Chapter 7

Lactoferrin in the Battle against Intestinal Parasites: A Review

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Abstract

Lactoferrin is an iron-binding glycoprotein of the innate immune system, which is present in some mammalian fluids and secreted into the mucosae; it is also produced by the secondary granules of the polymorphonuclear neutrophils and secreted at infection sites. Lactoferricins (Lfcins) are peptides derived from the N-terminus of Lf. Lf avoids the iron availability to parasites in the body fluids due to its high avidity for iron, maintaining together with transferrin the free-iron concentration in about 10^{-18} M, which is too low to support the pathogenic invader survival. Intestinal parasitic diseases affect people worldwide, mainly in developing countries with poor hygienic conditions; for example, parasites such as *Entamoeba histolytica*, *Giardia intestinalis*, and *Cryptosporidium parvum* infect the human intestine when are orally ingested as cysts. Human and bovine Lf have been found parasiticidal in experiments *in vitro* and in animal models. Interestingly, Lf synergizes with metronidazole, the main drug used against *E. histolytica* and *G. intestinalis*. The aim of this chapter is to show the benefits of using Lf and Lfcins against intestinal parasitic diseases.

Keywords: antimicrobial, intestinal mucosa, iron, lactoferrin, parasiticide

1. Introduction

Lactoferrin (Lf) is an iron-binding nonheme glycoprotein that possesses an exceptional high iron-binding affinity and retains iron at acidic pH. Lf is mainly devoted to chelate iron in fluids and secretions; in addition, Lf is immunomodulatory. Based on its iron content, Lf can exist in two forms: iron-loaded (holoLf, with one or two ferric ions) and iron-free (apoLf). Lf is a constituent of the mammalian innate-immune defense system. In mucosae, Lf displays antimicrobial activity against a wide range of pathogens [1–5]. Lf is synthesized by the mammary gland and secreted into colostrum and milk, participating in the primary immune response in newborns [5–8]. In humans, Lf concentration ranges from 7 to 15 mg/ml in colostrum and 1.2 mg/ml in mature milk. Lf is also present in tears, saliva, and exocrine secretions of mucosal

surfaces located in the respiratory, reproductive, and intestinal tracts [9–12]. Lf has been found in tissues of the stomach, lung, liver, bone marrow, cartilage, and bones [13–16]. In the gastrointestinal tract, Lf concentration varies from 0.75 μg/ml in duodenal juice, 0.71–1.07 μg/ml in whole gut lavage fluid, or 0.3–0.7 μg/g in feces [17].

Lf is also synthesized during the transition from promyelocytes to myelocytes of white cells; thus it is a major component of the secondary granules of polymorphonuclear (PMN) neutrophils present in blood [18]. These cells store Lf (3 μg Lf/10^6 neutrophils) and they release it at the sites of microbial invasion which are of low pH due to the pathogens activity [2, 7, 11, 19]. Lf concentration in plasma is relatively low (0.0004–0.002 mg/ml) and derives from neutrophils; however, in patients with sepsis neutrophils are activated and degranulated, secreting into the bloodstream significant levels of apoLf (~0.2 mg/ml) [9]. Lf in feces is also due to the neutrophils action and its concentration noticeably increases in bowel inflammatory diseases (BID) due to pathogenic bacteria, such as ulcerative colitis and Crohn's disease. Thus, Lf is used in a test as an inflammatory marker in intestine, test that discriminates between people suffering BID from those that only have irritable bowel syndrome (IBS), who show normal values of Lf [20]. A test of latex agglutination using anti-Lf antibodies demonstrated that cases with either shigellosis or bacterial urinary infections revealed a high Lf titer which was positively correlated with the number of PMN. In contrast, cases with parasitic infections such as *Entamoeba histolytica* or *Schistosoma haematobium* were characterized by a relatively lower inflammatory process as expressed by mild Lf titer which was also correlated with the PMN count [21]. Ascites Lf can also offer a promising biomarker for bacterial peritonitis, and Lf in pancreatic juice and stone could provide pathophysiological information [22].

2. Structure and biological properties of lactoferrin

Lf was initially identified from bovine milk [23], and simultaneously isolated from bovine [24] and human [25] milk more than 55 years ago. Both glycoproteins (hLf and bLf) share 70% in amino acid sequence [26] and are monomeric, with an approximated molecular weight of 80 kDa; both are highly cationic with a basic isoelectric point (8.5–9). Tertiary structure of Lf consists in two main N and C lobes that are in turn organized in domains N1, N2, and C1 and C2. Both lobes are linked at N1 and C1 domains by a three-turn alpha chain [27, 28] and are able to bind one ferric ion ($Kd = 10^{-23}$ M); this ion derives from the diet or from iron-charged transferrin (holoTf) [29]; Tf is a similar glycoprotein present in plasma and lymph but it has lower affinity for iron than Lf. HoloLf structure is conformationally more rigid and stable compared with apoLf [30–32].

In the N1 terminus of Lf, there is a region lacking iron-chelating activity, known as a lactoferricin (Lfcin) domain, characterized by its strong cationic charge. Lfcin can be obtained from Lf by enzymatic proteolysis with stomach pepsin; the antibacterial properties of Lf are due to this Lfcin domain [33–35]. Several Lfcins have been employed against pathogens, and they are termed according to the residues number they contain. Moreover, antimicrobial peptides have been synthesized and can be used in combination with drugs [36]. Synthetic Lfcin17-30 and lactoferrampin (Lfampin265-284), and a fusion peptide of both, Lfchimera,

have been assayed against multiresistant bacteria, and also those that form biofilms [37–39]. Lfchimera also has been tested against parasitic protozoa [40–42].

Microbes that colonize mucosal surfaces in the different body tracts will likely be exposed to different concentrations of Lf, to different complexes of Lf with other proteins, and to different levels of Lf derivatives [43]. As a plus of the beneficial effects of Lf in the intestinal tissues, many studies report its property as growth-promoting on bifidobacteria [44]. All these findings suggest that Lf and Lfcins can be of potential use as adjuncts to conventional antibiotics and drugs in the pharmacological use against pathogens.

