A SEMINAR PAPER
ON

LACTOFERRIN: A MULTIFUNCTIONAL MARVELOUS PROTEIN IN MILK

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A SEMINAR PAPER
ON
LACTOFERRIN: A MULTIFUNCTIONAL MARVELOUS PROTEIN IN MILK [1]

By
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ABSTRACT

Lactoferrin is a multifunctional iron glycoprotein which is known to exert a broad-spectrum primary defense activity against microbsfound in milk. Colostrum, the first milk produced after a baby is born, contains high levels of lactoferrin, about seven times the amount found in milk produced later on. Lactoferrin is also found in fluids in the eye, nose, respiratory tract, intestine, and elsewhere.Numerous functions have been reported and continue to be reported for the protein, some of which are related to its iron-binding properties. Its extensive antimicrobial activities were originally attributed to its ability to sequester essential iron, however, it is now established that it possesses bactericidal activities as a result of a direct interaction between the protein orlactoferrin-derived peptides. This paper reviews the properties of lactoferrin, the isolation from milk and also biological functions as well as its antimicrobial activities of lactoferrin and discusses the potential mode of action of lactoferrin-derived cationic peptides against Gram-negative bacteria in the light of recent studies.Apart for emphasizing on the specific beneficial properties of lactoferrin from each of these sources, the general antimicrobial, immunomodulatory and anticancer activities of lactoferrin are also discussed here. The new perspectives in the studies on the antimicrobial activity of Lf appear to be linked to its potential prophylactic and therapeutical use in a considerable spectrum of medical conditions, taking advantage of the availability of the recombinant human Lf. But the historical evolution of our knowledge on Lf indicates that its antimicrobial activity must be considered in a general picture of all the biological properties of this multifunctional protein.

Keywords: Lactoferrin;structure; isolation; immunity and host defense; cancer; antimicrobial activity.

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Chapter I
INTRODUCTION

1.1 Background

Milk proteins are the most important source of bioactive peptides, the health benefits of milk protein-derived peptides have been a subject of growing commercial interest in the context of health-enhancing functional foods (Jagat *et al*., 2015). Lactoferrin is a multifunctional iron-binding transferrin family protein found mainly in the milk of several speciesandby-products derived from the dairy industry, such as whey. Lactoferrin is primarily extracted from bovine milk and it is subsequently added into many commercial products such as nutritional supplements, infant formula, cosmetics and toothpaste (Wang *et al.,* 2017). Lactoferrin from human and bovine milk shows 69% homology in their amino acid sequence. As described in several review articles (Vorland, 1999; Shimazaki, 2000; Lonnerdal and Iyer, 1995, Leavy and Viljoen, 1995, Baker *et al.,* 2002; Baker and Baker, 2004, 2005), lactoferrin was first discovered in bovine milk in 1939 by Sorensen and Sorensen and was first isolated from milk, simultaneously in three separate laboratories, in the year of 1960. This red or salmon-pink protein was initially called as "red protein" in milk and then referred to as lactotransferrin or lactosiderophilin because of its high similarity to transferrin and siderophilin in blood and ovotransferrin in egg. The name "lactoferrin" was being proposed by Blanc and Isliker in 1961 as stated in an article by Masson and Heremans (1971).The primary differences between human and cow milk are their carbohydrate and mineral contents and protein composition. Lactose and oligosaccharides contents of human milk (71 g/liter and 10-25 g/liter, respectively) are higher than that of bovine milk (46 g/liter and 1-2 g/liter, respectively) as described by Jenness (1988). The differences in protein composition of human and cow milk shown in Fig.1also indicate the presence of high amount of lactoferrin in human milk (Jenness, 1982). Lactoferrin was subsequently being isolated in the milk of all mammalian species investigated, with the exception of the dog and the rat (Masson and Heremans, 1971). Current sequence databases contain amino acid sequences for the lactoferrin's of nine species, human, pig, horse, cow, buffalo, sheep, goat, camel and mouse.

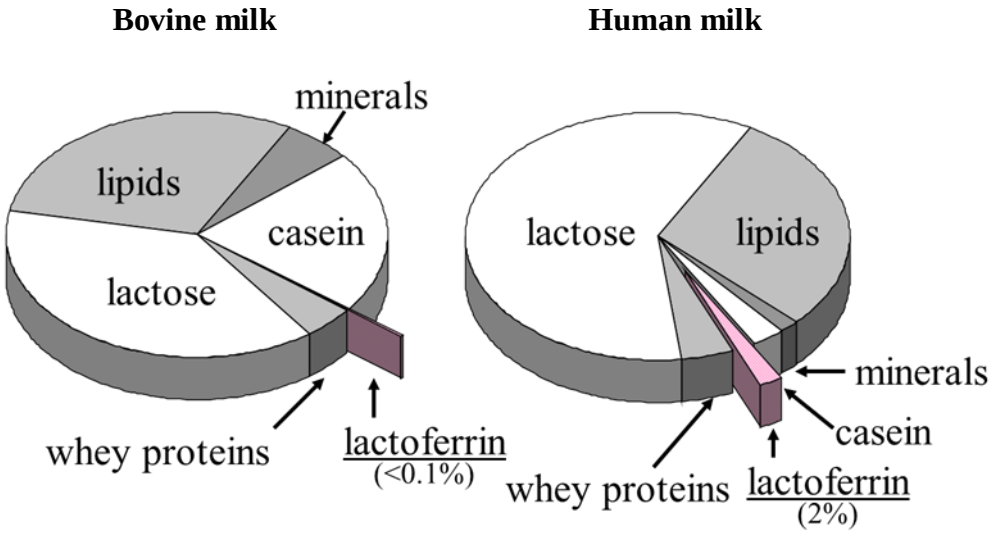

Fig. 1. Lactoferrin contents of total milk solid (Shimazaki *et al.,* 2000).

The protein exhibits several biological activities normally associated with a host defense system (Nuijens *et al*., 1996; Shimazaki, 2000; Brock, 2002). The antimicrobial activity is one of the most pivotal, as well as the first, discovered functions of lactoferrin, and it has been well established for many years (Arnold *et al*., 1977). However, lactoferrin not only inhibits the growth of microorganisms but it also acts as a growth promoter for other microorganisms (Menozzi *et al.*, 1991).

1.2 Objectives:

After completing this article, readers will be able:

- To explore the properties of lactoferrin.
- To review the isolation of lactoferrin from milk or whey.
- To highlight the bio-functionalities of lacteferrin.

Chapter II
METHODOLOGY

Scientific approach requires a close understanding of the subject matter. This paper mainly depends on the secondary data. Different published reports of different journals mainly supported in providing data in this paper. This paper is completely a review paper. Therefore no specific method has been followed in preparing this paper. It has been prepared by browsing internet,studying comprehensively various articles published in different journals, books, proceedings, dissertation available in the libraries of BSMRAU and personal communication. The author would like to express her deepest sense of gratitude to her major professor and course instructors for their efficient and scholastic guidance, precious suggestions to write this manuscript from its embryonic stage. All the information collected from the secondary sources have been compiled systematically and chronologically to enrich thispaper.

Chapter III

REVIEW OF MAJOR FINDINGS AND DISCUSSION

3.1Properties of lactoferrin

3.1.1 Structure

The determination of amino acid sequence of lactoferrin by Metz-Boutigue *et al*., 1984, confirmed its belonging as archetypal members of the transferrin family. All the transferrin family proteins are glycoprotein, with a molecular weight of about 80 kDa (670-690 amino acid residues), and typically exhibit 50-70% pairwise sequence identity. The sequence identity between lactoferrins and serum transferrins is ~60%, and between lactoferrins from different species, it is ~70% (Baker, 1994). Human lactoferrin has a molecular weight of 82.4 kDa and is composed of 702 or 692 amino acid residues whereas bovine lactoferrin has a molecular weight of 83.1 kDa and is composed of 689 amino acid residues as reviewed by Shimazaki, 2000.

