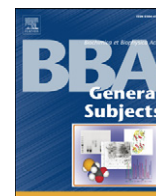




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## Review

Lactoferrin a multiple bioactive protein: An overview <sup>☆</sup>Isui Abril García-Montoya <sup>1</sup>, Tania Siqueiros Cendón <sup>1</sup>, Sigifredo Arévalo-Gallegos <sup>1</sup>, Quintín Rascón-Cruz <sup>\*</sup>

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## ABSTRACT

**Background:** Lactoferrin (Lf) is an 80 kDa iron-binding glycoprotein of the transferrin family. It is abundant in milk and in most biological fluids and is a cell-secreted molecule that bridges innate and adaptive immune function in mammals. Its protective effects range from anticancer, anti-inflammatory and immune modulator activities to antimicrobial activities against a large number of microorganisms. This wide range of activities is made possible by mechanisms of action involving not only the capacity of Lf to bind iron but also interactions of Lf with molecular and cellular components of both hosts and pathogens.

**Scope of review:** This review summarizes the activities of Lf, its regulation and potential applications.

**Major conclusions:** The extensive uses of Lf in the treatment of various infectious diseases in animals and humans has been the driving force in Lf research however, a lot of work is required to obtain a better understanding of its activity.

**General significance:** The large potential applications of Lf have led scientists to develop this nutraceutical protein for use in feed, food and pharmaceutical applications. This article is part of a Special Issue entitled Molecular Mechanisms of Iron Transport and Disorders.

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## 1. Introduction

Lactoferrin (Lf), first reported almost 50 years ago [1–3], is a non-hemic iron-binding protein. It is a member of the transferrin family, along with serum transferrin, ovotransferrin, and melanotransferrin [4], all of which function in iron transport, and inhibitors of carbonic anhydrase, Saxyphillin, and Pacifastin are also members of this superfamily. Lf is produced by mucosal epithelial cells in various mammalian species including humans, cows, goats, horses, dogs, and rodents, and it is also produced by fish [5]. This multifunctional glycoprotein is found in mucosal secretions including tears, saliva, vaginal fluids, semen [6], nasal and bronchial secretions, bile, gastrointestinal fluids, urine [7], milk and colostrum [8]. The most abundant antimicrobial proteins include lysozyme, collectin [9,10] and lactoferrin. Lf possesses a greater iron-binding affinity, and it is the only transferrin with the ability to retain this metal over a wide range of pH values, including resistance to proteolysis. The most striking physicochemical feature of Lf is its very high affinity for iron. In both Lf and related transferrins (Tfs), two Fe<sup>+3</sup> ions are bound very tightly (K<sub>d</sub> ~ 10<sup>22</sup> M) but reversibly to LF, with two synergistically bound CO<sub>3</sub><sup>2-</sup> ions [11,12]. Because of its wide distribution in various tissues, Lf is a highly multifunctional protein. Indeed, it is involved in many physiological

functions, including regulation of iron absorption and immune responses; it also exhibits antioxidant activity and has both anticarcinogenic and anti-inflammatory properties. However, its antimicrobial properties are its most widely studied function [8,12–16]. The antimicrobial activity of Lf is driven mostly by two mechanisms. The first involves iron sequestration in sites of infection, which deprives microorganisms of this nutrient and causes a bacteriostatic effect. The second mechanism is the direct interaction of the Lf molecule with the infectious microorganism. In some cases, positively charged amino acids in Lf can interact with anionic molecules on certain bacterial, viral, fungal and parasite surfaces, causing cell lysis. The physiological capabilities of Lf in host defense combined with current pharmaceutical and nutritional needs have led to the classification of Lf as a nutraceutical protein, and for several decades, investigators have looked for the most convenient way to produce it. Three basic approaches are currently being used. First, native Lf can be commercially purified from the milk and colostrum of several mammals. Second, recombinant Lf (rLf) can be generated from bacterial, fungal and viral expression systems. Third, transgenic plants and animals have been generated with express rLf.

## 2. Characteristics of the Lf sequence

The nucleotide sequence of human milk Lf (hLf) was first determined by Ray et al. in 1990 [18] and compared with the amino acid sequence of human lactotransferrin determined previously [17]. Lf genes are highly conserved among species, with an almost identical organization and an mRNA of about 1900–2600 bp. A homology search in sequence databases revealed nucleotide sequences for the Lfs of 13 species: 3

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primates, 7 even-toed ungulates, 1 pig, 1 cat, and 1 mouse. Pairwise sequence identities ranged from a minimum of ~78% to nearly 100%. The main outliers in this group were Lfs from primates, in which human and chimpanzee are quite similar (95–98%) when compared to orangutan (79%). On the other hand, horse and camel; pig, cow, yak, water buffalo, bull, and goat Lfs share 81 and 78% of sequence identity respectively when compared to human Lf as outgroup. The sequence relationships given above show that the Lfs form a highly conserved sequence family, and also sequence identity between Lfs and other Tfs is relatively high at 60–65% [19]. A characteristic feature of Lfs is their highly basic character, with a pI typically greater than 9; this property is typically predictable from their sequence. Structurally, the feature that most readily distinguishes Lfs from Tfs is the peptide linker between the two lobes, thought to have evolved from an ancient duplication event [20], which also contains several proline residues.

