EFFECT OF LACTOFERRIN ON PEDIATRIC INFECTIONS

Theresa J. Ochoa

KU Leuven
Biomedical Sciences Group
Faculty of Medicine
Department of Immunology, Microbiology and Transplantation

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Effect of lactoferrin on pediatric infections

Theresa J. Ochoa, MD

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Jury:
Supervisor: Prof. Dr. Jan Jacobs
Co-supervisor: Prof. Dr. Veerle Cossey
Chair examining committee: Prof. Dr. John Creemers
Chair public defence: Prof. Dr. Dominique Bullens
Jury members:
Prof. Dr. Jaan Toelen
Prof. Dr. Christine Vanhole
Prof. Dr. William McGuire
Prof. Dr. Paolo Manzoni
Dr. Marijn Vermeulen

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Theresa J. Ochoa

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Composition of Examination Committee

Promoter:	
Prof. Dr. Jan Jacobs	Department of Microbiology, Immunology and Transplantation, KU Leuven, and Unit of Tropical Bacteriology, Institute of Tropical Medicine, Antwerp, Belgium
Co-Promoter:	
Prof. Dr. Veerle Cossey	Department of Development and Regeneration, KU Leuven, and Neonatal Intensive Care Unit, University Hospitals, Leuven, Belgium
Chair examining committee:	
Prof. Dr. John Creemers	Department of Human Genetics, KU Leuven, Belgium
Chair public defence:	
Prof. Dr. Dominique Bullens	Department of Microbiology, Immunology and Transplantation, KU Leuven, Belgium
Jury members:	
Prof. Dr. Jaan Toelen	Department of Development and Regeneration, KU Leuven. Department of Paediatrics, University Hospitals Leuven, Belgium
Prof. Dr. Christine Vanhole	Department of Development and Regeneration, KU Leuven. Neonatal Intensive Care Unit, University Hospitals Leuven, Belgium
Prof. Dr. William McGuire	Centre for Reviews and Dissemination and Hull York Medical School, University of York, England
Prof. Dr. Paolo Manzoni	Division of Pediatrics and Neonatology, Degli Infermi University Hospital, Biella, Italy
Dr. Marijn Vermeulen	Sophia Children´s Hospital, Department of Neonatology, Erasmus University Rotterdam, The Netherlands

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Introduction

Infections represent an important cause of morbidity and mortality in children especially in resource limited countries.[1] Multiple preventive interventions have been designed to decrease mortality and disability related to infections in children. One of the most important and cost-effective interventions to reduce infant mortality is exclusive breastfeeding.[2,3] Lactoferrin is one of the major factors present in milk responsible for these protective effects.[4] We hypothesize that lactoferrin given as an oral supplement to infants in resource-limited countries will improve their health by mimicking its protective roles in breast milk, thereby decreasing the incidence and severity of common pediatric infections due to its antimicrobial and immunomodulatory properties.

This thesis focuses on the effect of lactoferrin on enteric infections (Chapter 1) and on neonatal infections (Chapters 2-5). In this introductory part we will review the morbidity and mortality associated with enteric and neonatal infections, then we will review the effect of breast milk on prevention of these infections, and finally we will focus on the structure, biological functions, and mechanisms of action and on the pre-clinical and clinical studies of lactoferrin. This introduction is partially based on our recent review on lactoferrin and prematurity (Ochoa & Sizonenko 2017)[5].

PERU

The studies related to this PhD thesis have been conducted in Lima, Peru, where I live and work. Peru is an upper middle income country with a large geographic diversity and high socio-economic and healthcare heterogeneity. Box 1 describes the main development and health indicators of Peru.

Box 1: Peru: Current development and health indicators.

PERU

- Peru is located in South America along the Pacific Ocean.
- Is an upper middle income country. Currently has a high human development index of 0.741
- The Andes Mountains divides the country in three regions: the coastal region, the mountain region and the Amazon jungle region.
- The population is 32 million: 56% is in the coast, 30% in the mountains and 14% in the jungle; 21% of the population is rural.
- Under-five mortality rate: 14 per 1000 live births
- Infant mortality rate: 11 per 1000 live births
- Fertility rate: 2.1
- Breastfeeding in infants < 6 months: 64%
- Under-five chronic malnutrition: 12% overall, 25% in rural areas.
- Prevalence of anemia in children 6-35 months of age: 42% overall, 49% in rural areas.

(https://www.inei.gob.pe/)

ENTERIC INFECTIONS

Diarrheal disease still represents a high-burden public health problem in resource-limited countries, despite advances in understanding and management achieved in recent decades. Diarrhea is one of the leading causes of death in young children; the World Health Organization (WHO) and the United Nations Children´s Fund (UNICEF) estimate that approximately 0.5 million diarrheal deaths occur yearly in children younger than 5 years of age, mainly in sub-Saharan Africa and South-East Asia.[1] Moreover, multiple episodes of acute diarrhea and persistent diarrhea seriously affect growth, nutritional status and cognition.[6-9]

The main pathogens associated with diarrhea in children are viruses (rotavirus, norovirus and enteric adenovirus) and bacteria (*Shigella, Campylobacter*, diarrheagenic *Escherichia coli*, *Salmonella*). However, there are important differences on the distribution of pathogens by age and by the methods used for diagnosis.

Among the interventions implemented to decrease the incidence, severity and deaths associated with diarrhea, the most important have been the use of low osmolarity oral rehydration solutions and zinc, water and improve sanitation, and the introduction of rotavirus vaccine; the latter being the most significant intervention to decrease diarrhea mortality. However, the effectiveness of rotavirus vaccine in resource-limited countries is not as good as in resource-rich countries[10], probably related to differences in the intestinal microbiome, the co-administration of other oral vaccines and passive transfer of rotavirus antibodies to the infants.[11] Thus, diarrhea remains as an important morbidity in young children in resource-limited countries.

NEONATAL INFECTIONS

Three million neonates die each year, mainly in resource-limited countries.[12] The main causes of death in the neonatal period are prematurity, birth asphyxia (or complications at birth) and neonatal infections.[1,12] Approximately 99% of neonatal deaths occur in low- and middle-income countries, with 78% of all neonatal deaths occurring during the first week of life.[13]

Neonatal infections are responsible for 13% of all neonatal mortality and 42% of deaths in the first week of life.[14] The rates of infection are higher in infants with lower birth weight; infants born with less than 1500g, so-called *very low birth weight infants* (VLBW), have the highest rates of sepsis. [12,15] The increased risk of infections in preterm infants is due primarily to the immaturity of their immune systems, in addition to alteration of their skin and mucosa barriers, invasive procedures, parental nutrition, use of broad-spectrum antibiotics, among other factors.[12] Thus, preterm infants require additional immune protection.

In addition to sepsis, premature and low birth weight infants are at higher risk for developing necrotizing enterocolitis (NEC). Important risk factors for NEC are intestinal immaturity, infant formula feedings, bacterial colonization and inflammation.[15-17] Infants that develop NEC have worse neurodevelopment.[18,19]

Given the high incidence and high morbidity and mortality of sepsis and NEC in preterm infants, many interventions (fluconazole prophylaxis, intravenous immunoglobulin and colony-stimulating factors) have been designed to reduce the rates of infection in this vulnerable population.[20,21] More recently, there is good evidence on the effect of prebiotics and probiotics; however, there are still some concerns on the choice and safety of these formulations.[22] Selected nutritional components (L-glutamine, L-arginine) may also help reduce the rates of infection and NEC[23]; nevertheless, new preventive interventions are still needed.