3. Importance of iron in infections and the role of lactoferrin

Due to the iron toxicity, all organisms need to regulate its concentration and maintain iron homeostasis [45, 46]. This transition element is mainly linked to proteins, like the heme group in hemoglobin, as cofactor of enzymes, bound to other proteins like iron-chelating proteins, or stored in ferritin [9, 45, 47, 48].

To multiply and cause disease, parasites must acquire iron within their vertebrate hosts. However, mammals have evolved a universal strategy against microbial invaders, consisting in the expression of iron-sequestering systems for dropping the free iron concentration that pathogens need to survive inside a host. The iron-chelating property of Lf and Tf in fluids leads to a concentration of 10^{-18} M, a quantity too low to sustain the microbial life [9, 49, 50]. In addition, infections are often associated with a reduction in the circulating iron in fluids, a host response known as hypoferremia of infection [10]. So, pathogens must have systems needed to gain the iron retained in human proteins such as Lf; if not, they succumb by the iron restriction. This is the reason by which Lf is microbiostatic.

Furthermore, Lf can damage the functional integrity of the microbial surface and being bactericide [1]; diverse authors have shown that bLf and hLf display activity against Gram-positive and Gram-negative bacteria, including antibiotic multiresistant bacteria [35, 51–53]. Lf is also able to affect and kill certain unicellular parasites, such as *Toxoplasma*, *Entamoeba*, and *Giardia*. In consequence, Lf can be parasiticide [54–56].

On the other hand, Lf is considered a modulatory molecule of both the innate and adaptive immune systems. Lf is able to modify the production of humoral mediators and the activity of cell components involved in specific immune responses, such as the increase of T-cell proliferation and maturation [57–60]. Lf is capable of modulating the response of macrophages to induce a Th1 response essential to combat intracellular pathogens [61–64]. Effects of Lf on inflammation correlate with a decrease of the proinflammatory mediator tumor necrosis factor (TNF), interleukin (IL)-6, and IL-1 and, in some cases, with an increase of anti-inflammatory interleukins, IL-4 and IL-10 [65–68]. Lf from neutrophils decreases the TNF release and modulates the recruitment and activation of phagocytes to sites of inflammation. Also the peptide Lfcin has shown anti-inflammatory effect [69]. In addition, several researchers have proven that orally administrated bLf prevents cancer progression, which

could be due to an improvement of immunity against the tumor cells, or a direct interaction with these cells, or to both effects [16, 70].

4. Lactoferrin against intestinal infections caused by parasites

The identification of natural compounds with antiparasitic activity has always been a pivotal aim of parasitology research. Alternative therapies against parasites have been explored mainly in chronic infections, or when drugs cause adverse effects, or when microbes are resistant to all treatments. As a consequence to be part of the mammalian natural defense, Lf has been searched as an antimicrobial in assays *in vitro,* and a minimal inhibitory concentration (MIC) has been established for each microorganism tested. Experimental infections have also been performed *in vivo* in animal models in which different doses of Lf and administration via have been employed, and the reduction of lesions is evidenced. In a wide range of bacteria and in less number of fungi and parasites, Lf has been tested as microbicide, in some cases with promissory results. It has been shown that Lf inhibits the growth of protozoan parasites, such as *Toxoplasma gondii* [55], *Plasmodium falciparum* [71], *Trypanosoma cruzi* [72], and *E. histolytica* [73, 74]. *T. cruzi* is an emerging parasite responsible for frequent outbreaks of acute cases of Chagas disease contracted orally and causing high mortality [75]. In this chapter, the interactions of some intestinal protozoa with the innate immune-system protein Lf are discussed, as examples of the Lf parasiticidal action. **Table 1** shows the cases of parasites affected by Lf and its natural or synthetic derived peptides.

4.1. Entamoeba histolytica and amoebiasis

Amoebiasis is a parasitic disease caused by the protozoan *E. histolytica* and a major medical problem in developing countries. This infection is responsible for 50 million cases of tissue invasion and 60,000 deaths per year [76]. Amoebiasis is primarily spread in food and water contaminated by human feces [77, 78]. Only about 10% people show invasive symptoms and the rest of them can remain asymptomatic due to the host defense. In addition, *Entamoeba dispar*, a morphologically indistinguishable noninvasive amoeba, is involved in many asymptomatic cases. Distinguishing *E. histolytica* from *E. dispar* requires molecular or enzymatic characterization [79].

Furthermore, the pattern of amoebic infection, the presence of antibodies, manifestations of disease, an approach to investigations, and strategies for management remain complex [80]. *E. histolytica* trophozoites (amoebae) can damage the large intestine causing ulcers and sometimes they move to the liver, forming abscesses that could be fatal if not treated. *E. histolytica* can also affect nonhuman primates in captivity or wild life [81, 82]. The *in vitro* studies of amoebic pathogenesis have demonstrated three essential processes in the interaction of *E. histolytica* with target cells: (1) adherence of amoebae to cells, which is mediated in virulent strains by a GalNAc-inhibitable amoebic adhesin; (2) contact-dependent target cell lysis, and (3) amoebic phagocytosis of target cells [83, 84]. Many factors have been involved in promoting the invasiveness, pathogenicity, and virulence of *E. histolytica* [85].

Noteworthy, incidence of amoebiasis remains high nowadays when compared to the last century, in spite of the high efficacy of metronidazole treatment. However, this drug causes nausea, vomiting, and other side effects, in addition to be found mutagenic in bacterial cultures, and carcinogenic to experimental animal models [86, 87]. These findings, and the obtaining of resistant strains to metronidazole *in vitro*, encourage us to the development and/

