Chapter 11

Milk minor constituents, enzymes, hormones, growth factors, and organic acids

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Abstract

Milk and derived products contain essential nutrients, such as proteins, lactose, minerals, vitamins, and enzymes. Additionally, despite of their low concentrations in milk, many other minor constituents present important physiological and/or technological roles (e.g. hormones, growth factors). Dairy industries face many challenges regarding milk processing. Also, the full knowledge on these constituents' physiological roles and effects on health is still lacking. Technological advances, innovative research approaches using metabolic engineering, systems and synthetic biology, and novel production methods will allow the production of higher amounts of such constituents, to produce them in such a way that recovery is easier and to produce differentiated compounds, thus revolutionizing the fields of personalized nutrition and functional foods. This chapter focuses on the description of the minor milk constituents, their applications and main technological challenges. Future perspectives and concerns related to these constituents are discussed.

Keywords: *milk minor constituents, enzymes, hormones, growth factors, organic acids, challenges, dairy industry, human nutrition, functional food, personalized nutrition*

1. Introduction

Milk and derived products contain several essential nutrients and protective agents, such as enzymes and growth factors (Fox and Kelly, 2006a). However, lately a decrease in milk consumption in the Western societies has been observed, mainly due to negative effects on health that have been claimed regarding its intake (Haugh *et al.*, 2007). The high content of saturated fatty acids in milk has been pointed out as being responsible for negative effects contributing to heart diseases, weight gain and obesity (Insel *et al.*, 2004). Nevertheless, this issue is controversial since there are many milk components that promote health benefits including oleic acid, conjugated linoleic acid, omega-3 fatty acids, proteins, vitamins, minerals and bioactive compounds, and various milk proteins and their peptides have been suggested to possess anti-cancer activity (Duarte *et al.*, 2011; Rodrigues *et al*, 2009; Rodrigues and Teixeira, 2009). Regarding the milk fat, an increase of the mean gastric emptying time has been observed when comparing the consumption of whole with half-skimmed milk, thus whole milk promotes an increase of the gastrointestinal transit time. Therefore, the consumption of whole milk may be valuable for regulating appetite and it has not been proven that moderate consumption of milk fat is related to an increase of developing certain diseases. The relationship between milk or milk products consumption and possible negative health effects is still not fully explored. For example, the interaction between carbohydrates and protein in milk exposed to heat may result in products whose effects have not been accurately assessed. The association between food and health is well established (Kussmann and Fay, 2008) and some studies have shown that variable risk factors seem to be of greater significance for health than previously anticipated (Yusuf *et al.*, 2004). Prevention of disease may in the future be just as important as treatment of diseases (Torres *et al*., 2010). Indeed, currently many consumers are extremely conscious of health-properties of food, and the market for healthy food and food with special health benefits is increasing (Haugh *et al.*, 2007).

Milk is a complex matrix made up of components, which *per se* may have negative or positive health effects, respectively. The concentration in milk of several nutrients, including the minor constituents, can be manipulated through feeding regimes and it can be increased or diminished towards a healthier product. The development of functional and healthy foods has become one of the most exciting fields of research in recent years (Pouliot and Gauthier, 2006; Michaelidou, 2008; Michaelidou and Steijns, 2006; Kaput, 2008). The relative ease with which milk can be converted into a wide variety of products makes it an extremely useful base material. In some cases, milk undergoes relatively limited processing, consisting of heat treatment to increase the product microbial shelf life and homogenization to increase the physical shelf life through retarding fat separation (Huppertz and Kelly, 2009). Other well known processes involve the acid-induced coagulation of milk to produce yoghurt, or the enzymatic coagulation of milk to manufacture cheese. In addition, milk may be spray-dried or used as a base from which constituents, e.g. proteins, fats or minor constituents are isolated. As a result of the widespread applications and use of milk and milk products in human nutrition they have been the subject of intensive research in the last century.

This chapter focuses on the description of the minor constituents, enzymes, hormones, growth factors and organic acids in milk, as well as their applications and technological challenges. Finally, future perspectives and concerns related to these constituents will be discussed.

2. Milk minor constituents

Milk is often described as a colloidal suspension, containing emulsified globules of fat, a heterogeneous family of major and minor proteins, the carbohydrate lactose, minerals, vitamins, enzymes (Huppertz and Kelly, 2009) and many other minor components that hold important physiological and/or technological roles such as immunoglobulins, hormones, growth factors, cytokines, nucleotides, peptides,

polyamines, enzymes and other bioactive peptides (Haug *et al.*, 2007). The lipids in milk are emulsified in globules coated with membranes. The proteins are in colloidal dispersions as micelles. The casein micelles occur as colloidal complexes of protein and salts, primarily calcium (Keenan and Patton, 1995). Lactose and most minerals are in solution. Milk composition has a dynamic nature that varies with several factors (Ontsouka *et al.*, 2003; Huppertz and Kelly, 2009):

(a) genetics (e.g. species, breed and individual);

(b) stage of lactation;

(c) health status of the individual animal; and

(d) environmental factors (e.g. feed, climate or method of milking).

Specific milk proteins are involved in the early development of immune response, whereas others take part in the non-immunological defense (e.g. lactoferrin) (Rodrigues *et al.*, 2009). In addition to the major constituents, milk also contains a number of organic and inorganic compounds in small or trace amounts (e.g. peptides, amino acids, antioxidants, salts, nucleotides and vitamins), some of which affect both the processing and nutritional properties of milk. Table 1 summarizes the amounts of some of the most important minor milk constituents in milk from several species.

2.1. Salts and Minerals

Milk salts are essentially phosphates, citrates, chlorides, sulphates, carbonates and biocarbonates of sodium, potassium, calcium and magnesium. Since milk contains organic and inorganic salts, the level of salts is by no means equivalent to the ash content (Huppertz and Kelly, 2009). The milk salt composition is also influenced by a number of factors, including species, breed, stage of lactation, health status, climate, and feed.

Although salts comprise less than 1% of the milk they influence its rate of coagulation and other functional properties. Calcium, magnesium, phosphorous and

citrate are distributed between the soluble and colloidal phases. Their equilibrium is altered by heating, cooling and by a change in pH. The solubility of calcium phosphate is strongly temperature-dependent and, unlike for most other compounds, decreases with increasing temperature (Pouliot *et al.*, 1989). In their studies, these authors found that the levels of calcium and phosphate in the milk serum decrease progressively with temperatures increasing in the range of 4^{o} - 90ºC. Heat-induced decreases in levels of serum magnesium and citrate were also observed but to a smaller extent. Levels of sodium and potassium in milk serum were not affected by heat treatment. Severe heat treatments (above 90ºC) may result in irreversible changes in the mineral balance in milk (Holt, 1995).

In addition to the major salts, milk also contains trace elements. Some elements come into the milk from feeds, but milking utensils and equipment are important sources of such elements as copper, iron, nickel and zinc. Mineral and vitamin contents of goat and sheep milk are mostly higher than in cow milk (Park *et al.*, 2007).

2.2. Vitamins

The fat-soluble vitamins A, D, E and K of milk are associated with the milk fat fraction, while the water-soluble vitamins B complex and C are associated with the water phase. Vitamins are unstable and processing can therefore reduce the effective vitamin content in milk. During processing, the fat-soluble vitamins are retained by the cream, while the water-soluble vitamins remain in skim milk or whey (Michaelidou and Steijns, 2006).

Several researchers suggested that slight deficiencies in B vitamins may constitute risk factors for vascular and neurological diseases and cancers (Brachet *et al.*, 2004). A combined deficiency of folates and vitamin B12 has been associated with the development of dementia and Alzheimer's disease among the eldery (Seshadri *et al.*, 2002). Furthermore, folate, B6 and B12 influence the homocysteine metabolism. As elevated levels of plasma homocysteine constitute a risk factor for developing

cardiovascular disease, an increase in folate intake would be beneficial (Graham and O'Allaghan, 2000). Apart from the prevention of cardiovascular diseases, folates possess a protective role against child birth defects (Forssen *et al.*, 2000; Molloy, 2002). Also, there is growing evidence that a low folate status is linked to an increased cancer risk, particularly colon cancer (Rampersaud *et al.*, 2002). Based on the evidence that B vitamins are beneficial for human health, they have been included in the list of nutraceuticals (Hugenholtz *et al.*, 2002).

At an industrial scale, lactic acid bacteria (LAB) are used to increase the production levels of B vitamins in dairy products. Recent developments in LAB metabolic engineering include the re-routing of complex, biosynthetic pathways leading to the production of metabolites with a health benefit for the consumer, as is the case of the B vitamins. These advances could lead to novel functional foods with great potential for the application of LAB (Kleerebezem and Hugenholtz, 2003). Novel dairy foods, enriched through fermentation using multivitamin-producing organisms with mutations in methylene tetrahydrofolate reductase, could compensate the B vitamin-deficiencies known worldwide and could specifically be used in dietary foods for specific consumer groups (Sybesma *et al.*, 2004). Using fermentation processes for the natural enrichment of foods presents key advantages over the enrichment of food through the addition of chemically synthesized vitamins. It enables the use of naturally occurring molecules in physiological doses and in an environment most suited for optimum biological activity (Michaelidou and Steijns, 2006). This is of extreme importance for dairy products, since some bioactive molecules do not work alone requiring their specific binding proteins or other milk proteins for biological activity. Additionally, it is important to notice that the use of these fortified fermented foods generally is not limited by legislation. However, as in all cases of food fortification, a special care should be given to possible adverse effects related to the excess intake of vitamins.

2.3. Immune components

Milk plays an important role in mammalian host defense (Stelwagen *et al.*, 2009). Present in colostrums and milk of all lactating species, immunoglobulins (Ig) provide immunological protection of the offspring against microbial pathogens and toxins. Depending on the species one can find different types of immunoglobulins and concentrations. In colostrum, the concentration of immunoglobulins is particularly high, with IgG being the major immunoglobulin class present in ruminant milk, in contrast to IgA being the major immunoglobulin present in human milk. Immunoglobulins are transported into mammary secretions via specialized receptors. In addition to immunoglobulins, both colostrum and milk contain viable cells, including neutrophils and macrophages, which secrete a range of immune-related components into milk. These include cytokines and antimicrobial proteins and peptides, such as lactoferrin, defensins and cathelicidins. Mammary epithelial cells themselves also contribute to the host defense by secreting a range of innate immune effector molecules. A detailed understanding of these proteins and peptides offers great potential to add value to the dairy industry. This is demonstrated by the widespread commercial applications of lactoferrin isolated from bovine milk (Rodrigues *et al.*, 2009). Furthermore, some immunoglobulins may influence milk processing, as is the case of IgM that plays an important role in the creaming of cow's milk (Huppertz and Kelly, 2009). Sheep and goat milk are also important sources of minor milk proteins with immune effects including immunoglobulins, lactoferrin, transferrin, ferritin, proteose peptone, calmodulin (calcium binding protein), prolactin, and folate-binding protein. Non-protein nitrogen (NPN) contents of goat and human milks are higher than in cow milk (Park *et al*., 2007).

