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Lactoferrin in Relation to Biological Functions and Applications: A Review

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ABSTRACT

Lactoferrin (LF) is an iron-binding glycoprotein member of the Transferrin (TF) family which present in milk, other exocrine secretions and neutrophil granules in mammals, it is highly conserved among human and other mammals' species. First identified in milk as the red milk protein due to its iron content, LF has since attracted much interest over the past fifty years. LF is considered to be an important host defense molecule and has a diverse range of physiological functions such as antimicrobial/antiviral activities, immunomodulatory activity and antioxidant activity. During the past decade, it has become evident that oral administration of LF exerts several beneficial effects on the health of humans and animals, including anti-infective, anticancer and anti-inflammatory effects. This has enlarged the application potential of LF as a food additive. The technology of producing bovine LF on a large scale was established over 20 years ago. This review summarizes our current understanding of the properties of physico-chemical of LF, physiological functions and technological characteristics as well as nutritional and applications relationships that explain the roles of LF in host defense.

Key words: Milk proteins, lactoferrin, lactoferricin, biological multi-functions, iron binding

INTRODUCTION

Colostrum and milk is a vital nutritional source for the offspring of all mammals, including humans. In addition to its nutritional value, it is a rich source of proteins including Lactoferrin (LF) (Jenssen and Hancock, 2009). Also, milk contains various protective proteins that, if properly activated, can contribute to the preservation of milk (IDF, 1988). Milks from different species differ significantly as to their protective proteins content. Thus human milk contains almost 10 times more LF than doe's bovine milk (Reiter, 1985).

Also, whey proteins are used as common ingredients in various products including infant formulas, specialized enteral and clinical protein supplements and sports nutrition products with the expectation of the therapeutic potential of whey proteins and peptides. LF, one of the major whey proteins, is a red iron-binding protein present mainly in external secretions such as breast milk and in polymorphonuclear neutrophils. This protein plays an important role in the defense mechanism of mucosal surfaces, since in an iron-depleted state it has bacteriostatic properties (Masson and Heremans, 1966). LF is released from polymorphonuclear neutrophils on activation of these cells and its presence in body fluids is proportional to the flux of neutrophils (Rado *et al*., 1984; Guerrant *et al*., 1992; Martins *et al*., 1995; Parsi *et al*., 2008).

LF belongs to the family of iron-binding proteins and exhibits a wide spectrum of antimicrobial and immunotropic properties. It is particularly resistant to proteolytic degradation in alimentary tract, in contrast to other milk proteins, e.g., casein. In any case, LF-derived peptides also possess potent antibacterial activities which absorbed from the intestine by means of specific receptors located on brush border cells. Administered orally, LF stimulates both local and systemic immune response. It plays a role in the absorption of nutrients. The protein can deliver such metal ions as iron, manganese and zinc and facilitate the absorption of sugars (Artym and Zimecki, 2005).

LF has a truly multifunctional protein that has been studied extensively over the past decades. It is best known for its ability to bind iron which eventually led to the discovery of its biological functions, including antimicrobial, antiviral, antioxidant, antiinflammatory activities, immunomodulation, modulation of cell growth and inhibition of several bioactive compounds, such as lipopolysaccharide (LPS) and glycosamino-glycan (Baveye *et al.*, 1999; Chierici, 2001; Jenssen and Hancock, 2009). It also provides a defense against gastro-intestinal infections, participates in local secretory immune systems (Valenti *et al.*, 1998; Vorland, 1999; Steijns and van Hooijdonk, 2000), in synergism with some immunoglobulins (Igs) such as IgG and other protective proteins, supplies an iron-binding antioxidant protein in tissues and possibly promotes growth of animal cells, such as lymphocytes and intestinal cells (Lonnerdal and Iyer, 1995; Meulenbroek and Zeijlemaker, 1996).

The alteration of the activity of these antimicrobial factors in cow's milk could have an impact on the shelf life of raw milk and on the development of additional health and functional foods based upon these factors. The composition of different milk samples is usually not uniform; therefore, the concentrations of several milk constituents change during the lactation period and differ from one mother to the next. There are several factors that are known to influence the concentration of milk constituents in predictable ways (Levieux *et al.*, 2002; Cheng *et al.*, 2008). These include lactation stage, breastfeeding routine, parity, age and other maternal characteristics such as regional differences and, in some situations, season of the year and maternal diet.

On the other hand, Igs (antibodies) are protective proteins that are important in the transfer of passive immunity from the mother to the child. The young of many mammalian species are born without an effective immune system, therefore the Igs and LF exhibit antimicrobial activity and protect the neonate from infection until their own immune system has developed. The increasing commercial interest in exploiting the therapeutic value of LF and IgG has stimulated the need for reliable assays for their determination at the endogenous level in milk (Hurley *et al.*, 1993; Elagamy *et al.*, 1996; Indyk and Filonzi, 2005).

The *in vitro* activity of LF also includes transcriptional activation of several genes (Oh *et al.*, 2004). Tomita *et al.* (1991) found that pepsin-hydrolysate of LF (LFhyd) has more potent antimicrobial activity than the native protein and they purified the active peptide from LFhyd. The antimicrobial peptide derived from Lfhyd was named lactoferricin (LFcin) (Bellamy *et al.*, 1992a). Interestingly, LFcin and its derivatives exhibit various biological activities, like LF. Therefore, the LFcin-region seems likely to be an important functional domain of LF (Wakabayashi *et al.*, 2003).

LF content in milk varies depending on the species. The amount of LF is lower in cows' milk (i.e., 0.1-0.4 mg mL^{-1}) than in human milk (i.e., 1-3 mg mL^{-1}). However, a factory scale technology to produce large amounts of bovine LF at high purity from cow's milk was established over 20 years ago (Law and Reiter, 1977).

Recently it has been recognized that oral administration of LF exerts various health beneficial effects such as anti-infective activities not only in infants but also in adult animals and humans (Tomita *et al.*, 2002; Teraguchi *et al.*, 2004; Weinberg, 2007). Weinberg (2007) reported that recombinant bovine and human LF is available for development into nutraceutical, preservative and pharmaceutical products. Among conditions for which the products were being investigated were: angiogenesis, bone remodeling, food preservation, infection in animals, humans, plants, neoplasia in animals, humans, inflammation in intestine, joints, wound healing as well as enhancement of antimicrobial and antineoplastic drugs and prevention of iron induced oxidation of milk formula.

PROPERTIES OF PHYSICO-CHEMICAL OF LACTOFERRIN HISTORY AND CONCENTRATION OF LF IN DIFFERENT SPECIES

LF is an iron-binding glycoprotein of the TF family which was first fractionated as an unknown "red fraction" from cow's milk by Sorensen and Sorensen (1940) and later in human milk by Schafer (1951). The red protein from both human and bovine milk was defined as a lactotransferrin-like glycoprotein or lactosiderophilin because of its high similarity to TF and siderophilin in blood and ovotransferrin in egg which in United Kingdom (Groves, 1960), in Sweden (Johansson and Hjerten, 1960b), in France (Montreuil *et al.*, 1960). LF was first isolated from cow's milk and then from human milk (Ensminger and Esminger, 1986; Wood, 1988; Spik *et al.*, 1998; Shimazaki, 2000a, b).

LF was present in large quantities by several groups not only in milk secreted by the mammary gland but also in various exocrine mammalian secretions such as tears, saliva, seminal fluid, cervical mucus, bronchial secretions and in some white blood cells (i.e., neutrophilic leucocytes) (Levay and Viljoen, 1995; Steijns, 2001). Because LF is predominantly found in the products of the exocrine glands of the digestive, respiratory and reproductive systems, it is thought that LF has a role in the host non-specific defense against invading pathogens.

LF concentration varied between 31.78 and 485.63 (μg mL^{-1} in milk from normal animals (Cheng *et al.*, 2008). It is concentration is higher than 2 mg mL^{-1} in human milk (Nagasawa *et al.*, 1972) and in the range of 0.02-0.2 mg mL^{-1} in bovine milk (Masson and Heremans, 1971; Suzuki *et al.*, 1977). The LF concentration in guinea-pig, mouse and horse milk is in the range of 0.2 to 2 mg mL^{-1} as well as that in rat, rabbit and dog milk is lower than 0.05 mg mL^{-1} (Masson and Heremans, 1971).