Parasite	Protein and/or peptides	Experiments performed	References
Amoebozoa *Entamoeba histolytica*	**hLf bLF Lfcin4-14 LFcin17-30 LFampin265-284 LFchimera**	***In vitro* assays** Viability assays; *E. histolytica* trophozoites were incubated with the Lfs or peptides.Viability was established.Also, synergy of Lf with metronidazole was assayed.	[40, 73, 74]
	bLF	***In vivo***Murine intestinal amoebiasis model;Mice were intracecal inoculated with *E. histolytica* trophozoites, and then intestinal amoebiasis was developed. bLF was orally administered. Viability and infection of mice was determined.	[99]
	bLF	***In vivo***Amoebic liver abscess; mice were intraportal inoculated with *E. histolytica* trophozoites until liver abscess development, and then hepatic amoebiasis was developed. hLF was orally administered.Viability and infection of mice was determined.	[108]
Metamonada *Giardia intestinalis*	**hLf bLf Lfcin4-14**	***In vitro* assays** Viability assays; *G. intestinalis* cultures were incubated with hLf, bLF, and natural Lfcins. Viability was assessed.	[54]
	bLF LFcin17-30 LFampin265-284 LFchimera	***In vitro* assays** Viability assays, clinical isolates of *G. intestinalis* were incubated with bLF and the synthetic bLF derived peptides.Viability of cultures was determined.	[42]
	bLf	**Clinical trials** bLF versus placebo were administered to children for the prevention of diarrhea by *G. intestinalis.*	[103]
Apicomplexa *Cryptosporidium parvum*	**hLf bLf hLfcin bLfcin**	**Infectivity assay on host cell cultures** Preincubation of sporozoites with Lf or peptides and then, infection of Caco-2 cells.	[135]
Microsporidia *Encephalitozoon intestinalis*	**hLf bLfcin4-14**	**Spore germination assay on host cell cultures** Intestinal epithelial cells were infected with clinical isolates of *E. intestinalis* and then, were treated with hLf or bLFcin4-14.	[143]
Fungi *Candida albicans*	**pLf**	***In vivo* assay** Oral administration with porcine Lf-rich milk in mice pups infected with *C. albicans*	[147]
Apicomplexa *Toxoplasma gondii*	**bLF Lfcin**	***In vitro* assays** Viability assays; *T. gondii* was incubated with the Lfs or peptides. Viability and infectivity established. ***In vivo* assays** Infection of mice and pretreated or treated with bLF administered orally. Infectivity, parasitemia, and survival of mice were determined.	[148, 149] [150, 151]
Helminths *Haemonchus contortus*	**cLf**	***In vitro* assays** Effect of cLf on egg hatching and worm motility inhibition	[152]

Table 1. Parasites affected by lactoferrin and its natural or synthetic derived peptides.

or identification of new antiamoebic drugs that could replace metronidazole or synergize with it allowing a diminution in the dose of drug necessary for an effective treatment [88–90]. Up to date, there is no direct evidence of a protective role of Lf in human intestinal and hepatic amoebiasis. However, results from studies *in vitro* and in experimental animal models allow us to consider the use of Lf for both types of amoebiasis.

4.1.1. Studies in vitro *of use of lactoferrin against* E. histolytica *trophozoite growth*

Our group of research fractionated human milk and tested each fraction against amoebae in an axenic culture to search an effect of Lf, lysozyme, and secretory immunoglobulin A (sIgA); we also sought any combined effect among these molecules, and tested human, bovine, and swine milk against the parasite. For that, trophozoites of the strain HM-1:IMSS were treated with 5–20% of each milk, with 10% of human milk fractions, or with 1 mg/ml of isolated human milk Lf or sIgA, or chicken egg white lysozyme. From milks, only human and bovine milk were amoebicidal showing a concentration-dependent effect, which increased in the absence of iron. Human milk protein fractions (Lf, lysozyme, and sIgA) were amoebicidal, and Lf showed the major effect [74]. Regarding the mechanism of action, Lf bound to the amoebic membrane causing cell rounding, lipid disruption, and damage.

In another work, the microbicidal action of hLf, bLf, and Lfcin4-14 was established on the viability of *E. histolytica* trophozoites. Both Lfs and Lfcin were able to kill amoebae in a concentration-dependent manner. The effect was modulated according to the culture age, pH, and temperature and prevented by Fe^{2+} and Fe^{3+}. Mg^{2+} and Ca^{2+} prevented the killing effect of Lf but not of Lfcin. Parasites obtained from the stationary phase were more susceptible to Lf than those from the exponential phase. A synergistic effect was observed with metronidazole, decreasing about fivefold the concentration necessary to kill most amoebae [73, 74]. This observation is important, since as we mentioned before, metronidazole has been found toxic and mutagenic at the used concentrations. These data suggest that both Lfs and bLfcin might be used in amoebiasis if they are administered with low doses of metronidazole to have less toxicity of this drug. After that, we used the synthetic peptides Lfcin17-30, Lfampin256-284, and Lfchimera to search for an effect against *E. histolytica*. At 50 μM of each peptide, Lfcin and Lfampin showed a moderate amoebicidal effect, with 45–50% of amoeba viable at 24 h culture. However, at 50 μM Lfchimera, about 75% of amoebae were killed, whereas at 100 μM all parasites died. These data indicate that N-terminal Lf-peptides, mainly Lfchimera, have amoebicidal activity in a time- and concentration-dependent manner [40].

4.1.2. Effect of lactoferrin on a murine intestinal-amoebiasis model

Infection with *E. histolytica* may be confined to the intestinal lumen, or can result in invasion of the colonic mucosa (intestinal amoebiasis, IA). Pathologic changes of this mucosa initially are nonspecific but are followed by ulceration [77]. In a study with 3000 patients, it was found that the clinic-pathologic forms of the disease were: ulcerative rectocolitis (95%), typhloappendicitis (3%), amoeboma (1.5%), and fulminating colitis with toxic megacolon (0.5%) [91].

In addition to the studies *in vitro*, human breast milk and saliva secretions have been well documented to possess antiamoebic activity and, in addition to the sIgA antibodies, Lf could be one of the active molecules in IA [92–95]. Around 35 years ago, it was suggested that Lf present in milk could be involved in protecting against IA in some population groups. Prospective studies carried out on Turkana and Maasai African nomads that consume milk as the major item in their diet showed amoeba seronegativity or freedom from intestinal infection with *E. histolytica*, respectively, in contrast to similar nomads having a mixed diet. In both studies, the milk-drinker group showed iron deficiency, probably due to the poor supply of iron in the milk, and it was proposed that the low intestinal content of iron affected the growth of *E. histolytica*. Noteworthy, it was also proposed that Lf and Tf present in the milk may actively compete with amoeba for intestinal iron [92, 96]. Likewise, newborns are protected against infectious agents including amoeba while they are being breastfed. In a study carried out with 322 Egyptian infants of 2–6 months old, the group who had been breastfed since birth showed significantly lower incidence of parasitic infections than the other group who only received formula (38.5% versus 75.2%, respectively). Reduction in infections by *Cryptosporidium* spp., *E. histolytica*/*E. dispar*, *G. lamblia,* and *Blastocystis* spp., as well as mixed parasite infections, was observed. These studies suggested that cattle and breast milk contain components that can combat intestinal infections in humans [97].

