8 Milk-derived Bioactive Components from Fermentation

O.A. Alhaj¹ and A. Kanekanian²

¹Department of Food Science & Nutrition, College of Food and Agricultural Sciences, King Saud University, Kingdom of Saudi Arabia
²Centre for Nutrition, Dietetics and Food Science, Cardiff Metropolitan University, Cardiff, Wales, United Kingdom

8.1 Introduction

Nowadays, foods are no just considered by consumers as an energy supply to satisfy hunger and provide some nutritional needs, but are also expected to offer additional benefits beyond the traditional nutritional needs such as the prevention and minimisation of nutrition-related diseases and improvement of physical and mental well-being of the consumer (Menrad 2003; Mosilhey 2003). Milk is a complete food for newborn mammals during the early stages of rapid development (Shah 2000). Milk from different mammals including bovine (Ozer & Kirmaci 2010), camel (Alhaj & Alkanhal 2010), goat (Park & Haenlein 2006) and sheep (Park et al. 2007) were considered to be important functional sources and could provide a number of particular health benefits due to the presence of bioactive components.

Milk and dairy products contain a complex mixture of carbohydrates, fats, proteins, vitamins and minerals; these components were found to have bioactive properties with potential to improve human and animal’s health. Bioactive components could refer to the ‘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’ (Park 2009).

Milk contains 3.3–3.6% protein, of which casein is the major part (comprising about 80% of the protein). Casein exists in colloidal form with an average diameter of about 120 nm containing 93% proteins and 7% inorganic salts, mainly calcium and phosphate (Léonil et al. 2000). Casein micelles are classified into four major caseins: α-casein, constituting 40–45% of the casein complex with several variants, αs1, αs2, αs3, αs4 and αs5; β-casein, constituting 30–35% of the casein complex and classified into variants A, B and C; γ-casein is part of the β-casein chain with varying chain length and is classified into more than three variants; and κ-casein constitutes only 11–15% of the casein complex. These percentages differ between animal species such as cow and camel milk (Alhaj & Alkanhal 2010). Whey protein is the second major protein which is the soluble part of milk proteins that represent 0.6% of the milk.
proteins, in which 0.3% is β-lactoglobulin (β-lg), and is divided into the two variants A and B, and 0.07% is α-lactalbumin (α-la). The rest represent the other serum proteins such as bovine serum albumin (BSA), immunoglobulins, lactoperoxidase, lysozyme and lactoferrin.

### 8.2 Bioactive components in milk fat

A high consumption of milk and milk products is known to be one of the causes of coronary heart disease due to their high fat content, cholesterol and saturated fatty acids (SFA). Recent studies reported that out of 12 major fatty acids (FA), milk contains only three SFAs that are expected to be associated with raising total cholesterol in plasma (German & Dillard 2006). Numerous studies have reported that milk fat contains a number of bioactive components with certain biological function affecting human health which include sphingolipids and their metabolites, butyric acid and essential FAs, including conjugated linoleic acid (CLA), vitamins A, D, E and K, β-carotene (pro-vitamin A) and etheric lipids which have been shown to have a role in brain development (Ebringer et al. 2008). These components have been shown to have beneficial activities include anticancer (Parodi 1999; Vesper et al. 1999; Parodi 2001), antimicrobial (Possemiers et al. 2005), anti-inflammatory (German & Dillard 2006), anti-atherogenic (Mozaffarian et al. 2006), reduction of serum low-density lipoprotein (LPL) cholesterol (Vesper et al. 1999) and enhancement of immune system (Sugano et al. 1999).

#### 8.2.1 Conjugated linoleic acid (CLA) as a bioactive component

CLA is the most important bioactive component in milk fat and has received particular interest from researchers. The concentration of CLA in camel milk fat was reported to be higher (4.56 mg g⁻¹ fat) than that in bovine milk fat (Cardak et al. 2003; Alkhudair 2009). This compound has recorded a high content of up to 30 mg g⁻¹ fat in dairy products (O’Shea et al. 1998). A higher content of up to 53 mg g⁻¹ fat has also been reported (Collomb et al. 2006). The variation in CLA content in milk and dairy products could be attributed to several factors such as forage type (Chilliard et al. 2000; Bouattour et al. 2008), seasonal variation (Lock & Garnsworthy 2003), lactation stage (Jahreis et al. 1999) and animal species (cow, goat, camel, ewe, buffalo, etc.).

Among mammals, buffalo milk fat recorded the highest concentration of CLA of 4.83 mg g⁻¹ fat due to the high fat content in buffalo milk (Van Nieuwenhove et al. 2004). In this case, CLA content is believed to decrease according to the following order: buffalo > camel > sheep > ewe > cow > goat milk fat (Jahreis et al. 1999; Van Nieuwenhove et al. 2004; Alkhudair 2009).

CLA was found to be naturally occurring in milk through two different pathways. The primary pathway is through endogenous synthesis in the mammary gland which involves Δ⁹-desaturase of vaccenic acid in the lactating cows (Griinari et al. 2000). The second pathway is by bioconversion of polyunsaturated fatty acids in the rumen by some lactic acid bacteria such as Lactobacillus delbrueckii ssp bulgaricus and Lactobacillus acidophilus (Lin et al. 2005). In another study, five strains of lactic acid bacteria (Lactobacillus acidophilus DSM 9126, L. delbrueckii subsp. bulgaricus DSM 20080, Bifidobacterium longum subsp.
Infantis DSM 20088, B. angulatum DSM 20098, Propionibacterium freudenreichii subsp. freudenreichii DSM 20271) were able to increase the concentration of CLA in camel and cow milk (Alkhudair 2009). The predominant CLA in milk and dairy products were reported to belong to the CLA isomer 9cis, 11trans octadecadienoic acid, which comprise more than 80% of the total CLA intake in the human diet (Lawson et al. 2001; Collomb et al. 2006).

CLA is reported to possess a number of health benefits in animals and human (Bhattacharya et al. 2006). Studies reported that butter supplemented with CLA has shown to lower serum cholesterol concentration in rodents (Lock et al. 2005) and reduce body fat mass, body weight, plasma total cholesterol and LDL in human subjects (Gauvillier et al. 2005). Similar studies have been shown to reduce cancer risk in rats (Ip et al. 1999; Parodi 1999; Corl et al. 2003) as well as having an antitumour activity in human breast cancer cells (Tanimahanasamut et al. 2004); the possible mechanism of actions were reported by Kelley et al. (2007). Long-term feeding (14 weeks) of CLA may enhance immune system and increase the mucosal IgA immune response in rats (Ramírez-Santana et al. 2009). CLA was also found to work as anti-obesity supplement to reduce body weight in mice (Kanaya & Chen 2010). In another study on mice, dietary CLA was found to reduce allergic airway inflammation (Jaudszus et al. 2008) and reduce inflammation associated with collagen-induced arthritis (Huebner et al. 2010).

In contrast, a diet rich in CLA and butter was reported not to have an anti-inflammation effect (Raff et al. 2008) and have no effect on blood pressure and isobaric arterial elasticity on healthy young men (Raff et al. 2006).

8.3 Oligosaccharides as bioactive components in milk

Lactose is known to be the main source of sugar in milk; other minor forms of carbohydrates were also found in milk including neutral and acidic oligosaccharides, glucose, fructose and glucosamine (Fox & McSweeney 1998). Oligosaccharides are a class of carbohydrates and constitute 3–10 monosaccharide units (Mussatto & Mancilha 2007). A large amount and variety of neutral and acidic oligosaccharide were isolated from milk and colostrum of human (Kunz et al. 2000, 2001) and domestic farm animals (Urashima et al. 2001; Nakamura & Urashima 2004; Fukuda et al. 2010). Differences were reported between the oligosaccharide structure of human milk and domestic animal milk (Urashima et al. 1997). Similarly, significant homology and heterogeneity changes were also found between bovine and camel milk oligosaccharide (Fukuda et al. 2010).

For example, Sialyl -3’-galactosyllactose and sialyllacto-N-novopentaose-a have been found in camel milk but not in human milk/colostrum (Fukuda et al. 2010; Alhaj et al. 2013). On the other hand, 3’-GL, LNNH, 3’-SL, 6’-SL and MSLNNH oligosaccharides are present in both dromedary camel milk and human milk (Alhaj et al. 2013). These oligosaccharides, Sialyllacto-N-novopentaose-a, LNNH and MFLNNH, containing Gal(b1–4)GlcNAc (N-acetyllactosamine), are categorised as Type II oligosaccharides (Alhaj et al. 2013). However, only type II oligosaccharides were found in camel’s milk. Hence, the presence or absence of Type I oligosaccharide is believed to be the main significant difference between human and camel or bovine milk oligosaccharide such as those based on a mixture of monosaccharides with β 1–3 and β 1–4 glycosidic bonds and other compounds (Fukuda et al. 2010).
The number of oligosaccharides identified in human milk was about 50 compared to that in bovine and caprine milk of about 30 (Saito et al. 1984; Martínez-Férez et al. 2006). Recently, thirteen oligosaccharides were characterized in Bactrian camel colostrums/milk (Fukuda et al. 2010), and Seven oligosaccharides Dromedary camel milk (Alhaj et al. 2013). Recently, 10, 3, 7 oligosaccharides were characterised in Bactrian camel colostrums/milk (Fukuda et al. 2010), as well as dromedary camel milk (Alhaj et al. 2013). The concentration of oligosaccharide in animal’s milk was found to decrease during lactation compared to human milk (Finke 2000; Martin et al. 2001; Fukuda et al. 2010) however, although oligosaccharides in late lactation were reported to increase in bovine milk (Martin et al. 2001) and decrease in human milk (Kunz et al. 2001).

Human milk oligosaccharides are thought to be beneficial to the gut of breast-fed infants in terms of being important growth-promoting factors for the bifidus flora (Gori Cambrodon and Gudial-Urbano 2001). The 3’-GL oligosaccharide is present in dromedary camel milk and considered as a prebiotic component, which may suggest that dromedary camel milk oligosaccharides contains prebiotic and could be used as a food additive with this function (Urashima et al. 2009; Alhaj et al. 2013). The potential health benefits of milk oligosaccharides and their mechanisms are reported by Kunz & Rudloff (2006) and Qiang et al. (2009). These benefits are summarised as follows: stimulation of the growth of bifidobacteria and lactobacilli in the gastrointestinal tract; inhibition of the growth of pathogenic micro-organisms in the vagina; reduction of obesity and diabetes Type 2; reduction of cardiovascular risk; improvement of immune system; protection against cancer and tumours in man; influence availability and absorption of minerals; and reduction of diarrhoea.

8.4 Milk proteins as a source of bioactive peptides

Bioactive peptides have been defined as ‘peptides produced in vivo or in vitro by enzymatic hydrolysis of food proteins with biological functions or physiological effects’ (Gill et al. 1996). Later, Meisel (2004) defined bioactive substances from food as being ‘components (genuine or generated) of consumption ready food which may exert regulative activities in human beyond basic nutrition’. The most recent definition of bio-functional or bioactive peptides was introduced by FitzGerald & Murray (2006) as ‘peptides with hormone-or drug-like activity that eventually modulate physiological function through binding interactions to specific receptors on target cells leading to induction of physiological responses’.

Various foods were found to contain bioactive peptides encrypted in their primary structure such as dairy product, wheat gluten, eggs, rice, corn, soybean proteins and fish (Kitts & Weiler 2003). Moreover, milk is the most important source of bioactivities because most of the known bioactive peptides are derived from milk protein fragments due to minor proteins and peptides secreted into milk in active form by the mammary gland (Meisel 2004). For a long time, milk proteins were considered to only provide nutrition components such as essential amino acids for young mammals (Hambræus 1992). In the last decades, several studies have shown that milk proteins are an important nutritional and functional source and could possess a range of biological functions, depending on the amino acid sequence of the bioactive peptide. Milk proteins are not only important because of their high nutritional value but because of their availability in the markets in large amounts at moderate cost (Léonil et al. 2000).
Bioactive peptides have received special attention from researchers. A number of natural bioactive peptides have already been found in bovine milk reported and were discussed by Xu (1998) including: ‘epidermal growth factor (EGF), transforming growth factor (TGF), nerve growth factor (NGF), insulin and insulin like growth factors I and II (IGF- I and IGF- II)’. Similarly, camel milk was also reported to contain naturally occurring antibodies (Hamers-Casterman et al. 1993). However, bovine milk proteins are currently recognised as the main source of bioactive peptide, specially caseins since they provide a large number of bioactive peptides (Meisel 2004).