Colostrum milk contains more LF than mature milk. It should be noted that LF is the second most abundant whey protein in human milk as shown in Fig. 1. While its concentrations in other secretory fluids are shown in Table 1 (Shimazaki, 2000a).

LF was significantly associated with stage of lactation ($r = 0.557$) and daily milk production ($r = -0.472$). Nevertheless, there was no significant relationship with parity. Moreover, milk LF concentration tended to be correlated with the somatic cell count score ($r = 0.375$). This finding suggests that milk LF may be helpful as an indicator for intramammary infection in dairy cows (Cheng *et al.*, 2008).

Harmon *et al.* (1975) reported that the LF concentration of milk was significantly associated with somatic cell count (SCC), levels of bovine serum albumin, stage of lactation and milk production. Still, LF had a negative relationship with milk production. Tsuji *et al.* (1990) reported

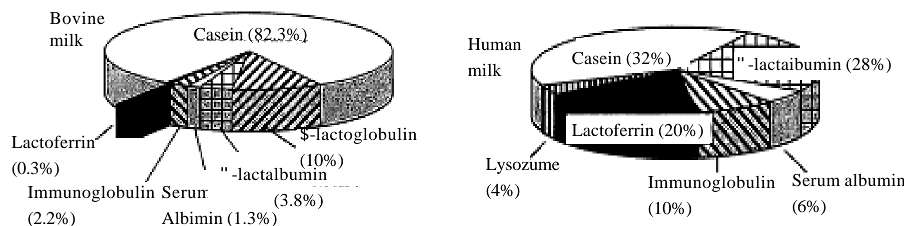

Fig. 1: Milk protein fractions content (%) of human and bovine milk. (Total protein concentration in human milk is 1 g 100^{-1} mL and that in bovine milk is 3.2 g 100^{-1} mL)

Table 1: Occurrence of lactoferrin in human external fluids

External fluid	Amounts reported
Saliva	>7-10 mg mL^{-1a}
Tear	>2.2 mg mL^{-1a}, 0.1 mg mL^{-1b}, 0.7 mg mL^{-1c}, 2.2 mg mL^{-1d}
Seminal plasma	>0.4-1.9 mg mL^{-1a}
Synovial fluid	>10-80 mg mL^{-1a}
Nasal	0.1 mg mL^{-1b}
Hepatic bile	10-40 µg mL^{-1b}
Pancreatic	0.5 mg mL^{-1b}
Gastric	0.5-1.0 mg mL^{-1b}
Urine	1 µg mL^{-1b}
Blood	0.1-2.5[e]
Neutrophils	3.45 µg/10^6 cell[f]
Granulocytes	4 µg/10 cell[g]

[a]: Steijns and van Hooijdonk (2000), [b]: Masson *et al.* (1966), [c]: Takayanagi *et al.* (1986), [d]: Kijlstra *et al.* (1983), [e]: Malmquist *et al.* (1978), [f]: Moguilevsky *et al.* (1987), [g]: Bezwoda and Mansoor (1989)

that the highest LF content in colostrum was observed in second lactation. After the third lactation, no differences in LF content were observed. Hagiwara *et al.* (2003) reported that the concentration of milk LF was significantly related to the age of cows but not to the stage of lactation; however, because those samples were from different mammary gland quarters and the sample number was relatively small.

SYNTHESIS OF LACTOFERRIN

Teng *et al.* (2002) reported that the LF synthesis can be continuous (exocrine fluids), under hormonal control (genital tract, mammary gland) [213]. LF is secreted in the apo-form from epithelial cells in most exocrine fluids such as saliva, bile, pancreatic and gastric fluids, tears and milk (Montreuil *et al.*, 1960). LF is mainly synthesized by glandular epithelial cells; its concentration in humans may vary from 1 g L^{-1} (mature milk) to 7 g L^{-1} (colostrum). The mean concentration is 30 mg L^{-1} in mature bovine milk. In addition, LF is synthesized during the transition from promyelocytes to myelocytes and is thus a major component of the secondary granules of PMNs (Masson *et al.*, 1966). LF levels of biological fluids may increase greatly and constitute a marker for inflammatory diseases (Mann *et al.*, 1994; Legrand *et al.*, 2008) noticeable this is in plasma, where the LF concentration can be as low as 0.4-2 mg L^{-1} under normal conditions but increases to 200 mg L^{-1} in septicemia.

ISOLATION AND PURIFICATION OF LACTOFERRIN

Acid precipitated casein has been used as the starting material for isolation of LF from bovine milk (Groves, 1960). However, the whey fraction of milk or colostrum is better source to obtain LF on laboratory scale (Law and Reiter, 1977) and cheese whey is another source used to obtain LF on a large scale.

Other methods used include affinity chromatography with immobilized materials such as heparin (Blackberg and Hernell, 1980), blue dye (Bezwoda and Mansoor, 1986), **β**-lactoglobulin (Ena *et al.*, 1990), antilactoferrin antibody (Kawakami *et al.*, 1987) or single-standard DNA (Hutchens *et al.*, 1989b). Also, metal-chelate affinity chromatography (Hutchens *et al.*, 1989a) and hydroxyapatite column chromatography (Itagaki *et al.*, 1993) have been used to purify LF.

ELISA and other immunodiffusion and immunoelectrodiffusion methods have been employed using anti-lactoferrin antiserum. In order to measure the LF concentrations in dairy products such as cheese, treatment at pH 4.0 to release LF to casein is necessary.

LF is isolated and purified on an industrial scale (approximately 20-30 tons annually worldwide) from cheese whey and skim milk. The concentration of LF in cheese whey is roughly 100 mg L^{-1}. Since LF exists as a cationic protein (isoelectric point of LF is alkaline) in whey, it is readily adsorbed to a cation-exchange chromatography resin and then eluted using salt solutions. The eluted crude LF is desalted and concentrated using ultrafiltration and diafiltration membranes, after which it is subjected to pasteurization. Purified LF powder with a purity of 95% or higher is finally obtained by freeze-drying. In an alternative process, microfiltration and spray-drying are performed instead of pasteurization and freeze-drying, respectively. Pasteurization has come to be considered as very important in order to inactivate not only bacteria but also viruses such as foot and mouth disease virus. While making efforts to develop a practical method for the pasteurization of LF which is stable against heat treatment under acidic conditions (Abe *et al.*, 1991) while heat treatment at a neutral pH causes denaturation of the protein. It is considered that heating at a pH 4 and to a temperature of 90-100°C for 5-10 min as well as the UHT method are suitable and practical methods for the pasteurization of LF. This pasteurization process was patented and it has been applied to the manufacture of a wide variety of commercial products containing LF. A pepsin hydrolysate of LF is produced by treatment with porcine pepsin under acidic conditions (Saito *et al.*, 1991). After hydrolysis has been completed, pepsin is inactivated by heat treatment. Then the reaction mixture is filtered and concentrated by reverse osmosis. Finally, the hydrolysate of LF is obtained by pasteurization and freeze-drying for use in infant formula.

In addition, LFcin can be purified from this LF hydrolysate by two-step hydrophobic chromatography. The peptide is eluted with an acidic buffer, the eluted solution is concentrated by reverse osmosis and finally, LFcin is produced by freeze-drying as a powder with over 95% purity. This production process for LFcin has also been patented (Bellamy *et al.*, 1992b).

Abd El-Gawad *et al.* (2003) found that immuno-precipitation of rbLF with anti-bLF in the medium at pH 7.5 showed two forms of LF, LF-a with a molecular weight of 84 kDa and LF-b with 80 kDa, while the immuno-precipitation at pH 8.0 produced one single band of LF-b with 80 kDa.

Adam *et al.* (2008) employed SDS-PAGE and Experion to isolate LF from the colostrum and normal milk samples with technique ion exchange chromatography, using monolithic column. Coomassie blue staining followed and the expressive band of LF at molecular weight 77 kDa was detectable (Fig. 2a line 2 and 3). Similarly to Coomassie blue staining by silver stained, the majority bands of LF were also visible (Fig. 2b). The result of the electrophoretic analysis is shown in Fig. 2c. Both techniques show that many other proteins are presented in the samples (Fig. 2).