2.4. Bioactive peptides

Due to the advances in biological research tools, milk and whey proteins have been recognized to contribute to human health through latent biological activity

(Rodrigues and Teixeira, 2009). These proteins are hydrolyzed by certain proteolytic enzymes releasing the so-called bioactive peptides that are capable of modulating specific physiological functions (Michaelidou, 2008).

Bioactive peptides can be obtained from precursor proteins through enzymatic hydrolysis by digestive enzymes derived from microorganisms or plants, or through fermentation of milk with proteolytic starter cultures (Korhonen and Pihlanto-Leppala, 2006). These peptides have been shown to exert various activities affecting the digestive, cardiovascular, immune and nervous system. Specifically, antihypertensive, antioxidative, antithrombotic and hypocholesterolemic peptides can affect the cardiovascular system. Opioid peptides, with agonist or antagonist activity, may regulate the nervous system. Mineral-binding, anti-appetizing and antimicrobial peptides exert their action on the gastrointestinal system. Immunomodulatory and cytomodulatory peptides are of special interest for the immune system. The occurrence and biological activity of these peptides in milk and its derivatives has been extensively reviewed (Clare and Swaisgood, 2000; FitzGerald and Meisel, 2000; Meisel and FitzGerald, 2000; Korhonen and Pihlanto-Leppala, 2006; Kilara and Panyam, 2003; FitzGerald *et al.*, 2004; Lopez-Fandino *et al.*, 2006).

Among the bioactive peptides from milk, those with blood pressure lowering effects are receiving special attention (FitzGerald *et al.*, 2004). Some antihypertensive products based on milk peptides with clinically proven health benefits are currently available in the market (Lopez-Fandino *et al.*, 2006). Sheep and goat milk proteins are important sources of bioactive ACE-inhibitory peptides and antihypertensive peptides (Park *et al*., 2007). Goat milk is being regarded as an appealing research area, as it has been less explored than bovine milk, but also because novel peptidic angiotensin-converting enzyme (ACE) inhibitors have been found in goat milk hydrolysates (Geerlings *et al.,* 2006). As sheep milk is usually converted to cheese, and mostly to traditional cheeses, ovine cheese varieties can be regarded as a valuable source for ACE-inhibitory peptides. The presence of hypotensive peptides, naturally formed in

cheese, has been found to depend on a balance between their formation and their degradation (Ryhanen *et al.*, 2001). Further research has to be conducted to evaluate the long-term physiological effects of consuming such peptides.

Another group of milk bioactive peptides that has aroused the interest of the scientific community are the caseinophosphopeptides (CPPs), since they have been suggested to enhance vitamin D-independent bone calcification in rachitic infants (Mellander, 1950). Its mechanism of action seems to be related to the presence, in their amino acid sequence, of a cluster of three phosphoserine residues followed by two glutamic acid residues. This sequence produces, at the intestinal pH, a negative core responsible for mineral binding (Ca, Zn, Mg) and for resistance of these peptides to proteolytic gastrointestinal enzymes. These two structural features support the hypothesis that CPPs could increase Ca passive diffusion and utilization *in vivo* by increasing Ca solubility at physiological pH of the distal small intestine. However, results from *in vivo* studies are still controversial, as there are many factors that could affect Ca availability, such as the various dietary compounds present at the same time in the intestinal lumen (FitzGerald, 1998; Vegarud *et al*., 2000; Erba *et al*., 2001).

Components that are able to transmit biochemical messages have attracted particular scientific attention as potential bioactive ingredients in a range of biomedical and functional foods, since they have been shown to be potent growth stimulants and mediators for a range of mammalian cells, both *in vitro* and *in vivo* (Smithers, 2004). Among them, non-peptide trophic factors play an important role in maintaining gastrointestinal mucosal mass and modulating the immune system via multiple mechanisms (Playford *et al.*, 2000). These factors include glutamine, polyamines, and nucleotides.

2.5. Polyamines

The interest in naturally occurring polyamines in milk, such as putrescine, spermidine and spermine, has been increasing in the last decades (Michaelidou,

2008). Polyamines consist of flexible polycations that are fully charged under physiological pH conditions. They fulfill a number of roles in cellular metabolism and are essential for cell growth and proliferation (Löser, 2000; Eliassen *et al.*, 2002; Gugliucci, 2004; Larqué *et al.*, 2007). Besides being involved in DNA, RNA and protein synthesis, the most important function of polyamines is the mediation of the action of all known hormone and growth factors.

The polyamine requirements that cannot be met by biosynthesis have to be satisfied by exogenous polyamines consumed from the food (Jeevanandam *et al.*, 1997). It has been suggested that gut maturation is sustained by dietary polyamines; therefore its supplementation in formula-fed infants may prove beneficial. Dietary polyamines may therefore decrease cow's milk allergen absorption and reduce the risk of food allergy (Dorhout and Muskiet, 1999).

Furthermore, polyamines may be important for the fidelity of the enhanced DNA transcription and RNA translation, that occurs in response to infection and during tissue repair, gut growth after surgery, and in gut barrier functions (Grimble and Grimble, 1998). Additionally, dietary polyamines might become important with ageing as cell proliferation slows with age (Nishimura *et al.*, 2006). On the contrary, there are situations where diets low in polyamines may be beneficial such as in the treatment of some tumors (Gugliucci, 2004; Larqué *et al.*, 2007). Accordingly, the effect of polyamines on human health may vary among people.

2.6. Nucleotides

Nucleotides, nucleosides and nucleobases belong to the non-protein-nitrogen (NPN) fraction of milk and are known to have a specific physiological impact in early life (Michaelidou, 2008). Nucleosides and nucleobases, the preferred forms for absorption in the intestine, are suggested to be the acting components of dietary and/or supplemented nucleic acid-related compounds in the gut. Schlimme *et al.* (2000) reviewed the composition and biological activity of these minor constituents in bovine

milk and colostrum. Due to the properties and roles of dietary nucleotides, an increased interest in their use for infant nutrition has been registered, and supplementation with ribonucleotide salts in the manufacture of infant and follow-on formulae has been allowed by the European Commission (Gil and Rueda, 2002; Yu, 2002; Aggett *et al.*, 2003; Alles *et al.*, 2004).

Recently it has been found that modified nucleosides may inhibit cell proliferation and activate apoptosis. Food-derived inducers of apoptosis may be of significance as exogenous anti-carcinogens in the control of malignant cell proliferation, where the intestinal tract could be the primary target site for a possible selective apoptotic stimulant against malignant cells (Schlimme *et al*., 2000).

Furthermore, dietary nucleotides influence biosynthetic processes and modulate gene expression (Sanchez-Pozo and Gil, 2002). Functions of the system and the brain also appear to benefit from food supplementation with nucleosides and nucleotides (Yamamoto *et al.*, 1997), although its effects in the gut seem to depend on the type of damage.

2.7. Proteose-peptones

The potential exploitation of selected milk proteins as ingredients in functional food products has been the reason for an increasing interest in their fractionation (Rodrigues and Teixeira, 2009; Zuniga *et al*., 2009). Heating of skimmed milk (95°C, 30 min) followed by acidification promotes the denaturation of whey proteins and their co-precipitation with caseins, which are insoluble at pH 4.6 (Girardet and Linden 1996). In spite of these drastic conditions, a heterogeneous fraction called proteose peptone (PP) remains soluble (Huppertz and Kelly, 2009). The PP fraction of milk appears to consist of two groups of proteins/peptides, i.e. those that are indigenous in milk (e.g. osteopontin, proteose peptone 3 (PP3)), and those that result from the action of proteolytic enzymes (primarily plasmin, on caseins). The principal components of the

PP fraction have been designated as components 3, 5, and 8 (PP3, PP5, PP8) (Innocente *et al*., 1999).

The primary structure of PP3 includes a polypeptide backbone of 135 amino acid residues containing five phosphorylated serines, two threonine-linked O-glycosylations, and one N-glycosylation, with an apparent molecular mass of 28 kDa (Sorensen *et al*. 1997). Also, a glycoprotein with apparent molecular mass of 18 kDa is associated with component PP3, corresponding to the 54-135 fragment released by plasmin hydrolysis in milk (Sousa *et al.*, 2007).The PP3 is extremely hydrophobic and particularly interesting because of its functional properties, such as its emulsifying power, strong affinity for oil–water interface, strong foaming properties, and biochemical role (Rodrigues *et al*., 2003). Although not many studies have been conducted on the biological functions of PP3, some researchers demonstrated its immunostimulation (Sugahara *et al*., 2005) and prebiotic effects (Etienne *et al*., 1994), as well as its role in caries prevention (Aimutis, 2004; Grenby *et al*., 2001).

2.8. Branched chain amino acids and other amino acids

Milk is rich in essential amino acids and branched chain amino acids (Haug *et al*., 2007). These amino acids have unique roles in human metabolism; in addition to provide substrates for protein synthesis, suppress protein catabolism and serve as substrates for gluconeogenesis; they also trigger muscle protein synthesis and promote protein synthesis (Wolfe, 2002; Layman, 2003; Etzel, 2004).

Branched chain amino acid leucine in particular triggers muscle protein synthesis which is sensed by the insulin signaling pathway (Etzel, 2004). The stimulated insulin secretion caused by milk, is suggested to be caused by milk proteins, and as shown by Nilsson et al. (2007) a mixture of leucine, isoleucine, valine, lysine and threonine resulted in glycemic and insulinemic response resembling the response seen after ingestion of whey. A combination of milk with a meal with high glycaemic load (rapidly digested and absorbed carbohydrates) may stimulate insulin release and

reduce the postprandial blood glucose concentration (Frid *et al.*, 2005). A reduction in postprandial blood glucose is favorable, and it is epidemiological evidence suggesting that milk may lower the risk of diseases related to insulin resistance syndrome (Pereira *et al.*, 2002).

