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Lactoferrin: A Powerful Antimicrobial Protein Present in Milk

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Abstract

Lactoferrin (Lf) is an iron-chelating glycoprotein present in milk and mucosal secretions, a component of the mammalian innate immune system. Lf is microbiostatic and microbicidal. Lf can reduce the bacterial expression of virulence factors, such as those involved in biofilm production and protease secretion. The high identity among mammalian Lf sequences facilitates its use in human and veterinary medicine. Lf of bovine origin is the principal Lf used due to its commercial availability through purification from milk whey; recombinant Lfs (bovine, human, and porcine) have been used as well. Lf is a stable protein that retains its physicochemical characteristics under gastric pH conditions, and in most cases it is bioactive even after digestion; thus, the incorporation of Lf into diets facilitates its administration to animals. The aim of this review is to examine original research in which the effects of bovine and porcine Lf on pathogens of domestic animals have been demonstrated through *in vitro* and *in vivo* assays, with the purpose of ascertaining the benefits that Lf provides in the treatment of infectious diseases.

Keywords: Lactoferrin; Lactoferricin; Nutraceutical; Pathogens; Antimicrobial

Abbreviations: bLf: Bovine Lactoferrin; bLfcin: Bovine Lactoferricin; BW: Body Weight; CFU: Colony Forming Unit; hLf: Human Lactoferrin; hLfcin: Human Lactoferricin; s.c: Subcutaneous; I.m.m.: Intramammary; I.p.: Intraperitoneal; I.v.: Intravenous; Lf: lactoferrin; bLfampin: bovine Lactoferrampin; Lfcin: Lactoferricin; LPS: Lipopolysaccharide; NF: Nanoformulation; MBC: Minimal Bactericidal Concentration; MIC: Minimal Inhibitory Concentration; MNV: Mouse Norovirus; FIV: Feline Immunodeficiency Virus; PBMC: Peripheral Blood Mononuclear Cells; HA: Hemagglutinin; WPC: Whey Protein Concentrate; P.I.: Post Infection; PFU: Plaque Forming Unit; pLf: Porcine Lactoferrin; pLFcin: Porcine Lactoferricin; r-pLf: Recombinant Porcine Lactoferrin; PRV: Porcine Pseudorabies Virus

Introduction

Animal health is disturbed by several types of pathogens, and sickness is a major constraint on efficient production of animal-derived foods in addition to causing suffering in livestock and pets. Antimicrobials remain vitally important for treating and/or preventing infections. The appropriate use of antibiotics may cure sick animals, speed their recovery, improve animal welfare, and reduce the risk of the infection spreading to non-immune animals or, in the case of zoonotic diseases, to humans [1]. Presently, the emergence of multi-resistant strains is a cause of concern in the medical field; thus, developing alternatives to antimicrobials for minimizing losses associated with infectious diseases is an evident need of the livestock industry [2].

Lactoferrin (Lf) is a therapeutic alternative against pathogens since it is a safe nutraceutical protein commercially available from milk whey, no resistance to it has been found, and it does not affect the microbiota. Thus, Lf could be used effectively in veterinary medicine as a substitute or adjunct therapy to antimicrobials in the treatment of infectious diseases. Lf is a mammalian cationic non-haem glycoprotein, 78-80 kDa in size, and is present in many body secretions such as those from the digestive, respiratory, and reproductive systems (Figure 1). Bovine Lf (bLf) is present at a high concentration in colostrum (2-5 mg/ml) and at a lower concentration in mature milk (0.1-0.3 mg/ml) [3,4]. Additionally, Lf is produced by the secondary granules of neutrophils, which release this protein at infection sites [5]. Lf is designated holoLf when bound with iron (mono- or diferric) and apoLf without iron. The Lf molecule is highly conserved among mammals (see multiple sequence aligment in https://www.ebi.ac.uk/Tools/msa/clustalo/).

Lf exerts a microbiostatic effect by chelating free Fe^{3+} in the fluids and mucosae. In addition, Lf is bactericidal, by binding to lipopolysaccharide (LPS), porins, and other outer membrane (OM) proteins in Gram-negative bacteria [6]; likewise, Lf can bind to teichoic acid in Gram-positive bacteria. All these actions cause disruption of the bacterial membrane, leading to cell lysis [7,8] bLf affects biofilm production [9-11], diminishes the release of toxins [12], and interferes with the adhesion of bacteria to host cells [9,13-16]. Moreover, Lf synergizes with antibiotics, potentiating their antimicrobial effect [17-19]. In viral diseases, bLf prevents infection by binding to the target [20]. An important feature of Lf is the production of cationic peptides called lactoferricins (Lfcins) derived from the cleaving of the N-terminal end by gastric pepsin; thus, Lfcins are produced when Lf is ingested from milk [21]. Several Lfcins have been assayed as antimicrobials; they are named according to the range of amino acids they contain. Synthetic Lfcins have been obtained from the N-terminus sequences of Lf. A chimaera peptide, obtained by fusing bovine Lfcin17-30 and lactoferrampin (Lfampin) 265-284, has also been tested as an antimicrobial [22].

