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; hLf: Human Lactoferrin; hLfcin: Human Lactoferricin; s.c: Subcutaneous; I.m.m.: Intramammary; I.p.: Intraperitoneal; I.v.: Intravenous; Lf: lactoferrin; bLampin: 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; PUF: 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 microflora. 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 ingested from milk [21]. Several Lfcins have been assayed as antibacterials; 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].
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].

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Source of bLf/bLfcin and iron-saturation condition</th>
<th>Results</th>
<th>Reference</th>
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<tbody>
<tr>
<td>S. aureus 6538P</td>
<td>bLf and bLf hydrolysate from Morinaga Milk Company, 26% iron [1]; [ ]* 5-20</td>
<td>- bLf without iron had a weak effect on viability, a maximum inhibition was obtained at 20 mg/ml</td>
<td>[34]</td>
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<td></td>
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<td>- Saturated bLf potentiated bacterial growth</td>
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<td>S. aureus SHY97-3923, SHY97-3906, SHY97-3432, SHY97-3433 [2]; PC-1, NCTC 9789, 2076, 22260, ST79/741, 3804, RN9, FAR8 and FAR10 [3]</td>
<td>bLf from Besnier, San Juan Capistrano (USA), bLfcin was obtained by enzymatic digestion; [ ]* 0.38-25</td>
<td>-MIC bLf ≤ 25 (µM)</td>
<td>[36]</td>
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<td>-MIC Lfcin=256 (µg/ml)</td>
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<td>- bLf synergized with penicillin-G in all strains except SHY97-3906 and SHY97-3433</td>
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<td>- Change in protein expression of culture incubated with bLf or bLf+penicillin-G</td>
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<tr>
<td>E. coli, S. aureus, coagulase-negative staphylococci [4], P. aeruginosa and K. pneumoniae [5]</td>
<td>bLf purified from cheese whey (expanded bed absorption chromatography method), 4% iron; [ ]* 0.67, 1.67, 2.67</td>
<td>- Major inhibitory activity of bLf vs E. coli since 1.67 mg/ml</td>
<td>[37]</td>
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<td>- Three S. aureus isolates were susceptible to bLf at 0.67 (mg/ml)</td>
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<td>- Bacteriostatic effect and concentration-dependent was observed at 16h</td>
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<td>V. parahaemolyticus 17802 [6], O3:K6 [7], 727 [8]; V. cholerae O1 and no-O1</td>
<td>bLf from DMV International (USA); bLfcin, bLfampin and bLfcin chimaera were prepared [9]; bLf 20% iron; [ ]* 0.001, 0.01, 0.02, 0.04</td>
<td>- bLf and bLf chimaera inhibited the V. parahaemolyticus growth in &gt;50%, bLfcin and bLfampin in 10-15%</td>
<td>[38]</td>
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<td></td>
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<td>- bLf decreased the V. cholerae growth in &gt;90%</td>
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</table>
-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; $[^{1}]$ 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)
| bLF 0.005 % iron; $[^{1}]$ 0.0 to 2 |

MIC bLF= 4.88-7.31 µM

$^{[6]}$

$bLf$ from NutriScience (USA); $^{[1]}$: 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 manheimiosis, 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 chimera was used. Moreover, bLfcin chimera showed a synergistic effect with ampicillin, principally against a multidrug-resistant strain of V. parahaemolyticus (Figure 2) $^{[38]}$.

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 $^{[44]}$.