2.9. Taurine

Taurine is an essential amino acid for preterm neonates and for specific consumer groups that are at risk for taurine deficiency, such as patients requiring long-term parenteral nutrition (including premature and newborn infants); diabetes patients, those with chronic hepatic, heart or renal failure (Lourenco and Camilo, 2002; Li *et al*., 2005). It is suggested that during parenteral nutrition, supplementation of 50 mg taurine per kg body weight may be required. Park and collaborators (2007) reported that normal cow milk contains 0.6 mg of taurine per 100 ml (1 μmol/100 ml), while cow colostrum has 8 mg/100 ml. Human mature milk contains significantly more taurine (30 μmol /100 ml) than cow milk or milk from other species, such as sheep (14 μmol/100 ml), but similar to goat (56 μmol/100 ml) (Park et al., 2007; Belewu and Adewole, 2009). Taurine contents of milk can be increased through feeding although there are some issues with its degradation as was suggested by Kim and Park (2003) in their patent (US Patent 6645519).

Taurine is the most abundant intracellular amino acid in humans. It may be synthesized in the body from methionine and cysteine, but in healthy individuals milk in the diet is the usual source of taurine. It is implicated in numerous biological and physiological functions, such as bile acid conjugation and cholestasis prevention, antiarrhythmic / inotropic / chronotropic effects, central nervous system modulation, retinal development and function, endocrine/metabolic effects and antioxidant/anti-inflammatory properties (Lourenco and Camilo, 2002). This essential amino acid has been shown to have endothelial protective effects (Fennessy *et al*., 2003), it may

function principally as a negative feedback regulator, helping to dampen immunological reactions before they cause too much damage to host tissues or to the leukocytes themselves (Park *et al*., 2002), and it has been shown to be analgesic (Li *et al*., 2005; Silva *et al*., 1993).

2.10. Glutathione

Milk is a good source of glutathione that acts in the organism as an antioxidant. Glutathione is a tripeptide of the sulphur amino acid cysteine, plus glycine and glutamic acid. It can be oxidized forming oxidized glutathione, and in this reaction it may remove reactive oxygen species (ROS), thereby regulating the level of ROS in the cells. Glutathione participates in the regulation of insulin production in pancreatic cells, as ROS inhibit expression of the pro-insulin gene. Glutathione appears to have different important roles in leukocytes, as a growth factor, as an anti-apoptotic factor in leukocytes and to regulate the pattern of cytokine secretion (Sprietsma, 1999). Moreover, glutathione is central for antioxidative defense in the lungs, which may be very important in connection with lower respiratory infections including influenza (Cai *et al.*, 2003).

3. Milk enzymes

Indigenous enzymes are milk constituents that originate from four main sources, blood plasma, secretory cell cytoplasm, milk fat globule membrane (MFGM), and somatic cells (leucocytes) (Fox and Kelly, 2006a). Around 70 indigenous enzymes were identified in normal bovine milk (Fox, 2003). Table 2 summarizes the concentrations and activities of some indigenous enzymes present in the milk of several species. Moreover, Table 3 presents examples of some of these enzymes that have been well characterized regarding their activity and significance. Additionally, in Table 4 some indigenous enzymes that have been suggested to play a key role in the

manufacture and/or quality of milk and its derivatives are presented. The best characterized enzymes in milk include *N*-acetyl-β-D-glucosaminidase (NAGase); acid phosphatase (AcP); alkaline phosphatase (AIP); amylase; catalase; γ-glutamyl transferase (γ-GGT); glutathione peroxidase (GSH); lactoperoxidase (LPO); lipoprotein lipase (LPL); lysozyme; plasmin; ribonuclease (RNase); sulphydryl oxidase (SHOx); superoxide dismutase (SOD); and xanthine oxidoreductase (XOR) (Kelly and Fox, 2006). Most of the indigenous enzymes in milk have no obvious physiological role in biosynthesis and secretion of milk (Fox and Kelly, 2006a). Moreover, since these enzymes have no essential beneficial effect on the nutritional or organoleptic attributes of milk, their destruction by heat is one of the purposes of many dairy processes. Besides indigenous enzymes, milk also contains proteases and lipases that are produced by contaminating bacteria during handling and processing. Even when several heat treatment steps are used to prepare milk products, these will not be enough to inactivate all the enzymes and extreme heat treatments will have adverse effects on the products (Chen *et al*., 2003). Proteinases and lipases surviving pasteurization and spray-drying treatments can cause important changes in the functionality and flavor of milk products (Renner, 1988; Visser, 1981; Deeth, 2006).

3.1. Lactoperoxidase

Lactoperoxidase (LPO; EC 1.11.1.7) belongs to the peroxides family of enzymes and is secreted from mammary, salivary and other mucosal glands, acting as a natural antibacterial agent. It has the ability to catalyze certain molecules, including the reduction of hydrogen peroxide (Bjorck, 1978). This enzyme catalyzes peroxidation of thiocyanate and some halides (such as iodine and bromium), which ultimately generates products that inhibit and/or kill a range of bacterial species (Kussendrager and van Hooijdonk, 2000; Pruitt, 2003).

Several isozymes of LPO have been reported as a result of the differences in the level of glycosylation and deamination of glutamine (Gln) or asparagine (Asn) (Fox and Kelly, 2006a). LPO mass is 78.0 kDa and its primary structure contains 612 amino acids (Cals *et al*., 1991). The molecule is highly structured, with 65% β-structure, 23% α-helix and 12% unordered structure (Sievers, 1980). LPO binds Ca^{2+}, which has a major effect on its stability, including its heat stability. At a pH below 5.0, the Ca^{2+} is lost, with a consequent loss of stability.

During the pasteurization process, LPO is not inactivated, suggesting its stability as a preservative (Table 3). This enzyme's biological function has been mainly associated with its antimicrobial activity, and to date there is no report on other direct functions such as immunomodulation or cancer prevention (Rodrigues and Teixeira, 2009).

3.2. Catalase

Catalase (H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6) catalyses the decomposition of hydrogen peroxide in water and oxygen, and also oxidizes reducing agents. The catalase activity in milk varies with feed and stage of lactation, and especially during mastitis its level is markedly increased (Johnson, 1974). It has been reported that this indigenous enzyme is concentrated in the cream (specific activity is 12 times higher than in skimmed milk), thus the MFGM (Milk Fat Globule Membrane) is usually used as the starting material for isolating catalase from milk (Kitchen *et al*., 1970).

Catalase has a molecular mass of 250 kDa (Ito and Akuzawa, 1983a) and three isozymes have been found in the catalase isolated from cream (Ito and Akuzawa, 1983b). Furthermore, catalase is relatively heat labile (Farkye and Imafidon, 1995) and its inactivation has been evaluated as a possible index of thermization of milk (almost completely inactivated by heating at 65ºC for 16 s) by Hirvi and Griffiths (1998).

3.3. Xanthine oxidoreductase

Xanthine oxidoreductase (XOR; EC 1.13.22; 1.1.1.204) is a milk indigenous enzyme capable of oxidizing xanthine and hypoxanthine with the concomitant reduction of O_2 to H_2O_2 (Fox and Kelly, 2006a). For its catalytic activity, XOR has been found to require FAD+ (Massey and Harris, 1997; Harrison, 2004). XOR is concentrated on the MFGM, in which it is the second most abundant protein representing 20% of the protein of the MFGM. This enzyme is a dimer with two identical subunits (146 kDa), each containing 1332 amino acid residues in the case of the bovine milk enzyme. Each XOR monomer contains one atom of molybdenum (Mo), one molecule of FAD+ and two Fe_2S_2 redox centres. NADH acts as a reducing agent.

Milk is a good source of XOR, at least part of which is transported to the mammary gland via the blood stream. Human milk contains XOR, although its levels vary markedly during lactation. The XOR activity in human milk is low because 95 to 98% of the enzyme molecules lack Mo (Atmani *et al.*, 2004). Also, the level of XOR activity in goat, sheep and buffaloes milk is low (Pandya and Khan, 2006). The level of XOR activity in milk can be increased by supplementing the diet with Mo (Fox and Kelly, 2006a).

3.4. Proteinases

Proteinases correspond to the group of proteolytic enzymes that act internally on polypeptide chains, rather than cleaving off single amino acids or dipeptides from the ends of polypeptide chains (Chen *et al*., 2003). Furthermore, this group of proteinases is generally classified in four sub-groups on the basis of the mechanism of action of the enzyme:

(a) serine proteinases, such as plasmin;

(b) cysteine (or sulphydryl) proteinases, such as cathepsin B;

(c) aspartic (or acid) proteinases, such as cathepsin D; and

(d) metallo-proteinases, such as thermolysin.

Two particular indigenous milk proteinases have been studied in detail, namely plasmin and cathepsin D (Kelly and Fox, 2006). These indigenous proteinases arise from mammary tissue cells, blood plasma or leucocytes (Fox and Kelly, 2006a).

3.4.1. Plasmin

The principal milk indigenous proteinase is plasmin (EC 3.4.21.7) (Table 3). The plasmin system has five elements: plasmin, plasmin inhibitors, the inactive zymogen plasminogen, plasminogen activators (PAs) and inhibitors of plasminogen activators (Grufferty and Fox, 1988). This system enters milk from blood and plasmin activity increases during a mastitic infection and in late lactation. Plasmin in milk occurs mainly as the inactive precursor plasminogen (Rollema *et al*., 1981). Plasminogen is activated through proteolysis by PAs, which are serine proteinases (Fang and Sandholm, 1995). In milk, plasminogen, plasmin and PAs are associated with the casein micelles and are concentrated in rennet-coagulated cheese curds and casein, while the inhibitors of PAs and plasmin are soluble in the milk serum (Fox and Kelly, 2006a).

Due to changes in practices in the dairy industry, such as improved bacterial quality, extended storage and the introduction of high-temperature processed milk (plasmin is very heat stable), plasmin has gained an increased significance since its relationship with microbial proteases provides a means to control its levels to benefit the quality of dairy products (Kelly and McSweeney, 2003).

Bovine plasminogen is a single-chain glycoprotein containing 786 amino acid residues, with a molecular mass of 88 kDa. Plasminogen is converted to plasmin by cleavage of the Arg557–Ile558 bond by specific proteinases. Three elements of the plasmin system (plasmin, plasminogen and PAs) have been reported to have very similar and relatively high heat stabilities. For plasmin and plasminogen, this high heat stability is attributed to protection by milk proteins, such as casein (Grufferty and Fox, 1988). Plasmin contributes to primary proteolysis in cheese, especially high-cooked varieties in which the coagulant is extensively denatured; it may cause age gelation of

ultra high temperature (UHT) sterilized milk; and reduces the yield of cheese and casein owing to the loss of proteose peptones in whey (Rollema *et al*., 1981).