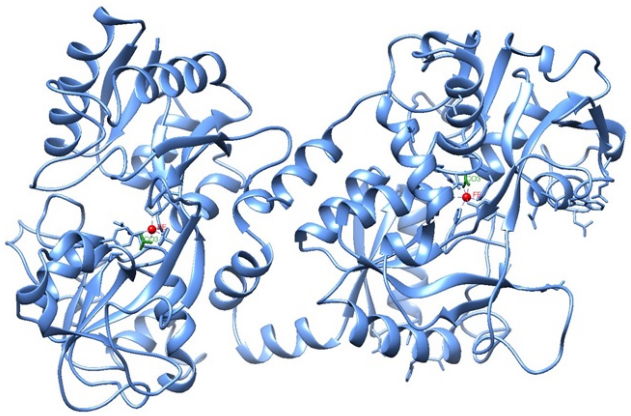

Figure 1: Tridimensional structure of bovine lactoferrin [PDB: 1BLF]. The bLf molecule is represented in blue ribbon diagram, the Fe atoms are represented in color red and the carbonate ions in color green.

Lf has been described as modulator of the immune system, particularly regulating the production of proinflammatory cytokines [23]. bLf has shown beneficial effects when tested in patients with cancer [24-26], and some studies have shown that Lf can promote wound healing and bone growth [5]. bLf showed an immunostimulant effect in calves [27,28], chickens [29], and fishes [30,31]. To explain the physiological effects of bLf, it has been analyzed its bodily distribution in rodents and pigs. In newborn pigs, both bLf and bLfcin were absorbed in the small intestine by enterocytes and travelled to the peripheral circulation [32]. Additionally, the transport of bLf through the blood-brain barrier and the blood-cerebrospinal fluid barrier in Wistar rats was demonstrated [33]. In this review, we discuss the results obtained with bLf and porcine Lf (pLf) in relation to several species of pathogens in assays performed *in vitro* and *in vivo*. The potential use of Lf as a tool for prevention and treatment of animal diseases is also analyzed.

Role of Bovine Lactoferrin (Blf) and Lactoferricins (Blfcins) in the Veterinary Field

In vitro assays

As a first approach to discovering the properties of bLf against pathogens in veterinary medicine, researchers conducted susceptibility tests *in vitro*, mainly using apobLf (Table 1). For example, both human and bovine Lf showed antibacterial effects against *Staphylococcus aureus*. Tests on agar plates showed that both apoLfs exhibited weak zones of inhibition, whereas holo forms were ineffective [34]. In dairy herd mastitis infection, *S. aureus* is an important pathogen in terms of economic losses to producers because of decreased milk production, costly pharmacologic treatments, medical veterinary fees and the discarding of milk due to the presence of pathogens or antibiotic residues [35]. The bactericidal and synergistic effects of bLf in combination with penicillin G on the growth of *S. aureus* was evaluated. Additionally, alterations in bacterial structure were observed with bLf, similar to those observed with high concentrations of penicillin G alone [36]. In addition, it was reported that apobLf could inhibit the growth of *S. aureus*; the results served as experimental evidence for further in vivo research [37].

Pathogen	Source of bLf/bLfcin and iron-saturation condition	Results	Reference
S. aureus 6538P	bLf and bLf hydrolysate from Morinaga Milk Company, 26% iron(1); [ ]* 5-20	-bLf without iron had a weak effect on viability, a maximum inhibition was obtained at 20 mg/ml	[34]
		- Saturated bLf potentiated bacterial growth	
S. aureus SHY97-3923, SHY97-3906, SHY97-4320, SHY97-4343(2); PC-1, NCTC 9789, 2076, 22260, ST79/741, 3804, RN9, FAR8 and FAR10(3)	bLf from Besnier, San Juan Capistrano (USA), bLfcin was obtained by enzymatic digestion; [ ]* 0.38-25	-MIC bLf ≤ 25 (μM)	[36]
		-MIC Lfcin=256 (μg/ml)	
		-bLf synergized with penicillin-G in all strains except SHY97-3906 and SHY97-4343	
		-Change in protein expression of culture incubated with bLf or bLf+penicillin-G	
E. coli, S. aureus, coagulase-negative staphylococci(4), P. aeruginosa and K. pneumoniae(5)	bLf purified from cheese whey (expanded bed absorption chromatography method), 4% iron; [ ]* 0.67, 1.67, 2.67	-Major inhibitory activity of bLf vs E. coli since 1.67 (mg/ml)	[37]
		-Three S. aureus isolates were susceptible to bLf at 0.67 (mg/ml)	
		-Bacteriostatic effect and concentration-dependent was observed at 16h	
V. parahaemolyticus 17802(6), O3:K6(7), 727(8); V. cholerae O1 and no-O1	bLf from DMV International (USA); bLfcin, bLfampin and bLfcin chimaera were prepared(9); bLf 20% iron; [ ]* 0.001,0.01, 0.02, 0.04	-bLf and bLf chimaera inhibited the V. parahaemolyticus growth in >50%; bLfcin and bLfampin in 10-15%	[38]
		-bLf decreased the V. cholerae growth in >90%	