Bioactive peptides could be produced in large quantities by the proteolysis of individual caseins and whey proteins (Meisel 1998). In general, most of the milk-protein-derived bioactive peptides contain 3–20 amino acid residues per molecule (Korhonen & Pihlanto 2003). Some angiotensin converting enzyme (ACE) -inhibitory peptides were found to contain 23 amino acids (Otte et al. 2007b). These bioactive peptides have single or multifunctional properties and naturally exist in the milk (Meisel & Bockelmann 1999). Some of these bioactive peptides are fully active through protein structure, while others are usually inactive in their precursor state and can only be liberated in vivo by gastrointestinal digests or in vitro by digestive or microbial proteolytic enzymes (Meisel & Bockelmann 1999; Korhonen & Pihlanto 2003).

These bioactive peptides can also be generated during the manufacturing process of milk by naturally occurring enzymes (Gobbetti et al. 2002). Some types of ripened cheeses are good for example for ‘naturally occurring functional foods’ because some bioactive peptides could be naturally generated during their manufacturing process by the microbial proteolysis of the cheese culture (Meisel 2004). However, the liberated bioactive peptides may become inactive after further proteolysis by the cheese culture during long ripening periods (Meisel et al. 1997). For example, medium-aged Gouda has double the ACE-inhibitory activity than that found in longer-ripened Gouda (Smacchi & Gobbetti 2000). Milk could also be the best carrier of these bioactive peptides because feeding fermented milk containing these bioactive peptides demonstrated more bioactivity effect than feeding them purely water. For example, long-term feeding of milk fermented with strains of Lactobacillus helveticus containing Val-Pro-Pro and Ile-Pro-Pro peptides to spontaneously hypertensive rats (SHR) attenuated blood pressure more than feeding them purely in water. This is due to the presence of other factors in fermented milk that affect blood pressure such as mineral availability (Sipola et al. 2001).

### 8.4.1 Health benefits of milk proteins and their derived bioactive peptides

It has been reported that bioactive peptides derived from milk proteins could provide several health benefits including: ACE-inhibitory action (Yamamoto et al. 1994; Clare & Swaisgood 2000; Lv et al. 2003; Minervini et al. 2003; Gobbetti et al. 2004; Quan et al. 2008; Alhaj et al. 2012); opioids peptides (Fiat et al. 1993; Rokka et al. 1997; Clare & Swaisgood 2000; FitzGerald & Meisel 2003, p. 676); metal binding (Holt 1994); reuduction of the risk of obesity and development of diabetes Type 2 (Erdman et al. 2008; Warensojo et al. 2010) and diabetes Type 1 (Agrawal et al. 2003); immunostimulating peptides (Migliore-Samour et al. 1989; Coste et al. 1992); antimicrobial peptides (El-Agamy et al. 1992; Liepke et al. 2001; Benkerroum et al. 2004; McCann et al. 2006); inhibitory effect against hepatitis C.
virus (Almahdy et al. 2011); peptides inhibitory to HIV-1 Proteinase (Gobbetti et al. 2002); antithrombotic peptides (Jollès & Henschen 1982; Mazoyer et al. 1992; Fiat et al. 1993); bone formation (Narva et al. 2004); anticancer (LeBlanc et al. 2005); and lowering of cholesterol level in blood (Nagaoka et al. 2001), in vivo in rats (Elayan et al. 2008) and in vitro (Alhaj et al. 2010). The mechanisms of these health benefits were reviewed by Gobbetti et al. (2002) and Alhaj & Alkanhal (2010).

The most-favoured bioactive peptides applied in foodstuffs formula to provide health benefits are ACE-inhibitory peptides, caseinophosphopeptides and immunomodulating peptides (Meisel & Bockelmann 1999).

8.4.2 Peptide synthesis

Peptide synthesis has been applied to the preparation of bioactive peptides; the selection of the most suitable method mainly depends on the length and the quantity of the desired peptide (Korhonen & Pihlanto 2003). Methods used for peptides synthesis have been reviewed by Gill et al. (1996). Three approaches are currently available for peptide synthesis. (1) Total enzymatic synthesis, which is relatively inexpensive and is currently limited to short peptides. (2) In contrast, recombinant DNA technology is the most suitable method for large peptides and proteins and could also be used for products consisting of up to several hundred amino acids (Wetzel & Goeddel 1987; Harford 1988). The application of this method requires an expensive research and development phase but, once the system is established, large quantities of the product could be obtained from very inexpensive starting materials via fermentation (Gill et al. 1996). (3) Chemical synthesis is the most widely used method on a laboratory scale (Korhonen & Pihlanto 2003). Two types of this method are currently available: liquid phase synthesis, which is used for the large-scale synthesis of relatively short peptides; and solid-phase synthesis, an expensive method because its instruments and reagents are restricted to this method (Gill et al. 1996). However, the latter method is still the most powerful method for synthesis of peptides composed of about 10 to >100 residues on a small scale and used for the rapid production of peptide libraries for screening purposes (Valembois et al. 1992).

8.4.3 Milk protein hydrolysate

The determination of casein and whey proteins fractions and their degradation products was a major task in the field of dairy science for several years and can provide valuable information (Molina et al. 2000; Veloso et al. 2002; Tanabe et al. 2003). It is known that protein hydrolysate can be made by breaking down the peptide bonds in proteins by individual or combined proteolytic enzymes to form a mixture of different length of peptide chains and some amino acids. However, some regions in the primary structure of casein are partially protected from proteolytic breakdown, therefore considered as strategic zones (Fiat & Jollès 1989). These regions exhibit different biological effects because they contain overlapping peptide sequences (Gobbetti et al. 2002). Peptides from casein hydrolysates were found to be more effective as an ACE-inhibitor than whey-protein-derived hydrolysate peptides.
Milk-derived Bioactive Components from Fermentation

(Pihlanto-Leppälä et al. 1998; Otte et al. 2007a). However, the inhibition of ACE by milk protein peptides is the most-studied mechanism among scientists.

Currently, protein hydrolysates can be classified into three major groups according to their degree of hydrolysis, determining their applications: hydrolysates with a low degree of hydrolysis are used to improve the functional properties of the product; hydrolysates with a variable degree of hydrolysis are mostly used to enhance taste; and hydrolysates with a high degree of hydrolysis are mostly used as nutritional supplements and nutraceutical compounds in functional food production (Pedroche et al. 2004).

Biologically active peptides were reported by FitzGerald & Meisel (2003, p.675) and Korhonen & Pihlanto (2003) to be released from milk proteins in vivo and in vitro as: (1) in vivo digestion by gastrointestinal proteinase/peptidases; (2) in vitro enzymatic hydrolysis with digestive enzymes such as proteinase and exopeptidases; and (3) proteinase and peptidase enzymes produced by bacteria during the processing of dairy products including fermentation.

**Bioactive peptide formation in the gastrointestinal tract**

The formation of bioactive peptides from proteins and oligopeptides in dairy products can be produced by digestion (Hernández-Ledesma et al. 2004). In the gastrointestinal tract, long oligopeptides are further degraded by digestive enzymes, leading to the release of possible bioactive peptides (Korhonen & Pihlanto 2003). On the other hand, the biological activity of partially hydrolysed bioactive peptides could be decreased or eliminated if further digestion takes place by intestinal proteinases or brush border peptidases (Meisel & Bockelmann 1999). Various bioactive peptides were detected in vivo in the human and animal intestine after ingestion of milk, yogurt or bovine casein. In humans, caseinophosphopeptides (CPPs) were found in the stomach and duodenum of adults after ingestion of milk or yogurt (Chabance et al. 1998). Meisel et al. (2001) reported the presence of CPPs in the distal small intestine (ileum) of adults after milk and CPP meals ingestion. Similarly, β-Casomorphins were found in the intestinal contents of humans after bovine casein ingestion (Svedberg et al. 1985). In animals, the opioid peptide β-Casomorphins were found in the intestinal contents of minipigs (Meisel 1986) and of pre-ruminant calves after milk feed (Scanff et al. 1992).

**Bioactive peptide absorption in the gastrointestinal tract**

The health benefits of bioactive peptides that are either already present in food or produced in vivo by the action of intestinal proteinases could be exhibited locally at the luminal side of the gastrointestinal tract (Meisel & Bockelmann 1999), or they reach their organ target to offer the health benefit. In the latter case, bioactive peptides should survive/remain intact during their passage through the gastrointestinal proteinases/peptidases, luminal intestinal peptidases and serum peptidases (FitzGerald & Meisel 2003, p. 687).

The route for providing health effect is explained in Figure 8.1). When bioactive peptides pass through the gastrointestinal tract, they should be absorbed through the gastrointestinal epithelial membrane into the bloodstream to end up in the target organ to exhibit their biological effect (Korhonen & Pihlanto 2003). However, it seems that the number and size of
the bioactive peptides derived from casein after milk and yogurt digestion decrease in adult humans between the stomach and the end of duodenum (Chabance et al. 1998).

It has been reported that peptides with a short chain such as di- and tripeptides were found to be easily absorbed in the intestine and reach the target organ without further hydrolysis by digestive enzymes (Meisel & Bockelmann 1999; Shah 2000), especially peptides containing proline (Kim et al. 1972). For example, small ACE-inhibitory peptides (Val-Pro-Pro and Ile-Pro-Pro) can pass through the intestine to reach peripheral target sites without being decomposed by digestive enzymes (Yamamoto 1997). The absorption of these two peptides through the gastrointestinal tract was detected in vitro (Masuda et al. 1996) and in vivo (Satake et al. 2002). ACE-inhibitory peptides were found to remain stable in commercial products (Hernández-Ledesma et al. 2004) and in yak milk casein hydrolysate (Mao et al. 2007). These short ACE-inhibitory peptides were shown to remain intact, and no further hydrolysis by digestive enzymes in SHR intestine will take place; they are absorbed directly and exhibit ACE-inhibitory activity in the abdominal aorta (Masuda et al. 1996). However, peptides with longer-sequence (12) amino acids such as CGP and other antithrombotic peptides were found to be absorbed through the adult gastrointestinal tract after yogurt and milk intake (Chabance et al. 1998).

Several studies have shown that amino acids produced by protein hydrolysates are absorbed faster and more completely than the intact proteins (Lopes et al. 2005). The absorption of small peptides have been reported by Gill et al. (1996), Tholstrup (2006) and reviewed by Fricker & Drewe (1996). However, after protein digestion, peptides could be absorbed through the gastrointestinal membranes by different mechanisms such as passive diffusion through the enterocytes and paracellular pathway or through cytotic mechanisms via dipeptide carrier or secretion back into the intestinal lumen by p-glycoprotein (Fricker & Drewe...
Not all bioactive peptides are absorbed or pass through to the bloodstream in human adults due to the prevention of digestion processes and intestinal barriers (Chabance et al. 1998). Some peptides could express their biological activity in the gastrointestinal tract without being absorbed, such as caseinophosphopeptides (CPPs) (Narva et al. 2003). Other researchers reported the potential effect of a small quantity of casein hydrolysate peptides on lipid metabolism when entering the circulation system through the lumen (Asato et al. 1994).

Production of bioactive peptides in milk by enzymatic hydrolysis

Basically, bioactive peptides can be produced not only by starter cultures but also by enzymatic hydrolysis during the manufacture of cheese, yogurt and other dairy products. Enzymatic hydrolysis is used as a successful method to produce active hydrolysate. This method is considered to be the most common way to produce bioactive peptides and has been shown to improve the nutritional and functional properties of milk proteins (Léonil et al. 2000; Gauthier & Pouliot 2003; Korhonen & Pihlanto 2006). Controlled protein hydrolysis can also be used to produce bioactive peptides (Mao et al. 2007). The physicochemical properties of protein hydrolysate have been reviewed by Gauthier & Pouliot (2003).

However, several studies have reported on the use of numerous proteolytic enzymes in vitro to release the bioactive peptides from their parent milk protein by hydrolysis with pancreatic proteinases, especially trypsin. Nagaoka et al. (2001) was the first to identify novel hypocholesterolemic peptides Ile-Ile-Ala-Glu-Lys from β-lg by tryptic hydrolysis. The antioxidant activity of camel α-la by enzymatic hydrolysis was recently studied by Salami et al. (2009) and shown to be greater than that of bovine α-la. Some peptide length and sequences such as Ile-Val-Gly-Arg-Pro-Arg-His-Gln-Gly (9 amino acids) were found not to possess ACE-inhibitory activity when administered intravenously to spontaneously hypertensive rats (SHR). However, when subjected to trypsin hydrolysis and converted into shorter peptides e.g. Ile-Val-Gly-Arg-Pro-Arg, it showed ACE-inhibitory activity after oral administration to SHR (Li et al. 2004), indicating that trypsin hydrolysis was essential to release the ACE-inhibitory peptide from protein chain. Moreover, the level of bioactivity was found to essentially depend on the degree of hydrolysis of protein (Alhaj et al. 2010).

