present in koumiss and kefir is relatively low. D(−) and L(+) lactic acid are two different lactic acid isomers. WHO recommends 100 mg D(−) lactic acid/kg body weight for a human being, and an adult whose weight is 60 kg should take in no more than 1 kg D(−) lactic acid every month (Zhang and Cheng 1997). As for koumiss and kefir, the percentage of L(+) lactic acid is approximately 100%, but the content of L(+) lactic acid differs with different starters in kefir. Studies show that lactic acid content of kefir made in Australia is 6–8 g/kg and the percentage of L(+) lactic acid is 94.3%, while the percentage of L(+) lactic acid in Germany is 93.1% (Zhang and Cheng 1997). The percentages of D(−) lactic acid in different milk products are shown in Table 10.3. From the table we may see that the percentage of L(+) lactic acid is higher in kefir than in other fermented milk.

Table 10.3. Percentages of lactic acid in different fermented milk

Products	L(+) Lactic Acid (%)	D(−) Lactic Acid (%)
Kefir	95–98	2–5
Buttermilk	94–97	3–6
Sour milk	96–98	2–4
Fresh cheese	86–96	4–14
Yogurt	40–75	25–60
Cheese	50–90	10–50

Adapted from Zhang and Cheng (1997).

Peptides

Protein is one of the three major constituents of our diet. In addition to their nutritional function, proteins contribute significantly to the sensory attributes of foods. The functional properties of proteins are important for their characteristics in food processing. Proteins in milk are of excellent quality biologically and both the caseins and whey proteins are well endowed with essential amino acids. Diets based on casein, milk, and fermented milk show the maximum increment of body mass in rats. The highest values of the protein utilization index are found in animals fed on fermented milk products. The favorable protein utilization and body mass increment in animals on fermented milk diets are attributed to a better digestibility of proteins in these products.

Fermentation of milk proteins by lactic acid bacteria and yeasts may make the products totally digestible and result in the release of peptides and amino acids. Many organisms possess enzymes, such as proteinases and peptidases, that are able to hydrolyze proteins. Fermented milk (yogurt, kefir, koumiss, sour milk) compared with unfermented milk gives higher values of nonprotein nitrogen and free amino-nitrogen content after pepsin digestion in vitro. Some kefir grains and koumiss starters have high proteinase activity, which increases the possibility that bioactive peptides may be present in koumiss and kefir. Initial studies on the peptide content of a kefir drink have shown that kefir contains a large number of peptides and that the majority of kefir peptides have molecular weights of ≤5,000 kDa (Edward 2005).

The peptides and amino acids released by bacteria may affect the nutritional potential and biological value of the final products. Amino acids may not be directly contributory to the flavor and aroma of fermented milk; however, they act as precursors of a number of reactions that produce carbonyl compounds. Caseins are the main source of amino acids. The contribution of caseins to the provision of essential amino acids depends on the type of proteinase, and the proteinase of the cell membrane is a key enzyme in that process because it is the only enzyme capable of initiating the degradation of casein to oligopeptedes (Emilina et al. 2006). Endopeptidase activity was found in strains of *S. thermophilus* and *Lactococcus lactis* spp. *Lactis*, and aminopeptidase in *Lactobacillus. bulgaricus* and *L helveticus* (Chopin 1993). In multiple component starters it is difficult to characterize the interaction between them. There are few data about the pathway of bioactive peptides and the relationship between the casein and bacteria growth. There is also little information available about the changes in free amino acid concentration in the growth medium after the cultivation of multiple-strain starters. Studies showed that proteins were broken down by acetic acid bacteria and yeasts with the existence of lactic acid. During the first hours lots of free amino acids appeared, and then free amino acids would accumulate. The contents of proline, leucine, lysine, and histidine increased significantly (Zhang and Cheng 1997). Two active peptides isoleucyl-prolyl-proline (Ile-Pro-Pro) and valyl-prolyl-proline (Val-Pro-Pro) have been isolated consistently from casein digests by *L. helveticus* (Paraskevopoulou et al. 2003). These peptides are mainly of low molecular weight and some of them become apparent only after the products of the bacterial activity upon the casein fractions are further subjected to proteolysis by pepsin and trypsin in the digestive tract.

Peptides formed during koumiss and kefir fermentation process have also been shown to have a variety of bioactive and physiological activities, including stimulation of the immune system in animal models and lower blood pressure in spontaneously hypertensive rats and in humans with mild hypertension (Sipola et al. 2002). Tryptophan, one of the essential amino acids abundant in kefir, is well known for its relaxing effect on the nervous system. Although hypertension is considered a disease of

mature and old age, precursor conditions leading to hypertension are often present at a very young age. Furthermore, hypertension secondary to a number of conditions, such as kidney, endocrine, and neurological diseases, are frequent in childhood and for this reason it is important to consider all preventive and therapeutic possibilities that may be useful at a young age, including the effect of bacterial fermentation upon koumiss and kefir.

PROBIOTICS

The word *probiotic*, derived from the Greek language, means "for life" and has had many definitions in the past. Recently, the definition of probiotic has been expanded to a mono- or mixed-culture of live microorganisms, which applied to man or animal (e.g., as dried cells or as a fermented product), beneficially affect the host by improving the properties of the indigenous microflora (Yan and Jian 2007). Interest in the role of probiotics for human health goes back at least as far as 1908 when Metchnikoff suggested that man should consume milk fermented with lactobacilli to prolong life (Analie and Bennie 2001). At present it is generally recognized that an optimum balance in microbial population in our digestive tract is associated with good nutrition and health. The microorganisms primarily associated with this balance are lactobacilli and bifidobacteria. Factors that negatively influence the interaction between intestinal microorganisms, such as stress and diet, lead to detrimental effects in health. Increasing evidence indicates that consumption of probiotic microorganisms can help maintain a favorable microbial profile and results in several therapeutic benefits. The most popular dairy products for the delivery of viable *Lactobacillus acidophilus* and *Bifidobacterium bifidum* cells are yogurt, koumiss, and kefir, and consumption of probiotic bacteria via food products is an ideal way to reestablish the intestinal microflora balance.

Furthermore, the lactic acid bacteria (LAB) in koumiss and kefir (see Table 10.4) may produce an array of substances, such as organic acids, hydrogen peroxide, antimicrobial peptides, and deconjugated bile acids (Ana et al. 2007). These compounds have antimicrobial effects called *bacteriocins*. Bacteriocins are considered to be safe nonartificial antimicrobials, because they are assumed to be degradable by proteases.

Koumiss and kefir are made by fermentation with a mixed microflora, which contains different lactic acid bacteria and yeasts. *L. brevis*, *L. acidophilus*, *L. casei*, *L. plantarum*, and *Streptococcus lactis* are the predominant lactic acid bacteria (Motaghi et al. 1997). In general, lactic acid bacteria are more numerous (10^8–10^9) than yeasts (10^5–10^6) and acetic acid bacteria (10^5–10^6) in kefir grains, although fermentation conditions can change this pattern. These probiotics in koumiss and kefir have been shown to be inhibitory toward many of the commonly known foodborne pathogens. Production of organic acids by probiotics may lower the pH and alter the oxidation-reduction potential in the intestine, resulting in antimicrobial action. Combined with the limited oxygen content in the intestine, organic acid inhibits especially pathogenic Gram-negative bacteria. Probiotics may prevent the intestinal infections by inhibiting the growth of pathogens and may reduce serum cholesterol within the intestine (Analie and Bennie 2001).

At the same time, the yeasts in koumiss, such as *Saccharomyces cerevisiae*, *Candida* sp., produce alcohol, which may have antibiotic function. Studies on 94 koumiss samples showed that 81 samples had single-spore yeast and the amounts of single-spore yeast were in positive correlation to the height above sea level (Huo et al. 2002). Almost every koumiss sample from Inner Mongolia contains single-spore yeasts. The various yeasts in koumiss play an important role in the fermentation process and in the formation of bioactive components.

The role of probiotics in lowering serum cholesterol is not yet understood. The hypothesis is that reduction of cholesterol may be due to a coprecipitation of cholesterol with deconjugated bile salts at lower pH values as a result of lactic acid production by the probiotics (Kailasaphaty and Rybka 1997). Furthermore studies showed that probiotics can inhibit carcinogens and/or procarcinogens; they may inhibit the growth of bacteria that convert procarcinogens to carcinogens and reduce the intestinal pH to reduce microbial activity.

OTHER COMPONENTS

Fatty Acids

There is limited information about the composition of fatty acids in koumiss and kefir. It must be stressed that mare and cow milk fats contain an extremely

Table 10.4. Bacteria and yeast found in kefir grains, kefir, and koumiss

Product	Bacteria and Yeast
Kefir	***Lactobacilli***
	Lactobacillus kefir, L. delbrueckii, L. kefirnofaciens, L. rhamnosus
	L. kefirgranum, L. casei, L. parakefir, L. paracasei
	L. brevis, L. fructivorans, L. plantarum, L. hilgardii
	L. helveticus, L. fermentum, L. acidophilus, L. viridescens
	Lactococci
	L. lactic subsp. lactis, L. lactis subsp. cremoris
	Streptococci
	S. thermophilus, S. filant, S. durans
	Enterococci
	E. durans
	Leuconostocs
	Leuc. mesenteroides ssp. *dextranicum*
	Leuc. mesenteroides ssp. *cremoris*
	Acetic Acid Bacteria
	Acetobacter sp., A. pasteurianus, A. aceti
	Yeast
	Kluyveromyces lactis, K. marxianus ssp. *bulgaricus, K. marxianus* ssp. *marxianus*
	Saccharomyces florentinus, S. globosus, S. unisporus, S. carlsbergensis
	Candida kefyr, C. pseudotropicalis, Torulaspora delbrueckii
	Other Bacteria
	Bacillus sp., Bacillus subtilis, Micrococcus sp., Escherichia coli
Koumiss	*Lactobacillus salvarius, L. buchneri, L. pantarum*
	L. acidophilus, L. delbrueckii, L. casei
	Lactobacillus curvatus, Lactobacillus agilis, Lactobacillus zeae
	Lactobacillus coryniformis subsp. *coryniformis, Lactobacillus acetolerans*
	Lactobacillus kefiranofaciens, Lactobacillus sake

From Edward (2005), Svetla et al. (2005), Zhang et al. (2005).

wide range of fatty acids. Most of these are present in the form of various glycerides, but over 400 individual fatty acids have been identified in mare and cow milk. Obviously it is impossible to assign a physiological role to all. Studies show that the concentrations of fatty acids C_{16} and below in koumiss and kefir are broadly similar to unfermented milk, while $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ contents of koumiss and kefir are significantly higher. Studies show that the percentage of C_{14}–C_{18} is 88%, including 33% linoleic acid and linolenic acid. The benefits of $C_{18:3}$ to humans include anticarcinogenic properties, prevention of cardiovascular diseases and hypertension, and improvement of vision (Zhang et al. 2006).

The linoleic acid concentration of mare milk and cow milk is 8.50 and 2.39 mg/100 g, respectively. Several studies have reported that the addition of LAB (see Table 10.5) to dairy products may contribute to the production of free fatty acids by lipolysis of milk fat. Moreover, LAB also has the ability to produce conjugated linoleic acid (CLA) from linoleic acid. The CLA has several beneficial health effects including reduced risk of carcinogenesis, atherosclerosis, and obesity; improved hyperinsulinemia; and prevention of catabolic effects of the immune system (Seppänen et al. 2002).

Vitamins

Mare milk contains vitamins A, C, E, B_1, B_2, B_{12}, and pantothenic acid. Furthermore, some bioactive components are the end products of probiotics during the fermentation. For koumiss, the probiotics are mainly composed of lactic acid bacteria and

Table 10.5. The CLA formation strains of lactic acid bacteria

Strains	Codes
Lactobacillus acidophilus	CCRC 14079; AKU 1137; AKU 1122; IAM 10074; ATCC 43121, CGMCC 1.1854
L. plantarum	4191; AKU 1009; AKU 1122; JCM 8341; ZS 2058
L. brevis	IAM 1082;
L. paracasei ssp. paracasei	IFO 12004; JCM 1109; AKU 1142; IFO 3533
L. casei	E5; E10; Y2; F 19
L. pentosus	AKU 1148; AKU 1142; IFO 12011
L. rhamnosus	AKU 1124
L. reuteri	ATCC 23272; ATCC 55739
Lactobacoccus lactis	ATCC 19435; M 23; 40;
Bifidobacteria breve	NCFB 2257; NCFB 2258; NCTC 11815; NCIMB 8815
B. adolescentis	NCFB 230; NCFB 2204; NCFB 2231
B. infantis	NCFB 2205; NCVB 2256

yeasts. They also produce antibacterial products and stimulate the synthesis of vitamin B_1, B_2, and B_{12}.

The beneficial yeast and bacteria (lactic acid bacteria and acetic acid bacteria) in kefir can produce vitamin B_1, B_2, B_{12}, and K during fermentation. Furthermore kefir is an excellent source of biotin, a B vitamin that aids the body's assimilation of other B vitamins, such as folic acid, pantothenic acid, and B_{12}. The benefits of maintaining adequate B vitamin intake range from regulation of the kidneys, liver, and nervous system to helping relieve skin disorders, boost energy, and promote longevity.

Minerals

In addition to beneficial bacteria and yeast, koumiss and kefir also contain minerals, which can help the body with healing and maintenance functions. Kefir contains an abundance of calcium and magnesium, which are important minerals for healthy bones and nervous system. The contents of minerals in koumiss are relatively low, but the ratio of calcium and phosphorus is 2:1, which is good for humans.

Koumiss and kefir are the outcome of intense bacterial activity of the starter cultures, leading to production of lactic acid and biologically active compounds, adding nutritional and physiological value. Even though there are many studies on koumiss and kefir and their properties and the results are still controversial, few studies have been carried out in human trials. With the improvement of living standards, people pay more attention not only to the comprehensive nutritional value, but also to the digestible efficiency. Koumiss and kefir differ from other fermented milk products because they are not produced by the metabolic activity of a controlled culture. They are made by fermentation with a mixed microflora, which is confined to several types of LAB and yeasts.

In summary, koumiss and kefir have a long history as healthy foods, implying that these two products have some bioactive compounds that would be beneficial for human health. Recent applications of modern research methodologies demonstrated that the microorganisms and their end products in the course of fermentation processes can intervene in a variety of biological activities that have positive impact on the health and well being of all human age groups.

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11
Bioactive Components in Whey Products

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INTRODUCTION

Whey proteins such as β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) are cheese byproducts. Whey proteins represent about 20% of milk proteins. Whey proteins are comprised of 50% β-Lg; 12% α-La; 10% immunoglobulins; 5% serum albumin, and 0.23% proteose peptones, lactoferrin (LF), and lactoperoxidase (LP) (Horton 1995; Siso 1996). Individual whey protein components and their peptide fragments show various bioactivities including antimicrobial and antiviral actions, immune system stimulation, anticarcinogenic activity, and other metabolic features. Several peptides, which are inactive due to the encryption within the sequence of the parent proteins, may act as regulatory compounds by providing a hormonelike activity (Gobetti et al. 2002).

Cheese whey is the liquid remaining after the precipitation and removal of milk casein curd. The whey proteins are not coagulated by acid and are resistant to the actions of rennet or chymosin.

Liquid cheese whey can be processed into different forms including condensed whey, acid- or sweet-whey powders, demineralized whey powder, and delactosed whey powder, which provide significant benefits for preservation, manipulation, and transport (Kosikowski 1979; Siso 1996). Whey protein concentrate (WPC) and whey protein isolate (WPI) derived from whey processing have a high protein content, since the protein portion from the liquid whey is recovered and concentrated (Kosikowski 1979). The most commonly used methods for protein recovery are ultrafiltration and diafiltration. These methods offer cost reduction, high processing speed, and the absence of denaturation or protein-structure modification (Evans and Gordon 1980; Kosikowski 1979).

Whey proteins can be used as simple protein supplements as well as food ingredients due to their unique effect on food texture (Kinsella and Whitehead 1989a; Kosikowski 1979; Siso 1996). Extensive investigations have been conducted on the functionalities of whey proteins, such as gelling, foaming, water-holding, emulsifying, and stabilizing (Burrington 1998; DeWit 1998; Huffman 1996; Szczesniak 1998). Understanding and controlling their functional properties is the key to whey protein utilization.

The β-Lg is the major component of whey proteins. The β-Lg is a small (18.3 kDa) globular protein made up of 162 amino acid residues with two disulfide (-SS-) groups and one sulfhydryl (-SH) group. The α-La is the second abundant component in whey protein. A number of β-Lg– and α-La–derived peptides have significant angiotensin-converting enzyme (ACE)–inhibitory activity and antimicrobial activity (Bruck et al. 2003; Pellegrini et al. 1999, 2001). Other bioactivities of β-Lg– and α-La–derived peptides include antiviral (Berkhout et al. 1997; Neurath et al. 1997a,b; Oevermann et al. 2003; Superti et al. 1997; Wyand et al. 1999), anticarcinogenic (Duringer et al. 2003; Fast et al. 2005a,b; McIntosh et al. 1995), hypocholesterolemic (Nagaoka et al. 2001), and opioidlike activities (Horikawa et al. 1983; Teschemacher 2003; Teschemacher et al. 1997; Yoshikawa et al. 1986).

Bovine serum albumin (BSA) has been given little attention in respect to its role in the functional properties of whey protein concentrates and makes up only about 5% of the protein in whey protein

concentrates. BSA is one of the proteins with good essential amino acid profiles. Free fatty acids, lipids, and flavor compounds can be easily absorbed to BSA (Kinsella and Whitehead 1989b). Its lipid-binding properties are a primary biological function (Fox and Flynn 1992). Lipid-binding properties of BSA could play a role in mediating lipid oxidation, since BSA has been shown in vitro to protect lipids against phenolic-induced oxidation (Koisumi and Nonaka 1975; Smith et al. 1992).

LF is a glycoprotein (80 kDa) that is a member of the transferring family in milk (Metz-Boutique et al. 1984). LF consists of a single polypeptide chain comprising 673 amino acid residues that form two homologous domains. LF has been shown to have a number of other physiological and biological functions. LF plays an important role in iron uptake in the intestine and is considered to be an important host defense molecule (Levay and Viljoen 1995; Wakabayashi et al. 2006). LF plays an important role in innate defense against antimicrobial activities, antiviral activities, antioxidant activities, immunomodulation, and cell growth modulation (Baveye et al. 1999; Chierici et al. 1992). The antimicrobial peptide derived from hydrolyzed LF also exhibits various bioactivities (Bellamy et al. 1992). LF makes an important contribution to the host defense system by eliminating pathogens, viruses, and fungi. The biological activities of LF include iron transport (Nagasako et al. 1993), antimicrobial activity (Farnaud and Evans 2003), antifungal activity (Bellamy et al. 1994), antiviral activity (van der Strate et al. 2001), anticancer activity (Sekine et al. 1997c), immunomodulating effects (Shinoda et al. 1996), and antiinflammatory activity (Legrand et al. 2005).

Immunoglobulins (IGs) are a family of globular proteins with a number of bioactivities, including antimicrobial and protective activities. IGs form a globular structure in water. IGs have several bioactive functions, such as protection of the gut mucosa against pathogenic microorganism and passive immunity to the ruminant neonate in colostrum (Butler 1983; Korhonen et al. 2000). Among IGs, immunoglobulin G (IgG) antibodies show multifunctional activities, including complement activation, opsonization, and agglutination in bacteria. In addition, IgG helps reduce infection by binding to specific sites on the surfaces of most infectious agents or products (Lilius and Marnila 2001). In bovine colostrum and milk, IgG (subclasses IgG1 and IgG2) is the major immune component (Blakeslee et al. 1971; Butler et al. 1971). However, the levels of immunoglobulin A (IgA) and immunoglobulin M (IgM) are low. In the transition from colostrum to mature milk, IG levels decrease sharply during the first 5 days postpartum (Guidry et al. 1980; Oyeniyi and Hunter 1978).

Whey contains components that provide significant nutritive elements, immunological protection, and bioactive substances (Warner et al. 2001). Whey proteins can be used as simple protein supplements as well as novel healthy ingredients in the food industry. Whey proteins provide beneficial health-promoting functions. These include promotion of health conditions and prevention of diseases.

It is clear that bioactive components in whey have potential benefits in terms of nutritional, functional, and bioactive food and pharmaceutical products. In this chapter, we summarize the beneficial physiological effects of the bioactive components in whey proteins, including β-Lg, α-La, and other minor elements and their derived peptides.

BIOACTIVITIES OF WPC, WPI, AND THEIR DERIVATIVES

Inhibition of ACE Activity in WPC, WPI, and Their Derivatives

Recovered and concentrated whey proteins are usually dried in the form of powders such as WPC and WPI. Figure 11.1 shows a commercial whey

Figure 11.1. Commercial whey protein product (WPI) with powders in display.

protein powder. About 50% of cheese whey is treated and transformed into various foods, pharmaceutical products, and other bioproducts in the form of liquid whey, powdered whey, WPC and WPI (Marwaha and Kennedy 1988).

Whey proteins including WPC and WPI are hydrolyzed enzymatically into peptides but are often inactive in the sequence of the parent protein (Foegeding et al. 2002; Meisel 1998). Certain bioactive peptides in whey proteins may protect against hypertension through ACE-inhibitory activity, which controls high blood pressure and hypertension through the dilation of blood vessels (Belem et al. 1999; Masuda et al. 1996; Mullally et al. 1997a; Pihlanto-Leppälä et al. 1998; Walsh et al. 2004). ACE is effective in the regulation of blood pressure because it catalyzes the formation of angiotensin II from angiotensin I. Two amino acids are removed from angiotensin-I, yielding the octapeptide angiotensin-II, which is a very potent vasopressor. Inhibition of the angiotensin-II synthesis lowers blood pressure. ACE inhibition is measured by the concentration of substance needed to inhibit 50% of the original ACE activity (IC_{50}). A lower IC_{50} value indicates higher efficacy. The ACE-inhibitory and hypocholesterolemic activities of whey proteins and their derivatives are listed in Table 11.1.

Whey protein exhibits a greater hypocholesterolemic effect in comparison with other proteins such as casein or soybean protein. The hypocholesterolemic effect of whey proteins has been reported in previous research (Sautier et al. 1983). The level of serum high-density lipoprotein (HDL) cholesterol was reduced in rats that ingested WPI for 49 days (Sautier et al. 1983). Serum total and HDL cholesterol levels had a significant positive correlation with Tyr and Glu and a negative correlation with Cys and Ala in the WPI. In another study, consuming a diet of 20% WPC for 45 days significantly lowered the cholesterol level in rats (Jacobucci et al. 2001).

Anticarcinogenic Activity in WPC, WPI, and Their Derivatives

The anticarcinogenic activity of whey proteins and their individual components is well documented (Badger et al. 2001; Gill and Cross 2000; McIntosh et al. 1995; Tsuda et al. 2000). Whey protein offers protection against colon and mammary tumors (Hakkak et al. 2000; Rowlands et al. 2001). WPC also provides anticarcinogenic and anticancer activities due to increase of glutathione (GSH) concentration, which stimulates immunity originating antitumor effects in low-volume tumor cells (Bounous 2000) or the decrease of tumor cells with higher concentration of GSH (Parodi 1998). In addition, hydrolyzed WPI protected against oxidant-induced cell death in a human prostate epithelial cell line (RWPE-1) (Kent et al. 2003). The anticarcinogenic activity of whey proteins and their derivatives is summarized in Table 11.2.

Immune System Modulation in WPC, WPI, and Their Derivatives

Whey proteins have been reported to enhance nonspecific and specific immune responses through both in vitro and in vivo experiments (Gomez et al. 2002). Dietary supplementation with a whey-based product increased the lymphocyte level of glutathione (GSH), which is a naturally found peptide providing intracellular defense against oxidative stresses (Grey et al. 2003). GSH is naturally found in all cells of mammals. When illness occurs, GSH is depleted because of said stress. The content of amino acid precursors such as cysteine, glutamate, and glycine in the synthesis of GSH contributes to immunoenhancing effects. Thus, the ingestion of whey proteins plays an important role in the production of GHS because whey proteins are rich in cysteine and glutamate (DeWit 1998; Wong and Watson 1995). Both WPC and WPI are effective cysteine donors for GSH replenishment, during immune deficiency states. The immune system modulating capacity of whey proteins and their derivatives is summarized in Table 11.3.

Whey proteins have a high protein quality score. Whey proteins help improve muscle strength since their overall amino acid composition is rather similar to that of skeletal muscle. Among the essential amino acids, branched-chain amino acids (BCAAs)—leucine, isoleucine, and valine—are neutral amino acids with interesting and clinically relevant metabolic effects (Laviano et al. 2005). BCAAs constitute about 26% of the total protein (Bos et al. 2000; Layman 2003) and about 50% of the essential amino acids in milk protein (Jenness 1974). Accordingly, the high concentration of BCAA, and leucine in particular, in dairy products may be an important

Table 11.1. Angiotensin-converting enzyme (ACE)–inhibitory and hypocholesterolemic activities of bioactive components in whey products

Components	Bioactive Functions	Reference
Whey protein concentrates (WPC), whey protein isolates (WPI), and their derivatives	Conversion of angiotensin-I to octapeptide angiotensin-II, which is a very potent vasopressor	Belem et al. 1999 Masuda et al. 1996 Mullally et al. 1997a
	Control of high blood pressure and hypertension through the dilation of blood vessels	Pihlanto-Leppälä et al. 1998 Walsh et al. 2004
	Reduction of serum high-density lipoprotein (HDL) cholesterol	Sautier et al. 1983
	Reduction of cholesterol level	Jacobucci et al. 2001
β-lactoglobulin and its derivatives	Enhancement of ACE-inhibitory activity of hydrolyzed tryptic peptide of β-Lg, f142–148	Mullally et al. 1997a Mullally et al. 1997b
	Most potent β-Lg-derived ACE-inhibitory peptide, Ala-Leu-Pro-Met-His-Ile-Arg	Ferreira et al. 2007 Mullally et al. 1997b
	ACE-inhibitory activity of f19–20, f19–21, f19–22, f19–23, f19–24, and f19–25	Hernandez-Ledesma et al. 2007
	Radical-scavenging activity due to the presence and position of Trp, Tyr, and Met	
	Hypocholesterolemic activity of f71–75 in animal studies	Hartmann & Meisel 2007 Morikawa et al. 2007
	Reduction of micellar cholesterol solubility and suppression of cholesterol absorption	Nagaoka et al. 2001
	Hypocholesterolemic activity of β-lactotensin	Yamauchi et al. 2003
	Reduction of total and LDL + VLDL cholesterol levels in serum	
α-lactalbumin and its derivatives	α-La–derived peptide obtained by pepsin treatment	Mullally et al. 1996
	Antihypertensive effect of α-lactorphin	Nurminen et al. 2000
	Inhibition of the production of angiotensin-II in blood	
	ACE-inhibitory activity of f50–52	Pihlanto-Leppälä et al. 2000
Immunoglobulins	Reduction of plasma cholesterol and subsequent decrease of blood pressure in hypercholesterolemic patients	Sharpe et al. 1994

factor in the repartitioning of dietary energy from adipose tissue to skeletal muscle (Fouillet et al. 2002; Garlick and Grant 1988; Ha and Zemel 2003).

The BCAAs, especially leucine, possess several characteristics that make them unique among the essential amino acids. BCAAs are a substrate for muscle protein synthesis (Buse and Reid 1975; Dardevet et al. 2000). Many researchers have reported that the dietary BCAAs (especially leucine) supplementation affected positively the limitation of muscle protein loss during aging (Dardevet et al. 2000; Katsanos et al. 2006; Rieu et al. 2006). The transamination of BCAAs helps synthesize carbon frames for muscles. The carbon skeletons from the transamination are used as a metabolic energy for

Table 11.2. Anticarcinogenic activity of bioactive components in whey products

Components	Bioactive Functions	Reference
Whey protein concentrates (WPC), whey protein isolates (WPI), and their derivatives	Protection against colon and mammary tumors	Hakkak et al. 2000 Rowlands et al. 2001
	Increase of GSH concentration to stimulate immunity originating antitumor effects	Bounous 2000
	Decrease of tumor cells with higher concentration of GSH	Parodi 1998
	Protection against oxidant-induced cell death in a human prostate epithelial cell line	Kent et al. 2003
β-lactoglobulin and its derivatives	Anticarcinogenic properties by binding mutagenic heterocyclic amines	Yoshida et al. 1991
	Protection against the development of putative tumor precursors in the hindgut wall	McIntosh et al. 1998
α-lactalbumin and its derivatives	Induction of apoptosis disrupting the chromatin organization in cell nuclei	Duringer et al. 2003
	Restriction of cell division in mammalian intestinal cell lines	Ganjam et al. 1997
	Potent Ca^{2+}-elevating and apoptosis-inducing agent	Madureira et al. 2007
	Antiproliferative effects in colon adenocarcinoma cell lines	Sternhagen and Allen 2001
Lactoferrin and lactoferricin	Potent anticancer agent in treating the development and progression of tumors	Gill and Cross 2000 Masuda et al. 2000
	Anticarcinogenic activity due to iron-binding capacity	Wakabayashi et al. 2006
	Reduction of the risk of oxidant-induced carcinomas	Weinberg 1996
	Reduction of the risk of colon adenocarcinomas	Tsuda et al. 1998
	Effectiveness on the inhibition of the intestinal carcinogenesis in rats	Sekine et al. 1997c
	Anticarcinogenic activities in various organs such as esophagus, lung, tongue, bladder, and liver	Iigo et al. 1999 Sekine et al. 1997b
Bovine serum albumin	Anticarcinogenic effect of enzyme-hydrolyzed BSA against genotoxic compounds	Bosselaers et al. 1994
	Effectiveness of BSA against human breast cancer cell line MCF-7	Laursen et al. 1990

muscle synthesis. Leucine in the BCAAs is the most potent amino acid in stimulating muscle protein synthesis (Anthony et al. 2000a,b). The BCAAs-enriched parenteral nutrition increased whole-body protein synthesis in patients with intraabdominal adenocarcinoma (Hunter et al. 1989; Tayek et al. 1986). The BCAAs-enriched parenteral nutrition also affects the improvement of whole-body leucine kinetics, fractional albumin synthesis rate, and leucine balance. These results provide the evidence that the BCAAs stimulate protein metabolism. Another study reports that the administration of BCAAs in vivo enhances muscle protein synthesis via activation of the mRNA binding step in the initiation of translation (Anthony et al. 2000ab).

Oral administration of leucine seems to stimulate insulin secretion through adjusting the concentration of circulating insulin (Greiwe et al. 2001; Malaisse 1984; Peyrollier et al. 2000). Oral administration of leucine increased rapidly serum insulin in food-

Table 11.3. Immune system modulation of bioactive components in whey products

Components	Bioactive Functions	Reference
Whey protein concentrates (WPC), whey protein isolates (WPI), and their derivatives	Enhancement of nonspecific and specific immune responses	Gomez et al. 2002
	Increase of the lymphocyte level of GSH	Grey et al. 2003
	Immunoenhancing effects by cysteine, glutamate, and glycine in the synthesis of GSH	DeWit 1998 Wong and Watson 1995
	Effective cysteine donors for GSH replenishment	
Lactoferrin and lactoferricin	Modulation of antiinflammatory processes	Kijlstra 1990
	Stimulation of the immune system due to the increase in macrophage activity as well as induction of inflammatory cytokines, stimulation of proliferation of lymphocytes, and activation of monocytes	McCormick et al. 1991 Potjewijd 1999 Sorimachi et al. 1997 Wakabayashi et al. 2006
	Activation of monocytes, natural killer cells, and neutrophils	Ambruso and Johnson 1981 Gahr et al. 1991 Nishiya and Horwitz 1982
	Lipopolysaccharide-LF complex formation and subsequent inhibition of inflammation	Na et al. 2004 Baveye et al. 1999
	Induction of IL-8 secretion by epithelial cells to enhance immune systems, including cytotoxic lymphocyte activities	Kuhara et al. 2000
	Enhancement of mucosal immunity	Debbabi et al. 1998
Immunoglobulins	Improvement of immune activity	Li et al. 2006 Facon et al. 1993 Mehra et al. 2006
	Reduction of susceptibility to disease	Ormrod and Miller 1991
	Enhancement of immunological functions of gastrointestinal-associated lymphoid tissue cells	Ishida et al. 1992
	Function of antigen-binding and protein G–binding activities of IgG1 in intestinal tracts	Ohnuki and Otani 2006

deprived rats (Anthony et al. 2002). However, the serum insulin level returned to food-deprived control values within 1 hour even though serum and intramuscular leucine concentration remained elevated.

Leucine seems to be the major regulating factor of amino acid and protein metabolism including donors for glutamine synthesis, and interorgan signalers (Lal and Chugh 1995; Lobley 1992). BCAAs are the only amino acids not degraded in the liver and their metabolism occurs primarily in the skeletal muscle (Etzel 2004). BCAAs are directed to protein synthesis and energy production (Layman 2003). The BCAAs provide various nutritional effects, which are to improve muscle performance, help lose weight in obesity, reduce catabolism in trauma patients, and improve clinical outcomes in patients with advanced cirrhosis (Bianchi et al. 2005). Development of food products containing dairy protein fractions with a high proportion of BCAAs might provide health benefits (Etzel 2004). The bioactivity of BCAAs is listed in Table 11.4.

BIOACTIVITIES OF β-LACTOGLOBULIN, α-LACTALBUMIN, AND THEIR DERIVATIVES

Inhibition of ACE Activity in β-Lg, α-La, and Their Derivatives

Various α-La– and β-Lg–derived peptides show inhibitory effects against ACE (see Table 11.1). The

Table 11.4. Bioactivity of branched-chain amino acids in whey products

Components	Bioactive Functions	Reference
Branched-chain amino acids (BCAAs, namely leucine, isoleucine, and valine)	Improvement of muscle strength	Laviano et al. 2005
	No degradation in the liver	Etzel 2004
	Directly used for protein synthesis and energy production	Layman 2003
	Repartitioning of dietary energy from adipose tissue to skeletal muscle	Fouillet et al. 2002 Garlick and Grant 1988 Ha and Zemel 2003
	Substrate for muscle protein synthesis	Buse and Reid 1975 Dardevet et al. 2000
	Positive limitation of muscle protein loss during aging	Dardevet et al. 2000 Katsanos et al. 2006 Rieu et al. 2006
	Stimulation of muscle protein synthesis by providing metabolic energy via transamination	Anthony et al. 2000a Anthony et al. 2000b
	Whole-body protein synthesis in patients with intraabdominal adenocarcinoma	Hunter et al. 1989 Tayek et al. 1986
	Improvement of whole-body leucine kinetics, fractional albumin synthesis rate, and leucine balance	
	Enhancement of muscle protein synthesis via activation of the mRNA binding step in the initiation of translation	Anthony et al. 2000a Anthony et al. 2000b
	Stimulation of insulin secretion through adjusting the concentration of circulating insulin	Greiwe et al. 2001 Malaisse 1984 Peyrollier et al. 2000
	Rapid increase in serum insulin in food-deprived rats	Anthony et al. 2002
	Regulating factors of amino acids and protein metabolism	Lal and Chugh 1995 Lobley 1992
	Loss of weight in obesity, reduction of catabolism in trauma patients, and improvement of clinical outcomes in patients with advanced cirrhosis	Bianchi et al. 2005

hydrolysis of α-La and β-Lg using proteolytic enzymes such as pepsin, trypsin, or chymotrypsin results in high ACE-inhibitory activity, while intact unhydrolyzed β-Lg shows very poor ACE-inhibitory activity (Mullally et al. 1997ab). The active peptides are usually short (<8 amino acids) and a typical tryptic peptide of β-Lg, f142–148 has an ACE IC_{50} value of 42.6 μM. In addition, the α-La– and β-Lg–derived peptides hydrolyzed from whey protein concentrates using trypsin with different degrees of hydrolysis characterize the most potent β-Lg–derived ACE-inhibitory peptide, Ala-Leu-Pro-Met-His-Ile-Arg, reported to date (Ferreira et al. 2007; Mullally et al. 1997b).

The ACE-inhibitory and radical-scavenging activities of the β-Lg–derived peptides, Trp-Tyr f19–20, Trp-Tyr-Ser f19–21, Trp-Tyr-Ser-Leu f19–22, Trp-Tyr-Ser-Leu-Ala f19–23, Trp-Tyr-Ser-Leu-Ala-Met f19–24, and Trp-Tyr-Ser-Leu-Ala-Met-Ala f19–25, have also been investigated (Hernandez-Ledesma et al. 2007). The IC_{50} values of the peptides vary from 38.3 to 90.4 μM, with the exception of Trp-Tyr-Ser (>500 μM). All β-Lg–derived peptides also exhibit radical-scavenging activity due to the presence and position of amino acids Trp, Tyr, and Met.

A typical α-La–derived peptide obtained by pepsin treatment shows ACE-inhibition activity with

an IC_{50} value of 733 μM (Mullally et al. 1996). The peptide α-lactorphin is a tetrapeptide of Tyr-Gly-Leu-Phe and originates from milk α-lactalbumin. Other studies show ACE-inhibitory activity in similar α-La–derived peptides such as Tyr-Gly-Leu (f50–52) (Pihlanto-Leppälä et al. 2000). In another study, the antihypertensive effect of orally administered doses of α-lactorphin on conscious spontaneously hypertensive or normotensive rats were studied to identify the dose efficacy (Nurminen et al. 2000). In rats, the α-lactorphin dose dependently lowered blood pressure without affecting heart rate. These peptides can inhibit the production of angiotensin-II in blood. Systolic blood pressure decreased significantly at 10 μg/kg dose, suggesting a mild pharmacological effect. The maximal reductions in systolic and diastolic blood pressure were 23 ± 4 and 17 ± 4 mm Hg, respectively, with a 100 μg/kg dose.

The hypocholesterolemic effects of the hydrolyzed β-Lg using proteolytic enzymes have also been reported (Hartmann and Meisel 2007; Morikawa et al. 2007; Nagaoka et al. 2001). A tryptic peptide from β-Lg (f71–75; Ile-Ile-Ala-Glu-Lys) has shown hypocholesterolemic activity in animal studies. The tryptic peptide reduces micellar cholesterol solubility, thereby suppressing cholesterol absorption in the jejunal epithelia due to significantly higher levels of fecal steroid excretion. Another enzyme hydrolyzed peptide (β-lactotensin) from β-Lg (f146–149) shows hypocholesterolemic activity in mice (Yamauchi et al. 2003). The β-lactotensin was administered orally for 2 days at a dose of 30 mg/kg in mice fed the high-cholesterol/cholic acid diet for 4 days. The β-lactotensin reduced 22.7% of the total and 32.0% of the LDL + VLDL cholesterol levels in serum.

In conclusion, bioactive components in α-La– and β-Lg–derived peptides are helpful to protect against hypertension through ACE-inhibitory activity and to regulate blood pressure.

Antimicrobial and Antiviral Activity of β-Lg, α-La, and Their Derivatives

The antimicrobial activity of the components in α-La– and β-Lg–derived peptides has been thoroughly reviewed (Clare et al. 2003; Hartmann and Meisel 2007; Madureira et al. 2007). The peptide fragments of β-Lg by trypsin digestion show bactericidal effects. The antibacterial in vitro activity of peptides from the bactericidal domains of β-Lg is strong with respect to relevant foodborne pathogens including *S. aureus, L. monocytogenes, Salmonella spp.*, and *E. coli O157* (Pellegrini et al. 2003). Peptide fragments such as f15–20, f25–40, f78–83 and f92–100 of β-Lg by trypsin digestion show antimicrobial effects against Gram-positive bacteria; β-Lg fragments are negatively charged and their bactericidal activity is restricted to Gram-positive bacteria (Pellegrini et al. 2001). The modulated peptides of β-Lg via targeted amino acid substitution illustrate bactericidal activity on both Gram-positive and -negative organisms including *E. coli* and *Bordetella bronchiseptica* (Pellegrini et al. 2001). In addition, peptide fragments of β-Lg by other enzymes such as alcalase, pepsin, or trypsin, show antimicrobial effects against pathogenic bacteria (El-Zahar et al. 2004; Pihlanto-Leppälä et al. 1999). The antimicrobial and antiviral activities of α-La– and β-Lg–derived peptides are listed in Table 11.5.

Native α-La does not show antibacterial activity against Gram-positive or -negative bacteria (Pellegrini et al. 1999). However, the peptides of α-La by digestion with trypsin and chymotrypsin produce active hydrolysates against bacteria. The digestion of α-La by trypsin produces two antibacterial peptides, f1–5 and f17–31 disulphide-bonded to f109–114. Treatment using chymotrypsin results in f61–68 disulphide bound to f75–80. These peptides are active against Gram-positive bacteria due to the negative charge on the surface of the α-La–derived fragments (Pellegrini et al. 1999). In addition, the injection of α-La peptides has been found to have direct immune modulating activity against *Klebsiella pneumoniae* in rats (Fiat et al. 1993).

The antiviral activity of α-La and β-Lg has been reviewed in several papers (Chatterton et al. 2006; Madureira et al. 2007). The treatment of a chemically modified β-Lg, 3-hydroxyphthaloyl-β-Lg inhibits the human immunodeficiency virus type 1 (Berkhout et al. 1997; Neurath et al. 1997a,b; Oevermann et al. 2003). In addition, the 3-hydroxyphthaloyl-β-Lg is effective against human herpes simplex virus types 1, bovine parainfluenza virus type 3, and porcine respiratory corona virus (Oevermann et al. 2003).