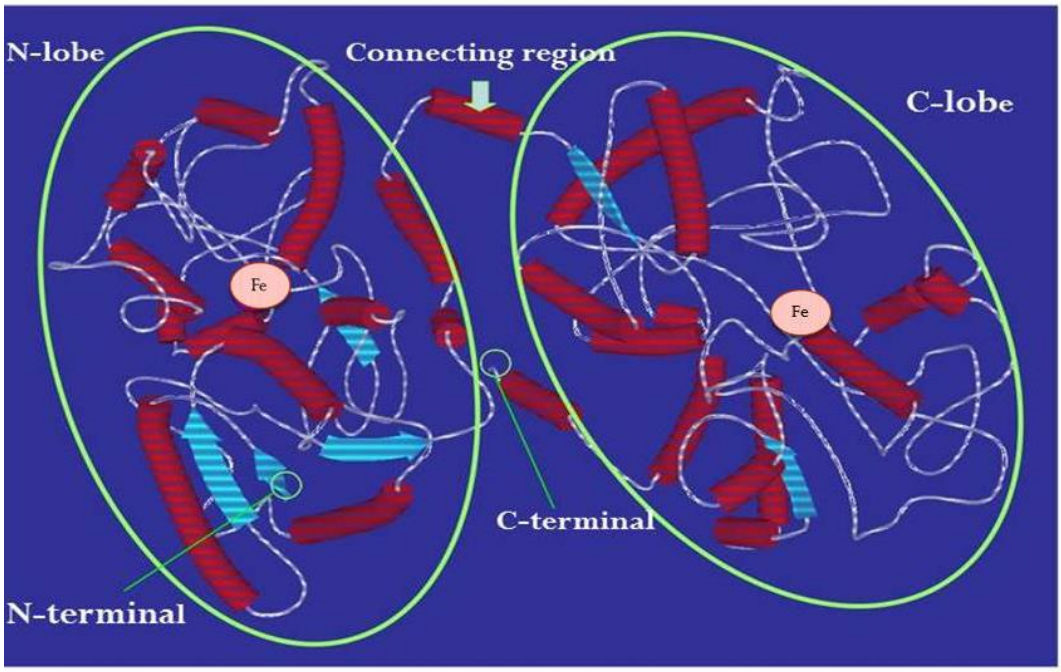

Fig. 2. A three dimensional structure of bovine lactoferrin showing two lobes and connecting region.

(Source: PhD Dissertation from Rahman, 2007).

Lactoferrin consists of a polypeptide chain which forms two globular lobes, each with two domains. The iron-binding site is located in each lobe and the iron atoms are coordinated by four amino acid ligands: two tyrosines, one histidine, and one aspartate. In the binding site a carbonate or bicarbonate ion adjacent to an arginine side chain also participates (Anderson *et al.*, 1987). Spik *et al.*, 1988 havereported differences in the glycan composition of lactoferrin from different species such as human, mouse, bovine and goat. Each lactoferrin contains 1–4 glycans depending on the speciesand the characteristics of the carbohydrate moiety are specific to each lactoferrin. The cationic N-terminus of lactoferrin is of special interest because it has been reported that its antibacterial activity resides there (Bellamy *et al.*, 1992). The thermal stability of lactoferrin has been well reported (Rüegg *et al*., 1977; Kawakami *et al.*, 1992; Sánchez *et al.*, 1992c; Paulsson *et al*., 1993; Paulsson and Elofsson, 1994; Mata *et al*., 1998). Differences have been found in the thermo resistanceamong the species with, for example, bovine lactoferrin being less resistant than the human counterpart (Sánchez *et al*., 1992). Slight differences have also been found in the stability of human lactoferrin isolated from milk and human recombinant lactoferrin expressed in mice, these differences being probably due to the different glycosylation pattern between both proteins (Conesa *et al*., 2007). It has been reported that unglycosylated lactoferrin is much more susceptible to degradation (Van Berkel *et al.*, 1995), and some studies have revealed that the susceptibility of lactoferrin to tryptic proteolysis depends also on the nature of the glycans bound to the protein (Van Berkel *et al*., 1996). More specifically, human lactoferrin has been shown to be more resistant to proteolysis than bovine lactoferrin due to the different glycans bound to them. Lactoferrin has N-glycosidically-linked glycans and number of potential glycosylation sites is five for bovine lactoferrin and three for human lactoferrin (Van Veen *et al*., 2004).

Table 1 shows theamino acids sequence of antibacterial peptides derived from human and bovine lactoferrin. The primary structure of human and bovine lactoferrin is determined by Baker *et al.* (2000) and Moore *et al.* (1997), respectively. The sequence of amino acid residues is based on the intact form of human or bovine lactoferrin.

Table 1. Amino acids sequence of antibacterial peptides derived from human and bovine lactoferrin

Antibacterial peptides	Sequences in lactoferrin	Primary structure
Human lactoferricin	1-47	GRRRRSVQWCAVSNPEATKCFQWQRN MRKVRGPPVSCIKRDSPIQCI
Bovine lactoferricin	17-41	FKCRRWQWRMKKLGAPSITCVRRAF

(Source: Wang *et al.*, 2017).

3.1.2 Localization and concentration

The concentration of lactoferrin in milk varies greatly among species and also at various stages of lactation as described by Lonnerdal and Iyer, 1995 and Shimazaki, 2000. For example, human milk and milk from other primates, pigs, and mice are high in lactoferfin, whereas milk from species such as the cow and other ruminants is very low in lactoferrin. Species that have low concentrations of lactoferrin in their milk usually have higher levels of transferrin in their milk, whereas species like the human have a very little transferrin in their milk (Masson and Heremans, 1971). Human colostrum contains higher than 5 g/L of lactoferrin as compared to 2-3 g/L in mature breast milk. Lactoferrin content in bovine colostrum is approximately 0.8 g/L; whereas bovine milk contains only 0.03-0.49 g/L (Table 2). The higher amount of lactoferrin in colostrum is helpfulto provide protections to breast-fed infants against bacterial infection and inflammation (Artym & Zimecki, 2005).

Table 2. Major sources and concentration of lactoferrin (LF)

Fluid	Lactoferrin concentration	Selected reference
Human colostrum	5.80 ± 4.30	Montagne, 2001
Bovine colostrum	0.82 ± 0.54	Kehoe, 2007
Camel colostrum	0.81 ± 0.31	Konuspayeva, 2007
Goat colostrum	0.39 ± 0.07	Hiss, 2008
Human milk	2.00 - 3.30	Montagne, 2001
Bovine milk	0.03 - 0.49	Cheng, 2008
Camel milk	0.06 - 0.89	Konuspayeva, 2007
Goat milk	0.17- 0.59	Chen, 2004

(Source: Wang *et al*., 2017).

Lactoferrin has also been found in the other exocrine secretions of human. Lactoferrin present in milk is synthesized by the epithelial cells of mammary gland (Vorland, 1999). Lactoferrin is present in biological fluids including milk, saliva and seminal fluid (Cheng *et al*., 2008). It is also present in mucosal surfaces and in some granules of polymorphonuclear leukocytes. The most abundant source of lactoferrin is human and bovine milk.