In contrast to the well-documented antiamoebic potential of Lf *in vitro*, almost nothing is known about its effect on an intestinal model of infection. The only study of this type has been addressed by our group in a murine model of cecal amoebiasis with high success [98]. The model uses mice of the C3H/HeJ strain, which has a spontaneous mutation in the toll-like receptor 4 gene, Tlr4Lps-d, making these mice more resistant to endotoxin. Intracecal inoculation with virulent *E. histolytica* cultured trophozoites results in an inflammatory and ulcerative disease highly reminiscent of human IA, starting with tiny erosions of the surface epithelium at 5 days, which evolve to deeper and extensive destructive lesions of the cecal wall at 21 days, including flask-shaped ulcers, intestinal perforations, and intramural abscesses formation, without evidence of tissue invasion by amoebae. In this model, we found that a simple oral dose of bLf to mice controls the infection already established in the cecum [99]. Details of this experiment are included below paragraphs.

Germ-free mice of the C3H/HeJ strain were intracecally infected by 10^6 virulent amoebae (strain HM1:IMSS). Fourteen days post challenge, by which time amoeba-induced lesions are expected [98], a group of mice was orally treated with bLf (20 mg/kg), daily for 7 days. At 21 days, all mice were sacrificed and the ceca exscinded, fixed, and embedded in paraffin (**Figure 1**, upper cartoon). Finally, tissue sections were stained with hematoxylin-eosin for histological analysis. The results showed that infected mice receiving bLf cured IA in 63.14% as neither trophozoites nor tissue damage were found in sections of the ceca (**Figure 1A**). The rest of treated mice showed partial resolve of the infection, evidenced by reduction in the number of amoebae and tissue damage, compared with the untreated mice, which had inflamed and vascularized ceca with abundant mucus, amoebae, and microhemorrhages (**Figure 1B**). Intriguingly, a similar protocol of treatment with 200 mg/kg did not resolve the infection, which could be due to the formation of immune complexes between bLf and sIgA antibodies present in the intestinal lumen, and/or formation of anionic aggregates that occur

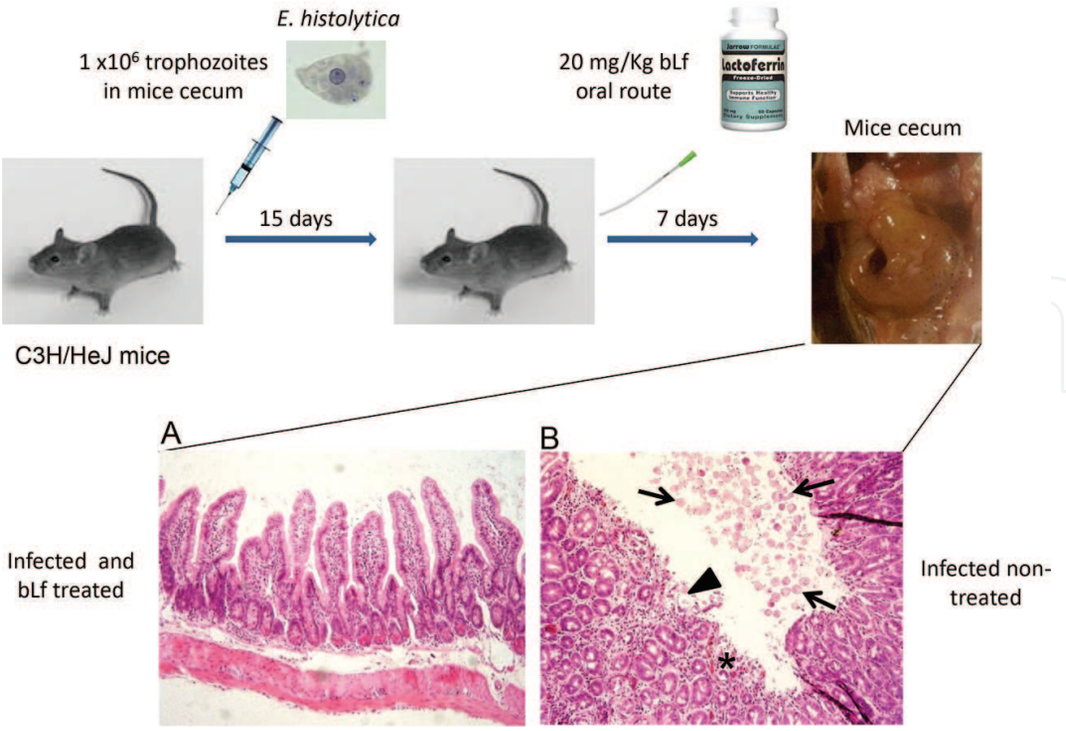

Figure 1. Treatment protocol with bLf against intestinal amoebiasis in a murine model. Above: Mice strain C3H/HeJ was intracecally infected with virulent E. histolytica trophozoites. Two weeks post-infection, mice were treated daily by oral route with 20 mg/kg bLf for 1 week. Upon completion of treatment, the mice were sacrificed and the ceca processed for histological analysis. Below: The treated mice showed absence of infection (left tissue section) compared to the ceca of infected but untreated mice (right tissue section), which showed many trophozoites in the lumen (arrows) and extensive damage of the intestinal epithelium, with loss of epithelial integrity (arrow head) and micro-hemorrhages (asterisk).

when high amounts of Lf are prepared in high salt concentrations [100]. It is worthy of noting that resolution of the IA by bLf correlated both with an increased production of total sIgA and an anti-inflammatory response, determined in cecum tissue extracts or tissue sections, respectively. The average of total sIgA levels in cured mice was twofold higher than that observed in the infected ones, and also higher in completely cured mice when compared to sIgA levels in mice with partial resolution of the infection. Also, whereas high expression of proinflammatory INFγ and TNFα as well as of regulatory IL-10 and TGFβ cytokines were observed in the ceca of infected mice, only high expression of IL-4 was observed in the bLf treated and cured mice. The immune-regulatory activity of Lf has been well documented, mainly downregulating the inflammatory response and reestablishing intestinal homeostasis, but also upregulating the humoral response [101, 102].