Trypsin is synthesised in the pancreas as trypsinogen; when this inactive proenzyme enters the small intestine it is converted into its active form as trypsin by enterokinase to hydrolyse proteins and polypeptides into smaller peptides and amino acids (Lehninger 1982, p. 688). Trypsin has a narrow specificity and cleaves proteins at bonds, preferentially the carboxylic side of the basic amino acid residues of lysine and arginine (Antal et al. 2001; Pedroche et al. 2004). The optimum pH of trypsin is 7–8 but it functions best at pH 7.7.

Other endoproteinases enzymes were reported to be used individually or in combinations to generate bioactive peptides, including: pepsin, chymotrypsin, papain, thermolysin, actinase, pancreatin, elastase, alcalase and carboxypeptidase, and proline specific endopeptidase are also used (Pihlanto-Leppälä et al. 1994; Abubakar et al. 1998; McDonagh & FitzGerald 1998; Gobbetti et al. 2002; Gauthier & Pouliot 2003; Mizuno et al. 2005; McCann et al. 2006).

In general, digestive enzymes are preferred for producing bioactive peptides for traditional batch hydrolysis on a large scale than proteinases produced by micro-organisms through fermentation because the process is rapid and cheap and a very small quantity of enzyme is
required to hydrolyse large amount of proteins in a short time. Some of the disadvantages of the digestive enzymes are being difficult to control, the narrow specificity and the need to add more enzyme due to their inactivation during the process. It has been stated by Amiot et al. (2004) that during hydrolysis, enzymes could be inhibited by peptides (Alder-Nissen 1986) and by peptide–peptide interactions (Pouliot et al. 2000) and this could also affect the overall hydrolysis level.

In contrast, proteinases produced by cultures can be costly, time consuming to produce and show low activity because the culture is nutritionally fastidious and the need to release their nutrients from the media to keep their growth. This type of hydrolysis is mainly controllable and produces favourable peptides, and their hydrolysis is gradual and non-specific because the culture produces more than one enzyme.

Formation of bioactive peptides in milk by microbial proteolysis

Biologically active peptides can be isolated in large numbers from bacterial, fungal, plant and animal origins (Gill et al. 1996). A number of starters, non-starter bacteria and yeasts were used to produce bioactive peptides from milk proteins. Researchers have reported the ability of some lactic acid bacteria, but not all tested strains, to produce bioactive peptides from milk proteins. The most-used lactic acid bacteria are the probiotic ones which produce bioactive peptides from different dairy products including Lactobacillus helveticus + Saccharomyces cerevisiae, Lactobacillus acidophilus, Lactobacillus casei TMC0409 + Streptococcus thermophilus TMC 1543, Lactobacillus delbrueckii subsp. bulgaricus SS1 + Lactococcus lactis subsp cremoris FT4, Lactobacillus strain GG, Bifidobacterium longum BB536, B. breve and Bifidobacterium animalis subsp. lactis (Bb-12).

Bioactive peptides can be produced using single or combined cultures such as the formation of ACE-inhibitory peptides after milk fermentation using a single strain such as Lactobacillus helveticus (Yamamoto et al. 1994) or two dairy strains such as Lactobacillus delbrueckii subsp bulgaricus and Lactococcus lactis subsp cremoris (Gobbetti et al. 2000). Yogurt and cheese cultures were also shown to produce different bioactive peptides in milk such as ACE-inhibitory peptides during fermentation and ripening (Gomez-Ruiz et al. 2002; Fuglsang et al. 2003; Donkor et al. 2007; Ong et al. 2007; Alhaj et al. 2012).

Combining cultures with different digestive enzymes has also been used to produce bioactive peptides. For example, UHT milk fermented with Lactobacillus GG leads to the production of several oligopeptides, resulting in the formation of bioactive peptides only after further digestion with pepsin or trypsin (Rokka et al. 1997).

Fermentation or co-fermentation is the most effective way to produce bioactive peptides in large quantities by microbial proteolysis, such as in milk-based fermented dairy product. For example, the production of the product Yakult® undertakes fermentation process for 7 days to cultivate the number of Lactobacillus casei Shirota bacteria and increase the concentration of bioactive peptides in the product (Yakult, personal communication, 2007). The long storage of 3–4 weeks with the microbial culture might result in the disappearance of some peptides of interest, and new peptides may appear due to the microbial proteolysis (Gobbetti et al. 2000).

However, several peptides with bioactivity derived from milk proteins released by microbial proteolysis have been reported by researchers, include immunostimulatory, ACE-inhibitory and antioxidative peptides (FitzGerald & Meisel 2003, p. 675; Korhonen
Milk-derived Bioactive Components from Fermentation

& Pihlanto 2006). In another study, the identification of two ACE-inhibitory peptides Val-Pro-Pro and Ile-Pro-Pro from milk fermented with strains of Lactobacillus helveticus and Saccharomyces cerevisiae have been reported by Nakamura et al. (1995). Also, the identification of ACE-inhibitory dipeptide (Tyr-Pro) from yogurt-like product fermented by Lactobacillus helveticus CPN 4 strain were reported by Yamamoto et al. (1999).

Alhaj et al. (2012) identified nine ACE-inhibitory peptides from fermented camel milk using two strains of Lactobacillus spp. Lactobacillus helveticus solely released three peptides of the sequences (LSLSQF, SLSQF or SQF and KVLVPQP), three peptides of the sequences (TDLEN, DLEN or LEN and LHLPLPL) and one peptide of the sequence (KVLPVVPQVMPYQP). Lactobacillus acidophilus, on the other hand, solely released two peptides FQEPFPDPVR and VLPFQEPVPDPVRG. All identified peptides correspond to β-casein of Camelus dromedarius milk. The level of bioactivity was found to essentially depend on the degree of hydrolysis of protein (Alhaj et al. 2010). Lactic acid bacteria including lactococci and lactobacilli (26 strains) were shown to have a proportional relationship between ACE-inhibitory activity and peptides release (Fuglsang et al. 2003).

Proteolytic activity of lactic acid bacteria

The purpose of adding any lactic acid bacteria (LAB) to milk is mainly based on producing lactic acid as a major end-product and, to a lesser degree, to degrade caseins and whey proteins into smaller peptides and amino acids. The presence of nitrogen in several food ecosystems is therefore considered as a limiting growth factor for lactic acid bacteria (Laan et al. 1989). Some lactic acid bacteria such as Lactococcus lactis could use oligopeptides as the main source of nitrogen during their growth in milk (Juillard et al. 1995b). On the other hand, Lactobacilli use some amino acids as an alternative source of energy to enhance their growth, such as arginine which is already produced from the primary breakdown of milk casein during cheese ripening (Kask et al. 1999; Laht 2003). In milk, the concentrations of essential amino acids are very low (Mills & Thomas 1981). Caseins are therefore the main source of amino acids for lactic acid bacteria in milk (Juillard et al. 1995a) and cheese (El-Soda et al. 1992). In the case of yogurt, when Lactobacillus delbrueckii subsp. bulgaricus grows this strain produces peptides and amino acids from milk proteins to encourage the growth of Streptococcus salivarius subsp. thermophilus (Tamime & Robinson 1985, p. 277). The formic acid produced by S. salivarius subsp. thermophilus and formed by milk heat treatment could accelerate the growth of L. delbrueckii subsp. bulgaricus (Tamime & Robinson 1985, p. 279; Adams & Moss 2000, p. 326), so both yogurt cultures utilise each other’s metabolites for more efficient growth and acid production (Adams & Moss 2000, p. 326).

Although peptides are more favourable for the growth of lactic acid bacteria than mixtures of pure amino acids, the available amounts of free amino acids and small peptides in milk are only enough for the growth of 5–25% of the full grown culture (Juillard et al. 1995a). LAB have the ability to use milk casein as a source of amino acids and nitrogen because of their complex system (Shihata & Shah 2000). For example, Lactococcus lactis could cleave casein into amino acids and make it available for their growth through a three-step process (Wohlrab & Bockelmann 1992; Mierau et al. 1997). First, casein is degraded into peptides by extracellular proteinase (PrtP). In the second step, several of these released peptides are incorporated into the cell via an oligopeptide transport system (Opp) and finally, the released
amino acids from these peptides could be utilised for other processes such as protein synthesis and the generation of metabolic energy.

As mentioned earlier, LAB has a complex proteolytic system to release the required nutrients by the synthesis of various enzymes such as a cell-wall-bound proteinase and also produce a number of intracellular peptidases including one or two endopeptidases. Some of these enzymes include aminopeptidases, iminopeptidase, dipeptidase, tripeptidase, prolidase, carboxypeptidase and prolinase (Bockelmann et al. 1995; Law & Haandrikman 1997; Christensen et al. 1999; Shihata & Shah 2000). At least 16 peptidases responsible for the conversion of peptides to free amino acids have been identified and biochemically characterised from LAB (Christensen et al. 1999).

The proteinase and peptidase enzymes of lactic acid bacteria are either bound to the cell wall or are intracellular and can only contribute after cell lysis (Law & Haandrikman 1997). Endopeptidases gained much attention because of their ability to hydrolyse peptide bonds within the oligopeptide (Janer et al. 2005). Lactic acid bacteria require significant amounts of essential amino acids and peptides for their growth and therefore need to hydrolyse casein and whey proteins into smaller parts to enhance maximum cell growth in milk.

Yogurt culture (Lactobacillus delbrueckii subsp. bulgaricus and S. thermophilus) was found to have higher proteolytic activity than Lactobacillus acidophilus and Bifidobacterium spp. (Shihata & Shah 2000) and found to produce more β-D-galactosidase than probiotic bacteria (Shah & Jelen 1990; Shah 1994). This could explain ‘why yogurt bacteria grow faster than probiotic bacteria and why yogurt bacteria are used as the main starter bacteria and probiotic bacteria as an adjunct starter’ (Shah 2001). It has been shown that proteolysis enhances the growth of probiotic bacteria in fermented products. For example, it was reported that the presence of traditional yogurt starter cultures shortens the fermentation time, enhances the growth of probiotics, increases the population number, increases their proteolytic activities and reduces the oligosaccharide content; this is due to the supply of exogenous small peptides and essential amino acids, which are usually absent or present in very low concentration and do not support their optimal growth in milk (Law et al. 1976; Dave & Shah 1997a; Gomes et al. 1998; Shihata & Shah 2000; Wang et al. 2006) or could be due to the equipollence relationship between each other.

Nevertheless, lactic acid bacteria are capable of degrading different proteins and peptides to supply the required nutrients, while end-products depend on the proteolysis system of the bacterial species or enzyme capacity and specificity. However, any changes in lactic acid bacteria metabolism or growth conditions result in different-end products. Some lactic acid bacteria proteinases such as Lactobacillus sp. and Lactococcus sp. could cleave more than 40% of peptide bonds of β-casein, leading to the release of more than 100 different oligopeptides (Juillard et al. 1995a; Mierau et al. 1997). Similar hydrolysis was obtained with α-casein (Kunji et al. 1996). These oligopeptides will be further degraded into smaller peptides by LAB peptidase and become the main source of bioactive peptides in fermented milk. When these bioactive peptides or peptides related to the bioactive peptides sequence are liberated, the biochemical activity of microbial communities might be influenced (Gobbetti et al. 2002). These peptides should be carefully conditioned because they have the ability to inhibit the microbial proteolysis during ripening (Gobbetti et al. 1995). For example, peptides from fresh short-ripened and long-ripened cheeses have been shown to inhibit the endopeptidases
of *Lactobacillus*, *Streptococcus* and *Lactococcus* strains, but have less effect on aminopeptidase activity (Gobbetti *et al.* 1995; Smacchi & Gobbetti 1998).