3.1.3 The physico-chemical properties of human and bovine lactoferrin are shown in Table 3.

Table 3. Physico-chemical properties of human and bovine lactoferrin

Property	**Human lactoferrin**	**Bovine lactoferrin**
Molecular mass		
Sedimentation co-efficient	75,100	77,200 ± 1,300
SDS-PAGE	76,800 ± 1,600	76,000 ± 2,400
Iron titration	80,000	78,500
Isoelectric point		
Chromato focusing	6.8 – 8.0	8.2 – 8.9
Isoelectric focusing	5.8 – 6.5	9.5 – 10.0
Absorption spectra		
Apo-form at 280 nm	10.9	12.7
Holo-form at 470 nm	0.510	0.400
Glycosylation	Relatively high	Low
Protease sensitivity	Relatively low	High
IgA-complexes	Present	Absent
Iron-binding		
Equilibrium dialysis (k1 x 10^{-4})	26.0	3.73

(Source: Naidu, 2000).

3.1.4 Number of amino acid residues in bovine and human lactoferrin is shown in Table 4.

Table 4. Number of amino acid residues in bovine and human lactoferrin

	Bovine milk	**Human milk**
Alanine	67	63
Proline	30	35
Arginine	39	43
Lysine	54	46
Asparagine	29	33
Valine	47	48
Tryptophan	13	10
Cysteine	34	32
Threonine	36	31
Isoleucine	15	16
Serine	45	50
Glutamine	29	27
Glutamic acid	40	42
Phenylalanine	27	30
Methionine	4	5
Leucine	65	58
Glycine	48	54
Tyrosine	22	21
Aspartic acid	36	38
Histidine	9	9
Total number of residues	689	691

(Source: Pierce *et al*., 1991).

3.2 Isolation of lactoferrin from milk

3.2.1 Isolation and purification

Acid-precipitated casein has been used as the starting material for isolation of lactoferrin from bovine milk (Groves *et al*., 1960). However, the whey fraction of milk or colostrum is a better source to obtain lactoferrin on a laboratory scale (Law BA *et al*., 1977) and cheese whey is another source used to obtain lactoferrin on a large scale. As the isoelectric point of lactoferrin is alkaline, cation exchange chromatography has been used to isolate lactoferrin and metal-chelate affinity chromatography (Hutchens *et al*., 1989) and hydroxyapatite column chromatography (Itagaki *et al.,* 1993) have also been used to purify lactoferrin.

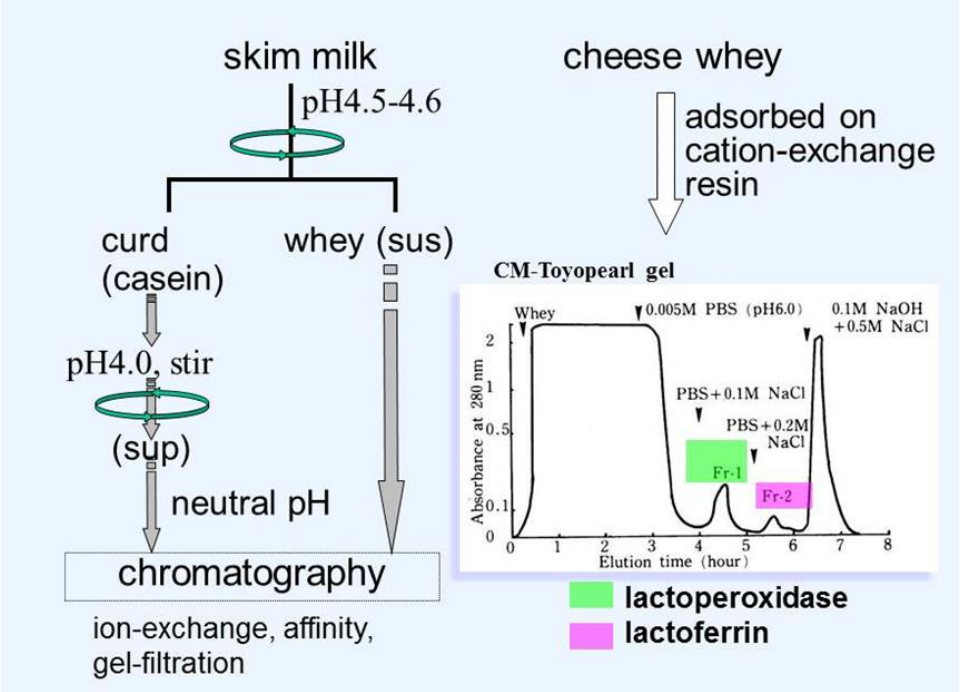

Fig. 3. Isolation of lactoferrin from milk source.

(Source: Moradian, 2014).

Care should be taken to test for contamination by lipopolysaccharide (LPS) prior to examining the biological activities of the isolated lactoferrin, as LPS is known to be a potent inflammatory mediator (Rietschel *et al.,* 1992). To assay the lactoferrin concentrations in secretory fluids or serum, ELISA and other immunochemical techniques such as single radial immunodiffusion and immunoelectrodiffusion methods have been employed using anti-lactoferrin antiserum. In order to measure the lactoferrin concentrations in dairy products such as cheese, treatment at pH4 to release lactoferrin bound to casein is necessary.

Table 5. Extinction coefficients (1%, 1cm path length) of lactoferrin

	Apo-type	**Holo-type**	
Bovine lactoferrin	12.7 (280 nm)	15.1 (280 nm)	0.46 (470 nm)
			0.58 (465 nm)
Human lactoferrin	10.9 (280 nm)	14.6 (280 nm)	0.51(470 nm)
	11.2 (280 nm)		

(Source: Baker *et al.*, 2000).

The extinction coefficients at 280nm and 465nm as shown in Table 5 can be used in quantification of lactoferrin when the sample solution contains no other substances which show absorbance at these wavelengths.

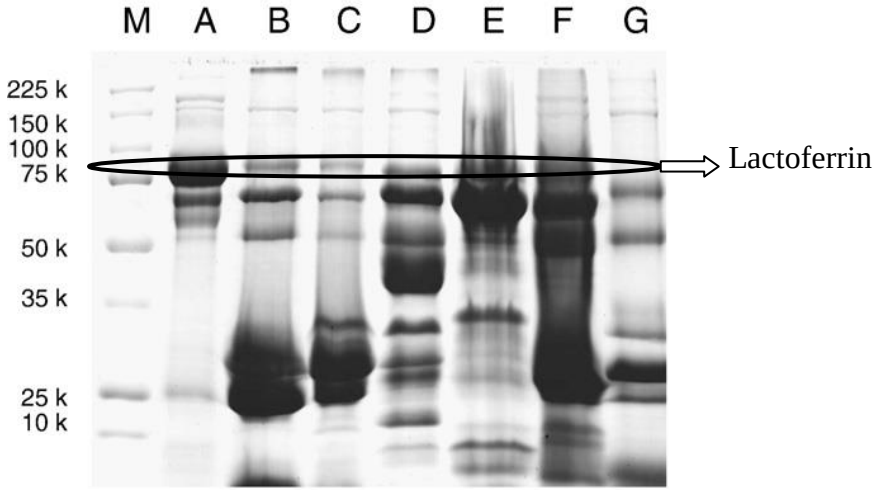

Fig.4. SDS-PAGE of milks from different species: (A) human, (B) sheep, (C) goat, (D) camel, (E) alpaca (F) elephant and (G) grey seal. (M) Molecular weight marker.

(Source: C. Conesa *et al*., 2008).