### 3. Molecular structure

Lf is an 80 kDa glycosylated protein of ~700 amino acids, with a high homology among species. It is comprised of a simple polypeptide chain folded into two symmetrical lobes (the N-lobe and C-lobe), which are highly homologous with one another (33–41% homology). The two lobes are connected via a hinge region containing parts of an  $\alpha$ -helix between amino acids 333 and 343 in human Lf (hLf) [7], which confers flexibility to the molecule [6,7,21]. The polypeptide chain includes amino acids 1–332, comprising the N-lobe, and 344–703, comprising the C-lobe, and it is made up of  $\alpha$ -helix and  $\beta$ -pleated sheet structures that create two domains within each lobe (domains I and II) [22]. Each lobe can bind a metal atom in synergy with the carbonate ion ( $\text{CO}_3^{2-}$ ). Lf is capable of binding  $\text{Fe}^{+2}$  or  $\text{Fe}^{+3}$  ions, but it has also been observed to be bound to  $\text{Cu}^{+2}$ ,  $\text{Zn}^{+2}$  and  $\text{Mn}^{+2}$  ions [23]. Because of its ability to reversibly bind  $\text{Fe}^{+3}$ , Lf can exist free of  $\text{Fe}^{+3}$  (apo-Lf) or associated with  $\text{Fe}^{+3}$  (holo-Lf), and it has a different three-dimensional conformation depending on whether or not it is bound to  $\text{Fe}^{+3}$  [19].

Apo-Lf has an open conformation, while holo-Lf is a closed molecule with greater resistance to proteolysis [7]. Because of the common structural framework among Lfs, it is possible to model their conformations using crystallographic data from other Lf species (Fig. 1a). The amino acids directly involved at the iron-binding site in each lobe are Asp60, Tyr92, Tyr192 and His253, while Arg121 is involved in binding the  $\text{CO}_3^{2-}$  ion (Fig. 1b). Lf is a basic, positively charged protein with a pI of 8.0–8.5. The primary structure of Lf shows the number and position of Cys residues that allow the formation of intramolecular

disulfide bridges; Asn residues in the N- and C-terminal lobes provide several potential N-glycosylation sites [25,26].

### 4. Antimicrobial activities of lactoferrin

Several functions have been attributed to Lf. It is considered to be a key component of the innate host defense system because it can respond to a variety of physiological and environmental changes [27]. The structural features of Lf provide additional functionalities beyond the  $\text{Fe}^{+3}$  homeostasis function common to all transferrins. Specifically, Lf exhibits strong antimicrobial activity against a broad spectrum of bacteria (Gram+ and Gram-), fungi, yeasts, viruses [14] and parasites [29], although it seems to promote the growth of beneficial bacteria like *Lactobacillus* and *Bifidobacteria* [28]. It also exhibits anti-inflammatory and anticarcinogenic activities [27] and has several enzymatic functions [30]. Lf plays a key role in maintaining cellular iron levels in the body. One of the first antimicrobial properties discovered for Lf was its role in sequestering iron from bacterial pathogens. This was believed to be the sole antimicrobial action of lactoferrin because apo-lactoferrin possessed antibacterial activity [31,32]. It was later demonstrated that lactoferrin can also kill microorganisms through an iron-independent mechanism [33] in which lactoferrin directly interacts with the bacterial cell surface [31,34,35].

#### 4.1. Antibacterial activity

The antibacterial activity of Lf has been documented in the past, both *in vitro* and *in vivo* for Gram-positive and Gram-negative bacteria and some acid-alcohol resistant bacteria. Table 1 shows the bacteria against which Lf has shown an inhibitory effect and the mechanism used by Lf to exert its effect. Particular attention should be given to bacteria listed in Table 1 because some of these are known to be resistant to antimicrobials, such as the strains of *Staphylococcus aureus*, *Listeria monocytogenes*, methicillin-resistant *Klebsiella pneumoniae* and *Mycobacterium tuberculosis*, among others. Lf has also been proven effective against strains of *Haemophilus influenzae* and *Streptococcus mutans* that were inhibited by an iron-independent interaction with the cell surface [39,45].

The sequestration of iron away from bacterial pathogens inhibits bacterial growth, limits the use of this nutrient by bacteria at the infection site and downregulates the expression of their virulence factors [60,65]. Lf's bactericidal function has been attributed to its direct interaction with bacterial surfaces. In 1988, it was shown that Lf damages the external membrane of Gram-negative bacteria through