Anticarcinogenic Activity of β-Lg, α-La, and Their Derivatives

The β-Lg provides some protection against carcinogenic properties by binding mutagenic heterocyclic

Table 11.5. Antimicrobial and antiviral activities of bioactive components in whey products

Components	Bioactive Functions	Reference
β-lactoglobulin and its derivatives	Antimicrobial activity of hydrolyzed peptides to *S. aureus, L. monocytogenes, Salmonella spp.* and *E. coli O157*	Pellegrini et al. 2003
	Antimicrobial effects of f15–20, f25–40, f78–83, and f92–100 of β-Lg by trypsin digestion against Gram-positive bacteria	Pellegrini et al. 2001
	Bactericidal activity of modulated peptides of β-Lg via targeted amino acid substitution on both Gram-positive and -negative organisms including *E. coli* and *Bordetella bronchiseptica*	Pellegrini et al. 2001
	Antimicrobial effects of peptides hydrolyzed by other enzymes such as alcalase, pepsin, or trypsin	El-Zahar et al. 2004 Pihlanto-Leppälä et al. 1999
	Effect of 3-hydroxyphthaloyl-β-Lg on inhibition of the human immunodeficiency virus type 1	Berkhout et al. 1997 Neurath et al. 1997a Neurath et al. 1997b
	Antiviral activity of 3-hydroxyphthaloyl-β-Lg against human herpes simplex virus types 1, bovine parainfluenza virus type 3, and porcine respiratory corona virus	Oevermann et al. 2003
α-lactalbumin and its derivatives	No antimicrobial of native α-La	Pellegrini et al. 1999
	Antimicrobial activity against Gram-positive bacteria f1–5 and f17–31 disulphide-bonded to f109–114 (by trypsin) f61–68 disulphide bound to f75–80 (by chymotrypsin)	Pellegrini et al. 1999
	Direct immune modulating activity against *Klebsiella pneumoniae* in rats	Fiat et al. 1993
Lactoferrin and lactoferricin	Capability of acting as an antimicrobial agent related to its iron chelating ability, thus depriving microorganisms of a source of iron	Arnold et al. 1980 Arnold et al. 1977
	Binding to Lipid A lipopolysaccharides of Gram-negative bacteria, and subsequent destruction of membrane potential and integrity	Appelmelk et al. 1994 Nibbering et al. 2001
	Elimination of *E. coli* endotoxins in the gut	Dohler and Nebermann 2002
	Antimicrobial activity against both Gram-positive and -negative bacteria including *E. coli, Salmonella typhimurium, Shigella dysenteriae, Listeria monocytogenes, Bacillus stearothermophilus,* and *Bacillus subtilis*	Batish et al. 1988 Payne et al. 1990 – Saito et al. 1991
	Antimicrobial activity against *H. pylori* and *H. felis* infections in mice	Dial et al. 1998
	Antimicrobial activity against *Carnobacterium viridans* at 4 °C	Al-Nabulsi and Holley 2005
	Antimicrobial activity of lactoferricin enzymatically derived from LF	Jones et al. 1994 Tomita et al. 1991
	Antiviral activity via the binding of LF to membrane and subsequent prevention of the penetration of viral particles into the cell membrane	van der Strate et al. 2001

Table 11.5. *CONTINUED*

Components	Bioactive Functions	Reference
	Antiviral effect against herpes simplex virus	Fujihara and Hayashi 1995 Marchetti et al. 1996
	Antiviral effect against cytomegaloviruses	Harmsen et al. 1995
	Antiviral effect against human immunodeficiency virus	Harmsen et al. 1995 Swart et al. 1996
	Antiviral effect against simian rotavirus, HSV-1, and so on	Lubashevsky et al. 2004 Marchetti et al. 2004 Superti et al. 1997
	Inhibition of the HIV-1 reverse transcriptase	Wang et al. 2000
	Attenuation of the inflammatory symptoms by influenza virus infection	Shin et al. 2005
	Antiviral effect against hepatitis C virus	Tanaka et al. 1999 Iwasa et al. 2002
Immunoglobulins	Reduction of microbial infections in IGs fortified infant formula	Li et al. 2006
	Effective protection against microbial infections in humans	Facon et al. 1993
	Effective against infections in calves caused by enterotoxigenic *E. coli*	Moon and Bunn 1993
	Antimicrobial activity against infections in humans caused by enteropathogenic	Freedman et al. 1998
	Enhancement of local immunity in the gastrointestinal tract	Larson 1992
	Ability to inhibit the adherence of enteropathogenic microorganisms not to be absorbed from intestinal tract	Goldsmith et al. 1983
	Antimicrobial activity against infections by *H. pylori*	Oona et al. 1997
	Preventive against dental caries caused by cariogenic streptococci	Loimaranta et al. 1999
	Preventive against enteric disease caused by viruses in piglets	Schaller et al. 1992
Lactoperoxidase	Antimicrobial activity due to ion acting as electron donor	Touch et al. 2004
	Catalysis of oxidation of thiocyanate (SCN^-) in the presence of H_2O_2 and subsequent production of intermediate product (hypothiocyanate, $OSCN^-$) with antimicrobial properties	Pruitt et al. 1990
	Properties of $OSCN^-$ including cell-permeable capacity, inhibition of glycolysis, and inhibition of NADH/NADPH-dependent reactions in bacteria	Reiter and Perraudin 1991
	Enhancement of bactericidal effect of H_2O_2 in the presence of LP	Reiter and Perraudin 1991
	Inhibition of dental caries via killing cell-mediated pathogens in oral cavity	Reiter and Perraudin 1991
Lysozyme	Induction of cell lysis by hydrolyzing β-1-4 linkages between N-acetylmuramic acid and 2-acetyl-amino-2-deoxy-D-glucose residues in bacterial cell walls	Vannini et al. 2004
	Active against Gram-positive and -negative bacteria	

amines (Yoshida et al. 1991). Other studies report that β-Lg enhanced protection against the development of putative tumor precursors in the hind gut wall due to higher sulphur amino acid content that protects DNA in methylated form (McIntosh et al. 1998) (see Table 11.2).

The α-La helps reduce the risk of incidence of several cancers. A recent study showed that a variety of folding forms of human α-La selectively entered tumor cells and induced an apoptosis disrupting the chromatin organization in the cell nuclei (Duringer et al. 2003). There are several evidences that α-La restricts cell division in mammalian intestinal cell lines (Ganjam et al. 1997), is a potent Ca^{2+}-elevating and apoptosis-inducing agent (Madureira et al. 2007), and delays initiation of cell apoptosis showing antiproliferative effects in colon adenocarcinoma cell lines after 4 days of growth with low concentrations (10–25 μg/mL) of such protein (Sternhagen and Allen 2001). Thus, α-La and its complexes can be considered potential therapeutic agents.

BIOACTIVITIES OF LACTOFERRIN (LF) AND LACTOFERRICIN

Antimicrobial and Antiviral Activity of Lactoferrin and Lactoferricin

LF has the potential capability of acting as an antimicrobial agent related to its iron-chelating ability, which deprives microorganisms of a source of iron. The iron-binding capacity of LF might remove iron, which ultimately is needed for the proliferation of microflora from the microbial environment (Arnold et al. 1977, 1980). The antimicrobial activity of LF depends on its concentration or the degree of iron saturation of the molecule, and on its interaction with mineral medium constituents (Payne et al. 1990). Antimicrobial activities of LF have been reviewed in numerous studies (Farnaud and Evans 2003). The concentration of LF in bovine milk is about 0.1 mg/L (Tsuji et al. 1990). LF can damage the outer membrane of Gram-negative bacteria via binding to Lipid A lipopolysaccharides (Appelmelk et al. 1994). It causes structural changes and subsequently helps destroy membrane potential and integrity. In vivo studies of animal models reveal that LF helps eliminate *E. coli* endotoxins in the gut (Dohler and Nebermann 2002). LF exhibits antimicrobial activity against both Gram-positive and -negative bacteria including *E. coli*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Listeria monocytogenes*, *Bacillus stearothermophilus*, and *Bacillus subtilis* (Batish et al. 1988; Payne et al. 1990; Saito et al. 1991). In another in vivo and in vitro test in mice, LF showed antimicrobial activity against *H. pylori* infections and the potential to clear *H. felis* infections (Dial et al. 1998). Recently, LF was found to exhibit antimicrobial activity against *Carnobacterium viridans* at 4 °C (Al-Nabulsi and Holley 2005).

Lactoferricin enzymatically derived from LF provides antimicrobial activity against various bacteria (Jones et al. 1994; Tomita et al. 1991). The antimicrobial effect of LF and lactoferricin is mainly due to the depletion of iron by chelating in the organisms. In addition, LF provides damage to the membrane of the Gram-negative bacteria by losing membrane potential and integrity via binding lipopolysaccharides (Appelmelk et al. 1994; Nibbering et al. 2001).

The antiviral activity of LF is due to the binding of LF to membrane in eukaryotic cells. This binding process prevents the penetration of viral particles into the cell membrane and subsequently suppresses the infection at an early stage of viral invasion. There have been several studies on the antiviral effect of LF against herpes simplex virus (Fujihara and Hayashi 1995; Marchetti et al. 1996), cytomegaloviruses (Harmsen et al. 1995), and human immunodeficiency virus (Harmsen et al. 1995; Swart et al. 1996). The antiviral effects of LF on several viruses have been comprehensively reviewed (van der Strate et al. 2001). LF plays a preventive role of simian rotavirus, HSV-1, and so on (Lubashevsky et al. 2004; Marchetti et al. 2004; Superti et al. 1997). LF increases the inhibition of the HIV-1 reverse transcriptase (Wang et al. 2000). In a murine pneumonia model of influenza virus infection, 0.06 g per body of orally administrated LF attenuated the inflammatory symptoms (Shin et al. 2005).

Two studies have investigated the effect of LF on the hepatitis C virus. The serum levels of both hepatitis C virus (HCV) RNA and alanine aminotransaminase in chronic hepatitis C patients were reduced with the ingestion of LF for 8 weeks (Tanaka et al. 1999). Ingestion of LF for 6 months decreased the serum level of HCV RNA in 25 patients with chronic

hepatitis C genotype 1b (Iwasa et al. 2002) (see Table 11.5).

Anticarcinogenic Activity of Lactoferrin and Lactoferricin

LF is a well-known potent anticancer agent in treating the development and progression of tumors (Gill and Cross 2000; Masuda et al. 2000; Wakabayashi et al. 2006). The iron-binding capacity of LF provides an anticarcinogenic activity. Free iron may act as a mutagenic promoter due to the induction of the oxidative damage to nucleic acid. Thus, the binding of LF to iron reduces the risk of oxidant-induced carcinomas (Weinberg 1996) and colon adenocarcinomas (Tsuda et al. 1998). It is also reported that LF is strongly effective on the inhibition of the intestinal carcinogenesis in rats (Sekine et al. 1997c). LF provides the chemopreventive effects for the prevention of carcinogenesis. Animal studies reveal that the oral administration of LF inhibited tumors in various organs such as esophagus, lung, tongue, bladder, and liver (Iigo et al. 1999; Sekine et al. 1997a,b) (see Table 11.2). The anticarcinogenic activity of LF is summarized in Table 11.2.

Immune System Modulation of Lactoferrin

LF may play a role in the immune modulation (Adamik et al. 1998). LF is a key factor in the modulation of antiinflammatory processes (Kijlstra 1990). LF plays an important role in stimulation of the immune system, likely due to the increase in macrophage activity as well as induction of inflammatory cytokines, stimulation of proliferation of lymphocytes, and activation of monocytes (McCormick et al. 1991; Potjewijd 1999; Sorimachi et al. 1997; Wakabayashi et al. 2006). In addition, the activation of monocytes, natural killer cells, and neutrophils also affects immune system simulation (Ambruso and Johnson 1981; Gahr et al. 1991; Nishiya and Horwitz 1982). The mechanisms for the immune modulation are due to the prevention of proliferation and differentiation of immune system cells (see Table 11.3). LF possesses a lipopolysaccharide-binding property. The lipopolysaccharide-binding property provides the immunomodulatory function (Na et al. 2004). Mixture of lipopolysaccharide and LF formed lipopolysaccharide-LF complex, which induced inflammatory mediators in macrophages. The lipopolysaccharide-binding capacity of LF prevents lipopolysaccharide from binding with monocyte CD14-receptors (Baveye et al. 1999).

There have also been other reports related to modulation of the immune systems. Oral administration of LF induces IL-8 secretion by epithelial cells to enhance immune systems, including cytotoxic lymphocyte activities (Kuhara et al. 2000). In addition, both LF and hydrolyzed forms enhance mucosal immunity (Debbabi et al. 1998).

BIOACTIVITIES OF IMMUNOGLOBULINS (IGs)

Inhibition of ACE Activity in IGs

Immunized milk has effects on reduction of plasma cholesterol and subsequently lowers blood pressure (Sharpe et al. 1994). Intake of 90 g of immune milk daily helped manage blood pressure in hypercholesterolemic patients (see Table 11.1).

Antimicrobial and Antiviral Activity of IGs

Enrichment of bovine IGs in infant formula may help reduce microbial infections (Li et al. 2006). A study reported that oral administration of the IgG-rich food immunized with enteropathogenic and enterotoxigenic microorganisms has been demonstrated to provide effective protection against microbial infections in humans (Facon et al. 1993). Bovine IGs were effective against infections in calves caused by enterotoxigenic *E. coli* (Moon and Bunn 1993) as well as infections in humans caused by enteropathogenic (Freedman et al. 1998). In addition, IgG contributes to local immunity in the gastrointestinal tract (Larson 1992). Considered the most important role of milk IgG has been its ability to inhibit the adherence of enteropathogenic microorganisms to the intestinal epithelial cells, because IgG is not absorbed from the human intestinal tract (Goldsmith et al. 1983).

Other studies showed that infant gastritis originated by *H. pylori* is well fought via a diet including immune milk containing specific anti–*H. pylori* antibodies (Oona et al. 1997). In a dental study, bovine antibodies were preventive against dental caries

caused by cariogenic streptococci (Loimaranta et al. 1999).

It is reported that IGs help decrease viral infections (Li et al. 2006). Antibody concentrates derived from milk collected from cows immunized with several inactivated human rotavirus were preventive against enteric disease caused by viruses in piglets (Schaller et al. 1992), and in therapeutics of child infections caused thereby (Sarker et al. 1998) (see Table 11.5).

Immune System Modulation of IGs

IGs are recognized to provide protection against diseases in the newborn through passive immunity. Enrichment of IGs in infant formulae and other foods helps provide consumers, including newborns, with improved immune activity (Li et al. 2006). Feeding pregnant cows with systemic immunization has been used to increase the levels of antibodies against immunizing bacteria, and it also reduces susceptibility to disease (Ormrod and Miller 1991). Other research showed that the effect on cows of a vaccine containing a lipopolysaccharide–protein conjugate derived from *E. coli* J5 enhanced the level of serum antibody (Tomita et al. 1995).

The immunological activity of bovine IgG against human pathogens is reported to be similar to that of IgG in human milk, demonstrating the benefit of hyperimmune bovine milk in the human diet (Facon et al. 1993; Mehra et al. 2006). Oral administration of milk IgG significantly enhanced the immunological functions of gastrointestinal-associated lymphoid tissue cells (Ishida et al. 1992). These facts indicate that the oral ingestion of IgG may trigger the active immune responses in animals. Most of the antigen-binding and protein G-binding activities of cow's milk IgG1 might functionally act in intestinal tracts (Ohnuki and Otani 2006) (see Table 11.3).

BIOACTIVITIES OF MINOR COMPONENTS IN WHEY

Bioactivities of Lactoperoxidase (LP)

LP is an effective bactericidal agent in mammals (DeWit and Van Hooydonk 1996). The antimicrobial activity of LP depends on the ion acting as electron donor. LP catalyzes the oxidation of thiocyanate (SCN^-) in the presence of H_2O_2 and produces an intermediate product with antimicrobial properties (Touch et al. 2004). The amount of SCN^- in cow milk ranges from 0.1 to 15 mg/kg. LP-catalyzed reactions yield hypothiocyanate ($OSCN^-$) which is responsible for its antibacterial activity (Pruitt et al. 1990). The anion $OSCN^-$ is thought to mediate bacterial killing, as it is cell-permeable and can inhibit glycolysis, as well as nicotinamide adenine dinucleotide (NADH)/nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reactions in bacteria (Reiter and Perraudin 1991).

Hydrogen peroxide is produced in endogenous form by many microorganisms as well as by specific generator systems, such as via oxidation of ascorbic acid, oxidation of glucose-oxidase, oxidation of hypoxanthine by xanthine oxidase, and manganese-dependent aerobic oxidation of reduced pyridine nucleotides by peroxidase (Wolason and Sumner 1993). H_2O_2 has a bactericidal effect, but the inhibition of microbial growth is more efficient in the presence of LP (Reiter and Perraudin 1991).

In addition, LP is reported to inhibit dental caries via killing cell-mediated pathogens in the oral cavity (Reiter and Perraudin 1991).

Bioactivities of Bovine Serum Albumin (BSA)

BSA is a large globular protein having about 66 kDa molecular weight, and its structure is compact and rigid, which is stabilized by 17 disulfide bonds (Peters 1985). It is relatively stable to denaturation and precipitation by heat and alcohol during purification (Xu and Ding 2004). Many researchers investigated biological, physicochemical, and structural properties of BSA and tried modified BSA to prepare a desirable model protein for the food systems (Peters 1985; Shin et al. 1994). In addition, the physicochemical properties of BSA could be modified for diverse functional applications.

BSA has the ability to inhibit tumor growth. There are a couple of reports that BSA has an anticarcinogenic activity. The study of antimutagenic effect in BSA, whey protein, β-Lg, and pepsin-hydrolyzed casein indicated that only the enzyme-hydrolyzed casein and BSA were effective against genotoxic compounds (Bosselaers et al. 1994). Another study of in vitro incubation with human breast cancer cell line MCF-7 in BSA media has provided adequate evidence on modulation of activities of the autocrine

growth regulatory factors (Laursen et al. 1990) (see Table 11.2).

BSA also has an activity to enhance the immune system. BSA has been used as a component of cell media to regenerate plants from cultured guard cells and to provide for enhancement of production of plasminogen activator. Denatured BSA might reduce insulin-dependent diabetes or autoimmune disease (see Table 11.3).

Bioactivities of Lysozyme

Lysozyme is an antimicrobial enzyme that induces cell lysis by hydrolyzing β-1-4 linkages between N-acetylmuramic acid and 2-acetyl-amino-2-deoxy-D-glucose residues in bacterial cell walls (Vannini et al. 2004). Lysozyme is active against Gram-positive and -negative bacteria. Lysozyme shows a synergistic action with LF by inducing damages on the outer membrane of Gram-negative bacteria (see Table 11.5).

CONCLUSION

Functional ingredients derived from milk, including whey, have a proven beneficial effect on human health. Whey proteins and their derivatives provide important nutrients; immune system modulation; and bioactivities, including the inhibition of ACE activity, antimicrobial activity, antiviral activity, anticarcinogenic activity, and hypocholesterolemic effects. They have potential for use as health-enhancing nutraceuticals for specific supplemental formulae for several chronic diseases.

Recently, advances in separation, isolation, and concentration techniques have made it possible to apply bioactive whey-based ingredients in nutraceuticals and functional foods. In the future, whey protein nutraceuticals will play a complementary and/or a substitutional role in synthetic drugs for disease control. In order to be used for functional ingredients for human health, there is a strong need for further clinical trials to support human health claims of bioactivities in whey proteins, since most experimental results have been obtained from animal studies.

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12
Probiotics and Prebiotics as Bioactive Components in Dairy Products

Young Jin Baek and Byong H. Lee

INTRODUCTION

After Elie Metchnikoff wrote a book entitled *Prolongation of Life* in 1907, which can be regarded as the birth of probiotics, many different forms of fermented milk have been consumed to the present day. Many lactic acid bacteria (LAB) are mainly associated with various habitats such as fermented dairy products and plant materials, but other habitats such as soil, silage, and the human oral cavity, intestinal tract, and vagina are also known.

Probiotics are live microbial food supplements, which benefit the health of consumers (Fuller 1989) or live microorganisms that, when administered in adequate amounts, confer a beneficial effect on the hosts (Parkes 2007). Although significant evidence does exist about the positive effects of probiotics on human health, the mechanisms underlying are not always understood, and more studies are required to confirm the claims. The best known are the lactic acid bacteria *Lactobacillus acidophilus, Lactobacillus casei*, and bifidobacteria types, which are used in yogurts and other dairy products such as acidophilus milk, buttermilk, frozen desserts, and milk powder. These nonpathogenic and nontoxigenic bacteria retain viability during storage and survive passage through the stomach and small bowel. Probiotics have been consumed in functional foods beyond traditional nutrients. A number of health benefits of probiotics are included in the balancing intestinal microflora, antidiarrheal properties, reduction in serum cholesterol, reduction of fecal enzymes, improvement in lactose metabolism, enhancement of immune system response, anticarcinogenic properties, improved bioavailability of nutrients, antimicrobial activity, enhancement of bowel motility/relief from constipation, improvement in inflammatory bowel disease, and suppression of *Helicobacter pylori* infection in the stomach.

Later, the study of these beneficial bacteria caused the introduction of another new term, *prebiotics*, which are substances, usually poorly metabolized, that stimulate the growth of intestinal probiotic bacteria (Gibson and Roberfroid 1995; Saier and Mansur 2005; Park and Floch 2007). Once a prebiotic reaches the colon, it must selectively promote the growth and/or stimulate the metabolic activity of health-promoting bacteria and decrease potentially harmful bacteria.

This chapter reviews the potential beneficial effects of probiotics and prebiotics in preventing and treating certain diseases as well as mechanism of action, selection criteria, and regulation. Some advances on techniques of strains identification and modification will be also briefly discussed.

LACTIC ACID BACTERIA (LAB) AND FERMENTED MILK

Characteristics of Lactic and Probiotic Bacteria

After the first discovery of LAB by Louis Pasteur in 1857 during the alcohol fermentation, Tissier at the

Pasteur Institute in 1899 isolated *Bacillus bifidus (Bifidobacterium)*, a type of anaerobic lactic acid bacteria from the stools of breast-fed infants. This discovery led to widespread research on infant nutrition and intestinal flora in the pediatric field. In 1900, the Austrian pediatrician Moro discovered *Bacillus acidophilus* (*Lactobacillus acidophilus*) from the feces of breast-fed infants (Ballongue 1993). Metchnikoff in 1904 discovered the presence of *Bacillus bulgaricus* (*Lactobacillus bulgaricus*) from Bulgarian yogurt. *Lactobacillus casei* was isolated from cheese by Orla-Jensen in 1916 and from indigenous microflora in the human GI tract by Shirota in 1929.

LAB strains are the major representatives of probiotics, both in dairy food, such as fermented milk, and the pharmaceutical market. LAB are associated with various habitats, particularly those rich in nutrients such as various food substrates and plant materials, which they are able to ferment or spoil. Other habitats include soil, water, manure, sewage, and silage. Some LAB strains inhabit the human oral cavity, the intestinal tract, and the vagina and may beneficially influence these human ecosystems.

LAB produce lactic or acetic acid and some carbon dioxide from carbohydrates during fermentation. General characteristics are Gram-positive, nonmotile, non–spore-forming, coccus- and rod-shaped with less than 50% of G+C content. *Bifidobacterium* species currently belongs to *Actinomyces* with higher than 60% of G+C content (Biavati and Mattarelli 2001). The genera include *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus*, and *Bifidobacterium*, etc. Among many probiotic strains shown in Table 12.1, major strains like *Lb. acidophilus*, *Lb. casei*, and *Bifidobacterium* spp. are discussed in detail in the following sections.

Table 12.1. Commonly used as probiotic strains

Genera	Species	Subspecies/Strain
Lactobacillus	*acidophilus delbrueckii*	LA-1, LA-5, NCFM, *bulgaricus* Lb12
	casei	Shirota, Immunitas
	crispatus	
	fermentum	RC-14, KDL
	helveticus	B02
	johnsonii	La1
	paracasei	CRL431, Lp01
	plantarum	299v, Lp01
	reuteri	SD2112, MM2
	rhamnosus	GG, GR-1, LB21, 271
	salivarius	
Bifidobacterium	*adolescentis*	ATCC 15703, 94-BIM
	bifidum	Bb-11
	breve	
	longum	HY8001 (Korea Yakult Drink Yogurt), BB536
	laterosporus	
	lactis	CRL 431
	infantis	Bb12, B94
	thermophilum	744
Propionibacterium	*freudenreichii*	JS
Bacillus	*subtilis cereus*	*toyoi*
Escherichia	*coli*	Nissle 1917
Enterococcus	*faecium*	SF68
Streptococcus	*salivarius*	*thermophilus*
Saccharomyces	*cerevisiae*	*boulardii*

Adapted from O'Sullivan et al. (1992), Ouwehand et al. (2002), Cabana et al. (2006), Shah (2007).

LACTOBACILLUS ACIDOPHILUS

Lb. acidophilus is a Gram-positive, catalase-negative, rod-shaped bacterium with no gas from glucose or gluconate; thiamine is not required with adolase activity (Gilliland 1996). It is facultative with best growth obtained in the absence of excess oxygen. Energy is obtained through homofermentative metabolism of a variety of carbohydrates to mainly D-lactic acid. The optimum growth is between 35–38 °C but grows at 45 °C, with no growth at below 15 °C. This becomes an advantage when this organism is added to nonfermented milk, because little or no acid development will occur during refrigerated storage of the product. *Lb. acidophilus*, being a normal inhabitant of the small intestine, is resistant to bile. Growth occurs at initial pH values of 5–7 with an optimum of 5.5–6.0. As acidity varies from 0.3 to 1.9% lactic acid, some of the characteristics enable it to provide potential health and nutritional benefits for human and animal hosts. Therefore, *Lb. acidophilus* is the most commonly suggested organism for dietary use.

LACTOBACILLUS CASEI

This species possesses many of the same characteristics as *Lb. acidophilus*, which have potential for health and nutritional benefits (Gilliland 1996). Although its optimum growth temperature is 37 °C, unlike *Lb. acidophilus*, it grows at 15 °C with no growth at 45 °C. There is no gas from glucose, but gas from gluconate and ribose. Four subspecies are currently recognized. It also ferments a number of carbohydrates homofermentatively to L(+)-1actic acid. It is a normal inhabitant of the small intestine and is resistant to bile. The genetic basis for ecological flexibility in *Lb. casei* is not fully understood; however, comparative genomic analyses have suggested extensive gene loss and gene acquisitions during evolution of *Lactobacillus*, presumably via bacteriophage or conjugation-mediated horizontal gene transfers (HGTs), and these may have facilitated their adaptation to diverse ecological niches (Makarov and Koonin 2007).

BIFIDOBACTERIUM sp.

Bifidobacteria are obligate anaerobes in the *Actinomycetales* branch of the high–G+C (>60%) Gram-positive bacteria with distinct fructose-6-phosphate "shunt" metabolic pathway (Biavati and Mattarelli 2001). However, *Bifidobacterium* spp. has similar characteristics to LAB: Gram-positive; non–spore-forming; nonmotile bacilli; catalase-negative; and irregularly shaped rods with club-shaped or spatulate extremities, many of which form branched cells. They are sensitive to oxygen and have more strict growth requirements. As the obligate anaerobes, they are considered normal inhabitants of the small intestine and are bile resistant.

The major fermentation products are acetic acid and lactic acid in the molar ratio (3 : 2). This becomes an important factor to provide antagonisms toward undesirable microorganisms in the intestinal tract, because acetic acid is more toxic to microorganisms than lactic acid. Since *Bifidobacterium* are sensitive to oxygen, difficulty may be encountered in maintaining high levels of viability in products during storage unless anaerobic conditions are used. This makes it technologically more difficult for them to maintain the viability, and thus they are commonly not used as often as *Lactobacillus* (Tannock 1998; Ouwehand et al. 2002).

YOGURT

History

Although there is no precise record of the date for the first fermented milk, goats were first domesticated in Mesopotamia about 5,000 B.C., and goat milk stored warm in gourds in a hot climate naturally formed a curd. There is also evidence from wall paintings dating back to 2,500 B.C. that the Sumerians were in the habit of inoculating milk to induce fermentation (Kroger et al. 1989). There are several records about the soured milk of cows and goats in the Bible. The first Turkish name appeared in the 8th century as *Yogurut*. Yogurt was made originally from sheep, buffalo milk, and partly from goat and cow milk. Ancient physicians of the Middle East prescribed yogurt or related soured milk for curing disorders of the stomach, intestines, and liver, and for stimulation the appetite. There are records of the use as cosmetics by Persian women. The related fermented milk products are known under different names, such as *leben* (Egypt), *gioddu* (Italy), *matzun* (Armenia), and *dadhi* (India) (Rasic and Kurmann 1978). The beneficial effects of yogurt were put on

a scientific basis at the beginning of the 20th century (Fuller 1992).

Scientific Basis and Microflora of Fermented Milk

At the beginning of the 20th century, the Russian microbiologist, Elie Metchnikoff (1845–1916), Nobel Prize winner for the discovery of phagocytosis while working at the Pasteur Institute, suggested that lactic acid bacteria involved in yogurt fermentation, among which are *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*, suppress putrefactive-type fermentations of the intestinal flora. It was believed that bacteria present in yogurt control infections caused by enteric pathogens and regulate toxemia, both of which play a major role in aging and mortality. Metchnikoff was the first to give a scientific explanation for the beneficial effects of lactic acid bacteria present in fermented milk and played a key role in the process of yogurt. This observation provided a major boost to the manufacture and consumption of yogurt. However, subsequent investigation showed the inability of *Lb. bulgaricus* for value of yogurt. In spite of this, the study significantly influenced the spread of the product not only to Europe but also around the world (Shjortt 1999). Now, the health benefits derived by the consumption of foods containing *Lb. acidophilus, Bifidobacterium*, and *Lb. casei* are well documented. The microorganisms in the final product must be viable and abundant. The introduction of fruit in manufacture, followed by a wide range of flavored yogurts, promoted further growth in the consumption of the product.

Typical yogurt cultures such as *Str. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus* are claimed to offer some health benefits, but they are not natural inhabitants of the intestine. Currently, cultures for health benefits are being introduced in the yogurt industry. *Lb. acidophilus, Lb. casei*, and *Bifidobacterium* have been used significantly in the various countries. New fermented products containing *Lb. acidophilus, Bifidobacterium* spp., *Lb. casei Shirota, Lb. gasseri, Lb. rhamnosus GG, and Lb. reuteri* have been developed in Europe and Asia. However, *Lb. acidophilus, Lb. casei*, and *Bifidobacterium* spp. are most commonly used as probiotics.

The major trends of recent products have been targeted to improve the possibility of intestinal implantation and increase the nutritional-physiological value by supplementing the special cultures capable of synthesizing vitamins, mainly the B complex, as well as to improve the organoleptic properties. The most important trends are pleasure, practicality, and health concerns. In yogurt making with mixed culture of *Str. thermophilus* and *Lb. bulgaricus*, the milk coagulation time of milk in mixed culture is shorter than in single culture growth. In the first stage of incubation, *Lb. bulgaricus* stimulates the growth of *Str. thermophilus* by liberating the essential amino acids from the casein. In the second stage, the growth of *Str. thermophilus* is slowed down due to the adverse effect of lactic acid, and *Lb. bulgaricus* increases its growth rate by the stimulative action of *Str. thermophilus* through the formation of formic acid. A desirable starter strain ratio of *Str. thermophilus* and *Lb. bulgaricus* 1:1 to 2:1 is maintained in yogurt manufacture (Rasic and Kurmann 1978).

The Beneficial Effects of Yogurt

The four major beneficial effects of fermented milk are as follows (Mechnikoff 1908): 1) Substances in milk that are useful to our body, such as proteins, fats, lactose, vitamins, and minerals are provided in fermented milk, in much the same way as in natural milk. 2) The lactic acid produced is said to reduce gastric acid secretion, stimulate peristalsis, and prevent putrefaction in the intestine. A part of the lactic acid combines chemically with calcium to form lactic acid-calcium complex, which is a more easily absorbable form. Part of the milk proteins digested to peptones and peptides are more easily utilized, consequently improving liver function and stimulating intestinal secretion. Trace amounts of active substances produced also may promote or maintain a healthy balance of the intestinal flora or may improve intestinal metabolism. 3) In the case wherein the lactic acid bacteria are killed by gastric juice or bile, or when pasteurized fermented milk drinks are taken, the cellular components released are absorbed. They have a stimulatory effect on the immune system, augment the anticancer immunity, promote liver function, and may be associated with detoxication of harmful substances in the intestine. 4) If the viable lactic acid bacteria reach the intestine and succeed to multiply there, the substances produced during growth may improve the

balance of the intestinal flora and play a role in the detoxication of harmful substances produced by other bacteria.

PROBIOTICS

Definition and Milestone of Probiotics

The origin of the term *probiotic* is credited to Werner Kollath, who was cited by the German scientist Ferdinand Vergin in 1954. Kollath proposed the term *Probiotika* to designate "active substances that are essential for a healthy development of life" (Guarner et al. 2005). The word evolved to *probiotic*, derived from the Greek meaning "for life," and was first used by Lilley and Stillwell in 1965 to describe substances produced by one microorganism that stimulated the growth of other microorganisms (Fuller 1992). Thereafter Parker (1974) first used the term for "organisms and substances" with beneficial effects for animals by influencing the intestinal microflora (Havenaar and Huis in't Veld 1992), and eventually he introduced a new definition as "organisms and substances which contribute to intestinal microbial balance."

Fuller (1989) revised Parker's definition by removing the reference to substances; thus, his definition of a probiotic is "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." This concept was also equally applicable to human nutrition and medicine (Fuller 1991). This definition stressed the importance of viable microbial cells as an essential requirement in a particular mechanism of action to improve the intestinal microbial balance. Havenaar and Huis in't Veld (1992) broadened the definition of probiotic to be "a mono- or mixed culture of live microorganisms which applied to man or animal beneficially effects the host by improving the properties of the indigenous microflora" (Macfarlane and Cummings 1999). This definition developed the concept of probiotics in several ways. It did not restrict probiotic activity to the gut microflora but included the possibility of application to microbial communities at other sites, e.g., respiratory tract, urogenital tract, and skin. The probiotic may consist of a monoculture or a cocktail of cultures, and it also introduced the concept of human use (Shjortt 1999). A more appropriate definition for human nutrition has been outlined by Salminen et al. (1998) as "a live microbial food ingredient that is beneficial to health." The Joint Food and Agriculture Organization/World Health Organization Working Group (2002) defined the term *probiotic* as "live microorganisms, which, when administered in adequate amounts confer a beneficial effect on the hosts." The International Scientific Association for probiotics and prebiotics as well as the European Commission recently adopted this definition as the legal proposal (Reid et al. 2003).

Ecology of the Intestinal Flora

The gastrointestinal (GI) tract is a very complex system of organs in charge of digestion and excretion. The microbial community in the human GI tract is extremely complex, containing more than 400 different species in the intestine. In adults this complex ecosystem of the intestinal microflora in the colon is composed of some 10^{14} bacteria from hundreds of different species (Tanaka and Sako 2002).

The mucosal surface becomes the adherence and microbial colonization site in the small intestine. When compared to ca. 2 m^2 skin surface of our body, the area of our GI system, calculated to be 150–200 m^2 is enormous (Waldeck 1990). The GI tract is the largest immune organ in the body, responsible for over 80% of the body's antibody production (Saavedra 2007). Furthermore, GI features such as saliva, gastric acid, peristalsis, mucus, and intestinal proteolysis offer additional protection against invading pathogenic microorganisms.

In spite of rapid research advances in gut microbial ecology, the systematic understanding of this complex ecosystem and the microbial interactions is still limited. Bacteria composing the intestinal flora are broadly categorized in three major groups. One is the lactic acid bacteria group including *Bifidobacterium, Lactobacillus*, and *Streptococcus.* The other is the aerobic bacteria group including *Enterobacteriaceae, Staphylococcus, Bacillus, Corynebacterium, Pseudomonas*, and yeast. The obligate anaerobes such as *Bacteroides, Eubacterium*, anaerobic *Streptococcus, and Bifidobacterium* are the predominant group at about 10^9–10^{11}/g in feces. In contrast, *Lactobacillus*, coliform bacteria and *Enterococcus* are about at the level of 10^5–10^8/g, but *Megasphaera* or clostridia are less than 10^5/g as a minor group (Mitsuoka 1997).

Balance in the Intestinal Flora

The human fetus lives in a completely germfree environment in utero in the mother. On the first day after birth, *E. coli, Enterococcus, Lactobacillus, Clostridium*, and *Staphylococcus* are found in the stools of almost all newborns, with total bacterial counts reaching about 10^{11}/g. In breast-fed infants *Bifidobacterium* generally begins to appear about 3 days after birth and the previously appearing bacterial groups begin to decrease. On the fourth to seventh day *Bifidobacterium* becomes predominant with counts of 10^{10}–10^{11}/g. In contrast, the bacterial counts of *E. coli, Enterococcus, Staphylococcus, Bacteroides*, and *Clostridium* are reduced to about 1% of those of *Bifidobacterium*, with *Bifidobacterium* accounting for close to 100% of the overall flora. The intestinal bacterial flora become nearly stable the first 7 days after birth. As the weaning period approaches, the intestinal flora begins to resemble that of the predominant Gram-negative rod flora seen in adults (Mitsuoka 1997). In breast-fed babies, bifidobacteria, lactobacilli, and staphylococci are the predominant organisms in the feces, whereas in formula-fed babies, coliforms, enterococci, and bacteroides predominate (Walker and Duffy 1998).

It is necessary to contain high levels of potentially beneficial or health-promoting bacteria such as lactobacilli and bifidobacteria, but potentially harmful or pathogenic microorganisms must be kept at lower levels in the intestine. The composition and metabolic activity of the gut microflora are influenced by various environmental factors, namely diet, age, stress, health status, and medication, etc. The oral administration of live, beneficial probiotic bacteria is one way to increase the number of health-promoting organisms in the GI tract.

Probiotic Selection Criteria

Gilliland (1979) reviewed the general characteristics required for starter culture bacteria to survive and grow in the intestinal tract. One of the first barriers to survival is the gastric acidity encountered in the stomach. *Lb. acidophilus* and *Lb. casei* are resistant to the acidic conditions of artificial gastric juice at pH 3.0 at 37°, but *Lb. delbrueckii* ssp. *bulgaricus* is not. Strains of *Bifidobacterium* vary in their ability to survive transit through the stomach (Berrada et al. 1991). The LAB also should be able to survive the acid conditions of the stomach and the bile in the upper digestive tract to reach the small intestine. Initially, the candidate strains were selected solely upon their ability to displace and destroy pathogens in vitro (Reid and Bruce 2006).

At present, several additional factors appear to be important when choosing practical probiotic strains for human consumption. In order for a microorganism to be classified as probiotic, it must fulfill the following criteria, as shown in Table 12.2: 1) human

Table 12.2. Properties and benefits of good probiotic strains

Property	Benefit
Resistance to pancreatic enzymes, acid, and bile	Survival of passage through the intestinal tract
Adhesion to the intestinal mucosa	Immune modulation, pathogen exclusion, enhance healing of damaged mucosa, prolonged transient colonization
Human origin	Species-dependent health effects and maintained viability
Production of antimicrobial substrates	Antagonism against pathogenic microorganisms
Documented health effects	Proposed health effects are “true”, clinically validated and documented health effects of minimum effective dosage in products
Health	The assessment and proof of a “GRAS” strain, with a previous “history of safe use” and safety in food; nonpathogenic even in immunocompromised hosts
Good technology properties	Strain stability, production at large scale, oxygen tolerance

Adapted from Berrada et al. (1991), Collins et al. (1998), Ouwehand and Vesterlund (2003), Boyle et al. (2006), da Cruz et al. (2007), Szajewska (2007).

origin, 2) nonpathogenic properties, 3) resistance to technological processes, 4) stability in acid and bile, 5) adhesion to target epithelial tissue, 6) ability to persist within the GI tract, 7) production of antimicrobial substances, 8) ability to modulate the immune system, and 9) ability to influence metabolic activities (Szajewska 2007).

Additional criteria for probiotic selection should be taken into consideration for commercialization purposes. First and foremost, a good probiotic strain should display good technological properties, including being cheaply and easily culturable on a large scale (Ouwehand and Vesterlund 2003). Strains that are viable at higher temperatures as well as in oxygen-poor conditions are favored. Second, storage and packaging materials must adequately protect and preserve the therapeutic activity of probiotic foods. (da Cruz et al. 2007).

Although some recent studies show potential benefits in consuming nonviable probiotics, live bacterial strains provide better efficacy as probiotics. Also, beneficial effects to the host are observed when probiotics are consumed in adequate quantities (a minimum of 10^9 viable cells per day). Therefore, aspects such as shelf life and storage conditions must be determined and optimized to ensure the viability of cells at the time of consumption. Third, because multistrain probiotic concoctions may provide enhanced health benefits, many probiotic products often contain more than a single microbial strain. In these cases, strain selection must consider the species compatibility issues. Finally, based upon the intended form of the final probiotic product (capsules, tablets, powders, or liquids), special requirements with respect to species selection may be necessary to consider. For example, due to the heating step of the encapsulation process, thermostable strains are better choices for probiotics destined to be sold as capsules (Boyle et al. 2006).

By far, the most commonly selected probiotic strains are from the *Lactobacillus* and *Bifidobacterium* genera (Szajewska 2007). These strains generally fulfill the criteria as excellent probiotics and are frequently derived from the GI tract of healthy humans or from other nonhuman strains used in the fermentation of dairy products. Many other strains are also very good probiotics regularly found in many commercially available probiotic products. Some less common probiotic selections include yeast species such as *S. cerevisiae boulardii* (Ouwehand and Vesterlund 2003). Not surprisingly, most of these strains are some of the most abundant bacterial strains residing as part of the natural gut microf1ora, oral cavity, urogenital tract, and skin. Administering endogenously found microbial strains fulfills the main criteria of a good probiotic. Moreover, the end result is to maintain and/or restore the natural microf1ora of the GI tract.

Molecular Tools in Tracing GI Microbes and Starters

Approaches for assessing the safety of probiotic and starter strains have been recommended by Salminen et al. (2001) and imply the following characteristics: 1) the genus, species, and strain and its origin, which will provide an initial indication of the presumed safety in relation to known probiotic and starter strains; 2) studies on the intrinsic properties of each specific strain and its potential virulence factors; and 3) studies on adherence, invasion potential, and the pharmacokinetics of the strains. As the functional food industry continues to expand and the role of the human GI microbiota is increasingly recognized, accurate strategies for assessing bacterial changes in response to probiotics, prebiotics, and synbiotics are important (Gibson and Collins 1997). The traditional identification method of probiotics relying on phenotypic differences between organisms may result in irreproducible results, leading to unreliable data. Sequence analysis of 16S rRNA will greatly advance our knowledge in the true genetic diversity of the gut microbiota. Moreover, by utilizing diagnostic sequences within the 16S rRNA, it is possible to design gene probes to facilitate the precise identification and detection. Current probing strategies such as in situ fluorescent hybridization or quantitative dot-blot hybridization could be more extensively applied to gut microbiology (Gibson and Collins 1997). Amor et al. (2007) detailed the molecular approaches for the identification of probiotics and LAB in general.

BENEFICIAL EFFECTS OF PROBIOTICS

A number of health benefits for products containing probiotic organisms have been reported; the main reported effects are summarized in Table 12.3: 1) balancing intestinal microflora; 2) improving colo-

Table 12.3. The main reported health benefits for products containing probiotic organisms

Restoration and maintenance of healthy flora
Diarrhea, including travelers' and antibiotic induced
Inflammatory bowel disease (IBD), including ulcerative
Colitis and Crohn's disease
Constipation
Improving body's natural defense
Urogenital and vaginal infections
Enhancement of immune system functions
Inhibition of pathogenic and putrefactive bacteria
Cancer prevention
Allergy/atopic dermatitis prevention
Reduction of lactose intolerance
Cholesterol-lowering
Antihypertensive

Adapted from Lee and Salminen (1995), Tannock (1995), Holzapfel et al. (1998), Shah (2007).

nization resistance and/or prevention of infectious diarrhea (traveler's diarrhea, children's acute viral diarrhea), antibiotic-associated diarrhea, and irradiation-associated diarrhea; 3) reduction in serum cholesterol; 4) reduction of fecal enzymes, potential mutagens which may induce tumors; 5) improvement in lactose intolerance; 6) enhancement of immune system response; 7) nutritional benefits, such as improved calcium absorption, synthesis of vitamins, predigestion of protein, fat, and carbohydrates, and improved bioavailability of nutrients. Other less known effects include the following: 8) antimicrobial activity against gastrointestinal infections by some probiotic bacteria (Lee and Salminen 1995; Tannock 1995); and 9) enhancement of bowel motility/relief from constipation, adherence and colonization resistance, and maintenance of mucosal integrity (Holzapfel et al. 1998), alleviation of symptoms of food allergy, prevention of recurrence of superficial bladder cancer, and suppression of *Helicobacter pylori* infection in the stomach.

It is important to note that health benefits imparted by probiotic bacteria appear to be strain-specific, and not species- or genus-specific. None of the strains will provide all proposed benefits, not even strains of the same species, and not all strains of the same species will be effective against defined health conditions (Shah 2007).

Gastrointestinal Health

During the first days of life, the gut is enriched with bacteria, and the development of the microflora is rapid and depends on the following: genetic factors, delivery mode, mother's flora, type of feeding, and environmental surroundings (Saavedra 2007). Due to the instability of the baby's microflora, the resistance against pathogen colonization is weak; thus, the manipulation of the growing gut flora by probiotic intake may help development of various intestinal infections. Studies show that elevated numbers of *Bifidobacterium* spp. in a breast-fed infant's gut over formula-fed infants may be the cause of probiotic health benefits (Rubaltelli et al. 1998). Other studies have demonstrated that probiotics stimulate the developing immune system, which has lifelong positive effects to the host (Ouwehand et al. 2002). Probiotics are now being used as a supplement in some infant formulas (Ghisolfi et al. 2002). The significant clinical trials of probiotics in children have been compiled, but research data reveal controversy regarding the interpretation of their efficacy in children due to major differences in the study designs and recommendations (Cabana et al. 2006).

Disease/Disorder Prevention and Treatment

Common gastrointestinal diseases and disorders such as diarrhea (including travelers' and antibiotic-associated diarrhea), constipation, and inflammatory bowel disease (IBD) have all shown indications of improvement when treated with probiotics. Millions of people, especially children in developing countries, die every year due to diarrhea, an intestinal disturbance often caused by an imbalance of the gut's flora with rotavirus infection and *Clostridium difficile* overgrowth. Antibiotic-associated diarrhea is a major clinical problem occurring in up to 25% of patients, with diarrhea owing to *Clostridium difficile*. The clinical and economic costs of antibiotic-associated diarrhea are significant and better treatments are needed.