In general, *Lactobacilli* have complex nutritional requirements for organic substrates, amino acids and peptides, and subsequently possess stronger proteolytic activity than *Lactococcus* (Thomas & Mills 1981; Kandler & Weiss 1986) and *Bifidobacterium* spp. (Shihata & Shah 2000) and yet the released peptides are further hydrolysed by some peptidases (Meyer & Jordi 1987). On the other hand, *lactobacilli* have very limited capacity to synthesise amino acids from inorganic nitrogen sources, depending on amino acids present in the growth environment (Axelsson 1998; Christensen *et al.* 1999).

It has been reported that *Lactobacillus helveticus* ATCC 15009 requires all amino acids with the exception of four (alanine, cysteine, glycine and serine; Morishita *et al.* 1981). *Lb. helveticus* is therefore considered a good example of high proteolytic activity. This strain has a very efficient proteolytic system including general aminopeptidase, X-prolyl-dipeptidyl aminopeptidase, endopeptidase-proteinase and probably dipeptidase, tripeptidase, proline iminopeptidase and carboxypeptidase (Sasaki *et al.* 1995; Christensen *et al.* 1999).

**Proteolytic activity of *Bifidobacterium* spp.**

*Bifidobacterium* strains have low proteolytic activity when compared to other lactic acid bacteria (Klaver *et al.* 1993; Shihata & Shah 2000). *Bifidobacterium animalis* subsp. *Lactis*, as for many other *Bifidobacterium* species, are unable to synthesise some amino acids. In addition, milk does not contain sufficient amounts of free amino acids and small peptides for their growth (Abu-Taraboush 1996). They therefore need to hydrolyse milk proteins and utilise their products such as amino acids and peptides for their optimum growth. As a result, the level of the amino acids and peptides increase. So far, there has been very little information available in the literature regarding the proteolytic enzyme systems of *Bifidobacterium* spp. (Janer *et al.* 2005). *Bifidobacterium* spp., as with other lactic acid bacteria, do have some general aminopeptidase activity including a number of dipeptidase and possible iminopeptidase and tripeptidase (El-Soda *et al.* 1992; Shihata & Shah 2000). Cheng & Nagasawa (1983, 1984) isolated aminopeptidase and iminopeptidase activity from a cell-free extract of *B. breve*. The exopeptidase system of several *Bifidobacterium* strains was demonstrated by Minagawa *et al.* (1985) and El-Soda *et al.* (1992). Three kinds of aminopeptidase and carboxypeptidase were measured in five strains of *Bifidobateria* (Minagawa *et al.* 1985). The presence of aminopeptidase, dipeptidase, tripeptidase and carboxypeptidase activities from *B. longum*, *B. infantis* and *B. adolescentis* were demonstrated (El-Soda *et al.* 1992).

Recently, the proteolytic system of *Bifidobacterium animalis* subsp. *lactis* was studied by Janer *et al.* (2005), who demonstrated that this strain is able to produce intracellular endopeptidase (PepO), general aminopeptidase, X-prolyl-dipeptidyl aminopeptidase and proline iminopeptidase. Their study also showed that *Bifidobacterium animalis* subsp. *lactis* PepO enzyme mostly cleave peptides which bond on the N-terminal side of phenylalanine residues and were shown to have a post-proline secondary cleavage site. The number and specifications of the released bioactive peptides therefore depends on the selection of micro-organism species and growth conditions and the micro-organism’s proteolytic activity, bioactive peptide composition and dairy product type.
8.5 Production and purification of bioactive peptides

Researchers have developed several techniques for the production and purification of specific biologically active peptides from milk protein hydrolysates (FitzGerald & Meisel 2003, p. 686). The lack of suitable technologies has limited the industrial-scale production of bioactive peptides from food proteins (Korhonen & Pihlanto 2006). At the same time, the method applied for the industrial purification or enrichment should be carefully chosen as it can play an important role in maintaining their biological activities (Chatterton et al. 2006).

For example, heat treatment applied during processing can change the biological activity of the proteins by altering their structure and contributing to the lowering of their digestive properties (Chatterton et al. 2006). Nowadays, the technologies that are available for large-scale production has led to the introduction of some peptides with specific bioactivities to the market be used commercially. Some of these products have already been launched and are marketed internationally such as Calpis (reduction of blood pressure); BioPURE-GMP (prevention of dental caries, protection against viruses and bacteria); Capolac (helps mineral absorption); PeptoPro (improves athletic performance of muscle recovery); Vivinal Alpha (acids relaxation and sleep); and Recaldent (anticariogenic) (Korhonen 2009).

Membrane separation is the most common method for the isolation and purification of bioactive peptides from protein hydrolysate using microbial enzymes (Jelen & Lutz 1998); it is the best available technology used for peptide enrichment with a specific molecular weight range (Korhonen & Pihlanto 2007, p. 17). This technique has frequently been used for the enrichment of the ACE-inhibitory peptides from food proteins hydrolysate (Meisel et al. 2006, p. 285).

There are two available methods to perform the enzymatic hydrolysis however: either conventional batch hydrolysis or continuous hydrolysis using ultrafiltration membrane (Korhonen & Pihlanto 2003). Researchers have indicated several limitations for the traditional batch processing method compared to the continuous process, which are mainly related to their high use of enzyme and labour costs (Rios et al. 2004), their inefficiency for the frequency of start-up and shutdown procedures and need to recover and prepare the enzyme after each batch (Prazeres & Cabral 1994).

Enzymatic membrane reactors (EMR) have been used widely to produce hydrolysates with improved nutritional and/or functional properties through the total conversion of various food proteins (Prazeres & Cabral 1994; Martin-Orue et al. 1999). EMRs were developed to integrate several operations into a single operation; these operations include the enzymatic hydrolysis, product separation and/or concentration and enzyme recovery (Prazeres & Cabral 1994).

Ultrafiltration membrane reactors have been investigated for the continuous production of bioactive peptides, replacing the conventional batch method (FitzGerald & Meisel 2003, p. 687). This method has been shown to vastly improve productivity, efficiency and yield of the product (Deeslie & Cheryan 1981; Cheryan & Deeslie 1983) and shown to be easily scaled up (Korhonen & Pihlanto 2007, p. 17). This system was applied successfully for milk proteins for the continuous production of antithrombotic peptides from hydrolysed caseinomacropeptides (Bouhallab et al. 1992) and the continuous production of permeate enriched in CCPs or casomorphin precursors (Righetti et al. 1997). Bouhallab et al. (1992) reported the requirements for the continuous production of specific peptides sequences using membrane
reactors as follows: ‘i) highly specific enzymes; ii) better control of the enzymatic reaction
and iii) high selective membranes to separate the desired peptides from the reaction mixture’.

Bordenave et al. (2000) suggested the technique of combining ultrafiltration (UF) tech-
niques and enzymatic hydrolysis to produce a hydrolysate with a specified molecular size
distribution. Lieske & Konrad (1996) reported the improvement of foaming and emulsifying
properties of a papain hydrolysate when a selective UF step was added with a molecular
weight cut-off (MWCO) of 1 kDa to remove peptide fragments from whey protein concen-
trates. Other methods of ultrafiltration were reported for separating out small peptides from
high molecular mass residues and remaining enzymes using stepwise ultrafiltration with low
MWCO membrane (Korhonen & Pihlanto 2007, p. 17). A two-step ultrafiltration process has
also been used successfully to produce mixtures of polypeptides with molecular mass above
5 kDa and fractions rich in small components containing small peptides and amino acids
having molecular mass below 2 kDa (Turgeon & Gauthier 1990). The first UF (MWCO of
30 kDa) is to remove the whole unhydrolysed proteins and any remaining enzymes, while the
second UF (cut-off 1 kDa) is to remove small peptides and free amino acids (Lieske & Konrad
1996). Similarly, a two-step ultrafiltration membrane (30 kDa and 1 kDa) was used success-
fully for the enrichment of opioid peptides α-lactorphin and β-lactorphin (Pihlanto-Leppälä

Microfiltration (MF) is a membrane technique also used in the dairy industry. This tech-
nique is successfully used for the removal of pathogenic or spoilage bacteria from liquid milk
(Kelly & Tuohy 1997). The Tetra Pak Company (Pully, Switzerland) has developed a new
microfiltration machine for commercial use called Bactocatch process; this is the most com-
mon commercially used machine and is able to remove bacteria from skim milk (Elwell &
Barbano 2006). Two limitations should be considered when using this technique (Rysstad &
Kolstad 2006). First, this machine does not microfilter whole milk because milk fat glob-
ules have a similar particle size distribution to that of the cells and spores; milk fat must be
therefore removed first in order to microfilter the skim milk. Second, the particle size distribu-
tion of cells and spores could overlap with casein micelles; the pore size should therefore be
compromised to minimise the changes in milk composition. Despite these limitations, this
method could be used to replace the severe high-temperature pasteurisation and retain the
sensitive physiological compounds which are usually damaged during heat treatment. This
method could also be used for milk proteins separation (Jelen & Lutz 1998). and to extend
the shelf life of the processed milk (Rysstad & Kolstad 2006). This method could also be
ideal for camel milk, as its proteins are sensitive to severe high-temperature heat treatment
(Alhaj et al. 2011).

Ion exchange chromatography has been used for the isolation and enrichment of peptide
fractions from their protein hydrolysate. Ellegård et al. (1999) have developed new process-
ing methods to isolate high-purity CPPs from tryptic hydrolysate of caseinate followed by
acid precipitation, diafiltration and then using anion exchange chromatography. However, this
method of separation is quite costly and uneconomical for large-scale operations (Korhonen
& Pihlanto 2006). On the other hand, ion exchange chromatography was used to isolate and
purify caseinomacropetides (CMP) from whey proteins (Thomä-Worringer et al. 2006). The
isolation was performed by passing whey containing the CMP at low pH through a cationic
exchange column. The major whey proteins are usually adsorbed onto the cation exchanger
because of their positively charged molecules, while CMP pass through because of their highly negative charge (Doultna et al. 2003).

The isolation could also be carried out by passing the acidic whey containing CMP through an anionic exchanger; however, the CMP this time will be adsorbed onto the anion exchanger instead of whey proteins due to the charges on the protein (Tek et al. 2005). Using a similar technique, an anion exchange membrane system has been used for the in situ isolation and enrichment of cationic antibacterial peptides from lactoferrin (Recio & Visser 1999). This method, described by Korhonen & Phlanto (2007, p. 18), is based on concentrating the protein of interest within the chromatographic membrane and hydrolysing in situ with the desired enzyme. The resulting active peptides are retained on the column, whereas the fraction containing inactive peptides is washed out. Finally, the fraction containing the active peptides is obtained from the chromatographic membrane in a single elution step. The advantages of this process are that the isolation of the precursor protein is unnecessary and the enzyme used in the process can be recovered, reducing the cost of the final product.

8.6 Probiotic as bioactive component in milk

Consumer acceptance of probiotic fermented milk products has grown in Europe, North American and the Middle Eastern countries (IDF 1995). In the USA, the food industry has become the largest manufacturing industry (Klaenhammer 2000). There are now a variety of dairy products containing many different blends of lactic acid bacteria and probiotic cultures including yogurts, fermented milks, sour cream, cottage cheese, ice cream and frozen dessert. One of the earliest recognised functional foods was fermented milk (Metchnikoff 1908). In use for over 5000 years, their production enhances shelf life and improves the quality of milk as well as providing health benefits.

Dairy fermentations rely on a range of micro-organisms including lactic acid bacteria, propionic acid bacteria, yeasts and moulds to produce a very wide range of products from liquid fermented products to hard cheeses matured for several years before consumption. The addition of certain genera of probiotics such as Bifidobacterium and Lactobacillus was traditionally included in probiotic products to protect the human body against harmful effects (Vaughan & Mollet 1999) and improve the quality of the product itself by extending the shelf life, as was the case with producing different fermented products using mixed cultures of bifidobacteria and normal yogurt culture.

The consumption of probiotic products, especially drinks, is on the increase in many developed countries. The first fermented health drink containing probiotic bacteria was introduced by Yakult and has been available to the consumer since 1935 in Japan (Karimi & Pena 2003). Japan is now the largest market for functional foods and considered as the world leader in probiotic and prebiotic products, with more than 53 different types of milk products containing probiotic organisms on the market (Shah 2001). Probiotic products are restricted to fermented milk products in Europe, especially yogurt (Hilliam 2000). Australia, North America and many European countries are the other large markets for functional foods containing probiotics, prebiotics and synbiotics (mixtures of probiotic and prebiotic) that aim to improve the immune system and intestinal function. Interest in probiotic products has been growing in some parts of the Middle East recently, mainly for their associated health claims. It is
apparent that the growth in probiotics and functional foods are growing worldwide and faster than other health-promoting products; they have also been the focus of intensive research activity in recent years.