In conclusion, Lf might exert a protective effect against IA, through multiple mechanisms because of its multifaceted properties. Directly, Lf may perform amoebicidal activity disrupting the parasite membrane as suggested from the *in vitro* studies. Indirectly, Lf may boost the intestinal secretory immune response increasing the production of both, unspecific and specific antiamoeba IgA antibodies that could block the adherence of amoebae to the gut epithelium, or inhibit the growth of parasites by competing for local iron. Based on our studies aforementioned, and that the therapeutic use of Lf for treating infections causing diarrhea in humans is highly safe [103, 104], we suggest that oral daily treatment with a relatively low

dose of bLf for 1 week, either alone or in combination with metronidazole, could represent a new therapeutic strategy for curing the human intestinal infection caused by *E. histolytica*.

4.1.3. Effect of lactoferrin on the amoebic liver abscess

Intestinal amoebiasis may complicate by spreading of amoebae via the portal venous to the liver, or perforation of the intestinal wall, resulting in peritonitis or fistulas. Amoebic liver abscesses (ALA) may perforate into the peritoneal, pleural, or pericardial cavities. Hematogenous spreading of amoebae can also result in abscess formation in more distant sites, such as the brain [105]. ALA is the most important for no intestinal infection, due to its high frequency of occurrence and serious clinical concerns, since ALA occurs in up to 95% of fatal cases of amoebiasis. The abscess is composed of a thin capsular wall whose inner surface has "shaggy" appearance; microscopically, the abscess fluid is granular with eosinophilic debris and few or no cells. Smaller abscesses have been felt by some authors to form larger abscesses by coalescence; portal fibrosis and bile duct proliferation have been noted as part of a healing process [106].

ALA can be induced in animal models by intraportal inoculation of amoebae, and it presents a PMN infiltrate within the first 12 hours. As the neutrophils and hepatocytes lyse, the amoebae remain in debris of basophilic material. Later in the progression of abscess formation, these form a more organized capsule with collagen fibers and fibroblasts surrounded by macrophages and epithelioid cells. Experimental amoebiasis has been conducted to evaluate therapeutic regimens, immunology, or pathology of invasive amoebiasis [106, 107]. In this sense, we evaluated the therapeutic effect of bLf in a model of ALA in hamsters. Interestingly, hamsters treated intragastrically with Lf (2.5 mg/100 g body weight) over a period of 8 days, showed no clinical signs of disease and ALA was effectively decreased with only 0.63% detectable lesion, compared with 63% in untreated animals. Furthermore, liver function and blood cells approached normal levels in hamsters receiving bLf treatment [108]. These results suggest that bLf may aid in the therapy of amoebiasis, most likely without producing side effects in patients.

4.2. Giardia intestinalis

Giardia intestinalis (also known as *Giardia lamblia* or *Giardia duodenalis*) is a flagellated unicellular binucleated parasite that causes giardiasis, a diarrheal disease spread throughout the world [109]. Giardiasis is the most common cause of waterborne outbreaks of diarrhea. The prevalence of this parasitic disease commonly ranges from 20 to 30% of the population in developing countries or 3 to 7% in developed ones. Giardiasis is reported more frequently in young children (between 6 months and 5 years of age), and in chronically infected and immunesuppressed people, and also in susceptible travelers [109, 110].

Giardia species have two major stages in their lifecycle. First, infection with *G. intestinalis* initiates when the cysts are ingested in contaminated water or, less commonly, foods. The cyst is relatively inert, allowing prolonged survival in a variety of environmental conditions. Cysts excyst into trophozoites in the proximal small intestine, and then they attach to the lining of the small intestine and reproduce, interfering with the absorption of fats and carbohydrates from digested

foods, causing diarrhea and malabsorption [111]. After exposure to biliary fluid, some trophozoites form cysts in the jejunum and pass to the feces, allowing for completion the transmission cycle by infecting a new host [112, 113]. When the clinical signs of infection are present, they may include diarrhea, nausea, weight loss, and abdominal pain. Giardiasis is an established cause of failure to thrive in children; it also causes diminished cognitive functions and chronic fatigue. In adults, giardiasis may lead to postinfectious gastrointestinal disorders such as IBS and dyspepsia. In addition to diarrhea, *G. intestinalis* causes iron deficiency anemia, micronutrient deficiencies, protein-energy malnutrition, growth and cognitive retardation, and malabsorption. A few cases of *Giardia* associated with tumor masses have also been reported, but cause-to-effect relationship between giardiasis and cancer has yet to be established [114].

When giardiasis develops symptoms, a standard treatment mainly consists of metronidazole therapy. However, in addition to this drug causes side effects in patients, it has been associated with significant failure rates in clearing parasites from the gut [109]. Also, an increasing incidence of nitroimidazole-refractory giardiasis has been reported in travelers from India [115]. A correct fluid and electrolyte management is critical, mainly [22] in patients with large-volume diarrheal losses, and children with acute or chronic diarrhea in whom Giardia organisms have been identified [116–118]. In some patients, giardiasis resolves within a few days, whereas in others the symptoms last for years, even in the presence of circulating antigiardia antibodies in serum, or sIgA antibodies at mucosal sites and the cell-mediated immunity. Because of its biological features, it is likely that nonimmune factors play a role in the susceptibility or duration and severity of the disease. Both humoral and cell-mediated immune responses play a role in giardiasis, but the mechanisms involved are poorly known [119]. For example, human milk kills *G. duodenalis* trophozoites independently of specific sIgA antibodies [120]. The giardicidal factors present in milk are conjugated bile salts and unsaturated and free fatty acids [121–124]. Also, human neutrophil defensins and indolicidin were giardicides when they were added to the culture medium [111, 125].