		-$MgCl_2$ abolished the bLF chimera and bLf effect; ferric iron reduced the bLF effect	
		-bLF chimera synergized with ampicillin, mainly against V. parahaemolyticus	
A. pleuropneumoniae BC52, S4074 and WF83[10]	bLf from NutriScience (USA); bLf 4.1% iron; [ ]* 0.0625 to 1.25	-MIC bLf=10-15 (μM); 0.8 (μM) decreased 24-42% the bacterial adhesion of serotype 1 to SBEC	[19]
		-bLf decreased 27% the biofilm production of S4074[11] and suppressed proteolytic activity on porcine gelatin, in all strains	
		-bLf synergized with oxytetracycline against all strains	
M. haemolytica Serotype A1[12]	bLf from NutriScience (USA)	MIC bLf= 4.88-7.31 μM	[6]
	bLf 0.005 % iron; [ ]* 0 to 2		
(1): also apo and holo hLf (from Sigma) were used, they show similar effect that apo and holo bLf; (2): isolates from clinical bovine mastitis; (3): β-lactam antibiotic-resistant strains; (4): five isolates from each; (5): two isolates from each, isolated from subclinical or clinical bovine mastitis; (6): reference strain; (7): pandemic strain; (8): multidrug resistant strain; (9): as reported by Bolscher's group investigation; (10): reference strain S4074 and the isolate BC52 belong to serotype 1, the reference strain WF83 is of serotype 7; (11): with holo-bLf, the biofilm production decreased (60-70 %) in all strains; (12): Two strains were used (field isolated and reference strain) SBEC: swine buccal epithelial cells. [ ]*: concentration expressed as mg/ml			

Table 1: *In vitro* assays using apobLf and its N-terminal derivatives against veterinary pathogenic bacteria.

Recently, our research group demonstrated the bactericidal effect of bLf on *Actinobacillus pleuropneumoniae* and *Mannheimia haemolytica*, aetiological agents of porcine pleuropneumonia and bovine mannheimiosis, respectively. The ability of bLf to reduce some bacterial virulence factors, such as those promoting adhesion to swine buccal epithelial cells and activity of secreted proteases in *A. pleuropneumoniae*, was demonstrated. In the case of *M. haemolytica*, two bLf binding proteins were described [6,19]. In respect to synthetic cationic peptides derived from bLf, they were tested against the pathogenic foodborne bacteria *Vibrio parahaemolyticus* and *Vibrio cholerae*; a significant decrease in bacterial growth was observed when bLf or bLfcin chimaera was used. Moreover, bLfcin chimaera showed a synergistic effect with ampicillin, principally against a multidrug-resistant strain of *V. parahaemolyticus* (Figure 2) [38].

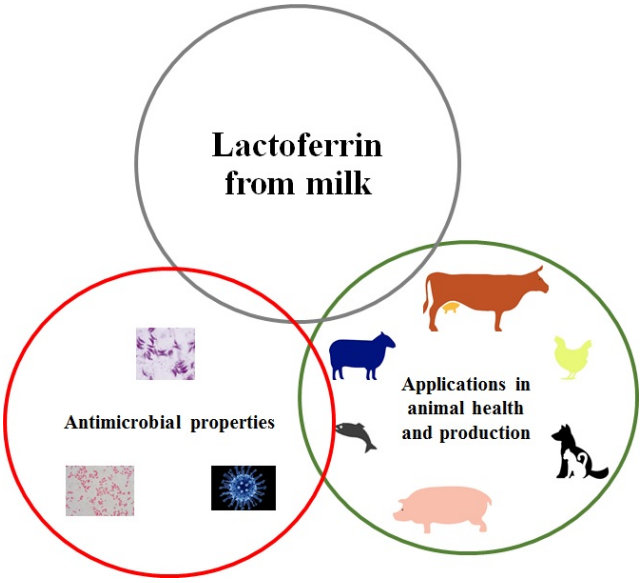

Figure 2: Overview of the bLf functions and potential in veterinary medicine.

Studies on the use of bLf on human parasitic protozoa have demonstrated its harmful effect on the parasites *in vitro*; therefore, the use of bLf could be extended to zoonotic parasites; a review of this field has been published elsewhere [39]. As is shown in Table 2, bLf and some of its derivatives, obtained by enzymatic digestion or synthetized, have been tested. A study proved the that bLfcin reduced the infectivity of *Toxoplasma gondii* and *Eimeria stiedai* when the sporozoites were preincubated with bLfcin, and penetration of mouse embryonal and rabbit hepatobiliary cells was decreased [40-42]. In domestic animals, the *T. gondii* infection can be asymptomatic depending on the parasite strain and host immune status; one of the clear clinical signs is abortion, especially in sheep [43]. On the other hand, *E. stiedai* inhabits epithelial cells of the bile ducts in rabbits, and its transmission is through the ingestion of sporulated oocysts [44].