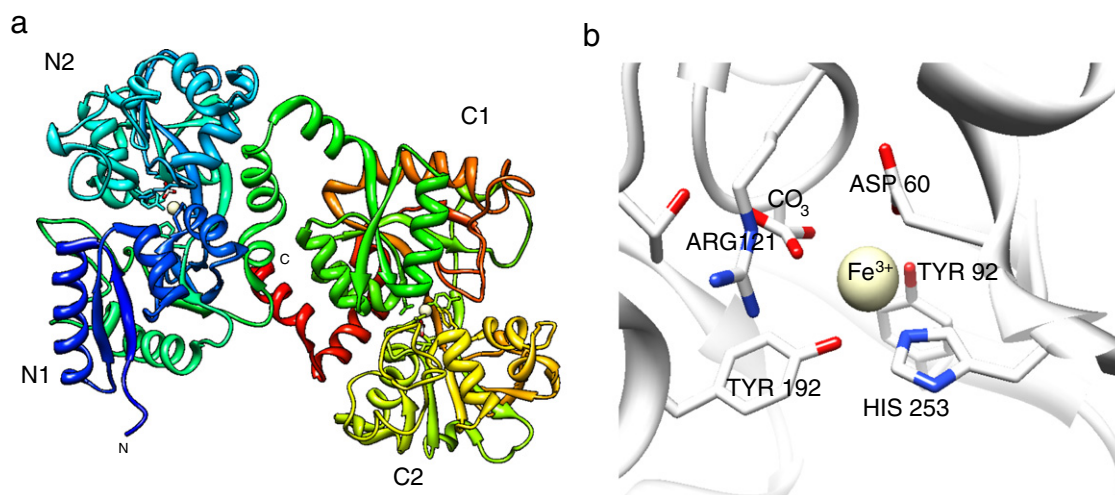


Fig. 1. Predicted structure of lactoferrin. a) From EU812318 (bLf) sequence using PDB ID: 1BIY (buffalo) as a template, showing two-lobe, four-domain polypeptide. b) Canonical iron-binding pocket site of lactoferrin.  $\text{Fe}^{+3}$  (cream)  $\text{CO}_3$  (gray and red). Modeled using Protein Model Portal [24], and viewed using Chimera software (<http://www.cgl.ucsf.edu/chimera/>).

**Table 1**  
Bacteria against which Lf has a reported effect.

	Target	Mode of action	Reference	
Gram-positive	<i>Bacillus stearothermophilus</i>	Iron sequestering	[36,37]	
	<i>Bacillus subtilis</i>	Iron-independent interaction with bacterial cell surface	[38]	
	<i>Klebsiella pneumoniae</i>	Iron-independent interaction with cell surface	[39]	
	<i>Listeria monocytogenes</i>	Altering bacteria virulence	[40,41]	
	<i>Staphylococcus aureus</i>	Iron sequestering	[42,43]	
	<i>Streptococcus mutans</i>	Iron-independent interaction with cell surface	[44–46]	
	<i>Streptococcus parasanguinis</i>	Altering bacterial growth	[47]	
	<i>Actinobacillus</i>	Proteolytic activity	[48]	
	<i>S. epidermidis</i>	Interaction with lipoteichoic acid on bacterial surface	[48]	
	<i>S. epidermidis</i>	Prevents biofilm formation—through iron sequestering	[48]	
	Gram-negative	<i>Chlamydomphila psittaci</i>	Interferes with cell adhesion	[50]
		<i>Haemophilus influenzae</i>	Altering bacteria virulence—degrading IgA1 and Hap	[39]
		<i>E. coli</i> enteropathogenic (EPEC)	Proteolytic activity of invasive mechanism	[49]
		<i>E. coli</i> enteropathogenic (EAEC)	Inhibit adherence of diffusely adherent	[51]
<i>E. coli</i> (DAEC)		Inhibit aggregative proliferation	[51]	
<i>Helicobacter felis</i>		Prevention of matrix interaction cell-host	[52]	
<i>Helicobacter pylori</i>		Iron-independent mechanism of inhibition	[53,54]	
<i>Legionella pneumophila</i>		Prevent intracellular proliferation	[55]	
<i>Pseudomonas aeruginosa</i>		Prevents biofilm formation	[56–58]	
<i>Shigella flexneri</i>		Disrupt bacterial type III secretion system	[59]	
<i>Vibrio cholerae</i>		Iron-independent mechanism of inhibition	[69]	
<i>Mycobacterium tuberculosis</i>		Augment cellular immunity	[61,62]	
<i>Mycoplasma bovis</i>		Prevents biofilm formation	[57]	
<i>Porphyromonas gingivalis</i>		Disrupt biofilm formation—cell membrane permeabilization	[63]	
<i>Samonella enteritidis</i>	Interferes with polysaccharide cell content	[64]		

an interaction with lipopolysaccharide (LPS) [66]. The positively charged N-terminus of Lf prevents the interaction between LPS and bacterial cations ( $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$ ) [67,68] and interferes with aggregative proliferation in *E. coli* [52]. The interaction between Lf and LPS or other surface proteins also potentiates the action of natural antibacterials such as lysozyme, which is secreted from the mucosa at elevated concentrations along with Lf [66]. It has also been demonstrated that the N-terminal lobe of Lf possesses a serine protease-like activity [39]. In *H. influenzae*, Lf is able to cleave proteins in arginine-rich regions, and the protease active site is situated in the N-terminal lobe, thus attenuating virulence and preventing colonization [69]. *In vitro* and *in vivo* studies have shown that Lf has the ability to prevent the attachment of certain bacteria to the host cell. The attachment-inhibiting mechanisms are unknown, but it has been suggested that Lf's oligomannoside glycans bind bacterial adhesins, preventing their interaction with host cell receptors [48,63]. Biofilm formation, which was proposed as a colonial organization adhesion method for *Pseudomonas aeruginosa*, is a well-studied phenomenon in patients suffering from cystic fibrosis. Through biofilm formation, bacteria become highly resistant to host cell defense mechanisms and antibiotic treatment [70–72]. It is well known that some bacterial strains require high levels of iron to form biofilms. Thus, Lf's function as an iron chelator has been hypothesized to effectively inhibit biofilm formation through iron sequestration [73,74].