It has been tested the effect of hLf, bLf, hLfcin, and bLfcin against *G. intestinalis*, *in vitro* [54]. On a molar basis, bLfcin had the most potent giardicidal activity, followed by hLfcin, bLf, and hLf; this effect was concentration-dependent and the activity estimated during 2 h of incubation. In addition, trophozoites from early stationary phase cultures were more susceptible to the parasiticidal effect. Intestinal factors and physiologic conditions present in the intestine did not have effect on the activity exhibited by Lfs and Lfcins. On the other hand, $MgCl_2$, $CaCl_2$, and $CoCl_2$ protected against the activity of hLf and bLf, but not of Lfcins against *G. intestinalis*. In the presence of ferric iron, neither Lfs nor Lfcins presented parasiticidal activity, indicating that iron has protective effects [54]. This finding is interesting, since Lfcins did not have any site for iron binding. Under electron microscopy, it was detected that giardias treated with Lfs and Lfcins showed striking and complex morphologic changes in plasmalemma, endomembrane, and cytoskeleton, and increased the electron density of lysosome-like peripheral vacuoles. Also, it was observed by confocal microscopy that Lfs and Lfcins are able to be bound by *G. intestinalis* membranes [126].

Recently, the effect of synthetic bovine Lfcins on the growth of *G. intestinalis* culture was studied. The peptides Lfcin17-30 and Lfampin265-284 and the fusion of both Lfchimera

showed microbicidal activity against *G. intestinalis* trophozoites. Apparently, the best effect was exerted by Lfchimera, since the first hour of incubation. Additionally, low concentrations of this peptide combined with low concentrations of metronidazole or albendazole had a better effect on the inhibition of *G. intestinalis* cultures than the drugs or peptides used alone. When the mechanism of action was explored by transmission and scanning electron microscopy, trophozoites treated with the synthetic Lfcins showed damage on membrane and internal structures [42].

The effect of bovine Lf has been also tested in patients. A randomized, double-blind, placebo-controlled trial was conducted, in a supplementation with bLf (0.5 g twice daily for 9 months), for the prevention of diarrhea in 26 children of 12–36 months of age, in Peru. In the comparison of results, the overall diarrhea incidence and prevalence rates were similar between the two groups (the Lf group versus the placebo group). However, there was a lower prevalence of colonization with *Giardia* species and better growth among children in the Lf group [103]. In conclusion, data from experiments *in vitro* and those from patients support the idea that Lf and Lfcins can be used in the defense against giardiasis.

4.3. Cryptosporidium parvum

Cryptosporidium parvum is an apicomplexan parasite of human and veterinary importance that causes diarrhea and gastroenteritis. Infection is common in children of developing countries with poor hygiene practices and no potable water supplies, where it has high seroprevalence rates and specific IgG seropositivity after 1 year of age, with recurrent infections and relapsing diarrhea [127–129]. The main risk factors are the ingestion of contaminated water, contact with infected persons or animals, and travel to endemic areas of the disease. The *Cryptosporidium* life cycle is divided into six major developmental phases; the infective sporozoites are produced after excystation of oocysts [130] that attach to the cell apical surfaces and become internalized within an intracellular but extra-cytoplasmic compartment, which is separated from the cytoplasm by an electron-dense layer that appears to be predominantly of host origin. In this compartment, parasite is protected from the hostile gut environment and supplied with energy and nutrients by the host cell through a feeder organelle, which is unique among apicomplexan parasites [131]. It has also been reported that *C. parvum* may have extracellular gregarine-like life stages [132].

In immunocompetent patients, diarrhea due to *C. parvum* is self-limited; however, cryptosporidiosis is recognized as an important disease in immunosuppressed people such as AIDS patients. By immunological and molecular techniques, researchers have identified over 25 putative virulence factors, which are proposed to be involved in aspects of host-pathogen interactions from adhesion and locomotion to invasion and proliferation [131, 133]. It has been investigated the increase of Lf in feces as an indicator of inflammation in healthy adult volunteers experimentally infected with oocysts, and in children with diarrhea that have naturally acquired *C. parvum.* Of the 21 specimens taken post challenge, only one of 14 *Cryptosporidium*-seropositive patients had Lf titer >1:50. In contrast, 12 of 17 specimens from children with only *Cryptosporidium* infection had mild to moderate elevation of fecal Lf. These results suggest that there may be a mild subclinical inflammatory component in

cryptosporidiosis in children with diarrhea. Also, that Lf increase is a good tool to detect inflammation in cryptosporidiosis [134].

Currently, there are no consistently effective parasite-specific pharmaceuticals or immunotherapies for control of cryptosporidiosis. Thus, several alternative therapies have been studied to combat this disease, among them, some natural compounds from the innate immune system. Some *in vitro* assays have been performed to demonstrate whether bLf, bLfcin, and a bLf pepsin hydrolysate (bLfh) have some effect against *C. parvum*. For that, freshly excysted sporozoites were incubated for 15 min in MEM containing 10 μg/ml of Lf or its derivative peptides; further, an infection to Caco-2 cells was done. The authors found that only the bLfh and bLfcin were highly parasiticidal decreasing sporozoite viability by 45–69% when compared to the control. In addition, these compounds strongly reduced sporozoite infectivity to the cells. The viability percentage was similar when the bLfh and bLfcin were used [135]. From these experiments, we can deduce that it would be remarkable the use of bLf derivatives to prevent or cure the infection by *C. parvum*.