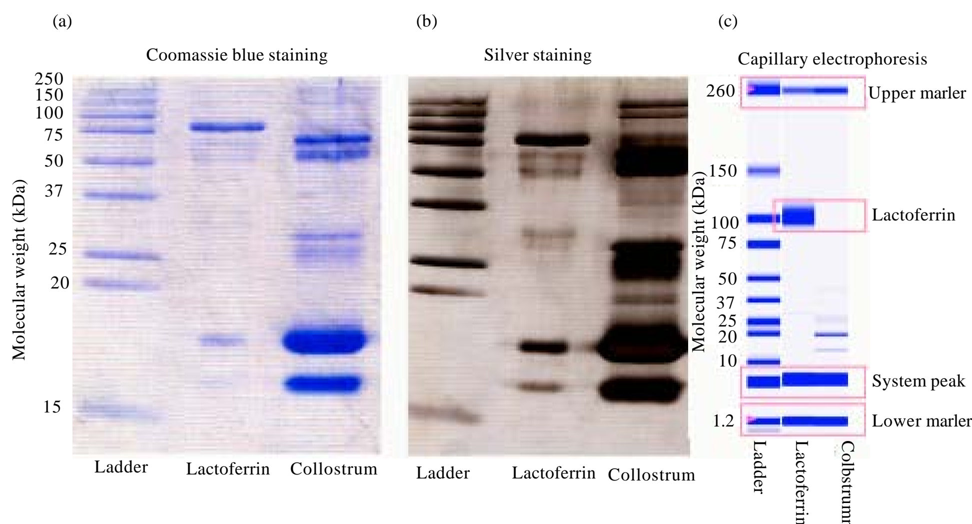

Fig. 2: SDS-PAGE with (a) Coomassie blue staining, (b) Silver staining and (c) Experion capillary electrophoresis of ladder, lactoferrin standard (100 μg mL^{-1}) and milk sample

STRUCTURE OF LACTOFERRIN

LF is a glycoprotein with a molecular weight of average about 80 kDa which shows high affinity for iron. The molecular structure and amino acid sequence of hLF were discovered in 1984. LF was then classified as a member of the TF family, due to its 60% sequence identity with serum TF. Human LF (hLF) has a molecular weight of 82.4 kDa and is composed of 702 (Metz-Boutigue *et al.*, 1984) or 692 (Powell and Ogden, 1990; Rey *et al.*, 1990) amino acid residues, the sequence identity is 69%. Bovine LF (bLF) has a molecular weight of 83.1 kDa and is composed of 689 amino acid residues (Pierce *et al.*, 1991).

Three dimensional structures of hLF (Farnaud and Evans, 2003), bLF (Moore *et al.*, 1997), horse LF and buffalo LF (Sharma *et al.*, 1998) have been determined by X-ray crystallographic analysis. The three dimensional structures of bovine and human LF are very similar but not identical which are shown in Fig. 3.

Three different isoforms of LF have been isolated. LF-α is the iron binding form but has no ribonuclease activity. On the other hand, LF-β and LF-γ demonstrate ribonuclease activity but they are not able to bind iron (Furmanski *et al.*, 1989). LF is comprised of a single polypeptide chain containing 703 amino acids folded into two globular lobes. These lobes, also called C-(carboxy) and N-(amino) terminal regions are connected with α-helix. Each lobe consists of two domains known as C_1, C_2, N_1 and N_2 The domains create one iron binding site on each lobe. LF molecules contain (according to the species and protein) varying numbers of sites for potential glycosylation, mostly on the surface of the molecule. The most common saccharine is mannose; around 3% are hexoses and 1% hexosamines. The degree of glycosylation varies and determines the rate of resistance to proteases or to very low pH (Anderson *et al.*, 1987). Jameson *et al.* (1998) observed two structures for LF: an open conformation, originally described for the iron-free LF and a closed conformation, mainly observed with the iron-saturated molecule. The conformational transition could be involved in basic functions such as transportation and

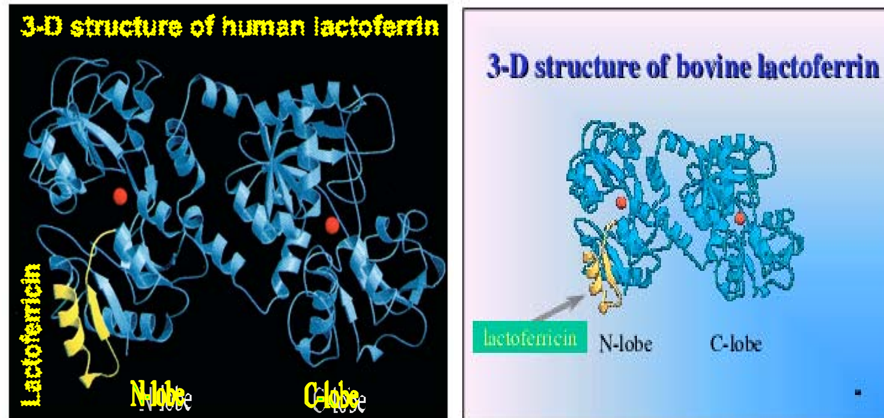

Fig. 3: Three dimensional structures of diferric human LF (Farnaud and Evans, 2003) and bovine LF (Moore *et al.*, 1997). The location of lactoferrcin within the protein is shown in yellow and the two ferric ions are in red

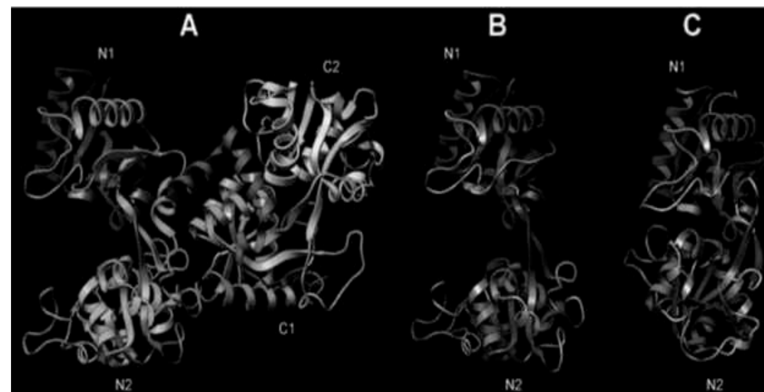

Fig. 4: Structure of hLF. (A) Ribbon diagram showing the polypeptide folding of iron-saturated hLF. The N-t lobe is on the left; the polypeptide chain is colored from the N- to the C-terminal end according to a red-shift. (B) Open and (C) closed structures of the N-terminal lobe of hLF (α-helices are colored in magenta and b-sheets in blue). Domains N1, N2, C1 and C2 are indicated. Domains, referred to as N1 and N2, or C1 and C2, delineate a deep cleft within which the iron binding site is located. The a/b fold of each domain consists of a central, mostly parallel b-sheet, with a helix packed against it. The helical N-terminus faces the interdomain cleft, making it somewhat positively charged and one of the helices, H5 from the N2 (or C2) domain, serves as the binding site for the essential carbonate anion at the metal binding site (Legrand *et al.*, 2008)

catalysis. According to crystallographic data, the domains move essentially as rigid bodies (Fig. 4) that close over the bound metal or open to release it.

There are three forms of LF according to its iron saturation: apo-LF (iron free), monoferric form (one ferric ion) and holo-LF (binds two Fe^{3+} ions). The tertiary structure in holo-LF and apo-LF is different (Jameson *et al.*, 1998).

The LF fraction separated from colostrum and milk of buffalo, cow (Local, Friesian and Brown-Swiss breeds), ewe (Rahmani and Ossemi breeds), goat as well as camel were patterned using SDS-PAGE as a single band almost of similar mobility as standard LF. Also, the LF fractions of different species were subjected to reverse phase HPLC and only one peak was apparent. LF concentrations varied considerably from one species to others (Abd El-Gawad *et al.*, 1996).

Immunoprecipitation of recombinant bovine LF with anti-bovine LF in the medium at pH 7.5 showed two forms of LF, the first named LF_a (84 kDa) and the second one LF_b (80 kDa) while the immunopercipitation at pH 8 produced on single band of LF_a (80 kDa) (Abd El-Gawad *et al.*, 2001a).