PROTECTIVE EFFECTS OF BREAST MILK

Breast milk, as a result of millions of years of evolution, is the perfect nutrition for infants. Human milk has the right type and amount of proteins, lipids, carbohydrates and micronutrients for each stage and requirement of the child. In addition, human milk also works as an immune-protective food that aids the immature neonatal immune system. Breast milk promotes the proliferation of a diverse and balanced microbiota. Microorganisms stimulated by breast milk (*Bifidobacteria*, *Lactobacillus* and *Bacteroides*) promote health and activate several immunological functions in the neonate, such as tolerance and mucosal barrier homeostasis which impairs translocation of pathogens across the gastrointestinal tract.[24,25]

It has been demonstrated that breast feeding is an effective intervention for protection from diarrhea, prevention of all causes of infant mortality and decrease rates of infection and NEC in preterm infants. [3,26-35]

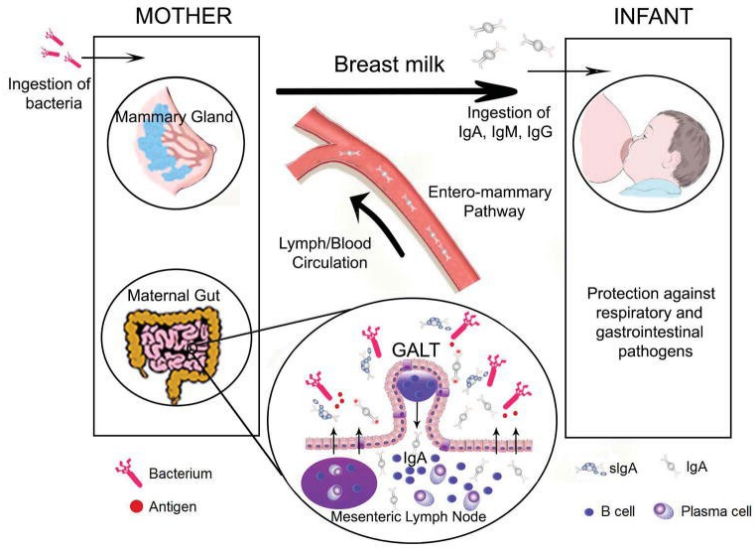

Figure 1. Immunomodulatory factors transmitted though breast milk. There is an integration between the mucosal immunity of the mother and the newborn. The mammary gland produces IgA antibodies specific for enteric and respiratory pathogens (Turin & Ochoa 2014)[27]

Human milk has multiple anti-infective, anti-inflammatory, and immunoregulatory components responsible for these protective effects. Factors produced by the mother's acquired and innate immune system and transmitted through milk, include secretory antibodies (sIgA), oligosaccharides, lactoferrin, lysozyme, leukocytes, cytokines among others[36-38] (Figure 1).

The composition of breast milk changes according to the infant´s age, nutritional requirements and need for passive protection. Breast milk is "the gold standard for protective nutrients".[2] Some of these innate immune factors are different in mothers´ milk of term and preterm infants.[39,40] The recognition of these bioactive milk factors and their distinctive mechanisms by which they protect provides models for new therapeutic and preventive approaches in pediatrics.

Previous studies have demonstrated that the consumption of breastmilk and colostrum [first form of milk produced by the mammary glands in the first 5 days of life, high in antibodies and proteins] offers protection against sepsis and NEC in VLBW infants.[41-46] A recent meta-analysis of 44 studies found that human milk provides clear protection against NEC and possible reduction of LOS and retinopathy of prematurity (ROP). The study concluded that any volume of human milk is better than exclusive preterm formula, and the higher the dose of human milk the greater the protection.[47]

LACTOFERRIN

Lactoferrin (LF) is an iron-binding protein that belongs to the transferrin family; it was first identified in whey milk by Sorensen & Sorensen in 1939.[48] Lactoferrin is present in most exocrine secretions including milk, tears, saliva, intestinal mucus and genital secretions, and in the specific granules of neutrophils, as part of the innate immune system.[4,49] Many of the biological functions of lactoferrin are related to its iron binding capacity and to the surface structure of the molecule.[50,51]

Structure

Lactoferrin is a ≈ 80kDa protein; it consists of a single polypeptide chain of 690 amino acid residues that is folded into two globular lobes, the N-lobe and the C-lobe (representing the N-terminal and the C-terminal halves). Each lobe is divided into two domains (N1 and N2, and C1 and C2, respectively). Each lobe has an iron binding site located between the two domains.[50] Depending on the iron status, lactoferrin can adopt two conformational states: it can be either "closed" when iron-bound (Fe2LF) or "open" when iron-free (apo-state) (Figure 2). The closed, metal-bound form is stable and rigid; in contrast the metal-free state is flexible, alternating between open and closed forms.[50] Lactoferrin binds iron better than transferrin at low pH. This iron-binding capacity produces local iron deprivation, which is important in preventing bacterial growth and biofilm formation. In addition, lactoferrin serves to regulate or inhibit iron absorption in infants.[51]

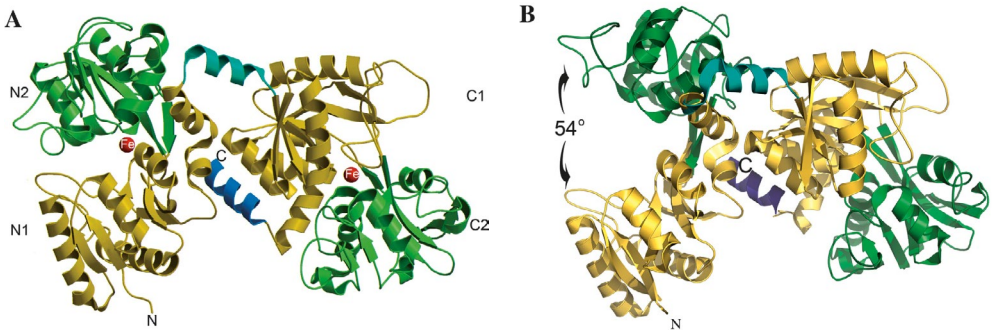

Figure 2. Lactoferrin structure. (A) Iron-bound form with N1 and N2 domains closed over the iron atom (red circle), the same for C1 and C2. (B) Iron-free form (Apo-LF). The N-lobe structure is open (Adapted from Baker 2012).[50]

Lactoferrin is highly positively charged, with an isoelectric point of 9–10. This cationic characteristic is responsible for its ability to bind different structures and molecules such as nucleic acids, proteins, cells and others.[50] Lactoferrin has a hot spot of positive charge located on the N-terminus (Figure 3). This part is responsible for binding LPS, DNA, heparin and glycosaminoglycans. The delta-lactoferrin isoform, which is expressed intracellularly, has a deletion on this N-terminal cationic portion of lactoferrin. Studies have demonstrated that lactoferrin can enter various eukaryotic cells and influence gene transcription.[4]

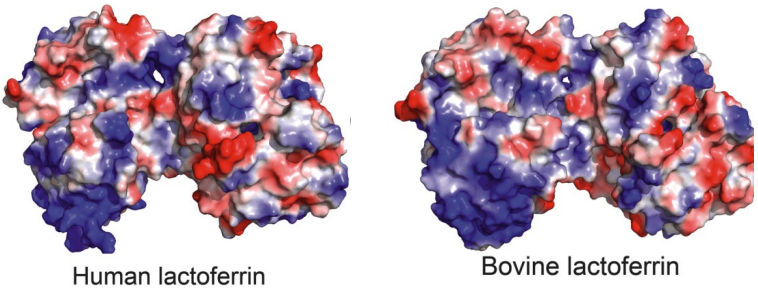

Figure 3. Surface charge on lactoferrin. Blue=positive; red=negative. A major hot spot of positive charge is found on the N-terminus of the molecule in both human and bovine LF (Adapted from Baker 2012).[50]

There are several peptides derived from lactoferrin with antimicrobial, antifungal and antitumor activities.[4] Human lactoferricin includes residues 1-49, whereas bovine lactoferricin includes 17-49. Lactoferricin occurs naturally, since it has been identified as a breakdown product in the human gut. Bovine lactoferricin is more bacteriostatic than human lactoferricin.[4] New lactoferricin-derived peptides and chimeric peptides have been produced with potent cytotoxic activity in cancer cells.[52]

The glycosylation patterns of lactoferrin vary between species and can probably influence its function. Human lactoferrin has three N-glycosylation sites, bovine has five potential sites. Most of the lactoferrin glycan terminate with a sialic acid

residue bind to galactose, the same moiety that is targeted by many viruses and bacterial proteins.[53] Thus, some of the reported antiviral and antibacterial activities of lactoferrin may well be mediated through its glycans.

Lactoferrin gene

The lactoferrin gene is located in the human chromosome 3p; it is organized into 17 exons.[54] Expression of the lactoferrin gene is both constitutive and inducible; it is differentially regulated through multiple signaling pathways such as steroid hormone, growth factor, and kinase cascade pathways. Other factors that may regulate lactoferrin expression in the mammary gland are prolactin and changes in cell shape and actin cytoskeleton.[55,56]

Lactoferrin gene single nucleotide polymorphisms (SNP) and isoforms of lactoferrin have been reported in humans and other species.[57] However, few studies have looked for an association between lactoferrin SNPs and disease. Lactoferrin polymorphisms have been linked to localized juvenile periodontitis[58,59], dental caries[60] and travelers´ diarrhea.[61] There are no studies describing the association of SNPs in the lactoferrin gene and concentration of this protein in human breast milk. However, studies in goats[62] and in cows[63] have found a correlation, giving support to the concept that lactoferrin concentration in breast milk may vary related to certain SNPs.