Pathogen	bLf/bLfcin features	Results	Reference
T. gondii RH and S-273	bLfcin and C-terminal fragment[1] [ ]* bLfcin: 0.1-1.0; C-terminal:1.0; bLf: 1.0	-bLfcin caused 96% mortality of the parasite	[40]
		-Infectivity in MEC decreased <10%	

		-In mice infected with *T. gondii* pre-incubated with bLfcin, the cysts number in brain was fivefold less than control mice	
T. gondii RH	bLf(2) 1.14% iron-saturated [ ]* 0.0001; 0.001; 0.1 and 1.0	-MPM infected with T. gondii pre- incubated with bLf, a 30 kDa tyrosine kinase was induced	[41]
		-Tyrosine-phosphorylation seems to be associated with the bLf inhibitory activity	
T. gondii Beverley; *E. stiedai* isolated from rabbit	bLfcin(3) [ ]* 0.1, 1.0	-bLfcin decreased MEC infectivity by *T. gondii* sporozoites and reduced the infection to RHC by *E. stiedai*	[42]
B. caballi and *B. equii*	bLf and bLfcin(4); bLf native with ~38% iron-saturated, apo-bLf 0% and holo-bLf with ~70% iron [ ]* 2.5, 5.0	-apo-bLf suppressed the *B. caballi* growth at least in 50%	[45]
		-The effect did not depend on the direct interaction between the protozoan surface and apo-bLf	
E. histolytica HM 1:IMSS	bLf, bovine, human and swine milk(5) [ ]* 1	-Bovine and human milk was amoebicidal, this effect was concentration and iron dependent	[53]
		-apo-hLf caused cell lysis	
		-The mechanism involved was caused by the binding of proteins to amoeba membrane	
E. histolytica HM 1:IMSS	bLf, apo-hLf and bLfcin(6) (fragment 4-14) [ ]* 1.0, 2.5	-bLf and hLf were amoebicidal	[54]
		-Effect was concentration-dependent and modulated by environmental conditions	
		-bLf was more effective than bLfcin	
		-All components synergized with metronidazole vs amoebae	
E. histolytica HM 1:IMSS	bLfcin, bLfampin and bLf chimera(7) [ ]* 0.025, 0.050, 0.075, 0.10	-bLfcin and bLfampin had a moderate amoebicidal effect	[55]
		-bLfcin chimera showed the highest microbicidal activity	
		-The microbicidal effect of Lf peptides was iron-independent	
G. duodenalis	bLf, bLfcin, hLf, Lfcin(2) [ ]* bLfcin and hLfcin: 0-0.024; bLf and hLf: 0-2.5	-LD50: bLfcin: 8 (µg/ml); bLf: 1.2 (mg/ml); hLfcin: 16 (µg/ml); hLf: 1.5 (mg/ml)	[59]
		-bLfcin 12 (µg/ml), bLf 2 (mg/ml), hLfcin 24 (µg/ml) and hLf f 2.5 (mg/ml) decreases trophozoite viability around 20%	
C. parvum Iowa	bLf, bLfh and bLfcin(8)	- bLfh and bLfin B were parasiticidal	[60]
		-An inhibitory activity on sporozoite infectivity in vitro was observed	

(1): obtained by enzymatic digestion; (2): bLf and bLfcin from Morinaga Milk, Japan; hLf from Sigma, USA and hLfcin synthesized by Quality Controlled Biochemicals; (3): purified from cow's milk; (4): bLf from Morinaga Milk Industry, Japan and bLfcin obtained by enzymatic digestion; (5): bLf from Morinaga Milk, Japan; also hLf, human sIgA and chicken egg-white lysozyme from Sigma, USA; (6): bLf from Morinaga Milk, Japan, apo-hLf and bLfcin from Sigma, USA; (7): synthesized at the department of Oral Biochemistry, ACDA, The Netherlands; (8): bLf from LKT Labs, USA, bLfh and bLfcin from Sigma, USA. Also bovine indolicidin, chicken lysozyme, honey bee-venom phospholipase A2, Bacillus cereus phosphatidylinositol-specific phospholipase C, LL37 (human cathelicidin), human β-defensin 1 and 2 were employed. bLfh: bovine lactoferrin hydrolysate; LD50: 50% lethal dose; MEC: mouse embryonal cells; MPM: mouse peritoneal macrophages; PV: parasitophorus vacuole; RHC: rabbit hepatobiliary cells. [ ]*: concentration expressed as mg/ml

Table 2: *In vitro* assays of bLf against pathogenic protozoan parasites.

Babesia caballi and *Theileria equi* (*Babesia equi*) are haemoprotozoa causing equine piroplasmosis, a tick-borne disease that affects all equid species (horses, donkeys, mules, and zebras). A study was conducted on the effect of bLf with different iron-saturation levels as well as an Lf hydrolysate (bLfh) on parasite viability [45]. The IC_{50} (concentration that inhibits 50% of parasites in blood) value was 2.7 mg/ml apobLf for *B. caballi*, but no effect was observed against *B. equi*; this result was similar even when the culture medium was treated with a heparin column to remove the bLf. The inhibitory effect of bLf may have been caused by the inactivation of a growth factor in the culture medium. Unfortunately, this study was discontinued and it is not possible to propose a well-supported mechanism for the action of bLf against these parasites.