Probiotics may offer potential effective therapy for antibiotic-associated diarrhea by restoring intes-

tinal microbial balance. A number of different probiotics have been evaluated in the prevention and treatment of antibiotic-associated diarrhea in adults and children, including the nonpathogenic yeast, *Saccharomyces boulardii*, and multiple lactic-acid fermenting bacteria such as *Lb. rhamnosus* GG (LGG). A careful review of the literature supports the efficacy of *S. boulardii* in the prevention of antibiotic-associated diarrhea recurrent *C. difficile* infection in adults, whereas LGG is useful in the treatment of antibiotic-associated diarrhea in children. Not enough data exist currently to support the use of other probiotic preparations in these conditions. Further study of probiotics, including large, well-designed, randomized controlled dose-ranging trials, comparative trials, and cost-benefit analyses are necessary (Lawrence et al. 2005; Harsey and Pharm 2008).

A review by Szajewska (2007) looking at six meta-analyses found that treatment with probiotics can beneficially effect acute diarrhea in infants. Particularly, treatment with probiotics seems to 1) reduce diarrhea duration; 2) be strain-dependent, most effective *Lactobacillus GG* and *S. boulardii*; 3) be dose-dependent, great with higher doses: 4) significantly affect watery diarrhea and viral gastroenteritis; 5) be more evident with earlier initiation of probiotics; and 6) be more evident in children in European countries. The potential impact of the use of probiotics is further supported by studies using combination probiotics such as *Lb. rhamnosus* GG strain, along with *Bif. bifidum, Lb. reuteri* DSM 20016 to effectively treat acute bacterial and rotaviral diarrhea without adverse effects (Shornikova et al. 1997; Guandalini et al. 2000) and dealing with probiotics for the prevention of antibiotic-associated diarrhea (Saran et al. 2002). A meta-analysis on treatment of *Clostridium difficile* disease (McFarland 2006) showed that three types of probiotics (*S. boulardii, Lb. rhamnosus* GG, probiotic mixtures) significantly reduced the development of antibiotic-associated diarrhea. The prevention of traveler's diarrhea appears to depend on many factors including the destination of travel, and therefore more controlled studies need to be performed to assess the probiotics efficiency. Another study showed significant self-reported improvement of constipation and stool consistency after a week of consumption of *Lb. casei* Shirota (Koebnick et al. 2003). Patients who took a malted milk drink containing *Bif. infantis* 35624 reported alleviation of abdominal pain and discomfort, bloating/distension, and bowel movement difficulty (O'Mahony et al. 2005).

Both types of IBD, ulcerative colitis and Crohn's disease, may develop when the normal host-bacterial relationship is deregulated. Clinical studies tend to show that inflammation of the colon alone or of both the colon and distal small intestine, respectively, in ulcerative colitis and Crohn's disease are due to a loss of tolerance to resident intestinal bacteria. Probiotics have not been reported to heal patients affected by IBD, but may lead to substantial improvement in the quality of patient life by prolonging remission (Kruis et al. 2004). Five interrelated probiotic mechanisms of action concerning IBD are proposed (Mahida and Rolfe 2004): 1) enhanced barrier integrity by increased mucus secretion, 2) antibacterial activity, 3) stimulation of immune response by increased SlgA production, 4) competitive exclusion of bacterial adhesion/translocation, and 5) other immunomodulatory actions. Effective treatment for the management of remission of colitis has been shown by using an eight-strain formula of bacteria called *VSL#3* (Mimura et al. 2004). The possibility of treating colitis with a strain of *E. coli* called *Nissle* 1917 has also been suggested (Tromm et al. 2004).

Substantial experimental evidence exists to suggest that probiotics and prebiotics may be beneficial in the prevention and treatment of colon cancer, but there have been few conclusive human trials. Probiotics may have the potential to inhibit the development and progression of neoplasia by decreasing intestinal inflammation, enhancing immune function or antitumorigenic activity, binding to potential food carcinogens including toxins, and reducing bacterial enzymes that hydrolyze precarcinogenic compounds, such as β-glucuronidase (Geier et al. 2006).

Much evidence is also available to prove that the growth and attachment of pathogenic *Helicobacter pylori* that causes peptic ulcers could be inhibited by probiotic strains (Midolo et al. 1995; Pinchuk et al. 2001). In mice *Lb. salivarius* inhibited the colonization of *H. pylori* and *Lb. salivarius* given after *H. pylori* implantation also eliminated the colonization (Kabir et al. 1997). lactobacilli have been demonstrated to have in vivo and in vitro inhibitory effects on *H. pylori* infection (Bhatia et al. 1989; Park et al. 2001).

Oral Health

Few studies have been carried out in this area, but some researchers believe that probiotics have a promising role in oral health. A certain strain of *Streptococcus salivarius* called K12 in lozenge form has been shown to be an effective treatment for halitosis in preliminary studies without showing any obvious health concerns (Burton et al. 2006). In studies examining the administration of probiotics for oral Candida, treatment with probiotics reduced counts of oral Candida in the elderly and may be a new strategy for controlling oral yeast infections (Hatakka et al. 2007). Nikawa (2007) also found a significant reduction of the oral carriage of *Streptococcus mutans* by ingesting yogurt containing *Lb. fermentum*, compared with the placebo yogurt. Other preliminary findings include effects of probiotics on an imbalanced oral ecosystem and periodontal disease (Kang et al. 2006). Meurman and Stamatova (2007) have shown an effect of probiotics on halitosis and definite inhibition on the production of volatile sulfur compounds. In addition, a reduction of gingivitis and gum bleeding was observed by Krasse et al. (2006) with the application of *Lb. reuteri*. The oral health applications of probiotics or replacement therapy with *Str. mutans* strains of attenuated virulence and increased competitiveness were more than 700 bacterial taxa in the oral cavity (Aas et al. 2005; Devine 2007). However, because data on oral probiotics are scarce and insufficient, further studies are required to identify the resident oral probiotics and clarify the mechanism of their colonization and the eventual effect on the oral environment (Meurman and Stamatova 2007).

Urogenital and Vaginal Health

Infections that involve urogenital microbial flora imbalance such as yeast vaginitis, bacterial vaginosis, and urinary tract infection can be recurrent. Current available antimicrobial treatments can often lead to diarrhea, depression, headaches, renal failure and super infections. Moreover, antimicrobial resistance tends to decrease the effectiveness of this therapy over time. Research is presently being carried out to evaluate the possibility of using the intestinal tract as a delivery system for urogenital probiotics. Lactobacilli have been shown to inhibit the growth of *Candida albicans* and/or its adherence on the vaginal epithelium in small sample sizes (Falagas et al. 2006). Reid (2005a) showed a reduction in and better treatment of urogenital infections using a combination of two different *Lactobacillus* strains. Presently, the only strains clinically shown to have an effect are *Lb. rhamnosus* GR-1 and *Lb. reuteri* (Commane et al. 2005); when used intravaginally once weekly or twice daily orally, they have reduced recurrences of UTI and restored a normal *Lactobacillus*-dominated vaginal flora (Reid and Bruce 2006).

Immunomodulation and Skin Health

The effect of probiotics on the immune system is well documented. It was shown that probiotics may influence the immune mechanisms of the host by effects on mucosal barrier mechanisms and on the functional maturation of the immune system. Vaalara (2003) reported that several in vitro studies suggested that cell-wall components, such as lipoteichoic acid and peptidoglycan from Gram-positive bacteria, are potent immune modulators (Huang et al. 2004; Zhang et al. 2005). Niers et al. (2007) also addressed the in vitro immune responses to probiotics, specifically prevention of allergic diseases and immunomodulation of neonatal dendritic cells. It was found that *Bifidobacterium* genus (only selected species) prime in vitro cultured neonatal dendritic cells to polarize T cell responses and may therefore be candidates to use in primary prevention of allergic diseases. The number of people afflicted with atopic diseases such as eczema (dermatitis), allergic rhinitis, or asthma is increasing in western societies. These diseases are caused by a hereditary predisposition to developing certain hypersensitivities upon exposure to specific antigens and might be in part attributed to reduced microbial exposure in early life. Reduced symptoms of the atopic syndrome in infants have been observed when treated with probiotics. A randomized placebo-controlled trial was performed with *Lactobacillus* GG, prenatally with pregnant women and postnatally for 6 months with their infants. The mother or partner had at least one first-degree relative with atopic eczema, allergic rhinitis, or asthma. The study yielded a half-reduced frequency of the atopic eczema in the probiotic group compared to that of the placebo group (Kalliomaki et al. 2001). Reduced symptoms of the atopic eczema and dermatitis syndrome in food-

allergic infants have also been observed when treated with probiotics (Pohjavuori et al. 2004).

It is believed that the intestinal microflora can reduce the allergic response by enhancing antigen exclusion and inducing IgA response (Abrahamsson et al. 2007). Majamaa and Isolauri (1997) propose that probiotics increase the mucosal barrier of patients with atopic dermatitis and food allergy when infants are treated with LGG-fortified formula. Significant benefits with probiotics and prebiotics (galactooligosaccharides) were found in the prevention of allergic diseases such as atopic eczema (Kukkonen et al. 2006).

Lactose Intolerance

Two major types of lactose intolerance are encountered in the population worldwide. Primary or adult-type maldigestion is due to a decrease in β-galactosidase (also called lactase) in childhood or teenage years. Secondary-type lactose maldigestion is thought to be caused by a loss of small intestinal mucosa (which results in severe diarrhea, bowel resection, etc.). Symptoms associated with lactose intolerance include abdominal pain, bloating, flatulence, and diarrhea. People suffering from lactose intolerance can often tolerate certain fermented milk products like viable yogurt. Microbial β-galactosidase present in yogurt survives gastric passage and supports cleavage of lactose. Moreover, the high viscosity of yogurt compared to milk increases the time for microbial or human β-galactosidase to hydrolyze lactose. One of the health benefits of probiotic organisms is probably generally accepted relief of the symptoms of lactose intolerance. However, reduced levels of lactose in fermented products due to partial hydrolysis of lactose during fermentation are only partly responsible for this greater tolerance for yogurt.

Overproducing β-galactosidase mutants are studied to improve probiotic efficacy to treat symptoms of lactose malabsorption (Ibrahim and O'Sullivan 2000). Nonfermented milk containing cells of *Lb. acidophilus* also can be beneficial for lactose maldigestors (Kim and Gilliland 1983). *Lb. acidophilus* can survive and grow in the intestinal tract. It is reasonable to expect, however, that additional β-galactosidase may be formed after ingestion of milk containing this organism. Probiotic supplementation also modified the amount and metabolic activities of the colonic microbiota and alleviates symptoms in lactose-intolerant subjects (He et al. 2008). The changes in the colonic microbiota might be among the factors modified by the supplementation that lead to the alleviation of lactose intolerance.

Cholesterol-Lowering and Antihypertensive Effects

Cholesterol plays a vital role in many functions of the body, but too much cholesterol in the blood will cause arterial clogging and increase the risk of heart disease and/or stroke. Many studies have proposed a hypocholesterolemic effect when fermented products and probiotics are consumed, especially with selected strains of lactic acid bacteria. Fermented milk containing *Lb. acidophilus* Ll was found to lower serum cholesterol. This would translate to 6–10% reduction in risk for coronary heart disease (Anderson and Gilliland 1999). Xiao et al. (2003) also showed total serum lowering with a yogurt containing *Bif. longum* BL1. The mechanisms of action proposed for the reduction of cholesterol include the inhibition of exogenous cholesterol absorption in the small intestine by assimilation by the bacteria, as well as suppressing bile acid resorption by bacterial hydrolase activity (De Smet 1998).

As probiotic bacteria are reported to deconjugate bile salts, deconjugated bile acid does not absorb lipid as readily as its conjugated counterpart, leading to a reduction in cholesterol level. *Lb. acidophilus* is also reported to take up cholesterol during growth, and this makes it unavailable for absorption into the bloodstream (Shah 2007). The detailed genetic organization of bile salt hydrolases from *Bifidobacterium* strains (Kim et al. 2004) and bile salt biotransformations by human intestinal bacteria (Ridlon et al. 2006) were studied. It is important to consider that many bacteria inhabiting the large bowel have not yet been identified and are difficult to culture routinely.

One consequence of this is that we do not know what the global effects of prebiotics are on the structure of the microbiota. When using prebiotics to selectively modify the composition of the microbiota the prebiotics on their own can only enhance the growth of bacteria that are already present in the gut. However, different people harbor different bacterial species, while the composition of the microbiota can

be affected by a variety of other factors, such as diet, disease, drugs, antibiotics, age, etc. (Macfarlane 2006).

Antihypertensive effects have also been associated with the consumption of probiotic bacteria (Aihara et al. 2005). There are a number of products in the market manufactured by international food/food ingredients companies aimed at exploiting the functional food potential of milk protein–derived hypotensive peptides (IPP, VPP, FFVAPFEVFGK) and whey peptides (Fitzgerald et al. 2004; Donkor et al. 2007; Ahn et al. 2007). These products are already in the market either in the form of fermented milk drinks or milk protein hydrolysates.

Additional Health Benefits

Because the natural microflora is sensitive to dietary intake, poor eating habits will cause an imbalance in the bacterial community, allowing for pathogenic infection. Other potential factors that may disrupt the body's microbiota include stress and disease, chlorinated water and alcohol consumption, antibiotics in food products, and medical treatment (Leduc 2002). Probiotic intake may ensure the maintenance of the microflora that prevents pathogen colonization. Lactic acid bacteria are able to produce various compounds that are toxic to pathogens. For example, they can produce bacteriocins, hydrogen peroxide, acetic acid, lactic acid, formic acid, and many others. These compounds can lower the pH of the intestine, which inhibits pathogens. The majority of health benefits observed are associated with the consumption of live probiotic bacteria, but few studies have demonstrated that some health benefits can be provided by dead probiotic bacteria. Cross et al. (2001) reported that heat-killed preparations of lactic acid bacteria regulate the immune system by stimulation of IFNa, IL-12 and IL-8 in in vitro cell culture. Matsuzaki et al. (1996) reported that heat-killed cells of *Lb. casei* YIT9018 exhibit strong antitumor activity and have an effect on the prevention of lung metastases in mouse and guinea pig.

PREBIOTICS

Definition and Milestone of Prebiotics

Prebiotics are defined as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Bomba et al. 2002). They are recognized for their ability to increase levels of health-promoting bacteria in the intestinal tract of humans or animals. Some studies have shown that prebiotics target the activities of bifidobacteria and/or lactobacilli (Tannock 2002).

Many criteria have to be met for a food ingredient to be considered a prebiotic. First, it should not be hydrolyzed or absorbed in the stomach or small intestine of the host. Otherwise, the bacteria would no longer have access to the compound and therefore no benefits would be encountered. Secondly, the prebiotic must be selective for beneficial commensal bacteria in the colon. It must be verified that the food ingredient does not also stimulate pathogenic strains in the gut. Finally, its fermentation by commensal bacteria should induce beneficial effects to the host (Bomba et al. 2002). At the present time, the only prebiotics known are carbohydrates, disaccharides, and polysaccharides.

Production of Prebiotics

A wide range of prebiotics have been isolated from plant materials, including β-glucans from oats; inulin from chicory root; and oligosaccharides from beans, onions, and leeks (Su et al. 2007). A study using disaccharides demonstrated an increase in probiotic bacteria and reduced the number of putrefactive bacteria and potential pathogens (Ballongue et al. 1997). The use of inulin, a natural oligosaccharide found in some plants, significantly changed the composition of the mucosa-associated flora (Langlands et al. 2004). Industrial production processes have been established to extract the nondigestible oligosaccharides from natural sources, by hydrolyzing polysaccharides or by enzymatic and chemical synthesis from disaccharide substrates.

As shown in Table 12.4, with the exception of soybean oligosaccharides and raffinose (which are produced by direct extraction) and lactulose (which is produced by alkali isomerization reaction), they are either synthesized from simple sugars, such as sucrose or lactose, by enzymatic transglycosylation reactions, or formed by controlled enzymatic hydrolysis of polysaccharides, such as starch or xylan (Sako et al. 1999)

These processes usually produce a range of oligosaccharides differing in their degree of polymeri-

Table 12.4. Nondigestible oligosaccharides with bifidogenic functions commercially available

Compound	Molecular Structure[a]	Production Method
Cyclodextrins	$(Gu)_n$	Starch by cyclodextrin glycosyltransferase (CGTase) and alpha-amylase
Fructooligosaccharides	$(Fr)_n$–Gu	Transfructosylation from sucrose by beta-fructofuranosidase or inulin hydrolysate
Galactooligosaccharides	$(Ga)_n$–Gu	Transgalactosylation from lactose by beta-galactosidase
Gentiooligosaccharides	$(Gu)_n$	Transgalactosylation of starch by beta-glucosidase
Glycosylsucrose	$(Gu)_n$–Fr	Transglucosylation of sucrose and lactose by cyclomaltodextrin glucanotransferase
Malto/Isomaltooligosaccharides	$(Gu)_n$	Transgalactosylation of starch by pullanase, isoamylase, alpha-amylases/transglucosidase
Isomaltulose (or palatinose)	$(Gu–Fr)_n$	Transglucosidase of sucrose
Lactosucrose	Ga–Gu–Fr	Transglycosylation of lactose and sucrose by beta-fructofuranosidase
Lactulose	Ga–Fr	Isomerization of lactose by alkali
Raffinose	Ga–Gu–Fr	Extraction of plant materials by water or alcohol
Soybean oligosaccharides	$(Ga)_n$–Gu–Fr	Extraction of soy whey
Xylooligosaccharides	$(Xy)_n$	Xylan hydrolysis by xylanase or acids

[a]Ga, galactose; Gu, glucose; Fr, fructose; Xy, xylose.

Adapted from Crittenden and Playne (1996), Sako et al. (1999), Mussatto et al. (2006).

zation and sometimes in the position of the glycosidic linkages. Residual substrates and monosaccharides are usually present after oligosaccharide formation, but such sugars can be removed by membrane or chromatographic procedures to form higher-grade products that contain pure oligosaccharides (Crittenden and Playne 1996).

Worldwide, there are 13 classes of food-grade oligosaccharides currently produced commercially (Table 12.5). Both the volume and diversity of oligosaccharide products are increasing rapidly as their functional properties become further understood. Detailed production methods for various oligosaccharides have been reviewed by Nakajima and Nishio (1993) and Playne (1994).

Beneficial Effects of Prebiotics and Synbiotics

Because of the survivability and colonization difficulties that abound with probiotics, the prebiotic approach offers an attractive alternative. Prebiotics exploit selective enzymes production by those gut microorganisms that may impart health benefits to the host. While some peptides, proteins, and certain lipids are potential prebiotics, nondigestible carbohydrates have received the most attention. Certain carbohydrates, oligo- and polysaccharides, occur naturally and meet the criteria of prebiotics (Gibson and Roberfroid 1995). Some nondigestible carbohydrates have a number of functional effects on the GI tract, which have been used to validate emptying, modulation of GI tract transit times, improved glucose tolerance, reduced fat and cholesterol absorption via binding of bile acids, increased volume and water-carrying capacity of intestinal contents, modulation of microbial fermentation with increased short-chain fatty acid (SCFA) production, and decreased pH and ammonia production (Roberfroid 1996). The combination of these effects could potentially result in improved host health by reducing intestinal disturbances (constipation and diarrhea), cardiovascular disease, and intestinal cancer.

The combination of probiotics and natural stimulating agents consists as a practical means to increase the effectiveness of probiotic preparations for

Table 12.5. Market volume of oligosaccharides

Class of Oligosaccharide	Estimated Production in 1995 (t)	Major Manufacturers	Trade Names
Galactooligosaccharides	15,000	Yakult Honsha (Jp)[a]	Oligomate
		Nissin Sugar Manufacturing Com. (Jp)	Cup-Oligo P7L and others
		Snow Brand Milk Products (Jp)	TOS-Syrup
		Borculo Whey Products (NL)[b]	
Lactulose	20,000	Morinaga Milk Industry Co. (Jp)	MLS/P/C
		Solvay (Ger)[c]	
		Milei GmbH (Ger)	
		Canlac Corporation (Can)[d]	
Lactosucrose	1,600	Ensuiko Sugar Refining Co. (Jp)	Nyuka-Origo Newka-Oligo
		Hayashibara Shoji Inc. (Jp)	
Fructooligosaccharides	12,000	Meiji Seika Kaisha (Jp)	Meioligo
		Beghin-Meiji Industries (Frn)[e]	Actilight
		Golden Technologies (U.S.A.)	NutraFlora
		Cheil Foods and Chemicals (Kor)[f]	Oligo-Sugar Raftilose & Raftiline
		ORAFTI (Bel)[g]	Fibruline
		Cosucra (Bel)	
Palatinose	5,000	Mitsui Sugar Co. (Jp)	ICP/O, IOS
Glucosyl sucrose	4,000	Hayashibara Shoji Inc. (Jp)	Coupling Sugar
Maltooligosaccharides	10,000	Nihon Shokuhin Kako (Jp)	Fuji-Oligo
		Hayashibara Shoji Inc. (Jp)	Tetrup
Isomaltooligosaccharides	11,000	Showa Sangyo (Jp)	Isomalto-900
		Hayashibara Shoji Inc. (Jp)	Panorup
		Nihon Shokuhin Kako (Jp)	Biotose & Panorich
Cyclodextrins	4,000	Nihon Shokuhin Kako (Jp)	Celdex
		Ensuiko Sugar Refining Co. (Jp)	Dexy Pearl
Gentiooligosaccharides	400	Nihon Shokuhin Kako (Jp)	Gentose
Soybean oligosaccharides	2,000	The Calpis Food Industry Co. (Jp)	Soya-oligo
Xylooligosaccharides	300	Suntory Ltd. (Jp)	Xylo-oligo
Chitosan oligosaccharides	50	Hubei Yufeng Bioeng. Co. Ltd. (China)	Chitosan-oligo

[a]Japan.
[b]The Netherlands.
[c]Germany.
[d]Canada.
[e]France.
[f]Korea.
[g]Belgium.
Adapted from Crittenden and Playne (1996), Blanco et al. (2007).

therapeutic use. Stimulating agents include substrates named *prebiotic* and *potentiated* probiotics.

It is important to consider that many bacteria inhabiting the large bowel have not yet been identified and are difficult to culture routinely. One consequence of this is that we do not know what the global effects of prebiotics are on the structure of the microbiota. Another important factor to bear in mind when using prebiotics selectively to modify the microbiota composition is that prebiotics on their own can only enhance the growth of bacteria that are already present in the gut. However, different people harbor different bacterial species, and the composition of the microbiota can be affected by a variety of other factors, such as diet, disease, drugs, antibiotics, age, etc. (Macfarlane 2006).

There are also preparations, referred to as *synbiotics*, on the market that combine probiotics and prebiotics to enhance health benefits. Synbiotics are defined as "a preparation containing microorganism strains and synergistically acting compounds of natural origin that increase the probiotic effects on the small intestine and the colon" (Bomba et al. 2002).

A study showed that the *Lb. paracasei* combined with fructooligosaccharides had a synergic effect, increasing the bacterial populations in the feces of weanling pigs (Nemcova et al. 1999). The synergetic components include phytocomponents (Yadva et al. 1995), essential oils from the plant Origanum (Srivopoulou et al. 1996), nonspecific substrates such as lactose (Corrier et al. 1990), whey (Edens et al. 1991), microbial metabolites from fermented milk (Hosoda et al. 1996), antibiotic vaccines (Promsopone et al. 1998), and trace elements such as zinc (Holm et al. 1996).

PROPOSED MECHANISMS OF PROBIOTICS AND PREBIOTICS ACTION

Several different mechanisms of action by which probiotics elicit beneficial effects have been described (Fig. 12.1). First, probiotics produce

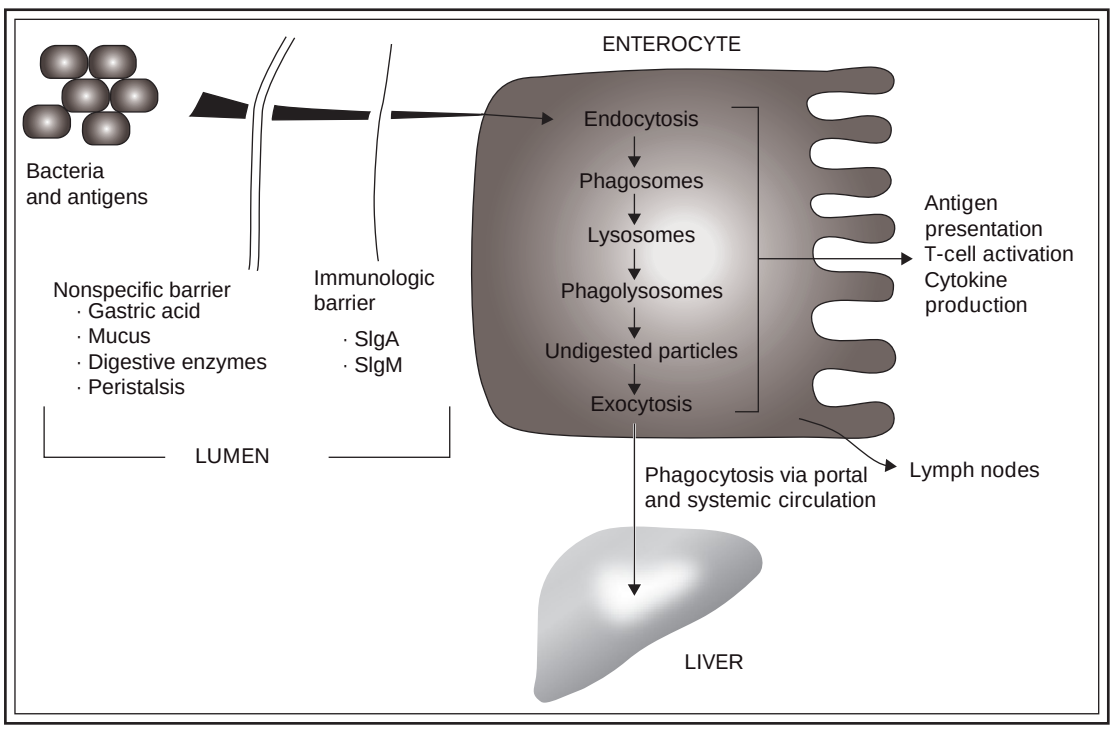

Figure 12.1. Barriers to antigenic absorption in the intestine (redrawn with permission of Broekaert and Walker 2006).

short-chain fatty acids, which are metabolic end products of carbohydrate fermentation and are hypothesized to antagonize other organisms. Probiotics have also been described to decrease the expression of cytokines involved in inflammatory pathways. Bacteria and antigens have to face nonimmunologic as well as immunologic barriers before enterocyte invasion. Once endocytosis is surmounted, bacteria have several opportunities to spread. Presentation of bacterial antigen leads to T cell activation and cytokine production. Bacteria are also phagocytosed via the portal and systemic circulation and transported to lymph nodes as well as other organs such as the liver (Fig. 12.1) (Broekaert and Walker 2006, 2007).

Many probiotic products have been developed to improve our GI tract health, but little is known about the interactions that take place in the GI tract, and therefore the precise mode of action is difficult to predict. However, in general, it is likely that probiotics affect the host cells (immune system) and intestinal microbes (competition), whereas prebiotics affect microbes directly because host cells cannot utilize prebiotic compounds. This gives microbes an opportunity to compete for prebiotic compounds, while still influencing the host immune system indirectly, as shown in Figure 12.2 (Tannock 2005).

The effects of probiotics and prebiotics on the health of all areas where human microflora inhabit are being investigated and explored. Probiotics, prebiotics, and synbiotics are now moving into the mainstream of medical therapy. This evolution has been facilitated by our ever-increasing understanding of the action mechanism of these agents and by the development of molecular methods for analyzing and identifying complex bacterial communities within the mammalian intestine.

The drastic increase of inappropriate use of antibiotics and bacterial resistance, along with renewed interest in biotechnological methods to prevent

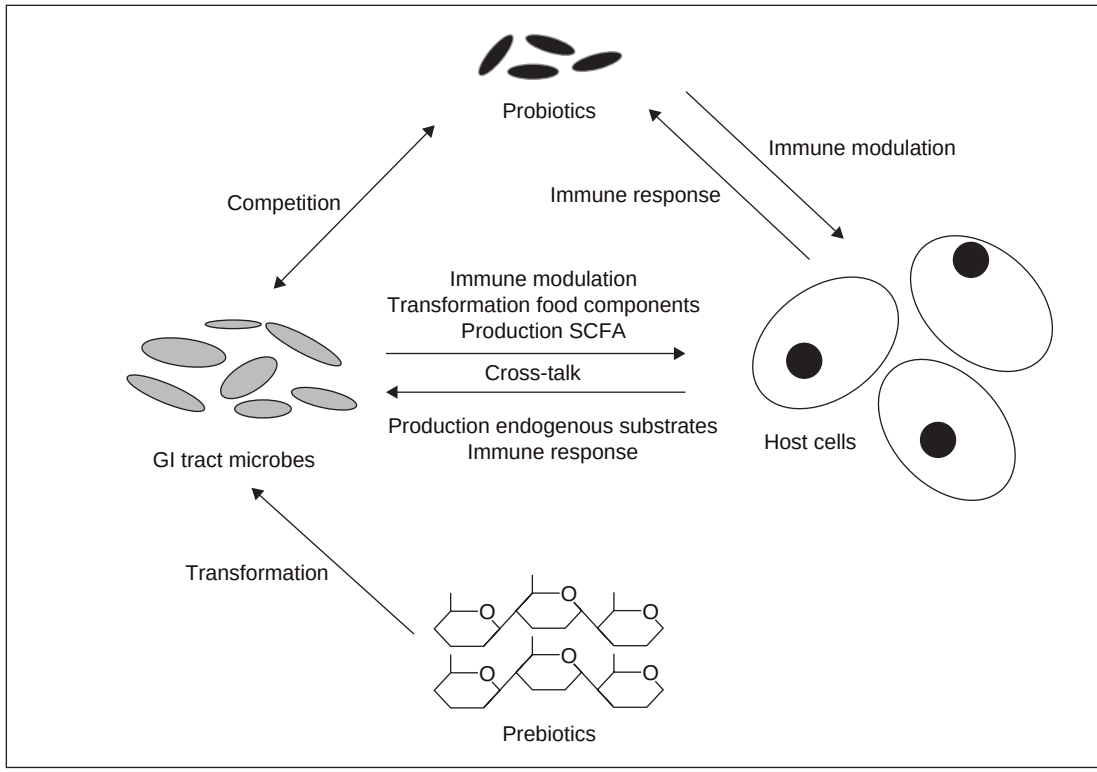

Figure 12.2. Host-microbe interactions and the hypothetical impact of pre- and probiotics on these interactions (redrawn with permission of Zoetendal and Mackie 2005).

infections, makes probiotics, prebiotics, and synbiotics a very interesting field for research and development.

RISKS ASSOCIATED WITH PROBIOTIC USE

Despite their overall good safety record, there are risks to specific populations of people associated with the use of probiotics. In most countries, probiotics are regulated as food supplements rather than pharmaceuticals. Therefore, at present, there are no requirements to demonstrate safety or efficacy of these products for human use. This fact has raised concerns about the absolute safety of probiotic use in humans.

A number of studies have already shown a potential for probiotics to cause bacterial and fungal sepsis in humans (Boyle et al. 2006). Most likely, these effects would not affect the normal healthy population, but should be considered when used by specific subgroups of persons "at risk." For instance, infection and toxicity by probiotics has never been documented, but subjects with underlying disease conditions that predispose to infection might be exposed to a putative risk. Likewise, unrestricted stimulation of the immune system by probiotics could be detrimental for patients suffering autoimmune diseases. The risk of transfer of antibiotic-resistance properties from probiotics to virulent microorganisms should also be evaluated (Guarner 2007).

There is general consensus that the consumption of probiotics, even in dosages as high as 10^8 cfu per day, failed to exhibit any toxicity. The safety assessment proposed (Donohue and Salminen 1996) also takes intrinsic properties of probiotic strains into consideration, in addition to metabolic products, toxicity, mucosal effects, dose-response effects, clinical assessment, and epidemiological studies. In view of their frequent association with human infection and possible easy acquisition of antibiotic resistance, the use of enterococci as probiotics is still a matter of some concern (Adams and Marteau 1995; Bonten et al. 2001).

It is important to note that all these cases have been reported in patients with underlying immunodeficiency or chronic diseases (cardiac or cancer), and in neonates (Boyle et al. 2006). However, there have not been any reports of sepsis in healthy individuals. Also, since many *Lactobacillus* strains are naturally resistant to vancomycin, there is a concern regarding the transfer of antibiotic resistance to pathogenic organisms. To date, the ability to transfer vancomycin-resistance genes of the *Lactobacillus* to other genera has never been observed (Boyle et al. 2006).

CONCLUSIONS AND PERSPECTIVES

Fermented dairy products by lactic acid bacteria and probiotics are enjoyed by consumers with increased popularity as convenient, nutritious, stable, natural, and healthy products. Today, probiotics have been extended to use outside of human nutrition in fermented functional foods resulting from the microbial production of bioactive metabolites, such as certain vitamins, bioactive peptides, organic acids, fatty acids, etc.

Probiotic foods in situ in the gut through the introduction of a probiotic microorganism may offer the host some added protection against diseases. Bioactive probiotics and derived components can be produced either directly through the interaction of ingested live microorganisms with the host or indirectly as a result of ingestion of microbial metabolites produced during the fermentation process. Although still far from fully understood, several probiotic mechanisms of action have been proposed, including competitive exclusion, competition for nutrients, and/or stimulation of an immune response.

To battle the increase of health care costs, a preventative approach to medicine with the development of probiotic, prebiotic, and synbiotic products is being advanced. Widespread antibiotic resistance has become a cause for concern, and new promising antimicrobial treatment options must be explored. Increases in the prevalence of many diseases are fueling the need for safer and more effective therapies.

Probiotics have the potential to shed some light on each of these current issues. They have the potential to reduce health care costs, prevent and treat some human illness, and satisfy the modern consumer through the balancing and restoration of natural gut microflora. To date, most of the benefits of probiotics for human consumption have been demonstrated in controlled clinical conditions. Yet,

there still seem to be frequent inconclusive and/or contradictory findings concerning the true benefit of probiotic consumption. The symbiotic nature of the host-microbe interactions of the gut microbiota is so complex that studying the relationship will most definitely lead to a better understanding of how probiotics truly function. Unraveling the probiotic mechanisms of action could open a new era for further enhancement of various probiotic strains through modern recombinant DNA techniques. Applications of molecular tools are also key toward unequivocally determining the effects of pro-, pre- and synbiotics on gut microbial ecology.

Looking ahead, this field holds immense promise for the future in delivering novel therapies in different fields. Although probiotics have a very promising future, there is a need for probiotic regulation concerning safety, optimal doses, and frequency of administration. Synbiotics are metamorphosing from little-known entities to well-known agents helpful in the maintenance of good health. It has now become imperative that the clinicians assimilate knowledge about probiotics and prebiotics and use them for the benefit of their prevention. In the future, more prominent, well-conducted studies should force government regulatory bodies, industries, and academic institutions to acknowledge the probiotic potential for health as well as to improve their safe commercialization worldwide.

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Section III
Other Related Issues on Bioactive Compounds in Dairy Foods

13
Regulatory Issues and Functional Health Claims for Bioactive Compounds

Peter Roupas, Peter Williams, and Christine Margetts

INTRODUCTION

As research into the health benefits of food components has expanded, both food manufacturers and consumers have developed a greater understanding of the relationship between food and health. Bioactive or health-promoting components may be naturally present in the food product or extracted from waste or food streams for addition to foods not naturally containing them. Addition of such components may also be used for fortification of foods that naturally contain these components to compensate for losses that may occur during processing. The development of the functional food category or foods with health properties beyond the commonly accepted nutritional benefits is rapidly evolving in all markets (Baroke 2007).

Milk and its products have long been regarded as a good source of nutrition (Barker 2003; Bryans et al. 2007; Miller et al. 2007). Dairy products and dairy ingredients are also widely used in many other manufactured foods. Current research undertaken into milk composition reveals it is a significant source of bioactives (Fosset and Tome 2003; Huth et al. 2006; IDF 2007a,b; Shortt and O'Brien 2004). Traditional dairy products such as milk beverages and yogurt are also becoming widely used as vehicles to deliver nondairy bioactives, such as phytosterols or omega-3 fatty acids (Baroke 2007; Berry 2008; Decker 2008).

This increased ability to enhance the health benefits of food products challenges traditional views and laws on fortification. The desire for food manufacturers and marketers to utilize new knowledge on the health-giving qualities of their products to garner market advantage (Baroke 2007; O'Brien 2007), by making explicit health claims, challenges what is currently permitted by food regulatory codes.

Food regulations vary from country to country and the increased movement of food across borders and even within large jurisdictions like the European Community, means that a particular food, ingredient, or bioactive may have to meet several sets of regulations, in spite of attempts to create a global code with Codex Alimentarius.

All new foods require authorization by relevant regulators but the process each must go through depends on whether the ingredient or food has a traditional history of consumption in that jurisdiction. Nontraditional foods (those that do not have a long history of human consumption) or new foods may be subjected to additional and separate "novel" food regulatory processes.

Regulatory authorities (e.g., Food Standards Australia New Zealand, U.S. Food and Drug Administration, European Food Safety Authority) are debating how to deal with health claims, particularly in marketing and labeling of foods. The levels and types of substantiation required for health claims to be made for foods and bioactive ingredients are also evolving. This chapter provides an overview of the current major approaches taken by larger jurisdictional groups such as the U.S., European Union, and Japan to regulate health claims.

DAIRY FOODS

Clinical, observational, and mechanistic data have demonstrated that dairy products and several of their constituents have a variety of metabolic roles (Smilowitz et al. 2005). Furthermore, market trends indicate that milk-based beverages and other dairy products such as yogurt and cheese are ideal vehicles for newly discovered bioactive food ingredients targeting lifestyle diseases (Huth et al. 2006; Mattila-Sandholm and Saarela 2003; Saarela 2007).

Oral administration of probiotics has been reported to be effective in preventing/treating diarrhea, reducing lactose intolerance and allergic diseases, treating irritable bowel syndrome, and reducing blood cholesterol levels (Desmond et al. 2005; Salminen et al. 2005). The physiological effects of probiotics can occur through either the direct effect of the live microbial cells, known as the *probiotic effect*, or indirectly via the metabolites produced by these cells, which is referred to as the *biogenic effect*. Cheese has also been shown to be an excellent delivery vehicle for probiotics and biogenic substances (Hayes et al. 2006) and for a variety of naturally occurring health-promoting components such as conjugated linoleic acid (CLA) and omega-3 fatty acids (McIntosh et al. 2006). Probiotics comprise approximately 65% of the world functional food market.

Because there was no international consensus on methods to assess the efficacy and safety of probiotic bacteria, recent initiatives from the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have resulted in the development of a legislative framework for probiotic bacteria, which is based on scientific criteria (in vitro trials, safety considerations, in vivo studies for the substantiation of effects) for the evaluation of health claims (Pineiro and Stanton 2007).

Drinking yogurt and lowfat milk are the most commonly used vehicles for the delivery of bioactive food ingredients. Probiotic yogurt drinks are preferred for the delivery of plant sterols; lowfat milk is commonly used to deliver omega-3 fatty acids. Drinks containing combinations of dairy and fruit juices, with added bioactive components, are also becoming common in the U.S. and European Union (EU) markets (Sharma 2005).

In addition to being a major provider of important nutrients for humans, including calcium, protein, and riboflavin, it is now recognized that ingredients derived from milk and whey can provide functional food products with other beneficial effects on human health. Casein and whey proteins and their derivatives have been shown to possess biological activities, including peptides able to exert antihypertensive (Pihlanto-Leppälä 2000; López-Fandiño et al. 2006) and other biological effects, lactoferrin and lactoperoxidase able to exert antimicrobial effects, and growth factors used in sports health and for tissue repair applications including wound repair (Playne et al. 2003). The currently identified bioactive components in bovine milk or colostrum, their physiological activities, and their concentration in bovine milk have recently been reviewed (Rowan et al. 2005; Huth et al. 2006).

Dairy foods with added dairy-derived or nondairy functional ingredients, or nondairy foods with added dairy ingredients may be considered by regulatory authorities to be "novel" or "nontraditional" foods i.e., foods that do not have a significant history of human consumption in that market. Such foods may raise human safety concerns and will need to undergo a risk-based assessment before being allowed into the food supply (Healy 2003).

An issue of concern to the dairy industry relates to recent regulations on the labeling of foods with respect to their levels of trans fatty acids (TFA). With the scientific evidence associating trans fatty acid intake with an increased risk of coronary heart disease (CHD), the U.S. Food and Drug Administration (FDA) has issued a ruling that requires the declaration of the amount of TFA present in foods, including dietary supplements, on the nutrition label. For the purpose of nutrition labeling, TFAs are defined as the sum of all unsaturated fatty acids that contain one or more isolated (i.e., nonconjugated) double bonds in a transconfiguration. The FDA will also be conducting consumer research to determine consumer understanding of various TFA labeling possibilities (Moss 2006). Denmark and New York City have imposed mandatory restrictions on these types of fats. The recent results from the TRANSFACT study (Chardigny et al. 2008) have shown differences (in women) between natural ruminant-derived trans fats and industrially produced hydrogenated trans fats with respect to biomarkers of CHD. The different effects of natural, ruminant-

derived trans fats and industrially produced trans fats on biomarkers/risk factors for CHD may result in further regulatory changes as new scientific knowledge is generated (Lock et al. 2005).

HEALTH CLAIMS

Linking the consumption of functional foods or food ingredients with health claims should be based on sound scientific evidence, with the "gold standard" being replicated, randomized, placebo-controlled, intervention trials in human subjects. The assessment of scientific safety and efficacy of a food or ingredient by the relevant regulatory authority is likely to require data on the characterization and source of the ingredient, manufacturing processes, concentration in the food product, and related safety considerations. The evidence for confirmation of efficacy may include in vitro data and in vivo animal trials using levels of defined biomarkers as measures of a physiological effect, but more than one human intervention trial in different population groups is also likely to be essential. Evaluations will probably include a number of quality criteria, such as the quality of the study design, conduct, and analysis, and decisions need to be based on the totality of the evidence. Not all foods on the market that are claimed to be functional foods are supported by enough solid data to merit such claims (Hasler 2002).

Legislation concerning health claims has progressed at a slow pace in many countries. After several years of debate, the European regulations on nutrition and health claims came into force in early 2007. This law sets out the conditions on the use of health claims, establishes a system for scientific substantiation, and should result in a European list of permitted claims by early 2010 (Richardson et al. 2007). Under these regulations, health claims fall into two categories: structure-function claims (e.g., calcium builds strong bones) and disease risk reduction claims (e.g., decrease in the risk of heart disease). The latter category will be required to have substantiated scientific evidence and must have special approval. Member states are compiling national lists of claims and will submit them to the European Commission. After consultation with the European Food Safety Authority (EFSA), the final Community list of permitted claims should be adopted by early 2010 (De Jong 2007).

In Australia and New Zealand, the binational regulator—Food Standards Australia New Zealand (FSANZ)—is currently developing a new standard that will permit scientifically substantiated claims for foods that meet certain nutrient profiling criteria (FSANZ 2008a). The proposed new standard will encompass two types of claims—nutrition content claims and health claims—and there will be two levels of health claims: general-level health claims and high-level health claims. The level of a claim will determine how the claim is regulated, including the evidence required for substantiation.

General-level health claims refer to the presence of a nutrient or substance in a food and its effect on normal health function. High-level health claims are those that make reference to a serious disease or biomarker, and these will need to be preapproved by FSANZ. Five high-level claims have already been accepted for inclusion in the new standard, including two related to calcium, vitamin D, and osteoporosis, and calcium and enhanced bone density. In the future, manufacturers will be able to make applications for approval of other high-level health claims, which will need to be scientifically substantiated using a defined substantiation framework.