3.3 Biological activities

Since its discovery and isolation from milk, lactoferrin has intrigued and puzzled researchers. Initially, the biological functions of lactoferrin were suggested to be related with its iron-binding property. The protein was also thought to be involved in the delivery of iron into milk due to its high concentration in the milk of some species. However, it is becoming increasingly evident that lactoferrin is a multifunctional protein to which several physiological roles have been attributed. Every year or two, a new and surprising function of lactoferrin is popping-up.

Apart from the antimicrobial activity, lactoferrin has been also proposed to exert other biological functions such as regulation of iron transport, antitumoral, anti-inflammatory, immuno-modulatory, transcriptional regulation and proteolytic and enzymatic activities, reviewed by Farnaud and Evans (2005).

3.3.1 Natural Antibiotic

Although human lactoferrin was believed to have antibacterial activity against *E coli*, subsequent studies reveal this is not correct (Ellison *et al*., 1991). Bovine lactoferrin is at best bacteriostatic (Yamauchi *et al.*, 1993). Conversely, bovine lactoferricin rapidly disrupts the outer membrane of *E. coli.* (Yamauchi *et al*., 1993). If human or bovine lactoferrin are incubated with lysozyme, another natural peptide antibiotic present in human milk, rapid killing of *E. coli* takes place. (Yamauchi *et al.,* 1993). If recombinant human lactoferrin, lysozyme and *E. coli* are incubated under conditions that simulate fluid in the small intestine, bacterial destruction occurs within 2 hours (Edde L *et al*., 2001). In neonates, there is also a synergy between recombinant human lactoferrin and pharmaceutical antibiotics that eradicate coagulase-negative staphylococci and *Candida albicans* (Venkatesh MP *et al.,* 2008). Under in vitro and in vivo conditions, antibiotics and lactoferricin more effectively kill *Pseudomonas aeruginosa* (Sánchez-Gómez *et al.*, 2011).

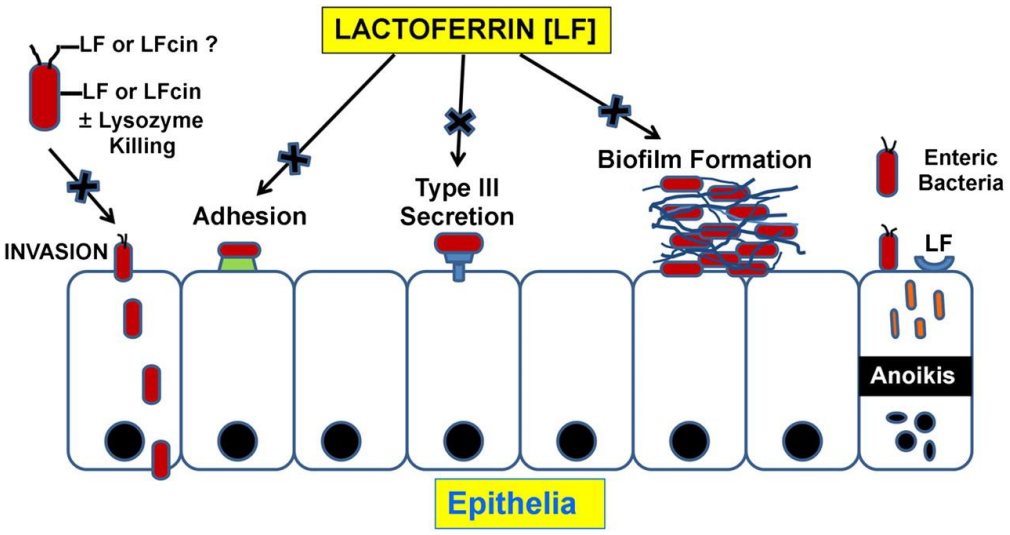

Fig. 5. LF-mediated restriction of bacterial invasion of intestinal epithelia.

(Source: Michael *et al*., 2012).

3.3.2 Immunomodulation activity

Lactoferrin is reported to regulate the immune system by stimulating and/or inhibiting cytokine release and modulating the activity of immune system cells (Yamauchi, 1998). To date there has been no direct in vivo evidence for a regulatory role of lactoferrin in the immune system, although knockout animals for lactoferrin have been produced (Ward *et al.*, 2004). Involvement of lactoferrin in the regulation of the immune systemwas suggested in 1980 (Breton-Gorius *et al*., 1980), when a total absence of lactoferrin in neutrophils, but normal lactoferrin content in glandular secretion(Gordon *et al.*, 1989), was observed for a patient suffering recurrent infections.

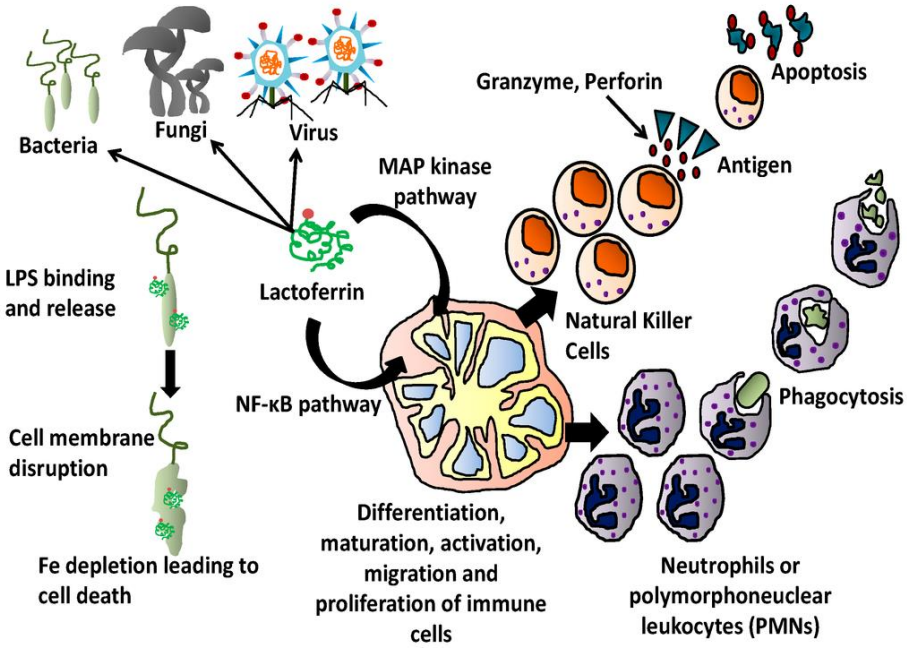

Fig. 6. Role of lactoferrin in the activation of immune cells.

(Source: Dashper *et al*., 2012).

More recently, human lactoferrin-transgenic mice have been shown to clear bacteria significantly better than congenic littermates (Guillen *et al.*, 2002). This effect is the consequence of directinhibition of the growth of *Staphylococcus aureus*, and of the enhancement of the T helper (Th) type 1 response due to overexpression and constitutive presence of lactoferrin in animal tissues. Furthermore, the susceptibility totuberculosis of b-2-microglobulin knockout mice was abolished by lactoferrin treatment (Schaible *et al*., 2002). Oral administration of lactoferrin also proved host-protection effects against microbial infections (Van Hooijdonk *et al*., 2000), during lethal bacteraemia in mice (Ochoa *et al.*,

2003) and against oral candidiasis (Takakura *et al*., 2004). Finally, orally administered lactoferrin was shown to protect piglets against septic shock (Lee *et al*., 1998).