Types of lactoferrin

Comparison between bovine and human lactoferrin.
Almost all mammals have lactoferrin. Human and bovine lactoferrin have a sequence identity of 69% based on the protein sequence alignment. They consist of 690 and 689 amino acids, respectively.[64] The 3-D structures of bovine and human lactoferrin are very similar (Figure 3). One of the main differences is the concentration. Lactoferrin concentration in bovine milk is very low (1.5 mg/mL in colostral whey and 20-200 μg/mL in milk); ten to five time less than in human milk.[65] Although differences in structural and biochemical properties exist, their bioactivity, as assessed in vitro or in animal models, is quite comparable.[65,66] Large quantities of bovine lactoferrin is commercially available and is currently used for many applications. However, commercial bovine LF may be different from native bovine LF isolated from bovine milk.[67]

Recombinant human lactoferrin.
With the development of genetic engineering techniques, several expression systems have been developed to produce recombinant human lactoferrin. Some examples are talactoferrin, a recombinant human lactoferrin produced in *Aspergillus niger* var awamori[68] that has been tested in clinical trials in adults and neonates with sepsis[69,70]; a recombinant human lactoferrin produced in rice[71] also tested in a trial of pediatric diarrhea[72]. More recently, a recombinant human lactoferrin with mammalian glycosylation pattern has been developed in the Chinese hamster ovary (CHO) cell line[73] and in multiple lines of transgenic dairy animals (cows and goats), increasing the production level.[74]

In all recombinant human lactoferrins the amino acid sequence and structure is similar; however, there are different glycosylation patterns based on the species where they are produced. The glycosylation seems not to influence the structure of the molecule and many biological functions. For example, human lactoferrin, talactoferrin and bovine lactoferrin have similar -but not identical- bioactivities tested *in vitro* and in tissue cultured cell. [75,76]

Mechanisms of action

Antimicrobial effects of lactoferrin.
Lactoferrin activity against infections is related to its "direct" antimicrobial effect on the microorganism and to its "indirect" effect based on its interaction with the immune system, enhancing its antibacterial and antifungal activities.

Lactoferrin protects against bacteria in different ways: it sequesters iron that is essential for bacterial growth; binds to the lipid A portion of lipopolysaccharide (LPS) on the cell surface of Gram-negative bacteria, disrupting the bacterial cell membrane;[77,78] lactoferricin (the N-terminal peptide fragment of bovine lactoferrin after exposure to pepsin) is bactericidal in vitro for Gram-positive and Gram-negative bacteria and yeast; lactoferrin decreases the ability of these pathogens to adhere or to invade mammalian cells by binding to, or degrading, specific virulence proteins.[79] These mechanisms support the hypothesis that lactoferrin could protect infants from infection by preventing the attachment of pathogens in the gut. However, lactoferrin also has an impact on non-gut infections (*e.g.* central line associated bloodstream infections): it has been demonstrated that lactoferrin binds to lipoteichoic acid of Gram-positive organisms; and has antifungal activity against a range of yeast and molds, alone or in combination with other antifungal drugs.[80] (Table 1).

Table 1. Selected *in vivo* and *in vitro* studies showing lactoferrin´s antimicrobial effect

Pathogen	Lactoferrin effect
Gram-negative bacteria	• Protects mice from a lethal dose of parenterally administered *E. coli*[81] • Protects against endotoxin-induced lethal shock in piglets[82] • Neutralizes endotoxin[83] • Protects rats from gut-related *E. coli* systemic infections[84]
Gram-positive bacteria	• Interacts with *Staphylococcus aureus, Streptococcus pneumoniae* and *Listeria monocytogenes*[85] • Enhances clearance of *E. coli* and *S. aureus* injected intravenously (IV) in mice[86] • Decreases bacterial counts and modulates the immune response of *S. aureus* system infection in piglets[87]
Candida spp	• Decreases size and number of infectious foci of *Candida albicans* in different organs in mice[88] • Synergistically with lactoferoxidase works against *C. albicans*, reducing the volume of the mycelia and changing the size and shape of the cell[89]

Anti-inflammatory and immunomodulatory properties of lactoferrin.
Lactoferrin is a "multifunctional molecule in immunity". [90] It can have a direct effect on the pathogens and at the same time protect against the excessive and damaging host responses in mammals.[49,90] After lactoferrin binds to cell surface receptors, it can elicit a signal pathway or the translocation of lactoferrin into the nucleus to influence gene transcription. This process is part of lactoferrin's immunomodulatory role.[91]

Lactoferrin modulates iron homeostasis during inflammation; it also directly regulates the inflammatory response, probably following its release from neutrophils.[51] Lactoferrin binds to bacterial endotoxin (LPS) and as a result there is a reduction on LPS-mediated upregulation of inflammatory cytokines. Lactoferrin sequesters "free" iron at inflammatory foci, thus preventing catalysis of the production of damaging free radicals.[92,93]

Lactoferrin downregulates pro-inflammatory cytokines in intestinal epithelial cells infected with invasive and noninvasive *E. coli* strains, which may represent an important natural mechanism in regulating epithelial cell responses to pathogenic bacteria, and in limiting cell damage and the spread of infection.[94] In addition, lactoferrin protects against barrier dysfunction, induced by infection or inflammation, in human intestinal cells. Bovine lactoferrin can restore the tight junction morphometry and inhibit cell apoptosis induced by TNF-α.[95]

Lactoferrin has several up-regulatory mechanisms: (1) stimulates maturation of T-lymphocytes, promoting either Th1 or Th2 cytokine profiles; (2) recruitment and activation of antigen-presenting cells, initiating the inflammatory cascade; (3) production of interleukin-18, type I interferons and increased natural killer cell activity, as part of its gut-associated immune functions.[49,90,96]

Much of the impact on extraintestinal manifestations of infections is likely to be related to immunomodulatory effects of oral lactoferrin. Zimechi[97] and Artym[98,99] showed that in mice oral bovine lactoferrin upregulates cellular and humoral immune responses. Similar enhanced immune responses have been demonstrated in humans. Healthy adults have been shown to have oral lactoferrin mediated increases in total T-cell activation, helper T-cell activation, cytotoxic T-cell activation, and antioxidant capacity.[100] Patients undergoing thyroid surgery who were treated with oral lactoferrin had improved immune responsiveness.[101] Children with HIV infection upregulated phagocytosis and intracellular pathogen killing, as well as Toll-like receptor 2 expression when given oral lactoferrin.[102]

In summary, lactoferrin reduces inflammation by decreasing production of tumor necrosis factor alpha and other proinflammatory molecules, and by regulating the immune response, protecting against severe inflammation related to infection and septic shock, and protecting against gut barrier dysfunction. This concept supports the hypothesis that lactoferrin could protect infants from infections and neurodevelopment impairment by decreasing both direct and indirect (inflammation-related) injury.

Lactoferrin protection against enteric infections in infants.
Lactoferrin may protect infants against enteric pathogens based on several previous *in vitro* and *in vivo* studies (Table 2).

Table 2. Lactoferrin´s effect against enteric pathogens

Pathogen	Lactoferrin effect
Intestinal bacteria	• Binds to diarrheagenic *E. coli**, *Shigella flexneri* and *Salmonella* Typhimurium[103-105] • Binds to *E. coli* colonization factors[106], inhibits hemagglutination[107] and inhibits adherence of ETEC to epithelial cells *in vitro* and to intestinal mucosa of germfree mice *in vivo*[108] • Inhibits adherence of EPEC, DAEC and EAEC to tissue culture cells[109,110]
Intestinal viruses	• Induces suppression of rotavirus attachment and replication *in vitro*, with high neutralization values[111,112] • Interferes with feline and murine calicivirus infection by blocking viral attachment and replication[113,114] • Prevents adenovirus replication[115] with antiviral activity located in the N terminus[116,117]
Intestinal parasites	• Causes ultrastructural changes and kills Giardia,[118,119] and prevents the formation of infective cyst[120] • Binds to and kills *Entamoeba histolytica* trophozoites[121,122] and *Cryptosporidium parvum* sporozoites[123] • In the hamster model of amebic liver abscess decreases the infection in the liver[124]

* Diarrheagenic *E. coli*: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorragic *E. coli* (EHEC), diffusely adherent *E. coli* (DAEC) and enteroaggregative *E. coli* (EAEC).