LACTOFERRCIN (LFCIN) STRUCTURE

Limited proteolysis leads to the release of LF fragments: N-t and C-t lobes, the N-2 domain (Legrand *et al.*, 1984) and LFcin (Bellamy *et al.*, 1992b). Lfcin (Lfcin-B from bovine LF and LFcin-H from human LF) is a 25 amino acid peptide (residues 17-42) including two Cys residues linked by a disulfide bridge and containing many hydrophobic and positively charged residues. The secondary structure of Lfcin is markedly different from the same sequence in intact LF (Gifford *et al.*, 2005). The long α-helix observed in the LF structure is replaced by a single β-sheet strand. This structure seems to be better suited for making contact with bacterial membranes. In biological fluids, LF exists in an iron-free form that is very susceptible to proteolysis. It cannot be overlooked that a posttranslational process of maturation by proteolysis leads to the release of LF-derived active peptides in biological fluids (Goldman *et al.*, 1990).

METABOLISM OF LACTOFERRIN

Levay and Viljoen (1995) observed that there are two ways in which LF can be eliminated from the organism: either through receptor-mediated endocytosis of phagocytic cells (macrophages, monocytes and other cells belonging to the reticuloendothelial system) with subsequent iron transfer to ferritin or through direct uptake by the liver. Endocytosis performed by Kupffer cells, liver endothelial cells and hepatocytes contributes to LF removal. Moreover, Hutchens *et al.* (1991) achieved that the kidneys seem to be involved in the removal of LF from the circulation since LF and its fragments, mainly of maternal origin, have been found in the urine of breast-fed infants.

RECEPTORS OF LACTOFERRIN

In fact, most molecular targets on the host cells are multiligand receptors and, interestingly, as reviewed hereinafter, many of them were reported as signaling, endocytosis and nuclear targeting molecules. Ling and Schryvers (2006) showed that the search for specific LF receptors, comparable with that of TF, has consistently mobilized the energy of researchers. Surprisingly, LF receptors with the highest specificity were discovered on bacteria, whereas specific mammalian receptors were only encountered on enterocytes (Suzuki *et al.*, 2005).

LF binding proteins have been found on the small-intestinal brush-border membranes of the mouse and piglet (Mazurier *et al.*, 1985; Kawakami *et al.*, 1990), on the amniotic membrane (Otsuki *et al.*, 2000) and on the surface of cells (Legrand *et al.*, 1992) such as hepatocytes, erythro-leukemic cells, monocytes, peritoneal macrophages, activated T-cells, platelets as listed by Brock (1997).

LF binding molecules have been characterized in many types of microorganisms. In *Toxoplasma gondii,* two LF binding proteins were recently identified as the ROP4 and ROP2 antigens (Dziadek *et al.*, 2007). In viruses, interactions of LF with the V3-loop of gp120 and proteins E1-2 of the Human Immunodeficiency Virus (HIV) and the hepatitis C virus, respectively, have been proposed (Swart *et al.*, 1996; Yi *et al.*, 1997). Strong interactions of bovine LF with adenovirus polypeptides III and IIIa that bind to integrins of host cells have also been demonstrated (Pietrantoni *et al.*, 2003).

Concerning bacteria, many studies reported LF binding to cells and its subsequent bactericidal effect. On Gram-positive *Staphylococcus aureus* and *Streptococcus uberis,* both glycosidic and proteic LF binding sites were evidenced but not further characterized (Naidu *et al.*, 1992; Moshynskyy *et al.*, 2003). In the case of Gram-negative bacteria, although evidence was provided that LF binds to the lipid-A moiety and/or the negative charges in the inner core of LPS with a high affinity (Appelmelk *et al.*, 1994), it is unlikely that LF/LPS interactions occur when LPS is integrated in the cell wall of bacteria. Interestingly, it has been hypothesized that LF may use porins as anchoring sites on the surface of bacteria (Erdei *et al.*, 1994; Sallmann *et al.*, 1999).

Also, a number of gram-negative bacterial species (in the families *Neisseriaceae* and *Moraxella*) have surface receptors capable of specifically binding LF and biochemical and genetic evidence has confirmed the existence of two LF binding proteins, LbpA and LbpB (Schryvers *et al.*, 1998; Prinz *et al.*, 1999; Wong and Schryvers, 2003). A receptor on *Trypanosma cruzi* has also been observed (Lima *et al.*, 1988). Unlike the mammalian receptors, the bacterial receptors are species-specific (Ling and Schryvers, 2006).

GLYCOSYLATION OF LACTOFERRIN

All Lfs contain biantennary N-acetyllactosamine-type glycans, α, 1-6 fucosylated on the N-acetylglucosamine residue linked to the polypeptide chain (Spik *et al.*, 1988). Human LF may also possess additional poly-N-acetyllactosamine antennae that may be α, 1-3-fucosylated on N-acetylglucosamine residues, whereas the LF of other species contains additional high-mannose-type glycans (Coddeville *et al.*, 1992). Both the number and location of the glycosylation sites vary among species. Furthermore, heterogeneity in the number of glycosylated sites is observed in individuals. The role of the glycan moiety seems to be restricted to a decrease in the immunogenicity of the protein and its protection from proteolysis (Spik *et al.*, 1988; Van Veen *et al.*, 2004).

The obtained data by Abdel-Salam *et al.* (2003) indicated that recombinant bLF (rbLF) from cell lysate contained more in the mannose-rich and less in the complex form, also, N-glycosylation may play an important role in the transport of rbLF in the presence of glycosidase inhibitors. Moreover, Abd El-Gawad *et al.* (2003) reported that rbLF was correctly folded and transported efficiently to the Golgi apparatus to become complex glycosylation and ultimately secreted into medium.

Impaired N-glycosylation may be played important role in the transport of recombinant bovine LF in the presence of the glycosides inhibitors (Abd El-Gawad *et al.*, 2001b).

HEAT TREATMENTS EFFECTS ON LACTOFERRIN

The question of heat stability is very important when LF is used as a bioactive component of foods. Since LF is reported to be easily inactivated by heat treatment, it has been found that apo-LF is resistant to denaturation by heating (90-100°C/5min) at pH 4 and could pasteurize LF by heat treatment or sterilize it by a UHT method without any significant loss of biological properties

(Abe *et al*., 1991). Heat denaturation data obtained by differential scanning calorimetry (Ruegg *et al*., 1977) and by fluorometry (Baer *et al*., 1979) have also been reported.

The thermal stability of bovine apo-LF and iron saturated LF have been investigated in relation to antibacterial activity and/or bacterial interaction. UHT treatment (135°C/4 sec) abolished the ability of iron-saturated LF to bind to bacteria as well as the bacteriostatic activity of apo-LF but standard pasteurization regimes used in dairy industry had practically no effect on LF structure. However a heat treatment at 137°C/8s do have only little effect on the ability of LF to stimulate cell proliferation. Kinetic approaches allowed Kussendrager (1994) to conclude that thermal stability of LF is affected by environmental conditions such as pH, salts and whey protein. Consequently the parameters of the heat-induced denaturation of LF have to be determined under conditions of the application of interest.

LF was estimated in camel's milk from Kazakhstan, where two species of camels (*Camelus bactrianus, Camelus dromedarius*) and their hybrids cohabit. The concentrations of LF were determined according to three variation factors: region, season and species, the mean values in raw camel's milk were 0.229±0.135 mg mL^{-1}. The seasonal effect was the only significant variation factor observed with the highest values in the spring. The LF concentration varied in 1 week postpartum milk from 1.422 to 0.586 mg mL^{-1} (Konuspayeva *et al*., 2007).

BIOLOGICAL FUNCTIONS OF LACTOFERRIN

The interest in LF has been primarily in regard to potential capability of acting in iron transport and as an antimicrobial agent related to its iron chelating ability, thus depriving microorganisms of a source of iron. It has been shown to have a number of other physiological and biological functions (Shinmoto *et al*., 1992; Kussendrager, 1993; Brock, 1995; Lonnerdal and Iyer, 1995; Adamik and Walszczyk, 1996; Shinoda *et al*., 1996).

Although human and bovine LF differs, there is increasing evidence that both serve similar biological functions (Adamik and Walszczyk, 1996; Shinoda *et al*., 1996; Miyauchi *et al*., 1997).