At the molecular level, altered expression of cytokines, mostly pro-inflammatory interferon γ (IFN-γ), interleukin(IL)-1b, IL-6 and TNF-α, and granulocyte-macrophagecolony-stimulating factor (GMCSF) have beendetected in the presence of exogenous lactoferrin (Kruzel *et al*., 2002),with a decrease of IL-5 and IL-10 production. At the cellular level, there seems to be an increasednumber of natural killer (NK) cells (Yamauchi *et al.,* 1998), increased phagocytosis-enhancing effect (Szuster-Ciesielska *et al*., 1995), an increased recruitment of neutrophils in blood (Kuroseet al., 1994) and modulation of myelopoiesis (Broxmeyer *et al.,* 1987).

3.3.3 Lf and allergies

In vivo studies showed lactoferrin protection against skin and lung allergies (Elrod *et al.,* 1997; Griffiths *et al.,* 2001). Lactoferrin is overexpressed in patientswith allergies (Elrod *et al.,* 1997), a process which involves theactivation of mast cells and basophils, and IL-1b andTNF-α-triggered migration of antigen-presenting cells (Zweiman *et al.,* 1990). In skin allergies, a mechanism by which lactoferrin binds to keratinocytes and inhibits the release of TNF-α fromthe cells has been proposed (Cumberbatch *et al.,* 2003). Another explanation has been found in the ability of lactoferrin to destabilize tryptase, a potent pro-inflammatory protease releasedfrom mast cells (Kimber *et al.,* 2002). Lactoferrin apparently displaces tryptase from heparin, which is known to maintain enzymaticactivity. It was recently shown that inhibition occurs following lactoferrin uptake by mast cells and interaction not only with tryptase but also with chymase and cathepsin G (He *et al.,* 2003). Recently, these authors also showed an inhibition of anti-immunoglobulin (Ig) E induced histamine and tryptase release from human colon mast cells by lactoferrin (He *et al.,* 2004).

3.3.4 Stimulation of a beneficial gut microflora

The bacterial flora of breastfed infants is different from that of formula-fed infants; breastfed infants have fewer potentially pathogenic bacteria such as *E. coli*, *Bacteroides*, *Campylobacter*, and *Streptococci*, but more *Lactobacilli* and *Bifidobacteria* (Kleesen *et al.,* 1995). Although antimicrobial components in human milk inhibit the growth of pathogenic bacteria, it can also stimulate the growth of beneficial bacteria, thus they have prebiotic activity. Lactoferrin can promote the growth of *Lactobacilli* and *Bifidobacteria*, but by

decreasing intestinal pH can also limit the growth of several pathogens (Olga Senkovich et al., 2010). One possible substance identified was N-acetyl-glucosamine (György et al., 1971). Subsequently, several oligosaccharides have been shown to have this activity (Newburg DS *et al.*, 1997), but it is also possible that milk proteins also have such prebiotic activity.

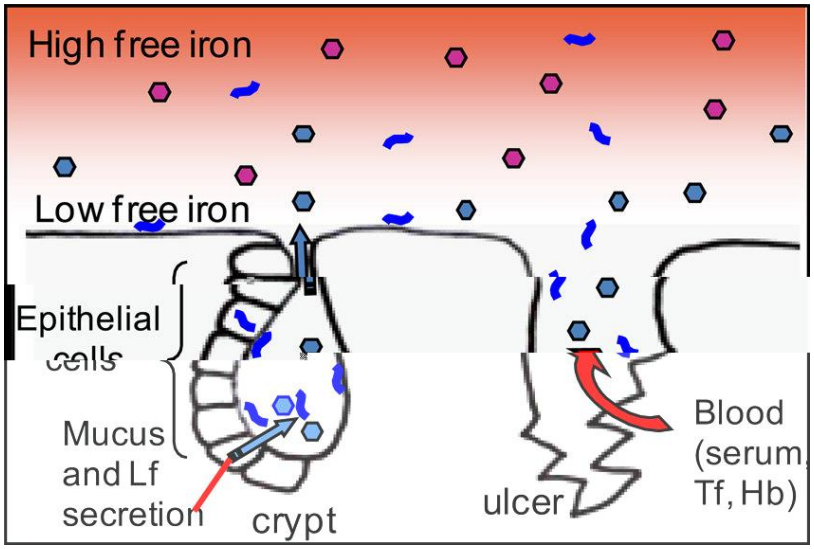

Fig.7. Free iron in the gastric mucosa. Lf, lactoferrin; Tf, transferrin; Hb, hemoglobin. Blue hexagons indicate iron-free Lf; red hexagons indicate iron-saturated Lf; curved blue bars indicate *Helicobacter pylori* cells.

(Source: Olga Senkovich *et al.*, 2010).

3.3.5 Anti-inflammatory role

In addition to controlling bacterial burden by direct antimicrobial action, evidence suggests that lactoferrin may limit the inflammation associated with microbial challenge. Animal studies have shown that lactoferrin administration protects against gastritis induced by *Helicobacter pylori* (Dial and Lichtenberger, 2002).

3.3.6 Role in iron homeostasis

Iron is required for many metabolic functions in the body, but it can be harmful in excess, promoting microbial growth and free radical-induced cellular damage. The iron-binding activity of lactoferrin is suggested to sequester free iron in the gut, thus controlling microbial pathogenesis and iron-induced cellular oxidative damage (Sanchez and Brock, 1992).

Iron and metal chelating ability by lactoferrins

Lactoferrin iron-saturation affects several physico-chemical properties of the proteins, thus directly influencing their biological functions. Concerning the colour of lactoferrin powders, apo-lactoferrin appear whitish while the native- and holo-lactoferrin are salmon pink with the colour intensity depending upon the degree of iron saturation (Steijnsand van Hooijdonk, 2000) as shown in Fig. 8.

(a) (b) (c)

Fig. 8. Appearance of lactoferrin. (a), (b) and (c) indicates lyophilized form of native (iron content around 30%), apo-type (iron content 0%) and holo-type (iron content 100%) bovine lactoferrin. Native lactoferrin is slightly pink, apo-type is white and holo-type is deep red in color. (Source: Luigi Rosa *et al*., 2018).

These three lactoferrin forms show similar secondary structures while the tertiary structure is different for holo-lactoferrin as well as the iron saturation increases their thermal stability as shown in Table 6.

Table 6. Thermal stability of apo- and holo-bovine lactoferrin (bLf)

Bovine lactoferrin	Temperature (°C)
Apo-bLf	67 ± 2
Holo-bLf	87 ± 3

(Source: Luigi Rosa *et al.*, 2018).

Lactoferrin also display high resistance to proteolytic degradation by trypsin-like enzymes proportional to the degree of iron saturation, resulting in iron-saturated lactoferrin more resistant than the iron-depleted forms (apo-lactoferrin) (Brines and Brock 1983). Moreover, other metal ions such as Al (III), Cu (II), Mg (II), Mn (III), Zn (II) and Ca (II) are chelated by lactoferrin by the two iron binding sites, even if at lower affinity than Fe (III) (Baker, 1994).

Moreover, two further Zn ions are sequestered by the C-lobe (Jabeen *et al.* 2005) as well as Ca (II) by carboxylate groups of sialic acid (Rossi *et al.* 2002).