A view has been expressed that the ethical responsibility for marketing sound health messages for dairy products rests with the industry. Although there are regulatory processes in place to protect the consumer, these sometimes can be circumvented. Making a health claim requires a certain level of proof for it to be accepted, and the food industry should strive to meet these challenges. Consideration must also be given to potential negative effects, such as the denigration of the product category by the use of unsubstantiated health claims that may mislead the consumer (MacNeill 2003). Consumers often express concern that health claims are just another sales tool, and the use of poorly substantiated claims could increase the current levels of consumer skepticism about all attempts to communicate the health benefits of food (Health Canada 2000; Food Standards Agency 2004).

A cautionary note with respect to making less than fully substantiated claims for foods lies in the recent litigation brought by a group of overweight children against the McDonald's Corporation that sought compensation for obesity-related health problems. While many derided this lawsuit as representing the worst excesses of the tort liability

system, others have drawn parallels to tobacco litigation. Food-related litigation raises the question of where accountability for the economic and public health consequences of food-related disorders properly rests (Mello et al. 2003).

It is anticipated that technological advances in the food industry, in conjunction with extensive clinical trials and governmental control, will eventually guarantee the credibility of health claims and ensure consumers' confidence in functional foods (Arvanitoyannis and Van Houwelingen-Koukaliaroglou 2005).

COMMUNICATION OF HEALTH MESSAGES TO CONSUMERS

The new EU legislation on nutrition and health claims emphasizes that the wording of claims should be understandable and meaningful to the consumer and they will be permitted only if the average consumer can be expected to understand the beneficial effects expressed in the claims (Leathwood et al. 2007).

Recent trends in the U.S. and the EU indicate that regulators will require further research to test how consumers are likely to interpret and use any health claim. In a recent study of television food advertisements in the U.S., 14.9% made a weight-related nutritional claim. The authors concluded that practitioners and policymakers should be aware of the prevalence of food advertisements and their potential impact on knowledge and behavior and should consider working more closely with food manufacturers to encourage the creation and promotion of weight-friendly foods. Furthermore, it was suggested that nutrition educators could help by teaching consumers critical thinking skills that may relate to food advertisements (Henderson and Kelly 2005). Similar conclusions were reached in a study of food advertisements in a series of women's magazines (Hickman et al. 1993).

Consumer perceptions of nutrition- and health-related food claims attached to food products have been investigated in a large-scale, cross-national, Internet-based survey. Participants were questioned in Germany (n = 1620), the UK (n = 1560), Italy (n = 1566), and the U.S. (n = 1621). Nutrition and health claims relating to six health benefits (increased concentration, decreased overweight, fatigue, infection, stress, and cardiovascular disease) and five claim types (marketing, content, structure-function, disease risk reduction, and product) were studied. Considerable variations in consumer perception were found according to country of origin and benefit being claimed, but not in relation to claim type (Trijp and Lans 2007).

It has been reported that young consumers are not interested in the effects of eating habits on health, but concern over consumption habits and health increases in older people. Young and middle-aged male consumers read only the energy value and nutritional information on the food label; female consumers read all the information on the labels of products that they purchased. Increase in educational level has also been reported to increase the preference for healthier foods (Isleten et al. 2007).

The use of health claims on the Internet and the level of compliance of these claims with existing regulations in Australia and New Zealand has been studied recently (Dragicevich et al. 2006). These data showed that 14.5% of food product websites carried a health claim, and 40.7 and 37.0% of products previously identified as carrying claims on product labels or in magazines, respectively, had Internet claims. Many of the claims (19.7%) were high-level or therapeutic claims not permitted by current food standards. The authors concluded that health claims were not being made more frequently on websites compared with product labels, but there was a greater prevalence of high-level and therapeutic claims made on the Internet. In the future, food standards enforcement will need to give greater priority to monitoring the use of health claims on the Internet (Dragicevich et al. 2006). A similar study by the same group of magazine advertisements has also found that many of the claims were high-level claims (29%) or therapeutic claims (8%), which are not permitted by current food standards (Williams et al. 2007). In this study 17% of the advertisements with health claims were for dairy foods.

Food labels are an important tool to assist consumers in making healthy food choices. In addition to mandatory nutritional labeling information, manufacturers have a variety of options on food/supplement packages to communicate the nutrition/health benefits of their products (Agarwal et al. 2006).

The FDA food labeling regulations aim to ensure that manufacturers aid consumers in making dietary choices by eliminating "hollow" health claims. Of particular concern are health claims made by one

brand when the claim is inherent to the product category, but has not been featured previously in advertisements or on packaging. There is concern that consumers will use information provided by one brand about such an attribute to infer that the other brands in the product category do not possess the attribute and thus be misled. Results from three experiments show that this practice can mislead consumers and affect consumer inferences, use of the target attribute, and choice in favor of the brands displaying the attribute. Furthermore, it was shown that improved consumer education can be achieved without the deception associated with narrow (brand-specific) health claims by using broader (category-defined) claims (Burke et al. 1997).

A study of consumers (Urala et al. 2003) has been carried out to evaluate whether product-related health claims in foods are advantageous or disadvantageous. Claims were made for six functional components and two control products. In general, all claims were perceived as neutral or as advantageous. Increasing the strength of the claim did not automatically increase the perceived benefit. Gender, trust in different information sources, and the frequency of use of so-called functional foods affected the perceived benefit. Women perceived the claims to be more beneficial than did men. Trustful respondents perceived the claims as more advantageous than did skeptical respondents, and the users of functional foods perceived health claims to be more advantageous than did nonusers. In addition, personal motivation affected the perception of the claims. With less familiar functional components, the strength of the claim increased the perceived benefit, whereas with familiar components, claims mentioning the reduced risk or prevention of a disease did not increase the perceived advantage.

A further study by the same group (Urala and Lahteenmaki 2004) quantified the attitudes behind consumers' willingness to use these products. Functional food-related statements formed seven factors describing consumers' attitudes toward functional foods: perceived reward from using functional foods, confidence in functional foods, necessity for functional foods, functional foods as medicines, absence of nutritional risks in functional foods, functional foods as part of a healthy diet, and the health effects of functional foods versus their taste. These attitude subscales differentiated between consumers in their reported willingness to use functional foods. The best predictor for willingness to use functional foods was the perceived reward.

One dilemma with health claims is that too much information can confuse consumers and too little information can mislead them. A controlled study has been used to examine the effectiveness of various front-sided health claims when used in combination with a full health claim on the back of a package. The results indicated that combining short health claims on the front of a package with full health claims on the back of the package leads consumers to more fully process and believe the claim (Wansink 2003).

A similar approach has been used in a recent study comparing claims about reduced risk of osteoporosis made on milk or a calcium-fortified orange juice packaging. This study investigated whether splitting a claim (a brief claim at the front of a package directing consumers to the full health claim at the back) and/or endorsement of the claim (by a regulatory body) affected the acceptance of the claim by the consumer. Split health claims produced more positive responses than not-split claims in several areas: they created a higher level of satisfaction with the labeling, they produced a higher level of trust, and they communicated better the health risk of the claim. Endorsement of the claim did not influence responses, possibly because of either the small print of the approval statement or the low awareness of the regulatory body among consumers, but belief in the claim was significantly higher on the milk product compared to the juice (Singer et al. 2006).

Consumers' main skepticism regarding functional foods resides in the veracity of health claims and in the often inadequate control of their claimed properties. It is very important that health claims on food products can be understood by the consumer. However, there is no clear understanding of how consumers use health claims and their likely impact on consumer food behavior or health. More research is needed, but a review of previous studies allows some common conclusions to be drawn. Health claims on foods are seen by consumers as useful, and when a product features a health claim they view it as healthier and state they are more likely to purchase it. Consumers are sceptical of health claims from food companies and strongly agree that they should be endorsed by government. Consumers do not make clear distinctions between nutrition content

claims, structure-function claims, and health claims. They generally do not like long and complex, scientifically worded claims on foods; they prefer split claims—with a short succinct statement of the claim on the front of pack and more detail provided elsewhere (Williams 2005a). There is also some evidence that the use of health claims improves the quality of dietary choices and knowledge of diet-disease relationships (Williams 2005b).

Dairy foods have long been promoted using health messages—dairy products have been promoted by governmental authorities wanting to improve public health and by dairy industry bodies promoting dairy foods. The health messages that have been used for the promotion of dairy foods include nutrient content messages ("a good source of calcium"), lowfat messages and health claims ("calcium reduces the risk of osteoporosis"). The changes in legislation permitting the use of (some) health claims beyond structure-function claims ("calcium helps promote bone health") on food labels and in advertisements aimed at consumers will expand the repertoire of messages available to communicate the benefits of dairy foods. However, health claims will not replace nutrient content and lowfat messages, and all three types of health messages are likely to be widely used to promote dairy foods in the future (Lawrence 2005).

NUTRIENT PROFILING

Nutrient profiling of foods is defined as the science of categorizing foods based on their nutrient composition. For regulatory agencies, nutrient profiles can be the basis for disallowing nutrition or health claims and for regulating advertising to children. The EU and Australia/New Zealand have adopted nutrient profiling as the basis for regulating nutrition and health claims, whereas the U.S. approach has emphasized positive nutrients, and the European approach has focused on the foods' content of fats, trans fats, sugars, and sodium. The Australian approach proposes a mixed scoring system of disqualifying nutrients (e.g., high salt, sugar, fat) balanced with positive scores for protein, fiber, fruit, and vegetable content (FSANZ 2008b).

Several nutrient profiling approaches are being used in different countries. It has been reported that 23 nutrient profiling systems have been developed (Garsetti et al. 2007). One approach uses thresholds where an upper limit is set for negatively perceived nutrients. Because positive/healthy nutrients are not taken into account, this system does not reflect the whole nutrient composition of a food, and as such would not recognize the importance of dairy foods in helping to meet nutritional requirements. Another approach is the use of scoring systems that allow the inclusion of both positive and negative nutrients, and these provide a more balanced view of the nutrient composition. However, a potential problem with this system is that if energy, total fat, saturated fat, and sugars are all included in such a model, this can lead to multiple scoring of some nutrients such as fat. A further approach is based on nutrient density. Nutrient density is the ratio of the amount of a nutrient in a food to the energy provided by that food. This approach differentiates between energy-dense, but nutritionally poor foods and foods that are both energy-dense and nutrient-dense. Dairy foods are naturally nutrient-dense, and their contribution to nutrient intake is well represented by this approach (IDF 2007c).

Nutrient profiling or scoring criteria can pose some challenges for dairy product manufacturers. Dairy products such as cheese and butter are naturally high in nutrients that would result in their disqualification from being able to make a health claim. For example, using the values for energy, saturated fat, sugar, and sodium initially proposed by FSANZ, most cheeses could not carry a health claim in Australia or New Zealand because of their inherently high energy, saturated fat, and sodium content. This is despite the fact that the National Health and Medical Research Council's (NHMRC) *Dietary Guidelines for Australian Adults* recommend that adult diets include milk, yogurt, and cheese (Lederman 2007). Subsequent modifications to the profiling system have included a separate set of criteria for cheeses with a calcium content of more than 320 mg/100 g (FSANZ 2008b).

The development of competing nutrient profile systems by researchers, regulatory agencies, and the food industry in the EU, the U.S., and elsewhere, has been marked by different priorities, pressures, and concerns. However, the development of nutrient profiles needs to follow specific science-driven rules. These include the selection of reference nutrients and reference amounts, the creation of an appropriate algorithm for calculating nutrient quality scores, and the validation of the chosen scheme

against objective measures of a healthy diet (Drewnowski 2007).

The application of nutrient profiling also aims to avoid a situation where nutrition or health claims mask the overall nutritional status of a food, which could mislead consumers when they are attempting to make decisions in the context of a balanced diet (Reuterswärd 2007).

REGULATION OF FUNCTIONAL FOODS AND FOOD SUPPLEMENTS IN JAPAN

A policy of Foods for Specified Health Uses (FOSHU), by which health claims on some selected functional foods are legally permitted was established in 1993 by the Japanese Ministry of Health and Welfare. Since 1984, when the concept of "functional food" was first proposed there, the science of regulating and labeling functional foods in Japan has been progressing along a unique path of development. Their unique approach is seen in the development of functional foods by minimizing undesirable as well as maximizing desirable food factors, for example, hypoallergenic foods developed from food materials by removing allergens (Arai 2000).

The concept of functional foods is well understood in Japan as a result of research initiated on the health benefits of foods in 1984. The Ministry of Education organized a national research and development project to evaluate the functionalities of various foods. Researchers from diverse scientific fields defined new functions of food, successfully incorporating previously recognized functions of nutrition, sensory/satisfaction, and physiological effects of ingredients in foods.

Some food manufacturers and distributors unfortunately capitalized on such food functionalities to promote "health foods" by violating the laws with claims for druglike effects. In 1991, the Ministry of Health and Welfare's successor, the Ministry of Health, Labor and Welfare (MHLW), introduced the FOSHU system to control such exaggerated and misleading claims. The other reason for such enforcement was an increase in the population of elderly people and lifestyle-related diseases, including obesity, diabetes mellitus, high blood pressure, cerebro- and cardiovascular diseases, and cancer.

In 2001, a new regulatory system, "foods with health claims" (FHC) with a "foods with nutrient function claims" (FNFC) system and newly established FOSHU was introduced. The MHLW further changed the FOSHU, FNFC, and other systems in 2005. Such changes included new subsystems of FOSHU such as 1) Regular/Specific FOSHU (including disease risk reduction claims), 2) standardized FOSHU, and 3) qualified FOSHU (Ohama et al. 2006).

Regular/Specific FOSHU (including reduction of disease risk) refers to foods intended for consumer products, where safety and efficacy regarding health claims have been proven by a series of safety/stability tests and clinical trials, and have been approved by the MHLW to make health claims for the specific product.

Standardized FOSHU was introduced in February 2005 and represents foods that contain certain effective ingredients that are proven to meet the standards and specifications for a specific health claim, ingredient, and/or quality standard. Food that has an accumulation of scientific evidence (more than 100 cases of past approvals as FOSHU) can be approved as a Standardized FOSHU upon sole review of MHLW, without needing an individual review by the examination council.

Qualified FOSHU, also introduced in February 2005, refers to foods with certain effectiveness, but whose scientific data are less conclusive than those required for the existing FOSHU standard.

There are three requirements that are essential for the approval of a FOSHU application. These are 1) scientific evidence of the effectiveness of the product, proven by clinical studies, 2) additional safety studies or other evidence to prove that there are no side effects following oral intake, and 3) an exact determination of the specific effective component (Anon 2007).

The regulatory range of FOSHU has been broadened to accept capsules and tablets, in addition to conventional foods. The MHLW regulatory system, FHC, consists of the existing FOSHU system and the newly established FNFC.

FNFC refers to foods that are intended for consumption as supplements and are defined as "food products with supplemental nutritional components that are likely to be deficient in the elderly and other persons who deviate from normal eating habits due to an irregular lifestyle." Vitamins (A, B1, B2, B6,

B12, C, D, E, niacin, folic acid, biotin, and pantothenic acid) plus five minerals (calcium, iron, zinc, copper and magnesium) have been placed in this group (Anon 2007). Examples of claims regarding these substances include: "Calcium is a nutrient which is necessary to form bones and teeth," and "Vitamin D is a nutrient which promotes calcium absorption in the gut intestine and aids in the formation of bones." The upper and lower levels of the daily consumption of these nutrients are also determined.

The claims of the Japanese FNFC are equivalent to the nutrient function claims standardized by the Codex Alimentarius. The enhanced function claim and the disease risk reduction claims were proposed by both the Codex Alimentarius and an Economic Union project in 1999. The structure function claim, which is similar to the enhanced function claim, was enacted by the Dietary Supplement Health and Education Act in the U.S. in 1994. Most of the statements of the Japanese FOSHU system are close to the structure/function claims in the U.S. or the enhanced function claims of the Codex Alimentarius (Shimizu 2003).

Food for Specified Uses (FOSU) or Food for Special Dietary Uses (FOSDU) is one of the categories of Food with Health Claims that is not regulated by the Food Hygiene Law, but rather by the Health Promotion Law. FOSU refers to foods that have been deemed appropriate for specified dietary purposes, such as infant nutrition, pregnant and lactating women nutrition, and the maintenance of general health and recovery from illness. Individual consumer products are examined on a case-by-case basis and must be approved before being permitted to display that the food is appropriate for special dietary uses (Anon 2007).

In Japan, gastrointestinal health is the category having the largest number of product approvals, such as for probiotics, prebiotics, and dietary fiber. Such products accounted for approximately 60% of the 289 approved products by the end of 2001 (Arai et al. 2002), where probiotics occupied about one-third of the gastrointestinal health category. Table 13.1 illustrates some examples of health claims in the Japanese market that have been approved for certain probiotic-type FOSHU products.

CODEX ALIMENTARIUS

The Codex Alimentarius (Latin for "food law" or "food code") is a collection of internationally recognized standards, codes of practice, guidelines, and other recommendations relating to foods, food production, and food safety under the aegis of consumer protection. Officially, it is maintained by the Codex Alimentarius Commission, a body established jointly by the Food and Agriculture Organization (FAO) of

Table 13.1. Examples of health claims approved on FOSHU products containing probiotic microorganisms

Commercial Products	Health Claims
Meihi Milk Products (Bulgarian Yogurt)	Due to the effects of *Lactobacillus* LB 81, this yogurt regulates the balance of intestinal bacteria that lead to and maintain a good intestinal condition.
Yakult	Due to the effects of the Yakult strain (*Lactobacillus casei* strain Shirota), which can reach the intestine alive, Yakult maintains the intestine in good health by increasing beneficial bacteria, decreasing harmful bacteria, and improving the intestinal environment.
Morinaga Milk Industry (Bifidus Yogurt)	This yogurt contains living bifidobacteria (*Bifidobacterium longum* BB536). It helps increase intestinal bifidobacteria, improve the intestinal environment, and regulate the intestinal conditions.
Takanashi Milk Products (Onaka-He-GG)	Due to the effects of *Lactobacillus rhamnosus* GG, this organism can reach the intestine alive. This yogurt increases beneficial bacteria and decreases harmful bacteria. It improves the intestinal environment and regulates the intestinal condition.

Short (2004), Hicky (2005).

the United Nations and the World Health Organization (WHO) in 1963 to protect the health of consumers and ensure fair practices in international food trade. Codex standards are used by many countries as benchmarks when developing local food regulations (WHO/FAO 2006).

The Codex Alimentarius position is that health claims should be permitted provided that all of the following conditions are met (CAC 2004):

1) Health claims must be based on current relevant scientific substantiation and the level of proof must be sufficient to substantiate the type of effect claimed and the relationship to health as recognized by generally accepted scientific review of the data and the scientific substantiation should be reviewed as new knowledge becomes available. The health claim must consist of two parts:
   (i) Information on the physiological role of the nutrient or on an accepted diet-health relationship; followed by
   (ii) Information on the composition of the product relevant to the physiological role of the nutrient or the accepted diet-health relationship unless the relationship is based on a whole food or foods whereby the research does not link to specific constituents of the food.
2) Any health claim must be accepted by, or be acceptable to, the competent authorities of the country where the product is sold.
3) The claimed benefit should arise from the consumption of a reasonable quantity of the food or food constituent in the context of a healthy diet.
4) If the claimed benefit is attributed to a constituent in the food, for which a Nutrient Reference value is established, the food in question should be:
   (i) A source of, or high in, the constituent in the case where increased consumption is recommended, or,
   (ii) Low in, reduced in, or free of the constituent in the case where reduced consumption is recommended. Where applicable, the conditions for nutrient content claims and comparative claims will be used to determine the levels for "high," "low," "reduced," and "free."
5) Health claims should have a clear regulatory framework for qualifying and/or disqualifying conditions for eligibility to use the specific claim, including the ability of competent national authorities to prohibit claims made for foods that contain nutrients or constituents in amounts that increase the risk of disease or an adverse health-related condition. The health claim should not be made if it encourages or condones excessive consumption of any food or disparages good dietary practice.
6) If the claimed effect is attributed to a constituent of the food, there must be a validated method to quantify the food constituent that forms the basis of the claim.
7) The following information should appear on the label or labeling of the food bearing health claims:
   A statement of the quantity of any nutrient or other constituent of the food that is the subject of the claim.
   The target group, if appropriate.
   How to use the food to obtain the claimed benefit and other lifestyle factors or other dietary sources, where appropriate.
   If appropriate, advice to vulnerable groups on how to use the food and to groups, if any, who need to avoid the food.
   Maximum safe intake of the food or constituent where necessary.
   How the food or food constituent fits within the context of the total diet.
   A statement on the importance of maintaining a healthy diet.

PROCESS FOR THE ASSESSMENT OF SCIENTIFIC SUPPORT FOR CLAIMS ON FOODS (PASSCLAIM)

The European Commission concerted action PASSCLAIM aimed to produce a generic tool for assessing the scientific support for health-related claims for foods and food components (Prentice et al. 2003) as a result of the attention being paid to claims for foods, especially those related to the newly discovered effects of dietary components on body functions. The PASSCLAIM project, which ran from 2001 to 2005, built upon the principles defined in publications arising out of the EU DG XII Functional Food Science in Europe (FUFOSE) project.

The main thrust of the Consensus Document on Scientific Concepts of Functional Foods in Europe, produced as the final deliverable from the FUFOSE Concerted Action, was to suggest the outline of a scheme to link claims for functional foods to solid scientific evidence. FUFOSE suggested that claims for "enhanced function" and for "reduced risk of disease" are justifiable only when they are based on appropriate, validated markers of exposure, enhanced function, or reduction of disease risk.

The objectives of PASSCLAIM were:

- To produce a generic tool with principles for assessing the scientific support for health-related claims for foods and food components that are eatable or drinkable
- To critically evaluate the existing schemes that assess the scientific substantiation of claims
- To select common criteria for how markers should be identified, validated, and used in well-designed studies to explore the links between diet and health

The criteria for the scientific substantiation of health claims on foods defined by the PASSCLAIM project were as follows:

- The food or food component to which the claimed effect is attributed should be characterized.
- Substantiation of a claim should be based on human data, primarily from intervention studies.
- When the true end point of a claimed benefit cannot be measured directly, studies should use markers.
- Markers should be biologically valid (i.e., they should have a known relationship to the final outcome), and be methodologically valid with respect to their analytical characteristics.
- Within a study, the target variable should change in a statistically significant way.
- A claim should be scientifically substantiated by taking into account the totality of the available data and by weighing up of the evidence.

Potential health claims can be based not only on modifications of target body functions (for obesity: body fat deposition), but also on other relevant associated functions (for obesity: energy intake, energy expenditure, and fat deposition) and should be evaluated using valid methodologies. According to the PASSCLAIM consensus document, the substantiation of health claims should take into account the totality of the available data; however, it should be based on human data, primarily from intervention studies with an appropriate design and a relevant end point (Riccardi and Giacco 2005).

Regardless of the different approaches to the use of health claims on foods taken around the world, their common theme is that any health claim will require scientific validation and substantiation. There is also broad consensus that any regulatory framework should protect the consumer, promote fair trade, and encourage innovation in the food industry. There is a need to have uniform understanding, terminology, and description of types of nutrition and health claims. The two broad categories defined within PASSCLAIM were 1) Nutrition Claims, i.e., what the product contains, and 2) Health Claims, i.e., relating to health, well-being, and/or performance, including well-established nutrient function claims, enhanced function claims, and disease risk reduction claims (Richardson et al. 2003).

U.S. FOOD AND DRUG ADMINISTRATION (FDA)

The U.S. Food and Drug Administration's regulatory authority over health claims was clarified in 1990 legislation known as the Nutrition Labeling and Education Act (NLEA). This law established mandatory nutrition labeling for most foods and placed restrictions on food label claims characterizing the levels or health benefits of nutrients in foods. NLEA set a high threshold for the scientific standard under which the FDA may authorize health claims; this standard is known as the significant scientific agreement (SSA) standard. An alternative to the FDA review of health claims was established in subsequent legislation (the FDA Modernization Act, 1997) which provided a U.S. government scientific body, other than the FDA, to establish that there is SSA for a substance/disease relationship (Rowlands and Hoadley 2006).

The FDA has approved 12 health claims for foods, as shown in Table 13.2. Some of these claims are applied to dietary supplements and also conventional foods. The Code of Federal Regulations and in Appendix C of the Food Labeling Guide describe full details of these health claims, which can be found in the FDA website (www.cfsan.fda.gov). In addition to approved claims, the FDA has specified

Table 13.2. Health claims approved by the FDA

Approved Claims
Calcium and osteoporosis
Dietary lipids and cancer
Dietary saturated fat, cholesterol, and coronary heart disease
Dietary sugar alcohols and dental caries
Fiber-containing grain products, fruit, and vegetables, and cancer
Folate and neural tube defects
Fruits and vegetables and cancer
Fruits, vegetables, and grain products that contain fiber, particularly soluble fiber, and risk of coronary heart disease
Plant sterol/stanol esters and the risk of coronary heart disease
Sodium and hypertension
Soluble fiber from certain foods and the risk of coronary heart disease
Soy protein and the risk of coronary heart disease

the requirements for the food making the claim, the food claim requirements, and model claim statements.

Courts have since extended the scope of health claims to include qualified health claims (QHC) that are health claims not substantiated on evidence that meets the level of SSA standard, but include a qualifying statement intended to convey to the consumer the level of evidence for the claim. FDA has responded by developing an evidence-based ranking system for scientific data to determine the level of evidence substantiating a health claim, and established a system with four different levels of substantiation (Rowlands and Hoadley 2006). However, it appears consumers are confused by the system of qualified health claims. They find it difficult to understand the meaning of the different types of qualified claims and may even interpret the qualification levels to refer to the overall safety of the product rather than an evaluation of the strength of the scientific substantiation (IFIC 2005).

GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS

The U.S. FDA had set the bar too high for health claims and was forced by the courts to implement a more reasonable standard, but the response, Qualified Health Claims, has failed to gain the confidence of the public because of the confusing wording of the claims demanded by FDA. The Dietary Supplement Health and Education Act (DSHEA) was the product of a compromise, with a lower threshold for demonstration of safety (reasonable expectation of no harm) that would be met by consumer self-policing and assumption of some risk. FDA has thwarted this effort by raising the bar for New Dietary Ingredient Notifications (NDIN) to what appears to be the higher threshold for the safety of food ingredients (reasonable certainty of no harm). The FDA apparently sees these two safety thresholds as a distinction without a difference (Burdock et al. 2006). As a result, increasing numbers of dietary supplement manufacturers, unwilling to gamble the future of their products to a system that provides little hope for the FDA's response of "no objection," have committed the additional resources necessary to obtain Generally Recognized As Safe (GRAS) status for their supplements (Burdock and Carabin 2004; Burdock et al. 2006; Noonan and Noonan 2004).

The pressure on FDA and Congress for change is again building with increased dissatisfaction among consumers as the result of confusing labels. A second force for change will be a need to uncouple the FDA mandated substance-disease relationship and return to the substance-claim relationship to allow for progress in nutrigenomics and metabolomics, which will result in an increasing number of substance-biomarker claims (Burdock et al. 2006).

CONCLUSIONS

Scientific discoveries and increasing interest in the potential health benefits of foods and food components have resulted in a range of content, structure-function, and health claims. The clinical and epidemiological evidence on the way each particular dietary component fosters growth and development, healthy functioning, and disease prevention is expanding. However, defining a single ideal diet is complicated by the many factors that may influence biological processes.

The diversity of findings in the literature may reflect the multifactorial nature of these processes. New and emerging genomic and proteonomic approaches and technologies offer the prospect of identifying molecular targets for dietary compo-

nents, thereby possibly determining the mechanisms by which specific individual dietary constituents modify the genetic and epigenetic events that influence the quality of life. Expanded knowledge on unique cellular characteristics with molecular targets for nutrients thus may be able to be used to develop strategies to optimize nutrition and minimize disease risk (Milner 2002).

This increasing emphasis on food and its component ingredients to reduce disease risk and promote health requires the development of accurate biomarkers for predicting outcomes of food-based interventions. Improved knowledge of human genomics and the ability to use microarray technology to screen for biomarkers at the gene level may provide the opportunity for individuals to be diagnosed for and informed of their own particular disease risk profile.

It remains to be seen whether these technologies will provide sufficient understanding of food-gene interactions to permit more certain health claims rather than better therapeutic treatments (Roberts 2002). The application of such personalized nutrition from the earliest stage of life, including in utero, will present significant challenges for the substantiation of health claims in the postgenome era (McGinty and Man 2007).

Manufacturers will face increasing demands to provide high-quality scientific data before approvals for health claims are granted. They will also need to consider the economics of the time and expense of scientific substantiation/clinical data to support claims, even if these result in higher consumer confidence in the resulting claims.

Consumer research shows that dairy foods like yogurt are viewed as desirable and credible carriers of functional ingredients, particularly over indulgent foods such as chocolate (Kleef et al. 2005). Dairy foods are also less likely to face the disqualifying criteria for health claims of addition of a healthy ingredient into a less-than-healthy food vehicle, or the high threshold values for less than healthy nutrients (e.g., high sugar content) now being included in codes by some regulatory bodies.

Dairy foods and ingredients have a natural advantage over new/novel foods from a regulatory viewpoint because they are generally considered as "traditional" foods—that is, there is a long history of human consumption. However, the regulatory landscape on adding bioactive ingredients, whether from dairy streams or from nondairy sources, into dairy foods is rapidly evolving, and the dairy industry will need to be aware of potential regulatory challenges within the countries where they want to market their products.

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14
New Technologies for Isolation and Analysis of Bioactive Compounds

Sumangala Gokavi

INTRODUCTION

Milk is increasingly changing from being simply the raw material for cheese, butter, and milk powders, to being a source of a diverse range of bioactive molecules, often with health-promoting attributes (Bauman et al. 2006). Extracting beneficial bioactive compounds from milk offers great potential to add value to dairy products. Isolation and analysis of biologically active compounds from milk represents a constant challenge to researchers all over the world. When a new compound is found to be bioactive, new technologies are being developed to isolate the compound. In addition, new analytical methods are developed to study the isolated compound in detail. Exploitation of the benefits provided by bioactive components will rely upon cost-effective processing, isolation, and analysis technologies. Because purified individual milk proteins exhibit better functionality than in their native protein mixtures, there is a great interest in developing easier methods to prepare pure casein and whey proteins on a large scale (Imafidon et al. 1997). This chapter discusses different technologies or methods developed for isolation of bioactive compounds from milk, and their purification and quantification.

Milk is a rich source of protein. Casein and whey proteins are the two main protein groups in milk; caseins comprise about 80% of the total protein content in bovine milk and are divided into α-, β- and κ-caseins. Whey protein is composed of β-lactoglobulin (β-Lg), α-lactalbumin (α-La), immunoglobulins (IgGs), glycomacropeptides (GMPs), bovine serum albumin, and minor proteins such as lactoperoxidase, lysozyme, and lactoferrin (LF). Each of the subfractions found in casein and whey has its own unique biological properties. Milk proteins can be degraded into numerous peptide fragments by enzymatic proteolysis and serve as sources of bioactive peptides such as casomorphins, angiotensin-converting enzyme (ACE)–inhibitory peptides, and phosphopeptides found in fermented milk products and in cheeses. Milk also contains antioxidative factors such as superoxide dismutase, catalase, glutathione peroxidase and growth factors such as Transforming Growth Factor Beta (TGF-β1 and TGF-β2), Epidermal Growth Factor (EGF), and nonprotein components such as oligosaccharides. The growing demand for cost competitive and multifunctional ingredients by food manufacturers means that the selection of processing/isolation technology for their manufacture is becoming increasingly very important. For these reasons several alternative procedures have been proposed and developed for industrial-scale isolation of bioactive compounds. These industrial processes and methods have been reviewed extensively by Imafidon et al. (1997). The methods for isolation and/or analysis of these compounds are discussed in the following sections.

ISOLATION AND QUANTIFICATION OF MAJOR WHEY PROTEINS

The rapid development of membrane and gel filtration techniques in the 1970s provided new possibilities for a large-scale concentration of whey proteins

and the manufacture of whey protein concentrates and isolates (Korhonen et al. 1998a). Also, manufacture of demineralized whey powders has become possible on a large scale through application of diafiltration or ion exchange chromatography. Techniques for the isolation of individual whey proteins on a laboratory scale by salting-out, ion-exchange chromatography, and/or crystallization has been available for a long time. Several pilot and industrial-scale technological methods have been developed for isolation of several individual whey proteins in purified form (Yoshida and Xiuyun 1991; Yoshida and Ye 1991; Burling 1994; Fukumoto et al. 1994b; Mitchell et al. 1994; Outinen et al. 1995, 1996; Konrad and Lieske 1997).

Gesan-Guiziou et al. (1999) developed a process for the preparation of purified fractions of α-La and β-Lg from whey protein concentrates (WPC) (Fig. 14.1), which is comprised of the following successive steps: clarification-defatting of WPC, precipitation of α-La, separation of soluble β-Lg, washing the precipitate, solubilization of the precipitate, concentration, and purification of α-La. This process was evaluated for its performance both on a laboratory scale with acid whey and then on a pilot scale with Gouda cheese whey. In both cases soluble β-Lg

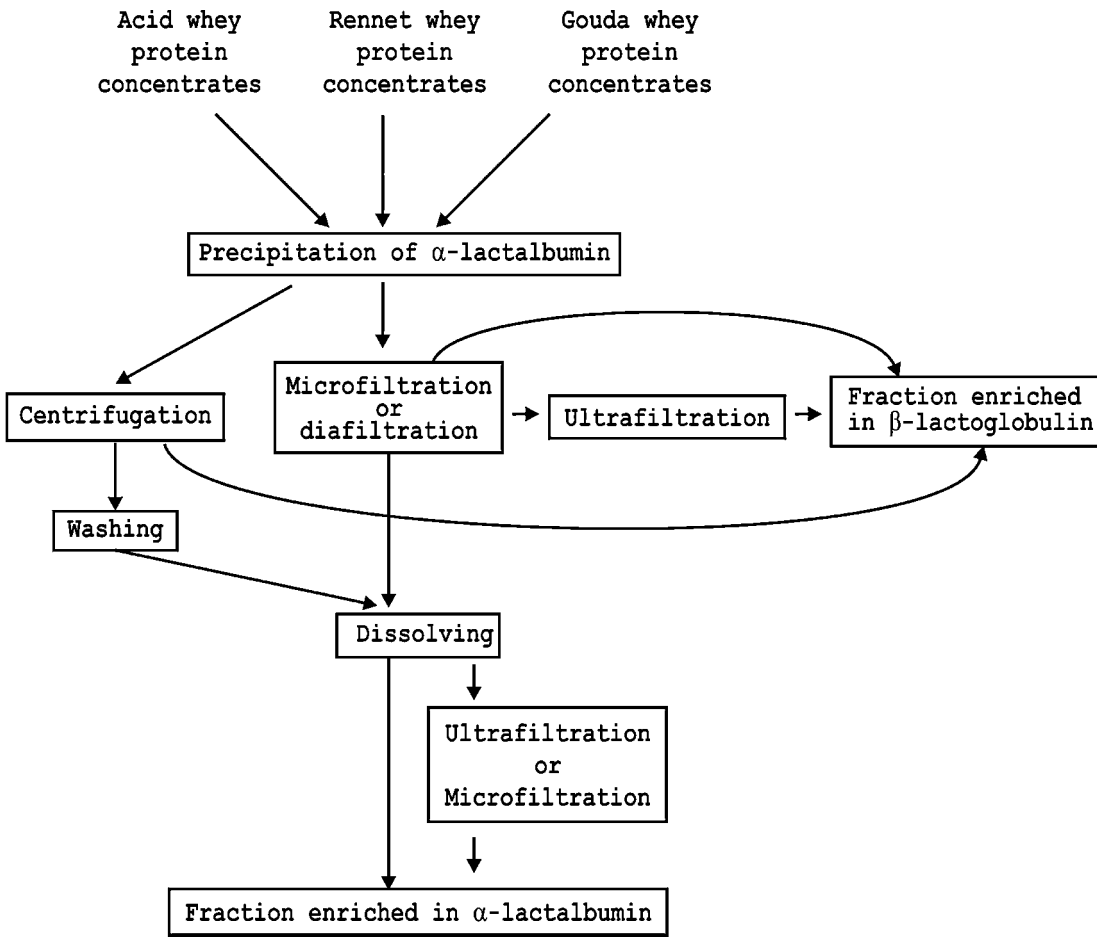

Figure 14.1. Overview of the process for the manufacture of fraction enriched with α-lactalbumin and β-lactoglobulin (Gesan-Guiziou et al. 1999).

was separated from the precipitate using diafiltration or microfiltration and the purities of α-La and β-Lg were in the range of 52–83% and 85–94%, respectively. The purity of the β-Lg fraction was higher using acid whey, which does not contain caseinomacropeptide, than using sweet whey.

Isolation and Analysis of β-Lactoglobulin (β-Lg)

Different laboratory and industrial-scale procedures for isolation of β-Lg have been available for some time. These include salting out with ammonium persulphate and polyethylene glycol, electrophoresis on different supports, column chromatography, high-performance liquid chromatography (HPLC), and isoelectric focusing (IEF), both with carrier ampholyte (CA) on granulated gel or immobilized pH gradient (IPG) in polyacrylamide (Conti et al. 1988). The flat bed IEF technique was extremely versatile and was recognized as one of the most powerful tools for the separation of whey proteins at the degree of purity required for structural studies (Conti et al. 1988).

Precipitating β-Lg based on its isoelectric point, after concentration of the whey source material using ultrafiltration followed by demineralization by diafiltration or electrodialysis, forms the basis of the earliest commercially feasible methodology (Bramaud et al. 1997). Bounous and Gold (1990, 1991), Bounous et al. (1994), and Bounous (1996) have described processes for the production of undenatured whey protein concentrates (containing β-Lg and α-La), that have a variety of biological actions. These patented processes are primarily based on microfiltration and ultrafiltration methods used in isolation, or in combination.

The most promising cost-effective technologies for isolation of β-Lg include liquid chromatography and methods of selective aggregation and precipitation of α-La from a whey source concentrated by ultrafiltration, under specified conditions of pH and temperature, leaving β-Lg in solution and unaffected by the pH/temperature treatment (Chatterton et al. 2006).

Preparative fractionation of bovine whey containing the new β-Lg genetic variant H was performed by HPLC gel filtration on a TSK G2000 SWG column (21.5 × 600 mm) (Conti et al. 1988). 1.0 g of lyophilized whey was dissolved in 6 mL of elution buffer (0.05 M phosphate, pH 6.6) and filtered through a Millipore membrane (pore size 0.45 μm). The sample was injected in aliquots of 2 mL each. Flow rate was 2.5 mL/min. UV-50 Varian detector was used at 280 nm. Volume of fractions collected was 2 mL. The fractions corresponding to each peak were pooled and concentrated in an Amicon cell with a YM5 membrane. The fractions were further purified using preparative IEF-IPG. Two polyacrylamide gel slabs (T = 5%) were cast and run in the same experimental conditions in order to keep one slab intact to show the separation of two β-Lg variants over the whole width of the plate. Each polyacrylamide gel slab (110 × 115 × 5 mm with a sample application slot of 93 × 9 × 3 mm) contained 0.916 mL Immobiline at pK 3.6, 1.350 mL at pK 4.6, 1.726 mL at pK 6.2 in the dense solution, and 1.726 mL at pK 4.6, 1.726 mL at pK 6.2, 0.3 mL at pK 9.3 in the light solution, to give a final pH gradient between 4.7 and 5.7. The linear pH gradient was obtained mixing the light and dense solutions with a P-3 peristaltic pump (Pharmacia) at a flow rate of 4 mL/min. The other experimental conditions were according to the LKB Application Note no. 323. The recovery of two proteins from the polyacrylamide gel was carried out by electro-elution on ISCO model 1750 electro-elution apparatus with a 0.005 M Tris-HCl buffer, pH 8.6. The two proteins were freeze-dried after diafiltration on an Amicon cell with 0.1% acetic acid, and about 25 mg of the H variant and 13 mg of the B variant were obtained. The purity of the two β-Lg variants (B and H) separated by preparative IEF-IPG was checked by analytical IEF with mixed Immobiline-CA. The dense solution for one gel contained: 0.334 mL Immobiline at pK 4.6 and 0.151 mL at pK 9.3, 0.035 mL Ampholine pH 3.5–5.0 and 0.035 mL Ampholine pH 4–6 in a final volume of 7 mL of 5% acrylamide and bisacrylamide; for the light solution the only difference consisted in the amount of Immobiline used: 0.657 mL at pK and 0.604 mL at pK 9.3. A linear pH gradient between 4.5 and 5.5 was obtained mixing the dense and light solutions with a P-3 pump (Pharmacia) at a flow rate of 2 mL/min. Focusing conditions without prerun, staining, and destaining of the gel were according to LKB application note no. 324.

Isolation and Analysis of α-Lactalbumin (α-La)

The starting material for enrichment and purification of bovine α-La is whey. Many processes in the dairy industry are based on membrane technology. This technique has also been exploited to enrich α-La. This can be achieved by either performing microfiltration to remove β-Lg or performing ultrafiltration using a 50 kDa cut-off membrane, thereby passing α-La into the permeate (Uchida et al. 1996). Mehra and Kelly (2004) have used a two-membrane cascade membrane filtration scheme and obtained enriched fractions of α-La.

Another method used enzymes such as trypsin or chymotrypsin to selectively degrade β-Lg which resulted in fraction enriched with α-La (Kaneko et al. 1992). A protease of microbial origin has also been used for this purpose (Kaneko et al. 1994). Yukio et al. (1992) used ion exchange chromatography, where chymosin whey was adjusted to pH 5 or higher, and where α-La did not bind to the ion exchange matrix, and was therefore easily eluted. The fraction was then adjusted to pH 4.0 and ultrafiltered on a narrow molecular mass cut-off membrane to separate glycomacropeptide from α-La, thus obtaining concentrated α-La.

Rialland and Barbier (1988) recovered α-La from whey by heating whey protein concentrate (WPC) to a temperature of 75 °C and acidifying it using a cation exchange resin in (H^+) form.

The ion exchange columns and resins are very expensive. Hence, the majority of isolation procedures for α-La utilize isoelectric precipitation, often in combination with heat treatment. This method is cheap and relatively easy to perform, and whey protein is first desalted, and the pH is adjusted to pH 3.8–5.5. The resulting solution is heat treated at between 55 and 70 °C for more than 30 seconds to permit aggregation of part of the whey protein. Thereafter, the solution is cooled to 55 °C to permit flocculation of the aggregates that consisted of α-La. The α-La is then isolated by microfiltration (Pearce 1995). De Wit and Bronts (1997) reported a similar method in which the protein was destabilized by exposing whey protein to a calcium-binding ion-exchange resin. The pH was then adjusted to between 4.3 and 4.8 and incubated between 10 and 50 °C. The protein was then fractionated to isolate α-La. By combining isoelectric precipitation and heat treatment, a new method was designed, composed of heat treatment of a 15% (w/w) whey protein concentrate at 60–80 °C, at neutral pH followed by cooling to 45 °C, and pH adjustment to 4.2–4.5. α-La was then isolated leading to an α-La/β-Lg ratio of more than 0.43 (Hakkaart et al. 1992). α-La is sensitive to calcium, and adjustment of the pH to around the isoelectric point of α-La results in formation of the molten globule form of the protein. Mild heat treatment causes the protein to precipitate. Unfortunately, the drawback of this approach is that the structure of the protein is irreversibly altered compared with that of the more gentle methods of purification. As a result, the bioactivity of the protein could be impaired (Chatterton et al. 2006).

ISOLATION AND ANALYSIS OF CASEIN FRACTIONS

The principal casein components (α_{s1}-, α_{s2}-, β- and κ-Cn) exist in strong association with each other as a micellar complex stabilized by van der Waals forces, hydrophobic interactions, hydrogen bonding, and electrostatic and steric stabilization. Several techniques have been reported for isolating individual caseins. To obtain a respective homogeneous protein, some form of chromatography is required (Imafidon et al. 1997). An extensive review on methods of isolation and purification of casein fractions has been published by Imafidon et al. (1997).

Differences in the solubility of the casein fractions in urea solution are commonly used to separate various components. Hipp et al. (1952) obtained different casein fractions using this principle. Ion exchange chromatographic separation of bovine milk proteins has been reviewed by Swaisgood (1992). Covalent chromatography of caseins on thiol sepharose after reduction of disulfide bonds can be used to purify κ-Cn and α_{s2}-Cn (Chobert et al. 1981).