3.4.2. Cathepsin D

The second proteinase identified in milk was cathepsin D, which is presumably a lysosomal enzyme (Larsen *et al*., 1996). As with plasmin, cathepsin D is part of a complex system, including inactive precursors (Hurley *et al*., 2000). The major form of cathepsin D in milk is the inactive zymogen, procathepsin D (the proenzyme of cathepsin D), although milk also contains low levels of the mature forms of the enzyme (Larsen *et al.*, 2000).

The level of cathepsin D in milk is correlated significantly with the somatic cell count (SCC), although it is not clear whether this reflects increased production of cathepsin D and/or increased activation of precursors (Hurley *et al*., 2000). Cathepsin D has a pH optimum of 4 and a molecular mass of 36 kDa and can degrade all milk proteins except β-lactoglobulin (Larsen *et al*., 1996). It is completely inactivated by heat treatment of 70ºC for 10 min at pH 4 in acetate buffer and by pasteurization at 65ºC for 30 min in skim milk (Chen *et al*., 2003). Because of this relatively low heat stability, even in milk, cathepsin D has not been regarded as an important enzyme in pasteurized milk and milk products.

3.5. Lipases and esterases

Lipolytic enzymes can be defined as carboxylesterases that hydrolyse acylglycerols (Beisson *et al*., 2000). Those that hydrolyse acylglycerols of less than 10 carbon-chain fatty acids are the esterases, or carboxylases (EC 3.1.1.1); those that hydrolyse acylglycerols of over or equal 10 carbon-chain fatty acids are the lipases, or triacylglycerol acylhydrolases (EC 3.1.1.3). Esterases are active in aqueous solutions, while true lipases are more active at lipid–water interfaces rather than in the aqueous

phase and most are also capable of hydrolyzing esterase substrates (Chen *et al.*, 2003).

Lipoprotein lipase (LPL) accounts for most of the lipolytic activity in bovine milk (Olivecrona *et al*., 2003) and shares about 30% sequence identity with pancreatic lipase, which is regarded as a typical lipase (Fox and Kelly, 2006a) (Table 3). LPL is a dimer of glycoprotein chains (two N-linked oligosaccharides), each of 42 kDa, and contains 8.3% carbohydrate (Olivecrona *et al*., 2003). This enzyme is synthesized in mammary gland secretory cells and its level in bovine milk is dependent on the breed, stage of lactation, diet and nutrition, the season and milk production level (Deeth, 2006).

LPL can form large aggregates, regardless of ionic strength, and yet retain an active conformation (Olivecrona and Bengtsson, 1984). Moreover, this enzyme is relatively unstable to heat. High-temperature, short-time (HTST) pasteurization (72ºC for 15 s) inactivates most, if not all, of the enzyme in milk (Deeth, 2006), and therefore LPL causes little, if any, lipolysis in pasteurized milk and products derived from pasteurized milk (Chen *et al*., 2003). In most milk samples, LPL causes hydrolytic rancidity only if the MFGM is damaged, e.g., by agitation, foaming, cooling/warming, freezing or homogenization (Chen *et al*., 2003).

Esterases are distinguished from lipases by their preference for soluble rather than emulsified ester substrates. Milk contains several esterases (Kitchen, 1985; Chen *et al.*, 2003), the most significant of which are acylesterases (EC 3.1.1.7), cholinesterase (EC 3.1.1.8) and carboxylesterase (3.1.1.1).

3.6. Amylase

Amylase in milk is indigenous and α-amylase is the principal enzyme, with a lesser amount of β-amylase; the enzymes partition mainly into skimmed milk and whey (Fox and Kelly, 2006a). α-amylase in milk is similar to salivary amylase. Amylase is

quite labile to heat and loss of amylase activity was proposed as a reliable index of the intensity of heat treatment applied to milk. Since bovine milk contains no starch and only low levels of oligosaccharides, the function of amylase in milk is unclear.

3.7. Alkaline phosphatase

Alkaline phosphatase (AlP; EC 3.1.3.1) is a membrane-bound glycoprotein that is widely distributed in animal tissues and in microorganisms (Table 3). It is a very important enzyme in clinical chemistry, being its activity in various tissues an indicator of diseased states. Nevertheless, its physiological roles are still unclear.

The AlP activity of bovine milk varies considerably between individuals, and throughout lactation; activity varies inversely with milk yield but is independent of fat content, breed and feed (Fox and Kelly, 2006b). AlP is concentrated in cream and is released into buttermilk on churning.

AlP is a homo-dimer of two identical sub-units, each of molecular mass 85 kDa; it contains four atoms of Zn which are essential for activity and is also activated by Mg^{2}+ (Fox and Kelly, 2006b). AlP is inhibited by metal chelators; the apo-enzyme may be reactivated by the addition of one of a number of metals, which is used as the principle of methods to determine very low concentrations of zinc in biological systems. It is also inhibited by inorganic phosphate.

3.8. Acid phosphatase

Acid phosphomonoesterase (AcP; EC 3.1.3.2) in milk has an optimal pH of 4.0 and is very stable to heating (for complete inactivation heating at 88ºC for 10 min is required). The enzyme is not activated by Mg^{2+} (as is AlP), but it is activated slightly by Mn^{2+} and is very strongly inhibited by fluoride (Fox and Kelly, 2006b).

About 80% of the AcP in cow milk is found in the skimmed milk but the specific activity is higher in cream. There is only one isozyme of AcP in milk that is strongly attached to the MFGM and is not released by non-ionic detergents (Kitchen, 1985).

Furthermore, about 40% of the AcP in skim milk partitioned into the whey on rennet coagulation.

The AcP isolated from skim milk is a glycoprotein with a molecular mass of 42 kDa and an isolelectric point of 7.9. It is inhibited by many heavy metals, oxidizing agents, orthophosphates and polyphosphates and is activated by thiol-reducing agents and ascorbic acid; it is not affected by metal chelators (Andrews, 1976). This enzyme contains a high level of basic amino acids and no methionine.

Although AcP is present in cow milk at a much lower level than AlP, its greater heat stability and lower pH optimum may make it technologically significant. The suitability of AcP as an indicator enzyme for super pasteurization of milk has been reported although it is not as useful as other alternatives (e.g. γ-GGT or LPO) (Andrews *et al*., 1987).

3.9. Ribonuclease

Ribonucleases (RNase) catalyze cleavage of the phosphodiester bond between the 50-ribose of a nucleotide and the phosphate group attached to the 30 position of ribose of an adjacent pyrimidine nucleotide, forming a 20, 30 cyclic phosphate, which is then hydrolyzed to the corresponding 30-nucleotide phosphate. RNases of various origins and with different biological functions have been characterized. RNase occurs in various tissues and secretions, including milk (Fox and Kelly, 2006b).

RNase in cow milk is optimally active at pH 7.5 and is more heat-stable at acid pH values than at pH 7. Little or no RNase activity survives UHT sterilization (121ºC for 10 s) but about 60% survives heating at 72ºC for 2 min (Meyer *et al*., 1987) or at 80ºC for 15 s (Griffiths, 1986). RNase activity in raw or heat-treated milk is stable to repeated freezing and thawing and to frozen storage for at least a year (Meyer *et al*., 1987). It has been suggested that RNase can inhibit bacteriophage, which can inhibit the growth of starter cultures in cheese making. Otherwise RNase has no technological significance in milk, while it may have significant biological functions.

3.10. *N*-acetyl-β-D-glucosaminidase

N-acetyl-β-D-glucosaminidase (NAGase; EC 3.2.1.30) hydrolyzes terminal, non-reducing *N*-acetyl-β-D-glucosamine residues from *N*-acetyl-β-D-glucosaminides, including glycoproteins and fragments of chitin. However, NAGase is not specific for *N*-acetyl-β-D-glucosaminides; since it can also hydrolyse *N*-acetyl-β-D-galactosaminides.

NAGase is thought to be a lysosomal enzyme that originates principally from mammary gland epithelial cells and, to a lesser extent, from somatic cells. More than 95% of NAGase in cow milk is in the skimmed milk. The enzyme is optimally active at 50ºC and pH 4.2 (Fox and Kelly, 2006b).

NAGase is inactivated by high temperature-short time (HTST) pasteurization and it has been proposed as a suitable indicator enzyme for assessing heat treatment in the range 65–75ºC during 15 s (Andrews *et al*., 1987).

3.11. Lysozyme

Lysozyme (EC 3.1.2.17) is a widely distributed enzyme that lyses certain bacteria by hydrolyzing the β(1-4)-linkage between muramic acid and N-acetyl-glucosamine of mucopolysaccharides in the bacterial cell wall. Although lysozyme is a lysosomal enzyme, it is found in soluble form in many body fluids and the lysozyme in milk is usually isolated from whey, indicating that it is in solution, like other lysosomal enzymes such as cathepsin D (Fox and Kelly, 2006b)..

The bovine milk lysozyme presents an optimum pH of 6.4, a molecular weight of 18 kDa and its amino acid composition and immunological properties are considerably different from those from other species (e.g. human or equine milk). The amino acid sequence of lysozymes is highly homologous with that of α-lactalbumin. All lysozymes are relatively stable to heat at acid pH values but are relatively labile at pH above 7.

More than 75% of the lysozyme activity in bovine milk survives heating at 75ºC during 15 min or 80ºC during 15 s and therefore it is not affected by HTST pasteurization.

Lysozyme's most probable physiological role is to act as a bactericidal agent. One might expect that, owing to its bactericidal effect, indigenous milk lysozyme would have a beneficial effect on the shelf-life of milk; nevertheless such effects do not appear to have been reported. Exogenous lysozyme may be added to milk for many cheese varieties (e.g., Gouda, Edam, Emmental, Parmigiano Reggiano) as an alternative to KNO_3 to prevent the growth of *Clostridium tyrobutyricum* which causes late gas blowing and off-flavors.

3.12. γ-glutamyl transferase

γ-glutamyl transferase (γ-GGT; EC 2.3.2.2) catalyses the transfer of γ-glutamyl residues from γ-glutamyl-containing peptides. In milk, γ-GGT is found in the membrane material in skim milk (70%) or in the MFGM, from which it can be dissociated by detergents or organic solvents. The enzyme has a molecular mass of 80 kDa and consists of two subunits of 57 and 25 kDa, both of which are glycoproteins (Baumrucker, 1980). The enzyme, which associates strongly, is optimally active at pH 8.5–9 and 45ºC and has an isoelectric point of 3.9. It is strongly inhibited by diisopropylfluorophosphate, iodoacetamide and metals (Farkye, 2003). γ-GGT activity in human and bovine milk varies during lactation, being highest in colostrum.