Entamoeba histolytica is a parasitic extracellular protozoan that causes human amoebiasis, mainly in developing countries [46]. However, *E. histolytica* has been reported in non-human primates (NHP) such as *Cercopithecus aethiops* (vervet), *C. albogularis* (Sykes' monkey) and *Papio anubis* (olive baboon) [47-50]. Although the prevalence of *E. histolytica* is low in NHP, it represents a risk of zoonosis for zoo workers who coexist with NHP. Additionally, in some countries, the general population are at risk due to humans and NHP

sharing the same water sources and foods. *E. histolytica* can damage the large intestine, causing abscesses, with occasional migration of amoebae to the liver, lungs and brain [51]. Metronidazole continues to be the choice therapy for amoebiasis. However, this drug causes nausea, vomiting, and other adverse side effects in addition to being mutagenic *in vitro* and carcinogenic in experimental animals; thus, for long-term therapies, it should be used carefully [52]. For these reasons, research has been focused on providing alternatives for therapy and prophylaxis against *E. histolytica*. For example, our research group reported that apo human Lf (apohLf) and apobLf eliminate *E. histolytica* trophozoites in *in vitro* cultures and proposed a mechanism that could be involved [53]. Afterwards, we assayed bLfcin4-14 as an antiamoebic, although it was less effective than bLf. In addition, a synergistic effect of apobLf with metronidazole was found against the parasite [54]. We also tested three synthetic bLf peptides (Lfcin17-30, Lfampin265-284 and Lf chimaera) on the viability of *E. histolytica*; the chimaera showed the best microbicidal activity [55].

Giardia duodenalis is a cosmopolitan parasite that affects domestic and wild mammals, the faecal-oral route is its main transmission via. This protozoan leads to diminishing the epithelial permeability, then an inflammatory response and absorptive changes that correlate with brush border injury are produced. It has been reported that assemblages A and B are able to infect humans [56]. Interestingly, these assemblages have been reported from wild animals under conditions of captivity [57], and from cattle [58]. bLf, bLfcin, hLf and human Lfcin (hLfcin) showed a lytic effect on *Giardia* trophozoites. When the addition of metal ions on bLf and bLfcin lytic effect was evaluated, the activity decreased, in a very similar manner to that observed when $Fe2(SO_4)_3$ was added [59]. This result suggests that, *in vivo*, the giardicidal effect of Lf could be dependent on the dynamics of intestinal micro-environment. It would be remarkable the use of bLf to prevent or cure the infection by E. histolytica, *G. duodenalis*, and probably other parasitic protozoa in NHP, since bLf does not cause adverse effects as metronidazole does.

Cryptosporidium parvum is a parasite that causes neonatal diarrhoea in calves and lambs and was recognized as an AIDS-defining illness during the 1980s. A less intense infection in Caco-2 cells was found when *C. parvum* sporozoites were preincubated with bLf for 15 min, and the percentage viability of the protozoan also decreased when bLfh and bLfcin4-14 treatments were used [60].

The antiviral effect of Lf and its mechanisms of action have been studied with different viruses in human and veterinary medicine. Two main mechanisms are known by which Lf inhibits viral infection: 1) directly binding to viral particles and 2) blocking virus receptors in the host cell. A panel of experimental assays has been established to study these mechanisms; the activity of Lf is screened by incubating cells with Lf before they are infected with viral particles [61]. The antiviral activity of Lf has mainly been studied in viruses that cause human diseases or can be transmitted from animals to humans: HIV, cytomegalovirus, hepatitis B and C virus, adenovirus, poliovirus, hantavirus, Sindbis virus, Semliki Forest virus, avian influenza A (H5N1), influenza virus A H1N1, respiratory syncytial virus, herpes simplex virus type 1 and type 2, echovirus, enterovirus, and rotavirus [62]. On the other hand, as Lf is a food component, it can be easily consumed by people to prevent common viral infections. However, further basic and clinical studies will clarify the usefulness of Lf in this field [63]. Table 3 summarizes some studies of bLf against viruses.

Virus	bLf	Results	Reference
Bovine herpesvirus 1 (alphaherpesvirus)	bLf (Sigma)	- 90-99% viral inhibition (5 and 2.5 mg/mL of bLf).	[64]
	10, 5, 2.5, 1.25, and 0.625 mg/ml	- Decrease in blastocyst development of treated embryos was statistically different from the untreated controls.	
Murine norovirus	bLf (Morinaga Milk Industry)	- Cytotoxicity was completely inhibited in all of the wells treated with 15 and 20 µg/well of bLf.	[65]
		- Virus titre in the culture medium significantly decreased with bLf [2.5-20 µg / well]	
		- MNV titre in cells was significantly reduced	
		- Expression of both IFN-α and IFN-β mRNAs in infected cells significantly increased in the bLf-treated cells.	
Avian influenza A (H5N1)	bLf (Armor Proteins) native and esterified protein (20, 40 and 80 µg/ml)	- Native lactoferrin seems to be the most active antiviral protein among the tested samples.	[66]
		- Esterified LF reached maximum antiviral influence at 80 µg/ml.	
Influenza A (H1N1, H3N2, H5N1, H7N1)	bLf (Morinaga Milk Industries, Zama City, Japan) and derived peptides.	- Concentrations of bLf ranging from about 0.05 pM to 6 nM could prevent HA activity.	[67]

		- bLf-derived peptides were better inhibitors than the entire protein.	

Table 3: *In vitro* assays of bLf against pathogenic viruses.