Diethylaminoethyl (DEAE)-celluloses have been used in column chromatographic methods for separation and purification of bovine caseins. Unfortunately, the amount of pure caseins recovered is relatively low due to the size of the column and overlapping effects during elution (Wei and Whitney 1985). To overcome these problems, Wei and Whitney developed batch fractionation procedures for isolating bovine casein using DEAE-cellulose.

Murphy and Fox (1991) described a technique for the preparation of α_s-/κ-Cn—and κ-Cn–rich fractions. The technique used ultrafiltration through 300,000 D cut-off membranes at 4 °C. Sodium caseinate (1%), at pH 7, equilibrated for 3 hours at 0 to 4 °C, was passed through the ultrafiltration unit. The β-Cn-enriched permeate was concentrated to the required degree using ultrafiltration with 100,000 Dalton MW cut-off membrane at 50 °C. Most β-Cn can be removed from the retentate by diafiltration to allow recovery of the α_s-+κ-Cn-rich fraction. These fractions were then freeze-dried and weighed to calculate the yield of the respective fractions. The β-Cn prepared by this procedure was about 80% homogeneous; the major contaminant was γ-Cn. Similarly, the α_s-+κ-Cn fractions were contaminated with about 15% β-Cn. Although the fractions were heterogeneous, up to 500 g of these protein fractions can be prepared by this technique, thereby creating an excellent starting material for further purification and the study of their functional properties.

Recently, Turhan et al. (2003) developed a method for fractionation of caseins by anion-exchange chromatography using food grade buffers. Casein was prepared by microfiltration of skim milk and fractionated using Q Sepharose fast anion exchange beads (Amersham Biosciences, Piscataway, NJ, USA). These were packed into a 1 cm diameter column (C 10/10). The bed height of the column was 8 cm, resulting in a column volume of 6.28 mL. A peristaltic pump (Tris; ISCO, Lincoln, NE, USA) was used to control the flow rate. Effluent from the column passed through an absorbance detector set at 280 nm (UA-5; ISCO). Four food-grade buffers studied were 1) mixture of 20 mM Tris, 65 μM dithiothreitol, 4 M urea, 2) mixture of 25 mM L-cysteine, 4 M urea, 3) mixture of 25 mM L-cysteine, 20 mM triethanolamine, 4 M urea and 4) mixture of 20 mM triethanolamine, 4 M urea. L-cysteine was successfully used as a reducing agent instead of traditional toxic agents, such as dithiothreitol or β-mercaptoethanol, enabling development of the first food-grade buffer system for casein fractionation.

ISOLATION AND ANALYSIS OF BIOACTIVE PEPTIDES

There are a number of methods by which bioactive peptides with biological activity can be produced. The most common methods are food processing using heat, alkali, or acid conditions that hydrolyze proteins: enzymatic hydrolysis of food proteins, and microbial activity of fermented foods. Biologically active peptides are released by limited hydrolyses of well-known proteins. So far the most common way to produce bioactive peptides has been through enzymatic digestion. For example, ACE-inhibitory peptides are most commonly produced by trypsin (Maruyama and Suzuki 1982). However, other enzymes and various enzyme combinations of proteinases-including alcalase, chymotrypsin, pancreatin, pepsin, and enzymes from bacterial and fungal sources have been used to produce bioactive peptides. Microbial enzymes have also been successfully used to produce ACE-inhibitory peptides most commonly found in cheese (Maeno et al. 1996).

After hydrolysis of milk proteins, the peptides in hydrolysates are fractionated and enriched by means of various methods. Ultrafiltration membranes have been successfully used to concentrate specific peptide fractions. Bouhallab and Touze (1995) used an ultrafiltration membrane reactor for the continuous extraction of permeates enriched with bioactive fragments in order to produce antithrombotic peptide (Fig. 14.2). Membranes containing negatively charged materials have been used to enrich cationic peptides with antibacterial properties from cheese whey (Recio and Visser 1999).

Isolation of mineral binding peptides includes selective solubilization and precipitation methods by the use of different solvents, chelating complexes (calcium/barium), and pH and ionic strengths (Gaucheron et al. 1996). Peptides of different molecular sizes and ionic and hydrophobic characteristics are obtained by dialysis or filtration techniques and by the use of selective chromatography by ionic, affinity, hydrophobic interactions, and chelating column techniques. Figure 14.3 outlines the possible isolation methods of mineral binding peptides from milk proteins (Veragud et al. 2000).

Chemical measurements and analytical techniques are the critical components of the molecular understanding of the biological process, where many bioactive peptides are involved. There are some methods, which have already been proved to be applicable for the identification and characterization of bioactive peptides derived from milk proteins. These methods are outlined in Figure 14.4 (Schlimme and Meisel 1995).

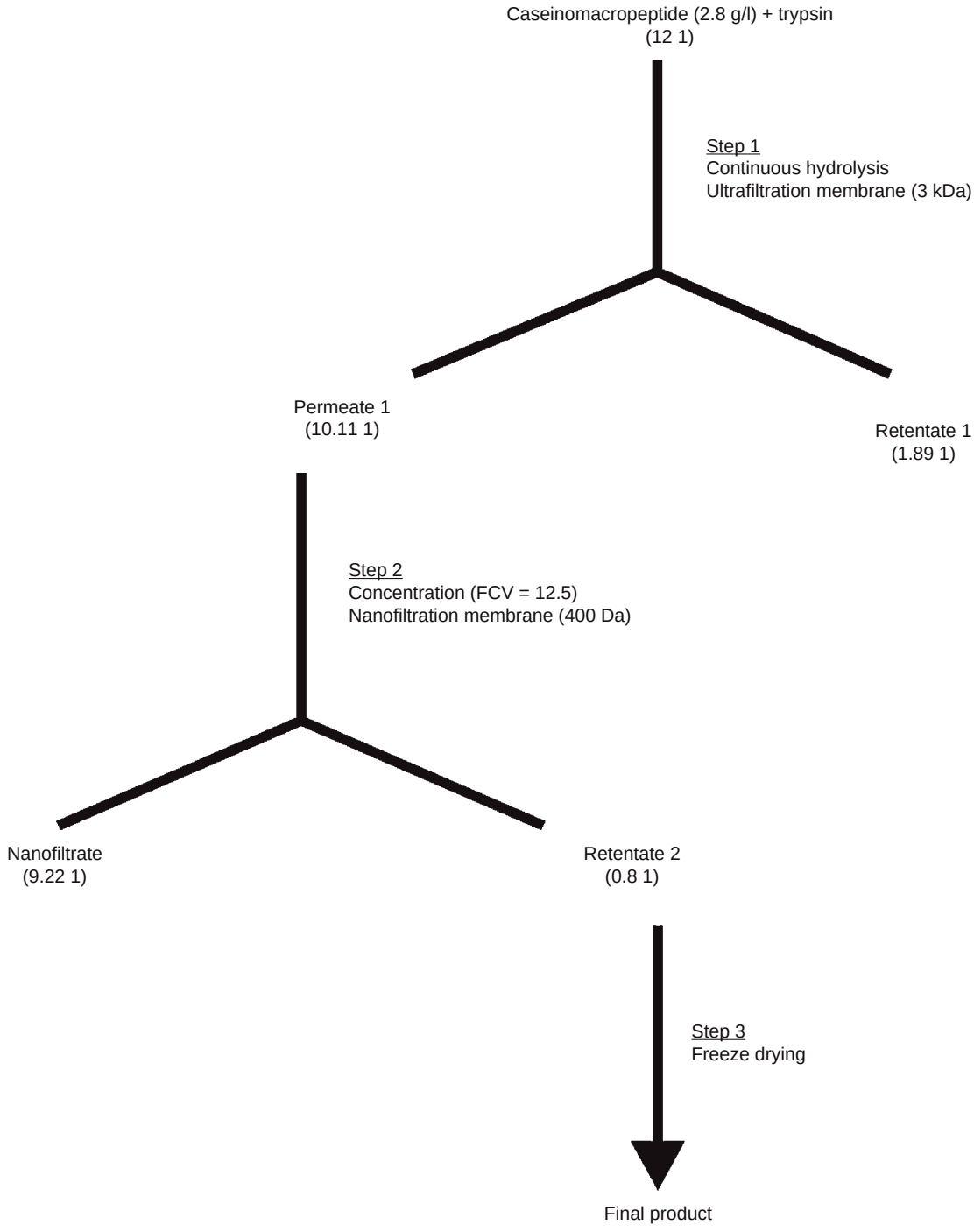

Figure 14.2. Continuous production of antithrombotic peptides (Bouhallab and Touze 1995).

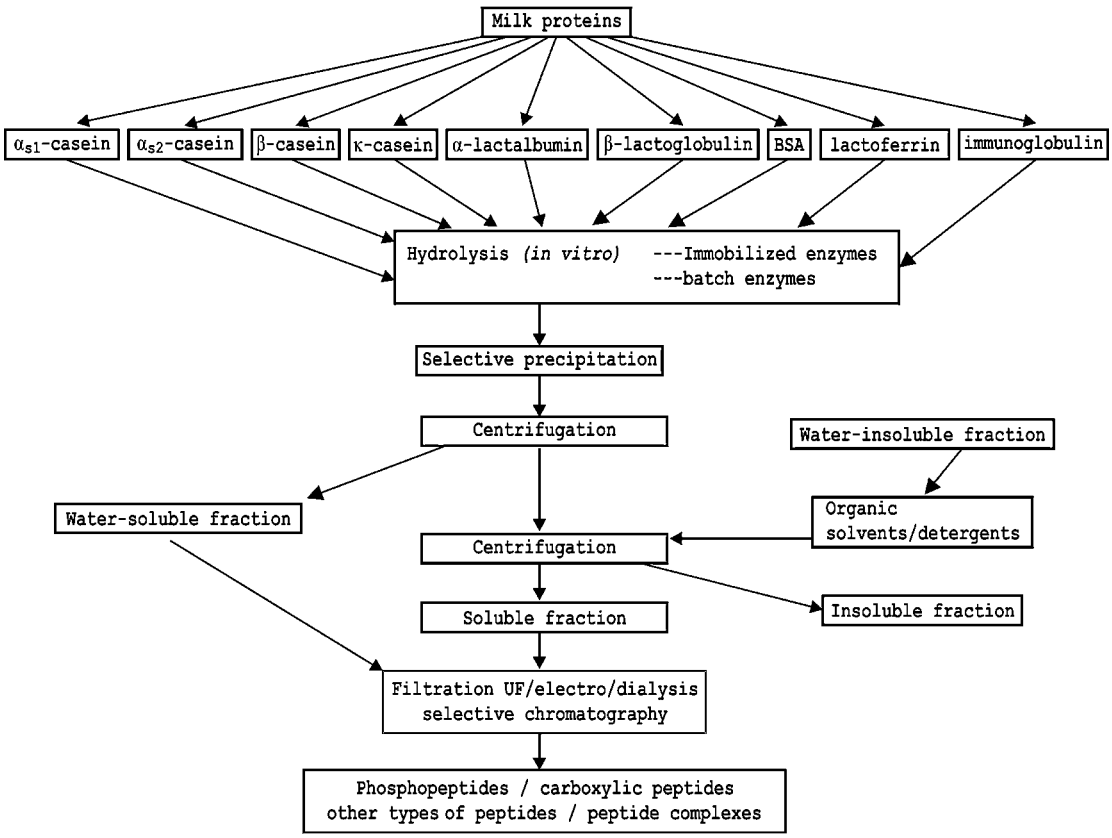

Figure 14.3. Possible isolation methods of mineral binding peptides from milk proteins. BSA = bovine serum albumin; UF = ultrafiltration (Veragud et al. 2000).

Ryhanen et al. (2001) have reported a method for the analysis of ACE-inhibitory peptides from cheese. The freeze-dried cheese sample (about 40 mg) was dissolved in 1 mL of deionized water containing 0.5 mL of trifluoroacetic acid (TFA)/L, and filtered through a 0.45 mm filter. This solution was used to fractionate the ACE-inhibitory peptides present in the cheese extract by HPLC. Portions of 100–200 mL were applied to a reversed-phase column (Super-Pak Pep-S 4.0 × 250 mm, 5 µm, pre-column Pep-S 4.0 × 10 mm,5 µm; Pharmacia, Uppsala, Sweden). Solvent A was TFA (0.5 mL/L), and solvent B acetonitrile (900 mL/L containing TFA 0.5 mL/L). A linear gradient was applied from 50 to 600 mL solvent B/L over 45 minutes at a flow rate of 1 mL/min. The eluate was monitored at 214 and 280 nm, and the fractions were collected on a peak basis and dried in a vacuum. This step was repeated 5–10 times, and the fractions from the different chromatographic runs were combined and dried in a vacuum. When necessary, the fractions were dissolved in 0.5 mL TFA/L and applied on a Nucleosil 300-5-C18 column (4.0 × 250 mm, 5 µm; Macherey Nagel, D-52348 Duren, Germany), and eluted as in the first step. The amino acid composition of the peptides and peptide mixtures was analyzed by the Pico-Tag method (Millipore Corporation 1987). The peptides showing ACE-inhibitory activity were sequenced by automated Edman degradation using a protein/peptide sequencer (Perkin-Elmer Abi 499 Precise, Foster City, CA 94404, USA).

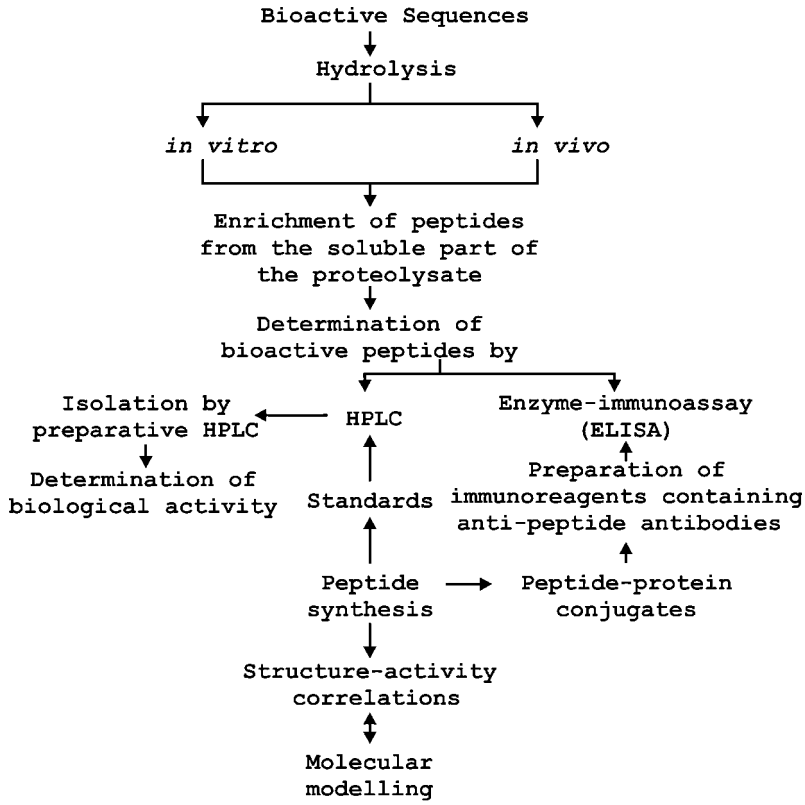

Figure 14.4. Outline of identification and characterization of bioactive peptides derived from milk proteins (Schlimme and Meisel 1995).

ISOLATION AND ANALYSIS OF ANTIOXIDATIVE FACTORS

Lindmark-Mansson and Akesson (2000) have reviewed methods for isolation and analysis of different antioxidative factors in milk.

Superoxide Dismutase (SOD)

Hill (1975) partly purified SOD from skim milk after precipitation of casein with rennet. The whey was concentrated, the solution was treated with ethanol and chloroform, and the precipitated proteins were removed by centrifugation. SOD was purified from the supernatant by gel chromatography and ion exchange chromatography.

The most commonly used assay of SOD in milk is based on measuring the inhibition of the reduction of cytochrome C by the superoxide anion, produced enzymatically in the xanthine-xanthine oxidase (XO) reaction (Korycka-Dahl et al. 1979). Since endogenous XO occurs in milk and may interfere with the SOD determination, Granelli et al. (1994) improved this method where XO is reduced by ultrafiltration in the samples prior to analysis.

Catalase

Catalase has been purified from bovine milk by several purification steps, including n-butanol extraction, ammonium sulphate treatment, ethanol-chloroform fractionation, DEAE-Sephacel column chromatography and Sephacryl S-300 gel filtration. Optimum pH and temperature for enzyme activity are pH 8.0 and 20 °C (Ito and Akuzawa 1983).

A method for determining catalase by a disk-flotation procedure has been described by Gagnon et al. (1959). This method is based on the liberation

of oxygen due to the action of catalase on hydrogen peroxide. Catalase activity can also be assayed by the polarographic method in which oxygen released from H_2O_2 is quantitated with an oxygen electrode (Hirvi et al. 1996).

Glutathione Peroxidase (GSHPx)

Bhattacharya et al. (1988) purified human milk glutathione peroxidase 4500-fold using acetone precipitation and purification by repetitive ion-exchange and gel filtration chromatography. Of the GSHPx activity in human milk, 90% could be precipitated by anti–plasma-GSHPx immunoglobulin G. Thus, most if not all GSHPx activity in human milk is due to the plasma form of the enzyme. In two examples of human milk, 4% and 13% of total selenium was calculated to be bound to GSHPx (Avissar et al. 1991).

Paglia and Valentine (1967) reported a method for measuring GSHPx activity. It is an indirect assay, which requires a peroxide source and a coupled reaction maintaining the concentration of the initial GSH substrate. The GSHPx activity is quantified indirectly in a sample by its ability to cause an increased rate of loss of NADPH compared to the rate of loss seen in a reagent blank.

Lactoferrin (LF)

Skim milk and cheese whey that have not undergone rigorous heating can be sources of LF. LF is denatured by heat treatment depending on the conditions. Pasteurized milk is not a suitable source of LF. LF has a cationic nature according to its amino acid composition, because of which it can be purified by cation-exchange chromatography such as carboxymethyl (CM)-Sephadex (Yoshida et al. 2000), and this purification method is the most popular procedure for LF purification by LF-supplying companies. Skim milk (pH 6.7) or cheese whey (pH 6.4) is filtered and applied to a cation-exchange chromatography column without pH adjustment. The column is washed with a low-concentration (1.6%) NaCl solution, by which lactoperoxidase is eluted. Then LF is eluted with a high-concentration (5%) NaCl solution. The LF is concentrated by ultrafiltration and is separated from NaCl by diafiltration. After low heat treatment, LF is freeze-dried to make a powder. Alternatively, LF is spray-dried after sterile filtration. Several other chromatographic methods have been evaluated for purification of LF (Wakabayashi et al. 2006).

The concentration of LF in milk and supplemented foods must be measured to monitor the stability of LF during processing and storage. Different analytical methods such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Ronayne de Ferrer et al. 2000), HPLC (Hutchens et al. 1991), and mass spectrometry (Natale et al. 2004) can quantify LF in food samples. However, these methods cannot discriminate between intact LF and denatured LF. Because immunological methods can discriminate the tertiary structures of proteins, these methods would be suitable for monitoring intact LF in food samples. A single radial immunodiffusion assay (Masson and Heremans 1971), the Rocket assay (Nagasawa et al. 1972), and enzyme-linked immunosorbent assay (ELISA) (Soderquist et al. 1995) have been used to measure LF in supplemented products. ELISA kits for bovine LF and human LF are commercially available from several suppliers such as Bethyl Laboratories (Montgomery, AL, USA) and Merck/EMD Biosciences (San Diego, CA, USA), and are generally used.

Tsuji et al. (1990) have described a method for analysis of lactoferrin in colostrum. The fat fraction was removed from colostrum by centrifugation at 10,000 rpm for 10 minutes at 4 °C to yield fat-free secretion; the fat-free fraction was employed in the lactoferrin assay. The single radial immunodiffusion method was used to determine the lactoferrin content of fat-free samples. Immunodiffusion was carried out within 24 hours at room temperature on glass plates covered with 1% agarose in 0.05 M potassium phosphate-buffered saline, pH 7.5, containing 0.l% NaN_3 and 2% antibovine lactoferrin rabbit serum. Purified lactoferrin and antiserum were prepared from colostrum of Holstein-Friesian cows obtained within 24 hours after parturition. Colostrum was centrifuged at 3000 rpm for 10 minutes. One liter of the skimmed colostrum was dialyzed against distilled water in the presence of 0.01% NaN_3 for 3 days at 4 °C with several changes of distilled water. The dialyzed skimmed colostrum was loaded onto a column of CM-Sephadex C-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) (2×20 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 8 (Buffer A). Lactoferrin was eluted with a linear gradient of NaC1 (0.1 to 0.7 M) in Buffer A after

unbound proteins and weakly bound proteins were washed out sequentially with 100 mL of Buffer A and with 100 mL of Buffer A containing 0.05 M NaCl. The combined fraction containing lactoferrin was dialyzed against Buffer A at 4 °C overnight and again loaded onto a column of CM-Sephadex C-50 (1 × 5 cm). Lactoferrin was eluted with a linear gradient of NaC1 (0.2 to 0.5 M) in Buffer A; at this step, 192 mg of nearly homogeneous lactoferrin were obtained. To obtain antiserum for lactoferrin, a rabbit was injected 5 times at weekly intervals with 1 mL of a mixture that contained an equal volume of purified lactoferrin and Freund's adjuvant. Using purified lactoferrin as standards, lactoferrin content of each colostrum sample was determined in triplicate.

An immunoassay method to quantify LF was reported (Yamauchi et al. 2004). An automated latex assay was developed using F(ab′)2 fragments of anti-LF rabbit IgG-coated polystyrene latex beads and an automated multipurpose analyzer. The latex assay employs agglutination of the antibody-coated latex particles in the presence of the antigen. This method enabled the quantification of LF in LF-supplemented products such as infant formula in a simple, rapid, highly sensitive, and precise manner. A reagent for the automated latex assay of bovine LF and human LF is commercially available as a kit (Cosmo Bio, Tokyo, Japan).

An automated SPR-biosensor assay for the quantification of lactoferrin in protein isolates, milk, colostrum, and lactoferrin-supplemented infant formula was developed by Indyk and Filonzi (2005). They used Biacore® Q optical biosensor from Biacore AB (Uppsala, Sweden).

GLYCOMACROPEPTIDE (GMP)

Two methods have been developed for GMP preparation. One method is to separate whey proteins from casein or κ-casein and hydrolyze the casein or κ-casein with rennet. Dosako et al. (1991) used this method to prepare GMP from sodium caseinate. Coolbear et al. (1996) compared different methods such as precipitation, gel filtration, and ion exchange for preparing GMP from κ-caseins and determined that these GMPs were virtually identical by using reverse-phase chromatography and gel electrophoresis.

The other method is purification of GMP directly from cheese whey made by a rennet process. When this method is used, the primary challenge is separation of GMP from the other whey proteins. Shammet et al. (1992) and Eustache (1977) prepared GMP by precipitation of the other whey proteins with trichloroacetic acid or phosphotungstic acid, respectively, followed by dialysis or ultrafiltration.

In other methods, WPC is heated to temperatures above 85 °C in order to flocculate the whey proteins, adjusting to the isoelectric point, and separating by centrifugation or filtration. The GMP, which is heat stable, is collected in the supernatant or filtrate (Nielsen and Tromholt 1994). Berrocal and Neeser (1993) heated WPC to 90 °C at pH 6, with added calcium and 25% ethanol. The solution was acidified to pH 4.5 and flocculent material was removed by centrifugation leaving a supernatant containing GMP. GMP has negative charge even at low pH whereas the other whey proteins are positively charged. This chemical nature of GMP is used to isolate GMP using Ion exchange. Whey at pH 3 is contacted with a cation exchanger (Kawasaki and Dosako 1994). The GMP is not adsorbed by the cation exchanger and may be concentrated and desalted by ultrafiltration. Alternatively, whey at pH less than 4 is contacted with an anion exchanger (Kawasaki et al. 1994).

Etzel (1999) employed a copper-containing metal affinity adsorbent as well as a cation exchanger to produce GMP from whey. Erdman and Neumann (1999) used a polystyrene weak anion exchange resin in the alkaline form to capture GMP from an acidified whey solution.

ANTIBODIES

The extensive research on understanding the underlying mechanisms of immunity has drawn attention of scientists to develop immune milk preparations for the prevention or treatment of microbial infections in humans and domestic animals. The rapid development of modern fractionation technologies, based on membrane separation and chromatography, has helped to isolate immunoglobulins (Igs) from bovine colostrum and milk on a large scale (Kothe et al. 1987; Abraham 1988; Stott and Lucas 1989; Korhonen et al. 1998b). Basically, the methods

for development of Ig-based preparations are based on either the concentration or isolation of Igs occurring naturally in colostrum or milk, or the hyperimmunization of pregnant cows during the "dry" period with antigens from pathogens in order to raise specific antibodies in the colostrum and milk. Immunization of cows with high doses of specific microbial antigens results in increased amounts of specific antibodies in the mammary secretions (Korhonen et al. 2000). The repeated systemic inoculation of cows with an immunogen at the end of the lactation period and during the dry period (Linggood et al. 1990; Beck 1990; Stolle 1990) is the most common method.

The isolation and purification of Igs from colostrum or cheese whey is based on ultrafiltration (UF) or a combination of UF and chromatography (Korhonen et al. 2000). The inexpensive method for the commercial production of crude Ig preparations would be the combination of different membrane technologies. However, specific chromatographic techniques need to be applied for increased recovery rate of Igs from whey and to increase the Ig concentration of the final preparation. Al-Mashikhi and Nakai (1988) and Fukumoto et al. (1994a) have been successful in preparing IgG from ultrafiltration-treated whey using immobilized chelate chromatography. Akita and Li-Chan (1998) developed the most suitable immunoaffinity chromatography process using immobilized egg yolk antibodies for the isolation of bovine IgG subclasses IgG1 and IgG2.

ISOLATION AND ANALYSIS OF MILK OLIGOSACCHARIDES

Different methods are being developed to produce milk oligosaccharides: 1) production of human milk oligosaccharides by fermentation of genetically engineered bacteria, 2) concentration/fractionation technologies such as membrane filtration, and 3) expression of human milk oligosaccharides in transgenic animals (Mehra and Kelly 2006).

Tanaka and Matsumoto (1998) produced galactooligosaccharides (GOS) from lactose by enzymatic transgalactosylation using β-galactosidases, where enzymatic synthesis leads to the production of heterogeneous mixtures of GOS structures with varying chain lengths and linkages.

Pelletier et al. (2004) have described processes for production of sialyloligosaccharides in situ in waste streams from cheese processing and from other dairy sources using α-(2,3)-trans-sialidase enzymes.

Processes for large-scale production of human milk oligosaccharides by fermentation of genetically engineered bacteria have been developed. Two fucosyltransferase genes of *Helicobacter pylori* were engineered into *E. coli* cells to express fucosyltransferases for production of LeX oligosaccharides (Dumon et al. 2004).

Recently, Martinez-Ferez et al. (2006) described a method for isolation of goat milk oligosaccharide fraction using membrane filtration technology. They used a two-stage tangential ultrafiltration-nanofiltration of goat milk, with 50 and 1 kDa molecular mass cut-off membranes, respectively (Fig. 14.5). The membrane cut-off gives an approximate idea of the membrane pore size. When a membrane is said to have a cut-off of 50 kDa, it means that it rejects 90% of some standard (dextrans, polypeptides, etc.) with a molecular weight of 50 kDa tested by the manufacturer. The mode of operation consisted of two separated, consecutive continuous diafiltration steps. This configuration was selected since it allows the washing out of low size molecules through the membrane. The cumulated permeate from the first stage was collected and employed as initial retentate in the second one. For both stages, Milli-Q™ water at 30 °C was added to the feed tank at the same rate as the permeate flux, thus keeping feed volume constant during operation. The velocity of recirculation was set at 3.3 m/s in order to mitigate fouling effects. The transmembrane pressure was 90 kPa during the first stage and 375 kPa during the second one. The temperature was kept at 30 °C to avoid precipitation of α-lactalbumin and coagulation of caseins. In order to regenerate the membranes after operation, a cleaning procedure was performed consisting of an initial rinse with demineralized water, followed by recirculation of a 20 g/L sodium hydroxide containing 0.1 g/L sodium dodecyl sulphate solution for 30 minutes and a final rinse with demineralized water until neutrality. A virtually lactose- and salt-free product was obtained containing more than 80% of the original oligosaccharide content. The amounts of oligosaccharide and lactose obtained from goat, cow, sheep, and human milk are given in Table 14.1.

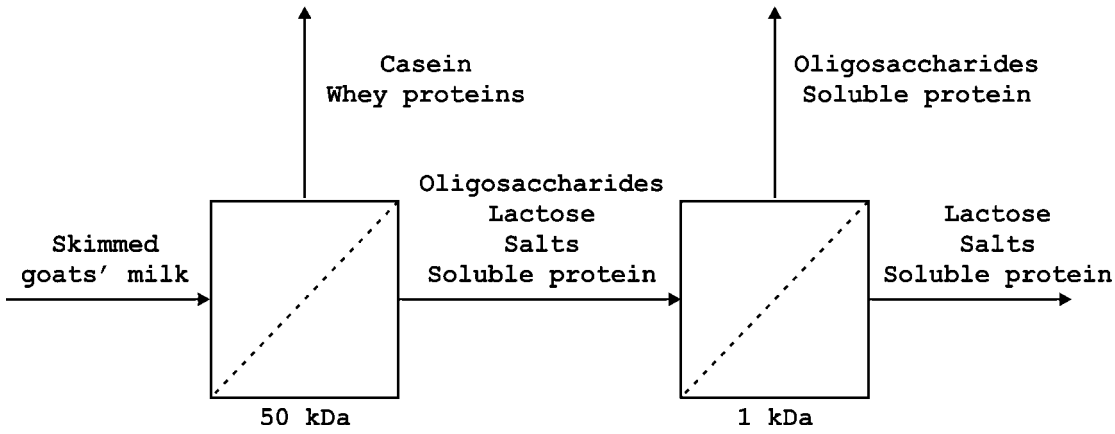

Figure 14.5. Scheme of the two-stage filtration process for the isolation of caprine milk oligosaccharides (Martinez-Ferez et al. 2006).

Table 14.1. Total amount of oligosaccharides and lactose in mature caprine, bovine, ovine, and human milk

Origin	Oligosaccharide (g/L)	Lactose (g/L)
Caprine milk	0.25–0.30	45
Bovine milk	0.03–0.06	46
Ovine milk	0.02–0.04	48
Human milk	5–8	68

Martinez-Ferez et al. 2006.

Several methods of quantifying milk oligosaccharides have been reviewed by Mehra and Kelly (2006). Kunz et al. (1996) reported a method to separate and characterize neutral and acidic lactose-derived oligosaccharides without prior derivatization or reduction using high-pH anion exchange chromatography, and pulsed amperometric detection (HPAEC-PAD). Thurl et al. (1996) used gel permeation chromatography and separated a crude milk oligosaccharide fraction into acidic oligosaccharides, neutral oligosaccharides, and lactose. After this separation step, neutral and acidic oligosaccharides were analyzed by HPAEC-PAD. The concentrations of 14 neutral oligosaccharides and 6 acidic oligosaccharides and N-acetylneuraminic acid were determined using the internal standards stachyose and galacturonic acid, respectively. Shen et al. (2001) developed a sensitive and highly reproducible method of high-performance capillary electrophoresis with UV detection by absorbance at 205 nm. This method requires only simple sample preparation and use of a regular UV detector. It is highly useful in defining variations in human milk acidic oligosaccharides. The technique has been reported to have detection sensitivity three orders of magnitude higher (lower limit of detection approximately 20–70 femtomoles) (Mehra and Kelly 2006).

Chaturvedi et al. (2001) employed reverse-phase high-performance liquid chromatography (RP-HPLC) to study the concentrations of individual neutral (fucosylated) oligosaccharides during human lactation. The neutral oligosaccharides from each sample were isolated, perbenzoylated, resolved, and quantified by RP-HPLC.

ISOLATION AND ANALYSIS OF NUCLEOTIDES

Nucleotide levels in milk have been analyzed by different authors principally using HPLC as the analytical technique. Janas and Picciano (1982) initiated the use of this technique for the analysis of nucleotides in human milk. Sugawara et al. (1995) quantified three nucleosides and six nucleotides. Finally, Perrin et al. (2001) quantified five nucleotides and five nucleosides in formula milk. These results are summarized in Table 14.2. A simple, reliable, effective and fast method using acid hydrolysis and quan-

Table 14.2. Values found in the literature on the analysis of nucleotides in milk

Nucleotides	Authors	Samples	Analysis	Concentration
5′-UMP	Janas and Picciano 1982	Breast milk	HPLC	321 µg/100 mL
	Sugawara et al. 1995	Breast milk	HPLC	0.23 µmol/100 mL
	Perrin et al. 2001	Starter formula	HPLC	63 mg/kg
5′-AMP	Janas and Picciano 1982	Breast milk	HPLC	143 µg/100 mL
	Sugawara et al. 1995	Breast milk	HPLC	0.23 µmol/100 mL
5′-GMP	Perrin et al. 2001	Starter formula	HPLC	21 mg/kg
	Janas and Picciano 1982	Breast milk	HPLC	163 µg/100 mL
	Sugawara et al. 1995	Breast milk	HPLC	n.d
	Perrin et al. 2001	Starter formula	HPLC	11 mg/kg
5′-CMP	Janas and Picciano 1982	Breast milk	HPLC	321 µg/100 mL
	Sugawara et al. 1995	Breast milk	HPLC	4.29 µmol/100 mL
	Perrin et al. 2001	Starter formula	HPLC	108 mg/kg
5′-IMP	Janas and Picciano 1982	Breast milk	HPLC	290 µg/100 mL
	Sugawara et al. 1995	Breast milk	HPLC	n.d.

5′-UMP = Uridine 5′monophosphate; 5′-AMP = Adenosine 5′monophosphate; 5′-GMP = Guanosine 5′monophosphate; 5′-CMP = Cytidine 5′monophosphate; 5′-IMP = Inosine 5′monophosphate.
Cubero et al. 2007.

tification by capillary electrophoresis (CE) was developed by Cubero et al. (2007) for the routine electrophoretic determination of nucleotides in breast milk. Breast milk samples of 1 month lactation were collected from healthy mothers (ages 25–35 years) and stored at −20 °C. The duplicated samples were dissociated by acidic hydrolysis ($HClO_4$) and the CE assay was performed in an uncoated fused-silica capillary (75 µm i.d. × 375 µm o.d.; Polymicro Technologies, LLC, USA) using an alkaline (borate) electrophoretic separation system. It benefits from the important advantages of capillary electrophoresis such as the small demand on sample size, simplicity of operation, low solvent consumption, and short analysis time. The method gave good recoveries of 5′-mononucleotides. Under the conditions used, the actual CE analysis time was less than 20 minutes. The physiologically and nutritionally important nucleotides were detected at concentrations of 387 µg/100 mL for UMP-5P, 385.3 µg/100 mL for AMP-5P, 67 µg/100 mL for CMP-5P, 172 µg/100 mL for TMP-5P and 315 µg/100 mL for GMP-5P.

CONCLUSIONS

The need to increase the utilization of milk can be met partly by isolating and purifying its bioactive components that have undergone lots of research to prove their beneficial bioactivities. This need will necessarily depend on the type of isolation and purifying technology used. Isolation technologies provide a wide range of value-added milk protein products. It is possible to modify the protein conformations with the different processing conditions, thereby tailoring milk proteins to have different functionalities.

In the future, more milk bioactive components will become available as new and cost-effective purification technologies are developed. As the nutritional value of the many milk components are identified, the focus on fractionation technologies will grow.

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15
Potential for Improving Health: Immunomodulation by Dairy Ingredients

Tadao Saito

INTRODUCTION

The animal's immune system is composed of two interrelated components: *humoral immunity* composed of soluble protective components and *cellular immunity* composed of leukocytes. Humoral-mediated immunity of the mammary gland consists primarily of antibodies in the form of immunoglobulins (Ig). Milk Ig plays an important role in immune protection of the mammary gland and the neonate (Butler 1974).

Milk contains a wide variety of factors that contribute to the protection of the newborn and the mammary gland from several diseases. Antibodies as Ig are a very important component of the disease-resistance function of mammary secretions until its own immune system becomes mature. Most major organic components found in milk (except Igs or serum albumin) are biologically synthesized by the mammary epithelial cells. The concentration and variety of Igs in colostrum against pathogens can be raised by immunizing cows with pathogens or their antigens. Although the immune system also includes the complement system, the complement is particularly low in milk and complement-Ig interactions are not expected to be significant in mammary secretions (Hurley 2003).

This chapter discusses antiinfectional and antiinflammable components such as milk proteins and milk oligosaccharides, including Igs found in milk, in the context of their diversity of origin, structure, transfer, and function.

MILK PROTEINS

Bovine milk proteins are an essential source of amino acids for neonates. They are also a source of biologically active peptides with antimicrobial, opioid, mineral-binding, antihypertensive, antithrombotic, and immunomodulating activities. Whey proteins and peptides derived from the enzymatic proteolysis of casein and whey proteins are known to modulate a variety of immune functions, including lymphocyte activation and proliferation, cytokine secretion, antibody production, phagocytic activity, and granulocyte and natural killer (NK) cell activity (Gauthier et al. 2006).

T-helper lymphocyte activation and proliferation are key elements of both the humoral and cellular immune responses against pathogens. These cells are divided into Th1- and Th2-lymphocytes, based on their cytokine profiles. Interferon-gamma (IFN-γ), tumor necrosis factor (TNF), and interleukin-2 (IL-2) produced by Th1-like lymphocytes contribute to cell-mediated immunity. Th2-like lymphocytes contribute to humoral immunity by secreting cytokines such as IL-4, 5, 6, and 10 (Janeway et al. 1999).

Recently, some whey protein isolates (WPIs), their enzymatic digests, and peptides prepared from the enzymatic digests were reported to stimulate the proliferation of murine-resting and Concanavalin A-stimulated splenocytes in vitro (Mercier et al. 2004; Saint-Sauveur et al. 2008).

General food allergies occur in about 5–10% of the infant and small-child populations. Cow's milk

protein allergy (CMPA) is the most common allergy in young human infants, with an incidence of 2–6%. The atopic disease is associated with a broad spectrum of IgE-mediated reactions, which are mostly expressed as immediate symptoms, such as urticaria, rhinoconjunctivitis, asthma, vomiting, and diarrhea. Cow milk proteins are recognized by the immune system of some newborn infants as foreign proteins, thus causing allergic reactions. Several studies demonstrated that most children with milk protein allergy synthesize antibodies principally against α-casein and β-lactoglobulin. The casein:whey protein ratio in native cow's milk is 80 : 20. Lara-Villoslada et al. (2005) pointed out that the balance between caseins and whey proteins in cow's milk may determine its allergenicity, and a reduction of α-casein might result in a reduction of allergenicity.

Immunoglobulin (Ig)

One of the major functions of the immune system is the production of soluble proteins, which circulate freely and exhibit properties that contribute specifically to immunity and protection against foreign material. These soluble proteins are the antibodies, which are a class of proteins called *immunoglobulins*. Recently, Hurley (2003) reported in detail immunoglobulins in mammary secretions.

Concentration of Immunoglobulins in Milk

About 20% of the total protein of bovine milk belongs to a group of proteins generally referred to as *whey* or *serum proteins*. In dairy industry companies, the whey proteins are frequently called *lactalbumin*. Whey proteins are prepared from skimmed milk by addition of appropriate acid (such as HCl, lactic acid, etc.) to pH 4.6 at 20 °C. Acid whey contains the proteose-peptone (PP) components. Igs are precipitated along with the caseins by saturated NaCl. Rennet whey contains the caseinoglycopeptides (CGP, glycomacropeptide [GMP]) produced from κ-casein by chymosin (rennet) reaction and small amounts of casein. Small casein micelles remain in the ultracentrifugal (UF) serum, especially if Ca^{2+} are not added.

Whey contains two well-defined groups of proteins: lactalbumins, which are soluble in 50% saturated $(NH_4)_2SO_4$ or saturated $MgSO_4$, and lactoglobulins, which are salted-out under these conditions. The lactoglobulin fraction also contains mainly immunoglobulins (Igs). Table 15.1 shows the protein composition of mature bovine milk (Swaisgood 1993; Tremblay et al. 2003). The concentrations of each protein component represent averages of values calculated from the literature. The content of immunoglobulins in normal bovine milk is about 0.8 g/L.

Mature bovine milk contains about 0.6–1.0 g Ig/L, and colostrum contains up to 10% Ig. The concentration level of Ig decreases rapidly after parturition. Ig in milk can be divided into five classes or isotypes: IgG, IgA, IgM, IgE, and IgD. Three subclasses of Ig are also present: IgG_1, IgG_2, and IgG_3 Bovine milk contains four kinds of Ig components: IgG_1 (59 mg/dl), IgG_2 (20 mg/dL), IgA (10 mg/dL) and IgM (5 mg/dL) (Hurley 2003; Larson 1992). Colostrum is an extremely rich source of Ig, but all Igs decrease within a few days to a total Ig concentration of 0.7–10 mg/mL, with IgG_1 representing the major Ig class in milk throughout the lactation period.

Bovine IgG_1 is the most abundant antibody in bovine colostrum and transported from the plasma of the cow to the colostrum via an active transport mechanism, mainly during the last 3 weeks before parturition. More than 90% is IgG_1 in the colostrum, although blood contains almost equal amounts of IgG_1 and IgG_2 (Hurley 2003). This fact suggests that bovine IgG_1 plays a physiologically important role

Table 15.1. Protein composition of mature bovine milk

Protein (Abbreviation Name)	g/kg of Milk[1]
α_{s1}-Casein (α_{s1}-Cn)	11.5
α_{s2}-Casein (α_{s2}-Cn)	3.0
β-Casein (β-Cn)	9.5
κ-Casein (κ-Cn)	3.4
γ-Casein (γ-Cn)	1.2
Total casein	28.6
α-Lactalbumin (α-La)	1.2
β-Lactoglobulin (β-Lg)	3.1
Serum albumin (BSA)	0.4
Immunoglobulin (Ig)	0.8
Proteose-peptones (PP)	1.0
Whey protein	6.5
Total protein	35.1

[1]The density of milk is 1.03 g/mL (Tremblay et al. 2003; Swaisgood 1993).

in calf intestinal tracts, in addition to protecting the host against pathogenic organisms. As the placental IgG transport in cows is markedly less efficient than that in humans, passive immunization through colostrum and milk postpartum is extremely important for calves.

Molecular Structure of Immunoglobulin

Figure 15.1 shows the whole structure of an intact immunoglobulin (Ig) and its enzymatic hydrolyzing point by papain digestion (Benjamini et al. 2000).

All monomeric Ig molecules consist of a similar basic structure composed of four subunit polypeptides, including two identical heavy (large) chains and two identical light (small) chains, with a total molecular mass of approximately 150–170 kDa. Both heavy and light chains are composed of domains referred to as *variable* (V_H, V_L) and *constant* ($C_{H1–3}$, C_L) regions. Disulfide (SS) bonds link each heavy and light chain pair, as well as linking the two heavy chains, resulting in a Y-shaped molecule with two antigen-binding sites. The number and position of SS bonds linking heavy chains are known to vary with the isotype of Ig. Igs are glycoproteins with sugar chains linked to the constant C_{H2} regions of the heavy chains. The N-terminal portion of the Ig molecule is the antigen binding region. Antigen binding occurs through interactions of the antigen with the variable regions (V_H and V_L) of heavy and light chains.