Many roles have been proposed and continue to be proposed, for LF (Fig. 5). Although some of these are clearly related to its iron-binding properties, for example its ability to provide bacteria with a source of iron and therefore act as a promicrobial, others appear to be independent of iron binding. The antimicrobial activity of LF is well established. For many years this activity was attributed to the ability of LF to sequester iron thereby depriving potential pathogens of this

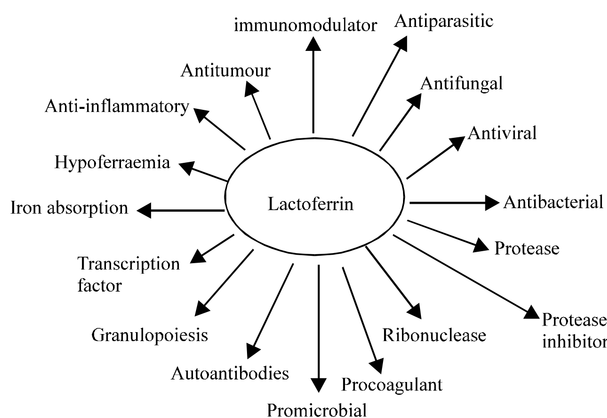

Fig. 5: Proposed roles of lactoferrin by Brock (2002)

essential nutrient. However, LF is now known to possess a second type of antimicrobial activity, bactericidal as opposed to bacteriostatic, the result of a direct interaction between the protein and the bacterium (Brock, 2002).

LACTOFERRIN AND HOST DEFENSE

Legrand *et al.* (2005) revealed that the LF can both positively and negatively influence immune system cells and cells involved in the inflammation reaction, caused by its iron binding properties and interactions with target cells and molecules. In one way, LF may support the proliferation, differentiation and activation of immune system cells and strengthen the immune response. On the other side, LF acts as an anti-inflammatory factor. Also, LF may prevent the development of inflammation and subsequent tissue damage caused by the release of pro-inflammatory cytokines and reactive oxygen species.

Machnicki *et al.* (1993) and Haversen *et al.* (2002) noted that the protective effect of LF is manifested in a reduced production of some pro-inflammatory cytokines such as tumor necrosis factor (TNFα) or interleukins IL-1β and IL-6. Also, an increased amount of anti-inflammatory interleukin IL-10 has been reported in several cases. Iron is essential as a catalyst for the production of reactive oxygen species. Therefore, LF can diminish the harmful influence of reactive oxygen species produced by leukocytes at the sites of inflammation (Ward *et al.*, 2005).

LACTOFERRIN AND IRON BINDING

Anderson *et al.* (1987) and Baker and Baker (2005) demonstrated that the iron binding site has the same composition and geometry in both lobes of LFs and TFs which comprises four protein ligands (2 Tyr, 1 Asp and 1 His) that provide three negative charges to balance the 3+ charge of Fe^{3+}, together with the side chain of an Arg residue whose positive charge balances the negative charge of a CO_3^{2-} anion.

In the "natural state" bovine LF is only partly saturated with iron (15-20%) and has a salmon pink colour. Iron-depleted LF with less than 5% iron saturation is called apo-LF, whereas iron-saturated LF is referred to as holo-LF. In breast milk the LF found is essentially apo-LF. The affinity of LF for iron is very high, about 260 times that of blood serum TF. The iron-binding capacity of LF is dependent of the presence of bicarbonate. The binding site appears to be optimized for the binding of ferric iron and bicarbonate but other cations may bound in the cleft; Al^{3+}, Ga^{3+}, $Cu^{2+,}$ Mn^{3+}, Co^{3+} and Zn^{2+} etc. (Baker, 1994; Brodie *et al.*, 1994). Also, Bagby and Bennett (1982) reported that the LF can bind metal ions other than iron including copper, zinc, aluminum, gallium, vanadium and calcium. Calcium ion-dependent oligomerizetion of LF has been reported, LF forms a tetramer in the presence of calcium ions (10 mM).

LF molecule can bind two Fe^{3+} ions, one within each lobe and the HCO^{3-} ion is necessary for iron binding (Legrand *et al.*, 1988; Spik *et al.*, 1994). Normally, LF contains iron ions and the degree of iron saturation is 10 to 30% in milk. TF releases iron ions at pH 4 but LF holds iron ions at pH>2 and it is reported that the iron binding strength of LF is 260 folds stronger than that of TF (Aisen and Liebman, 1972). Also, Baker and Baker (2005) reported that the iron release depends on the destabilization of the closed form, in the absence of receptor binding (as is the case for TF) while Mazurier and Spik (1980) decided that the release is triggered by lowering the pH. It has been reported that the stability of binding or the strength of iron binding to LF varies depending on species, in the following order: human and equine LF>bovine LF>bovine TF (Shimazaki *et al.*, 1993).

A lot of substances have been found to bind with LF including many kinds of small molecules and biopolymers (Brock, 1997), such as trypan blue dyes (Malmquist and Johansson, 1971), some kinds of drugs (Atkinson and Begg, 1988), ferritin (Pahud and Hilpert, 1976), immunoglobulins (Watanabe *et al.*, 1984; Ena *et al.*, 1990), albumin (Ena *et al.*, 1990), β-Lg (Ena *et al.*, 1990), DNA (Hutchens *et al.*, 1989b), LPS (Elass-Rochard *et al.*, 1995; Wang *et al.*, 1995), lipids (Appelmelk *et al.*, 1994), agar/agarose (Johansson and Hjerten, 1960a), carrier ampholytes (Shimakaki *et al.*, 1991) and heparin (Zou *et al.*, 1992; Van Berkel *et al.*, 1997).

The iron content and iron saturation of different LFs of buffalo, cow (Local, Friesian and Brown-Swiss breeds), ewe (Rahmani and Ossemi breeds), goat as well as camel were markdleyblower in colostrum than in normal milk (Mahfouz *et al.*, 1997).

ANTIMICROBIAL ACTIVITY

Kirkpatrick *et al.* (1971) observed that LF affects the growth and proliferation of a variety of infectious agents including gram-positive and negative bacteria, viruses, protozoa and fungi. Legrand *et al.* (2005) and Valenti and Antonini (2005) explained that the LF is considered to be a part of the innate immune system. It also takes part in specific immune reactions but in an indirect way which due to its strategic position on the mucosal surface. It represents one of the first defense systems against microbial agents invading the organism mostly via mucosal tissues.

It has been widely accepted for many years that LF displays antimicrobial activity against many different infectious agents. This activity was originally attributed to its ability, in common with TF, to sequester iron with a high affinity and unlike TF, retain its bound iron under acidic conditions. LF inhibits the growth of many Gram-positive and -negative bacteria, some yeast and molds (Table 2) and some kinds of parasites. LF is known to act synergistically with lysozyme (Carlsson and Bjorck, 1987) and IgA (Akin *et al.*, 1994) in various secretory fluids. The bacteriostatic effects of LF are thought to be due to its ability to sequester environmental iron (Arnold *et al.*, 1980) because some kinds of bacteria, such as *E. coli*, secrete chelators to enhance iron uptake.

The antimicrobial activity of rbLF, its peptic digest LFcin-B recombinant glycosylated human lysozyme (rhLz) and mixture of LFcin-B+rhLz+EDTA against *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli, Salmonella typhimurium, Salmonella enteritidis,* was investigated by El-Sayed *et al.* (2003).

Yekta *et al.* (2010) studied the ability of bovine and human lactoferrin, two natural antimicrobial proteins present in milk, to inhibit *E. coli* O157:H7 growth and attachment to a human epithelial colorectal adenocarcinoma cell line (Caco-2). The direct antibacterial effect of bLF on *E. coli* O157:H7 was stronger than that of hLF. Nevertheless, both lactoferrins had bacteriostatic effects even at high concentrations (10 mg mL^{-1}), suggesting blocking of LF activity by a yet undefined bacterial defense mechanism.

Table 2: Lactoferrin-susceptible and -resistant microorganisms

Lactoferrin-susceptible microorganisms
Streptococcus mutans[a], *Streptococcus pneumoniae*[a], *Vibrio cholera*[a], *Pseudomonas aeruginosa*[a], *Staphylococcus aureus*[b], *Escherichia coli*[b], *Salmonella typhimurium*[b], *Salmonella montevideo*[b], *Listeria monocytogenes*[b], *Candida albicans*[b]
Lactoferrin-resistant microorganisms
Staphylococcus aureus[a], *Staphylococcus epidermidis*[a], *Streptococcus pyogenes*[a], *Streptococcus lactis*[a], *Lactobacillus casei*[a], *Pseudomonas aeruginosa*[b], *Escherichia coli* 126 : B 16[a], *Escherichia coli* O111[a], *Enterobacter cloacae*[a], *Salmonella newport*[a], *Shigella sonnei*[a]

[a]: Arnold *et al.* (1980), [b]: Ellison (1994)

Abdel-salam *et al.* (2006) shows that transformation of yeast cells with cDNA encoding bovine lactoferrin insert with eukaryotic expression vector occurred. Also, HPLC analysis data showed that the rbLf extracted from yeast media had the same retention time and molecular weight of the bovine lactoferrin standard. Recombinant lactoferrin extracted from medium demonstrated a greater inhibition effect than recombinant lactoferrin extracts from the cell lysates against *Bacillus subtilis* and *Escherichia coli*.