4.4. Fungi

4.4.1. Microsporidia

Microsporidia are unicellular, obligate intracellular fungal parasites that affect a variety of vertebrate and invertebrate hosts. The phylum Microsporidia comprises 150 genera with more than 1200 species, from which only seven genera infect humans [136]. These parasites have been found in water sources and in wild, domestic, laboratory, and food-producing farm animals; thus, microsporidia can also cause zoonotic diseases. In addition, microsporidiosis is an emergent infection because the parasites are opportunistic agents in patients with HIV, or in those immunosuppressed by organ transplant, or in children and old people, affecting the gastrointestinal tract, nasopharynx, lungs, eyes, and skin [136, 137]. In the gastrointestinal tract, infection of differentiated mucosal epithelial cells most likely results from impalement via spores containing a unique coiled tube used to impale target cells and inject the infectious sporoplasm [138]. Spores germinate in the lumen in close proximity to the target cells [136, 139, 140]. In addition to the unique way in which microsporidia infect cells, *Encephalitozoon cuniculi* spores enter nonprofessional phagocytes by phagocytosis and traffic into a late endosomal-lysosomal compartment; after being phagocytosed, spores germinate within the cell [141, 142]. The pathogenesis of intestinal disease is related to excess death of enterocytes as a result of cellular infection. Clinically, microsporidiosis most often presents with diarrhea and weight loss as a result of small intestinal injury and malabsorption [140]. *Enterocytozoon bieneusi* is the most common microsporidial cause of human intestinal disease. A second species, *Encephalitozoon intestinalis* (originally named *Septata intestinalis*) is associated with disseminated as well as intestinal disease, and the second most common cause of intestinal microsporidiosis. Therapeutic options are few; *E. intestinalis* responds well to albendazole, whereas no antiparasitic therapy has documented efficacy in *E. bieneusi* infections [140].

Leitch and Ceballos [143] [E-CE3] studied clinical isolates of *E. intestinalis*. A spore germination assay and a cultured intestinal epithelial cell-infection assay were used to determine if hLf and bLfcin, in addition to lysozyme and defensins, could inhibit the infection. In this assay, cells

were cultured on collagen-coated chamber slides, and at 7 days post confluence, monolayers were infected with 4×10^5 spores per well. After 24 hours p.i., the excess of spores was removed with Opti-MEM containing 1 mg/ml chondroitin sulfate and the wells refilled with medium. At 3 days p.i., cells were fixed and stained to visualize parasite sporogonial stages [144] and detect host cell and parasite nuclei. The *Encephalitozoon* species were unaffected in germination by Lf up to a concentration of 2 mg/ml, or by bLfcin. However, bLf was able to significantly diminish the infection to enterocytes.

4.4.2. Candida albicans

Besides microsporidia, numerous *in vitro* and *in vivo* studies have been conducted demonstrating the potent capacity of Lf and derived peptides to inhibit the growth and infectivity of other fungal pathogens that can affect mucous membrane of the upper digestive tract, mouth, and pharynx, such as *Candida albicans*. Candida organisms commonly colonize the human gastrointestinal tract as a component of the resident microbiota. Their presence is generally benign. However, high-level colonization by *Candida* could delay healing of inflammatory lesions and that inflammation promotes colonization. Both BID and gastrointestinal *Candida* colonization are associated with elevated levels of the proinflammatory cytokine IL-17. Because *Candida* is a frequent colonizer, these effects have the potential to impact many people [145]; in addition, *C. albicans* gut colonization in mice aggravates inflammation in allergic and autoimmune diseases, not only in the gut but also in the extra-gut tissues and underscores the necessity of investigating the pathogenic role of *C. albicans* gut colonization in immune diseases in humans [146]. Since research about the effect of Lf has been ample in *Candida*, it could be interesting to perform experiments to demonstrate that Lf can help against both the gut inflammation and the pathogen. Despite not being an intestinal pathogen, there is a work that reports the benefit of lactation of mice pups with porcine Lf-rich milk against an oral infection with *C. albicans* [147]. Thus, treated CD1 mice showed lower bacterial counts when compared with normal fed controls as well as a healthier architecture in the small intestine [148], suggesting that porcine Lf can be used as a selective decontamination of the digestive tract regimen.

4.5. *Toxoplasma gondii*

Toxoplasma gondii is an obligatory deadly intracellular parasitic protozoan transmitted by ingestion of uncooked infected meat; this parasite resides in every nucleated cell causing severe complications in immunocompromised hosts. Tanaka et al. [149] examined the effect of bovine Lfcin (LFcin-B), a peptide composed of 25 amino acid residues, on the viability and infectivity of *T. gondii* parasites, both *in vitro* and *in vivo.* After treatment of *T. gondii* with Lfcin at 100 μg/ml for 1 h, 65% of the parasites became oval in shape and had lost the ability to exclude the trypan blue dye, a vital staining. Interestingly, approximately 96% of the parasites treated with Lfcin at 1000 μg/ml for 0.5 h lost the dye exclusion ability. In contrast, more than 80% of the parasites incubated with bLf or a C-terminal peptide at 1000 μg/ml for 4 h retained the dye exclusion ability. On the other hand, the loss of infectivity of the parasites and/or cystozoites in cyst was confirmed by inoculation of mice. Five mice inoculated with 10^2 untreated parasites died within 9 days post challenge. Similarly,

parasites pretreated with bLf at 1000 μg/ml caused 100% mortality of inoculated mice within 9 days post challenge. In contrast, four of five mice inoculated with the same dose of parasites pretreated with Lfcin at 1000 μg/ml survived for more than 30 days post challenge. In the case of parasites pretreated with Lfcin at 100 μg/ml, one of five mice survived up to 30 days post challenge. In conclusion, this Lfcin peptide derived from bLf could be used against human toxoplasmosis. To study the effector pathway of *Toxoplasma* growth inhibitory activity induced by bLf in murine macrophage, the role of reactive oxygen intermediates (O^{2-}) and inorganic nitric oxide (NO) was examined by Tanaka et al. [150]. Production of O^{2-} was diminished in cultures of macrophages supplemented with bLf and the effect was dose and time dependent. Production of NO was enhanced in cultures of peritoneal macrophages supplemented with interferon-gamma, but not with bLf. Their findings suggested that this *Toxoplasma* growth-inhibitory activity induced by bLf in macrophages is not mediated by O^{2-} or NO molecules; it may be mediated by an L-arginine-dependent effector pathway that does not involve NO production. The same group of work [151] administered orally or intraperitoneally bLfcin (5 and 0.1 mg/mouse, respectively). Afterward, the researchers challenged mice with cysts of *T. gondii* at a dose of LD90. Although only a small number of mice were used, both administration routes of Lfcin prevented the death in the 100% mice.