8.6.1 What are probiotics?

The most widely used method to increase the number of beneficial and health-promoting bacteria in the intestinal tract is by the direct consumption of food containing live bacteria. Such bacteria are called probiotics (Fuller 1989; Salminen et al. 1998b). The word ‘probiotic’ (meaning ‘pro-life’) is derived from the Greek language (Fuller 1989) and has been used in several different ways over the years. Their use goes back to the Roman period where Pliny the Elder in 76 AD advocated the use of fermented milks for treating gastrointestinal disturbances (Stanton 2005).

Probiotics have different definitions depending on the interpretation of the researcher in understanding their mechanisms of action or their effect on health and well-being of the host (Salminen et al. 1999). The term probiotics was first introduced in 1965 to describe the growth-promoting factors produced by the fermentative micro-organisms (Lilly & Stillwell 1965). However, early attempts to classify probiotics were not generally accepted (Sperti 1971). In 1974 Parker proposed a definition for the probiotics as ‘organisms and substances that influenced the intestinal microflora and had beneficial effects on animals’ (Parker 1974). The term ‘substances’ is imprecise and could include antibiotics or the promotion of microbial growth (Gibson & Roberfroid 1995). In 1989 Fuller modified the definition of probiotics to ‘a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance’.

According to this definition, the term ‘probiotics’ was restricted to feed supplements beneficial to the intestinal tract of animals, and could not be used for living micro-organisms administered in food or for location other than gastrointestinal tract (Šušković et al. 2001). In 1992 Havenaar and Huis in’t Veld proposed the widening of Fuller’s definition to ‘a probiotics is a mono- or mixed culture of live micro-organisms which applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora’ (Havenaar & Huis in’t Veld 1992). The formal and most-used definition of probiotics was adopted by FAO/WHO in 2001 as: ‘live microorganisms which when administered in adequate amounts confer a health benefit on the host’.

The most recent approach for describing probiotics was introduced by Schrezenmeir and de Vrese (2001) as ‘a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonisation) in a compartment of the host and by that exert beneficial health effects in this host’. Probiotics means any live micro-organisms, bacteria or yeast that are capable of providing any health benefits to the host and can be administered as a component of functional food or as food supplement e.g. capsules or tablets.

The second generation of probiotics are genetically modified micro-organisms providing the host with some necessary components. For example, research has been undertaken to increase the immune response induced by genetically modified lactobacilli to produce antigens for Helicobacter or rotavirus (Mercenier et al. 2004). Lactococcus lactis strains were also genetically engineered to produce immunomodulatory cytokines (e.g. interleukins) at
mucosal surfaces (Steidler & Neirynck 2003). These are not commercial products, probably due to consumers’ resistance to genetically modified micro-organisms.

8.6.2 Probiotics concept

The concept of probiotics was first proposed by the Russian Nobel Prize winning scientist Elie Metchnikoff (Metchnikoff 1908) who suggested that longevity of Bulgarian peasants was due to the consumption of large quantities of fermented milk products. Metchnikoff has introduced his intoxication theory and stated that intestinal putrefaction and fermentation are the main cause of aging (toxicants). He believed that the consumption of a product containing bacillus can decrease the toxic effect of colonic microflora by influencing the microflora of the colon. The organism he identified was called Bulgarian bacillus and later Bacillus bulgaricus, and was probably what became known as Lactobacillus bulgaricus and is now called Lactobacillus delbrueckii subsp. Bulgaricus. This strain, together with Streptococcus salivarius subsp. thermophilus, is now a widely used culture to produce yogurt. In 1935, Rettger and colleagues became interested in the mechanisms of probiotics effects and began to investigate the use of intestine-derived species for medicinal use (Rettger et al. 1935). Because the products containing probiotics had not been subjected to large-scale trials of efficacy, its use was however not considered by the pharmaceutical industry (Tannock 2003).

Medication and treatment with antibiotics is effective and has been successfully used to treat many diseases for decades. In some cases, the emergence of antibiotic-resistant strains of some bacteria, such as the methicillin-resistant Staphylococcus aureus (MRSA), has been a challenge to the pharmaceutical industry. However, antibiotics are no longer developed by the pharmaceutical industry at a sufficient rate to be able to compete with the emergence of new stains of resistance bacteria (Bengmark 1998). Antibiotics are generally known to delay the recolonisation of the normal colonic flora (Rolfe 2000); probiotic products certainly cannot replace antibiotic treatments, but they can restore some of the lost microflora in the body and provide health benefits. These factors may support the concept of having fermented milk containing beneficial micro-organisms as a preventive approach rather than a curative method.

8.6.3 Characteristics of probiotics

Hundreds of species of probiotic strains have been identified as commensal organisms in the human intestinal tract, but there is no one set list of criteria for classifying a viable bacterial strain as a probiotic (Kailasapathy & Chin 2000). A joint report by both the Food and Agriculture Organization and World Health Organization Working Group (FAO/WHO 2002) pointed out the main criteria for probiotic strains in vitro (Table 8.1). Within probiotic bacteria, large variations can be found in probiotic species or even in its strains (Chung et al. 1999). The characteristics of probiotic bacteria should therefore match the human gut environment according to the claimed health benefit. Roy (2005) suggested that ‘the performance of strains should be evaluated individually prior to commercial use in new dairy fermented products’. Nevertheless, Lactobacillus acidophilus, Bifidobacterium spp. and L. casei can be classified as probiotics because they possess beneficial health effects in the host by modulating the intestinal microflora (Schrezenmeir & De Vrese 2001).
Yogurt is another type of fermented milk usually produced by adding the traditional lactic acid culture \((Lactobacillus\ delbrueckii\ ssp.\ bulgaricus\ and\ Streptococcus\ salivarius\ subsp.\ thermophilus)\) to milk to convert the lactose in milk into lactic acid. The fermentation time of yogurt is short (approximately 4 hours) depending on the incubation temperature and the viability of the culture, compared to that for probiotic bacteria which is up to 24 hours (Dave & Shah 1997a). The survival of yogurt culture through the human gastrointestinal tract is still controversial because it is known that the two main traditional yogurt starter cultures \((L.\ delbrueckii\ subsp\ bulgaricus\ and\ S.\ salivarius\ subsp.\ thermophilus)\) do not survive in the acidic conditions in the stomach or during their journey through the intestinal tract (Gilliland \textit{et al}. 1984; Salminen \textit{et al}. 1998a). Consequently, no health benefits can be gained by adding these cultures to the product. Studies in the last two decades have reported the inability of these yogurt cultures to survive for a long time, not adhering or surviving through the intestinal tract because they are not bile-resistant bacteria (Gilliland \textit{et al}. 1984; Salminen \textit{et al}. 1998a). These results partly agree with Garvie \textit{et al}. (1984) who reported that when yogurt was fed continuously to rats, the \textit{Lactobacillus\ delbrueckii\ ssp.\ bulgaricus\ and\ Streptococcus\ salivarius\ subsp.\ thermophilus} counts decreased which indicates that yogurt culture can only survive but not multiply in the gut of the rats.

Researchers have recently reported the viability of yogurt cultures during their passage through the gastrointestinal tract and their presence in human feces (Campo \textit{et al}. 2005; Mater \textit{et al}. 2005; Elli \textit{et al}. 2006) and their ability to enhance lactose intolerance (FAO/WHO 2002). Yogurt culture was considered as probiotic because they ‘clearly fulfil the current concept of probiotic at least for its beneficial effect on lactose digestion in vivo’ (Guarner \textit{et al}. 2005) and was shown to have measurable health benefits (Adolfsson \textit{et al}. 2004). Interpretation of these results must take into account the different experimental protocols such as sample size and duration of study, associated with several methodological problems. It can be concluded that the probiotic nature of yogurt culture cannot be ignored for their health benefits.

However, a list of 56 species of \textit{Lactobacillus} and 30 species of \textit{Bifidobacteria} has been reported by Gomes & Malcata (1999). The main probiotic organisms known worldwide include: \textit{Lactobacillus\ lactis}, \textit{L.\ delbrueckii\ ssp.\ bulgaricus}, \textit{L.\ brevis}, \textit{L.\ johnsonii}, \textit{L.\ reuteri}, \textit{L.\ Cellobriosus}, \textit{L.\ curvatus}, \textit{L.\ fermentum}, \textit{L.\ plantarum}, \textit{Lactococcus\ lactis\ ssp.\ cremoris}, \textit{Lactococcus\ lactis\ subsp.\ lactis}, \textit{Bifidobacterium\ bifidum}, \textit{B.\ animalis\ subsp.\ lactis (Bb-12)}, \textit{B.\ adolescentis}, \textit{B.\ infantis}, \textit{B.\ longum}, \textit{B.\ thermophilum}, \textit{Streptococcus\ salivarius\ subsp.\ thermophilus}, \textit{S.\ ceremoris} and \textit{S.\ diacetylactis}. Other microbial species besides lactic acid bacteria were used as probiotics such as \textit{Pediococcus\ acidilactici}, \textit{Enterococcus
faecium, *E. faecalis* Bacillus subtilis and *Propionibacterium* ssp. Some yeast has also been used as probiotic, for example *Saccharomyces boulardii* (Tamime *et al.* 2005, p. 39).

Several strains of probiotics have already been used commercially for the production of fermented milk products including *Lactobacillus acidophilus*, *L. casei* Shirota, *L. casei* Imunitass, *B. bifidum*, *B. infantis*, *B. longum*, *B. breve* and *B. animalis* subsp. *Lactis* (Reuter *et al.* 2002; Tamime 2002). A list of 19 fermented milk products containing *Bifidobacteria* in different markets worldwide was reported by Tamime *et al.* (1995), and has by now tremendously increased in number. Among *Bifidobacterium* species, *Bifidobacterium animalis* subsp. lactis is the most widely used species in probiotic applications (Masco *et al.* 2005). Most *Bifidobacterium* strains used in probiotic fermented milk are expected to belong to *B. animalis* but are declared on the label as *B. longum* by manufacturer (Klein *et al.* 1998; D’Aimmo *et al.* 2007). However, commercially available milk-based probiotic health drinks could contain a single strain such as Yakult® health drink (Japan) containing *L. casei* Shirota, or contain two strains such as Muller® health drink (Germany) containing *Bifidobacterium* sp. and *Lactobacillus acidophilus*. Other products could contain more than two strains such as food supplement VSL-3® (Italy) containing 8 lactic acid bacteria species.

Before delivering a product containing probiotic bacteria to the consumer, there are a number of common criteria used for isolating and defining probiotic and specific strains which include the following:

- non-toxic, non-allergic, non-pathogenic and preferably unaffected by antibiotics;
- beneficial to the host (human or animal), in other words able to provide one or more clinically documented health benefits;
- an intake of 300–400 g per week contains $10^6–10^8$ CFU g$^{-1}$ of the probiotic;
- able to survive in or colonise the gut;
- viable and stable during processing, storage and even beyond the consume-by date;
- enhance the shelf life and storage stability of fermented products;
- demonstrate stability and functionality after freeze- and spray-drying methods;
- able to adhere and compete with the normal microflora;
- amenable to cultivation on an industrial scale;
- do not produce harmful and putrefactive substances but are able to produce antimicrobial metabolites (including bacteriocins, carbon dioxide, hydrogen peroxide and organic acids such as lactic and acetic acids);
- remain viable when exposed to acid, bile and oxygen;
- resist degradation caused by intestinal enzymes such as lysozymes;
- maintain a good flavour and aroma profile; and
- are genetically stable.

These probiotic criteria are adopted from Fuller (1989); Macfarlane & Macfarlane (1995); Dave & Shah (1997a); Salminen *et al.* (1998a); Gismondo *et al.* (1999); Kailasapathy & Chin (2000); Shah (2001); Šušković *et al.* (2001) and Hernández *et al.* (2005).