#### 4.2. Antiviral activity

Lf possesses antiviral activity against a broad range of RNA and DNA viruses that infect humans and animals [6]. Initial work suggested that only enveloped viruses were affected by Lf, and that this activity was due to several factors, including inhibition of virus–host interaction in the herpes simplex virus (HSV) [75,76]; inhibition of intracellular virus trafficking in the hepatitis B virus (HBV) [77–79] and human cytomegalovirus (HCMV) [80,81]; or direct binding of lactoferrin to the viral particle in the hepatitis C virus (HCV) [82,83], feline herpes virus (FHV-1) [16], and hepatitis G virus (HGV) [84]. The human immunodeficiency virus (HIV) remains a major medical challenge because current treatments of the syndrome that it causes are not completely effective. *In vitro* studies show that, among human plasma and milk proteins, Lf exerts a strong activity against HIV. This effect is

due to inhibition of viral replication in the host cell [39,85,86]. Lf also binds to three of the many co-receptors of HIV [87] and the DC-SIGN receptor [88]. The interaction of Lf with surface nucleolin was shown to block the attachment and entry of HIV particles into HeLa P4 cells [87].

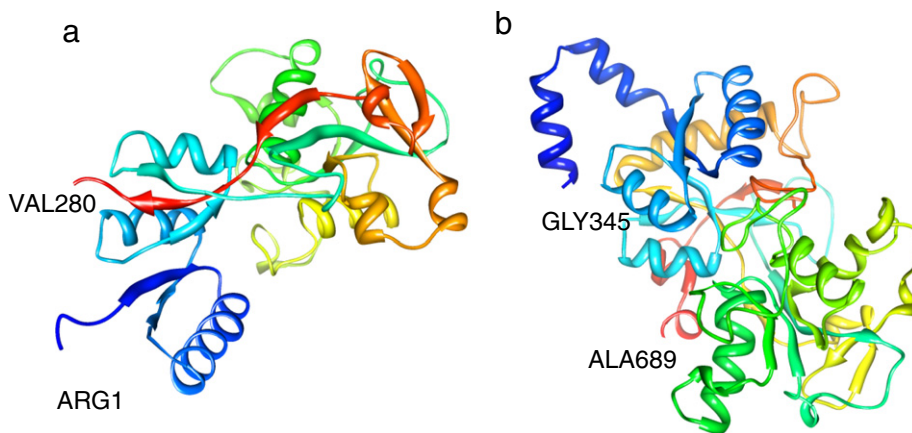
Fig. 2 illustrates structural in situ models of the C- and N-lobes. Interestingly, two large fragments of bLf, the C-lobe (aa 345–689) and the N-lobe (aa 1–280), have been shown to inhibit HSV infection [89]. The N-lobe the peptides 222–230 (ADRDQYELL) and 264–269 (EDLIWK) were also able to inhibit HSV-1 infection when chemically synthesized, albeit only when they were assayed together [89]. Additionally, smaller antibacterial peptides such as bLficin interfere with HSV-1 cell-to-cell movement [77].

Human respiratory syncytial virus (RSV) is inhibited by Lf at concentrations ten times lower than those found in human milk. Lf also acts against non-enveloped viruses such as adenoviruses and enteroviruses [90]. Several mechanisms of action have been proposed for the antiviral activity of Lf. One of the most widely accepted hypotheses is that Lf binds to and blocks glycosaminoglycan viral receptors, especially heparan sulfate (HS). The binding of Lf and HS prevents the first contact between virus and host cell, thus preventing infection [6]. The antiviral effect of Lf has also been observed in viruses that infect animals, such as the Friend virus complex, which causes erythroleukemia in rodents [92], the feline calicivirus [93], and the feline immunodeficiency virus [16]. It appears that Lf exhibits its antiviral activity at an early phase of the infection process [16,75,78,83,84,91,94–97]. Several viral pathogens have been shown to use the host cell surface as herpes simplex virus, as an attachment receptor during the infection process [98,99]. Results, also suggest that the GRRRRS sequence at the amino terminus of human lactoferrin acts synergistically with an RKVR sequence at positions 28–31 to form the predominate functional GAG-binding site of human lactoferrin [100].

#### 4.3. Antifungal activity

*Candida* can colonize mucosal surfaces in individuals, and it is considered to be analogous to a commensal organism that can also become an opportunistic pathogen. Kirkpatrick et al. conducted the first studies with *Candida* spp. in 1971 and attributed the antifungal effect of Lf to its ability to sequester  $\text{Fe}^{+3}$  [101,102]. Both hLf and bLf, as well as





**Fig. 2.** Predicted structure of antiviral-active peptides using PDB ID: 1FCK as template. a) From N-lobe 1–280 aa. b) From C-lobe 345–689 aa. Modeled using DeepView software [24], and viewed using Chimera software (<http://www.cgl.ucsf.edu/chimera/>).

the bLf-derived peptide lactoferricin, have well-documented *in vitro* activity toward human pathogenic fungi, especially *Candida albicans* and several other *Candida* species. Lf also has antifungal activity [103,104], and it was observed that Lf could kill both *C. albicans* and *C. krusei* by altering the permeability of the cell surface, as it does in bacteria [105,106].

Bovine Lf has been shown to be highly fungicidal for *C. tropicalis* and *C. krusei* and somewhat fungicidal for *C. albicans* and *C. guilliermondii*, while *C. glabrata* is almost resistant to Lf [107]. Lf exhibited activity against *Cryptococcus neoformans* and *C. albicans* via cytoplasmic and mitochondrial membrane permeabilization [108]. Although Lf's antifungal mechanism of action is through cell surface interaction rather than iron deprivation [109], several reports demonstrated its ability to cause cell wall damage [107,110,111]. In addition to direct interaction with the pathogen,  $Fe^{+3}$  sequestration is another important mechanism for fungicidal activity. In 2007, Zarembek et al. showed that  $Fe^{+3}$  sequestration by neutrophil apo-Lf is important for host defense against *Aspergillus fumigatus* [112]. Additionally, the *in vitro* antifungal activity of two peptides (hLF(1–11 aa), bLf N1-domain) derived from human lactoferrin (hLF) were compared, and dose-dependent antifungal activity was observed [113,114]. Lf shows an interesting antifungal effect on body tinea caused by *Trichophyton mentagrophytes*, against which it acts indirectly, facilitating clinical improvement of skin lesions after the peak of the symptoms. Treatment of guinea pigs with bLf reduces fungal infection on the skin of the back and limbs in tinea corporis and tinea pedis, respectively [115]. It has also been demonstrated that Lf can mediate its antifungal activity through the stimulation of host cell immune mechanisms both *in vitro* and *in vivo* [102].