Lactoferrin protection against infections and NEC in neonates
Lactoferrin may protect newborns against infection mainly by three mechanisms (Box 2)

Box 2. Mechanisms of lactoferrin protections against infections in infants*

Mechanism	Effect
Modulation of bacterial growth in the gastrointestinal tract	Enhances diversity of the intestinal microflora, and promotes higher concentrations of *Bifidobacterium* and *Lactobacillus*
Promotion of intestinal cell proliferation, differentiation and maturation	Decreases intestinal permeability and prevents bacterial translocation from the gut to the bloodstream
Regulation of the host immune response	Limits cell damage and the spread of infection

*Adapted from Embleton[96] and Ochoa & Sizonenko 2017[5]

There are several specific mechanisms of lactoferrin protection (Table 3). These protective effects are much more relevant in the premature infant who is at risk of infection, inflammation and oxidative stress injuries.[4,5,96]

Table 3. Potential protective mechanisms of lactoferrin in preterm infants against infection and NEC*

	Mechanism	Specific mechanisms or implications
1	Iron binding	Iron sequestration inhibits bacterial growth
2	Destabilization of microorganism´s cell membrane	Gram-negative bacteria: LF binds to the lipid A portion of LPS on the cell surface. Gram-positive bacteria: LF has anti-lipoteichoic acid activity Fungus: LF has anti-Candida cell wall activity
3	Binding to viral and bacterial host cell receptors	Decreases the ability of pathogens to adhere or to invade mammalian cells
4	Modification of pathogen´s virulence factors	Binds or degrades specific virulence proteins
5	Inhibition and disruption of biofilm formation	Critical for bacteria that exert virulence through biofilm formation
6	Bactericidal activity	Lactoferricin, an N- terminal peptide fragment of bovine-LF, kills bacteria
7	Intestinal flora modulation and maturation	Promotes growth of healthy gut bacteria
8	Promotion of intestinal cell proliferation, differentiation and maturation	Decreases intestinal permeability and gut barrier dysfunction
9	Regulation of the immune response	Protects against severe inflammation related to infection and septic shock.
10	Reduction of inflammation	Decreases production of TNF-α (tumor necrosis factor alpha) and other pro-inflammatory molecules

*Adapted from Ochoa & Sizonenko 2017[5]

Concentration of lactoferrin in breast milk

The content of lactoferrin varies in different organs/tissues; it is higher in the vagina, kidney and mammary gland.[55] Lactoferrin concentrations change with lactation stage; colostrum has the highest concentration (6-10 mg/ml), and it decreases significantly with days postpartum. The concentration on mature milk (after 1 month of age) is around 1mg/ml.[125] Multiple studies have demonstrated that lactoferrin concentration decreases with time; however, the role of environmental factors on lactoferrin concentration is not well defined. We have conducted a systematic literature review to investigate the factors that may influence

lactoferrin concentration. We included 70 publications from 29 countries and analyzed several factors such as maternal age, race, parity, nutritional status, infection, prematurity, among others. We found contradictory results or insufficient evidence.[126] One of the main limitations of the published studies are their small sample sizes and different, and sometimes suboptimal, methods they have used to measure lactoferrin.

There have been some small studies looking at the correlation of lactoferrin concentrations in breast milk and infection in children from Gambia[127], Nigeria[128], Israel[129], Australia[130] and Argentina[131]. However, none of these studies have been conducted in VLBW infants. Of interest, the study from Nigeria found lower lactoferrin concentrations in the breast milk of mothers with sick children,[128] supporting the hypothesis that the predisposition of infants to infection may be due to inadequate lactoferrin intake from breast milk.

Clinical studies of lactoferrin

Clinical studies of lactoferrin in adults

Several clinical trials of bovine lactoferrin have been conducted in adults for treatment of infections and cancer. Some of the most relevant trials using bovine lactoferrin are listed in Table 4 and trials using recombinant human lactoferrin are listed in Table 5. Most of these trials have shown a protective effect; however, some have contradictory results.

Table 4. Selected clinical studies of bovine lactoferrin in adult patients

N	Author	Year	Country	Clinical condition	Reference
1	Trümpler	1989	Switzerland	Neutropenia and bacteremia	132
2	Yamauchi	2000	Japan	Tinea pedis	133
3	Okada	2002	Japan	Chronic hepatitis C	134
4	Okuda	2005	Japan	*Helicobacter pylori*	*135*
5	Di Mario	2006	Italy	*Helicobacter pylori*	*136*
6	Ueno	2006	Japan	Chronic hepatitis C	137
7	Kozu	2009	Japan	Adenomatous colorectal polyp	138
8	Chan	2017	Philippines	Mild to moderate acne vulgaris	139
9	Rezk	2016	Egypt	Anemia in pregnant women	140
10	Lepanto	2018	Italy	Anemia in pregnant and non-pregnant women	141
11	Muscedere	2018	Canada	Prevention of infections in critical ill patients	142
12	Russo	2019	Italy	Recurrent vulvovaginal candidiasis	143

Table 5. Selected clinical studies of recombinant human lactoferrin in adult patients

N	Author	Year	Country	Clinical condition	Reference
1	Troost	2003	Netherlands	Indometacin induced enteropathy	144
2	Lönnerdal	2006	USA	Iron absorption	145
3	Parikh	2011	India	Advanced non-small-cell lung cancer	146
4	Ramalingam	2013	USA	Advanced non-small-cell lung cancer	147
5	Vincent	2015	Multicenter	Severe sepsis	69

Clinical studies of lactoferrin in children

Before the interest of lactoferrin for prevention of infections in neonates (discussed below), several studies of bovine lactoferrin supplementation have been conducted in infants since the ´80s to determine its effect mainly on fecal flora, iron status and infections.

In 2012 my team published a systematic review of clinical trials conducted in children. We found 19 clinical studies that have used human or bovine LF for different outcomes: iron metabolisms and anemia (6 studies), fecal flora (5 studies), enteric infections (3 studies), common pediatric diseases (1 study), immunomodulation (3 studies), and neonatal sepsis (1 study) (Table 6).[148] The major points of this review are that relatively few patients have been treated in a systematic way and that the effect of lactoferrin on iron uptake, hematologic indices, and fecal flora was minimal.

Relevant to my PhD work (Chapter 1) are the three previous trials on diarrhea. Egashira in Japan administered 100 mg of bovine lactoferrin containing products (milk, yogurt) to children less than 5 years of age attending nursery schools and found that although there was no difference in the incidence of rotavirus diarrhea, the frequency and severity of the vomiting and diarrhea among the rotavirus episodes were less in the lactoferrin group compared to the controls.[149] Zavaleta in Peru administered a rice-based oral rehydration solution containing recombinant human lactoferrin and lysozyme (from rice) to children 5-33 months old admitted to the hospital with diarrhea and dehydration. She found that the duration of the diarrhea episodes was shorter in the lactoferrin group than the control.[72] Our team conducted a pilot study in children aged 12–36 months in Peru. Children were randomized to receive 500mg of bovine lactoferrin twice a day or placebo daily for 9 months. We found no differences in diarrhea incidence or duration of the diarrhea episodes, however we found significant less colonization with Giardia and lower duration of Giardia carriage in the lactoferrin group.[150]

After the publication of our systematic review there have been two additional relevant trials of lactoferrin in children. A study in Taiwan (2011) in 172 children aged 2-6 years showed that the administration of a lactoferrin-containing formu-

la did not modify the incidence of enterovirus type 71 or rotavirus infection.[151] Next, a trial conducted in China (2016) enrolled 260 infants aged 4-6 months and randomized them to receive a lactoferrin-fortified infant formula or regular formula for 3 months. The authors found less incidence of respiratory and diarrhea illnesses in the lactoferrin group compared with the control formula.[152]

Table 6. Clinical studies of lactoferrin (LF) in children published before 2009 (Modified from Ochoa 2012†)[148]