*Bifidobacterium* spp

*Bifidobacterium* spp. are anaerobic, non-spore forming, Gram-positive, non-motile and catalase-negative with a range of cell morphologies including short curved rods, club-shaped...
rods and Y-shaped branches (Gomes & Malcata 1999). The optimum growth temperature is in the range 37–41°C. Bifidobacterium spp. are known to grow at temperatures of 20–45°C, although growth outside this range might also occur in some strains (Marks 2003, p. 38) depending on the habitat and the origin/source of the bacteria (Biavati & Mattarelli 2001). For example, Bifidobacterium animalis subsp. lactis from animal origin has been shown to have high thermal resistance, which makes it a good choice for yogurt production because a higher incubation temperature during milk fermentation can shorten yogurt production time (D’Aimmo et al. 2007); it is found to have slower growth rate at 30°C (Östlie et al. 2005). However, it was also shown that these bacteria would survive during incubation and storage with normal yogurt culture (Lamoureux et al. 2002; Matsumoto et al. 2004). Bifidobacterium spp. grows best at pH 6–7, but the vast majority are unable to sustain growth at low pH values of 4.5–5.0 or at higher pH values of 8.0–8.5 (Gomes & Malcata 1999). The survival of Bifidobacteria at certain pH values varies, for example, B. breve, B. longum, B. infantis and B. pseudolongum have all been shown to be able to grow at pH 4.0 in the presence of 0.3% of bile salts or in the presence of 300 μg mL⁻¹ of lysozyme, which is usually acts on Gram-positive bacteria by destroys the integrity of bacterial cell walls (Šušković et al. 1997). They are also resistant to at least 10 μg mL⁻¹ of H₂O₂ (Gagnon et al. 2004) and to a limited number of antibiotics (Temmerman et al. 2002).

However, the beneficial effect of probiotics is usually linked to their survival and the adverse effect they impose on Gram-negative and pathogenic micro-organisms by inhibiting their growth, such as the case with Escherichia coli (Yusof et al. 2000; Ibrahim et al. 2003; Gagnon et al. 2004) and Salmonella typhimurium S-285 (Yusof et al. 2000). In order to maintain good confidence in the use of probiotic strain, it is important to demonstrate the metabolism and characteristics of any particular strain during their production, shelf life and their ability to survive through the gastrointestinal tract. One of the Bifidobacterium strains from animal origin such as Bifidobacterium animalis subsp. lactis (Bb-12) produced and marketed by Chr. Hansen Ltd has been used in many fermented milk products. This strain already has eight clinically proven health benefits (Playne 2002) and has been shown to have some useful characteristics suitable for commercial product application such as resistance to freeze-drying (Modesto et al. 2004). It is stronger and more temperature tolerant compared with other Bifidobacteria strains from human origin such as B. bifidum, B. breve, B. infantis and B. longum (Lamoureux et al. 2002). This strain is bile-tolerant (Charteris et al. 1998) and resistant at pH 3 for 180 min, but declined slowly at pH 2 and no survival was found after 60 min at pH 1 (Pochart et al. 1992). This strain was found to have remarkably higher acid resistance than B. infantis and B. longum at pH 2 for 90 min in a dynamic model system of in vitro human upper GI tract (Mainville et al. 2005). This is in agreement with Vernazza et al. (2006), who reported that B. lactis (Bb-12) was more acidic resistant at pH 2 than B. adolescentis and B. infantis and two strains of B. longum. It has been reported (Alander et al. 2001; Gopal et al. 2003; Mättö et al. 2006) that Bb-12 has the ability to survive during passage through the gastrointestinal tract in human (79% of study subjects).

Experiments involving human subjects reported the survival of 23.5% of the administrated dose of Bifidobacterium animalis subsp. lactis in the samples taken from ileum (Pochart et al. 1992). In a similar study, this strain was detected in the faeces of all subjects after the second week of ingestion, whereas the total number was increased during the consumption of these bacteria and remained viable in the intestinal tract for 4–5 weeks (Collado et al. 2006). The
acid tolerance of *Bifidobacterium animalis* is expected to belong to the action of H^+-ATPase activity. Their resistance to antibiotics were reported by Zhou *et al.* (2005), Antunes *et al.* (2007), D’Aimmo *et al.* (2007) and Saarela *et al.* (2007), which makes it a successful strain for being unaffected by antibiotic therapy (Rolfe 2000). Other studies showed the high sensitivity of *Bifidobacteria* to oxygen, although their oxygen tolerance is species dependent (De Vries & Stouthamer 1969; Shimamura *et al.* 1992; Talwalkar *et al.* 2001). *Bifidobacterium animalis* subsp. *lactis* has been shown to be oxygen tolerant, explaining its tendency to survive in the presence of oxygen (Meile *et al.* 1997; Bolduc *et al.* 2006). The growth and metabolite production of *B. animalis* has been studied by Østlie *et al.* (2003, 2005) who showed that *B. animalis* has a heterofermentative nature and is able to produce acetaldehyde, ethanol, acetoin, acetic, lactic, pyruvate, orotic and succinic acids in different concentrations depending on temperature and fermentation time, but has low CO_2_ production ability. The above characteristics make *Bifidobacterium animalis* subsp. *lactis* a better choice for commercial probiotic formulations (Meile *et al.* 1997; Gueimonde *et al.* 2004; Masco *et al.* 2004; Bolduc *et al.* 2006).

**Lactobacillus** spp.

*Lactobacillus* spp are anaerobic, Gram-positive, non-sporulating, non-motile and rods or coccobacilli (Gomes & Malcata 1999). Several research works have been published on the sensitivity and the level of survival of some of the known probiotics such as *Lactobacillus casei* and *L. acidophilus*. Most probiotic species seem to resist low pH and other external factors, making them ideal for inclusion in fermented dairy products. They remain viable with varying degrees of survival in various food products and in human gut. Mishra & Prasad (2005) reported that *Lactobacillus casei* showed tolerance at pH 3.0, resistance to pH 2.0 for up to 90 minutes and 12 hours in the presence of 2% of bile salts. Coeuret *et al.* (2004) have also shown that *L. paracasei/casei* is resistant to 100 μg mL\(^{-1}\) lysozyme after 90 minutes, resistant to 12–14 out of 23 antibiotics and has an inhibitory effect on the growth of *S. aureus*, *Listeria monocytogenes*, *Salmonella* spp. and *E. coli*. *L. casei* Shirota strain has also been shown to have the ability to survive and resist the tough conditions through the passage of the gastrointestinal tract after the consumption of fermented milk containing \(10^{10}\) of the live strain (Yuki *et al.* 1999). This strain is also shown to have the ability to modulate the composition and metabolic activity of the intestinal flora (Spanhaak *et al.* 1998). It has also been shown that *L. acidophilus* has a high tolerance to phenol, up to 0.5%, a good survival rate at pH 4 (Šušković *et al.* 1997) and resistance towards certain antibiotics (Temmerman *et al.* 2002; Danielsen & Wind 2003). *L. acidophilus* was also reported to grow at a temperature of 45°C, but its optimum growth temperature is 35–40°C (Gomes & Malcata 1999). Currently, ingredient suppliers offer ambient stable probiotic cultures packaged in gel capsules that promote survival and resistance to pH, oxygen, moisture and temperature (Cruce & Goulet 2001).

Some *Lactobacillus* species such as *L. acidophilus*, *L. johnsonii* and *L. helveticus* were found to produce predominantly lactic acid, while other species such as *Lactobacillus fermentum*, *L. plantarum*, *L. casei* and *L. rhamnosus* were found to produce lactic acid, acetic acid, ethanol and formic acid (Lee *et al.* 1999). Lactic acid was found to be the main product of *Lactobacillus casei* Shirota (Sgouras *et al.* 2004). Varieties in metabolism production depend upon the homo or heterofermentative nature of the strain.
Coeuret et al. (2004) reported that Lactobacillus paracasei and L. casei inhibit the growth of Staphylococcus aureus, Listeria monocytogenes, Salmonella spp. and Escherichia coli at 15 and 37°C. Lactobacillus acidophilus M92 have been shown to inhibit the growth of Staphylococcus aureus 3048, S. aureus K-144, Escherichia coli 3014, Salmonella mumum, Bacillus cereus, B. subtilis ATCC 6633, Enterococcus faecium and Candida tropicalis (Šušković et al. 1997; Rolfe 2000). Furthermore, Lactobacillus casei Shirota were found to inhibit Helicobacter pylori and E. coli in vivo and in vitro (Sgouras et al. 2004).

8.6.4 Health benefits associated with fermented milk product consumption

The health benefits of consuming fermented milk containing lactic acid bacteria have been acknowledged for hundreds of years. Since that time a growing number of studies have confirmed that yogurt culture and probiotic bacteria really do offer several important health benefits, especially for specific groups of patients when regularly consumed in a large numbers (Macfarlane & Cummings 2002; Ouwehand et al. 2002). The final products should also have acceptable sensory attributes such as taste, aroma, colour and texture during their shelf life (Holzapfel et al. 1998).

The health benefits of consuming viable probiotic and yogurt cultures have been reviewed by Gill & Guarner (2004) and Adolfsson et al. (2004), respectively, while the consumption of fermented milk products containing specifically bifidobacteria and the potential health benefits were reviewed by Leahy et al. (2005). The health benefits of the regular intake of fermented probiotic drinks and yogurt are claimed to be the following.

1. Improves the intestinal microbial balance (Bergogne-Berezin 2000).
2. Produces lactase that prevents symptoms of lactose intolerance (Pettoello et al. 1989; Marteau et al. 2001) and reduces some forms of food allergies (Pelto et al. 1996).
3. Strengthens the immune system (Fiat et al. 1993; Marteau et al. 1997).
4. Reduces the risk of colon cancer (Rafter 2002) and protects against breast cancer in humans (Van’t Veer et al. 1991) and their anti-tumour effects in rats (Abd El-Gawad et al. 2004).
10. Yogurt consumption improves lactose digestion, enhances the immune responce, prevents allergic disorder and alleviates acute diarrhoeal disorder (Adolfsson et al. 2004; Guarner et al. 2005).
11. More recently, yogurt cultures (L. delbrueckii ssp. bulgaricus Lb1466 and S. thermophilus St1342) were reported to have ACE-inhibitory activity in vitro with or without adding probiotic bacteria such as L. acidophilus L10, L. casei L26 and B. lactis B94 (Donkor et al. 2007).
Some health benefits could be obtained by both viable and non-viable cells including lactose intolerance treatment which reduces acute gastroenteritis duration, anti-\textit{Candida} activity (Ouwehand \& Salminen 1998) and cholesterol (Hosono \& Tono-oka 1995). No firm conclusion has been drawn on the efficacy of viable cells against non-viable cells. However, Ouwehand \& Salminen (1998) indicated that the viability of probiotic micro-organisms could show a better effect than the non-viable micro-organisms. Nevertheless, non-viable probiotics still have some economic advantages in terms of longer shelf life and less requirements for refrigerated transport and storage, expanding the potential use of probiotic products in the strict handling conditions in developing countries (Ouwehand \textit{et al.} 2000b).

8.6.5 \textit{Factors stimulating the growth and survival of \textit{Bifidobacterium} spp.}

All definitions since 1989 (Fuller 1989) have emphasised the importance of viability of probiotic bacteria, and considered them as an essential factor for the health benefits of probiotics. This emphasis comes from the importance of maintaining the probiotic bacteria not only during its shelf life in the product but also in the gut environment until reaching the target site and delivering the health benefit(s). In general, the growth of \textit{Bifidobacterium} spp. in milk were reported to be poor, which might be due to the lack of growth factors such as free amino acids and small peptides in milk (Gomes \textit{et al.} 1998; Tamime \textit{et al.} 2005, p. 57) or lack of proteolytic activity of \textit{bifidobacteria} strains (Klaver \textit{et al.} 1993). However, the minimum nutritional requirement for bifidobacteria to grow is ‘a semi-synthetic medium containing only lactose, three amino acids including cysteine, glycine and tryptophan, several vitamins and nucleotides and some minerals’ (Gomes \& Malcata 1999). The growth of \textit{Bifidobacterium} in milk might be species- and milk-dependent, whereas the growth of \textit{B. longum} has been reported to be higher in camel milk than in bovine milk and vice versa for \textit{B. angulatum} 27535 (Abu-Taraboush \textit{et al.} 1998). According to Modler (1994), the growth of \textit{Bifidobacterium} spp. was found to be enhanced by two types of factors – bifidogenic and growth – as described in the following.

\textit{Bifidogenic factors}

Bifidogenic factors were defined by Modler (1994) and later modified by Gomes \& Malcata (1999) as ‘compounds, usually of a carbohydrate nature that survive direct metabolism by the host and reach the large bowel or cecum where they are preferentially metabolised by bifidobacteria as source of energy’. This factor is now known as prebiotic and defined by Gibson \& Roberfroid (1995) as ‘a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon’. According to this definition any dietary component that reaches the large intestine are candidate prebiotics such as non-digestible carbohydrates, some peptides and proteins as well as certain lipids (Gibson \& Roberfroid 1995).