#### 4.4. Antiparasitic activity

A clear understanding of the antimicrobial activities of Lf has been difficult to achieve because the mechanisms of action of Lf and the ecological niches of microbes often differ from one organism to another. The molecular mechanisms of Lf antiparasitic activity are even more complex.

Antiparasitic activities of Lf usually involve interference with iron acquisition. This activity has also been shown using peptides derived from the full molecule [116,117]. There is also evidence supporting the occurrence of a similar mechanism during amoebiasis, which is one of the leading causes of diarrhea in children under 5 years of age and is caused by *Entamoeba histolytica* [118].

Apo-Lf is the milk protein with the greatest amoebicidal effect against *E. histolytica in vitro* because it can bind to lipids on the trophozoite's membrane, causing membrane disruption and damage to the parasite [119,120]. Lf appears to act as a specific iron donor and could be expected to enhance infection by other parasites such as

*Trichomonas foetus* [121]. It was reported that bLF bound to components of *T. brucei*, and that bLF hydrolysate disrupted the sites responsible for binding to parasite proteins, causing  $Fe^{+3}$  deprivation [122]. Other *in vitro* studies show that serum transferrin as well as human and bovine Lf can bind the intracellular parasite *Toxoplasma gondii*, which causes toxoplasmosis and affects both humans and animals. However, Lf cannot prevent the parasite from entering the host. The mechanism of action in this case is inhibition of the intracellular growth of *T. gondii* in the host cells [123]. In animal models, a lactoferricin reduced infectivity of *T. gondii* and *Eimeria stiedai* sporozoites [124]. The effect of Lf on the hemoparasites *Babesia caballi* and *Babesia equi* depends on whether or not Lf is bound to  $Fe^{+3}$  [125]. *B. caballi* was found to be significantly suppressed by apo-Lf but was not inhibited by the other types of Lf, whereas none of the Lf types had an inhibitory effect against *B. equi* [126]. Lf also demonstrates additive or synergistic activity with clinically used antiparasitic compounds [116,118,119].

#### 5. Immunomodulatory and anti-inflammatory activity

Lf displays immunological properties that influence both innate and acquired immunities [127]. Its relationship with the immune system is evident from the fact that people with congenital or acquired Lf deficiency have recurring infections [128]. Oral administration of bLf seems to influence mucosal and systemic immune responses in mice [129]. Lf can modulate both specific and non-specific expression of antimicrobial proteins, pattern recognition receptors and lymphocyte movement related proteins [130]. The role that Lf plays in regulating innate immune responses confirms its importance as a first line host defense mechanism against invading pathogens, modulating both acute and chronic inflammation [131–134]. Most intriguing is the ability of Lf to induce mediators from innate immune cells that subsequently impact adaptive immune cell function. Lf's positive charge allows it to bind to negatively charged molecules on the surface of various cells of the immune system [135], and it has been suggested that this association can trigger signaling pathways that lead to cellular responses such as activation, differentiation and proliferation. Lf can be transported into the nucleus, where it can bind DNA [86,136] and activate different signaling pathways [7].

In addition to inducing systemic immunity, Lf can promote skin immunity and inhibit allergic responses. It activates the immune system against skin allergens, causing dose-dependent inhibition of Langerhans cell migration and the accumulation of dendritic cells in lymph nodes [6]. Leukocytes exposed to Lf modulate their cytokine production; proinflammatory cytokines, TNF- $\alpha$ , IL-6, and IL-1 $\beta$  can be modulated by Lf to increase [137–139] or decrease [131,139–141]. Production of these factors is dependent upon the type of signal recognized by the immune

system. At the cellular level, Lf increases the number of natural killer (NK) and adaptative (T strain CD4+ and CD8+) cells [142], boosts the recruitment of polymorphonuclear cells (PMNs) in the blood [143], induces phagocytosis [144], and can modulate the myelopoietic process [145]. It is well documented that IL-12 plays an important role in driving development of helper T-cell type 1 immunity [146,147]. Therefore, the role of Lf in the regulation of proinflammatory cytokines and IL-12 clearly demonstrates communication between innate and adaptative immune responses.