N	Reference	n	Main Results
1	Spik 1982 [153]	40	bLF in the feces averaged 200 mg/day. Ingested bLF and hLF are not completely destroyed and keep their ability to bind iron.
2	Moreau 1983 [154]	44	The establishment of *E. coli* strains in the digestive tract was not significantly different between the intervention and control groups. The *in vitro* bacteriostatic effect of LF + IgG on the growth of *E. coli* strains is not found *in vivo*.
3	Fairweather-Tait 1987 [155]	13	There was no overall difference on iron retention between the intervention (formula with LF) and control groups. No effect on iron bioavailability.
4	Balmer 1989 [156]	58	The addition of LF had little effect on the fecal microflora and did not move the fecal flora pattern in the direction of the breast-fed babies.
5	Schulz-Lell 1991 [157]	16	Supplementation of the adapted infant formula with bLF did not improve iron absorption.
6	Roberts 1992 [158]	51	High LF concentration (100mg/100ml) was able to establish a "bifidus flora" in half of the babies, but only at age three months.
7	Chierici 1992 [159]	51	The formula supplemented with the higher amount of bLF induced significantly higher serum ferritin levels compared to the unsupplemented formula at day 90 and day 150.
8	Lönnerdal 1994 [160]	50	There were no significant differences in hematological indices among the groups with bLF and selenium at 6 months of age.
9	Davidsson 1994 [161]	8	Fe absorption was significantly lower from breast milk than from LF-free breast milk. These results do not support a direct role for LF in the enhancement of Fe absorption from human milk at this age.
10	Wharton 1994[162]	ND	bLF had little effect on the fecal flora and did not move it in the direction of the breast-fed baby.
11	Hernell 2002 [163]	57	No significant differences in hematology or iron status were observed between groups (formula with and without bLF) at 4 and 6 mo of age.
12	Zuccotti 2006 [164]	22	Significant reduction in plasma viral load and increase in the percentage of CD4+ cell counts above baseline with bLF + antiretroviral treatment.

N	Reference	n	Main Results
13	Egashira 2007 [165]	298	The incidence of rotaviral gastroenteritis showed no significant difference between the two groups. The frequency and duration of vomiting and diarrhea were markedly decreased in the bLF group.
14	Zuccotti 2007 [166]	11	Immune modulation of the innate and adaptive immune responses; skewing of the CD8 T-lymphocyte differentiation pathway towards the mature, lytic forms, and a significant increase in phagocytosis and killing by CD13+ phagocytes with bLF.
15	Zavaleta* 2007 [72]	140	Significant decrease in duration of diarrhea in the intervention group (recombinant -hLF and lysozyme) compared to the control group and a significant increase in the number of children who achieved 48 h with solid stool.
16	King* 2007[167]	52	There were significantly fewer lower respiratory tract illnesses, primarily wheezing, and higher hematocrit levels at 9 months in the LF group.
17	Ochoa* 2008 [150]	52	Comparison of overall diarrhea incidence and prevalence rates found no significant difference between the 2 groups. There was a lower prevalence of colonization with *Giardia* and better growth among children in the bLF group.
18	Zuccotti 2009 [168]	ND	Significant skewing of CD8+T lymphocytes maturation; CD14+, toll like receptor (TLR) 2-expressing cells augmented, whereas CD14+/TLR4+ diminished; and IL10 production by CD14+ cells was reduced in children receiving LF + Curcumin.

† The Manzoni 2009 study[169] was excluded from this table, since it is presented in table 7; *Randomized, double-blind, controlled trial; hLF human lactoferin; bLF, bovine lactoferrin; ND, No data

Clinical studies of lactoferrin in neonates

Given the high morbidity and mortality of sepsis in preterm infants and recognizing the potential benefits of lactoferrin in neonates (Box 2), there has been a big interest in studying lactoferrin during the last decade, to determine its role in reducing the rates of infection in this patient group.

The first published study by Manzoni and colleagues (2009) in Italy[169] demonstrated a dramatic 68% decrease in the rate of sepsis in VLBW infants using bovine lactoferrin. The authors randomly assigned 472 infants to receive orally administered bovine lactoferrin (LF), LF plus the probiotic *Lactobacillus rhamnosus* GG (LF+LGG), or placebo for 30 days. The incidence of sepsis was significantly lower in the LF and LF+LGG groups compared with the placebo group (5.9% and 4.6% vs. 17.3%). Death from sepsis also was reduced in both treatment groups compared with placebo (0% and 0.7% vs. 4.8%). Compared with placebo, LF+LGG significantly reduced necrotizing enterocolitis, and LF significantly reduced threshold retinopathy of prematurity. No other secondary outcomes differed significantly, and no adverse effects were reported. The researchers continued enrolling infants and later evaluated the effect of lactoferrin on fungal infections[170] and NEC.[171]

In 2014 we published a literature search of published clinical studies and trials registered in international electronic registries.[172] In addition to the original Manzoni trial, we found 10 additional trials of lactoferrin for prevention of sepsis, with a worldwide representation (3 in Europe, 2 in North America, 2 in South America, and 1 in Africa, Asia and Oceania respectively). At the moment of writing, although most of these trials have been finished, not all have been published yet. In addition to the studies reported by our team, three additional studies from China have been published.[173-175]

Currently there are 10 trials of bovine lactoferrin for prevention of sepsis in neonates published in the literature (Table 7).[176] Our pilot trial (Chapter 2)[177] is included in this list; however, our second study (Chapter 3) is not, because it has been published just recently. The similarities, differences, strengths and weaknesses of each trial, including our recent trial, will be discussed in the Discussion section of this PhD thesis.

Table 7. Published clinical trials of lactoferrin (LF) for prevention of sepsis in neonates (Modified from Razak 2019)[176]

	Year	Author	Country	n	Population	Daily dose	Outcome: LOS* (LF vs. placebo)	% sepsis reduction
1	2009	Manzoni[169]	Italy	505	<1500g	100mg +/- LGG	5.9% vs. 17.3%	66
2	2014	Akin[178]	Turkey	50	<1500g	200mg	18.2% vs. 32.0%	43
3	2015	Kaur[179]	India	130	<2000g	80-140mg/kg	3.2% vs. 13.4%	76
4	2015	Ochoa[177]	Peru	190	500-2500g	200mg/kg	4.2% vs. 4.2%	0
5	2015	Dai[173]	China	70	26-33w	100mg +/- LGG	5.7% vs. 22.9%	75
6	2016	Sherman[70]	USA	120	750-1500g	300mg/kg **	10.2% vs. 16.7%	39
7	2016	Barrington[180]	Canada	79	<31w	100mg	17.5% vs. 20.5%	15
8	2016	Liu[174]	China	160	26-33w	250mg+/- LGG	2.5% vs. 6.3%	60
9	2017	Tang[175]	China	172	<37w	100mg +/- LGG	6.1% vs. 16.7%	63
10	2019	ELFIN[181]	UK	2203	<32w	150mg/kg	17.4% vs. 16.5%	+5

*Culture-confirmed late-onset sepsis; ** Recombinant human lactoferrin; all other trials used bovine lactoferrin

Lactoferrin digestion, gastric processing and proposed location of action

In order to perform clinical trials and before it becomes a standard of care, it is important to understand the uptake and metabolism of lactoferrin in the body as well as the safety profile.

The processing of lactoferrin in the gut is not fully defined. Lactoferrin is relatively resistant to proteolysis in the gastrointestinal tract during infancy. Lactoferrin has been shown to be internalized by intestinal cells via a specific receptor that binds both human and bovine lactoferrin.[67] This may explain the systemic effect of lactoferrin. Data on adults demonstrate that 60-80% of ingested lactoferrin survives gastric transit intact.[144] Studies in infants suggest better survival of the protein. Multiple studies have shown that breast fed infants have significant amounts of intact lactoferrin in feces compared to formula fed controls.[182-186] Even as late as 17 months of age approximately 2% of ingested lactoferrin survives to be excreted in feces.[185] As noted in Table 6, listing major findings in previous trials in children, biologically active bovine lactoferrin can be found in the feces of infants who are ingesting 1mg/day.[153] The infants excreted approximately 20% in feces during the 2 month trial. A second infant study found that those being fed bovine lactoferrin had significantly more lactoferrin in feces ($p<0.001$) than controls, but the levels were low 2.4-7.1mg/24hrs.[155] In summary, since lactoferrin has been found intact in the stool of breastfed infants, it is generally considered resistant against proteolytic degradation in the gut.[187]

In vitro digestion of lactoferrin by duodenal juice from a 3-year-old was slow - with 68% survival after 40 minutes of digestion.[188] In fact, lactoferrin was the most slowly digested protein in human milk. Studies in an intestinal enterocyte model have demonstrated that human and bovine lactoferrin are partially digested and can be taken up by the lactoferrin-receptor in the intestine and exert its functions.[67]

Furthermore, when lactoferrin is partially digested, biologically active fragments are produced. Multiple fragments of lactoferrin have been found in stool of infants.[189] Lactoferricin is a major peptide produced by pepsin digestion of human and bovine lactoferrin. Bovine lactoferricin as well as related peptides impairs attachment/invasion of several enteric pathogens. Bovine lactoferricin is more biologically active than is the human counterpart.[190] Thus, the action of lactoferrin and its antimicrobial peptides is likely to be in both the large and small intestine.