The influence of bifidobacteria viability by the addition of prebiotic has been reported in many studies (e.g. Rycroft \textit{et al.} 2001; Bruno \textit{et al.} 2002; Martínez-Villaluenga \textit{et al.} 2006; Vernazza \textit{et al.} 2006) where the viability, growth and activity of \textit{Bifidobacterium} spp. were found to be increased in milk containing oligosaccharides (Chick \textit{et al.} 2001).
It was also reported (Vernazza et al. 2006) that *Bifidobacterium* spp. include *B. lactis* Bb-12 (Chr. Hansen), *B. adolescentis*, *B. infantis* and two strains of *B. longum* were able to grow and utilise a number of prebiotic substrates in different degrees, especially galacto-oligosaccharide (GOS) and isomalto-oligosaccharide (IMO).

**Growth factors**

These factors are also referred to as bifidus factors (Klaver et al. 1993) and were defined by Modler (1994) as ‘compounds which promote the growth of bifidobacteria in vitro but cannot be delivered to the large bowel or cecum to selectively promote proliferation of bifidobacteria’. These factors could include many substrates such as: milk proteins and their tryptic hydrolysates; milk protein hydrolysate from microbial proteolysis; and other substrates, as described in the following.

*Milk proteins and their tryptic hydrolysates.* It has been reported that major whey proteins (α-la and β-lg) and casein fractions from human and bovine milk are excellent growth promoters for *Bifidobacterium* spp. (Petschow & Talbott 1990). Similarly, the viability of *Bifidobacterium* spp. in yogurt was improved by adding casein hydrolysate and whey protein concentrate containing amino acids and peptides such as tryptone (Dave & Shah 1998). The addition of whey peptide fractions containing caseinomacropeptide (CMP) and whey protein concentrate (WPC) were found to enhance the growth of *Bifidobacterium animalis* subsp. *lactis* (Janer et al. 2004), which was in agreement with Ibrahim & Bezkorovainy (1994) who indicated that the presence of α-la and β-lg enhanced the growth of *B. longum*. Whey proteins act as a source of amino acids and peptides and are a rich source in sulphur-containing amino acids, which are released during heat treatment, resulting in low redox potential (Dave & Shah 1998). It has also been reported (Poch & Bezkorovainy 1991) that κ-casein digested by trypsin was able to enhance the growth of *Bifidobacteria* spp. due to the presence of disulphide/sulphhydryl residues (cysteine-containing peptides). Bovine casein hydrolysate and yeast extract were considered to be the best growth promoters for all tested *Bifidobacterium* spp. and were better than human and bovine whey proteins and bovine serum albumin hydrolysate (Poch & Bezkorovainy 1988). Furthermore, *Bifidobacterium* spp. preferred the peptides produced by enzymatic hydrolysis of casein as a source of nitrogen more than free amino acids (Proulx et al. 1992; Juillard et al. 1995a). The length of these peptides plays a very important role for the growth and acid production of LAB (St-Gelais et al. 1993). For example, the growth of bifidobacteria was not found to be stimulated by peptides produced by tryptic hydrolysis of casein having molecular mass higher than 5000 Da (Proulx et al. 1994).

*Milk protein hydrolysate from microbial proteolysis.* The growth of bifidobacteria in milk was found to improve when co-cultured with proteolytic bacterial species to provide sufficient nitrogenous compounds compared with milk without the presence of these cultures (Klaver et al. 1993). For example, co-culturing bifidobacteria with *L. casei* could stimulate their growth and acid production because *Lactobacillus casei* hydrolyse milk proteins into peptides and make it available for the growth of bifidobacteria (Cheng & Nagasawa 1983, 1984). Similar results were obtained by Gomes et al. (1998) where the growth and acid production of *B. lactis* was found to be enhanced when cocultured with *L. acidophilus*. 

Other substrates. Many other compounds have also been regarded as growth factors which enhance the growth of probiotics, especially *Bifidobacterium* spp. (bifidogenic) as reported by Modler (1994) and Gomes & Malcata (1999). Compounds such as cysteine (Desjardins et al. 1990; Dave & Shah 1997b; Ravula & Shah 1998), threonine (Marshall et al. 1982), honey (Chick et al. 2001; Kajiwara et al. 2002; Haddadin et al. 2007), yeast extract (Poch & Bezkorovainy 1988; Ibrahim & Bezkorovainy 1994), casitone or a mixture of amino acids (Klaver et al. 1993), enzyme-treated chlorella, peptone and trypicase (Modler 1994), dextrin, maltose and extracts from carrots (coenzyme A) and ascorbic acid (Collins & Hall 1984; Klaver et al. 1993) have all been reported as bifidogenic.

8.6.6 The direct health benefits of probiotics: Mechanisms of action

Several mechanisms of action have been proposed regarding the health benefits of probiotics but are still not clearly understood. These mechanisms have been linked to the direct effect on the biological function of the human body and/or by inhibiting the growth of some pathogens. These proposed mechanisms could be categorised as in the following.

Probiotic population

Information about the minimum intake/dose of probiotic bacteria required to deliver the reported health claims is still unclear. However, it is known that probiotic strain(s) should be of a high population which is typically between $10^6$ and $10^9$ CFU g$^{-1}$ to exert the claimed health benefits. EU guidelines propose setting the minimum level of probiotic bacteria in such a product at $10^7$ CFU mL$^{-1}$ during the shelf life of the product (EU 2003). It is known that probiotic concentration in a product is not the only critical factor for providing health benefits; daily intake also has an important effect in maintaining such health benefits. Nowadays, consumers are aware of the probiotic properties of cultured milk and dosage specifications (Mosilhey 2003).

Microflora in the large intestine can be classified according to their biological nature, which includes proteolytic species, saccharolytic organisms and organisms that can metabolise gases (Fooks et al. 1999), or according to the dominance in the intestine. The majority are beneficial including the dominant bacteria such as *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Eubacterium* and *Peptostreptococcus*. The less-dominant bacteria, but still beneficial, include *Streptococcus*, *Enterococcus*, *Enterobacteriaceae* family and some *Clostridium* species. A number of aerobic and anaerobic bacteria such as *Veillonellae*, *Clostridium*, *Staphylococcus*, *Proteus* and *Pseudomonas* bacteria were also found to produce harmful nitrogen waste products such as amines, ammonia, phenols, urea, indoles, nitrites, hydrogen sulphide and secondary bile acids (Macfarlane & Macfarlane 1995; Lennart-Cedgård 1998; Gill & Rowland 2002). A reduction in the number of beneficial bacteria might allow injury to the intestine directly or contribute to diseases in the case of absorption (Clinton et al. 1988; Kikugawa & Kato 1986; Macfarlane & Macfarlane 1997; Aslam et al. 1992; Hawrelak & Myers 2004). Interestingly, the daily ingestion of *B. lactis* Bb-12 at a concentration of approximately $1-3 \times 10^{10}$ CFU g$^{-1}$ was found to increase the bifidobacterial number in faeces (Schiffrin et al. 1995; Alander et al. 2001). Recently, a double-blind feeding study was undertaken on 18 healthy elderly individuals
using a symbiotic preparation containing a high number of *Bifidobacterium lactis* BL-01 and *B. bifidum* BB-02 combined with an inulin-based prebiotic (Bartosch et al. 2005). In their study, the number of *Bifidobacterium bifidum*, total bifidobacteria and total lactobacilli increased significantly in the symbiotic group during feeding when compared to a placebo.

**Nutrient availability**

The availability of nutrients plays an important role in the survival and growth of micro-organisms. Nutrients are an important factor in determining gut flora composition; in healthy individuals, there is a natural balance between beneficial and harmful bacteria (Gopal et al. 2003). In fact, frequent drinking of probiotic products will reinforce the presence and the number of probiotic bacteria in the gut and consequently reduce the chance of pathogen survival due to the lack of nutrients that have mainly been utilised by probiotics, specifically the carbohydrates in the large intestine as reported by Freter et al. (1983). On the other hand, adding some prebiotic substrates such as oligosaccharides and inulin promote the growth of a limited number of bacteria (Gibson et al. 1995). In recent studies, the consumption of fermented milk containing GOS, *B. lactis* Bb-12 (Chr. Hansen) and syrup (Alander et al. 2001) or reconstituted milk powder containing GOS (Gopal et al. 2003) was found to significantly increase the faecal count of bifidobacteria in human subjects.

**Adhesion to the intestinal mucosa**

Probiotic bacteria that are capable of competing for limited nutrients and adhere to the intestinal mucosa and epithelial cell surfaces are the only bacteria that can stay in the intestine. In doing so, they compete successfully to eliminate other bacteria from the ecosystem (Macfarlane & Gibson 1994) through the production of antimicrobial substances, including short chain fatty acids and antimicrobial peptides, to create beneficial conditions in the gastrointestinal tract; these conditions also decrease the luminal pH that inhibit the growth of some bacteria. Consequently, surviving probiotic bacteria can compete with other bacterial populations to occupy the adherence sites in the intestinal mucosa.

*In vitro* studies reported that the adhesion of probiotic bacteria was found to depend on the concentration of the ingested probiotic bacteria (Tuomola & Salminen 1998); the normal microflora does not greatly affect probiotic bacteria adhesion (Ouwehand et al. 1999b). Furthermore, ingestion of probiotic bacteria is able to improve the binding of human gastrointestinal microflora and stabilise it (Fuller 1991; Ouwehand et al. 2000a; Gopal et al. 2003). *In vivo* studies reported that most probiotic bacteria can only colonise the human gut temporarily, unfortunately (Ouwehand et al. 1999a; Tannock 1999). The administered probiotic bacteria can be detected for days or few weeks after cessation of probiotic feeding in the faeces and will thereafter disappear gradually, indicating the transient colonisation of probiotic bacteria in the gut (Bouhnik et al. 1992; Ouwehand et al. 1999a; Brigidi et al. 2000). For example, *Lactobacillus casei* Shirota were found not to colonise the gut epithelium permanently and were only detected in the microflora for the time of administration only (Sgouras et al. 2004).

However, the probiotic strains have been shown to have a strong adhesion when compared to common dairy strains (Salminen et al. 1998a). *Bifidobacterium lactis* Bb-12 was found to
adhere well to the immobilised human ileostomy glycoproteins, human intestinal mucosa and bovine mucosa in vitro (Ouwehand et al. 2000a; He et al. 2001). The binding of this strain to the immobilised human ileostomy glycoproteins were found to be doubled when incubated simultaneously with Lactobacillus GG and Lactobacillus delbrueckii subsp. bulgaricus due to co-aggregation between strains (Ouwehand et al. 2000a). In the same study, other strains of probiotic did not significantly affect the adhesion of Bifidobacterium lactis Bb-12 to the immobilised human ileostomy glycoproteins, including Lactobacillus johnsonii La1 and Lactobacillus acidophilus La5. Furthermore, B. adolescentis, B. animalis, B. breve, B. bifidum and B. longum were also found to have the ability to adhere to human intestinal mucosa and bovine mucosa in vitro (He et al. 2001).

However, probiotic micro-organisms can adhere to gut epithelium specifically and non-specifically. The procedures of these adhesions have been described by Šuškovič et al. (2001) and Salminen et al. (1998b). The specific adhesion is usually defined as a lock and key function which occurs when the bacterial cell protein binds to a receptor on the epithelial cell. Non-specific adhesion is more general and based on physico-chemical factors such as hydrophobic or electrostatic interaction. However, in vivo non-specific adhesion may have no significant role in the colonisation of the epithelia, but it might have an important role in the colonisation of luminal contents (Šuškovič et al. 2001). For example, non-specific adhesion may enhance substrate uptake, consequently enforcing growth (Jonsson & Conway 1992). Some strains of lactobacilli including L. casei (BIO®, Danone), L. casei Imunitass (Actimel®, Danone), L. casei 01 (Starter culture, Chr. Hansen Ltd), L. casei Shirota (Yakult®, Yakult) and L. casei var. rhamnosus (Lactophilus®, Laboratoires Lyocentre) were reported to be considered as non-specific binding strains when compared to negative control E. coli B44 as an indicator of non-specific binding (Tuomola & Salminen 1998).