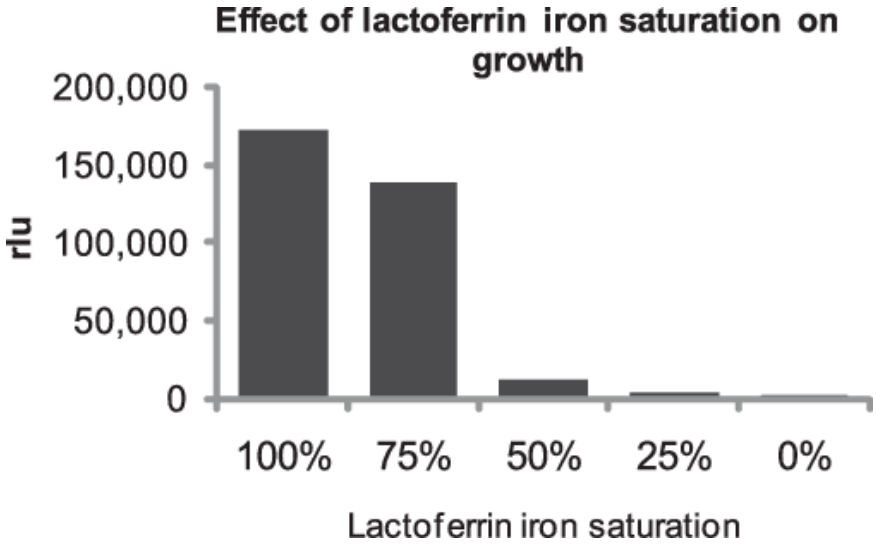

Fig. 9. Effect of lactoferrin iron saturation on *H. pylori* growth. Ferri-lactoferrin and apo-lactoferrin were added to TT18 at ratios that resulted in the saturation levels indicated. The total lactoferrin concentration was 9 g/ml. Inocula containing the same amount of strain 26695m were added to duplicate wells. Growth was assessed by measuring the ATP content. rlu, relative light units.

(Source: Olga Senkovich *et al.*, 2010).

3.3.7Antibacterial activity

Lactoferrin exhibits both bacteriostatic and bactericidal activity against a wide range of microorganisms including Gram-negative and Gram-positive bacteria. The main antimicrobial mechanism of lactoferrin against the microorganisms that require iron is due to the ability of lactoferrin to chelate this metal, thereby, depriving them of the source of this nutrient (Masson *et al.*, 1966). Moreover, lactoferrin interacts with the cell membrane of some bacteria, leading to changes in the permeability and causing the release of lipopolysaccharide from the Gram-negative bacterial outer membrane (Ellison *et al.*, 1988). Table 7 shows the bacteria against which lactoferrin has shown an inhibitory effect and the type of lactoferrin used.

Table 7. List of bacteria express lactoferrin-binding proteins or receptors

Bacteria	**Selected references**
Bordetella pertussis	Redhead *et al*., 1987
Mycobacterium pneumoniae	Tryon and Baseman, 1987
Listeria monocytogenes	Lee et al., 2005
Treponema spp.	Staggs, *et al*., 1994
Helicobacter pylori	Dhaenens *et al*., 1997
Staphylococcus aureus	Naidu *et al*., 1990, 1991, 1992
Streptococcus uberis	Moshynskyy *et al*., 2003
Neisseriaceae spp.	Lee and Schryvers, 1988; Schryvers and Morris, 1988; Schryvers and Lee, 1989
Streptococcus pneumoniae	Hammerschmidt *et al*., 1999
Moraxella spp.	Yu and Schryvers, 2002

Some of the bacteria listed in Table 7 are specially categorised as antimicrobial-resistant, such as the strains of *Staphylococcus aureus*, *Listeria monocytogenes* and methicillin resistant *Klebsiella pneumoniae*. Lactoferrin's bacteriostatic function is due to take up theFe3+ ion, limiting use of this nutrient by bacteria at the infectionsite and inhibiting the growth of these microorganisms as wellas the expression of their virulence factors (Reyes *et al.,* 2005). It was proved that theexternal membrane of Gram-negative bacteria damaged by lactoferrin through an interaction with lipopolysaccharide (LPS) in 1988 (Ellison *et al.,* 1991). The positively chargedN-terminus of LF prevents the interaction between LPS and the bacterial cations (Ca2+ and Mg2+), causing a release of LPS from thecell wall, an increase in the membrane's permeability and ensuing damage to the bacteria (Coughlin *et al.,* 1983). Lactoferrin's mechanism of action against Gram-positive bacteria isbased on binding due to its net positive charge to anionic molecules on the bacterial surface, such as lipoteichoic acid, resulting in areduction of negative charge on the cell wall and thus favoringcontact between lysozyme and the underlying peptidoglycan overwhich it exerts an enzymatic effect (Leitch *et al*., 1999).In vitro and in vivo studies have proved that lactoferrin has the ability to prevent the attachment of certain bacteria to the host cell.Attachment-inhibiting mechanisms are unknown, but it has been suggested that lactoferrin's oligomannoside glycans bind bacterial adhesins, preventing their interaction with host cell receptors (Drago *et al.,* 2006). Recent studies also showed that lactoferricin

inhibits the attachment of enteropathogenic *E. coli* (EPEC) to intestinal cells, which appears to be mediated by the serine protease activity of lactoferrin (Plaut *et al*., 2000).

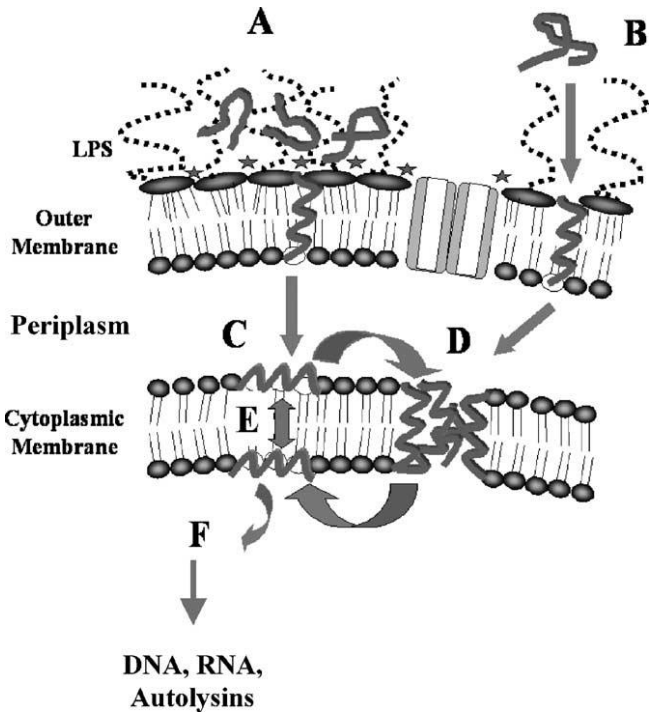

Fig. 10. Proposed mechanisms of interaction of cationic antimicrobial peptides with the cell envelope of Gram-negative bacteria (A) Peptides can bind to the negative charges of the outer membrane. (B) Only to the divalent cation binding sites on LPS in order to disturb the outer membrane and interact directly with the hydrophobic part of the membrane. (C) Once the peptide has crossed the outer membrane, it binds to the negatively charged surface of the cytoplasmic membrane and inserts further into the membrane interface .(D)Some peptides have been proposed to then either aggregate into a micelle-like complex, which spans the membrane. (E) Flip–flop across the membrane and (F) Interact in the cytoplasm with negatively charged molecules such as DNA and RNA. (Source: Hancock and Chapple, 1999).

Bovine lactoferrin has been shown to reduce the incidence of late onset sepsis in extremely preterm infants, but Food and Drug Administration approval of lactoferrin for use in the NICU has not taken place. Because lactoferrin is currently available only for scientific investigations, the feeding of a mother's milk is encouraged shortly after birth because the concentration of lactoferrin is highest in colostrum (Sherman *et al.,* 2014). Research reveals that formula containing bovine lactoferrin establishes a "bifidus flora" in infants (Roberts *et al*., 1992). Additionally, an enteral supplement of bifidobacteria reduces NEC in a neonatal

rat model of the disease (Caplan *et al*., 1999). Bovine lactoferrin influences the composition of fecal microbiota and that together with standard care practices in the NICU, modifies the fecal microbiome and reduces HAIs in very low birth weight(VLBW) infants (Sherman *et al.*, 2014).