Table 2: In vitro assays using apobLf and its N-terminal derivatives against human parasitic protozoa.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>bLF/bLfcin features</th>
<th>Results</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>T. gondii RH and S-273</td>
<td>bLfcin and C-terminal fragment$^{[1]}$ $[^{1}]$ bLfcin: 0.1-1.0; C-terminal: 1.0; bLF: 1.0</td>
<td>-bLfcin caused 96% mortality of the parasite</td>
<td>$^{[40]}$</td>
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<td></td>
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<td>-Infectivity in MEC decreased &lt;10%</td>
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<td>Table 2: In vitro assays of bLf against pathogenic protozoan parasites.</td>
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**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\textsubscript{50} (concentration that inhibits 50% of parasites in blood) value was 2.7 mg/ml apo-bLf and 5.0 mg/ml holo-bLf 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.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>bLf and bLfcin (mg/ml)</th>
<th>hLf and hLfcin (mg/ml)</th>
<th>Effect</th>
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</thead>
<tbody>
<tr>
<td><strong>T. gondii RH</strong></td>
<td>[1] bLf: 2 mg/ml, bLfcin: 0.0001; 0.001; 0.1 and 1.0</td>
<td>-MFP infected with <em>T. gondii</em> pre-incubated with bLfcin, a 30 kDa tyrosine kinase was induced. -Tyrosine-phosphorylation seems to be associated with the bLf inhibitory activity [41]</td>
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<tr>
<td><strong>T. gondii Beverly, E. stiedai isolated from rabbit</strong></td>
<td>bLfcin: 0.1, 1.0</td>
<td>-bLfcin decreased MEC infectivity by <em>T. gondii</em> sporozoites and reduced the infection to RHC by <em>E. stiedai</em> [42]</td>
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<tr>
<td><strong>B. caballi and B. equi</strong></td>
<td>bLf and bLfcin: 0% and holo-bLf with ~70% iron</td>
<td>-apo-bLf suppressed the <em>B. caballi</em> growth at least in 50% -The effect did not depend on the direct interaction between the protozoan surface and apo-bLf [45]</td>
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<td><strong>E. histolytica HM 1:IMSS</strong></td>
<td>[5] bLf, bovine, human and swine milk (mg/ml)</td>
<td>-Bovine and human milk was amoebicidal, this effect was concentration and iron dependent -apo-hLf caused cell lysis -The mechanism involved was caused by the binding of proteins to amoeba membrane [53]</td>
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<tr>
<td><strong>E. histolytica HM 1:IMSS</strong></td>
<td>bLf, apo-hLf and bLfcin (mg/ml)</td>
<td>-bLf and hLf were amoebicidal -Effect was concentration-dependent and modulated by environmental conditions -bLf was more effective than bLfcin -All components synergized with metronidazole vs amoeba [54]</td>
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<tr>
<td><strong>E. histolytica HM 1:IMSS</strong></td>
<td>bLf, bLfcin and bLf chimera (mg/ml)</td>
<td>-bLfcin chimera showed the highest microbicidal activity -The microbicidal effect of Lf peptides was iron-independent [55]</td>
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<td><strong>G. duodenalis</strong></td>
<td>bLf, bLfcin, hLf, Lfcin(2) [mg/ml]</td>
<td>-LD50: bLfcin: 8 (µg/ml); bLf: 1.2 (mg/ml); hLfcin: 16 (µg/ml); hLf: 1.5 (mg/ml) -bLfcin 12 (µg/ml), bLf 2 (mg/ml), hLfcin 24 (µg/ml) and hLf f 2.5 (mg/ml) decreases trophozoite viability around 20% [59]</td>
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<tr>
<td><strong>C. parvum Iowa</strong></td>
<td>bLfh and bLf chimera (mg/ml)</td>
<td>-bLfh and bLfin B were parasiticidal -An inhibitory activity on sporozoite infectivity in vitro was observed [60]</td>
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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 apoHLf 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 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 Fe(III) 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 bLh 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.

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

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

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<th>Table 3: In vitro assays of bLf against pathogenic viruses.</th>
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<td>- bLf-derived peptides were better inhibitors than the entire protein.</td>
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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 bLf.cin that could affect the total parasite load. Mice were orally infected with a low-virulent strain (type II), and then bLf.cin was administered. All infected mice that received bLf.cin orally i.p. (5 mg or 0.1 mg of bLf.cin, respectively) survived. Importantly, the enteric route decreased the number of cysts in cerebral tissue almost 14-fold with respect to untreated mice. Infected mice that were not treated with bLf.cin 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 bLf.cin 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 bLf.cin. 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 bLf.cin treatment, hepatomegaly and many abscesses were produced [42]. The authors mentioned that coccidian infection could be prevented in vivo, considering that bLf.cin is produced by bLf digestion in the stomach and the resulting peptide can travel to the intestine, where sporozoites excyst and infect the host enterocytic cells. To demonstrate this possibility, it would be interesting to assay oral treatment with bLf or bLf.cin before and during sporozoite infection. Recently, a research compared the effect of native bLf with a bLf nanof ormulation (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 statistically compared in eight 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 produced no 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 10^4–10^6 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 than 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 aopbLf 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 10^16 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 intrastragical challenged with pathogenic E. coli (2 × 10^6), S. aureus (2 × 10^6), 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 LFs (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 Pfcin 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 10^8 transgenic Lactobacillus as a food additive for 14 days and infected with 2 × 10^6
CPU 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 microbota decreased but bifidobacteria and lactobacilli increased. After the challenge with *E. coli* K88 or PRV, the mice fed with recombinant lactobacilli did not exhibit 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 later 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 Lcins 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 microbialicidal 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, LfL 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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**References**