Some studies however reported the ability of non-viable probiotic bacteria to adhere to tissue culture cells, concluding that viability is not necessary for adhesion (Hood & Zottola 1988; Coconier et al. 1993). In general, inactivation of probiotic bacteria by heat treatment was found to decrease the ability of many probiotic strains to adhere to intestinal mucosa, but few of them were found to increase their ability to adhere to intestinal mucosa after heat treatment. Ouwehand et al. (2000b) suggested that ‘this may be due to the changes in the cell envelop of the inactivated strains rather than due to the fact that they are dead’. They showed that the inactivation by heat treatment at 80°C and 100°C for 10 min were shown to increase the adhesion of Bifidobacterium lactis Bb-12 and Propionibacterium freudenreichii subsp. shermanii JS to immobilised intestinal mucosa due to the increased interaction between them after heat treatment. On the other hand, this treatment has reduced the adhesion of Lactobacillus rhamnosus GG and Lactobacillus johnsonii La1 and significantly reduced the adhesion of Lactococcus lactis subsp. cremoris and Lactobacillus casei Shirota. In another study, heat treatment at 100°C for only 30 sec reduced the adhesion of Bifidobacterium bifidum by 40% compared with viable bacteria (Fontaine et al. 1994).

8.6.7 Indirect health benefits of probiotics: Biogenic effect

This effect occurs as a result of metabolites produced by probiotics during the fermentation process, and can be divided into antimicrobial substances, intestinal bacterial enzyme activities, immune enhancement and bioactive peptides, as described in the following.
Antimicrobial substances

Most living micro-organisms need to produce antimicrobial compounds against each other to prevent any onset of infection that would inhibit or stop the growth of other micro-organisms. However, the antipathogenic effect of probiotic bacteria could be through decreasing the luminal pH in the human gut and by the secretion of numerous antimicrobial compounds that kill or inhibit the growth of many Gram-positive pathogens such as *Staphylococcus aureus* and *Clostridium perfringens* (Hernández et al. 2005) and Gram-negative pathogens such as *Salmonella typhimurium* and *Escherichia coli* (Sinha 1986; Yusof et al. 2000). Similarly, the shelf life of a product can be extended on the same principle by producing numerous antimicrobial compounds, and some of these components such as nisin have been used as food preservatives (Qiao et al. 1995). The most widely produced antimicrobial compounds by probiotic bacteria are the organic acids, especially acetic and lactic acids, which comprise 90% of the produced acids (Shah 2001). In addition to these, other acids are produced such as citric, butyric, hippuric, pyruvic, succinic, orotic and uric acids in various and small quantities by probiotic bacteria (Lankaputhra & Shah 1998a; Ouwehand et al. 1999a; Østlie et al. 2003) and yogurt culture (Fernandez-Garcia & McGregor 1994).

Other studies suggest that some *Bifidobacteria* strains could inhibit a number of pathogen through a non-pH related effect such as bacteriocins (Meghrous et al. 1990) and hydrogen peroxide (Gibson & Wang 1994). Carbon dioxide is also produced widely by probiotic strains; some of these components can be produced if the metabolism system of the bacteria is active during their stay or passage through the intestinal tract (Ouwehand et al. 1999a). These inhibitory compounds do not only reduce the viable number of bacteria, but also play an important role on affecting bacterial metabolism or toxin production (Rolfe 2000). However, effectiveness levels of these antimicrobial components on the growth of the pathogens vary, whereas combined components have more effect against pathogens than individual components. For instance the presence of hydrogen peroxide together with lactic acid has a greater inhibitory effect than hydrogen peroxide or lactic acid alone (Lankaputhra & Shah 1998b). Meanwhile, hydrogen peroxide produced by the yogurt culture (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*) is the main cause for the reduction of probiotic bacteria such as *Lactobacillus acidophilus* (Gilliland & Speck 1977; Dave & Shah 1997a) as well as reducing the population of Gram-negative *Pseudomonas* sp. (Price & Lee 1970) and Gram-positive *Staphylococcus aureus* (Dahiya & Speck 1968).

As mentioned in Section 8.6.3, *Bifidobacterium animalis* was shown to survive and maintain its viable count during incubation with yogurt culture for 28 day of storage (Lamoureux et al. 2002). Regardless of the beneficial effect of *Bifidobacteria* to some lactic acid bacteria when simultaneously grown, bacteriocins produced by lactic acid bacteria were also found to possess an antimicrobial effect and provide undesirable conditions against probiotic bacteria as well as pathogens (Gomez et al. 1997; Miteva et al. 1998; Hernández et al. 2005). However, some bacteria have self-protective mechanisms to limit the production of bacteriocin, as in the case of *Lactococcus lactis* producing nisin (Qiao et al. 1995).

In general, antimicrobial peptides (not casein peptides) showed a broad spectrum of antibacterial activity in addition to showing a rapid killing effect, often within minutes *in vitro*, against various targets including Gram-negative and Gram-positive bacteria, fungi, enveloped viruses, parasites and even tumour cells (Maloy & Kari 1995; Hancock & Lehrer
It has been reported (Kappeler 1998; Kappeler et al. 1999) that Peptidoglycan recognition protein (PGRP) was isolated from camel milk but does not exist in cow milk; this protein inactivates pathogens by binding to peptidoglycan structures in the cell wall. It is expected that camel’s PGRP and its hydrolysate may have potent antibacterial properties similar to lactoferrin and lactoferricin. Higher amounts of lysozyme, lactoferrin and immunoglobulins were found in dromedary camel milk than bovine or buffalo milk (Kappeler et al. 1999; El-Agamy 2000; Konuspayeva et al. 2007; Alhaj & Alkanhal 2010). PGRP was found in greater amounts in camel milk than other antibacterial proteins such as lactoferrin, lactoperoxidase or lysozyme (Kappeler et al. 1999).

**Intestinal bacterial enzyme activities**

The metabolic activity of the colonic microflora has received considerable attention from researchers because of their pathological role in exhibiting conditions such as pseudomembranous colitis and colonic carcinogenesis (Marteau et al. 1990). Their other physiological functions include fermentation, which is considered to be one of the most important functions since it enlarges the surface area for enzyme action (Mahé et al. 1994). Probiotic bacteria alter the microflora metabolism in a beneficial manner and decrease the mutagenicity of the intestinal cells (Lidbeck et al. 1992). A disorder in intestinal microflora causes dysfunction of intestinal metabolism, consequently increasing the effect of harmful enzymes that are thought to be the cause of several diseases such as rheumatoid arthritis and lower colon cancer (Goldin & Gorbach 1984; Salminen et al. 1998a; Gill & Rowland 2002).

Information about faecal enzyme activities showed that probiotic bacteria are also able to increase the activity of some beneficial enzymes such as β-galactosidase (lactase), the deficiency or lack of which causes lactose intolerance (Martini et al. 1991; Sanders 1993; Rolfe 2000; Marteau et al. 2001). Moreover, yogurt culture (Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus salivarius subsp. thermophilus) have also demonstrated a similar improvement in lactose digestion due to an increase in β-galactosidase activity in rats and humans (Garvie et al. 1984; Lin et al. 1991; Martini et al. 1991; Sanders 2000). Available evidence suggests that lactase activity varies between the available commercial yogurt brands, and some of these brands showed improvement in lactose digestion and tolerance (Wytock & DiPalma 1988). However, these variations in lactase activity could be due to differences in the lactase activity of the individual culture or temperature changes during product distribution from manufacturer to retailer (Wytock & DiPalma 1988). Alternatively, the disruption of microbial cell structure eventually increases lactase release by gastric acid (Martini et al. 1987).

Probiotic bacteria are also able to reduce the activity of some harmful enzymes such as β-glucuronidase, β-glucosidase, azoreductase, nitroreductase, dehydroxylase and steroid-7-α- dehydroxylase (Goldin et al. 1980; Goldin & Gorbach 1984; Rowland 1992; Pedrosa et al. 1995; Bengmark 1998; Gill & Rowland 2002). Hydrogen and methane production and faecal β-galactosidase and β-glucosidase activities could be used as fermentation capacity indicators (Marteau et al. 1990). Clinical studies have proved the metabolic activity alterations of lactic acid bacteria, whereas the faecal activities of β-glucuronidase, azoreductase and nitroreductase were significantly declined when humans were fed *L. acidophilus* of human origin (Goldin et al. 1980; Goldin & Gorbach 1984).
Similar results were obtained in another study where a significant reduction in all three enzyme activities was reported in healthy elderly subjects after they were fed with *Lactobacillus gasseri* for 12 days (Pedrosa et al. 1995). In another human study, no change was reported in the activity of faecal β-galactosidase, β-glucuronidase and azoreductase while nitroreductase activity was significantly decreased and faecal β-glucosidase increased after 3 weeks of feeding human volunteers fermented dairy products containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (Marteau et al. 1990). In contrast, drinking unfermented milk alone by healthy adults showed no significant alterations in the metabolic activity (Goldin & Gorbach 1984).

**Immune enhancement**

The immune system is a complex system with different functions, but the main roles of this system are to control the growth of the harmful micro-organisms and distinguish them from familiar bacteria and to expel any foreign bodies such as damaged tissue and tumour cells by producing antibodies and chemical agents (Meydani & Ha 2000; Saxelin 2004). The immune system is the first line of host defence by producing two protective responses. Firstly, innate immunity does not require stimulation and is not enhanced by repeated exposure; this type of immunity has a rapid (minutes rather then days) and non-specific response, particularly to pathogens and other foreign antigens because it is based on the pattern and receptor recognition. Secondly, adaptive humoral and cellular immune functions respond to specific antigens or pathogens and are enhanced by repeated exposure 4–7 days after the beginning of an infection (Szabo 1997; Meydani & Ha 2000). Probiotic bacteria play an important role in both acquired (specific) and innate (non-specific) immunity. For example, oral administration of probiotic bacteria can improve the immunity by increasing the natural killer lymphatic cells (NK cells), phagocytic activity, macrophages, cytokine production, enhancing the circulating IgA antibody and enhance B and T cell productions (Fuller 1989; Schiffrin et al. 1995; Gill 1998; Arunachalam et al. 2000; Borregaard et al. 2000; Kailasapathy & Chin 2000; Meydani & Ha 2000; Simmering & Blaut 2001).

**Bioactive peptides**

The proteolytic activity of probiotic bacteria on milk proteins gives rise to a range of peptides, some of which are bioactive. The range of biological functions depends on the amino acid composition and sequence of the peptide. Different probiotic bacteria have been added to milk or dairy products, individually or in combination, to provide several health benefits. As part of their metabolic products, these bacteria have also been shown to hydrolyse the major components of milk proteins and increase the number of peptides available for their growth (Alhaj et al. 2007). Some of these released peptides from caseins and whey proteins by bacterial proteolysis were found to provide several health benefits such as hypocholesterolemic effect (Nagaoka et al. 2001; Alhaj et al. 2010) and ACE-inhibitory peptides, the major regulator of blood pressure (Minervini et al. 2003; Gobbetti et al. 2004). Other bioactivities such as antioxidants, anticarcinogenic, anticariogenic, immunomodulating and other effects are covered in Chapters 1, 2, 4 and 7.
8.7 Conclusion

Functional foods are currently receiving interest globally and consumers now expect food to provide specific benefits above their basic nutritional value. Milk from different mammalian sources is an excellent nutritional source for human adults and infants. Research has reported that milk and dairy products provide particular health benefits to the consumer depending on the bioactive component in milk. Many bioactive components are found naturally in milk such as conjugated linoleic acid (CLA), oligosaccharide and some bioactive peptides. The production of bioactive peptides can be achieved from protein hydrolysis by digestive enzymes or through fermentation when using lactic acid bacteria, including probiotic.

Research into the identification, isolation and health benefits of bioactive components in bovine milk have been given extensive coverage during the last 20 years, while non-bovine milk including camel, buffalo, human, goat, sheep and yak milk need more investigation to confirm their proposed health benefits. Some health claims of milk bioactive components have been extensively investigated \textit{in vitro} and \textit{in vivo} such as ACE-inhibitory activity, cholesterol reduction and antimicrobial effect. Further research is needed for the \textit{in vitro} and \textit{in vivo} studies to provide firm evidence of the health benefits and claims associated with bovine and non-bovine milks.

References


Almahdy, O., EL-Fakharany, E.M., EL-Dabaa, E., Bun Ng, T. & Redwan, E.M. (2011) Examination of the activity of camel milk casein against Hepatitis C virus (Genotype-4a) and its apoptotic potential in hepatoma and hela cell lines. *Hepatitis Monthly, 11*, 724–730.


Milk-derived Bioactive Components from Fermentation