ANTIBACTERIAL ACTIVITY

The antibacterial activity of LF was initially ascribed to its ability to bind and sequester environmental iron, thereby depriving potential pathogens of this essential nutrient. The ability of LF to inhibit bacterial growth *in vitro* was indeed one of the earliest functions described for the protein. The antimicrobial activity of LF was demonstrated towards a number of bacteria and apo-LF was found to be bactericidal for *Streptococcus mutans* and *Vibrio cholerae* but not for *Escherichia coli* (Arnold *et al.*, 1977). Further studies have shown that LF was bactericidal only when in its iron-free state and that iron-saturated LF has a reduced antimicrobial activity (Arnold *et al.*, 1980; Kalmar and Arnold, 1988; Yamauchi *et al.*, 1993). Iron-independent killing by LF was, however, first demonstrated by Arnold and co-workers in 1982. Previous study in 1981 had shown that LF was bactericidal for *Streptococcus mutans* even when exogenous iron was added to the experimental media (Arnold *et al.*, 1981). Bortner *et al.* (1989) proposed that this iron-independent LF killing was a result of a direct interaction of LF with the bacterial surface. Lassiter *et al.* (1987) had previously suggested that the target site on bacteria would be anionic. Further studies have attempted to elucidate the mechanisms for this direct effect. LF was shown to interact with LPS of the Gram-negative bacterial membrane of *E. coli* (Appelmelk *et al.*, 1994), with the release of the LPS from the membrane. However, this release was blocked by addition of Ca^{2+} and Mg^{2+} ions. The presence of Ca^{2+} ions also inhibited the ability of LF to increase the susceptibility of *E. coli* to the antibiotic rifampicin (Ellison *et al.*, 1990). The importance of these divalent cations as modulators for the antimicrobial activity of LF has been further reported by Bortner *et al.* (1986, 1989) who showed that LF sensitive strain of *Legionella pneumophila* was protected from killing by addition of calcium chloride, magnesium nitrate and magnesium chloride, however addition of sodium chloride had no effect. Kalmar and Arnold (1988) showed that Mg^{2+} ions decreased LF killing, whereas the addition of Ca^{2+} or K^{+} ions had no affect on the antimicrobial activity of LF.

It has been discovered that LFcin, a cationic peptide generated by the pepsin digestion of LF, has more potent bactericidal activity than the native protein. There are two forms known at present: LFcin H (derived from human LF) and LFcin B (of bovine origin) (Bellamy *et al.*, 1992a).

The proteolytic activity of LF is considered to inhibit the growth of some bacteria such as *Shigella flexneri* or enteropathogenic *E.coli* through degrading proteins necessary for colonization. However, this can be disabled by serine protease inhibitors (Orsi, 2004; Ward *et al.*, 2005).

ANTIVIRAL ACTIVITY

LF has been shown to be effective in protecting against a number of different viruses (Marchetti *et al.*, 1996; Shimizu *et al.*, 1996).

Marchetti *et al.* (1996) reported that both human and bovine LF were effective against the herpes simplex virus type 1 (HSV-1) by inhibiting adsorption of the virus. This activity was independent of the iron with-holding, since both iron saturated and apo-LF was equally effective.

Swart *et al.* (1998) found that LF from both human and bovine sources was able to completely block HCMV replications and to inhibit HIB-1 induced cytopathic effects.

Evidence suggests that LF contributes to the host defense against viral infections. It has been reported that LF is effective against Hepatitis C Virus (HCV) (Tanaka *et al.*, 2000a; Yi *et al.*, 1997), Herpes Simplex Virus (HSV) (Hammer *et al.*, 2000), Feline Immunodeficiency Virus (FIV) (Sato *et al.*, 1996), Human Immunodeficiency Virus (HIV)-l and human cytomegalovirus (Harmsen *et al.*, 1995). With respect to the anti-HCV effect of LF, oral administration of bovine LF (1.8 to 3.6 g day^{-1}) was found to result in a decrease in serum alanine transaminase and HCV RNA concentrations in 4 of 7 patients (Tanaka *et al.*, 2000b). These effects are thought to be attributable to the interaction between LF and an HCV envelope protein (Tanaka *et al.*, 2000b) or between the glycan chains of LF and HCV (Valenti *et al.*, 1998).

The effects of recombinant Lf on the growth of human parainfluenza virus type 2 (hPIV-2) in $LLCMK_2$ cells were investigated by Yamamoto *et al.* (2010), using a recombinant, green fluorescence protein-expressing hPIV-2 (rghPIV-2), it was found that virus entries into cells were considerably inhibited by Lf but cell-to-cell spread was not inhibited.

ANTIINFLAMMATORY PROPERTIES

LF has a beneficial effect on infections and protects animals against a lethal dose of LPS (Zagulski *et al.*, 1989; Dial *et al.*, 2005). Additionally, LF plays antiinflammatory roles in noninfectious pathologies such as rheumatoid arthritis, inflammatory bowel disorders, neurodegenerative diseases and skin allergies. It has been shown that administration of LF protects against chemically induced cutaneous inflammation (Cumberbatch *et al.*, 2003) and no steroidal antiinflammatory drug-induced intestinal injury (Dial *et al.*, 2005). In collagen-induced and septic arthritis mouse models, peri-articular injection of human LF reduced inflammation (Guillen *et al.*, 2000).

ANTIOXIDANT ACTIVITY

Bannister *et al.* (1982) reported that the generation of hydroxyl radicals by the xanthine-xanthine oxidase reaction has been shown to be increased by iron-saturated LF. The antioxidant activity of LF has been attributed to its sequestration of free iron ions. Apo-LF sequesters any free iron in secretions, so it may protect mucus glycoproteins from active oxygen species generated in iron-catalyzed reactions (Clamp and Creeth, 1984). It is expected that such mucoprotective action would be overcome during infections. The suppression of lipid peroxidation has also been observed by Shinmoto *et al.* (1992).

ANTIPARASITIC ACTIVITY

LF acts against parasites in various ways. For example, the infectivity of *Toxoplasma gondii* and *Eimeria stiedai* sporozoites is reduced after their incubation with LF B. It is thought that LFcin breaches parasitic membrane integrity causing subsequent changes in interactions between the host and the parasite (Omata *et al.*, 2001). The competition for iron between the parasite and LF is the basis of its antiparasitic activity against *Pneumocystis carinii* (Cirioni *et al.*, 2000). In contrast, some parasites such as *Tritrichomonas foetus* are able to use LF as a donor of ferric ions (Tachezy *et al.*, 1996).

ANTIFUNGAL ACTIVITY

LF was first reported to have anti-fungal activity by Kirkpatrick *et al.* (1971). In combination with fluconazole, LF was shown to reduce the minimum inhibitory concentration at which

fluconazole killed a number of clinical isolates of Candida species, suggesting that LF may have a potential use in combination therapy against drug-resistant Candida infections (Kuipers *et al*., 1999). The synergistic action of LF with antibiotics, antifungals and antibacterial agents already in use increases their efficacy (Ellison and Giehl, 1991; Naidu and Arnold, 1994; Wakabayashi *et al*., 1996, 1998). The combined use of LF and antifungal against severe infections with *Candida* species is hence an attractive therapeutic option. Since fluconazole-resistant *Candida* species have been frequently reported in HIV-infected patients, the addition of LF which is one of the host specific defense factors present in saliva that exhibit antifungal activity, may delay the occurrence of resistant species (Kuipers *et al*., 1999). Al-Sheikh (2009) reported that lactoferrin showed significant antifungal effect on the three pathogenic *Candida* species viz., *C. albicans, C. krusei* and *C. tropicalis* while the addition of iron enhances the multiplication of *Candida* species.