γ-GGT has a role in the regulation of cellular glutathione (GSH) and may be involved in the transport of amino acids from blood into the mammary gland via the so-called γ-glutamyl cycle and thus may be involved in the biosynthesis of milk proteins (Fox and Kelly, 2006b).

3.13. Superoxide dismutase

Superoxide dismutase (SOD; EC 1.15.1.1) scavenges superoxide radicals. The H_2O_2 formed may be reduced to water and oxygen by catalase, peroxidase or a suitable reducing agent. SOD has been identified in many animal and bacterial cells being its biological function to protect tissue against free radicals of oxygen in anaerobic systems. There are four isoforms of SOD, Cu/Zn-SOD, extracellular (EC) SOD, Mn-SOD and Fe-SOD. Cu/Zn-SOD is the most common form in mammals and has been isolated from a number of tissues, including bovine erythrocytes. The enzyme, which is very stable in 9M urea at neutral pH, consists of two identical subunits of molecular mass 16 kDa (153 amino acid residues), held together by one or more disulphide bonds (Hara *et al*., 2003). Mn-SOD and EC SOD are tetrameric enzymes with subunits of molecular mass 20 and 35 kDa, respectively.

3.14. Sulphydryl oxidase

Sulphydryl oxidase (SHOx; EC 1.8.3-) catalyses the oxidation of SH groups of cysteine, GSH and proteins to disulphides. The enzyme is widely distributed in cell membranes, including those of the mammary gland, kidney, pancreas and intestine. SHOx is a glycoprotein (10% carbohydrate) containing 0.5 atoms of Fe per monomer (89 kDa). The enzyme is optimally active at pH 7 and 35ºC and is inhibited by metal chelators and SH-blocking reagents.

SHOx oxidizes reduced RNase and restores enzymatic activity, suggesting that its physiological function is the formation of specific disulphide bonds during the post synthesis processing of proteins. Its significance in the dairy industry is its ability to oxidize SH groups exposed and activated during high-temperature processing and which are responsible for the cooked flavor in such products (Swaisgood, 2003). Apparently, oxidation of the SH groups renders the product more stable to lipid oxidation, although SH groups *per se* are anti-oxidants.

3.15. Aldolase

Aldolase (EC 4.1.3.13) reversibly hydrolyses fructose 1,6-diphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. It is a key enzyme in the glycolytic pathway. Its presence in cow milk has been reported, and although most (66%) of the aldolase in milk is in the skim milk fraction (Kitchen *et al*., 1970), it is concentrated in the cream (Keenan and Mather, 2006). The aldolase is located in the cytoplasm of the mammary cells, from which the enzyme in milk presumably originates, although some may be from blood. Furthermore, it has been suggested that aldolase plays a role in flavor development in dairy products.

3.16. Glutathione peroxidase

Glutathione peroxidase (GSH; EC 1.11.1.9) is widespread in the cytoplasm of animal tissues, especially erythrocytes. Its function is to protect the cell against the damaging effects of peroxides, as part of an anti-oxidative system which includes SOD (Avissar *et al*., 1991). GSH is a tetrametric protein of four identical subunits (21 kDa), each of which contains one atom of selenium (Se). The molecule has been well characterized, including elucidation of its primary, secondary and tertiary structures (Liu and Luo, 2003).

GSH has no known enzymatic function in milk, in which it binds 30% of the total Se, an important trace element in the diet. The level of GSH in milk varies with the species and diet (Farkye, 2003).

4. Milk hormones and growth factors

A clear division between milk hormones, cytokines and growth factors is currently missing (Gauthier *et al.*, 2006). All these molecules are important for the growth, maturation or repair of different cell types in the neonate and/or adult. Moreover, all of these growth-promoting factors are signaling molecules released by

cells to communicate with each other. Briefly, hormones are substances that are released into the extracellular medium by the cells of a given tissue, to be transported to a new site of action (endocrine function), where they induce a specific response. The distinction between cytokines and growth factors is not straightforward since some growth factors (e.g. transforming growth factor beta; TGF-β) have also been reported as cytokines by many authors (Gauthier *et al*., 2006). Cytokines are proteins or glycoproteins produced by many cell types that have profound bioactive effects on other cells within a short distance at low concentrations (Playford *et al.*, 2004). As a result, the cytokine effects are local and these agents are involved in autocrine or paracrine functions. Examples of cytokines include the interleukins (IL) series, tumor necrosis factors (TNF) and interferon (IFN). Growth factors are proteins or polypeptides that bind to specific receptors triggering intracellular secondary messengers, ultimately resulting in cellular proliferation and/or differentiation (Michaelidou and Steijns, 2006).

Milk contains more than 50 growth factors and hormones, being their concentrations much lower than those of immunoglobulins or lactoferrin (Michaelidou and Steijns, 2006). Whereas bovine colostrums may contain high levels of cytokines such as interleukin-1β (IL-1β), IL-6, tumor necrosis factor (TNF-α), interferon-γ (INF-γ), and IL-1 receptor antagonist, their levels in mature milk are remarkably lower (Hagiwara *et al*., 2000). Whey has also been identified as a source of growth factors with potent and proven bioactivity (Smithers, 2004). Examples of growth factors identified in milk include insulin-like growth factor (IGF-I and II), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor (TGF-β) and betacellulin (Dunbar *et al.*, 1999; Elfstrand *et al.*, 2002; Pakkanen and Aalto, 1997; Rogers *et al.*, 1995). Since these compounds are potent growth stimulants and mediators for a range of mammalian cells, they constitute potential bioactive ingredients in a range of biomedical and functional foods (Smithers, 2004). Milk derived TGF-β might be exploited in functional foods for the infant or during therapies for

specific intestinal diseases or cancers (Donnet-Hughes *et al.*, 2000). Milk-derived products are already in clinical use for the treatment of inflammatory bowel disease; casein-based enteral feeds are used for the treatment of Crohn disease (Beattie *et al.*, 1998; Donnet-Hughes *et al*., 2000) and their efficacy might be due to the presence of growth factors (Playford *et al.*, 2000). Also, results from animal and human trials with a growth factor extract from Cheddar cheese whey targeting oral mucositis and chronic ulcers were very promising (Smithers, 2004). Dairy derived preparations appear to be an attractive therapeutic option (Regester and Belford, 1999) because they contain many different growth factors in a formulation that provides additive or synergistic activity and inherent protection against proteolytic digestion. Besides, such preparations have the advantage of being perceived as ‘‘natural’’ products, which may result in greater patient acceptance and compliance (Playford *et al.*, 2000).

4.1. Hormones

Milk contains several hormones at trace levels that have limited nutritive or diagnostic value. However, many studies regarding the hormones' physiological roles in human and bovine milk have been conducted (Koldovsky and Thornburg, 1987; Grosvenor *et al.*, 1993). Hormones in milk originate from the blood and are secreted in milk through an active transport within the mammary gland. Also, some hormones can be synthesized by the mammary gland and excreted to milk. Table 5 summarizes the main hormones that have been identified in bovine milk, as well as its concentration ranges (Jouan *et al*., 2006). Most hormones reported in milk are classified into four main groups, such as gonadal, adrenal, pituitary and hypothalamic hormones. Other molecules, such as proteins related to the parathyroid hormone have also been reported as hormones.

4.1.1. Gonadal hormones

The gonadal hormone group includes estrogens, progesterone and androgens, being the androgens the least studied hormones. Since the amount of hormones in milk and milk products is very low, their accurate quantification remains a challenge. Several techniques have been proposed for this purpose, such as colorimetry, spectrofluorometry, gas chromatography and high-pressure chromatography, and radioimmunoassay. The concentrations of estrogens, namely 17β-estradiol, estrone and estriol in milk and several dairy products have been reported (Wolford and Argoudelis, 1979). The cow milk fat fraction was found to contain 65% of 17β-estradiol and 80% of estrone. The occurrence of estrogens in both butter and skim milk clearly indicates a distribution of those steroids between the lipid and serum phases of milk. Estrone was found to be the predominant estrogen in milk, and it is well known that estrogen concentrations are related to gestation and reproductive cycle. Regarding progesterone levels in milk, they have been found to be related to pregnancy and parturition (Comin *et al.*, 2005). In cow milk, progesterone concentrations are determined by gas chromatography and are around 0.3-0.4 pg mL^{-1} (Darling *et al.*, 1974). The progesterone concentrations were found to be higher in cream than in skim milk. Ginther and co-workers (1976) determined the progesterone contents of several cow dairy products, namely 11.4 ng mL^{-1} in whole milk, 4.7 ng mL^{-1} in skim milk, and 58.8 ng mL^{-1} in cream.

4.1.2. Adrenal gland hormones

Adrenal gland hormones consist essentially of the glucocorticoids that have been identified in bovine milk in concentrations between 0.7 and 1.4 ng mL^{-1} (Gwazdauskas *et al.*, 1977). No remarkable differences have been found between whole and skim milk. Cortisol and corticosterone are the main glucocorticoids in blood plasma of cows (Tucker and Schwalm, 1977). During lactation, the glucocorticoid concentrations in milk only represent 4% of the blood plasma concentrations, suggesting that only a small amount is transferred to the milk. Glucocorticoids are not

concentrated in cream, as is the case of estrogens. In bovine milk, corticosteroids are equally distributed between caseins and whey protein fractions. Glucocorticoids possibly act in conjunction with other hormones to maintain lactation and their effects are mediated by specific receptors. Furthermore, there seems to be a reduction of the glucose uptake by the mammary gland due to the presence of glucocorticoids, thus suggesting that the milk production is regulated by these hormones.

4.1.3. Pituitary hormones

Two hormones have been identified in the category of pituitary hormones, namely prolactin and the growth hormone. Prolactin concentrations ranging 5 to 200 ng mL^{-1} were detected by radioimmunoassay in bovine milk (Malven and Mc Murty, 1974). It has been suggested that prolactin concentrations vary seasonally being higher in the summer, and a direct effect of storage temperature of the milk appears to affect this hormone concentration. Lower temperatures present a negative impact on prolactin concentrations. Kacsoh and collaborators (1991) reported that part of the prolactin content of milk is associated with the milk fat globules, thus 60% of the prolactin is removed from milk during the centrifugal separation of fat. This hormone is thought to originate from blood plasma and its biological functions are not well known. The growth hormone or somatotropin has been detected in milk at concentrations lower than 1 ng mL^{-1} (Torkelson, 1987). Regarding its biological functions, somatotropin is thought to be acting on the mammary gland by means of specific receptors. Furthermore, this hormone was also found to increase the concentration of insulin-like growth factor-1 (IGF-I) in epithelial cells of the mammary gland of lactating cows (Glimm *et al*., 1988).