Safety of intervention

Bovine lactoferrin is a functional food that is already consumed either as a food supplement or in dairy products. Purified bovine lactoferrin is commercially available and relatively inexpensive; multiple companies currently market lactoferrin as a nutritional supplement. Morinaga Milk Industry in Japan has been adding bovine lactoferrin to their yogurt, nutritional supplements, oral care products and infant formula since 1986. There is a need to determine whether it is efficacious.

Bovine lactoferrin has previously been shown to be safe in multiple studies involving infants. In addition to the 15 studies listed in Table 6, in which bovine lactoferrin has been given to healthy term children, there are 10 additional studies that have used lactoferrin in preterm infants (Table 7). No side effects have been noted related to use of bovine lactoferrin in infants in all trials, except in

the ELFIN study; among 1098 infants randomized to lactoferrin two infants had serious adverse events possibly related to the intervention (one child had blood in the stool and one child died after intestinal perforation secondary to NEC). [181]

The U.S. Food and Drug Adminsitration (FDA) has received multiple applications regarding bovine lactoferrin to be classified as GRAS (Substances Generally Recognized As Safe), under proposed Code of Federal Regulations Title 21, part 170 Food additives, eligibility for classification as generally recognized as safe (21 CFR 170.36). The FDA has concurred with the GRAS designation on multiple occasions. A substance used in food can be GRAS if its safety has been established by generally available scientific data and information that lead qualified experts to conclude that the ingredient is safe for its proposed use. Thus, although efficacy remains open to investigation in humans, bovine lactoferrin is readily available and being used in persons of all ages for its potential health benefits.

One potential risk is cow milk protein allergy. However, serious reactions to cow milk are rare and the incidence of cow milk protein allergy as defined by strict criteria (elimination from diet with subsequent rechallenge) is 2-3% during infancy, and the prognosis is good.[191] Lactoferrin may be less allergenic since the data suggest that milk allergy is directed primarily toward alpha-lactalbumin, beta-lactoglobulin and casein. There is a paucity of literature suggesting that lactoferrin is allergenic.[192] One study in mice found that human lactoferrin administered intranasally induced allergic airway inflammation in mice[193]; however, most studies in the literature, highlight the use of lactoferrin to decrease allergy in mice.[194,195]

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Research aims

IDENTIFICATION OF RESEARCH NEEDS, KNOWLEDGE GAPS, HYPOTHESIS AND RESEARCH AIMS OF THIS THESIS

Research needs and knowledge gaps

Although Peru is an upper middle-income country with a growing economy (see Box 1 in the Introduction), there is still a high burden of infectious diseases in the pediatric population in some areas of the country, especially in rural and peri-urban communities. In addition, there is large heterogeneity in the health care system. Public hospitals, even in the main cities like Lima, have crowded neonatal intensive care units with suboptimal infection control practices. Therefore, there is an urgent need to implement preventive strategies to decrease the risk and burden of infections in these vulnerable populations.

On the other hand, there is extensive knowledge on the antimicrobial and immunomodulatory properties of bovine and human lactoferrin from *in vitro* and *in vivo* studies and several clinical trials, as reviewed in the Introduction; however, there is a knowledge gap about the clinical applications of lactoferrin in specific pediatric infections and patient groups. There is a need of properly designed pediatric clinical trials with an adequate sample size.

First, there is a lack of knowledge about the effect of bovine lactoferrin supplementation on prevention of diarrhea in previously weaned children in resource limited settings. The morbidity and mortality that result from diarrheal disease after weaning represent child health issues of global importance. Prolonging exposure to milk protective factors such as lactoferrin, may be a cost-effective intervention to decrease diarrheal disease burden and its resulting adverse effects on growth and intellectual function. **Chapter 1** will address this.

Next, given the high incidence and high morbidity and mortality of sepsis in preterm infants, efforts to reduce the rates and impact of infections is urgently needed in neonatal care. Supplementing specific milk protective factors to preterm infants who do not receive sufficient quantities of human milk, is an excellent strategy that utilizes what has been learned by studying human milk to benefit one of the most susceptible populations. Multiple clinical trials of bovine lactoferrin supplementation in infants have started to address this problem. However, there still a gap on knowledge on the effect of bovine lactoferrin for prevention of sepsis in infants, related to the subgroup (birth weight group and different risk settings), dose of lactoferrin (fixed dose or dose per kilo), duration of the intervention (2-4-8 weeks), outcome determination (culture confirmed and/or culture negative clinical infection) and the long-term effect on growth and neurodevelopment. All are critically important questions that were not completely answered in the previous studies. **Chapters 2, 3 and 4** will address this.

Finally, multiple previous studies have demonstrated that the consumption of colostrum and maternal breastmilk offers protection against sepsis and necrotizing enterocolitis (NEC) in small infants, as reviewed in the Protective effects of

breast milk section in the Introduction. However, there is a gap in knowledge of the effect of direct human-lactoferrin intake on the protection against infections and death in preterm newborns. All previous studies have focused on bovine lactoferrin supplementation, not human lactoferrin intake from the mother. **Chapter 5** will address this.

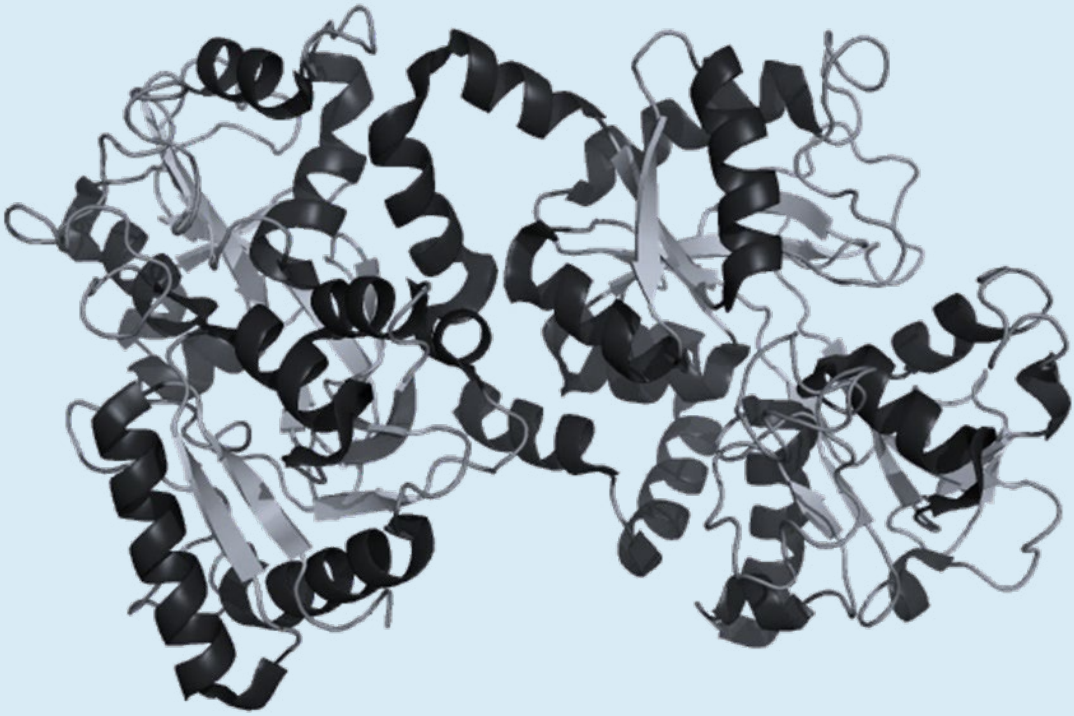
Hypothesis and Research Aims

General hypothesis

Based on the previous *in vitro* and animal studies, I hypothesize that lactoferrin given as an oral supplement to infants in resource-limited settings will improve their health by mimicking its protective roles in breast milk, decreasing the incidence and severity of common pediatric infections due to its antimicrobial and immunomodulatory properties.