The inhibition of replication in bovine herpesvirus 1 by bLf has been demonstrated; this is an alphaherpesvirus responsible for abortion, infertility, genital disease, and respiratory infection in cattle. bLf inhibited viral replication by 99% in MDBK cells, and with bLf combined with cidofovir, over 100% viral inhibition was obtained. Furthermore, the effects of bLf on bovine embryonic development were determined. Embryos could develop in the presence of bLf; however, bLf adversely affected blastocyst development; thus, the authors do not recommend the use of bLf as an antiviral supplement during *in vitro* culture of developing bovine embryos [64]. In another study, the effects of bLf against norovirus infection were evaluated *in vitro* using mouse norovirus (MNV) and RAW264.7 cells. Norovirus causes most acute nonbacterial gastroenteritis in humans of all ages worldwide. In this case, the MNV was used since there is no cell culture or animal model for testing human norovirus. Interestingly, when cells were infected with MNV in the presence of bLf, the cytotoxic damage to infected cells was completely inhibited, and the MNV titres were significantly decreased. It was concluded that bLf exerts protective effects against MNV infection through inhibition of both viral attachment and replication and may be useful as a preventive and/or therapeutic anti-norovirus agent [65].

In 2010, the antiviral effect of native and esterified whey protein fractions (α-lactalbumin, β-lactoglobulin and Lf) against avian influenza A (H5N1) virus was demonstrated in MDCK cells at a 100% level of infection. Lf seemed to be the most antivirally active protein in native whey, with inhibition between 34.98 and 70.92%, but esterification of bLf enhanced its antiviral activity from 69.28 to 99.42%. Because of this, it can be concluded that esterification of Lf is a potent tool that can enhance its antiviral activity [66]. To determine how bLf binds to influenza virus, researchers have performed docking studies focused on molecular dissection of bLf and the interactions of its molecular fragments with precise locations upon viral haemagglutinin (HA). The inhibition of influenza virus haemagglutination was demonstrated, and cell infection is entirely attributed to the bLf C-lobe. By far-Western blotting and sequencing, the strong binding of the bLf C-lobe to the HA2 region of viral HA has been well demonstrated, and three C-lobe fragments of bLf have been identified as virus haemagglutination and infection inhibitors at femtomolar concentrations [67].

In vivo assays

Due to the undeniable importance of *T. gondii* in cats and zoonosis, *in vivo* assays have been developed to search the effect of bLf and bLfcin that could affect the total parasite load. Mice were orally infected with a low-virulent strain (type II), and then bLfcin was administered. All infected mice that received bLfcin orally or i.p. (5 mg or 0.1 mg of bLfcin, respectively) survived. Importantly, the enteral route decreased the number of cysts in cerebral tissue almost 14-fold with respect to untreated mice. Infected mice that were not treated with bLfcin showed 80% death [68]. In assays with new drugs, after the in vitro approaches to the use of bLf, biological systems are commonly used as a basic tool to replicate diseases and apply treatments in research and development. For example, *T. gondii* and *E. stiedai* sporozoites were preincubated with bLfcin for 1 h, and mice and rabbits, respectively, were then infected with these parasites. In the study, the survival rate, clinical signs, and number of cysts in some tissues or typical lesions were compared between animals infected with sporozoites preincubated or not with bLfcin. In the case of *T. gondii*, all mice survived more than 30 days after infection without clinical signs, and cysts were found in the peritoneal cavity and brain tissue at necropsy. In rabbits infected with preincubated sporozoites, a low number of *E. stiedai* cysts in faecal samples between 16-35 days after infection was detected and cholestasis was observed at necropsy, whereas in infected rabbits without bLfcin treatment, hepatomegaly and many abscesses were produced [42]. The authors mentioned that coccidian infection could be prevented *in vivo*, considering that bLfcin is produced by bLf digestion in the stomach and the resulting peptide can travel to the intestine, where sporozoites excyst and infect the host enteroepithelial cells. To demonstrate this possibility, it would be interesting to assay oral treatment with bLf or bLfcin before and during sporozoite infection. Recently, a research compared the effect of native bLf with a bLf nanoformulation (NF) against the *T. gondii* RH strain. A human toxoplasmosis disease model was developed by inoculating 100 tachyzoites through i.p. route in Balb/c mice. Experiments included mice fed with a diet supplemented with the following treatments: bLf, NF, and sulfadiazine as standard drug; the effect was studied at days 10 and 15 p.i. and different parameters were assessed. The NF decreased the parasite load in various organs and helped survival of mice until day 25 p.i. From this study, the authors concluded that NF did not reduce the therapeutic potential of Lf; however, the NF enhanced its stability and showed anti-toxoplasmal activity. The results suggested that this NF of Lf could have advantages over the standard drug therapy against *Toxoplasma*, including that it not produced any side effects [69].

Regardin to virus research, bLf was tested as an adjuvant in vaccination of neonatal mice against H1N1 influenza virus. Bovine Lf was able to replace aluminium as an adjuvant; in addition, Lf enhanced the response to H1N1 (HA) in these mice [70].