4.1.4. Hypothalamic hormones

The group classified as hypothalamic hormones includes the gonadotropin-releasing hormone, luteinizing hormone-releasing hormone, thyrotropin-releasing hormone and somatostatin. All these hormones have been detected and quantified in

bovine milk mainly using radioimmunoassay. Gonadotropin-releasing hormone content in milk is 5-6 times greater than in blood plasma (Baram *et al*., 1977). The hormone might be from an extra-hypothalamic origin but it is more likely coming from an active transport by the mammary gland. Amarant and collaborators (1982) determined the luteinizing hormone-releasing hormone content in milk and colostrums. This hormone may originate from the blood and be concentrated in the mammary gland by an active process or be of an extra-hypothalamic origin. The same authors also measured thyrotropin-releasing hormone in milk and colostrum. As for the other hypothalamic hormones, their origin in milk is unclear. The occurrence of somatostatin in bovine milk has been demonstrated by enzyme immunoassay on fat and casein free milk (Takeyama *et al*., 1990). Its concentrations in milk vary between 10 and 30 pmol/L and it does not seem to be affected by parturition.

4.1.5. Other hormones

As previously mentioned, other molecules, such as proteins related to the parathyroid hormone, insulin, calcitonin, bombesin, erythropoietin and melatonin, have also been identified in milk although they are less known and characterized. Many authors have mentioned that parathyroid hormone-related protein is present in bovine milk (Budayr *et al.*, 1989; Ratcliffe *et al*., 1990). Curiously the concentrations of this hormone in both fresh and pasteurized milk have been found to be similar, thus suggesting that this hormone is heat-stable. The physiological functions of this hormone have not been clearly established. Produced by the mammary gland, this hormone might be involved in the transport of calcium from blood plasma to milk. Regarding insulin, its concentrations in milk were found to vary during pre-partum, post-partum and after parturition (Malvern, 1977). In colostrum its content is between 0.67 and 5.0 nM, which is 100-fold higher than the concentration in the blood plasma (Ballard *et al*., 1982). Moreover, calcitonin concentrations in human milk have been estimated at 700 ng mL^{-1} and this hormone has been found to inhibit the liberation of

prolactin (Koldovsky, 1989). As for bombesin, it is known to influence the gastric hormonal secretions following ingestion (Lazarus *et al.*, 1986). Satiety, blood sugar concentrations, gut acidity and concentrations of some gastro-intestinal hormones are known to be influenced by bombesin. Bombesin has been found in human milk, bovine milk, milk powder and whey (Koldovsky, 1989). Furthermore, no analytical data regarding the erythropoietin content in bovine milk are available, although this hormone has been identified in human milk (Grosvenor *et al*., 1993). Finally, melatonin is a hormone synthesized by the pineal gland in a diurnal pattern reflecting photoperiodicity. Melatonin has been found in human, bovine and goat milk (Eriksson *et al*., 1998; Valtonen *et al*., 2003) at a low concentration.

4.2. Growth factors

The most abundant growth factors in bovine milk and colostrum are insulin-like growth factor I (IGF-I), transforming growth factor (TGF)-β2, some members of the epidermal growth factor (EGF) family and fibroblast growth factor (FGF2) (Grosvenor *et al*., 1993; Pakkanen and Aalto, 1997). Table 6 summarizes the experimental data available on the content of some of these growth factors in bovine milk. The concentrations found in colostrum are generally higher than those in milk, except for betacellulin (BTC), where it appears to be equivalent (Pouliot and Gauthier, 2006). Quantitatively, the relative concentrations of growth factors in milk are IGF-I 〉 TGF-β2 〉 EGF ≈ IGF- II 〉 bFGF.

The main biological functions of milk growth factors have been extensively reviewed by Gauthier and co-workers (2006). Briefly, EGF and BTC are members of EGF family that were detected in milk products in sufficient amount to induce physiological effects (Dunbar and Goddard, 2000). They stimulate the proliferation of epidermal, epithelial and embryonic cells; inhibit the secretion of gastric acid and promote wound healing and bone resorption.

The TGF-β family comprises multifunctional growth and differentiation factors that act on most cell types with activities dependent on the cell type, stage of proliferation and environment (Massague, 1990), thus playing an important role in embryogenesis, tissue repair, formation of bone and cartilage, and in the control of the immune system. TGF-β2 is the predominant form of the TGF family members found in milk products, and although its physiological function is unknown some authors suggested that it could be a mediator of mucosal immunity or gut epithelial differentiation in neonates (Cox and Burk, 1991; Jin *et al*. 1991). TGF-βs stimulates the proliferation of some cells, especially in connective tissue, whereas they act as growth inhibitors of some other cells, such as lymphocytes and epithelial cells.

The insulin-like growth factors, IGF-I and IGF-II, stimulate the proliferation of many cell types (Jones and Clemmons, 1995). IGF-I stimulates cellular growth, development and differentiation. Furthermore, IGF-I stimulates glucose uptake and the synthesis of glycogen. Administration of IGFs to humans causes hypoglycemia (Guler *et al*., 1987), improvement in nitrogen balance (Clemmons *et al*., 1992), lowering of cholesterol and potassium (Moscatelli, 1987), and improvement in renal functions (Guler *et al*., 1989; Hirschberg *et al.*, 1993).

Fibroblast growth factor (FGF2) can exert multiple functions on a variety of cells. It has been reported to stimulate proliferation, migration and differentiation of endothelial cells, fibroblasts and epithelial cells (Chen *et al*., 2004). This growth factor also promotes angiogenesis, normal wound healing, tissue development, hematopoiesis and the synthesis of collagen and fibronectin. It has been suggested that FGF2 in milk might be bound to the heparan sulfate proteoglycan in the milk fat globule membrane (Hironaka *et al*, 1997).

5. Milk organic acids

Most work that has been recently conducted regards the presence of fatty acids in milk (e.g. conjugated linoleic acid (CLA), linoleic acid, myristic, plamitic, butyric and stearic) (Parodi, 1999; Mansbridge and Blake, 1997) and less attention has been given to the organic acids (e.g. citric, lactic, acetic) (Mullin and Emmons, 1997). Citric acid is the predominant organic acid in milk. During storage it disappears rapidly as a result of the action of bacteria. Other acids (lactic, acetic) are degradation products of lactose. The occurrence of orotic acid, an intermediary product in the biosynthesis of pyrimidine nucleotides, is specific for milk. Orotic acid, as well as total creatinine and uric acid are suitable indicators for the determination of the proportion of milk in foods. The contents of orotic acid, creatinine and uric acid in milk vary among the different species. For example, the content in orotic acid is low in buffalo milk (19.6 μg/100 ml) as compared to cow milk (52.6 μg/100 ml), while creatinine is higher (246 mg/100 ml *versus* 167 mg/100 ml) and uric acid is the same (0.26 mg/100 ml) (Park and Haenlein, 2006).

Raw milk produced under normal conditions develops acidity. It has long been recognized that highly acid milk does not putrefy. Therefore, allowing milk to develop acidity naturally preserves the other milk constituents. Fresh bovine milk is particularly suitable as a fermentation substrate for most microorganisms, since it contains 5% lactose, 3.3% protein, has a water activity near 1.0 and a pH of 6.7. Although milk from other species present different compositions of protein and lactose, they are also suitable for microbial growth (Park and Haenlein, 2006). Milk samples from normal healthy mammary glands contain many strains of bacteria (Haug *et al*., 2007).

Bacteria in milk are responsible for acid development. They produce acid by the anaerobic breakdown of lactose to lactic acid and other organic acids. A number of sugar fermentations depending on the microorganism involved and the end products have been reported in milk. For example, if only streptococci and lactobacilli are present, then the end product will be lactic acid. The lactic acid fermentation is the most important one in milk and is central to many processes (Mullin and Emmons, 1997). Lactic acid bacteria are saccharolytic and fermentative, and therefore are ideally

suited for growth in milk. In general, they will out-compete other microorganisms for lactose, and due to acidification they will produce an unfriendly environment for competitors. Therefore, when properly made, cultured dairy products (fermented milks) have long shelf-lives and, although growth of acid-tolerant yeast and molds is possible, growth of pathogens rarely occurs. Propionibacteria will ferment lactose producing lactic acid, propionic acid, acetic acid and carbon dioxide. Propionic fermentation is a mixed-acid fermentation and is used in the manufacture of Swiss cheese varieties. Yeasts, such as *Candida* and *Torula*, will produce ethanol and carbon dioxide. Alcohol fermentation can be used to prepare certain fermented milks and also to make ethanol from whey. On the other hand, coliform fermentation can also occur with the production of lactic acid, acetic acid, ethanol, carbon dioxide and hydrogen. This type of fermentation is an example of undesirable spoilage fermentation. Large numbers of coliform bacteria in milk indicates poor hygiene. The coliform fermentation disrupts lactic acid fermentation and also causes spoilage in cheese. The type of fermentation obtained will depend on the numbers and types of bacteria in milk, storage temperature and the presence or absence of inhibitory substances. Different bacteria may be used for fermentation, giving products special flavor and aroma, and with several potential health beneficial metabolites (Rossland *et al*., 2005). Fermented milks (buttermilk, kefir, coumis, aigar, among others) are nutritious foods and many have been reported as possessing medicinal properties (Vinderola *et al*., 2005; Branca and Rossi, 2002; De Vrese *et al*., 2001; Sanggaard *et al*., 2004).

Organic acids play an important role in fermented milks and cheese, and can be changed due to technical options in the process. Park and Guo (2006) reviewed the organic acid composition of several types of goat cheese depending on the processing. For example, for plain soft goat cheese they found differences in tartaric, formic and uric acid contents between fresh and frozen-thawed treatments. Freezing caused an increase in formic and uric acids and a decrease in tartaric acid. These changes in organic acid contents of the plain soft goat cheese are in contrast with the results

reported by Califano and Bevilacqua (1999) that found no significant effect of freezing on the variations in organic acid contents of cow milk Mozzarella cheese. Also, Park and Guo (2006) reported that aging (refrigerated storage at 4ºC for 28 days) of plain soft goat cheese promoted changes in only three acids (orotic, malic and butyric). In Montery Jack cheese, frozen and thawed cheese had higher acetic, butyric, citric, malic, propionic, and pyruvic acids compared to the unfrozen control cheese. The levels of acetic, butyric, malic, and orotic acids were elevated by aging time.