Fig. 11 shows the order of bacteria that identified in the feces of 3-week-old very low birth weight infants (VLBW infants –less than 1500 gram) treated with bovine lactoferrin or placebo and using pyro sequencing for sequencing identification and this figure implies that with the lactoferrin treatment the pathogenic bacteria was decreased but beneficial probiotic bacteria was increased. Good result was found with 4% compared with the treatment with 3% lactoferrin.

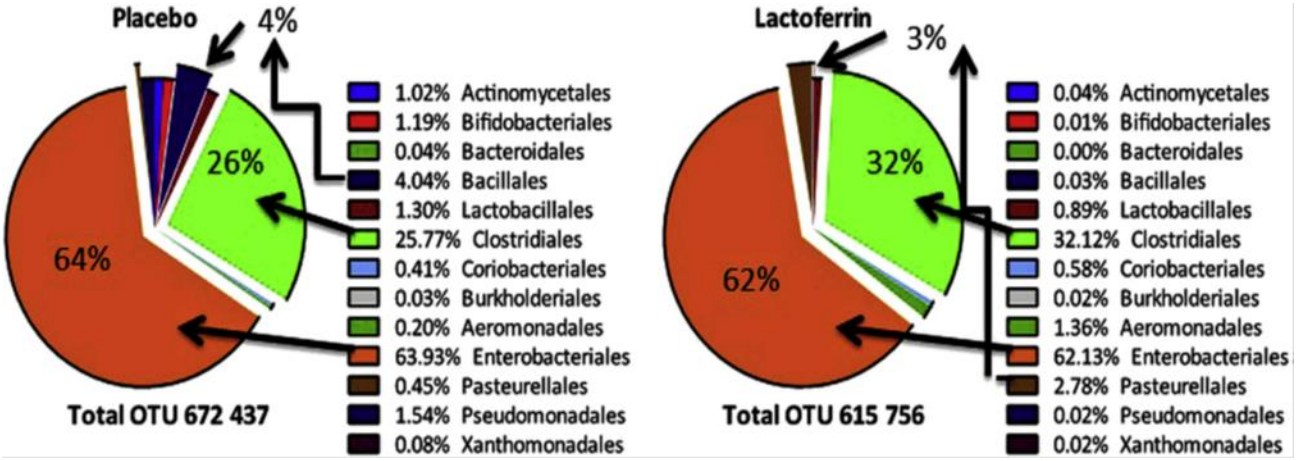

Fig. 11. Order classification of bacteria identified in the feces of 3-week-old very low birth weight infants (VLBW infants –less than 1500 gram) treated with bLf or placebo and using pyro sequencing for sequencing identification.

(Source: Sherman *et al.*, 2014).

3.3.8 Antiviral Activity

Lactoferrin has the capacity to bind certain DNA and RNA viruses (Yi *et al.*, 1997). Nevertheless, its main contribution to antiviral defense consists in its binding to cell membrane glycosaminoglycans. In this manner lactoferrin prevents viruses from entering cells and infection is stopped at an early stage (Ward *et al*., 2005). Such a mechanism has been demonstrated as being effective against the Herpes simplex virus (Fujihara and Hayashi, 1995; Marchetti *et al*., 1996), cytomegaloviruses (Andersen et al., 2001), and the human immunodeficiency virus (Harmsen *et al.*, 1995), respectively.The antiviral effect of lactoferrin has also been observed in viruses which infect animals, such as the Friend virus complex, which causes erythroleukaemia in rodents (Lu L *et al.*, 1987), the feline calicivirus (Addie *et al*., 2003) and felineimmunodeficiency virus.

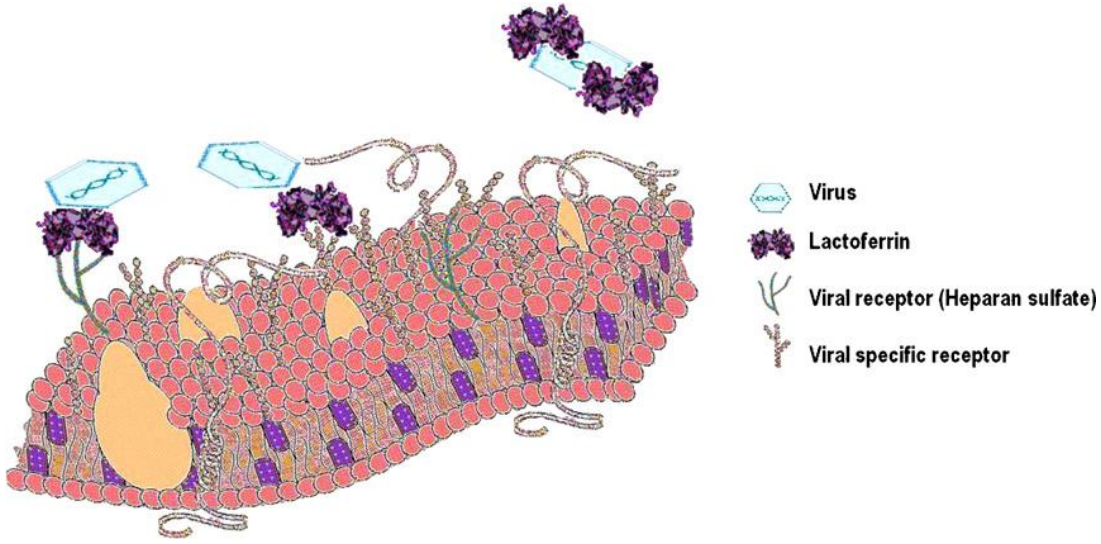

Fig. 12. Mechanism of antiviral action of lactoferrin (LF). LF can be linked to the viral particle and to glycosaminoglycans, specific viral receptors or heparin sulfate to prevent Internalization of the virus into the host cell.

(Source: González-Chávez *et al*., 2009).

3.3.9 Antifungal Activity

Lactoferrin has shown significant fungicidal activity. For example, *in vitro* incubation of bovine lactoferrin with six different species of *Candida* produced significant susceptibility in all six species (Xu *et al*., 1999). In 2003 it was shown that oral lactoferrin treatment of oral candidiasis caused by *C. albicans* reduces the level of the pathogen and promotes a cure (Takakura *et al*., 2003). Although the antifungal mechanism of action of lactoferrin is through a direct interaction with the pathogen, Fe3+ sequestration is another important mechanism. In 2007, Zarember *et al*. showed that Fe3+ sequestration by neutrophil apo-

lactoferrin is important for host defense against *Aspergillus fumigatus* (Zarember *et al.*, 2007). Lactoferrin shows an interesting antifungal effect on body tineas caused by *Trichophyton mentagrophytes*, against which it acts from a distance. Treatment of guinea pigs with bovine lactoferrin reduces fungal infection on the skin of the back and limbs in tinea corpus and tinea pedis, respectively (Wakabayashi *et al.,* 2000).

3.3.10 Development of the gut and its functions

Administration of lactoferrin has been shown to increase cell proliferation in the small intestine of experimental animals and to affect crypt cell development (Nichols *et al*., 1987). This mitogenic effect of lactoferrin has been hypothesized to be responsible, in part, for the rapid development of the intestinal mucosa of suckling newborns (Berseth *et al*., 1983). Weight gain in infants fed formula supplemented with bovine lactoferrin has been shown to be higher than in infants fed regular formula (Hernell *et al*., 2002), which agrees with this proposed function of lactoferrin. Further studies on the potential growth-stimulating effect of lactoferrin are needed.