## 6. Anticarcinogenic activity

The anti-tumor properties of Lf were discovered about a decade ago and have been confirmed by numerous laboratory studies which have shown that bovine lactoferrin (bLf) significantly reduces chemically induced tumorigenesis when administered orally to rodents [148]. Since then, human clinical studies are showing that ingestion of LF can have a beneficial effect against progression of cancer [150]. bLf possesses antimetastatic effects and inhibits the growth of transplanted tumors [149,151]. Similar to its role in inflammation, Lf has the ability to modulate the production of cytokines in cancer. Lf can induce apoptosis and arrest tumor growth *in vitro*; it can also block the transition from G1 to S in the cell cycle of malignant cells [7,152]. Additionally, treatment of tumors in mice with recombinant human Lf (rhLf) inhibits their growth, increases the levels of anticarcinogenic cytokines such as IL-18, and activates NK cells and CD8+ T lymphocytes [153,154]. Interestingly, bLf and hLf exert opposite effects on angiogenesis. Whereas orally administrated bLf inhibits angiogenesis in rats [155,156] and tumor-induced angiogenesis in mice [154], hLf exerts a specific pro-angiogenic effect [157]. Recently, colorectal cancer was inhibited by bLf in animal models, and human Lf reduced the risk of colon carcinogenesis as demonstrated by a clinical trial [158]. Increasing evidence suggests that inhibition of the Akt signaling pathway might be a promising strategy for cancer treatment [159]. In breast cancer, Lf is able to limit the growth of tumor cells, and addition of exogenous Lf to the culture media of breast cancer cell lines (MDA-MB-231) induced cell cycle arrest at the G1/S transition [160]. Additionally, Lf induced growth arrest and nuclear accumulation of Smad-2 in HeLa cells [161]. The ability of bovine Lf to induce apoptosis in THP-1 human monocytic leukemic cells has also been demonstrated [162]. Although the results achieved by several researchers point to a clear anti-tumor role for Lf, the mechanisms by which it exerts these effects are not fully understood. Thus, further work on this subject is required.

## 7. Enzymatic activity

Lf has the ability to function as an enzyme in some catalytic reactions. A remarkable similarity of certain motifs between Lf and

ribonuclease A has been observed [163]. Lf has DNA binding properties [164] and can act in transcriptional activation of specific DNA sequences [165] or as a mediator of signal transduction [166]. Lf has the highest levels of amylase and ATPase activities of all the milk proteins [167]; however, these are not its only enzymatic activities. Indeed, Lf exhibits a wide variety of activities, which can be attributed to variations in the nature of its protein characteristics; Lf has multiple isoforms, degrees of glycosylation, variations in tertiary structure (holo- or apo-Lf) and degrees of oligomerization [168,169]. The discovery of Lf's enzymatic properties has helped to elucidate its many physiological functions.

## 8. Bioactive peptides derived from lactoferrin

Lactoferrin was first isolated by Groves in 1960 and was recognized as a "red protein from milk" [2]. Moderate proteolysis led to a release of two Lf fragments, namely the N- and C-terminal lobes. Enzymatic treatment of bLf with pepsin produced a low molecular weight peptide with antibacterial properties against a large number of Gram-positive and Gram-negative bacteria, in addition to fungi. Bellamy et al. [170] identified a region of amino acids at the N-terminus that retains its biological activity when separated from the full molecule; this was termed lactoferricin (Lfc-B) and was shown to exhibit greater antimicrobial activity than Lf. The activity that is exerted by this region corresponds to residues 17–41 of bLf [171]. The region also includes two Cys residues linked by a disulfide bridge that contains many hydrophobic and positively charged residues.

The tertiary structure of Lfcin is markedly different when compared to its homologous region of intact Lf (Fig. 3a). An *in situ* model of hLfcin (Fig. 3a) and a solvated version (Fig. 3b) show structural differences at their N- and C-termini (Fig. 3a, b). A single  $\beta$ -sheet strand replaces the long  $\alpha$ -helix observed in the Lf structure; such a structure may be better suited to recognize bacterial membrane topology [172]. Additionally, the bLfcin peptide contains an alpha-helix (Fig. 3a–c). This region retains high homology among other species of mammals and corresponds to amino acids 12–48 [17].

It was found that minimal variations in the amino acid sequence change the antimicrobial activity of the peptide. For example, Lfampin 268–284 and Lfampin 265–284, chemically synthesized fragments from the N-terminal sequence of bLf, differ in only three amino acids (265Asp–Leu267–Ile268) but exhibit different strengths of antimicrobial activity [171]. Proteolysis of iron-free Lf could release Lf-derived active peptides in biological fluid [172].

## 9. Lactoferrin gene regulation

Lf has been identified in several tissues in both humans and animals. The Lf gene has been found at the chromosome level in a set

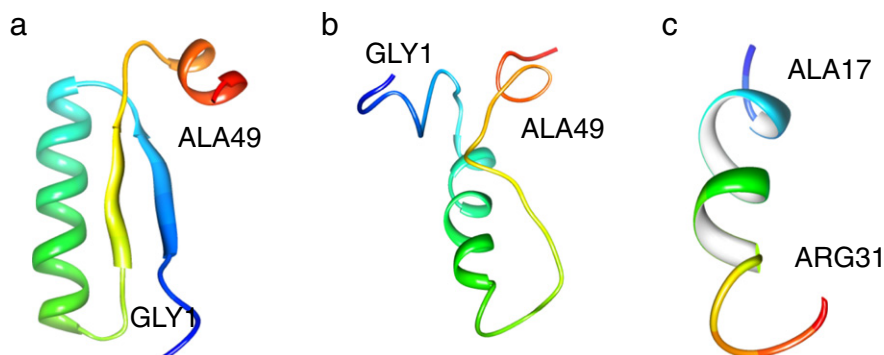


Fig. 3. Location of field and aqueous solvated structure comparison of the human and bovine lactoferricin. a) Domain of *in situ* hLfcin (residues 17–41; PDB ID: 1BLF) and, aqueous solvated b) hLfcin and c) bLfcin. Viewed using Chimera software (<http://www.cgl.ucsf.edu/chimera/>).

of different species; in humans it was found on chromosome 3, while in mice it was found on chromosome 9 [173], and its size ranged from 23 to 35 kb. Lf genes have extensive homology among species, with an identical organization in cows, pigs and mice (17 exons, 15 encoding Lf) [174,175]. The amount of Lf synthesized in the mammary gland is controlled by prolactin [176]. Its mRNA levels vary by tissue, suggesting tissue- or cell-specific regulation [177,178]. The Lf full coding regions of 60 different species were analyzed, and it was found that the length of the gene varies from species to species (from 2055 to 2190 residues) due to deletions, insertions and mutations in the stop codon [179]. Lf is expressed both constitutively and inducibly. It is constitutively expressed on mucosal surfaces, while in some tissues it is induced by external agents.