6. Future perspectives and concerns

Milk and milk products are commonly used in many food regimens, and although the operations for their processing are known and optimized, many challenges persist (Villamiel *et al*., 2009). Within modern societies the milk has to be treated in different ways to be kept for several days. This processing includes steps that may be of concern. In fresh milk each lipid globule is surrounded by an apical plasma membrane from the mammary epithelial cell. It is not known, although debated, whether the milk homogenization, when the fat globules with their globule membrane are broken up into many new small lipid droplets with just a small fragment of the originating membrane, might have health implications (Haug *et al.*, 2007). Additionally, proteins and peptides are heat sensitive, and their bioactivity may be reduced by pasteurization of milk. Heating of milk may also result in the formation of potentially harmful new products, e.g. reaction products between carbohydrates in milk with proteins (Lund *et al*., 2005). Also, the amount of some vitamins and antioxidants will be reduced by heating. Glutathione may easily be destroyed during storage (Ankrah *et al*., 2000). Therefore, the dairy industry has to deal with the important challenge of treating milk in such a way that preserves its vitamins, proteins and peptides. Most of the new technologies currently used are not completely new and they have been explored in the past but with limited success. However, the technical-scientific progress together with the consumer demands for minimally processed foods has led to their renaissance.

Some dairy industries currently filter milk using membrane processes instead of using pasteurization, and the use of non-thermal processing technologies may yield health benefits (Haug *et al.*, 2007). New technologies used in the milk processing for the inactivation of microorganisms and enzymes involve microwaves, high pressure, pulsed electric fields, microfiltration, innovative steam injection systems and combined technologies (Villamiel *et al*., 2009).

Regarding novel production methods it is important to mention that systems, synthetic and metabolic engineering will be in the near future key players for the development of improved and safe microbial cell factories that will enable the production of specific enzymes, vitamins or minor components (Park and Lee, 2008; Brenner *et al*., 2008). Using these tools it will be possible to produce higher amounts of such components, produce them in such a way that recovery is easier and produce differentiated compounds, thus revolutionizing the field of functional foods.

On the other hand, new perspectives also involve the development of novel formulations targeting specific consumer groups, such as newborns, elderly, diabetics, among others. Nowadays, the use of minor milk components to enrich food products and to develop new ones holds a big promise towards the emergent fields of personalized nutrition and functional foods (Stover and Garza, 2002; Kussmann and Fay, 2008; Kaput, 2008). Nevertheless, it is extremely important that the benefits claimed for such components are proved using living systems. There is still a lot of knowledge on the biological activity of the minor milk constituents lacking and new methodologies ought to be developed. Also, the study of the mechanisms of interaction of such components with cell receptors and/or specific genes is still limited and the omics techniques can be very useful to gather this sort of information (Ferguson, 2006; van Ommen and Stierum, 2002; Muller and Kersten, 2003).

Several challenges and concerns regarding the potential use and understanding of minor milk constituents, enzymes, hormones, growth factors, and organic acids, still need to be addressed. Some examples include technological concerns, such as the

impact of milk enzymes on the quality of fermented milks; the impact of technological developments in thermal processing on milk constituents, specifically on enzymes, relative to the sensory quality of milk; and the impact of membrane processes on enzyme activities in dairy products; and many others. On the other hand, interesting challenges ought to be pursued, such as those related to the commercial availability of certain milk enzymes which have not found widespread application in industry; the potential significance of enzymes and other minor constituents from colostrum; the potential nutraceutical significance of such milk constituents; the relationship between some enzyme activities and the sensory quality of stored milk and means to control the activity of some enzymes.

Finally, as milk composition varies broadly with several factors such as genetics (e.g. species and breed), stage of lactation, health status and environmental factors (e.g. feed, climate and method of milking), manipulating these factors constitutes a "natural" way of changing the minor constituents' contents in milk. Genetic manipulation of livestock has been limited to the permanent addition of genes of clinical interest. Nevertheless, researchers have been exploring the utility of genetically engineered cattle as a means of altering milk composition to improve the functional properties of milk, increasing marketability (Karatzas and Turner, 1997). Improvements would include increasing the concentration of valuable components in milk (e.g., casein), removing undesirable components (e.g., lactose), or altering composition to resemble that of human milk as a means of improving human neonatal nutrition. On the other hand, milk composition can be altered by nutritional management or through the exploitation of naturally occurring genetic variation among cattle (crossbreeding or selection). Additionally, on-farm methods including rumen modification, trait selection and rations can be used to change milk composition (Haenlein and Anke, 2011; Knowles *et al*., 2006). Through these methods it has been possible to increase concentrations of calcium, selenium, iodine, iron and cobalt/Vitamin B12 in milk. For example, the conjugated linoleic acid content in milk fat can be modified by feeding that

influences the pattern of fat precursors the mammary gland removes from blood for fat synthesis (Mel'uchov´a *et al*., 2008). Variation in conjugated linoleic acid content in milk of ruminants appears to be minimally influenced by the stage of lactation, parity and breed (Sanz Sampelayo *et al*., 2007). Nevertheless, diet is the most important factor influencing milk conjugated linoleic acid concentration. The conjugated linoleic acid concentration in milk is higher in pasture-raised animals than in those fed with dry diets, and it decreases with increasing growth stage of forage or maturity. Off-farm alternative approaches to manipulate milk composition might be less desirable. For instance, fortification at the processor with trace elements and vitamins by way of post-harvest or supplements is common, and usually inexpensive, but many consumers prefer not to consume these additives (Cox, 2008), some legal definitions of fresh milk preclude addition of fortificants, and some markets permit only 'unadulterated' foods (e.g., exported infant formula). Regardless of method, manipulations will be appropriate only if they suit typical farming practice and do not perturb other product qualities such as safety, shelf life, texture or taste.

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Table 1. Contents of some minor constituents in milk from several species (adapted from Csapo *et al.*, 2009, Dimitrov *et al.*, 2007, Haenlein and Anke, 2011; Park and Haenlein, 2006; Michaelidou, 2008; Belewu *et al.*, 2009).

MINERALS AND TRACE ELEMENTS (mg Kg^{-1})	Cow	Human	Goat	Sheep	Buffalo	Mare	Camel
Calcium (Ca)	1190-1220	330	1340-1500	1930	1830	485-1355	760–1965
Phosphorus (P)	930-1190	430	960-1210	1580	820	216-1205	490–1480
Potassium (K)	1520-1520	550	1700-1810	1360	1070	303-790	600–2110
Sodium (Na)	490-580	150	410	440	440	75-237	360–902
Magnesium (Mg)	120-130	40	93-160	180	180	29-118	40–209
Chlorine (Cl)	1000-1030	600	1500		580		
Sulfur (S)	320		29		157-314		
Zinc (Zn)	3.8-5.3	3.8	2.7-5.6	5.7	3.2-7.3	2.2-3.6	2.8-4.4
Iron (Fe)	0.5-0.8	2.0	0.7-4.0	0.8	0.4-13	0.5-1.1	0.-3.7
Copper (Cu)	0.2-0.6	0.6	0.5-1.0	0.4	0.07-2.6	0.2-0.7	0.11-1.5
Manganese (Mn)	0.2-0.3	0.7	0.3-1.0	0.07	0.38-0.66	0.05	0.2-1.9
Aluminum (Al)	0.6		1.12	0.5-1.8			
Cadmium (Cd)	0.004		0.005	0.03-0.06			

Cobalt (Co)	0.0008			0.004-0.09	0.7-1.6		
Chromium (Cr)	0.02			0.04-0.4			
Nickel (Ni)	0.02		0.288	0.01-0.4			
Barium (Ba)	0.2			1.7			
Lead (Pb)	0.03			0.006			
Selenium (Se)	0.0096-0.02	0.0152	0.013-0.247				
Fluorine (F)	0.1				0.4-18.5		
Iodine (I)	0.08/0.21	0.07	0.22		8.6-19.4		
Molybdenum (Mo)	0.06		0.116				
VITAMINS (mg Kg^{-1})							
Retinol, vit. A	0.38-0.52	0.57	0.55	0.84	0.102	0.34	
Thiamin, vit. B1	0.28-0.90	0.14-0.17	0.68	0.8	0.5		0.33-0.6
Riboflavin, vit. B2	1.2-2.0	0.2-0.36	2.1	3.56	1.0		0.42-0.8
Pyridoxin, vit. B6	0.42-0.63	0.11	0.46	0.8	3.8		0.52
Cobalamin, vit. B12	0.002-0.007	0.0003-0.0005	0.0007	0.007	3.4		0.002
Vitamin D	0.0003-0.0005	0.0004	0.0006	0.0018		0.0032	
Tocopherol, vit. E	0.31-0.9			1.1	0.334	1.128	

Ascorbic acid	3-23	35-50	12.9	41.6	23-30	17.2	24-52
Folic acid	0.01-0.1	0.055	0.01	0.05	0.1		0.004
Niacin	0.5-0.84	1.47-1.7	2.7	4.16			4-6
Pantothenic acid	2.6-4.9	1.84-2.23	3.1	4.08	1.5		0.88
Biotin	0.02	0.004	0.015		26.8		
IMMUNE COMPONENTS							
Immunoglobulins (%)	10.1-11.7	15.1-19.7				18.7-20.9	
IgA (milk: μg ml^{-1})	140	1000	30-80				
IgA (colostrum: mg ml^{-1})	3.9	17.35	0.9-2.4				
IgM (milk: μg ml^{-1})	50	100	10-40				
IgM (colostrum: mg ml^{-1})	4.2	1.59	1.6-5.2				
IgG (milk: μg ml^{-1})	590	40	100-400	350-500	450-600		1700
IgG (colostrum: mg ml^{-1})	47.6	0.43	50-60				
Lactoferrin (μg ml^{-1})	20-200	〈 2000	20-200	20-200	20-200	20-200	150-250
POLYAMINES (μmol L^{-1})							
Putrescine	1	0.4	5.12-6	0.4			
Spermidine	1-4.7	2.7	26-39.67	2.05			