Specific research aims

1. **Determine the effect of bovine lactoferrin supplementation on prevention of diarrhea in children**. I will test the hypothesis that bovine lactoferrin supplementation in young previously weaned children 12-24 months of age in resource limited settings will lower the frequency of symptomatic diarrheal illness. This hypothesis is based on multiple previous studies including our own data which have demonstrated that lactoferrin decreases the attachment of enteropathogens to host cells by disruption of bacterial cell surface anchored virulence proteins, among other mechanisms.
2. **Determine the effect of bovine lactoferrin on prevention of neonatal infections in infants <2500g**. I will test the hypothesis that oral administration of bovine lactoferrin early in life will prevent the development of late-onset-sepsis based on lactoferrin effect on modulating bacterial growth in the gastrointestinal tract, promoting intestinal cell proliferation, differentiation and maturation, and regulating the host immune response to infection.
3. **Determine the effect of bovine lactoferrin on prevention of late-onset sepsis and neurodevelopment improvement in preterm infants <2000g.** I will test the hypothesis that lactoferrin supplementation will decrease the rate of infections in preterm infants based on lactoferrin anti-infective properties mentioned above. I also hypothesized that lactoferrin supplementation will improve neurodevelopment by decreasing direct central nervous system (CNS) infection (meningitis) as well as indirect CNS injury through sepsis-related changes in perfusion and inflammation by regulating the immune response and reducing inflammation related to infection.
4. **Determine the effect of bovine lactoferrin on prevention of late-onset sepsis in infants <1500g**. I will test the hypothesis that lactoferrin supplementation will prevent serious infections and death in very low birth weight infants (<1500g) based on lactoferrin anti-infective, anti-inflammatory, and immune-modulating properties.
5. **Determine the effect of human lactoferrin on late-onset sepsis, NEC or death in infants <2000g**. I will test the hypothesis that higher intake of mother´s own milk lactoferrin early in life will prevent the development of late-onset sepsis, necrotizing enterocolitis (NEC) and death in low birth weight infants, based on the natural protective properties of lactoferrin present in human milk.



CHAPTER 1:

Effect of bovine lactoferrin on prevention of diarrhea in children

RANDOMIZED DOUBLE-BLIND CONTROLLED TRIAL OF BOVINE LACTOFERRIN FOR PREVENTION OF DIARRHEA IN CHILDREN

Authors:
Theresa J. Ochoa, MD[1,2,3], Elsa Chea-Woo, MD[2], Nelly Baiocchi, MD[2], Iris Pecho, MPH[4], Miguel Campos, MD, PhD[5], Ana Prada, MSc[1], Gladys Valdiviezo, RN[1], Angela Lluque, MSc[1], Dejian Lai, PhD[6], and Thomas G. Cleary, MD[3]

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[1] Instituto de Medicina Tropical, Universidad Peruana Cayetano Heredia, Lima, Peru
[2] Department of Pediatrics, Universidad Peruana Cayetano Heredia, Lima, Peru
[3] Center for Infectious Diseases, University of Texas School of Public Health, Houston, TX
[4] Department of Public Health, Administation and Social Sciences, Universidad Peruana Cayetano Heredia, Lima, Peru
[5] Department of Mathematics, Universidad Peruana Cayetano Heredia, Lima, Peru
[6] Division of Biostatistics, University of Texas School of Public Health, Houston, TX

ABSTRACT

Objective
To determine the effect of bovine lactoferrin on prevention of diarrhea in children.

Study design
We conducted a community-based randomized double-blind placebo controlled trial comparing supplementation with bovine lactoferrin versus placebo. Previously weaned children were enrolled at 12–18 months and followed for 6 months with daily home visits for data collection and supplement administration. Anthropometric measures were done monthly.

Results
555 children were randomized: 277 to lactoferrin and 278 to placebo; 65 dropped out; 147,894 doses were administered (92% compliance). Overall there were 91,446 child-days of observation and 1,235 diarrhea episodes lasting 6,219 days. The main pathogens isolated during diarrheal episodes were norovirus (35.0%), enteropathogenic *E. coli* (11.4%), Campylobacter (10.6%), enteroaggregative *E. coli* (8.4%), enterotoxigenic *E. coli* (6.9%) and Shigella (6.6%). The diarrhea incidence was not different between groups: 5.4 vs. 5.2 episodes/child/year for lactoferrin and placebo, respectively (p=0.375). However, the diarrhea longitudinal prevalence was lower in the lactoferrin group (6.6% vs. 7.0%, p=0.017) as well as the median duration of episodes (4.8 vs. 5.3 days, p=0.046), proportion of episodes with moderate or severe dehydration (1.0% vs. 2.6%, p=0.045) and liquid stools load (95.0 vs. 98.6) liquid stools/child/year, p<0.001). There were no adverse events related to the intervention.

Conclusions
Although there was no decrease in diarrhea incidence, longitudinal prevalence and severity were decreased with lactoferrin.

Keywords: lactoferrin, diarrhea, children, prevention, clinical trial.

INTRODUCTION

The WHO estimates 8.1 million deaths occur yearly in children (<5 years of age) with diarrhea accounting for 14% of deaths.[1] In addition to causing mortality, diarrhea has serious long term effects with multiple episodes and persistent diarrhea affecting growth, nutrition and cognition.[2] Breastfeeding is the most cost effective intervention for protecting children against diarrhea and all causes of mortality.[3] Exclusive breast-feeding, and to a lesser extent partial breastfeeding, protects against acute and persistent diarrhea.[4] Breastfeeding helps protect infants by serving as a source of nutrition uncontaminated by environmental pathogens. It is also generally assumed that protection is due to the multiple anti-infective, anti-inflammatory, and immunoregulatory factors transmitted through milk, including secretory antibodies, glycans, lactoferrin, leukocytes, cytokines and other components produced by the mother's immune system.[5,6]

Lactoferrin, the second most abundant protein in human milk, is also found in most exocrine secretions including tears, saliva, intestinal mucus and genital secretions, and in the specific granules of neutrophils. Lactoferrin has multiple putative activities (anti-microbial, anti-inflammatory, immunomodulatory).[7–9] It has been thought to protect against Gram negative enteropathogens by sequestration of iron essential for bacterial growth, binding to the lipid A portion of LPS on the cell surface, and disrupting the bacterial cell membrane.[10,11] In vitro lactoferrin decreases virulence of enteropathogens by decreasing their ability to adhere to or invade mammalian cells, and by binding to, or degrading, specific virulence proteins.[12–14] Human (hLF) and bovine lactoferrin (bLF) have similar bioactivity despite minor structural and biochemical differences, as assessed in vitro and in animal models.15,16 bLF has previously been shown to be safe in infants.[17–19]

Our hypothesis was that bLF would lower the frequency and severity of diarrhea in children related to its multiple anti-bacterial activities.[12–14,20,21] The primary objectives were to determine the effects of lactoferrin on prevention of diarrhea episodes and on growth in previously weaned children.

METHODS

A community-based randomized double blind placebo-controlled trial was conducted in children from Lima, Peru, comparing twice daily supplementation with bLF versus placebo administered for 6 months with monitoring of diarrhea and growth. Eligible children were previously weaned 12–18 months old. Exclusion criteria were a history of severe, persistent or chronic diarrhea, severe malnutrition, serious infections requiring hospitalization in the month prior, serious chronic illness, or a personal or family history of allergy to cow's milk or infant formula, eczema, allergic rhinitis or asthma.

We conducted a census in the District of Independencia to determine which households included a child ≤18 months old. Then, nurses conducted a food-intake survey to determine which children were weaned. Eligible families were visited by a study nurse who explained the protocol, answered questions, and obtained written informed consent from both parents.

Immediately after recruitment patients were assigned a study number that had been previously randomly assigned to bLF or placebo with fixed, equal allocation to each group and blocked randomization with block size of 4, prepared by a third party. Only the research pharmacist knew the randomization.