Applications of bLf to treat microbial infections in domestic animals, including zoonoses: Concerning domestic animal diseases that cause economic losses in livestock production, some researchers have tested bLf as an alternative to antibiotic treatment. For studying the effect of a combinatory therapy, given i.m.m., on bovine mastitis caused by *S. aureus*, 22 cows with clinical mastitis were treated with bLf (200 mg), cefazolin (250 mg), or bLf plus cefazolin. After seven days of i.m.m. administration, the cure rate (disappearance of clinical signs: swelling and firmness) with each antimicrobial was approximately 50%, in comparison with 80.7% for bLf+cefazolin. The anti-inflammatory effect of bLf was reported to result from down-regulation of TNFα and IL-6. Thus, the combination therapy was more effective than the antibiotic alone [71]. In another other study, the efficacy of bLf alone or in combination with penicillin G against experimental mastitis caused by *S. aureus* SHY97-4320 (highly resistant to β-lactam antibiotics) was tested [72]. Cows in late lactation were infected for periods of two weeks and one month. The infections were introduced through i.m.m. infusions of 10^3-10^4 CFU; later, each

mammary quarter was treated with 100,000 IU of penicillin, 1 g of bLf, bLf+penicillin, or buffer (control). The results showed that the bLf alone and the bLf+penicillin treatment were more efficient that the penicillin alone. Moreover, the infection did not become chronic. The combination of bLf+penicillin is relevant due to the marked antimicrobial resistance of the strain studied. In addition to the antimicrobial effect of bLf in the i.m.m. treatment, we must remember the role of bLf as an immune modulator acting on lymphocytes, macrophages and neutrophils in the mammary tissue. The authors stated that the beneficial results were probably multifactorial, since the molecular mechanism by which bLf improves the antibiotic efficacy was not yet completely clear; another topic of discussion could be the iron-saturated percentage of Lf [72].

Meanwhile, experiments have been conducted to establish bLf as a means of limiting the transmission of zoonotic pathogens. The potential of bLf to prevent colonization and excretion of EHEC O157:H7 (enterohaemorrhagic) in 3-month-old sheep was investigated [73]. The effect of apobLf at 1.5 g or 0.15 g every 12 h for 30 days was evaluated in 17 sheep. All animals were orally infected with 10^{10} CFU. Interestingly, both bLf dosages significantly reduced the number and duration of *E. coli* excreted in the faeces. Furthermore, the group that received a high dose of bLf showed a significantly higher Ab response against EspA and EspB (effector molecules) than the control group. With these findings, the authors suggested that bLf could play an important role in preventing colonization by EHEC on farms. Later, colonization and excretion of *E. coli* O157:H7 was analyzed in Holstein-Friesian calves fed with or without bLf for 50 days [74]. The calves were assigned to three groups for treatment: oral (3 g/day), rectal (0.3 g/day), and an untreated control, all infected rectally with 1010 UFC. Throughout the experiment, the excretion and bacterial content in the tissues - the jejunum, ileum with or without Peyer's patches, colon, caecum, rectum, and recto-anal junction - were determined. Additionally, the serum Ab responses against intimin, EspA and EspB were measured. The results showed a constant decrease in bacterial excretion with rectally administered bLf, to the point of total elimination; in contrast, the oral bLf group had an oscillating pattern of bacterial excretion. All groups developed serum responses, but no clear differences could be observed among the groups. A year later, the same research group conducted some variations of their previous experiments, emphasizing the ability of bLf to clear *E. coli* O157:H7 colonization in cattle. Six-month-old Holstein-Friesian calves were used; the animals were experimentally infected with an EHEC strain and received daily rectal treatment with bLf (1.5 g/day). The treatment (19 days) decreased faecal excretion of *E. coli* and eliminated the infection. Furthermore, specific IgA responses against EspA and EspB at the rectal mucosa were detected. Thus, these findings indicate that the use of bLf as a rectal treatment in calves carrying EHEC could be a tool to abolish further transmission, including transmission to humans [74].

Additionally of promising results in livestock, bLf has been employed in pets as well. Commonly, studies on the effects of drugs are first realized *in vitro*; however, as bLf is an innocuous protein, it was orally administered to cats diagnosed with intractable stomatitis due to feline immunodeficiency virus (FIV) infection. Lf suppressed buccal inflammation, improved the clinical symptoms, and decreased serum γ-globulin, a marker of inflammation [75]. Afterwards, the effects of bLf on proliferation, cell cycle progression and expression of cytokines in peripheral blood mononuclear cells (PBMC) were examined to clarify the anti-inflammatory effect. bLf at 10 and 50 μg/ml decreased ConA-induced proliferation as well as apoptosis progression in PBMC, in FIV-negative and FIV-positive cats. The addition of 500 μg/ml bLf after ConA significantly inhibited the expression of IFN-γ and IL-2 in FIV-positive cats. This study suggested that treatment with bLf could maintain the immune homeostasis of immunosuppressed FIV- positive cats [76].

Uses of porcine lactoferrin (pLf) and lactoferricin (pLfcin) in the veterinary field: The structure and functions of bLf and hLf have been well characterized, although little is known about pLf. Recombinant pLf (r-pLf) was purified using a fast protein liquid chromatography system; the glycosylation of *Pichia pastoris*-derived r-pLf was analyzed, and patterns similar to those of pLf were observed. In addition, bacteriostatic and bactericidal activities were tested in an *E. coli* reference strain. The MIC and minimal bactericidal concentration (MBC) of a pepsin-digested r-pLf hydrolysate against *E. coli* were 150 and 200 μg/ml, respectively, while intact r-pLf had an MIC of 750 μg/ml. The peptides obtained by pepsin digestion of r-pLf exhibited more antimicrobial activity than native r-pLf, apparently because they disrupt the cell wall and disintegrate the LPS molecules of the outer membrane [77].