Spermine	1-4	1	3.18-3.8	2.39			
NUCLEOSIDES (μmol L^{-1})							
Cytosine	2.4-5.8	4.3-7.8	8.8	6.7			
Uridine	14.7-73.1	0.5-6.9	17.9-76.3	67.8			
Inosine	1-6.5		12.8-60.6	41.2			
Guanosine	0.8	0.2-1	2.3-2.9	2.1			
Adenosine	1.4	3-5.3	2.4-3.4	8.8			
NUCLEOTIDES (μmol L^{-1})							
Cytidyl-5'-monophosphate	2.9-26.6	18.3-66	72.5	48.6			
Uridyl-5'-monophosphate	Traces	6.4-9.3	227.2	110.7			
Guanosyl-5'-monophosphate	1.8	1-1.5	Traces	Traces			
Adenosyl-5'-monophosphate	Traces	1.9-15.1	85.6	54.1			
ESSENTIAL AMINO ACIDS	**Cow**	**Human**	**Goat**	**Sheep**	**Buffalo**	**Mare**	**Camel**
Alanine (g Kg^{-1})	1.08	3.42	0.89	2.17		0.59	

Arginine (g Kg^{-1})	1.15-1.98	3.09	0.75	1.2-1.84		0.95	3.92
Cysteine (g Kg^{-1})	0.31	1.73	0.23	0.44	0.59	0.18	
Glycine (g Kg^{-1})	0.61	1.89	0.47	0.98		0.25	
Histidine (g Kg^{-1})	1.68-8.10	1.97-2.17	0.67-2.23	0.88-1.41		0.35	1.95-2.15
Isoleucine (g Kg^{-1})	1.58-3.38	2.33-4.53	1.24-2.15	2-2.66	5.71	0.62	2.94-5.71
Leucine (g Kg^{-1})	3.33-5.88	2.71-8.85	2.48-2.68	3.22-4.89	9.79	1.47	2.71-8.55
Lysine (g Kg^{-1})	2.90-5.12	5.36-6.09	2.06-5.48	2.6-4.51	7.50	1.16	3.75-4.26
Methionine (g Kg^{-1})	0.88-1.56	1.36-1.46	1.43	0.84-1.57	0.93	0.35	2.42-2.60
Phenylalanine (g Kg^{-1})	1.68-2.84	2.58-3.15	1.21-1.99	1.6-2.6	4.71	0.68	3.15-3.84
Threonine (g Kg^{-1})	1.41-2.68	3.56- 3.75	1.26-3.31	1.48-2.22	3.57	0.62	2.39-2.51
Tryptophan (g Kg^{-1})	0.84	5.22	5.11	0.46			12.53
Tyrosine (g Kg^{-1})	1.58	3.93	0.98	2.55	3.86	0.72	
Valine (g Kg^{-1})	1.75-4.48	4.35	1.57	2.2-3.10	6.74	0.75	3.39
Branched-chain amino acids (g Kg^{-1})	7.02- 13.64	1.78	5.29	7.44-10.77		2.81	
Taurine (μmol 100 ml^{-1})	1.0	30	56	14			

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Table 2. Concentrations and activities of some indigenous enzymes in milk from several species (adapted from Chen *et al*., 2003; Seifu *et al*., 2005; Fox and Kelly, 2006a; Park and Haenlein, 2006).

ENZYME **concentration and/or activity**	**Cow**	**Human**	**Goat**	**Sheep**	**Buffalo**	**Camel**
Acid phosphatase (AcP)	0.75 U ml^{-1}		1.4 U ml^{-1}			
Alkaline phosphatase (AIP)	1.8 -2.5 U ml^{-1}				1.2-1.7 U ml^{-1}	
			11-13 mg L^{-1}			
Amylase					1.2-1.7 U ml^{-1}	
Lactoperoxidase (LPO)	30 mg L^{-1}	1.5 mg L^{-1}			0.2-0.9 U ml^{-1}	1.5 U ml^{-1}
	1.5 - 2.7 U ml^{-1}	0.06 -0.97 U ml^{-1}	1.55-4.45 U ml^{-1}	0.77- 3.46 U ml^{-1}		
Lipoprotein lipase (LPL)	0.5-2.0 mg ml^{-1}	4-20 mg ml^{-1}				
Lysozyme	10-35 μg 100 ml^{-1}	4-40 μg 100 ml^{-1}	25 μg 100 ml^{-1}	23-50 μg 100 ml^{-1}	13-15.2 μg 100 ml^{-1}	228-500 μg 100 ml^{-1}
Plasmin	0.07– 0.3 μg ml^{-1}	0.07– 0.13 μg ml^{-1}				
Ribonuclease (RNase)	1000-2000 μg 100 ml^{-1}	10-20 μg 100 ml^{-1}	425 μg 100 ml^{-1}			
Sulphydryl oxidase (SHOx)	33 mg ml^{-1}					
Xanthine oxidoreductase (XOR)	120 (μl O_2 h^{-1} ml^{-1})	12(μl O_2 h^{-1} ml^{-1})	19-113 (μl O_2 h^{-1} ml^{-1})		120 (μl O_2 h^{-1} ml^{-1})	

Table 3. Indigenous bovine milk enzymes (adapted from Kelly and McSweeney, 2003; Fox and Kelly, 2006a; Huppertz and Kelly, 2009).

Enzyme	EC number	Source	Activity	Optimal pH	Optimal T (ºC)	Properties	Interest for Dairy Industry
Plasmin	EC 3.4.21.7	Blood Associated with casein micelle in milk	Serine protease Active on all caseins, particularly on β-casein and αs2-casein	7.5	37	Most plasmin survives pasteurization, though considerable inactivation occurs at higher temperatures Thermal inactivation depends also on the presence of β-lactoglobulin	Cheesemaking properties of milk deteriorate as a result of plasmin activity In the cheese itself, plasmin contributes to primary proteolysis Plasmin and plasminogen have a role in the physical instability or age gelation of UHT milk
Lipoprotein lipase	EC 3.1.1.34	Mammary gland Associated with casein micelle and some in the serum phase	Glycoprotein Liberates fatty acids from the 1 and 3 position in tri-, di- and monoglycerides	9.2	37	Heat-labile enzyme Very little activity survives pasteurization Complete thermal inactivation occurs for heat treatment exceeding 75ºC for 15 min	Technologically significant enzyme from the viewpoint of milk deterioration Lipolysis leads to the release of free fatty acids, which can result in the development of hydrolytic rancidity
Alkaline phosphatase	EC 3.1.3.1	Mammary gland Associated with phospholipid particles in the milk fat	Phosphomonoesterase Active against a wide range of substrates Hydrolyses most	9.0-10.5	37	Relatively heat-sensitive Its thermal stability is only slightly higher than that of non-spore forming pathogenic bacteria present in milk	Its thermal inactivation has been effectively used as a sensitive indicator for adequate pasteurization of milk However, this enzyme can be reactivated on subsequent storage, leading to false alkaline

		globule membrane	phosphate ester bonds and can dephosphorylate caseins under suitable conditions				phosphatase-positive test results
Lactoperoxidase	EC 1.11.1.7	Mammary gland Exists primarily in the milk serum	Glycoprotein Catalyses the oxidation of a donor compound (e.g. aromatic amine, polyphenol, aromatic acid)	8.0	-	Possesses antibacterial activity in the presence of H_2O_2 and thiocyanate through the catalysis of the oxidation of thiocyanate to hypothiocyanate Heat-stable enzyme. Thermal inactivation for temperatures up to 80ºC	Preservation of milk quality Indices of the thermal history of milk Antimicrobial activity Commercial source of enzymes

Table 4. Significance of indigenous bovine milk enzymes for the production and/or quality of milk and milk products (adapted from Kelly and Fox, 2006a,b)

Product	Enzyme	Significance
Raw milk	Lactoperoxidase	Antimicrobial effect
	Xanthine oxidoreductase	
Pasteurized milk	Plasmin	Possible contribution to instability
	Alkaline phosphatase	Index of processing
UHT milk	Plasmin	Possible contribution to gelation on storage
Cream	Lipase	Can cause rancidity
	Lactoperoxidase	Indicator of heat treatment
Milk powders	Lipase	Can cause rancidity
	Plasmin	Can survive drying and remain active
Yogurt	Lactoperoxidase	Possible inhibition of post-acidification
	Plasmin	Possible effect on gel structure and texture
Fresh cheese	Plasmin	Affects rennet coagulation in milk
Ripened cheese		
General	Plasmin	Contributes to primary proteolysis
	Lipase	Contributes to lipolysis
Swiss	Cathepsin D	May contribute to proteolysis due to inactivation of chymosin
Acid	Cathepsin D	May contribute to proteolysis due to low pH

Table 5. Bovine milk hormones (adapted from Jouan *et al.*, 2006)

Hormone	Concentration range (ng mL^{-1})
Estrogen	5×10^{-3} -10×10^{-3}
Progesterone	2-20
Glucocorticoids	0-50
Prolactin	5-200
Growth hormone	0-1
Somatostatin	10-30
Parathryroid hormone-related protein	40-100
Insulin	5-40
Calcitonin	700
Bombesin	0.25-450
Melatonin	5×10^{-3}-25×10^{-3}

Table 6. Bovine milk growth factors (adapted from Grosvenor *et al*., 1993; Gauthier *et al.*, 2006)

Growth factor	Concentration (ng mL^{-1})	Source	Primary activity	Amino acid residues	Molecular mass (g mol^{-1})	pI
EGF	〈 2.0	Wide range of tissues and body fluids	Stimulates proliferation of epidermal, epithelial and embryonic cells Inhibits secretion of gastric acid Promotes wound healing and bone resorption	53	6000	4.8
BTC	1.9			80	22 000	7.7
IGF-I	30.4	Primarily the liver	Stimulates proliferation of many cell types IGF-I is a stronger mitogen than IGF-II which stimulates primarily cells of fetal origin Influences the differentiation of some cells Causes hypoglycemia, improvement of nitrogen balance, lowering of cholesterol and potassium, and improvement of renal functions	70	7650	7.8
IGF-II	50-100	Variety of cells		67	7530	6.5
TGF-β2	13-71	Platelets and many other cells	Stimulates growth of cells, especially in connective tissue Inhibits other cells, such as lymphocytes and epithelial cells Important role in embryogenesis, wound healing, formation of bone and cartilage, and control of the immune system	425	25 000	7.7
PDGF	NA	Platelets and many other cells	Plays a role in embryonic development, proliferation of cells of mesenchymal origin, migration, angiogenesis and wound healing	250-300	30 000	9.6
FGF2	0.5-1	Wide range of cells	Important role in proliferation, differentiation and survival of many cell types Involved in angiogenesis, wound healing and hematopoiesis	146	16 400	9.6

EGF – estrogen growth factor; BTC – betacellulin; IGF – insulin growth factor; TGF - transforming growth factor; PDGF - platelet-derived growth factor; FGF - fibroblast growth factor; NA – not available