ANTICANCER ACTIVITY

Administration of bovine LF has been found to be effective in reducing the number of Aden carcinomas in the large intestine chemically induced in rats (Tsuda *et al*., 2000) and in inhibition of tongue carcinogenesis in rat (Tanaka *et al*., 2000c) and in suppression of the spontaneous development of jejunely polyps in mice (Tsuda *et al*., 2000) and in inhibition of lung metastasis of colon carcinoma cells in mice (Tsuda *et al*., 2000). It has been proposed that these effects may be due to an increase in cytotoxic cells in peripheral blood.

Tsuda *et al*. (2002) and Mohan *et al*. (2006) reported that the concomitant administration of bovine LF and carcinogens to rodents inhibits the induction of activating enzymes for carcinogenic heterocyclic amines, modulates lipid per oxidation and activates antioxidant and carcinogen detox-ification enzyme activities, blocking cancer development.

There is increasing evidence, based on animal studies, that LF may have therapeutic value in treatment of different types of cancer (Sekine *et al*., 1997; Uchida *et al*., 1999). LF has great potential therapeutic use in cancer disease prevention and/or treatment, namely as a chemo-preventive agent (Rodrigues *et al*., 2009).

CELL GROWTH PROMOTING ACTIVITY

LF is known to act as a negative feedback regulator of myelopoiesis (Broxmeyer *et al*., 1980) and as a growth factor for human lymphocytic cell lines in serum-free medium (Hashizume *et al*., 1983). It is reported that hLF has greater growth stimulatory activity than hTF (Hashizume *et al*., 1983). Also, LF shows promotion of nerve growth factor synthesis and secretion in mouse fibroblast L-M cells (Shinoda *et al*., 1993) and promotes endometrial cell proliferation (Yanaihara *et al*., 2000).

BONES REMODELING ACTIVITY

LF has been identified as a potent anabolic factor affecting osteocytes. It is stimulates osteoblast proliferation, enhances thymidine incorporation into osteocytes and reduces apoptosis of osteoblasts by 50-70%. A similar effect was also recorded in chondrocytes (Cornish *et al*., 2004). LF reduces or even inhibits osteoclas-togenesis in a concentration-dependent fashion. On the other hand, LF shows no influence on the bone resorption performed by mature osteoclasts (Lorget *et al*., 2002).

Cornish *et al*. (2004) showed that LF may affect bone cells through the inhibition of osteolytic cytokines such as TNFα or IL-1β, whose levels rise during inflammation. Thus, LF contributes to the stabilization of the osseous tissue. Because of these aforementioned properties, LF might be potentially useful in the treatment of diseases such as osteoporosis in the future.

ENZYMATIC ACTIVITY

LF has the ability to function as an enzyme in some reactions. LF is the milk protein with the highest levels of amylase, DNAse, RNAse and ATPase activities (Devi *et al.*, 1994; Kanyshkova *et al.*, 2003). However, these are not the only enzymatic activities of LF. The basis for LF various enzymatic activities is unknown. However, the variety of activities can be attributed to variations in the nature of the protein: multiple isoforms; degrees of glycosylation; tertiary structure (holo-or apo- LF) and the degree of oligomerisation. For instance, the LF molecule capable of hydrolysing RNA has an isoform that is incapable of Fe^{3+} binding (Furmanski *et al.*, 1989).

LF has demonstrated remarkable resistance to proteolytic degradation by trypsin and trypsin-like enzymes. The level of resistance is proportional to the degree of iron saturation (Brock *et al.*, 1976; Brines and Brock, 1983; Lyer and Lonnerdal, 1993). It is known that holo-LF shows greater resistance to protease digestion than apo-LF (Brock *et al.*, 1976) and LF is not cleaved by plasmin or chymosin.

The discovery of LF enzymatic activities has helped to explain several of its physiological mechanisms, such as protection against microbial pathogens where LF might inhibit growth partly through hydrolysis of viral, bacterial, fungal and parasitic nucleic acids.

A remarkable similarity in some motifs between LF and ribonuclease A has been revealed and LF is, indeed, capable of RNA hydrolysis. The ribonuclease activity varies depending on the type of RNA. mRNA is the most sensitive to LF, whereas tRNA is the least. The non-iron-binding isoforms of LF seem to be responsible for RNA degradation (Furmanski *et al.*, 1989; Devi *et al.*, 1994).

ANTIMICROBIAL ACTIVITY OF LACTOFERRIN-DERIVED PEPTIDES

The hydrolysates produced by pepsin cleavage of bovine and human LF were found to contain a potent bactericidal peptide, named LFcin B and LFcin H (Bellamy *et al.*, 1992a). The action of pepsin on LF produces peptides that have enhanced antimicrobial action as compared to LF (Jones *et al.*, 1994; Wakabayashi *et al.*, 1994; Facon, 1996; Facon and Skura, 1996; Dionysius and Milne, 1997; Tomita *et al.*, 1998). A peptide with 28 residues from the N-terminal of LF has been names LFcin B and has much stronger antibiotic activity than LF (Jones *et al.*, 1994). Multiple peptides have been isolated from pepsin hydrolysates of LF, with varying microbial activities (Dionysius and Milne, 1997) isolated 3 peptides with different structures and activities. Peptide I had essentially the same structure as LFcin B. The three peptides displayed varying antibacterial activity against a number of different spoilage and pathogenic organisms. Peptide I was most effective against *Listeria monocytongenes*. Antimicrobial activity shown in media is lost in food products and inhibited by 5 mM calcium and bile salts (Facon, 1996; Facon and Skura, 1996).

LFcin B has been shown also to be effective at concentrations as low as 3 μg L^{-1} against a number of different strains of yeasts and filamentous fungi (Bellamy *et al.*, 1994).

CATIONIC PEPTIDES

A wide variety of organisms produce antimicrobial peptides as a primary innate immune strategy (Hancock and Lehrer, 1998). Hundreds of such peptides have been isolated throughout nature, from single celled microorganisms, mammals, amphibians, birds, fish and plants (Hancock and Chapple, 1999), indicating their importance in the innate immune system (Bevins, 1994; Hancock and Diamond, 2000). Typically, these peptides are relatively short (less than 100 amino acids), positively charged, amphiphilic and are reported to be active against

bacteria, fungi, viruses and protozoa (Martin *et al.*, 1995). They display a great structural diversity and although their modes of action can vary and are not fully understood, their main site of action is thought to be the cell membrane. Several mechanisms have been proposed for cationic antimicrobial peptides. Hydrophobicity, cationicity and secondary structure have been implicated in the antimicrobial effect. Although certain peptide structural groups have been noted, including amphipathic-helices, -structures, extended structures and loops, no overall conservation of amino acids exists (Boman, 1995; Hancock, 1997).

LACTOFERRICIN

The presence of stable antimicrobial peptides resulting from the proteolysis of LF was shown more than a decade ago when Saito *et al.* (1991) demonstrated that limited acid proteolysis of bovine LF yielded a hydrolysate that had greater antibacterial activity than LF. Bellamy *et al.* (1992a, b) described the generation, after pepsin hydrolysis, of fragments from human and bovine LFcin that showed enhanced antimicrobial activity but no iron-binding capacities. The fragments were characterized and named human and bovine LFcin. Both peptides are derived from the N-terminal region of the N-lobe and have greater antibacterial activity than their parent proteins LFcin H corresponds to amino acid residues 1-47 from the N-terminal region of the protein (Fig. 2) and includes an 18-residue loop formed by an internal disulphide bridge. Residues 1-11 constitute a separate fragment which remains bound to the main loop by a disulfide bridge. The slightly more potent LFcin B from bovine LF comprising only residues 17-41, consists primarily of the 18-residue loop stabilized by a disulfide bridge.

BOVINE LACTOFERRICIN

LFcin B, like other antimicrobial peptides that display membrane-disruptive properties, contains a high proportion of basic amino acid residues. It has been demonstrated that this highly cationic portion of LF is responsible for the ability of LF to bind glycosaminoglycan (Mann *et al.*, 1994), heparin and LPS (Elass-Rochard *et al.*, 1995). LFcin B is proposed to exert its effect at the surface of the bacterial membrane (Bellamy *et al.*, 1993) and positive charges within the peptide are thought to aid interaction with membrane components. The greater the number of positive charges, the greater the number of interactions with negatively charged membrane components (Nikaido and Vaara, 1985; Hwang *et al.*, 1998). Numerous studies have been undertaken to identify which regions of the bovine peptide are important for its antimicrobial activity (Hoek *et al.*, 1997; Schibli *et al.*, 1999).