The Lf promoter contains an estrogen responsive element [REF], and is consequently positively regulated by estrogen. The chicken ovalbumin upstream promoter transcription factor (COUP-TF) binding element overlaps the ERE of the lactoferrin gene [180]. COUP-TF binds to this element and competitively inhibits binding of the estrogen receptor to the lactoferrin ERE, thereby inhibiting transcription of the lactoferrin gene. Another negative regulator of LF gene transcription is repressor of estrogen receptor activity (REA). The absence of REA increases the expression of estrogen-induced Lf by up to 100-fold [181].

Lf can also be induced by compounds other than estrogens, such as retinoic acid, which stimulates gene expression in embryonic cells [182]. Lf expression is upregulated by estrogen with a magnitude of response that is cell-type-specific, and it is also upregulated by retinoic acids. Transcription factors such as Ets, PU.1, C/EBP, CDP, and KLF5 also modulate Lf gene expression, mainly in myeloid cells [183]. Lf expression is also modulated by oxidative stress, in response to infection, or during the early steps of embryogenesis [184].

## 10. Clinical applications of lactoferrin

Lf can be isolated from cow's milk by various purification methods, or it can be expressed by recombinant methods [51,71,185]. Because of the multiple functions of Lf, it has been used for clinical trials and industrial applications. One of the first applications of Lf was in infant formula. Currently it is added to immune system-enhancing nutraceuticals, cosmetics, pet care supplements, drinks, fermented milks, chewing gums, and toothpaste. The ability of Lf to prevent nosocomial infection in infants was tested [186] and the results showed lower infection levels. Several studies showed that infants fed with infant formulas had less intestinal iron absorption than breastfed infants [187,188]. It was proposed that Lf also promotes the proliferation of lactic acid bacteria in the bowel, such as *Bifidobacterium* and *Lactobacillus*, which protect the host from harmful bacteria [186,189].

The activity of Lf and its bioactive peptides has been documented both *in vitro* and *in vivo* against a wide variety of pathogens. Clinical trials demonstrated the efficiency of Lf for use in treating infections and inflammatory diseases. For example, Lf was tested as a second treatment against *H. pylori* in patients with recurring infections. The patients supplemented with bLf showed a greater recovery from infection [190]. Lf also exhibits synergistic activity with antifungal agents, thus reducing the minimum inhibitory concentrations of these agents against *C. albicans* and *C. glabrata* [101,191]. As with antibacterials, Lf exhibits synergy with antiviral drugs in the treatment of hepatitis C, cytomegalovirus and HIV [6,79,192]. Lf delays the hypersensitivity response and limits the pathology caused by *M. tuberculosis* by increasing IL-12 and IL10 expression [193]. A clinical trial was conducted which demonstrated that ingestion of bLf could reduce the risk of colon cancer in humans [157]. Lf also offers applications in food preservation and safety because it can decrease bacterial counts in pork meat [194,195], retard lipid oxidation [196] and limit the growth of microbes.

Lf can also be used as a molecular marker; detection of urinary Lf via electrochemical immunosensors aids in the diagnosis of urinary tract infections [197]. Increased levels of Lf serve as a clinical marker of inflammatory Severe Acute Respiratory Syndrome or septicemia [198].

## 11. Production of native and recombinant lactoferrin

The development of commercial production strategies for the production of recombinant Lf as a safe, effective drug and nutraceutical protein is one of the major goals in both research and industry. Purification of Lf depends on the intrinsic properties and features of the molecule, such as its net positive charge, its ability to bind  $\text{Fe}^{+3}$  and its glycosylation state [6,199,200]. However, the need for larger amounts of Lf has led to the development of strategies to obtain a recombinant form of the protein (rLf).

In global biotechnology, there are now three major competing approaches for the production of rLf: production in transgenic animal milk, production in microscopic fungi, and production in plants. To date, several rLf expression systems have been developed (Table 2) that utilize both prokaryotic and eukaryotic organisms. The first expression systems utilized Bay Hamster Kidney Cells to express human lactoferrin [221].