Proteolytic treatment with protease:papain splits Ig molecules (MW 150 kDa) into three fragments of about equal size of molecular weight. Digestion of the IgG molecule with papain hydrolyzes the heavy chain at the hinge region (arrows in Figure 15.1) and

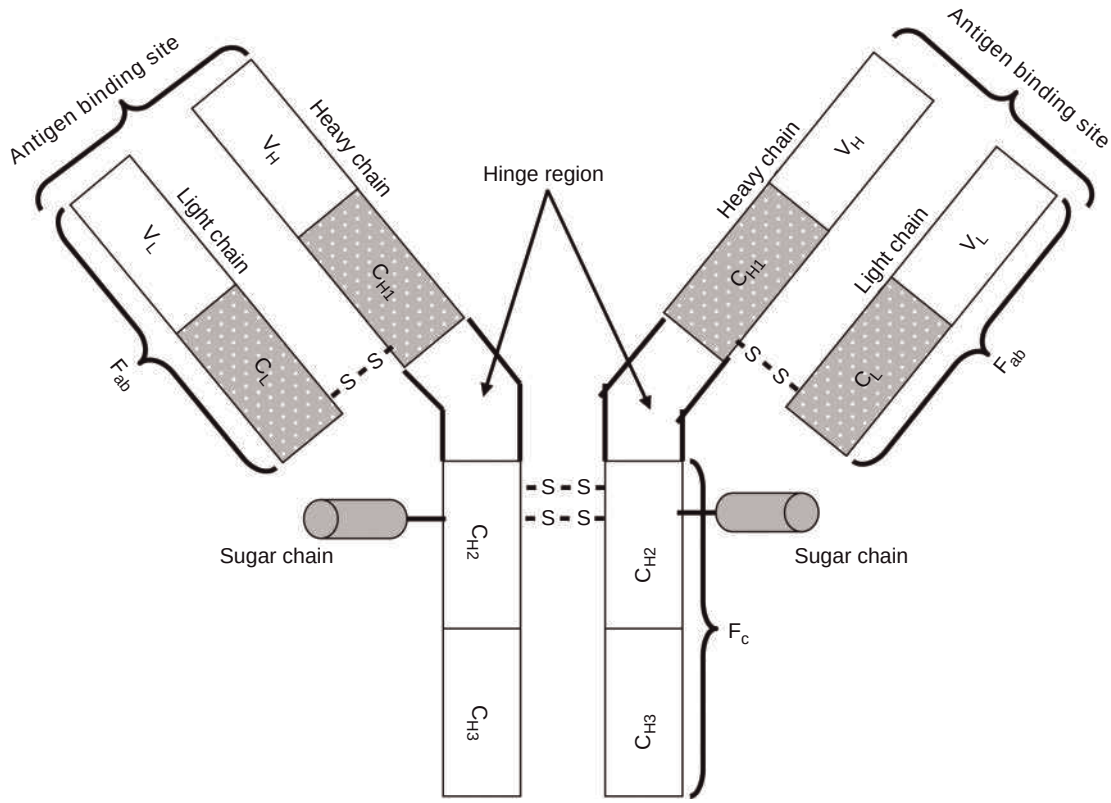

Figure 15.1. Chemical structure of immunoglobulin (Ig) molecule. V = variable region, C = constant region, L = light chain, H = heavy chain. Subscripts 1, 2, and 3 refer to the three constant regions of the heavy chains. Fab = antigen-specific portion of the Ig molecule, Fc = the cell-binding effector portion of the Ig molecule.

releases two identical antigen binding fragments and the constant portion of the molecule. Two of these fragments were found to retain the antibody's ability to bind antigen specifically, but they could no longer precipitate the antigen from solution. These two fragments are named *Fab* (fragment antigen binding) and are considered to be univalent, possessing one binding site each and being in every way identical to each other. The Fab consists of V_H and C_{H1} domains of the heavy chain and V_L and C_L domains of the light chain (either kappa or gamma) (Benjamini et al. 2000).

The third fragment could be crystallized out of solution, a property indicative of its apparent homogeneity. This fragment is called *Fc* (fragment-crystallizable). The Fc fragment cannot bind antigen, but is responsible for the biologic functions of the antibody molecule after antigen has been bound to the Fab part of the intact molecule.

The Fc portion of the antibody contains the portion responsible for many of the biological activities of the antibody molecule, including complement activation, recognition by Fc-receptors on leukocytes and epithelial cells, transport through epithelial cells, and recognition by bacterial Ig-binding proteins (Benjamini et al. 2000).

IgA consists of two such units (i.e., eight chains) linked together by secretory components (SC) and a junction component (J). IgM consists of linked four-chain units. The heavy and light chains are specific to each type of Ig. The physiological function of Ig is to provide various types of immunity (Sell and Max 2001).

Immunoglobulins in Mammary Secretions

Igs are not evenly distributed among fractions of milk. Ultracentrifugation (UF) of milk results in preferential association of IgM and IgA with the fat fraction and association of IgM and IgG_2 with the casein pellet. However, the major Ig portions for IgG_1, IgG_2, IgA, and IgM are found in the whey fraction (Frenyo et al. 1986). Some Igs are associated with the cell pellet in fractionated milk. Table 15.2 shows the concentration of Ig in blood, colostrum, and milk of cows and humans. IgG is the predominant Ig in blood. The predominant colostral Ig depends on the species and particularly on the route of transfer of passive immunity from mother to offspring. Concentrations of IgG are greatest in the colostrum of ruminants and are the major isotype in bovine milk. Estimates of concentrations of Ig in colostrum and milk are quite variable and can be affected by parity, genetics, and stage of lactation (Butler 1974, 1981; Devery-Pocius and Larson 1983; Hurley 2003).

Immunoglobulin G is found only in the monomeric form in blood or milk, whereas IgA and IgM

Table 15.2. Concentration of immunoglobulins and percentage of the major components in serum and mammary secretions in cow and human normal milk

Animal Species	Immunoglobulin	Concentration (mg/mL)			% Major Component Ig		
		Blood Serum	Colostrum	Milk	Blood Serum	Colostrum	Milk
Cow	IgG (total)	25.0	32–212	0.72	88	85	66
	IgG1	14.0	20–200	0.6			
	IgG2	11.0	12.0	0.12			
	IgA	0.4	3.5	0.13			
	IgM	3.1	8.7	0.04			
	FSC		0.5	0.2			
Human	IgG	12.1	0.43	0.04	78		
	IgA	2.5	17.35	1.00		90	87
	IgM	0.93	1.59	0.10			
	FSC		2.09	0.02			

Data compiled and calculated from cow (Butler 1981, 1983; Devery-Pocius and Larson 1983) and human (Butler 1974).

Data partially quoted from Table 9.2 in *Advanced Dairy Chemistry* Vol.1 (Hurley 2003).

Cow data from Holstein Friesian cow.

are present in polymeric forms in both blood and milk. Most serum IgA is monomeric, and most IgA in external secretions is di- or tetrameric IgA and contains the J chain, which links monomers together near the C-terminal of the heavy chains. The mass of dimeric IgA, including the J chain, is approximately 370 kDa (Brandtzaeg 1985).

Serum and milk IgM are complex molecules composed of five monomers of IgM linked by disulfide bonds and containing one J chain. The complex has a molecular mass of approximately 1000 kDa. The pentameric structure of IgM gives each molecule 10 antigen-binding sites. Transport of IgM through epithelial cells occurs via the same pIgR mechanism as secretory IgA, and much of secretory IgM in milk is associated with SC (Hurley 2003).

The primary Ig isotype in human colostrum and milk is IgA, consisting of 90% secretory immunoglobulin A (sIgA), and provides passive immunity based on previous maternal exposure to microbial pathogens. As IgA combines with high concentration of lactoferrin and a high activity of lysozyme, human milk shows a particularly high antimicrobial activity. The sIgA, which is resistant to digestion, enters the digestive tract of the infant and binds to enteric pathogens inhibiting their ability to produce infection (Hurley 2003).

Cell-Mediated Immunity

Mammary gland and milk leukocytes play important roles in mammary immunobiology and in immunity of the neonate (Newby et al. 1982). Leukocyte concentration in mammary secretions varies considerably with species, stage of lactation, and physiological and pathological states of the mammary gland. Somatic cell count in milk from bovine mammary glands with bacterial infections can increase rapidly within a few hours of infection (Harmon et al. 1976). In the dairy industry, milk leukocyte concentration (somatic cell count) has been the basis for recognizing and quantifying mammary gland inflammatory responses such as mastitis. Milk contains leukocytes primarily consisting of neutrophils, macrophages, and lymphocytes and also a small percentage of epithelial cells.

Neutrophils generally do not have a role in cellular immunity of the mammary gland but offer a phagocytic defense to infection. Milk macrophages are phagocytic and can ingest bacterial cells, milk components, and cellular debris. Phagocytosis by macrophages is increased by opsonization of antigens with specific antibodies. Macrophages play an important role in cellular-mediated immunity through their antigen processing in association with MHC-II (major histocompatibility complex) antigens and presentation functions (Sordillo et al. 1997). Specific immunity arises from the interaction of antigen-presenting cells.

Ohnuki et al. (2006) reported that mice were bred with diets consisting of ovalbumin alone (OVA, control diet) or mixtures of OVA and bovine milk IgG (IgG-added diets) and both the cellular and humoral immune properties of the mice were investigated. The number of IL-12$^+$CD11b$^+$ cells in spleens and the formation of superoxide by peritoneal macrophages were higher in mice given the IgG-added diet than in those given the control diet. In contrast, the numbers of interferon-γ^+CD4$^+$ and IL-4$^+$CD4$^+$ cells in Peyer's patches or spleens and the levels of total or OVA-specific intestinal IgA and serum IgG were significantly lower in mice given the IgG-added diet. They indicated that the oral ingestion of bovine milk IgG might stimulate some innate cellular immune systems, while suppressing humoral adaptive immune responses in mice.

Mizutani et al. (2007) reported that intact IgG_1 prepared from bovine colostrum and its digests by several proteases (except for peptic) significantly stimulated IgA formation, while intact IgG_1 and its digests (especially tryptic) enhanced IgG formation. Intact IgG_1 and both tryptic and cymotryptic digests noticeably increased mRNA expressions of cytokines secreted by type 2 helper T (Th2) cells such as IL-4, 5, and 6. These results suggest that intact IgG_1 and its gastrointestinal proteolytic digests may stimulate the production of IgA via the modulation of cell numbers and/or functions of B and Th2 cells.

Several researchers have reported that bovine milk IgG specific to intestinal microorganisms protects animals including humans against intestinal infections. Ohnuki and Otani (2007) reported that bovine milk IgG stimulated antibody responses in mouse spleen cell culture, whereas oral ingestion of bovine milk IgG suppressed the response in mice. It is unclear why bovine milk IgG has different effects on antibody responses in vitro and in vivo. Immunocompetent cells such as dendritic cells and macrophages possess several types of IgG receptors

(FcγR) on their surface. FcγRI and FcγRIII stimulated the formation of Ig when IgG binds to the receptor, whereas FcγRIIb inhibits it. Also, FcγRI may strongly interact with monomeric IgG, while FcγRIIb may interact little with antigen-free igG. Therefore, the different effects of bovine milk IgG on antibody responses in vitro and in vivo may be due to the difference in FcγR for milk IgG on immunocompetent cells (Ohnuki and Otani 2007).

LACTOFERRIN (LF)

Lactoferrin (Lf) is an iron binding glycoprotein, with the red color of the transferrin family, that is expressed in most biological fluids including milk and is a major component of the mammal's innate immune system (Lönnerdal 2003). Its protective effect ranges from direct antimicrobial activities against a large range of microorganisms, including bacteria, viruses, fungi, and parasites, to antiinflammatory and anticancer activities (Legrand et al. 2008).

Lf was first identified in bovine milk by Sørensen and Sørensen (1939) and subsequently isolated from human milk by Johanson (1960) and characterized by Montreuil et al. (1960). The red-colored Lf has also been called *lactotransferrin*, and its mechanisms of action not only have the ability to bind iron but also to interact with molecular and cellular components of both host and pathogens. Besides its antimicrobial activities, immunomodulatory properties were also reported. Lf is secreted in the apo-form from epithelial cells in milk (Montreuil et al. 1960). Lf is mainly synthesized by glandular epithelial cells, and its concentration in milk varies widely among animal species. The contents of Lf in human, pig, and mice milk are rich, but in other species such as cows and other ruminants Lf is generally low. In mature bovine milk, Lf is about 30 mg/L, whereas its concentration in human milk varies from 1 g/L (mature milk) to 7 g/L (colostrum).

Lf is a single-chain protein with a molecular mass of ca. 80 kDa. The cDNA and amino acids of bovine Lf have been reported by Mead and Tweedie (1990). The mature protein consists of 689 amino acids and has a 19-amino-acid signal peptide. The sequence of human Lf has been determined by both amino acid (Metz-Boutigue et al. 1984) and nucleotide (Rey et al. 1990) sequencing. The Lf gene appears in mammals and is highly conserved among species, with an identical organization (17 exons with 15 encoding Lf) and conserved codon interruptions at the intron-exon splice junctions (Teng et al. 2002).

Lf contains intramolecular disulfide (SS) bonds but no free sulphydryl (SH) groups. Lf has a high isoelectric point of pH 8.7, and has a tendency to associate with other molecules due to charge interactions. Lf consists of two globular lobes, which are linked by an extended α-helix, and the two domains have a similar amino acid sequence. Each lobe contains one iron-binding site and one sugar chain. However, the conformations of both lobes are different, and their affinity for iron is slightly different (Anderson et al. 1989).

All Lfs contain biantennary N-acetyllactosamine-type sugar chains, α,1–6 fucosylated on the N-acetylglucosamine residue linked to the polypeptide chain (Spik et al. 1988). Bovine Lf is characterized by having α-1,3-linked galactose residues at the terminal nonreducing position of the sugar chain. Human Lf also possesses additional poly-N-acetyllactosamine antennas, which may be α-1-3-fucosylated on N-acetylglucosamine residues, whereas the Lf of other species contains additional high-mannose-type glycans (Coddeville et al. 1992). Both the number and location of the glycosylation sites vary among species. The role of the glycan moiety seems to be restricted to a decrease in the immunogenicity of the protein and its protection from proteolysis (van Veen et al. 2004).

Lf is a potent modulator of inflammatory and immune responses, revealing host-protective effects not only against microbial infections but also in inflammatory disorders such as allergies, arthritis, and cancer. The up- or downregulating effects of Lf are related to its ability to interact with pro-inflammatory bacterial components, mainly LPS, or specific cellular receptors on a wide range of epithelial and immune cells. This results in the modulation of the production of various cytokines and of the recruitment of immune cells at the infected sites (Legrand et al. 2008).

Cell migration is critical for a variety of biological processes. Recently, Yamauchi et al. (2006) reported that bovine Lf reduces the number of infiltrating leukocytes during influenza virus infection (pneumonia) and suppresses the hyperreaction of the host. Lf decreases the recruitment of eosinophils, reduces pollen antigen-induced allergic airway inflammation

in a murine model of asthma (Kruzel et al. 2006), and reduces migration of Langherans cells in cutaneous inflammation. Finally, Lf can modulate fibroblast motility by regulating MMP-1 (matrix metalloproteinase) gene expression, involved in the extracellular matrix turnover and the promotion of cell migration (Oh et al. 2001).

Lf displays immunological properties influencing both innate and acquired immunities. Especially, oral administration of bovine Lf seems to influence mucosal and systemic immune responses in mice (Sfeir et al. 2004). Recently, Chodaczek et al. (2006) demonstrated that a complex of Lf with monophosphoryl lipid A is an efficient adjuvant of the humoral and cellular immune responses. Its stimulating effect on the immune system concerns mainly the maturation and differentiation of T lymphocytes, the Th1/Th2 cytokine balance and the activation of phagocytes.

Lf can stimulate the differentiation of T cells from their immature precursors through the induction of CD4 antigen under nonpathogenic conditions (Dhennin-Duthille et al. 2000). Oral delivery of Lf significantly increased the number of $CD4^+$ cells in lymphoid tissues (Kuhara et al. 2000). Lf induces a Th1 polarization in diseases in which the ability to control infection or tumor relies on a strong response; however, it may also reduce Th1 cytokines to limit excessive inflammatory responses. Oral administration of Lf increased the splenocyte production of IFN-γ and IL-12 in response to herpes simplex virus type 1 infection (Wakabayashi et al. 2004). The proposed mechanism is that oral Lf induces IL-18 production in the small intestine, then increases the level of Th1 cells.

Lf secreted from neutrophil granules during infection can bind to PMN (polymorphonuclear) and monocytes or macrophages and promotes the secretion of inflammatory factors such as TNF-α (Sorimachi et al. 1997). Lf also can activate macrophages through TLR4-dependent and -independent signaling pathways and induces CD40 expression and IL-6 secretion (Curran et al. 2006). As demonstrated during the infection of bovine mammary glands by *Staphylococcus aureus*, the binding of Lf to macrophages also enhances the phagocytosis of pathogens (Kai et al. 2002). It has been suggested that Lf plays a regulatory role during cytokine responses. At concentrations lower than 10-8 M, it has been reported that Lf is an inhibitor of cytokine responses in vitro, suppressing the release of IL-1, IL-2, and TNF (tumor necrosis factor) from mixed lymphocyte cultures (Crouch et al. 1992).

LYSOZYME (LZ)

Lysozyme (Lz) is present in human milk at a higher concentration than in the milk from other species including cows. Lz plays an important role in the protection of newborns due to its bacteriolytic function by hydrolyzing the β-1,4 linkages between N-acetyl glucosamine (GlcNAc) and N-acetylmuramic acids (MurNAc) in peptidoglycan heteropolymers of the prokaryote cell walls. Lz is also called 1,4-β-N-acetylmuramidase (EC 3.2.1.17), peptidoglylcan N-acetylmuramoyl hydrolase. The concentration of Lz in mammalian milk varies from <0.3 mg/100 mL in bovine milk (Chandan et al. 1968) to about 79 mg/100 mL in equine milk (Jauregui-Adell 1975). Human milk contains Lz about 10–12 mg/100 mL (Jollès and Jollès 1967). In bovine milk, Lz activity increases with somatic cell count and mastitis. In association with lactoferrin, Lz functions as a bacteriocidal agent in milk. Lz from bovine and human milk shows lytic action toward *E. coli*. Therefore, premature infants fed precolostrum containing a high level of Lz may have a lower incidence of gastrointestinal invasion by pathogenic bacteria. Lz is one of the important antiinfection factors naturally existing in the milk mentioned above (Farkye 2003).

LACTOPEROXIDASE (LPO)

Lactoperoxidase (LPO, EC 1.11.1.7) occupies about 1% of whey proteins in bovine milk at 10–30 μg/mL milk. It is the most abundant enzyme in milk. The other components of the LPO system are hydrogen peroxide (H_2O_2), halide ions, and the thiocyanate ion (SCN^-). The enzyme catalyzes the conversion H_2O_2 to H_2O. In 1991, the amino acid sequence of bovine LPO was determined as 612 amino acid residues with M.W. 69,502 Da. The first direct evidence for the expression of the LPO gene within the secretory cells of the lactating mammary gland was published by Cals et al. (1994).

The most significant reactions for the bovine LPO system are those related to catalase activity and to thiocyanate oxidation. The specific reactions are complex and involve multiple intermediates. LPO is present at high concentrations in bovine milk. In the

presence of adequate concentrations of added H_2O_2 and SCN^-, the LPO system inhibits the growth and metabolism of many species of bacteria. The activated LPO system can be utilized to preserve raw milk at places where refrigeration is not possible. To activate the system, raw milk is supplemented with SCN^- and sodium percarbonate ($2Na_2CO_3 \times 3H_2O_2$) and has successfully elongated the shelf life of milk in several countries (Björck 1994).

β-LACTOGLOBULIN (β-LG)

Bovine milk allergy, most common in early childhood, is an IgE-mediated reaction to bovine milk protein such as β-lactoglobulin (β-Lg). β-Lg occupies around 12% of the total milk protein and around 50% of whey proteins. The molecule consists of 162 amino acid residues and MW is about 180 kDa. β-Lg has four kinds of genetic variants (A, B, C, D), and both A and B variants are major. Among constituent amino acids, β-Lg has five residues of cystein; four residues exist with a disulphide (S-S) bond, but one residue exists in a free thiol (SH) form. Because the protein is not in human milk, β-Lg can be the main reason for allergic protein with bovine milk intake. It is known that a β-Lg exists as monomer below pH 3.0, dimer at pH 5.5–7.5 and as octamer at pH 3.5–5.2 (Sawyer 2003). β-Lg is a very resistant protein to enzymic digestion, particularly to pepsin, which is attributed to its unique structural stability at acidic pH. At pH values around 2.0 (optimum for pepsin activity), β-Lg is dissociated into monomers, but its folding is similar to neutral pH. The structure of β-Lg is basically a β-barrel where most hydrophobic amino acids, targets for pepsin, point inward and are not easily accessible to the enzyme. To prevent allergic symptoms and to remove the allergenic epitopes, extensive hydrolysis of the protein and ultrafiltration is introduced. Recently, Chicón et al. (2008) reported that the application of high pressure at 400 MPa during pepsin treatment of β-Lg reduced the antigenicity and IgE binding of β-Lg.

Prioult et al. (2004) showed that PHA-stimulated splenocytes secreted more IFN-γ in the presence of an acidic peptides fraction isolated from a β-Lg hydrolysate, but the secretion of IL-4 and IL-10 was unaffected. IFN-γ regulates pathogen recognition by increasing the quantity and diversity of peptides presented on the cell surface in the context of class I MHC (major histocompatibility complex), which enhances the potential for cytotoxic T cell recognition of foreign peptides and thus promotes the induction of cell-mediated immunity.

Saint-Sauveur et al. (2008) reported that enzymic digests of whey protein isolates (WPI) stimulated the proliferation of splenocytes in the presence and absence of ConA and significantly stimulated the secretion of IL-2 and IFN-γ; moreover, acidic or neutral peptide fractions stimulated splenocyte proliferation and cytokine secretion most.

CASEINS

Casein Phosphopeptide (CPP)

Casein phosphopeptides are phosphorylated peptides that can be prepared by protease digestion of calcium-sensitive caseins such as α_{s1}-casein. They possess a phosphoserine (SerP)-rich region, consisting of SerP residues, such as α_{s1}-casein Glu^{63}-SerP-Ile-SerP-SerP-SerP-Glu-Glu^{70} or β-casein Glu^{14}-SerP-Leu-SerP-SerP-SerP-Glu-Glu^{21}. The property of CPP allows them to form complexes with calcium ions or other mineral ions. For this reason, the effect of CPP in preventing precipitates and increasing absorption of calcium in the intestine was demonstrated both in vitro and in vivo. The usage of CPP has become widespread to promote remineralization for preventing osteoporosis, anemia, and caries.

Some CPP are known to have immunoenhancing activities. Hata et al. (1999) reported that CPP-III (β-casein f1–28 and α_{s2}-casein f1–32) stimulated proliferation of mouse spleen cells. The compounds also showed enhanced intestinal IgA levels by promoting the production of IL-6 using oral administration of the CPP-III-supplemented diet in mice. The critical role of β-casein f1–28 in IgA production has become clear, and the special sequence SerP-X-SerP was revealed to be essential for these immunoenhancing activities (Otani et al. 2001).

Recently, Tobita et al. (2006) reported that β-casein f1–28 of CPP-III stimulates both proliferation and IL-6 expression of mouse $CD19^+$ cells via Toll-like receptor 4 (TLR-4). When the spleen lymphocyte subset ($CD4^+$, $CD8^+$, and $CD19^+$ cells) from C3H/HeN mice was cultured with β-casein f1–28, it exerted a dose-dependent mitogenic effect on $CD19^+$ cells. The effect was significantly inhibited by treating with neutralizing antibodies for TLR-4. Reverse

transcription-polymerase chain reaction (RT-PCR) analysis showed that β-casein f1–28 exerted an IL-6–enhancing effect on $CD19^+$ cells. The authors reported that some phosphorylated exocellular polysaccharides (EPS) produced by lactic acid bacteria (LAB) are known to have mitogenic activity to mouse spleen B cells, which were the primary population of the $CD19^+$ cells (Kitazawa et al. 1998; Sato et al. 2004). Although the relationship between these phosphorylated mitogenic structures and TLR is still unclear, the phosphoserine residues of β-casein f1–28 may act similarly to the phosphate groups in these phosphorylated mitogens such as EPS.

CPP are now sanctioned in Japan as a food ingredient for specified health uses due to their function of increasing the bioavailability of calcium.

Caseinoglycopeptide (CGP)

κ-Casein is a unique casein component that contains only carbohydrate chains consisting of galactose, N-acetylgalactosamine, and N-acetylneuraminic acid. κ-Casein has about five sugar-chain binding sites in its C-terminus area. The chemical structures of the sugar chains of bovine κ-casein isolated from normal milk and colostrum were clarified by the author's research group (Saito et al. 1980, 1981, 1982; Saito and Itoh 1992). Chymosin (EC 3.4.23.4), which is an aspartic acid protease produced by the cow, cleaves the peptide bond between Met^{105} and Phe^{106} residues in κ-casein, resulting in the separation of para-κ-casein (f1–105) and caseinoglycopeptide (CGP, f106–169, GMP). CGP containing sugar moieties have the same peptide chain, but variable sugar and phosphorus contents. Several biological and physiological functions have been reported, such as opioid effects, calcium absorption–promoting effect, immunoactivating effect, inhibition of angiotensin-converting enzyme (ACE), and bifidus factors.

CGP was prepared from sweet cheese whey during processing of ripened cheese by using several techniques, such as ion-exchange chromatography (Saito et al. 1991) or ultrafiltration (UF) treatment. CGP has been found to inhibit adhesion of S*treptococcus* (*Str.*) *sobrinus*, *Str, sanguis, Str. mutans* and *Actinomyces viscosus* to erythrocytes and polystyrene surfaces (Neeser et al. 1988). It has also been shown to inhibit binding of cholera toxin to Chinese Hamster ovary cells at concentrations as low as 20 ppm (Kawasaki et al. 1992). *Vibrio cholerae* produce a potent enterotoxin (cholera toxin, CT) that is responsible for profuse watery diarrhea. CT is composed of one A subunit that is surrounded by five B subunits. The B subunits are responsible for binding the toxin molecule to ganglioside G_{M1}, which is present on mucosal enterocytes. Since CGP has a similar sugar structure to that of G_{M1}, it has been reported to exhibit the ability to bind CT and inhibit the toxin.

Similar activity was also observed with binding of *E. coli* heat-labile enterotoxins LT-1 and LT-II to Chinese Hamster ovary cells. Brück et al. (2003) reported that CGP reduced *E. coli*–induced diarrhea in infant rhesus monkeys. The postulated mechanism of effect was an antiadhesive capacity of the CGP to *E. coli*. Recently, Rhoades et al. (2005) reported that CGP reduced adhesion of verotoxigenic *E. coli* O157:H7 (VTEC) strains to <50% for the control to HT29 human colon adenocarcinoma epithelial cells and also reduced enteropathogenic *E. coli* (EPEC), *L. pentosus*, and *L. casei*.

CGP showed the ability to bind to *Salmonella enteritidis* and enterohemorrhagic *E. coli* O157:H7. The binding ability was decreased by a sialidase treatment and completely eliminated by periodate oxidation. In the case of Salmonella infection, CGP with xylooligosaccharide and CGP with carboxymethyldextran significantly suppressed IL-8 production, which was the index of infection. Nakajima et al. (2005) indicated CGP to be a promising agent for preventing intestinal infection.

MILK OLIGOSACCHARIDES (MO)

Human (breast) milk is a rich source of complex oligosaccharides synthesized within the mammary gland. The concentration of human milk oligosaccharide (HMO) varies widely due to the lactational stage decreasing from high amounts in colostrum (50 g/L) to an average of 10–15 g/L in mature milk (Kunz et al. 2000). Recently, Kunz and Rudloff (2008) discussed potential antiinflammatory and antiinfectious effects on HMO in detail.

Until now, more than 100 oligosaccharides have been reported in human milk. In neutral HMO, the major components are lacto-N-tetraose (LNT, Galβ1-3GlcNAcβ1-3Galβ1-4Glc, 0.5–1.5 g/L), and their monofucosylated derivatives, lacto-N-fucopentaose I (LNFPI: [Fucα1-2]Galβ1-3GlcNAcβ1-

3Galβ1-4Glc) or lacto-N-fucopentaose II (LNFPII: Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc, up to 1.7 g/L). The neutral tetrasaccharide LNT and two monofucosylated pentasaccharides are existing up to 50–70% of the total complex HOM (Kunz and Rudloff 2008).

In the HMO including sialic acid (sialylated sugars, acidic HMO), which contains only N-acetyl neuraminic acid (NeuAc, Neu5Ac or NANA), the content of 3′-sialyllactose (NeuAcα2-3Galβ1-4Glc) as a major acidic trisaccharide is about 1.0 g/L followed by isomers of three monosialylated lacto-N-tetraose derivatives [LS-tetrasaccharide a (LSTa): Galβ1-3[NeuAcα2-3]GlcNAcβ1-3Galβ1-4Glc; LS-tetrasaccharide b (LSTb): Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc and LS-tetrasaccharide c (LSTc): Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc] and disialylated lacto-N-tetraose [disialyl-lacto-N-tetraose (DSLNT): [NeuAcα2-3] Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc] (Kunz and Rudloff 2008).

The presence of different HMO in human milk depends on the activity of specific enzymes in the lactating gland. An α1-2-fucosyltransferase is expressed in about 77% of all Caucasians who are classified as secretors. Therefore, HMO from these women is characterized by the presence of α1-2-fucosylated components, such as 2′-fucosyllactose (Fucα1-2Galβ1-4Glc), lacto-N-fucopentaose I (LNFP-I) and more complex MO. In Lewis (a+b–) individuals, another fucosyltransferase combines Fuc residues in α1-4 linkages to a subterminal GlcNAc residue of type I (GlcNAcβ1-3Galβ1-) chains. In Lewis (a–b+) donors, who represent about 70% of the population, both fucosyltransferases (the secretor gene and the Lewis gene-dependent form) are expressed. Whether the obvious difference has an impact on infants' health, either immediately or in later life, is not well known.

In bovine colostrum, small amounts of free sugars are detectable, with 3′-sialyllactose (acidic trisaccharide) as the major components of bovine milk oligosaccharide (BMO, about 853 mg/L). Until now, the existence of 9 neutral MO and 11 acidic MO was reported, including the author's analysis (Saito et al. 1984, 1987). Although only N-acetylneuraminic acid is detected in HMO as sialyl derivative, both N-acetyl and N-glycolyl neuraminic acid are contained in BMO.

Besides the biological activity of HMO as growth factors for *Bifidobacterium*, antiinfection ability was known in some oligosaccharides of HMO. In human milk, MO structures are present and prevent the adhesion of certain microorganisms by acting as "soluble receptor analogs" due to a specific sugar sequence and linkages between those. The main factor of many infectious diseases such as diarrhea seems to be ability of microorganisms to adhere to the mucosal surface and later colonization and invasion in the case of *E. coli*, *H. jejuni, Shigella* strains, *Vibrio cholerae,* or *Salmonella* species. In many in vitro studies, MO can interfere with these specific host pathogen interactions.

Mysore et al. (1999) reported that some MO have recently been tested as antiadhesive drugs in animals. *H. pylori*–positive rhesus monkeys were treated with 3′-sialyllactose alone or in combination antiulcer drugs. Because two monkeys were permanently cured, the authors showed that the therapy is safe and can cure or reduce *H. pylori* colonization in animals. Clinical experience with MO as antiadhesive and antiinfection drugs is still very limited because of a lack of production of large amounts of complex milk-type MO for clinical studies to take in vivo data.

Recent observations indicate that HMO not only influence systemic processes such as leukocyte endothelial cell or leukocyte platelet interactions (Bode et al. 2004), but they also induce intestinal cellular processes (Kunz et al. 2003). Therefore, HMO seems to affect the gut in two ways: as growth factors influencing normal gut development and maturation and as antiinflammatory and immune components modulating the intestinal immune system.

Human milk is a rich source of sugars with structural similarities to the binding determinants of selectin ligands. Excessive leukocyte infiltration causes severe tissue damage in several inflammatory diseases. The initial step leading to leukocyte extravasation is mediated by selectins on activated condothelium and their oligosaccharide ligands on leukocytes (Springer 1990). Because HMO contain binding determinants for selectins, they exert the potential to affect leukocyte rolling and adhesion to endothelial cells.

The adhesion of monocytes, lymphocytes, or neutrophils isolated from human peripheral blood passing over TNF-α–activated endothelial cells was

reduced by up to 50% using sialylated HMO (Bode et al. 2004). The effects were more pronounced than those achieved by soluble sialyl-Lewis x (binding determinant for selectins). 3′-sialyllactose and 3′-sialyl-3-fucosyl-lactose were identified as active sugars in HMO. These results indicate that some special sugars in HMO may serve as antiinflammatory components and might contribute to the lower incidence of inflammatory disease in human milk-fed infants.

Lara-Villoslada et al. (2006) reported that milk oligosaccharides isolated from goat milk (GMO) reduced intestinal inflammation in a rat model of dextran sodium sulfate (DSS)–induced colitis. There is increased interest in the study of manipulation of the flora with pre- and probiotics regarding inflammatory bowel disease (IBD) such as ulcerative colitis (UC) and Crohn's disease. GMO rats showed less severe colonic lesions and a more favorable intestinal microbiota. The expression of genes involved in intestinal function, such as mucine-3, was down-regulated in DSS-control rats but returned to normal values in GMO rats. The authors concluded that GMO reduced intestinal inflammation and contributed to the recovery of damaged colonic mucosa.

MILK FAT GLOBULE MEMBRANE (MFGM)

Since the successful culture of *Helicobacter* (*H.*) *pylori* by Warren and Marshall (1983), this Gram-negative bacterium was recognized as one of the important pathogens in humans. The bacteria now are widely recognized as the pathogens in the development of gastritis, gastroduodenal ulcers, gastric cancer, and mucosa-associated lymphoid tumors. Many studies have examined the prevention of infection by this microorganism. Various compounds have reported to inhibit *H. pylori* binding to gastrointestinal epithelial cells, gastric mucin/mucus, and glycolipid receptors in vitro, including sialic acid (3′-sialyllactose), sulfated sugar chains, glycolipid, cranberry juice, milk glycoprotein, and lactic acid bacteria. Recently, Matsumoto et al. (2005) reported that glycopolypeptide (GPP) prepared from milk fat globule membrane (MFGM) in buttermilk promotes the exfoliation of *H. pylori* bound to gastric mucin and prevents the de novo adherence of this microorganism. The prevention ability of GPP did not correlate with sialic acid content, indicating that sialic acid content is not important in the exfoliation.

Buthyrophilin, the most abundant protein in MFGM, is a type 1 membrane glycoprotein. The molecule consists of two extracellular Ig-like domains and a large intracellular domain homologous to ret finger protein (RFP). Butyrophilin may have receptor functions such as modulating the encephalitogenic T cell response (Cavaletto et al. 2002).

SOLUBLE FORM OF CD14 (SCD14)

An innate immune system that can distinguish among self, nonself, and danger is a prerequisite for health. Pattern recognition receptors (PRRs), such as the Tll-like receptor (TLR) family, enable this system to recognize and interact with a number of microbial components and endogenous host proteins. Recently, Vidal and Donnet-Hughes (2008) discussed in detail how CD14, a soluble PRR in milk, contributes to the neonatal immune system.

In 1990, CD14 was first reported as a membrane receptor for the bacterial endotoxin lipopolysaccharide (LPS) and one of the PRRs. CD14 was originally characterized as a monocyte/macrophage differentiation antigen and expressed by a variety of cell types, including neutrophils, chondrocytes, B cells, dendritic cells, gingival fibroblasts, and human intestinal epithelial cells. Later, a soluble form of CD14 (sCD14) also was discovered in cell culture supernatants and in normal serum. sCD14 existed in substantial concentrations in serum, urine, saliva, tears, and breast milk (Labeta et al. 2000; Vidal et al. 2001). The primary sequence of human CD14 is highly homologous (61–73%) to the deduced amino acid sequence of those from mouse, rat, rabbit, and cow (Ikeda et al. 1997). The human and bovine CD14 cDNA encodes a protein of 356 and 373 amino acids, respectively.

In normal human plasma, sCD14 exists at concentrations of ca. 3 μg/mL, but in human milk, its concentrations are about tenfold higher. sCD14 has also been detected in bovine colostrum and normal milk (Filipp et al. 2001). Membrane CD14 is usually attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor that is added to the C-terminus in the endoplasmic reticulum. The origin of sCD14 is still unknown. However, the cleavage of the receptor from monocytes by proteases or

phospholipases, and direct secretion of full-length molecules were estimated.

sCD14 also forms a complex with LPS, like membrane CD14, and can activate both CD14-positive cells and CD14-negative cells, such as epithelial, endothelial, and smooth muscle cells. sCD14 in milk mediates the LPS-induced production of the proinflammatory cytokines IL-8 and TNF-α (Labeta et al. 2000). Direct immunoregulatory effects of sCD14 on activated T and B cells have also been reported. sCD14 inhibits the proliferation of activated T cells and their production of the Th1 cytokines IL-2 and IFN-γ and Th2 cytokine IL-4. It also induces a progressive accumulation of IκBα, an inhibitor of NF-κB. sCD14 may actually facilitate the intestinal response to specific microbial motifs such as LPS by activating intracellular signaling pathways such as NF-κB, a process necessary for maturation of neonate immune system.

CONCLUSION

Mammalian milk, including cow and human milk, contains a large variety of immunomodulating components such as milk proteins (immunoglobulin, Ig; lactoferrin, Lf; lysozyme, Lz; lactoperoxidase, LPO; and β-lactoblobulin, β-Lg); functional peptides derived from casein (phosphopeptide, CCP; caseinoglycopeptide, CGP); and peptides from milk fat globule membrane (MFGM), milk oligosaccharides (MO) in human (HMO), bovine (BMO) and goat (GMO) milk, and a soluble form of CD14. Those components have the potential to modulate the host immunity system after intake and to influence inflammatory processes. It may be true that milk is the only natural product with potentially several functions that was biologically synthesized by the mammary gland as "food." We hope that reduction or improvement of many intestinal diseases, such as IBD (inflammatory bowel disease), UC (ulcerative colitis), and Crohn's disease can be achieved through investigation of the mechanism of immunosuppression and biological therapy targeting specific milk components of the immune response.

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16
Potential for Improving Health: Calcium Bioavailability in Milk and Dairy Products

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INTRODUCTION

Calcium is the most abundant mineral in the human body. It is an essential nutrient required for important biological functions including nerve conduction, muscle contraction, cell adhesiveness, mitosis, blood coagulation, heart beat regulation, stimulation of hormone secretion, and building and maintaining healthy bones and teeth, and structural support for the skeleton. Getting enough calcium from diets is important because the body cannot make it. Furthermore, daily loss of calcium through skin, nails, hair, and sweat, as well as through urine and feces must be replaced through the diet. A failure to replace lost calcium forces the body to take out calcium from the bones to continue to perform its functions, which makes the bones weaker and more likely to break over time. Consequently, adequate body calcium reduces the risk of osteoporosis and other diseases such as hypertension, colon cancer, obesity, and kidney stones (Miller et al. 2001).

In the past, early humans obtained calcium through the consumption of a wide variety of plant species (Eaton and Nelson 1991). With domestication, cultivation of a few staple cereal and vegetable crops and the raising of livestock, modern man depends on animal products, particularly milk, for his calcium needs. People in regions of the world where dairy products are not traditionally part of diets continue to depend on plant sources for their calcium needs. Furthermore, there has been an enormous increase in the diversity of food sources of calcium through extensive fortification and supplements. Calcium adequacy can therefore be met through consumption of dairy products, fortified foods, or supplements.

Milk and processed dairy products are the main source of calcium in human diets worldwide, and provide more than 70% of all the calcium available in the food supply of Americans. In the U.S., milk and dairy products provide 83%, 77%, and 65–72% of the calcium in the diets of young children, adolescent females and adults, respectively, and it is therefore difficult to achieve dietary calcium recommendations without consumption of dairy products (Huth et al. 2006). Current dietary recommendations for calcium are 500 mg for children aged 1–3 years, 800 mg for children aged 4–8 years, 1300 mg for adolescents aged 9–18 years, 1000 mg for adults aged 19–50 years, and 1200 mg for adults aged 51 years and older (Institute of Medicine 1997). Unfortunately, very few Americans are able to meet the recommended daily calcium intakes and, even more worrisome, there is a continued trend toward less dairy consumption and more consumption of carbonated drinks (Nielsen and Popkin 2004; Striegel-Moore et al. 2006). The consumption of fewer dairy products has been paralleled by an increase in the incidence of various cancers, obesity, diabetes, hypertension, and cardiovascular diseases. Research findings in the past 2 decades have demonstrated the potentially beneficial roles of calcium and/or dairy products with regard to a number of disorders or chronic diseases including osteoporosis, hypertension, colon cancer, breast cancer, kidney stones, premenstrual syndrome, polycystic ovary syndrome,

insulin resistance syndrome, obesity, and lead poisoning (Nicklas 2003). Could there be a connection between the declining dairy consumption and the ever-increasing incidence of the listed disorders or chronic diseases? This chapter highlights recent research findings with regard to factors that affect calcium bioavailability of milk and dairy products and the health benefits of adequate body calcium.

CALCIUM FROM MILK AND DAIRY PRODUCTS

Milk is the food with the highest nutrient density for calcium. Calcium contents of milk and milk products are listed in Table 16.1. Milk calcium is about two-thirds bound to casein proteins and to a lesser extent to other milk proteins, phosphorus, and citrate. A small percentage of milk calcium also exists in an unbound state. Milk products such as skimmed milk, dried skimmed milk powder, and yogurt retain essentially all the original calcium present in the milk prior to processing. Cheddar cheese and other hard cheeses retain about 80% of milk calcium; butter retains the least percentage (Table 16.1). Dairy sources of calcium are also linked to improved bone mass and reduced fracture rates via enhanced intestinal calcium absorption as well as increased calcium retention by bone (Volek et al. 2003; Fisher et al. 2004; Matkovic et al. 2004). Further peculiarities and advantages of calcium in milk and dairy products have been summarized by Guéguen and Pointillart (2000).

Table 16.1. Calcium contents of milk and milk products

Food Item	Weight (g)	Common Measure	Calcium Content per Measure
Milk, canned, condensed, sweetened	306	1 cup	869
Milk, canned, evaporated, nonfat	256	1 cup	742
Milk, canned, evaporated, without added vitamin A	252	1 cup	658
Milk shakes, thick vanilla	313	11 fl oz	457
Milk shakes, thick chocolate	300	10.6 fl oz	396
Milk, nonfat, fluid, with added vitamin A (fat free or skim)	245	1 cup	306
Milk, lowfat, fluid, 1% milkfat, with added vitamin A	244	1 cup	290
Milk, reduced fat, fluid, 2% milkfat, with added vitamin A	244	1 cup	285
Milk, buttermilk, fluid, cultured, lowfat	245	1 cup	284
Milk, whole, 3.25% milkfat	244	1 cup	276
Cheese, ricotta, part skim milk	246	1 cup	669
Cheese, ricotta, whole milk	246	1 cup	509
Cheese, Swiss	28.35	1 oz	224
Cheese, pasteurized process, Swiss, with disodium phosphate	28.35	1 oz	419
Cheese, provolone	28.35	1 oz	214
Cheese, mozzarella, part skim milk	28.35	1 oz	207
Cheese, cheddar	28.35	1 oz	204
Cheese, cottage, lowfat, 2% milkfat	226	1 cup	156
Yogurt, plain, skim milk, 13 grams protein per 8 ounces	227	8-oz container	452
Yogurt, plain, low fat, 12 grams protein per 8 ounces	227	8-oz container	415
Yogurt, fruit, low fat, 10 grams protein per 8 ounces	227	8-oz container	345
Yogurt, plain, whole milk, 8 grams protein per 8 ounces	227	8-oz container	275
Frozen yogurt, vanilla, soft-serve	72	1/2 cup	103
Ice creams, French vanilla, soft-serve	86	1/2 cup	113
Ice creams, vanilla, light	66	1/2 cup	106
Ice creams, vanilla	66	1/2 cup	84
Butter, salted or unsalted	14.2	1 Tbsp	3

Source: USDA (2002).