HUMAN LACTOFERRICIN

Due to the limited supply of human milk and the difficulties in extracting the native peptide, less research has been undertaken on LFcin H. It is a 47 amino acid peptide produced under the same conditions as LFcin B though of lower antimicrobial potency. However, the larger concentration of LF observed in human milk, compared to the respective concentration in bovine milk, may make the antimicrobial activity of hLF and LFcin relevant *in vivo*. The peptide is similar to LFcin B in that it has a loop region held together by a disulphide bridge. It has been suggested that antimicrobial activity was independent of the presence of this bridge (Bellamy *et al.*, 1992a, b). Although the total and net positive charges carried by the LFcin H are higher than those carried by LFcin B, the proportion of basic amino acid residues is greater in the bovine peptide. The lower cationicity within the 18 residue-loop might be at the heart of the difference in antimicrobial activity exhibited by human LF-derived peptides.

NUTRITIONAL AND APPLICATIONS OF LACTOFERRIN

Milk proteins have many nutritional, functional and biological functions in humans and animals and could also be used in practical applications. LF is one such milk protein (Hyvnen, 2010). The LF-derived peptide Lfcin is released through proteolysis by pepsin (Korhonen and Pihlanto, 2006), has received much attention recently due to its various roles in host defense (Gifford *et al.*, 2005). bLF is a by-product of the dairy process and as such is readily available. rhLf can be expressed in milk, rice or microorganisms and is also commercially available. Both can be added to foods and different health products (Lonnerdal, 2009). The suggested applications for LF are for example from food preservatives to health-promoting foods and supplements, infant foods, iron supplements, paharmaceuticals, sport foods, nutritional foods, chewing gums, healthcare products, such as toothpaste, mouthwashes and cosmetics (Marnila and Korhonen, 2009). The Food and Drug Administration (FDA) of the United States has assessed LF and considers it to be generally safe (U.S. Food and Drug Administration, 2001). In the meat industry, LF can be used as spray applied to carcasses to decrease the growth of contaminant bacteria and to extend the shelf-life (Naidu, 2002). LF could replace food and feed additives as a preservative, because it is a natural protein of milk. LF has been used as a supplement in calf feed, to prevent neonatal diarrhea and to increase weight gain (Joslin *et al.*, 2002; Robblee *et al.*, 2003). Calves receiving supplemental bLF in colostrum and milk replacer had fewer days of diarrhea with less serious clinical signs than the control calves. LF can also stimulate carbohydrate absorption and increase small intestine epithelial cell size (Zhang *et al.*, 2001), resulting a faster growth rate of mice.

Iron-saturated LF may serve a nutritional function as a source of iron or LF may regulate iron absorption. It is reported that the clearance of LF intravenously injected into mice is due to a hepatic receptor that specifically binds oligosaccharides containing Fuc (α-1, 3) GlcNAc linkages (Prieels *et al.*, 1978). On the other hand, another mode of recognition of hLF which is avidly taken up by the mouse liver, has been proposed. It is suggested that the integrity of the protein moiety of LF is required for its effective uptake by the liver (Moguilevsky *et al.*, 1985). The effectiveness of bLF in prevention of intestinal infections and in inhibition of bacterial translocation from the gut lumen into host tissues has been demonstrated in experiments using mice (Teraguchi *et al.*, 1995).

There are two ways in which LF can be eliminated from the organism: either through receptor-mediated endocytosis of phagocytic cells (macrophages, monocytes and other cells belonging to the reticuloendothelial system) with subsequent iron transfer to ferritin or through direct uptake by the liver. Endocytosis performed by Kupffer cells, liver endothelial cells and hepatocytes contributes to LF removal (Levay and Viljoen, 1995).

The large-scale preparation of bLF from cheese whey or skim milk (up to 100 metric tones per year) and of recombinant hLF produced in microorganisms and plants makes LF available for human and animal (fish farming) health purposes and commercial applications. The first major application of bLF was the supplementation of infant formulas but it is now added to cosmetics, pet care supplements and immune system-enhancing nutraceuticals, including drinks, fermented milks and chewing gums. In all these media, LF is expected to exert its natural antimicrobial, antioxidative, antiinflammatory, anticancerous and immunomodulatory properties. Furthermore, clinical trials demonstrated the efficiency of LF against infections and in inflammatory diseases. For example, a recent clinical study concluded that the combination of LF and fluconazole at the threshold minimal inhibitory concentrations elicited potent synergism, leading to total fungistasis of *C. albicans* and *C. glabrata* vaginal pathogens (Naidu *et al.*, 2004). Also, LF was reported as a

potent molecule in the treatment of common inflammatory diseases (Legrand *et al.*, 2005). In addition, extensive clinical trials are underway in Japan to further explore its preventive potential against colon carcinogenesis (Tsuda *et al.*, 2002).

On the other hand, LF offers applications in food preservation and safety, either by retarding lipid oxidation (Medina *et al.*, 2002) or by limiting the growth of microbes. For example, incorporation of LF into edible films has a great potential to enhance the safety of foods since the film can function as a physical barrier as well as an antimicrobial agent. LF can be also directly used as a spray applied to beef carcasses (Taylor *et al.*, 2004).

Lastly, LF can be used as a clinical marker of inflammatory diseases since LF levels in blood and biological fluids may greatly increase in septicemia or during Severe Acute Respiratory Syndrome (Reghunathan *et al.*, 2005). In the same way, fecal LF levels quickly increase with the influx of leukocytes into the intestinal lumen during inflammation. Fecal LF is thus used as a noninvasive diagnostic tool to evaluate the severity of intestinal inflammation in patients presenting with abdominal pain and diarrhea (Greenberg *et al.*, 2002). This biomarker has been shown to be a sensitive and specific marker of disease activity in chronic inflammatory bowel disease (Kane *et al.*, 2003) and in Crohn's disease (Buderus *et al.*, 2004).

Several evidences indicating benefits of bLF supplementation in infant formula were reported, including an improvement in intestinal microbialflora (Kawaguchi *et al.*, 1989; Roberts *et al.*, 1992), enhanced serum ferritin (Chierici *et al.*, 1992) and hematocrit (King *et al.*, 2007) levels and reduced lower respiratory tract illnesses (King *et al.*, 2007). Addition of bLF also inhibits lipid oxidation of infant formula (Satue-Gracia *et al.*, 2000). Currently, bLF containing infant formulas are sold in Indonesia and Korea, as well as in Japan. Other bLF containing products include yogurt, skim milk, milk-type drinks, supplemental tablets, pet food and cosmetics (Wakabayashi *et al.*, 2006).The beneficial effects of these bLF containing products on the health have been proved in clinical and animal studies. The effect of yogurt on rotaviral gastroenteritis and the effect of tablets on chronic hepatitis C, rotaviral gastroenteritis and *Helicobacter pylori* gastric infection have been reported (Egashira *et al.*, 2007; Ishii *et al.*, 2003; Okuda *et al.*, 2005). The therapeutic effect of pet food on dermatitis in dogs and cats was also shown (Masada *et al.*, 1995).

Bovine colostrum can be freeze-dried and used as raw materials for human food supplements which participate to improve immunity of human bodies by raising the immunoglobulin and lactoferrin concentrations. The results led to the establishment of limits for the quality of colostrum (rich or poor in IgG), to develop a table of correspondence between IgG, lactoferrin and density that can be used as a practical method for rapid and cheap evaluation of colostrum (Bar *et al.*, 2010).

Industry continues to attempt to improve formula with the addition of compounds such as fatty acids, oligosaccharides, nucleotides and lactoferrin. However, human milk has such far reaching effects on the infant`s immune response that normal development depends heavily on its provision. All mothers should be encouraged and supported to continue breastfeeding for six months and beyond in order to promote the good health of their infants (Oddy, 2002).

CONCLUSIONS

LF has been the focus of intense research of late. Due to its unique antimicrobial, immunomodulatory and even antineoplastic properties, LF seems to have great potential in practical medicine. Nevertheless, much research and many experiments still need to be carried out in order to obtain a better understanding of its activity and interactions and to enable the full and safe utilization of this glycoprotein.

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