3.3.11 Role in Allergy and autoimmune Diseases

Lactoferrin appears to play a role in the allergic response as well (Elrod *et al*., 1997). Lactoferrin also appears to play a modulatory role in autoimmune responses and has even been suggested for possible usefulness in the treatment of certain autoimmune disorders (Zimecki *et al*., 1995).

3.3.12 Protection against cancer development and metastasis

A growing number of rodent studies have demonstrated a protective effect of lactoferrin against chemically induced carcinogenesis, tumor growth and/or metastasis in several organs, including the esophagus, tongue, lung, liver, colon and bladder (Tsuda *et al*., 2002). Lactoferrin can induce apoptosis and arrest tumour growth in vitro. It can also block the transition from G1 to S in the cell cycle of malignant cells (Öztas *et al.*, 2005). Treating tumours in mice with recombinant human lactoferrin inhibits their growth by 60% compared with a placebo and increases the levels of anti-carcinogenic cytokines such as IL-18, in addition to activating NK cells and CD8+ T-lymphocytes (Wang *et al.*, 2000). Lactoferrin's anti-cancer effect was recently observed through the immune expression of lactoferrin in human kidney cell carcinomas and in adjacent healthy tissue (Giuffrè *et al*., 2007). In vivo studies show that oral administration of lactoferrin results in the inhibition

of a T-cell-dependent tumour in head and neck squamous cell carcinoma (Wolf *et al.*, 2007).

Prostate cancer is the fourth most widespread cancer in the world, the second most common cancer in men, and the first in Europe and North America (Ferlay *et al.*, 2015). It is responsible of the death of 300000 patients per year worldwide and its incidence kept on increasing during the last two decades (Saman *et al.*, 2014). Although cryoablation, chemotherapy, radiotherapy, and radical prostatectomy can be efficacious against localized tumors, there is still no effective treatment for patients with recurrent or metastatic disease (Lu, 2009).Lactoferrin has been shown to have intrinsic antitumoral activity, making it particularly attractive as part of a gene medicine. Lactoferrin binds to specific receptors (Lf R1, Lf R2) or to transferrin receptors overexpressed on most cancers lines (Tuccari & Barresi, 2011). It has been shown to exert its anti-cancer effect through modulation of the mitogen-activated protein kinase signaling pathway and induction of cell cycle arrest, and can also induce apoptosis of cancer cells by activating the Fas signaling pathway in cancerous cells (Zhou *et al.*, 2008; Gibbons *et al*., 2011).

3.3.13Regulation of organ morphogenesis

One of the most recent novel activities described for lactoferrin is its regulatory function in bone morphogenesis. Lactoferrin was shown to prevent bone resorption in a rabbit mixed bone cell culture (Lorget F *et al.*, 2002). Subsequent experiments using cultured rodent tissue and organ cultures showed that lactoferrin promotes the growth and development of osteoblast cells by stimulating proliferation and decreasing apoptosis (Cornish J *et al.*, 2004). In addition, lactoferrin was shown to enhance osteoblast differentiation and inhibit osteoclast genesis. The growth promoting effects demonstrated for lactoferrin were far more potent than the response seen by established bone growth factors, including epidermal growth factor. Importantly, the anabolic effects on bone growth were substantiated by in vivo studies where subcutaneous administration of lactoferrin (4 mg daily for 5 days) to mice resulted in a fourfold increase in bone mass (Cornish J *et al.*, 2004). In a follow-up study, it was shown that the mitogenic response of lactoferrin in osteoblasts is mediated in part by binding and signaling through the low-density lipoprotein receptor-related protein-1 (LRP-1) (Oria R *et al,*. 1988). Interestingly, while LRP1 also mediates lactoferrin endocytosis into these cells, uptake of lactoferrin is not required for the mitogenic function of this protein, as lactoferrin promotes the growth of osteoblasts under conditions where endocytosis is abrogated.

Although the physiological relevance of these findings during normal bone development is unknown, these novel findings suggest that lactoferrin administration may have potential therapeutic implications for osteoporosis treatment (Grey A et al., 2004). Lactoferrin has also been demonstrated to have mitogenic effects on other cell types, including rat and human enterocytes (Hagiwara T et al., 1995), B and T lymphocytes (Mazurier J et al., 1989) and macrophages (Oria R et al,. 1988).

3.3.14Antiparasitic activity

Most of the studies on lactoferrin's antiparasitic activity have been performed in vitro, assaying molecular associations in the presence or absence of Fe3+. This activity has also been shown using peptides derived from the full molecule. Intestinal amoebiasis is caused by a protozoan infection and is one of the leading causes of diarrhoea in children under 5 years of age and the fourth leading cause of death in the world. The infection is caused by *Endameba histolytica*, which uses complex mechanisms to invade the intestinal mucosa and cause amoebic colitis (Gómez-Trejo *et al*., 2007). Apo-lactoferrin is the milk protein with the greatest amoebicidal effect against *E. histolytica* in vitro, as it can bind the lipids on the trophozoite's membrane causing membrane disruption and damage to the parasite (León-Sicairos *et al.,* 2006).Other in vitro studies show that human lactoferrin can bind the intracellular parasite *Toxoplasma gondii*, which causes toxoplasmosis and affects both humans and animals. However, lactoferrin cannot prevent the parasite from entering the host. Its mechanism of action in this case is inhibition of intracellular growth of *T. gondii* within host cells (Dzitko *et al*., 2007).In the case of the haemoparasites *Babesia caballi* and *Babesia equi*, lactoferrin's effect depends on whether or not it is bound to Fe3+ (Botteon *et al.,* 2002). *Babesia caballi* was found to be significantly suppressed by apo-lactoferrin but was not inhibited by the other types of lactoferrin; for *B. equi* none of the lactoferrin types showed an inhibitory effect (Ikada *et al*., 2005).

Chapter IV

CONCLUSIONS

- Lactoferrin was found to contain an antimicrobial sequence near its N-terminus which appears to function by a mechanism distinct from iron chelation. The antimicrobial sequence was found to consist mainly of a loop of 18 amino acid residues formed by a disulfide bond between cysteine residues 20 and 37 of human lactoferrin, or 19 and 36 of bovine lactoferrin. The identified domain contains a high proportionof basic residues, like various other antimicrobial peptides known to target microbial membranes and it appears to be located on the surface of the folded protein allowing its interaction with surface components of microbial cells.
- The isolated, "lactoferrin", was shown to have potent broad spectrum antimicrobial properties and its effect was lethal causing a rapidloss of colony-forming capability. Such evidence points to the conclusion that this domain isthe structural region responsible for the microbicidal properties of lactoferrin. The evidencealso suggests the possibility that active peptides produced by enzymatic digestion of lactoferrin may contribute to the host defense against microbial disease.
- The versatility of lactoferrin was the focus of this review. The advantages of this natural molecule prove its potential as a natural therapeutic agent that can be used in various fields of research including cancer. Since lactoferrin has been shown anti-bacterial and anti-fungal agent, it would be beneficial to use it in lotions and creams as a bactericidal and fungicidal agent. Its use can be extended to topical applications as well. An interesting aspect of using lactoferrin as an anti-cancer agent by delivering it to the body in the form of ice-creams, tablets and oral supplements as a natural products have been researched upon. With its role in being able to combat deadly viruses like Hepatitis C virus (HCV) and Hepatitis B virus (HBV) also poses a need for its use as an anti-viral agent for human immunodeficiency virus (HIV) and other potent viruses that cause health risks.

Chapter V

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