Not only are the calcium content of foods and the amount of calcium intake important, but so, too, are its absorption and bioavailability to the body. *Absorbability* is often used as a synonym for *bioavailability*, but the former is actually the first step in the process. *Bioavailability* is defined as the fraction of the ingested nutrient that is absorbed and utilized for normal physiological functions, including bone mineralization or storage (Ünal et al. 2005). Calcium in milk and its products appear to have a high bioavailability, and they are also a rich source of other valuable nutrients to the body. Several reasons advanced for such a high calcium bioavailability of dairy and dairy products are summarized in Table 16.2.

Intestinal calcium (Ca^{2+}) absorption occurs by two main mechanisms: a transcellular, metabolically driven transport, and a passive nonsaturable route, called the paracellular pathway. These mechanisms are regulated by hormones, nutrients and other factors (Perez et al. 2008). Calcium is absorbed predominantly in the small intestine. The paracellular Ca^{2+} transport is carried out through tight junctions (TJ) by an electrochemical gradient. The transcellular Ca^{2+} transport consists of three steps: 1) apical entry of Ca^{2+} through epithelial Ca^{2+} channels, 2) cytosolic diffusion bound to calbindins, and 3) extrusion across the basolateral membranes by the plasma membrane Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger (Perez et al. 2008).

Calcitriol (Na^{+}/Ca^{2+} $1,25(OH)_2$ D_3) stimulates the individual steps of transcellular Ca^{2+} transport. Calcitriol is the active form of vitamin D and is produced by hydroxylations of vitamin D in the liver and the kidney. Calcitriol molecules bind to their nuclear receptors (VDR), and the complex $1,25(OH)_2$ D_3-VDR interacts with specific DNA sequences inducing transcription and increasing the expression levels of Ca^{2+} channels, calbindins, and the extrusion systems (Perez et al. 2008). Calcitriol is upregulated during pregnancy, lactation, and growth, which explains the body's increased demand for calcium at these physiological stages. Calcium absorption declines with age and is believed to be due to a reduction in intestinal responsiveness to serum calcitriol rather than a reduction in serum concentrations of calcitriol (Scopacasa et al. 2004). Calcitriol production is also regulated by parathyroid hormone (PTH), which is in turn regulated by a fall in plasma calcium concentrations. PTH and calcitriol are both involved in bone resorption and increase the reabsorption of calcium by the renal tubule and therefore ensure that plasma calcium concentration varies very little. At the intestinal level, PTH seems to act indirectly on intestinal Ca^{2+} absorption by stimulation of renal 1α-hydroxylase, thereby increasing $1,25(OH)_2$ D_3-dependent absorption of Ca^{2+} from the intestine (Perez et al. 2008).

Bioavailability therefore depends on absorbability and incorporation of absorbed calcium into bone, and on urinary excretion and fecal loss of endogenous calcium. Absorption at the level of the intestine and incorporation of calcium into bone is influenced by hormones, nutrition, and physiological stages. Although certain types of foods promote the absorption and incorporation of calcium into

Table 16.2. Suggested factors responsible for bioavailability of calcium from milk and dairy products

Factor	Reference
Unique nature of casein transport complexes or casein micelles	Farrell et al. (2006)
Prevention of the formation of insoluble calcium salts in the intestine by casein phosphopeptides	Lee et al. (1980); Kitts et al. (1992)
Optimal Ca:P ratio	Cámara-Martos and Amaro-López (2002)
Lactose binding	Cámara-Martos and Amaro-López (2002)
Facilitation of uptake by casein phosphopeptides produced by enzymatic digestion of casein during food processing or fermentation	Combes et al. (2002)
Facilitation of uptake by casein phosphopeptides produced by gastrointestinal digestion	Tsuchita et al. (2001); Kato et al. (2002); Mora-Gutierrez et al. (2007)

bones, others result in calcium being mainly excreted in the urine. Phosphorus stimulates calcium incorporation into bone, but an excess of it may also cause undesirable ectopic calcification, while sulfate and chloride anions, organic ligands, and excess proteins all increase the loss of calcium. Vitamin D, as shown above, is crucial for maximizing gut absorption of calcium via vitamin D dependent calcium receptors. It is estimated that adequate vitamin D status increases calcium absorption to 30–40% of intake compared with only 10–15% absorption without adequate vitamin D (Lanham-New et al. 2007).

Components of milk including proteins, casein phosphopeptides (CPP), and lactose favor the intestinal absorption of calcium. These milk components keep calcium in a soluble form until it reaches the distal intestine where it can be absorbed by unsaturable routes that are independent of vitamin D. It is believed that the type of micelle formed affects the digestibility and precipitation of calcium in the gut and, subsequently its bioavailability (Lönnerdal 1997). As compared to human milk, cow's milk has a higher casein content, which also forms larger micelles and contains calcium phosphate in a colloidal state. This enhances its absorbability over human milk and infant formulae. The positive effect of caseins in the absorption of calcium was demonstrated in a study that found a greater percentage of dialyzed calcium in infant formulae, with casein and hydrolyzed protein as the principal proteins in comparison with formulae composed of either serum alone or 50% serum/50% casein or soy proteins (Roig et al. 1999).

Casein phosphopeptides, formed during intestinal digestion or bacterial fermentation of casein, improve calcium absorption by binding with the calcium and maintaining the cation in a soluble form, thus making it bioavailable and inhibiting its precipitation in the intestine in the form of calcium phosphate (Mykkanen and Wasserman 1980; Li et al. 1989). These CPPs therefore help maintain calcium in solution until it reaches the distal intestine where it can be absorbed by passive diffusion. As compared to whey proteins, feeding of casein substrate for release of CPP to growing pigs improved femur mineralization (Scholz-Ahrens et al. 1990). The observation was similar in vitamin D–deficient rats but not in vitamin D–sufficient rats (Scholz-Ahrens et al. 1990). In other studies, greater calcium absorption was observed in very young pigs fed milk than in those fed an isocalcium diet containing soybeans (Partridge 1981; Matsui et al. 1997). In comparing the intestinal absorption of calcium from two sources, calcium-CPPs and $CaCl_2$ in the absence and presence of phosphate, Erba et al. (2001) observed that the absorption of calcium from $CaCl_2$ solutions decreased by 90% and 97% with respect to absorption in the absence of phosphate when the Ca:P ratios were 1:1 and 1:2, respectively. With calcium-CCP, however, absorption decreased by only 25–50% and 55–60%, respectively, for the same Ca:P ratios. The positive effect of CCP could not only be the result of the enhancement of calcium solubility in the intestinal lumen but also to the possible protector effect of these CPPs against antagonistic interactions between calcium and other mineral elements. In humans, however, the efficiency of CPPs has been modest. A benefit was found only in those who had poor calcium absorption efficiency (Heaney et al. 1994). The whey proteins, α-lactalbumin and β-lactoglobulin also bind calcium. Despite spectacular effects of these proteins on the solubility of calcium in vitro, they have a much less dramatic effect on calcium absorption and retention in vivo (Guéguen 1993).

The beneficial effect of lactose on the absorption of calcium has been studied more intensely than the effects of any other milk component. Earlier findings by many investigators on the effects of lactose on calcium absorption are summarized in a review by Miller (1989) and recent findings by Guéguen and Pointillart (2000). Despite different schools of thought, it is now clear that lactose, like other slowly absorbed sugars, must be at the site of absorption and that it prolongs the passive, vitamin D–independent absorption of calcium in the ileum. The effects of this action may be spectacular (doubling absorption) if a high dose of lactose (15% to 30% of the diet) is given (Miller 1989; Rémésy et al. 1992). The effect of lactose has been clearly demonstrated in both in vitro studies and in short- and long-term trials in rats, but its significance for human calcium nutrition is much less clear. Lactose at physiological concentrations does not seem to significantly affect the absorption of calcium from milk except at a very high dose (50 g/day) (Cochet et al. 1983). Furthermore, calcium from yogurt, in

which lactose is partially hydrolyzed, or from most cheeses, which contains little or no lactose, is absorbed as efficiently as that from milk (Brommage et al. 1991; Guéguen 1992). Thus, lactose, at concentrations normally found in milk, seems to have little effect on calcium absorption in adult humans.

HEALTH-IMPROVING POTENTIAL OF CALCIUM

Consumption of dairy and other foods rich in calcium may increase calcium bioavailability. Such intake will ensure a balance in body needs for calcium and consequently helps lower the risk of the disorders discussed in the following sections, many of which are costly as well as responsible for morbidity and mortality.

Calcium and Osteoporosis

Osteoporosis is a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk (WHO 1994). Bone calcium content is the result of whole body calcium balance, which is determined by uptake in the gut and fecal and renal calcium excretion. A negative calcium balance occurs when the amount of dietary calcium absorbed and retained is insufficient to offset obligatory losses of calcium in the urine and feces. Over time, even a minor negative calcium balance leads to reduced bone mass.

A large body of scientific evidence indicates that an adequate intake of calcium or consumption of calcium-rich foods such as milk and its products throughout life helps to achieve optimal bone mass at an earlier age or at 30 years of age, to slow age-related bone loss, and to reduce osteoporotic fracture risk in later life (AMA 1997). An analysis of 139 investigations relating calcium and bone health and spanning 3 decades provides convincing evidence of the beneficial role of consuming calcium-rich foods or adequate calcium intake on bone health (Heaney 2000).

Bone thinning occurs as part of the natural process of aging. Intestinal malabsorption of calcium has been attributed to reduced biosynthesis of calcitriol and decreased sensitivity of the intestinal cells to it (Perez et al. 2008). A low vitamin D status of older individuals due to poor diets and lack of exposure to light exacerbates the problem. A recent study analyzed a total of 590 articles for quality indicators for the care of osteoporosis in vulnerable elders (Grossman and MacLean 2007). It suggests that if a vulnerable elder has osteoporosis, he or she should be prescribed calcium and vitamin D supplements, because this may decrease the risk of fractures (Grossman and MacLean 2007). Recognizing the beneficial effects of dairy foods on bone health and their overall nutrient contribution to the diet, it is concluded that a high dairy-food intake is cost efficient as well as cost effective to augment bone gain during growth, retard age-related bone loss and reduce osteoporotic fracture risk (Heaney 2000).

Calcium and Body Weight and Obesity

The prevalence of obesity in the U.S. and indeed in most western countries including Canada has been rising steadily since the 1960s (Flegal et al. 1998). Health risks increase dramatically with increasing weight or body mass index and a reduction in body weight reduces the potentially fatal risks of heart diseases and diabetes, stroke, and high blood pressure or hypertension (Manson et al. 1995).

Studies seeking epidemiologic explanations for the rising problem of obesity have identified dietary calcium intake as one factor that is negatively correlated with adiposity or body mass index (Zemel et al. 2000; Zemel 2002). In an agouti transgenic mouse model of obesity, weight reductions of 26%, 29%, and 39% were observed after supplementation of, respectively, 1.2% calcium carbonate, 1.2%, and 2.4% calcium from dairy products as compared to a low calcium group (0.4% calcium) (Zemel 2001). Shi et al. (2001) also reported significant reductions in body weight gains, fat pad mass, and basal intracellular calcium concentrations in the adipocytes in energy restricted aP2-agouti transgenic mice after consumption of a high-calcium or high-dairy diet. The decrease in total body weight and fat pad mass was greater with the high dairy diet and the rate of lipogenesis was suppressed, whereas that of lipolysis was enhanced, and more in the high dairy group than in the high-calcium group. For the apparent role of calcium and dairy products in modulating energy metabolism and risk of obesity in humans, Lin et al. (2000) found that dietary calcium:energy ratio was

a significant negative predictor of changes in body weight and body fat, and that increased total calcium and dairy calcium consumption predicted fat mass reductions independently of caloric intake for women at lower energy intakes. In a study that compared supplemental calcium and dairy products on weight loss during energy restriction in obese adults, Zemel (2002) reported a 26% and a 70% reduction in body weight in supplemental and dairy calcium groups, respectively, as compared to the control. Furthermore, a recent review of developments in calcium-related obesity research summarized the influence of calcium and dairy food intake on obesity (Major et al. 2008). Numerous lines of evidence were listed to support each of 13 propositions, with the conclusion that calcium and dairy food intake may influence many components of energy and fat balance, indicating that inadequate calcium/dairy intake may increase the risk of positive energy balance and of other health problems (Major et al. 2008).

The mechanistic basis for the changes in body weight and body fat as a result of high dietary calcium intake was elucidated by observations in agouti transgenic mice and is based on the prevention of calcium ion influx into adipocytes, which inhibits lipogenesis and stimulates lipolysis, thus resulting in fat loss and a concomitant reduction in body weight. Dietary calcium might alter lipid flux by lowering plasma concentrations of calcitrophic hormones (Vit D and PTH), which are known to modulate human intraadipocyte calcium concentrations and thereby affect the rate of lipogenesis and lipolysis (Shi et al. 2001; Zemel 2002). Human and animal studies have also demonstrated that a substantial increase in dietary calcium content promotes the formation of indigestible calcium soaps in the gastrointestinal tract, and it is thus possible that some of the reduction in adiposity of animals and people consuming high-calcium diets may be due to reduced absorption of dietary fat (Shahkhalili et al. 2001). The specific mechanisms explaining the high potency of dairy products relative to calcium supplementation still remains a mystery but seems not to be related to the bioavailability of calcium, because the bioavailability of calcium from dairy products is not considered to be greater than that of calcium supplied by nondairy foods (Parikh and Yanovski 2003). Probably, some bioactive component(s) of milk is responsible for the augmented weight loss and antiobesity effects of dairy products.

Calcium and Diabetes

The incidence of type 2 diabetes mellitus is increasing at an alarming rate (Mokdad 2003). Some of the potentially modifiable environmental risk factors for diabetes are obesity, change of lifestyle (exercise), and diet. A series of recent clinical and epidemiological studies have suggested that dietary calcium and dairy consumption may have beneficial effects on body weight and obesity, blood pressure, insulin resistance syndrome, and cardiovascular health (Liu et al. 2006).

Two prospective studies (the Nurses Health Study and the Black Women's Health Study) consistently found high calcium intake to be inversely associated with incidence of type 2 diabetes mellitus (Pittas et al. 2006: van Dam et al. 2006). Pooled data from the two studies revealed that the risk of developing type 2 diabetes mellitus was reduced by 18% in the highest compared to the lowest calcium intake group (Pittas et al. 2007). After combining data from several cross-sectional studies, Pittas et al. (2007) reported that the consumption of 3–4 servings per day of dairy was associated with a 29% reduction in the prevalence of insulin resistance syndrome or metabolic syndrome compared with the consumption of 0.9–1.7 servings per day. In a large 10-year prospective cohort study, Liu et al. (2006) found a moderate inverse association between dairy consumption, especially low-fat dairy consumption, and incidence of type 2 diabetes that was independent of age, body mass index (BMI), family history of diabetes, history of hypertension, physical activity, and diet factors related to type 2 diabetes.

For glucose intolerance and type 2 diabetes mellitus to develop, defects in pancreatic cell function, insulin sensitivity, and systematic inflammation are often present and there are some indications that calcium and vitamin D influence these mechanisms (Weyer et al. 1999; Hu et al. 2004; Pittas et al. 2007). Pittas et al. (2007) hypothesized that inadequate calcium intake or vitamin D insufficiency may alter the balance between the extracellular and intracellular beta cell calcium pools, which may consequently interfere with normal insulin release, especially in response to glucose load, resulting in development of type 2 diabetes mellitus.

Calcium and Cancer

Colorectal cancer is the third most common incident cancer worldwide and its geographical distribution worldwide seems to be related to dietary factors (Cho et al. 2004). Evidence from animal studies suggests that high dietary calcium intake may have a protective effect against colonic carcinogenesis (Lipkin 1999). Several other studies support a beneficial role for calcium against colon cancer (Lupton 1997; Holt et al. 1998; Holt 1999; Baron et al. 1999). Increasing food sources of calcium, specifically low-fat dairy foods, reduced the risk for colon cancer in an investigation of 70 patients with a history of developing polyps or noncancerous growth in the colon (Holt et al. 1998). Also, increasing calcium intake by 1200 mg/day in a double-blind trial of 930 adults with recent history of colorectal adenomas reduced the number of recurrent adenomatous polyps by 19% and the total number of tumors by 24% in less than a year (Baron et al. 1999). Furthermore, it has been suggested that a high calcium intake (>1000 mg/day) may reduce the risk of colorectal cancer from 15 to 40% (McCullough et al. 2003). A recent study conducted among more than 45,000 American women without a prior history of colorectal cancer suggested that an intake of greater than 800 mg calcium/day (from diet and/or supplements), compared to an intake of less than 400 mg calcium/day, was associated with a 25% reduction in the risk of colorectal cancer (Flood et al. 2005). In addition, meta-analysis of the epidemiologic literature and reports from prospective cohort studies also provide evidence that high dietary calcium intake does in fact have a negative association with colorectal cancer (Cho et al. 2004). A significant inverse association between milk consumption and the risk of colorectal cancer was also observed in both men and women (Cho et al. 2004).

Many factors have been related to altered breast cancer risk, and among the nutritional factors, calcium metabolism is believed to play an important role in the development of breast cancer. However, cohort studies of calcium-rich dairy foods and breast cancer have reported conflicting results ranging from positive association to some negative association; recent findings consistently report an inverse association between dietary calcium and breast cancer or risk of breast cancer (Shin et al. 2002). Epidemiologic studies relating calcium intake and the risk of breast cancer also reported a statistically significant inverse association between calcium intake and breast cancer (van't Veer et al. 1991). In a large prospective study involving women participating in the Nurses Health Study, Shin et al. (2002) found a negative association between dietary calcium intake and the incidence of breast cancer in premenopausal women. The consumption of dairy calcium was inversely associated with risk of breast cancer and resulted in a 31% reduction in risk of breast cancer, while the consumption of nondairy calcium had no significant association with risk of breast cancer. In a Finnish cohort study, Knekt et al. (1996) also reported a strong negative association between milk intake and the subsequent incidence of breast cancer. A higher intake of calcium and dairy products has also been reported to be associated with increased survival rates of women diagnosed with breast cancer (Holmes et al. 1999). In another study involving 972 women of all racial backgrounds, low-fat milk calcium was suggested to reduce the risk of ovarian cancer (Goodman et al. 2002).

The precise mechanism by which calcium modulates the development of cancers is not yet clear. Among the cell functions modulated by calcium, its role in cell division and the regulation of cell proliferation and differentiation may be particularly important. Calcium might have direct effects on differentiation and apoptosis, possibly related to the action of calcitrophic hormones, intracellular release of calcium, calmodulin activation, and subsequent phosphorylation of other cellular enzymes and activation of other signaling pathways (Lamprecht and Lipkin 2001). In addition, calcium is believed to exert its protective effect against colorectal cancer through the formation of insoluble soaps with bile acids and neutralizing their ability to irritate the epithelial surface of the colon and thereby induce an increase in proliferation rates (Hyman et al. 1998). Alternatively, calcium may act through pathways independent of its ability to bind secondary bile acids and seemingly to diminish directly proliferation rates (Flood et al. 2005).

Calcium and Hypertension

Hypertension or high blood pressure is an important risk factor for development of cardiovascular diseases, renal failure, and stroke. Aging is accompa-

nied by a progressive increase in the incidence and prevalence of hypertension (Zemel 2001). The prevalence of hypertension also increases with BMI (body mass index) such that BMI of 30–34.9 and ≥35 are associated with a 2.5-fold and 4.5-fold increase in hypertension prevalence in adults under the age of 55, respectively (Must et al. 1999).

Several well-designed epidemiological studies have identified an inverse association between dietary calcium and blood pressure levels or reduced risk of developing hypertension (Hamet 1995; McCarron 1992; Cappuccio et al. 1995). A prospective Nurses Health Study investigated the effects of dietary calcium and magnesium on blood pressure and found that dietary calcium intake equal to or above the recommended daily allowance of 800 mg/d reduced the risk of developing hypertension by 22% compared to those consuming under 400 mg/d (Morris and Reusser 1995). Further concordance came from a U.S. government-sponsored DASH (Dietary Approaches to Stop Hypertension) study on the effects of calcium and calcium-rich dairy products on blood pressure (Appel et al. 1997; Obarzanek and Moore 1999). They revealed that the consumption of three servings of dairy foods (predominantly lowfat milk) in combination with fruits and vegetables significantly reduced blood pressure in persons with high blood pressure (Appel et al. 1997; Obarzanek and Moore 1999).

An inverse association has also been found between calcium intake and pregnancy-related hypertensive disorders, specifically gestational hypertension (≥90 mmHg diastolic pressure appearing after 20 weeks of pregnancy) and preeclampsia (gestational hypertension + significant proteinuria). The inverse association between calcium intake and hypertensive disorders of pregnancy was first observed in Mayan Indians of Guatamala who traditionally soak their corn in lime (calcium carbonate) before cooking (Belizan and Villar 1980). A recent pooled analysis of 12 randomized trials involving calcium supplementation with at least 1 g/d of calcium versus placebo by Hofmeyr et al. (2007) also confirmed the negative association between calcium intake and the incidence of pregnancy-related hypertensive disorders.

Dietary calcium level is believed to modulate blood pressure by an almost similar mechanism that it uses in the regulation of adiposity, and its protective effect can be explained in part by the influence of calcitrophic hormones on intracellular calcium. Low dietary calcium results in the production of calcitrophic hormones, which in turn results in an intracellular influx of calcium ions into a variety of cells including the vascular smooth muscle cells. Increased intracellular calcium ion concentrations exert a pressor effect, serving to promote contraction, increase peripheral vascular resistance, and accordingly increase blood pressure (Zemel 2001). Diets with high dietary calcium, by virtue of suppressing the release of calcitrophic hormones, minimize intracellular influx of calcium ions and consequently lower peripheral vascular resistance and blood pressure.

Calcium and Cardiovascular Diseases

Cardiovascular diseases (stroke and heart attack) are the leading causes of death in the U.S. Several studies have reported a negative association between dietary calcium intake and risk factors for cardiovascular diseases such as body weight, obesity, and hypertension (see previous sections), and calcium intake is expected to play an active role in the development of cardiovascular diseases.

In a large Japanese Collaborative Cohort prospective study involving middle-aged men and women, Umesawa et al. (2006) found total calcium intake to be inversely associated with mortality from total (either hemorrhagic or ischemic) and ischemic stroke. Dairy calcium intake was also inversely associated with total stroke and total cardiovascular disease but not coronary heart disease. Men at the highest quartile of dairy consumption had a 47%, 54%, 47%, and 17% reduction in risk of total stroke, hemorrhagic stroke, ischemic stroke, and total vascular disease, respectively, relative to those at the lowest quartile of dairy consumption. The respective risk reductions in women were 43%, 49%, 50%, and 13%. There was however, no association between nondairy calcium intake and mortality from cardiovascular disease (Umesawa et al. 2006). Two other cohort studies, the Honolulu Heart Program and the Nurses Health Study, also reported a negative association between total calcium intakes, particularly dairy calcium intake, and the risk of stroke (Abbott et al. 1996; Iso et al. 1999). Kinjo et al. (1999) in another large cohort study involving 265,070 Japanese subjects, reported 26% and 15% reductions in risk of cerebral hemorrhage and ischemic stroke,

respectively, in people consuming dairy milk ≥4 times a week compared with once a week.

The mechanisms responsible for the inverse association between dietary or dairy calcium and risk of cardiovascular diseases are believed to be due to the independent effects of dietary calcium on the risk factors for cardiovascular diseases such as hypertension, obesity, and high blood cholesterol levels. High dietary calcium is believed to be hypotensive, hypocholesteromic and antiartherogenic, and can thus minimize the incidence of cardiovascular diseases. In addition, calcium has antiplatelet aggregation effect and can therefore directly reduce the incidence of both heart attack and stroke (Groot et al. 1980). Furthermore, whey peptides in milk and dairy products may have a hypotensive effect through inhibition of the angiotensin-converting enzyme (Abubakar et al. 1998).

Calcium and Kidney Stones

Kidney stones, otherwise known as nephrolithiasis, is a common disease of the kidney affecting an estimated 5–10% of the American population annually (Heller 1999). Most kidney stones (90%) are of the calcium oxalate type.

Two prospective observational studies demonstrated that increased dietary calcium, particularly from dairy sources, reduced the incidence of kidney stones in adults (Curhan et al. 1993, 1997). The majority of the calcium-containing kidney stones is usually associated with hypercalciuria or elevated calcium in the urine (>300 mg/d in men, 250 mg/d in woman or 4 mg/kg in both sexes) and based on this observation it was initially thought that high dietary calcium intake contributed to the recurrence of kidney stones in affected individuals. On the contrary, in the largest prospective epidemiological study ever published on the relationship between calcium and kidney stones (the New England Study), Curhan et al. (1993) reported an inverse relationship between dietary calcium intake and the risk of symptomatic kidney stones. In this study, the mean dietary calcium intake was significantly lower among men who later developed kidney stones than among those who remained free of stones (797 ± 280 versus 851 ± 307 mg, $P < 0.001$). The New England study also examined the relation of specific foods that are high in calcium content to the risk of kidney stones and found the strongest inverse associations between milk and dairy products and the risk of kidney stones (Curhan et al. 1993).

The protective effect of high dietary calcium intake against kidney stones was both surprising and unexpected, given that recurrent kidney stones are characterized by apparent hypercalciuria. Calcium restriction on the other hand increases the absorption of oxalate in the intestines in normal subjects and patients with kidney stones leading to an increase of 16% to 56% in urinary oxalate excretion or hyperoxaluria (Rao et al. 1982). The inverse relationship between high dietary calcium intake and the risk of kidney stones may be due to increased binding of oxalate by calcium in the gastrointestinal tract and restriction of its absorption in a form that leads to kidney stones. Urinary oxalate appears to be more potent than urinary calcium for stone formation, and calcium restriction could actually be more harmful in that it may lead to increased urinary oxalate excretion (Goldfarb 1988).

Calcium and Other Health Problems

Beneficial effects of adequate calcium (mainly supplements) intakes have been reported for other disorders including periodontal disease, premenstrual syndrome, polycystic ovarian syndrome, and lead intoxication (Bruening et al. 1999; Thys-Jacobs et al. 1998; Thys-Jacobs 2000; Nishida et al. 2000).

Premenstrual syndrome (PMS) also known as *premenstrual dysphoric disorder* is widely recognized as a recurrent, cyclical disorder related to the hormonal variations in the menstrual cycle, disrupting the emotional and physical well-being of millions of women during their reproductive lives (Thys-Jacobs 2000). Thys-Jacobs et al. (1989) reported a significant 50% reduction in PMS symptoms after supplementation of a group of women with 1000 mg/day of calcium in the form of calcium carbonate for 3 months. Similar results were reported by Penland and Johnson (1993). In another prospective and randomized double-blind placebo controlled clinical trial involving women with moderate to severe PMS symptoms, Thys-Jacobs et al. (1998) reported an overall 48% reduction in symptom score in a group supplemented with 1200 mg/day of calcium in the form of calcium carbonate. In a large prospective epidemiological study of calcium and vitamin D intake and risk of incident of PMS, Bertone-Johnson et al. (2005) reported a negative

association between calcium from food sources and risk of PMS and lack of association between calcium from supplements and risk of PMS. The most likely explanation for the temporal occurrence of PMS and the concomitant hypocalcemia during the same period is the relationship between the ovarian steroid hormones and the calciotrophic hormones. Ovarian steroid hormones, especially estrogens, are known to influence the actions of the calcitrophic hormones especially 1,25-dihydroxyvitamin D and parathyroid hormone (PTH) (Gallagher et al. 1980). Estrogen is believed to lower serum calcium levels by enhancing calcium deposition and suppressing bone resorption, and PTH appears to act in exactly the opposite manner. Thys-Jacobs and Ma (1995) proposed hypocalcemia resulting from the surge in ovarian steroid hormones during the late luteal phase as the primary cause of PMS.

Among known toxic heavy metals, lead is a ubiquitous environmental poison to any form of life. The most common sources of lead and subsequent lead exposure are through leaded gasoline, lead in cans made of lead solder, ceramics with lead glazes, and lead in paints, traditional medicines, folk medicines, and cosmetics (Fewtrell et al. 2003). Lead paints continue to be the major source, especially in many developed countries, causing childhood lead poisoning. Lead may have deleterious effects on hematopoietic, renal, neurological, reproductive, and skeletal systems (Bernard and Becker 1988; Ronis et al. 1998; Silbergeld 1991). A number of dietary factors influence susceptibility to lead intoxication including calcium intake and vitamin D status (Mahaffey 1985). An inverse association between calcium intake and blood lead levels was reported in humans, where calcium supplementation was found to significantly reduce blood lead levels in pregnant women whose diets were deficient in calcium (Farias et al. 1996; Hernandez-Avilla et al. 1996). A similar inverse association between lead and calcium intake and milk-based foods were also reported by Sorrell et al. (1977). The protective effect of high dietary calcium intake against lead poisoning is due to its inhibitory effect on intestinal lead absorption. Because the same transport system is used for the gastrointestinal absorption of both calcium and lead, the resulting competitive interaction significantly reduces the amount of lead that can be absorbed, thus reducing the incidence of lead poisoning (Vega et al. 2002).

CONCLUDING REMARKS

Despite the importance of milk and dairy products in providing valuable nutrients and sufficient body calcium needs, the last 2 decades have seen a general downward trend in milk consumption. Many population groups, particularly adolescent and older adults, consume significantly less calcium than recommended. Several reasons have been advanced for this trend, including substitution of soft drinks for milk, eating away from home, parental and peer influence, skipping meals, poor knowledge and attitudes, weight and fat concerns, taste, and lactose intolerance (Miller et al. 2001; Lanham-New et al. 2007; Major et al. 2008). Considering the importance of calcium to overall well-being and the adverse health and economic effects of low calcium intakes, strategies are needed to ensure adequate calcium intake by all age groups.

The beneficial health effects of adequate body calcium are clear but the exact mechanisms are still elusive. It is important that more research efforts are made for better understanding of the mechanisms. Finally, since milk calcium is more biologically available than calcium from other sources, the campaign for milk and dairy foods consumption should be intensified.

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17
Potential for Improving Health: Iron Fortification of Dairy Products

Young W. Park

INTRODUCTION

Iron deficiency anemia is still the most prevalent nutritional problem in the U.S. and world (Stoskman 1987; Zhang and Mahoney 1989b). Among the population groups, infants and children, adolescents, pregnant women, women at child bearing age, and the elderly are most vulnerable to iron deficiency (Baker and DeMaeyer 1979; Dallman et al. 1980).

Girls and women from age 9 to 54 years receive from their diets an average of 20% less than the recommended daily allowance of iron, with many below 30% or more (ARS, USDA 1972). In the UK, 40% of adolescents (94 out of 234) were iron depleted based on serum ferritin concentration of less than 10 ng/mL (Armstrong 1989). Maternal anemia may lead to fetal growth retardation, low-birth-weight infants, and increased rates of early neonatal mortality (Juneja et al. 2004). Iron deficiency anemia is a major cause of low birth weight and maternal mortality (DeMaeyer et al. 1989), and also is recognized as an important cause of cognitive deficit in infants and young children.

Iron is deficient in the milk of most dairy species, such as cows and goats. It is difficult to increase iron intake by dietary manipulation, because some frequently consumed foods contain very low iron. Iron fortification in various foods including milk and dairy foods is, therefore, needed to increase dietary iron levels. 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 (Juneja et al. 2004).

Dairy products are widely consumed, providing high-quality proteins, vitamins, and minerals except iron. Farley et al. (1987) reported that low levels of iron in dairy products decreases the iron density of diets when the proportion of dairy products in the diets increases. Thus, it is logical that fortifying dairy products with iron may increase dietary iron density in people who consume large amounts of dairy products. Increasing the content and bioavailability of iron in the diet, especially by iron fortification in milk and dairy products, can prevent iron deficiency and thereby have a potential for improving human health. This chapter reviews the scientific basis and research studies on iron supplementation and/or fortification in milk and dairy products for improving and enhancing human health.

POTENTIAL OF IRON FORTIFICATION IN IMPROVING HUMAN HEALTH

Iron fortification of various foods such as flour, bread, and cereals has been practiced as a means of correcting dietary iron deficiency. Milk has been known as nature's most complete food with all major and minor nutrients, but it is a poor source of iron, which cannot be significantly increased by oral administration of iron salts to lactating humans or animals (Pond et al. 1965).

There is little argument that iron added to milk not only shows excellent biological availability, but can prevent iron-deficiency anemia (Demott 1971). It has been demonstrated that iron salts can be added in amounts equivalent to 20 mg/L skim milk with no

adverse flavor effects, when iron-fortified dry milk is reconstituted to skim milk or used in the preparation of milk containing 2% fat and 10% total solids (Kurtz et al. 1973).

Iron addition to dairy products has been discouraged due to some negative changes in the quality of the products. However, if suitable sources for iron fortification were found, the quality of dairy products could be fortified and enhanced on the basis of iron content as well as calcium and vitamin D contents (Zhang and Mahoney 1989a). Various studies have been conducted to evaluate the potential of iron fortification in different milk and dairy products for improving human health.

Iron Fortification in Fluid Milk

Iron fortifications in whole milk as well as skim milk have been reported in several studies. Edmondson et al. (1971) investigated enrichment of pasteurized whole milk with various iron compounds such as ferric ammonium citrate, ferric choline citrate, ferric glycerophosphate, ferrous sulphate, ferrous ammonium sulphate, ferrous fumarate, and ferrous gluconate. They deodorized the milk by heating raw whole milk 72 °C for 16 seconds and "flashing" into a vacuum pan without heat in order to determine a proper method without developing undesirable flavors by the fortification. The additions of iron compounds were made immediately before pasteurization, which followed deodorization with a few treatments of iron additions after pasteurization.

Hegenauer et al. (1979) supplemented iron and copper in milk with various chemical forms of iron and copper complexes, such as ionic, chelated, and polynuclear forms, and evaluated these compounds' ability to promote lipid peroxidation during short-term incubation and long-term cold storage in raw and pasteurized milk. They sought a solution to this nutritional paradox by developing trace element complexes that display a useful balance of physical-chemical, nutritional, and organoleptic properties as new food additives. Raw whole milk and homogenized, pasteurized milk as well as raw skim milk were prepared, and effects of pasteurization, homogenization, and storage on product stability and quality were determined with respect to iron and copper supplementation of the fluid milk products.

In four experiments with fluid or spray-dried skim milk, Kurtz et al. (1973) fortified the products with ferrous chloride, ferric chloride, ferrous gluconate, and ferric ammonium citrate. Skim milk or reconstituted nonfat dry milk were fortified by adding an aqueous solution of an iron salt either before or after pasteurization, and the skim milk concentrates were fortified by adding the solution of iron salt either to the skim milk before pasteurization or to the concentrate after standardization to 45% total solids. Iron salts were fortified in amounts equivalent to 20 ppm iron in all different forms of the skim milk prepared, and all fortified products were organoleptically compared for the feasibility of fortification.

Wang and King (1973a) reported that ferric ammonium citrate is suitable for preparing an iron-fortified milk by direct addition of an aqueous solution to raw milk followed by normal processing. After 7 days' storage the iron-fortified milk was as acceptable to consumers as the nonfortified control sample and the contents of vitamin E, vitamin A, and carotene were not sacrificed. A normal serving of this iron-fortified milk would provide a substantial part or all of the RDA for iron in the diet.

In milk products, compatible and nonreactive iron compounds are attractive as fortifiers because they have less "iron taste" compared with soluble iron. Ferric pyrophosphate is considered one of these compounds due to its nonreactivity. However, low bioavailability and insolubility of nonreactive iron compounds make them practically infeasible for iron fortification. Juneja et al. (2004) demonstrated the superior utility of the superdispersed ferric pyrophosphate in milk and yogurt, in comparison with other iron compounds such as ferric pyrophosphate, ferrous sulphate, sodium ferrous citrate, and heme iron.

Iron Fortification in Dairy Products

Chocolate Milk

Kinder et al. (1942) proposed that foods containing cocoa and chocolate are suited to be fortified with iron because the added iron remains completely available and the cocoa and chocolate contain natural antioxidants to inhibit development of oxidative rancidity. As a result they were considered to serve as good carriers of added iron. An iron-enriched chocolate milk might be particularly helpful for children whose diets are deficient, because many children prefer the chocolate flavored product and chocolate milk is used widely in institutional feeding programs (Henderson 1971).

Whey Protein Powder and Whey Protein Concentrate (WPC)

Ferripolyphosphate (FIP), an aqueous complex of ferric chloride and sodium polyphosphate, when added to acid whey at pH 4.6, precipitated the proteins (Jones et al. 1975). The product, a powder free of lactose and milk salts, contained approximately 10% Fe, 13% P (or 30% P_2O_5), and 50% protein. The iron from FIP-protein powders and FIP (aqueous) was found to be highly assimilable (92–100%) relative to ferrous sulfate, when fed by direct addition to animal diets. Iron from FIP, solid gel, was less well utilized (50–60%), but nonetheless can be considered a good source of biologically assimilable iron.

Kim et al. (2007a) examined the iron-binding ability of whey protein concentrate (WPC). Heated (for 10 minutes at 100 °C) WPC (2% protein solution) was incubated with 2% each of Alcalase, Flavourzyme, papain, and trypsin for 30, 60, 90, 120, 150, 180, and 240 minutes at 50 °C. The highest iron solubility was noticed in hydrolysates derived with Alcalase (95%), followed by those produced with trypsin (90%), papain (87%), and Flavourzyme (81%). Iron-binding ability was noticeably higher in fraction 1 than fraction 2 in all hydrolysates of WPC.

Powdered Whole and Skim Goat Milk and Cow Milk

Four different levels of ferrous sulfate were fortified into powdered whole and skim goat milk and cow milk and fed to 63 weanling, male, Sprague-Dawley rats to examine iron availability from the powdered milk products (Park et al. 1986). Seven isocaloric and isonitrogenous experimental diets were formulated from whole and skim powdered goat and cow milk to compare the efficiency of body growth of rats and iron utilization from the two species of milk. They found that iron utilization was greater in rats fed on powdered goat milk than in those fed the cow milk counterpart.

Yogurt Drink

The utility of supplementation of superdispersed ferric pyrophosphate (SDFe) in yogurt was investigated by Juneja et al. (2004). The SDFe was mixed with a yogurt drink and then a 10% vitamin C solution was added, which resulted in iron and vitamin C contents ranging from 1.0 to 10 mg and 5.0 and 100 mg in the yogurt drink, respectively. They found that there were no unpleasant tastes for the yogurt drink fortified with 10 mg Fe by SDFe addition.

Infant Formulae

Since milk is deficient in iron content, iron is routinely added to cow milk–based infant formula. Iron absorption from breast milk, cow milk, and iron-supplemented formulae were compared, and an opportunistic use of change in total body iron was determined by hemoglobin, ferritin, and body weight in 132 infants (Saarinen and Siimes 1979). Experimental milk-based formulae were evaluated to study the effect of processing on availability of iron salts in liquid infant formula products (Theuer et al. 1973).

Cheeses

Iron fortification of cheese could increase average dietary iron intake about 14%. Zhang and Manoney (1989a) fortified 14 cheeses with iron to evaluate iron recovery and cheese quality by evaluating 2-thiobarbituric acid value and organoleptic analysis. Iron recoveries in the cheeses were 71 to 81% for $FeCl_3$, 52 to 53% for ferric citrate, 55 to 75% for Fe-casein complex, and 70 to 75% for ferripolyphosphate-whey protein complex. Aging cheese up to 3 months did not change TBA or oxidized off-flavor and cheese flavor scores. Ferripolyphosphate whey protein complex, Fe-casein, and $FeCl_3$ are potential iron fortification sources for cheese.

Zhang and Mahoney (1990) reported that a panel of 66 lay people did not detect differences in texture among the iron-fortified cheddar cheeses, but judged Fe-casein and control cheeses to have better cheese flavor. Fluorescent lighting slightly increased TBA values the same degree in all cheeses. The authors concluded that the quality of iron-fortified cheddar cheese was as good as the unfortified one.

EFFECTS OF IRON FORTIFICATION ON FOOD QUALITY OF MILK AND DAIRY PRODUCTS

The effect of iron fortification on quality of dairy products depends on the source and level of iron and the properties of the dairy products. Up to the early

1970s, dairy products have traditionally been protected from contamination with iron because it catalyzes oxidation of fat. Although skim milk powder is low in triglycerides, it contains about 50% of the phospholipids of milk, whereby iron fortification raises the possibility of developing an unpalatable product.

Many iron compounds that exhibit high bioavailability, including ferrous sulfate, have been shown to have an adverse effect on food quality by accelerating lipid oxidation or by producing an unfavorable color or flavor (Edmondson et al. 1971; Douglas et al. 1981; Bothwell and McPhail 1992).

Iron Fortification Effects on Sensory and Organoleptic Quality of Milk and Dairy Products

Contamination of metal ions to milk products has long been known to develop a characteristic "oxidized" odor and flavor as a result of lipid peroxidation of milk fat (Demott 1971; Wang and King 1973a; Shipe et al. 1978). However, methods of addition and types of compounds used for iron fortification made significant differences in flavor and sensory quality of the iron-enriched milk and dairy products.

In a study on enrichment of pasteurized whole milk with iron, Edmondson et al. (1971) found that ferric iron compounds uniformly resulted in lipolytic rancidity when milk was pasteurized at minimum to moderate temperatures below about 79 °C. This off-flavor was reduced acceptably, or completely eliminated simply by pasteurizing at 81 °C. They also observed that ferric compounds caused a rancid flavor, whereas the ferrous compounds resulted in an oxidized flavor with no rancidity (Table 17.1). Rancidity and oxidized flavors in the controls were insignificant.

Ferrous compounds normally caused a definite oxidized flavor when added to raw whole milk before pasteurization. This off-flavor was markedly reduced by deaerating the milk before adding the iron. Addition of the citrate salt followed by pasteurization at 81 °C is the more feasible method, since it would be difficult to add ferrous salt after deodorization and before pasteurization (Edmondson et al. 1971).

Table 17.1. Effect of added iron on rancid and oxidized flavors in pasteurized whole milk[a]

Iron Compound	Pasteur. Temp. (C)	Flavor Intensity[b]		pH
		Rancid	Oxidized	
Ferric ammonium citrate	71.0	4.0	0	6.45
	72.2	3.0	0.3	6.55
	73.3	3.2	0.3	6.51
Ferric glycerophosphate	72.2	3.5	0.8	
	73.3	1.5	1.2	
Ferric citrate	72.2	1.5	1.3	
	73.2	1.7	2.2	
Ferrous gluconate	71.0	0	3.5	6.64
	72.2	0.2	3.3	6.66
	73.3	0.2	3.5	6.64
Ferrous ammonium sulfate	72.2	0	2.8	
	73.3	0.2	3.3	
Ferrous sulfate	72.2	0	3.0	
	73.3	0	3.8	
Control	71.0	2.0	0	6.65
	72.2	0.8	0	6.66
	73.3	0.3	0	6.69

[a]Edmondson et al. (1971). Added iron = 40 mg/quart.

[b]Taste tests were made 1 day after processing. Flavor ratings are averages of six judges. Flavor rating scale: 0 = none, 1 = questionable, 2 = slight, 3 = distinct, 4 = strong.

In studying the effects of supplemental iron and copper on lipid oxidation in milk, Hegenauer et al. (1979) found that emulsification (ultrasonically homogenized) of milk fat prior to fortification greatly reduced lipid peroxidation (TBA values) by all different metal complexes tested. Compared under any conditions to the simple ferrous and cupric salts, the Fe^{+3} and Cu^{+2} chelates of nitrilotriacetate (NTA) and lactobionate produced significantly less lipid peroxidation at concentrations within the practical range of fortification (Table 17.2; Fig. 17.1). They also identified three stages in the oxidation of raw milk by ferrous iron (Fig. 17.1A): after a brief lag period of about 15 minutes, oxidation increased rapidly for about 30 minutes and continued slowly for the remainder of the observation period (3 hours). In contrast, oxidation catalyzed by chelates of ferric iron (NTA) was essentially unchanged after the initial reaction over a 3-hour period. The kinetics of oxidation of raw and homogenized, pasteurized milk has some similarities. Oxidation in homogenized milk supplemented with $FeSO_4$ also exhibited a brief lag: Oxidation thereafter plateaued after about 2 hours at 25 °C (Fig. 17.1B).