The transformation of the filamentous fungi *Aspergillus awamory* allowed for the expression of both hLf and murine Lf (mLf) [215,216]. As shown in Table 2, expression systems were developed in yeasts, bacteria, insects and plants, which have produced human recombinant Lf (rhLf) [205,207,212,217–219,230–242], caprine Lf (cLf) [208], bovine Lf (bLf) [201,203,206,214], equine Lf (eLf) [211], porcine Lf (pLf) [209,220], yak Lf [210], and Kumin Lf [204], as well as Lf peptides, including Lfc [202,203,206,214], which reached expression levels of 0.1 mg/L in a plant model [231] and 1200 mg/L in *P. pastoris* [212]. Use of viral vectors has allowed for the expression of Lf by insect infection, either in cell culture or directly in the organism, where the expression of both hLf and pLf has been successful. This practice has resulted in the production of transgenic *Spodoptera frugiperda* and *Bombyx mori*, which express 205  $\mu\text{g}$  of pLf per infected pupa [220] and up to 65 mg/L in larvae [218]. Lf has also been expressed in higher eukaryotic organisms, including both plants and animals. Using microinjection and direct infection with viral vectors in the mammary gland, transgenic animals have been created that produce milk containing recombinant Lf. These animals include goats [223,224], mice [225,226], rabbits [227] and cows [228,229]. Levels of up to 2 g/L have been achieved in transgenic goat milk.

Molecular farming, which involves the utilization of plants as bioreactors, is well depicted as a tool for the production of valuable therapeutic and industrial proteins. This process is advantageous because of the lack of contamination from human or animal pathogens and endotoxins in the final product. Furthermore, higher plants are able to synthesize proteins with the proper folding, glycosylation and functional activity, and plant cells can direct the protein of interest to environments that reduce its degradation. hLf was expressed in plant expression models (Table 2) and bLf was only expressed in tobacco plants [235]. However, none of these nuclear transformation methods are able to promote the accumulation of a large amount of rLf. Therefore, new promoters or regulatory elements as well as other transformation methods should be tested to increase accumulation and stability of recombinant proteins. The expression of Lf in plant models could significantly improve crop quality by increasing its resistance to some diseases; additionally, it provides a source for high quality Lf protein.

## 12. Concluding remarks

Lf is a multifunctional protein that takes part in a large number of important physiological processes. The beneficial effect of Lf in the treatment of various infectious diseases caused by bacteria,



**Table 2**  
Expression of recombinant Lf by various transgenic organisms.

Expression system	Lf origen	Expression level	Year/reference
<b>Bacteria</b>			
<i>E. coli</i>	blfc	10 mg/L	2007 [201]
	Lfc	60 mg/L	2006 [202]
	blfc	2 mg/L	2006 [203]
	Kumin Lf	17 mg/L	2010 [204]
<i>Lactobacillus casei</i>	hLf	10.6 mg/mL	2010 [205]
<i>Rhodococcus erythropolis</i>	blf C-lobe	3.6 mg/mL	2006 [206]
<b>Yeast</b>			
<i>Pichia pastoris</i>	hLf	115 mg/L	2004 [207]
	clf	2 mg/L	2007 [208]
	plf	12 mg/L	2002 [209]
	Yak Lf	40 mg/L	2006 [210]
	eLf	40 mg/L	2002 [211]
	hLf	1200 mg/L	2008 [212]
<i>Pichia methanolica</i>	plf	NR	2007 [213]
	blfc	90 mg/L	2007 [214]
<b>Fungi</b>			
<i>Aspergillus awamori</i>	hLf	2 g/L	1995 [215]
<i>Aspergillus oryzae</i>	hLf	25 mg/L	1992 [216]
<b>Insects</b>			
<i>Spodoptera frugiperda</i>	hLf	9.5 mg/L	1998 [217]
<i>Bombyx mori</i>	hLf	65 mg/L	2005 [218]
	hlf	13.5 µg/1–2 × 10 <sup>5</sup> cells	2006 [219]
	plf	205 µg/pupa	2005 [220]
<b>Cell lines</b>			
Baby hamster kidney (BHK)	hLf	20 mg/L	1991 [221]
Cell culture (human embryo kidney)	hLf	16 mg/L	2009 [222]
<b>Mammals</b>			
Goat	hlf	0.756 mg/L	2008 [223]
	hlf	2 g/L	2007 [224]
	hlf	2.5 mg/mL	1997 [225]
Mice	hlf	2.5 mg–200 µg/mL milk	1997 [226]
	hlf	2.3 mg/mL milk	2008 [227]
Rabbit	hLf	1 g/L milk	2002 [228]
Bovine	hlf	2.9 mg/mL	2006 [229]
<b>Plants</b>			
<i>Nicotiana benthamiana</i>	hLf N-lobe	0.6% soluble protein	2004 [230]
<i>Nicotiana tabacum</i>	hLf	NR	1994 [231]
	hLf	0.1–0.8% soluble protein	1998 [232]
	hLf	4.3% soluble protein	2003 [233]
Rice	blf	NR	2011 [234]
	hLf	1.6 mg/g seed	2004 [235]
	hlf	0.5% total biomass	2005 [236]
	hLf	2–4% soluble protein	2003 [237]
	hLf	0.1% soluble protein	2010 [238]
Potato	hLf	0.1% soluble protein	2000 [239]
Sweet potato ( <i>Ipomoea batata</i> )	hLf	3.2 µg/mg total protein	2006 [240]
	hLf	3.0% soluble protein	2003 [241]
<i>Panax ginseng</i>	hLf	NR	2002 [242]
Maize	hLf	NR	2001 [243]
Alfalfa ( <i>Medicago sativa</i> )	hLf	NR	2005 [244]
Barley	hLf	NR	2011 [245]

(h = human, b = bovine, c = caprine, p = porcine, e = equine, Lfc = lactoferricin, NR = not reported).

fungi, protozoa, and viruses in animals and humans is described above; nevertheless, much research and many experiments still need to be performed to obtain a better understanding of its activity. The usefulness of Lf has led scientists to develop this health-enhancing nutraceutical protein for use in food and pharmaceutical applications.

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