Lf also has shown antimicrobial properties in its nanoformulation using alginate chitosan calcium phosphate bLf nanocapsules (AEC-CCo-CP-bLf-NCs). Anand et al. [152] analyzed and compared the effect of bLf in its native as well as nanoformulation AEC-CCo-CP-bLf-NC against coccidian parasite *T. gondii*. The J7741 macrophage cell line culture model showed a significant increase in NO production and low parasitemia. In their *in vivo* BALB/c mice model, after treatment with NCs substantially increased the bioavailability of the protein and showed comparatively increased levels of reactive oxygen species, NO production, and Th1 cytokine which helped in parasite clearance. Regarding to the mechanism of action of NCs, immunoreactivity analysis showed accumulation of Lf in macrophages of various visceral organs, which are the site of parasite multiplication.

4.6. Helminths

Antipathogenic properties of camel milk have been investigated to substitute for drugs hence overcome drug resistance. Recently, Alimi et al. [153] investigated the antihelminthic activity of the chemical compounds of camel milk. *In vitro,* the antihelminthic effects of camel milk against *Haemonchus contortus* (nematode) from sheep were ascertained by egg hatching and worm motility inhibition, in comparison to milks from cow, ewe, and goat, and to the reference drug albendazole. Chemical compounds revealed that camel milk has higher contents of protective protein Lf and vitamin C than other species' milk; for example, the camel milk contains sevenfold more Lf than the cow milk. Camel milk showed ovicidal activity at all tested concentrations and completely inhibited egg hatching at concentrations close to 100 mg/ml (IC50 = 42.39 mg/ml). Also, camel milk revealed *in vitro* activity against adult parasites in terms of the paralysis and/or death of the worms at different hours post-treatment. After 8 h of exposure, camel milk induced 100% mortality at the highest tested concentration. In

contrast, there was 82.3% immobility of worms in albendazole at 8 h postexposition. Bioactive compounds such as Lf and vitamin C may be involved in such an effect.

5. High-scale production of lactoferrin

Lf is considered as a nutraceutical protein by certain countries. Because of its versatile properties on health and the null toxicity to humans, Lf can be added to different foods and nutritional supplements, in addition to be used in medicine as an immune modulator, antimicrobial, antiviral, and anticarcinogenic, among other properties, some of them unknown so far. Since the finding of the regulatory property of Lf, much research has been published about this molecule; nowadays we can found almost 7500 references in Pubmed for this glycoprotein.

Currently, Lf is one of the most studied proteins in order to have it commercially available and with full biological activity. Concerning this, Lf from different origins, mainly from human and bovine, but also from camel, buffalo, and other animals, has been obtained from milk and colostrum. To have a better quality and production of Lf, since 25 years ago Lf has been cloned in different vectors and expressed overall in eukaryotic systems which can glycosylate it, such as yeasts and fungi [154–156]. From these organisms, Lf has been highly purified as a recombinant protein and its biological role, mainly antibacterial, has been confirmed. In addition, human Lf has been cloned in transgenic cows and plants [156–159]. Interestingly, recombinant hLf expressed in cows enhanced systematic and intestinal immune responses in piglets, used as a model of infants [160]. In addition, when researchers analyzed the composition of meat from the offspring of hLF transgenic cows, which can express hLf protein in their mammary gland, they did not found any abnormality on the meat nutrient composition of hLF bulls [161]. Therefore, the ample use of Lf in the human health care is promissory.

Commercially available Lf is now offered by several companies for using in research, as a food supplement, as antibacterial or to increase immunity to improving health. Mainly skim milk and cheese whey that have not undergone rigorous heating can be sources of bLF. Because Lf has a cationic nature, it has been purified by cation exchange chromatography in bLF-supplying companies [162–164]. The Japanese Morinaga Milk Co. was the pioneer in research and development of bLf and in the addition of this protein to milk formula and other products are also in the use of Lf in clinical trials. Nowadays, there are numerous patents of Lf from companies that produce Lf of high quality. As examples, those of Nestle for infant memory and learning and promotion of brain maturation in children or another one to be used as antidiarrhea; Fonterra and Tatua, from New Zealand; Pharming Group from The Netherlands; Abial Biotech from Spain; Tatura-Bio from Australia, and NRL-Pharma from Japan, which produces enteric-coating Lf for use in adults in who the whole Lf molecule can rise the intestine. Highly purified Lf without LPS is produced by Taradon Laboratory. On the other hand, recombinant hLF for use in animal and human clinical studies has been produced in the fungus *Aspergillus niger* var. *awamori* by Agennix Inc., for the potential treatment of cancers, asthma, and chronic wounds; in transgenic cows by Pharming Technologies N.V. as a nutraceutical; and in rice by Ventria Bioscience for diarrhea and iron deficiency [164].

6. Conclusion and perspectives

Lf and its derived peptides Lfcins could be an option in the treatment of intestinal parasitic diseases, based mainly on results *in vitro* and in animal models. Research in this glycoprotein has generally been leaded with success, although it is necessary to deep in the understanding of the mechanisms of action of Lf against parasites. In general, drugs used in the therapy antiparasites cause toxic side effects, and/or the parasites can become resistant to them. Lf is an innocuous protein that could be used as adjunct with drugs, with the considerable advantage of using low doses of drugs due to the synergic effect of Lf. It would be required more studies in animal models and carry out strict clinical trials with methodologic accuracy and a large number of patients, in order to extend the use of Lf as a parasiticide.

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Abbreviations

ALA	Amoebic liver abscess
apoLf	Apolactoferrin
bLf	Bovine Lf
BID	Bowel inflammatory disease
INFγ	Gamma interferon
holoLf	Hololactoferrin
HIV	Human immune deficiency virus
hLf	Human Lf
IL	Interleukin
IA	Intestinal amoebiasis
IBS	Irritable bowel syndrome
Lf	Lactoferrin
Lfcin	Lactoferricin
MEM	Minimal essential medium
MIC	Minimal inhibitory concentration

PMN	Polymorphonuclear
p.i.	Post-infection
sIgA	Secretory immunoglobulin A
Tf	Transferrin
TGF?	Tumor growth factor beta
TNF?	Tumor necrosis factor alpha

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