Table 17.2. TBA reactivity of raw, raw emulsified, and raw skim milk supplemented with 1 mM Fe[a]

Iron Complex	Raw Milk	Emulsified Milk[b,d]	Skim Milk[c,d]
Ferrous sulfate	2.273	0.716	0.340
Ferric fructose	1.492	0.601	0.386
Ferric lactobionate	0.954	0.308	0.236
Ferric nitrilotriacetate	0.656	0.295	0.162

[a]Milk incubated with Fe (1 mM) and pH 6.8 Hepes buffer (20 mM) for 3 hours at 25 °C.

TBA observance value was at 532 nm.

[b]Raw milk ultrasonically emulsified for 60 s at 30 °C.

[c]Skim raw milk.

[d]Average value of duplicates from each of two incubation mixtures.

Adapted from Hegenauer et al. (1979).

Kurtz et al. (1973) observed that ferrous compounds were consistently inferior to ferric compounds in flavor scores (Table 17.3), which is similar to other reports on organoleptic studies. Scanlan and Shipe (1962) investigated the susceptibility of multivitamin mineral (MVM) milk to oxidation, and they concluded that ferrous iron is responsible for the catalytic oxidation of milk when iron compounds are used for mineral fortification of whole milk. Reisfeld et al. (1955) showed that the addition of ferric ammonium citrate to milk protected lipase against heat inactivation at a normal pasteurization temperature of 73.3 °C for 16 seconds.

Zhang and Mahoney (1989a) found that thiobarbituric acid values increased slightly in iron-fortified cheeses but were within the range reported by others for unfortified cheeses. They also reported that fortification with 40 μg Fe/g as Fe-casein did not affect oxidized off-flavor and thiobarbituric acid value (TBA) compared with unfortified cheese (Table 17.4).

Table 17.3. Flavor scores and number of oxidized-flavor criticisms of skim milk and nonfat dry milk (NDN) with or without added iron salts[a,b,c]

	Iron Salt Added				
Milk Product	None	Ferric Chloride	Ferric Ammonium Citrate	Ferrous Chloride	Ferrous Gluconate
Skim milk	36.6 (0)	36.5 (0)	36.7 (0)	35.1 (0)	35.3 (0)
Skim milk	36.4 (3)	35.7 (8)	35.5 (9)	33.7 (15)	34.4 (12)
NDM	36.5 (0)	36.3 (0)	36.4 (0)	35.1 (0)	36.0 (0)
NDM	36.2 (0)	—	36.3 (0)	34.6 (4)	35.2 (1)

[a]Kurtz et al. (1973). Iron salts added in amounts equivalent to 20 ppm iron in skim milk and in NDM giving 20 ppm iron after reconstitution to skim milk.

[b]Each row pertains to one experiment.

[c]Values in parentheses are the number of oxidized-flavor criticisms per 20 evaluations.

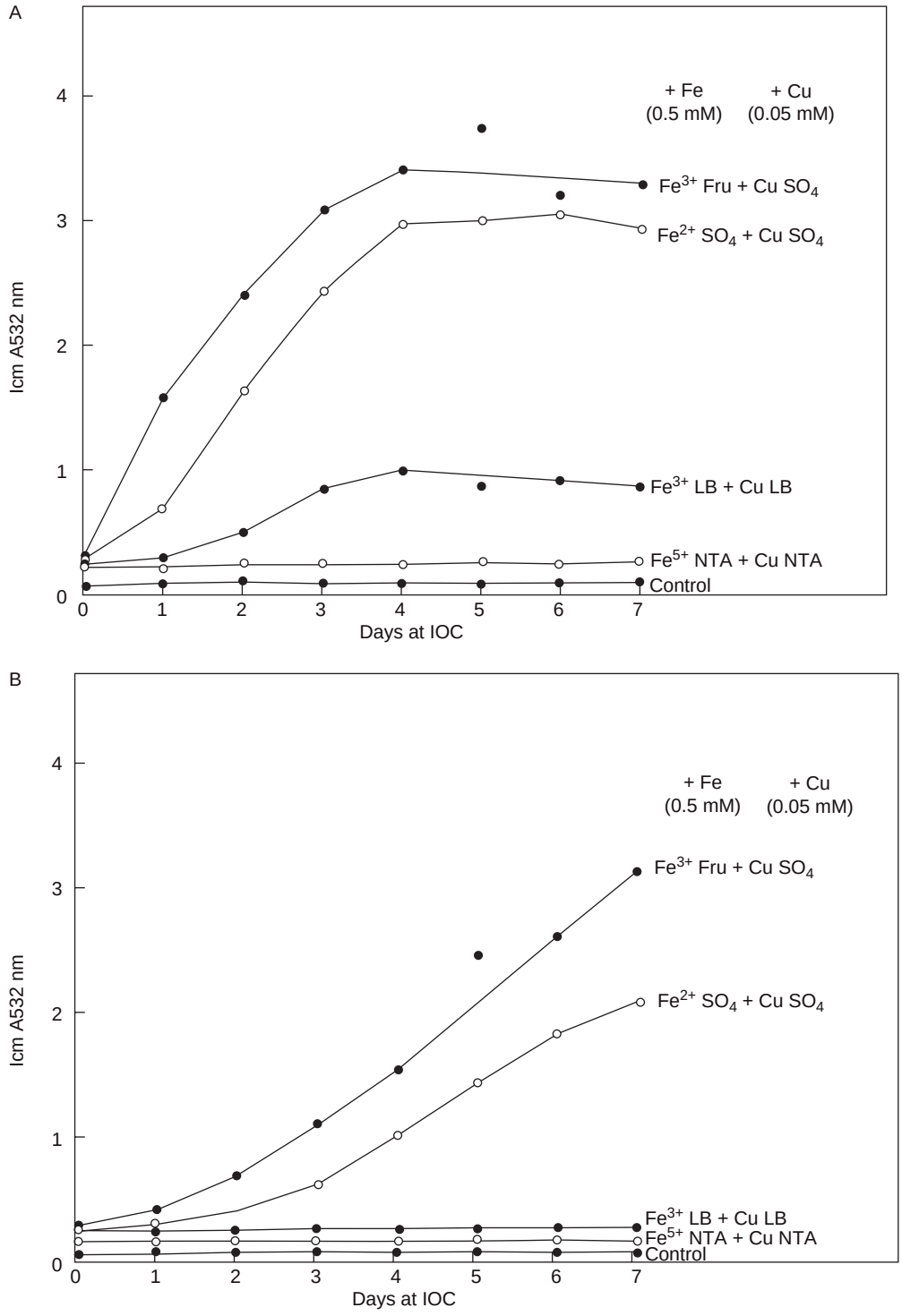

Figure 17.1. Oxidation of iron- and copper-supplemented milk during cold storage. One-quart cartons of (A) raw milk or (B) homogenized pasteurized milk were fortified by injection of sterile iron and copper solutions. TBA reactivity of oxidized product indicated by A532 nm (Hegenauer et al. 1979).

Table 17.4. Thiobarbituric acid (TBA) values and taste panel scores of iron-fortified cheese[1]

Iron Source	Control	Control + C	Fe-casein	Fe-casein + C	FIP-WP	FIP-WP + C
Cheese Fe, μg/g	2	2	74	81	93	81
TBA value						
7 d	0.01	0.06	0.26	0.22	0.32	0.15
1 mo	0.06	0.01	0.20	0.04	0.22	0.05
3 mo	0.06	0.01	1.03	0.21	0.34	0.02
Taste panel score[2]						
Oxidized off-flavor						
7 d	3.5[a]	4.6[abc]	6.4[c]	4.5[ab]	4.8[abc]	6.2[bc]
1 mo	3.7	5.3	5.5	3.8	3.8	5.5
3 mo	3.0[a]	6.1[b]	5.6[b]	3.5[a]	4.7[ab]	5.4[b]
Cheese flavor						
7 d	6.9[a]	5.4[ab]	4.4[b]	4.9[b]	5.4[ab]	4.7[b]
1 mo	6.1	3.7	4.7	5.2	6.2	5.0
3 mo	6.1[a]	2.9[c]	3.9[bc]	4.1[bc]	4.6[b]	3.6[bc]

[1]All iron sources are ferric iron. Adapted from Zhang and Mahoney (1989a).

[2]Taste panel scores were set from 1 to 10. For oxidized off-flavor, the higher scores indicate a stronger flavor. For cheese flavor, the higher score is better quality. The values are means of 11 judges.

C: cheese with annatto color; FIP-WP: ferripolyphosphate whey protein complex.

[a,b,c]Means with different letters are significantly different ($P < 0.05$).

Iron Fortification Effects on Color Stability of Milk and Dairy Products

In other food applications of fortified nonfat dry milk, the only adverse effect of added iron was an undesirable color shift when it was added to cocoa, tea, or coffee (Kurtz et al. 1973).

In studying color stability of various iron compounds, Juneja et al. (2004) tested superdispersed ferric pyrophosphate (SDFe), ferric pyrophosphate, ferrous sulfate, and sodium ferrous citrate by adjusting to 5 mg Fe/100 g distilled water, heated at 70 °C for 10 minutes and then kept at 40 °C. They found that there was no precipitation of SDFe solution after storage for 3 months, whereas commercial ferric pyrophosphate sedimented immediately, a brownish precipitate formed for ferrous sulfate, and solution with sodium ferrous citrate turned brown after 2 days.

The same researchers also prepared 100 mL liquid samples containing 2.6 g fat, 3.7 g protein, 17.2 g carbohydrate, 2.5 mg iron as sodium ferrous citrate or SDFe, and a small amount of vitamins and minerals, and then the samples were pasteurized at 120 °C for 20 minutes and stored in a dark place at 50 °C for 0, 1, 2, 3, and 4 months. They evaluated the color change (ΔE) after storage, and found that SDFe was the most stable and showed little color change.

Douglas et al. (1981) reported variations in color of iron-enriched chocolate milk as determined by the Hunter coordinates as shown in Table 17.5. They found that the L values for chocolate milk samples containing sodium ferric pyrophosphate (SFP), ferripolyphosphate (FIP), and ferrous fumarate (FF) were not initially changed appreciably from the control, whereas samples with other ferrous and ferric compounds showed pronounced darkening. Only a slight darkening occurred in the control, SFP and FIP samples after 2 weeks, while a much more pronounced darkening occurred in all other ferrous and ferric compounds.

The same authors also showed that the Hunter *a* value had no change from control initially for samples containing SFP, FIP, and FF, but there was a decrease in redness of samples containing other added iron compounds. The *a* value tended to decrease on aging with the greatest decrease in redness in the FF samples. Concerning the Hunter *b* values, no initial changes were observed for SFP-, FIP-, and FF-enriched sample groups, but a decrease was observed in other ferrous and ferric salt–enriched groups with a corresponding decrease in yellowness

Table 17.5. Hunter color coordinates of iron-fortified chocolate milk

	L		a		b		ΔE	
Iron Compound[1]	d1	d14	d1	d14	d1	d14	d1	d14
None (Control)	43.3	42.0	8.8	8.7	9.0	9.4	—	—
Sodium ferric pyrophosphate	43.0	42.6	9.0	9.0	9.1	9.8	0.37	0.78
Ferripolyphosphate[2]	43.4	42.0	8.8	8.6	9.0	9.2	0.10	0.22
Ferrous fumarate	43.3	40.8	8.8	6.4	8.8	7.9	0.20	3.00
Ferrous sulfate[3]	39.1	37.4	5.5	5.5	5.9	6.3	6.18	6.40
Ferric ammonium citrate[4]	39.6	38.0	5.5	5.3	6.1	6.3	5.74	6.10

[1]Iron added at 10 mg/0.95 liter.
[2]Ferripolyphosphate whey protein complex.
[3]Ferrous lactate.
[4]Ferric citrate, ferric choline citrate, and ferrous glycerophosphate.
Adapted from Douglas et al. (1981).

of the samples. The *b* value increased in all samples and the control after 2 weeks, except for the ferrous fumarate–enriched samples, which continued to decrease. The most significant color change with time resulted from the addition of FF as shown by the change in its total color difference (ΔE) (Table 17.5).

Iron Fortification Effects on Food Quality of Aging Dairy Products

In a study with fortification of several iron compounds in pasteurized whole milk, Edmondson et al. (1971) observed that the degree of rancidity, if present in freshly pasteurized samples, increased with storage time, whereas the oxidized flavor in the ferrous-fortified samples decreased during storage.

Although the concern about oxidative damage to dairy products catalyzed by iron has discouraged the utilization of fortification (Cook and Reusser 1983), cheddar cheese was successfully fortified with iron without any deterioration in product quality and was tested for extended aging periods for the product quality (Zhang and Mahoney 1990).

In evaluating the effect of iron fortification on quality of cheddar cheese with respect to fluorescent light and aging, Zhang and Mahoney (1990) found that all cheeses had similar low TBA values up to 12 months of aging. Trained panelists detected less oxidized off-flavor in the control and Fe-whey protein cheeses at 15 days but did not detect any differences among the cheeses thereafter. The panel judged as superior the flavor of the Fe-polyphosphate-whey protein, $FeCl_3$, and Fe-whey protein cheeses at 1 month, and of Fe-casein cheese at 9 months, but did not detect differences among the cheeses at 15 days or 4-, 7-, or 12-day aged cheeses.

The same authors noticed that the fluorescent light exposure slightly increased TBA values after 7 days, but the increase did not differ among the cheeses of different ages. The TBA values of the first 1-mm layer of cheese were higher than the second 1-mm layer in all cheeses after 28 days of light exposure. Compared with unfortified cheese, iron fortification did not affect TBA values under this light exposure.

POTENTIAL OF WHEY PROTEINS IN IRON-BINDING CAPACITY AND IRON CARRIER

Whey protein has recently gained immense recognition as a protein source in functional foods and infant formulae (Clemente 2000). Approximately 20% of total protein in bovine milk is whey protein, and the major fractions of whey protein are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), immunoglobulin (Ig), and BSA (bovine serum albumin) (Fox 1989; Walzem et al. 2002).

Although whey proteins are important ingredients for functional food, the major whey protein, β-Lg, can cause milk allergy in human infants due not only to the underdeveloped infantile gastrointestinal tract (Zeiger et al. 1986), but to the negligible amount of β-Lg in human milk in addition to the allergenic properties of bovine caseins and whey proteins (Park and Haenlein 2006). Cow milk allergy (CMA) is considered a common disease, with an estimated

prevalence of 2.5% in children during the first 3 years of life (Businco and Bellanti 1993), occurring in 12–30% of infants less than 3 months old (Lothe et al. 1982), with an overall frequency in Scandinavia of 7–8% (Host et al. 1988). Peptides derived from enzymatic hydrolysis of whey proteins have shown great potential for human health such as hypoallergenic and mineral-binding abilities, including calcium and iron.

Enzymatically Hydrolyzed Whey Proteins and Their Iron-Binding Ability

Before considering iron fortification, it would be more important to resolve the CMA problem of bovine whey proteins in manufacturing infant formulae. The enzymatic hydrolysis of whey proteins can offer a practical way to reduce its antigenic protein fractions (Heyman 1999). In addition, the hydrolysis of whey proteins can yield a variety of new peptides, which may provide many physiological benefits for humans (Otte et al. 1997). Whey protein hydrolysates have been extensively prepared and used to nutritionally support human patients who have various physiological insufficiencies and abnormalities (Halken and Host 1997).

Enzymatic hydrolysis of whey proteins results in a variety of peptides, many of which have been identified to exhibit considerable biological activities, including mineral carrier peptides (Kim and Lim 2004), angiotensin I converting enzyme (ACE)-inhibitory peptide (Gobbetti et al. 2007; Park et al. 2007), opioid peptide (Meisel and FitzGerald 2000), antithrombotic peptide (Chabance et al. 1995), immunomodulatory peptide (Mercier et al. 2004), anticarcinogenic peptide (Marshall 2004), and antibacterial peptide (Recio and Visser 2000).

Kim et al. (2007a) have shown that proteolytic enzymes extracted from the gastric gland (pepsin) of microbial origin (Alcalase), or from vegetable juices (papain), could be used for the hydrolysis of milk proteins. Enzymes from different origins, however, may have variations in their hydrolytic capacity to break down whey proteins, and thereby may influence the physicochemical characteristics of the hydrolysates (Bertrand-Harb et al. 2002), their biological activities (FitzGerald and Meisel 2000), and also the iron-binding ability of derived peptides.

Lactoferricin as a Hydrolysate of Whey Protein

Renner et al. (1989) reported that lactoferrin (LF) is the major iron-binding whey protein in milk of many species including humans, mares, and goats. Peptides derived from LF have antibacterial activities, which has drawn much attention during the last decade (Park et al. 2007). Bellamy et al. (1992) purified and identified the antibacterial domains of bovine LF f(17–41) and human LF f(1–47) as bovine and human lactoferricin (LFcin), respectively. Wakabayashi et al. (2003) also have shown that lactoferricin, a peptide derived through peptic hydrolysis of whey, has iron-binding capacity. Clinical infection results in a marked elevation in LF concentration in mammary glands.

Ovine LF was also hydrolyzed by the action of pepsin, and ovine LF hydrolysate activity was demonstrated from the corresponding region to the LFcin within the sequence of LF (Recio and Visser 2000). These peptides can help in the prevention of anemia, a widespread nutritional deficiency particularly common in children and women (WHO 2001). Chaud et al. (2002) showed that whey protein has a considerable binding ability for divalent and trivalent cations, whereby this attribute could be used together with protein hydrolysis to improve iron bioavailability for anemic subjects (Kim et al. 2007b).

Effect of Type of Enzyme on Iron-Binding Capacity

In the investigation of the iron-binding ability of whey protein concentrate (WPC) and its hydrolysates, Kim et al. (2007a) observed that iron solubility in WPC hydrolysates ranged from 81 to 95% and was higher than that noticed in WPC (Table 17.6). The highest iron solubility occurred in heated WPC hydrolysates derived with Alcalase (95%), followed by those produced with trypsin (90%), papain (87%), and Flavourzyme (81%).

The authors reported that iron-binding ability was noticeably higher in fraction 1 (F-1) than in fraction 2 (F-2) of all hydrolysates of WPC determined by reverse-phase HPLC (Fig. 17.2). The highest iron contents in F-1 were the WPC hydrolysates derived with Alcalase (0.2 mg/kg), followed by hydrolysates derived with Flavourzyme (0.14 mg/kg), trypsin (0.14 mg/kg), and papain (0.08 mg/kg), respectively.

Table 17.6. Iron solubility of whey protein concentrate (WPC) and its hydrolysates

Item	Iron Content (mg/kg)		Iron Solubility (%)
	Fraction 1	Fraction 2	
WPC	0.89 ± 0.02	0.62 ± 0.01	70 ± 0.3
WPC hydrolysate			
Alcalase	0.88 ± 0.03	0.84 ± 0.02	95 ± 0.5
Flavourzyme	0.86 ± 0.01	0.70 ± 0.03	81 ± 0.4
Trypsin	0.88 ± 0.02	0.79 ± 0.02	90 ± 0.2
Papain	0.87 ± 0.01	0.76 ± 0.01	87 ± 0.1

Kim et al. (2007a).

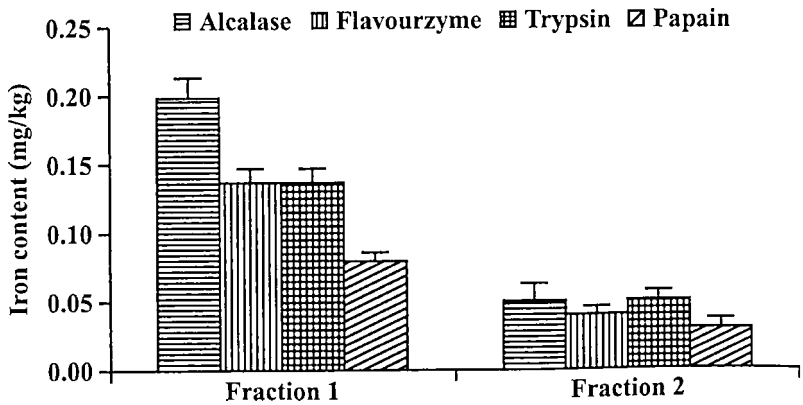

Figure 17.2. Mean iron contents (mg/kg) of fraction 1 and fraction 2 of enzymatic hydrolysates of heated whey protein concentrates (Kim et al. 2007a).

Iron concentrations in the F-2 of all enzymatic hydrolysates of WPC were low and ranged from 0.03 to 0.05 mg/kg. They postulated that F-1 may constitute a new class of iron chelates based on the reaction of $FeSO_4$. $7H_2O$ with a mixture of peptides obtained by the enzymatic hydrolysis of WPC. They concluded that Alcalase was more effective than other enzymes in producing a hydrolysate for the separation of iron-binding peptides derived from WPC.

BIOAVAILABILITY OF IRON AMONG DIFFERENT IRON-FORTIFIED MILK AND DAIRY PRODUCTS

The biological availability of iron in food is influenced by many factors including the oxidation state of the iron, type of iron compound, food to which iron is added, other foods in the diet, and physiological condition of the animal. Ferrous salts are better utilized than ferric presumably because the latter has to be reduced prior to when absorption would occur (Brown 1963; Fritz et al. 1970).

Comparison of Iron Bioavailability and Recovery among Different Iron Compounds

Natural iron in food is utilized less than iron salts, and iron absorption was greater from animal than vegetable foods (15–20 versus 5–10%) (Chodos et al. 1957). Ferric ammonium citrate and ferric choline citrate were better absorbed than ferrous sulfate by chicks (Fritz et al. 1970). Greater body weight gains and hematocrit values were observed in rats supplemented with ferrous sulfate in a dry

ration than in groups fed iron-fortified milk (Demott 1971).

In a feeding trial with rats, Douglas et al. (1981) examined iron bioavailability of different iron compounds by slope ratio analyses using hemoglobin depletion-repletion bioassay and changes in rat hemoglobin per unit of determined iron. The bioavailability of ferripolyphosphate-whey protein complex was essentially equal to the reference salt, ferrous sulfate. However, the sodium ferric pyrophosphate was lower ($P < 0.05$), and only 35% was available compared to ferrous sulfate (Fig. 17.3).

In a study on the assimilation of iron from iron-fortified milk by suckling pigs, Wang and King (1973b) prepared a radio-labeled milk diet by adding radioactive ferric ammonium citrate (FeAC) to a freshly prepared lot of fortified milk. After a 2-week adjustment period, animals were fed gradually increased modified milk up to 2–2.5 L/pig/day. At the end of the adjusting period and after a 12-hour fast each animal received 20 mg of radio iron–labeled ration (50 μci). The tracer dose was introduced directly into the stomach via a feeding tube. Blood, tissue, and organ samples were tested for iron uptake and assimilation. Hematological results revealed rather marked increases in hemoglobin and hematocrit after the adjustment period (Fig. 17.4). However, the levels for all three parameters were relatively constant during the experimental period. The ranges of the three parameters were the following: hemoglobin, 8.6–12.6 g/100 mL; hematocrit, 21–43%, RBC, $5.0–9.9 \times 10^6$, respectively.

The authors also reported the rate of incorporation of ^{59}Fe into RBC, expressed as percent of administered dose, where a plateau of constant radioactivity of RBC was reached 10–12 days after isotope administration (Fig. 17.5). The plateau was interpreted as the maximum incorporation of ^{59}Fe into RBC and the range of the ^{59}Fe in the RBC for the five piglets was 25.4–31.0%.

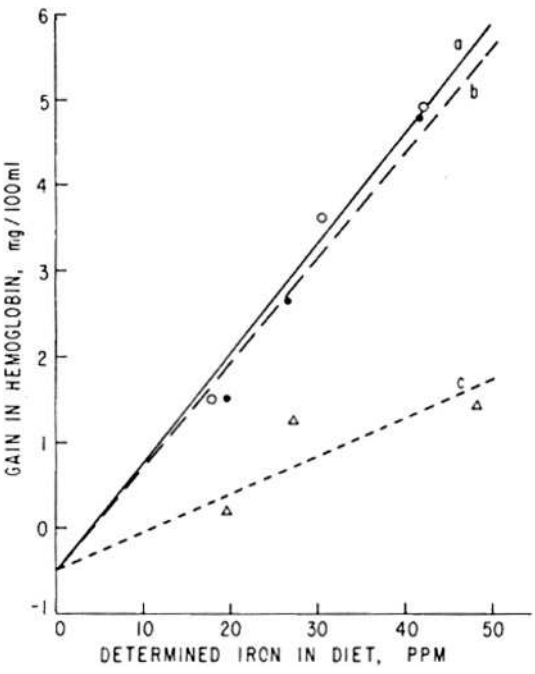

Figure 17.3. Slope ratio analysis of iron biological availability assay. a. Ferrous sulfate; b. ferripolyphosphate-whey protein complex; c. sodium ferric pyrophosphate (Douglas et al. 1981).

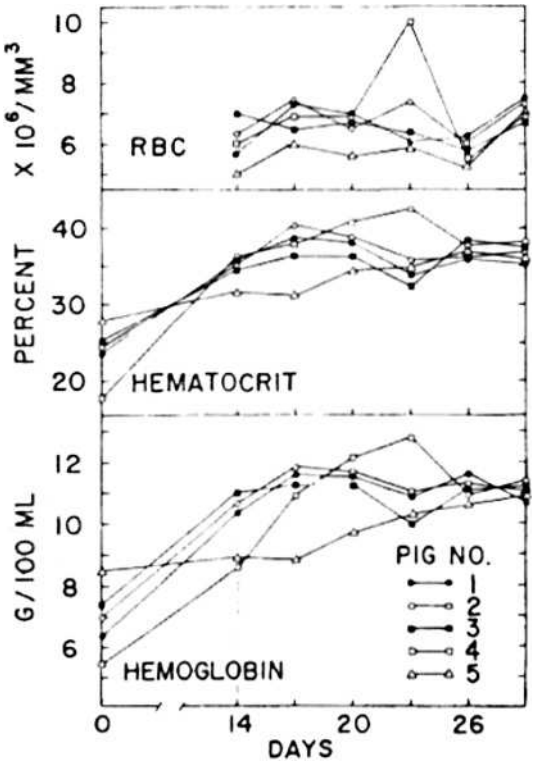

Figure 17.4. Effect of FeAC fortified-modified milk ration on values for hemoglobin, hematocrit, and red blood cells of five baby pigs. Labeled ration was administered on day 14 (end of adjusting period) (Wang and King 1973b).

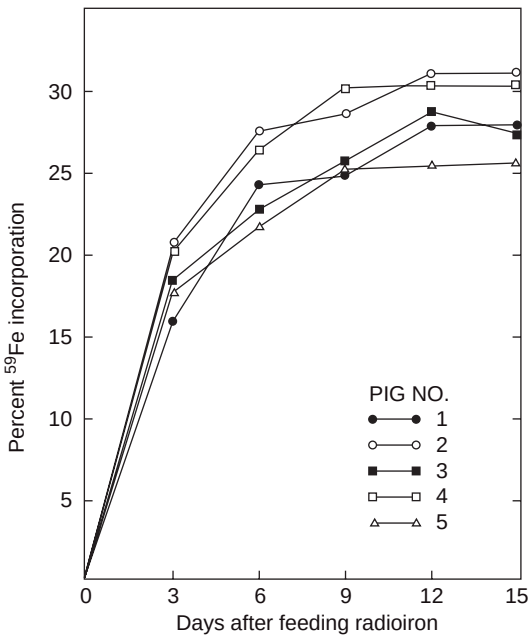

Figure 17.5. Rate of incorporation of ^{59}Fe into red blood cells of baby pigs fed FeAC fortified-modified milk. (Wang and King 1973b).

Bioavailability of iron in fortified milk and infant formula varies with iron sources and processing. In experiments with rats, Lönnerdal et al. (1985) reported that iron bioavailability of cow milk fortified with $FeCl_2$, $FeSO_4$, ferric nitrilotriacetate, or ferric lactobionate was similar. However, the rats absorbed less iron from milk fortified with ferric EDTA and the least from ferric citrate. Anderson et al. (1972) found that 65% of the $FeSO_4$ iron, 9% of the reduced iron, and 10% of the sodium iron pyrophosphate in fortified cereal plus milk were incorporated into hemoglobin.

Milk protein was attributed to the category of inhibitors of iron absorption by Morris (1983). It was postulated on the basis of the report by Cook and Monsen (1976), where substitution of beef by milk protein in a typical American diet reduced absorption of extrinsic labeled ^{59}Fe from 5.5 to 1.6%. In contrast, Carmichael et al. (1975) demonstrated that administration of nonfat milk enhanced the absorption of iron from ferric-nitrilotriacetate chelate and did not affect the absorption of iron from ferrous sulfate and ferric fructose in chickens and mice. Ranhotra et al. (1981) showed that bioavailability of iron from milk fortified with citrate phosphate iron complex was as high as iron from ferrous sulfate.

Park et al. (1986) compared iron bioavailability of goat milk with that of cow milk fed to anemic rats. They found that bioavailability of iron in goat milk was superior to that in cow milk when fed to anemic rats. For animals consuming whole goat milk supplemented with ferrous sulfate, the slope relating hemoglobin iron gained versus iron intake was 0.95. Respective bioavailabilities relative to ferrous sulfate were 54, 14, 28, and 14% for the whole goat, whole cow, skim goat, and skim cow milks.

Serum iron concentrations (SIC) in normal rats after oral administration of superdispersed ferric pyrophosphate (SDFe) and other iron compounds were determined (Juneja et al. 2004). Iron absorption determined by SIC curve averaged 113.4 μg/dL in the controls, which remained practically unchanged during the 5 days of experimental period after oral administration of the iron solutions of different iron compounds. They observed that during iron fortification, SIC rapidly increased and decreased after iron administration. The peak SIC at 30 minutes after oral administration reached 340.0 μg/dL for ferric pyrophosphate, 441.2 μg/dL for sodium ferrous citrate, and 444.4 μg/dL for ferrous sulfate 60 minutes postadministration, and then SIC rapidly declined in the controls (Fig. 17.6). The iron absorption peaks of SIC for the SDFe and commercial heme iron were delayed compared with those of other iron sources, reaching 388.8 and 471.6 μg/dL after oral administration. The SDFe group showed high SIC over 8 hours after oral administration (Fig. 17.6).

Zhang and Mahoney (1989b) tested iron bioavailabilities during 10-day feeding of cheddar cheeses, which were fortified with ferric chloride or iron-casein, ferripolyphosphate-whey protein, and iron-whey protein complexes in anemic and normal rats. They found the basal iron bioavailabilities (with normal adult female rats) for the respective iron sources were 5, 8, 6, and 7%, respectively, where the differences among the iron sources were not significant. The maximal bioavailability (with anemic weaning male rats) and basal bioavailability for the ferrous sulfate group was 85 and 5%, respectively.

In an iron and copper absorption trial, Campos et al. (2004) reported that the iron contents in liver

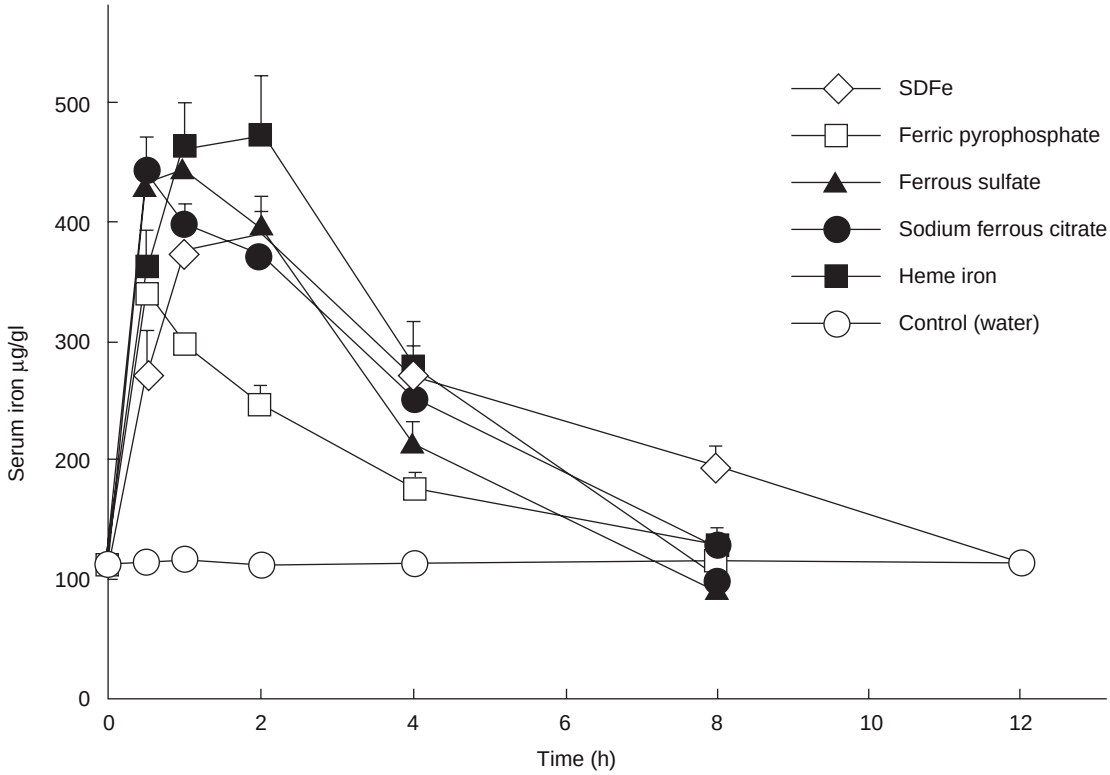

Figure 17.6. Serum iron level in normal rats after oral administration of SDFe and other iron compounds (2 mg Fe/kg body weight) (Juneja et al. 2004).

and spleen were higher in the standard diet and goat milk diet groups than in the cow milk diet group. The lower iron content in liver and spleen in rats fed with a cow milk diet may be explained by the observations of Hallberg et al. (1991), where they showed that the calcium derived from cow milk interferes with iron absorption in the diet, which could support the lower level of iron deposit in the organs. This effect did not occur in goat milk, which may not have calcium-iron absorptive interferences, because Park et al. (1986) showed that goat milk increased iron bioavailability.

In relation to the milk-based diets in resected animals, the copper deposits were higher in kidney, liver, sternum ($P < 0.001$), and spleen ($P < 0.05$) in rats fed a goat milk diet than those fed a cow milk diet. These results demonstrate that goat milk has beneficial effects on the metabolism of iron and copper in control and resected (resection of 50% of the distal small intestine) animals in comparison with those animals fed cow milk. The outcome of this study may indicate that goat milk has a good potential for use in human malabsorption syndrome of iron and copper. The beneficial effect of a goat milk diet on copper nutritive utilization may be attributed to the high medium-chain triglycerides (MCT) content (36%) with respect to a cow milk diet (21%) (Hartiti et al. 1995; Campos et al. 2004).

Hemoglobin Regeneration Efficiency

Hemoglobin regeneration efficiency (HRE) has been used to measure iron bioavailability by many researchers (Jones et al. 1975; Park et al. 1986; Zhang and Mahoney 1989b; Juneja et al. 2004). It is calculated as the percentage of consumed dietary iron incorporated into hemoglobin (Hb). The formula calculating HRE is as follows:

$$\text{HRE} = [\text{Final Hb-Fe (mg)} - \text{initial Hb Fe (mg)}] \times 100/\text{Dietary iron intake (mg)}$$

Jones et al. (1975) reported that ferripolyphosphate-protein powder is 92–100% efficient relative to ferrous sulfate in restoring hemoglobin levels (HRE) of iron-depleted rats and chicks. They also observed that the iron in sterile whole milk concentrates fortified with ferripolyphosphate-protein had 84–107% efficiency of iron utilization. No abnormal effects were found in rats fed a dietary intake of 720 ppm iron ad libitum from ferripolyphosphate-protein for a 90-day experimental period.

In studying iron bioavailability of goat milk in comparison with cow milk, Park et al. (1986) showed that the respective HREs for powdered whole goat milk, whole cow milk, skim goat milk, and skim cow milk were 50.6, 13.1, 26.0, 13.0%, indicating that iron bioavailability of goat milk was higher than cow milk (Table 17.7).

Although the goat milk diets contained less iron than the cow milk diets, the bioavailability of goat milk iron was higher than cow milk iron (Table 17.7). The differences in HRE between WGM and WCM or SCM were significant ($P < 0.01$), while those among WCM, SGM, and SCM were not different. All four diet groups had negative hemoglobin gain, which clearly shows goat and cow milk are deficient in iron. However, the degree of loss in hemoglobin was slightly greater in WCM than WGM, even though the WCM diet contained more iron. There were also significant differences in liver weight between WGM and WCM ($P < 0.01$) or SGM and SCM ($P < 0.05$) groups. Response of liver weight appeared to be parallel to that of whole body weight gain.

In the feeding trial with iron-fortified cheddar cheese to rats, Zhang and Mahoney (1989b) fed anemic weanling rats and normal adult rats to compare the HREs of iron-fortified basal experimental diets with those of iron-fortified cheddar cheese. Maximal and basal iron bioavailabilities were measured in anemic weanling rats fed low iron diets (about 22 mg iron/kg) and normal adult rats fed high iron diets (about 145 mg/kg) of iron density (32 mg iron/1000 kcal) found in some high iron human diets. Maximal iron bioavailabilities or HREs for ferric chloride or iron-casein, ferripolyphosphate-whey protein, and iron-whey protein com-

Table 17.7. Bioavailability responses of iron from goat and cow milk fed to anemic rats (Park et al. 1986)

Experimental Diet	WGM[a]	WGM[b] + $FeSO_4$	WGM[c] + $FeSO_4$	WGM[d] + $FeSO_4$	WCM[e]	SGM[f]	SCM[g]	SE[h]
Food intake, g/d	5.59	6.26	6.46	6.55	6.01	5.91	5.75	0.49
Total Fe intake, mg	0.468	0.776	1.072	1.625	1.164	0.638	0.734	0.046
Body weight								
Initial	70.6	70.7	73.4	71.4	71.4	72.0	74.0	1.01
Gain	23.0	30.1	28.7	30.2	18.8	31.2	26.3	0.78
Hemoglobin, g/dL								
Initial	5.78	5.42	5.82	5.75	5.71	5.74	5.79	0.11
Gain	−0.35	0.69	1.92	4.10	−0.44	−0.99	−1.02	0.24
Liver weight, g (wet)	3.52	3.65	3.75	3.76	2.93	3.51	3.23	0.05
Liver Fe, ppm (wet)	18.8	18.1	17.3	25.9	19.1	18.2	21.3	0.72
Hemoglobin Fe gain (mg)	0.237	0.518	0.805	1.333	0.153	0.166	0.098	0.055
HRE[i], %	51	67	75	82	13	26	13	

[a]Whole goat milk diet.
[b,c,d]Whole goat milk diets supplemented with 50, 100, 200 ppm ferrous sulfate, respectively.
[e]Whole cow milk diet.
[f]Skim goat milk diet.
[g]Skim cow milk diet.
[h]Standard error.
[i]Hemoglobin regeneration efficiency.

plexes were 85, 71, 73, and 72%, respectively, and for the respective iron-fortified cheddar cheese groups, they were 75, 66, 74, and 67%. The HRE and related hematinic values of the anemic rats fed iron-fortified cheeses are shown in Table 17.8. More than two-thirds of the dietary iron was incorporated into hemoglobin in anemic rats fed on iron-fortified cheese diets. There were no significant differences in HRE among the cheese diets. The HRE values of the iron-protein complexes were similar whether

Table 17.8. Bioavailability values for iron fortification sources and fortified cheeses fed to anemic rats[1]

		Iron-Fortification Sources				Iron-Fortified Cheeses			
Iron Sources	$FeSO_4$	$FeCl_3$	Fe-Casein	FIP-WP[2]	Fe-WP	$FeCl_3$	Fe-Casein	FIP-WP	Fe-WP
Diet number	2	5	7	9	11	13	14	15	16
Body weight, g									
Initial	87	91	87	88	91	88	87	92	88
Gain	41	35	39	38	39	40	40	41	40
Hemoglobin, g/dL									
Initial	5.35	5.30	5.37	5.40	5.37	5.42	5.33	5.40	5.37
Gain	6.05	6.61	4.69	5.04	5.28	4.78	4.17	4.44	4.38
Hb Iron gain, mg	1.89	1.95	1.46	1.56	1.67	1.53	1.31	1.48	1.40
Iron intake, mg	2.22	2.30	2.05	2.15	2.33	2.05	1.98	2.00	2.09
HRE[3], %	85	85	71	73	72	75	66	74	67

[1]Zhang and Mahoney (1989b).
[2]Ferripolyphosphate-whey protein.
[3]Hemoglobin regeneration efficiency.

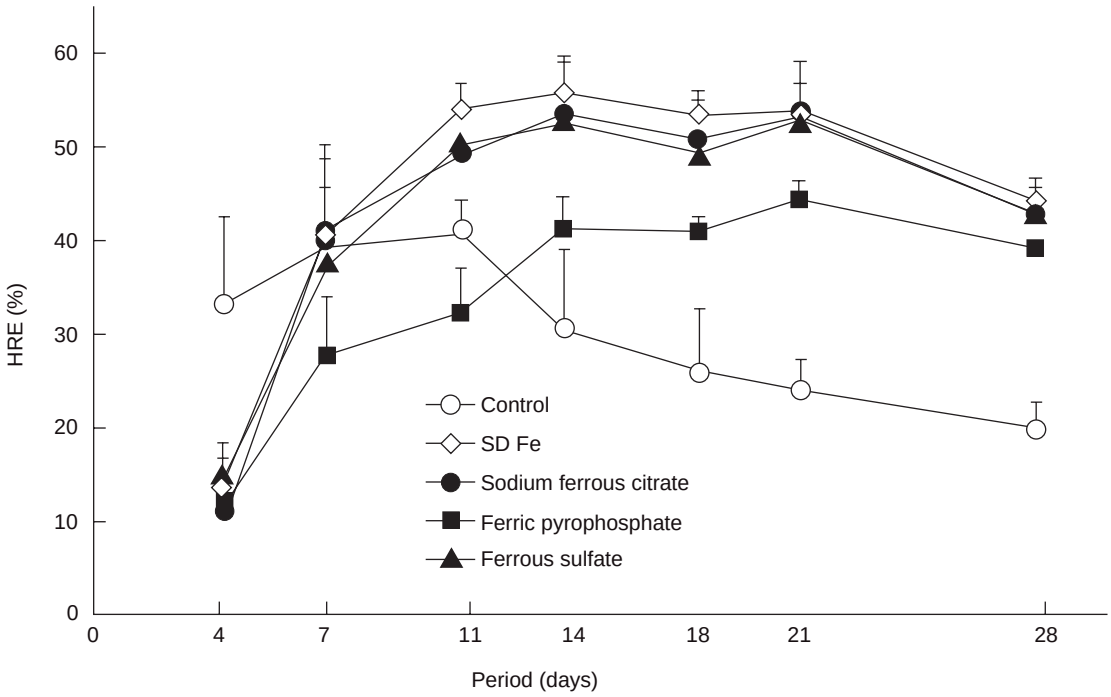

Figure 17.7. Hemoglobin regeneration efficiency (HRE) value in iron-deficient anemic and normal rats (dose: 3.5 mg Fe/100 g) (Juneja et al. 2004).

they were mixed directly into diets or in fortified cheese and then mixed into diets (Table 17.8). The HRE values were the same for diets supplemented with $FeSO_4$ or $FeCl_3$.

In evaluation of the relative biological values (RBV) of different iron compounds, Juneja et al. (2004) calculated RBV using HRE of superdispersed ferric pyrophosphate (SDFe), ferric pyrophosphate or sodium ferrous citrate divided by the mean HRE of ferrous sulfate. After two weeks of feeding trial of iron-fortified experimental diets, they found the respective HREs of the SDFe, ferric pyrophosphate, sodium ferrous citrate and ferrous sulfate were 55, 41, 53, and 53% (Fig. 17.7). The RBVs of iron sources were 1.05, 0.78 and 1.00 for SDFe, ferric pyrophosphate and sodium ferrous citrate, respectively. They concluded that SDFe exhibited the greatest value of HRE as well as RBV among the iron compounds tested (Fig. 17.7).

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