Later, the antimicrobial activity of r-pLf was evaluated in a transgenic mouse model, expressing r-pLf in their milk (120 mg/L). During the lactation stage fed normal mouse pups for 4 weeks. The pups were subsequently intragastrical challenged with pathogenic *E. coli* (2×10^6), *S. aureus* (2×10^8), or *Candida albicans* (2×10^6 CFU/mouse). Growth rate, intestinal mucosa condition, and circulating cytokines were examined. A reduction in the severity of illness and a lower death rate were observed in mice fed with r-pLf-enriched milk after the intestinal infection. In addition, these mice demonstrated significant inhibition of microbial survival in the intestinal tract after 3 days, and the number of pathogens cultured from blood was significantly lower during the initial 3 days after infection. The authors suggested that pLf could be used for the prevention of nosocomial pneumonia or sepsis [78].

By using bioinformatic tools, researchers compared the N-terminal 45-amino-acid sequences of Lf from several animal species to seek a putative antimicrobial domain. The identity percentage of the fragment from 1 to 45 between pLf and the other eight Lfs was as follows: bovine (48.9%), buffalo (46.7%), camel (44.4%), caprine (53.3%), equine (44.4%), human (42.2%), mouse (35.6%) and rat (33.3%). The first five amino acids of the porcine, bovine, buffalo, camel, caprine and equine Lf include two basic amino acids (Arg or Lys); human Lf contains four Arg. Afterwards, they generated a series of synthetic derivatives of porcine, bovine, and human Lfcins (20- and 9-residue peptides) to investigate their antimicrobial nature. The MIC and MBC of the various synthetized Lfcins were determined. Reference strains of *E. coli*, *S. aureus*, and *C. albicans* were used. When the MIC and MBC of the 20-residue Lfcins were compared, it was clear that bLfcin>pLfcin>hLfcin in effectivity against the pathogens tested. In addition, morphological changes in the microorganisms were visualized by SEM, and this technique revealed that treatment with the 20-residue pLfcin directly led to the disruption of the cell wall (*S. aureus*) and breakdown of the outer membrane (*E. coli*). Apparently, the specific differences in the first amino acids of the N-terminal sequence are very important for interaction and bactericidal ability [79].

Recently, a mouse model was used to assess the effect of r-pLf produced by *Lactobacillus* species (*L. casei*, *L. pentosus*, *L. plantarum* and *L. paracasei*). Mice were fed daily with 10^9 transgenic *Lactobacillus* as a food additive for 14 days and infected with 2×10^7

CFU of *E. coli* K88 or a $10^{-4.5}$ dilution ratio of the LD50 of porcine pseudorabies virus (PRV). In mice fed with recombinant lactobacilli the total viable counts of *E. coli* from microbiota decreased but bifidobacteria and lactobacilli increased. After the challenge with *E. coli* K88 or PRV, the mice fed with recombinant bacilli did not exhibited feeble body, loss of body weight, and death; compared with the control group, in the mice fed with recombinant *Lactobacillus* species the average daily weight gain increased, as well as total IgG, and total sIgA levels; additionally, they had higher IL-2 and TNF-α expression than the non-treated mice. A significant reduction was present in IL-4 levels. The mice fed with *L. pentosus* and *L. plantarum* showed the best results [80].

In another case, the use of r-pLf as a dietary supplement was studied in one-day-old chickens; the supplemented chickens showed substantial increases in body weight gain and survival rate for a period of 16 weeks. Also, the animals showed a normal jejunum and longer villi in this organ upon histological study when r-pLf was administered in combination with infectious bursal disease vaccination. r-pLf enhanced the Ab titre and promoted peripheral lymphocyte proliferation. Similarly, r-pLf also modulated the expression of IL-2, IFN-γ, IL-4 and IL-12 in ConA-stimulated peripheral T lymphocytes [81].

Conclusion and Perspectives

The purpose of this review was to collect, discuss and communicate the findings related to bovine and porcine Lf and Lfcin assays in veterinary medicine, particularly in relation to animal health. In human medicine, bovine and human Lf has been extensively studied as immunostimulants and against pathogens. Bovine Lf has a reasonable cost and is marketed without restriction. Meanwhile, through advances in biotechnology, r-pLf can also be employed as a food supplement, bringing benefits to the immune system and intestinal microbiota. The discovery of the beneficial effects of Lf has been analyzed from an *in vitro* perspective, but some experiments have also been done in animal models and domestic animals. Some of the microbicidal effects of Lf can be clearly attributed to its N-terminus end. The effect that Lf can have on extra- and intracellular environments is irrefutable, although its specific mechanisms of action remain to be elucidated. In animal production, such as pig and ruminant farming, bLf and pLf may be used for the prevention and control of outbreaks such as colibacillosis and pneumonic diseases. If we improve animal health, production parameters will benefit. Some other advantages are that Lf can be administered by different routes and is stable by the oral route; since though Lf can be partially digested by monogastric animals, the Lfcins produced are bioactive peptides that maintain antimicrobial activity. So, Lf is a multipotential and multifunctional glycoprotein with widespread applications in many animal species, including those of importance in the human food industry, for the control of animal diseases and zoonoses.

Competing Interests

The authors declare that they have no competing interests.

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