Community health workers visited each child 6 days/week (Monday through Saturday), twice daily (morning and afternoon) to give the coded preparations under supervision to ensure compliance. Children received 0.5g twice a day of bLF or placebo (diluted in 25 mL of water). The dose of lactoferrin was chosen based on the estimated amount consumed by a breastfed 12-month old infant.

The bLF preparation (Tatua Co-operative Dairy Co, Ltd, Morrinsville, New Zealand) is a freeze-dried protein purified directly from fresh bovine milk (iron saturation 10–20%). It is a salmon pink colored bland tasting powder produced under food grade conditions meeting ISO9001 standards. Maltodextrin (Montana S.A., Lima, Peru), a carbohydrate made from corn starch, was used as placebo. Both bLF and maltodrextrin were mixed with sugar, a strawberry flavor and pink food coloring agent, to make the preparations appear and taste identical. Screw top opaque plastic containers with a one month supply were prepared by a food processing company under good manufacturing practices (Montana S.A., Lima, Peru). Children received their normal diet including cow´s milk; however, commercially available cow´s milk does not provide a significant additional dose of bLF.

The physicians, nurses, community health workers, parents, and laboratory personnel were blinded to treatment assignment of each child throughout the study period. The data manager, statistician, and all investigators remained blinded to group assignment until the end of data analysis.

Diarrhea was defined as presence of ≥3 loose or watery stools in 24-hrs or ≥1 loose stool containing blood. An episode was considered to have started when a diarrhea day was preceded by at least 2 consecutive days without diarrhea and ended when the child had 3 consecutive days without any loose stool. All days between the start and ending day were considered part of the episode even if there was no diarrhea on a given day. Persistent diarrhea was defined as lasting for ≥14 days. Severe diarrhea was defined by the presence of ≥6 loose or watery stools in 24-hrs with vomiting, grossly bloody stools, documented fever (>39°C), or hospitalization for dehydration. Dehydration was assessed using WHO guidelines based on skin turgor, mental status and thirst, and was categorized as none, mild, moderate or severe. We used a Modified Ruuska-Vesikari score (MRV)[22] to deter mine severity. For assessment of bLF effect on growth, z-scores of height-for-age (HFA) and weight-for-height (WFH) were used, based on WHO 2006 growth standards.

The primary study outcome was diarrhea incidence during the 6-month intervention. The secondary outcome was HFA and WFH z-scores. Additional diarrhea outcomes were longitudinal prevalence, duration, severity, dehydration, and prevalence of loose stools.

The community health workers performed daily home visits to record data on diarrhea, hydration, and sign/symptoms suggesting possible allergy to study interventions. Community health workers received training in basic health issues in order to give health education. The community health workers and parents were instructed to bring the child to the emergency room or study clinic if severe diarrhea developed. During episodes a stool sample was collected and the child was treated with ORS and/or antimicrobials as clinically indicated. Zinc therapy is not routinely used in Peru. Monthly stool samples were collected in the absence of gastrointestinal symptoms (± 7days) to evaluate colonization.

Stools were analyzed at the Enteric and Nutrition Laboratory - Tropical Medicine Institute "Alexander von Humboldt" in Lima, for common enteropathogens using conventional microbiological procedures. Rotavirus and adenovirus were determined using immunochromatography (Operon, Huerva-Zaragoza, Spain). Norovirus was detected by PCR using previously described primers.[23] Diarrheagenic *E. coli* were diagnosed using a multiplex real time PCR.[24] Parasites were determined by direct microscopy, stains for *Coccidia* and concentration methods for *Strongyloides*. Children were evaluated monthly by a pediatrician at the Outpatient Clinic for a history, exam and growth measurements. Children were weighed nude on the same calibrated infant scale (to the nearest 0.01 Kg), and length measured supine using standard length-height measuring boards. Personnel who performed the growth measurements were standardized twice yearly.

Poisson regression was used to model the relationship between expected number of diarrhea episodes of treatment versus placebo group. For sample size calculation we used a formula developed by Signorini[25], based on one-sided hypothesis, which is consistent with our study. For sample size estimates we had assumed that there would be 3 diarrhea episodes/child/year in the placebo group, so that the number of children needed for a one sided test with a type I error (α) of 0.05 and a power (1-β) of 0.80 to detect a 25% reduction in the diarrhea episodes was 211 children in each group. We had projected that there would be a 30% dropout rate; we therefore planned to recruit 301 children/group. Partial information from dropouts has been used in the Poisson regression and in all analyses.

Analyses

Data were entered into an MSSQL database and were reviewed using SQL and VBS consistency checking programs. Patient, visit, result and episode analytical files were extracted to SPSS SAV binary format. Descriptive data tabulation comparing baseline and outcome variables between groups was made using SPSS V15.0. Statistical testing was made using R 2.13.1. Fisher Exact test for binary outcomes, Poisson test for rate outcomes, Student t-test for continuous outcomes

(log-transformed if significant to Levene test) were used. To summarize the comparisons, estimates of ratios or differences for proportions, rates or means with their 95% confidence limits were made. Extended models for testing multivariate hypothesis are described in the Results. The Data Safety Monitoring Board (DSMB) met every 6 months to review data for safety and study compliance. Children experiencing a severe adverse event were referred to the DSMB for their judgment about continuation of the study.

The study was approved by Institutional Review Boards of the University of Texas Health Science Center in Houston and Universidad Peruana Cayetano Heredia in Lima; by the Direccion de Salud Lima Ciudad; Instituto Nacional de Salud-Peru ; and Direccion General de Medicamentos, Insumos y Drogas-Peru; and was registered at Clinicaltrials.gov (NCT00560222).

RESULTS

The study was conducted from January 2008 through May 2011. The census of 52,144 households found 3,674 children in the targeted age range. The food-intake survey found 2,495 children still breastfeeding (67.9%) leaving 1,179 eligible children (Figure). A lower than expected enrollment rate together with a much lower than expected drop out rate and much higher than expected illness rate, resulted in 555 rather than 602 children enrolled; 277 were randomized to bLF and 278 to placebo. Eighty-nine baseline demographic and socio-economic characteristics and risk factors for diarrhea were compared by Kruskal-Wallis test; 8 had $p<0.05$, only WFH and diet intake of other micronutrients had $p<0.01$ (Table 1).

Table 1. Baseline demographic and socio-economic characteristics, and risk factors for diarrhea in bLF and placebo groups

		bLF	Placebo
Age at enrollment	Mean ± SD	15.76 ± 2.08	16.07 ± 2.09
Sex	Female, n (%)	137 (49.5%)	121 (43.5%)
Baseline anthropometry	Weight in Kg, mean ± SD	10.10 ± 1.21	10.43 ± 1.23
	Height in cm, mean ± SD	77.48 ± 3.22	78.03 ± 3.19
	Head circumference in cm, mean ± SD	45.98 ± 1.41	46.54 ± 1.46
	Height-for-age (HFA) z score, mean ± SD	−0.60 ± 0.92	−0.56 ± 0.99
	Weight-for-height (WFH) z score, mean ± SD	0.29 ± 0.93	0.51 ± 0.92
	Weight-for-age (WFA) z score, mean ± SD	−0.06 ± 0.91	0.12 ± 0.95
	Mass body index (MBI) in Kg/m2, mean ± SD	16.79 ± 1.35	17.09 ±1.34
	Mass body index (MBI) z score, mean ± SD	0.40 ± 0.93	0.63 ± 0.89

		bLF	Placebo
Breastfeeding	Breastfed prior to entry in study, n (%)	265 (96.4%)	266 (96.7%)
	Duration of exclusive breastfeeding in months, mean ± SD	4.74 ± 3.89	4.67 ± 3.85
	Weaning age in months, mean ± SD	10.18 ± 4.52	10.70 ± 4.59
Diarrhea history	N° of diarrhea episodes in the pre vious 6m, mean ± SD	2.01 ± 3.95	1.87 ± 2.14
	N° of prior persistent diarrhea episodes, mean ± S D	0.07 ± 0.32	0.12 ± 0.41
Household	N° of family members who live in the hous e, mean ± SD	6.09 ± 2.74	6.24 ± 2.96
	N° of children < 5y who live in the house, mean ± SD	1.59 ± 0.92	1.61 ± 0.81
	Family monthly income in US $, mean ± SD	242.02 ± 118.26	240.71 ± 127.86
	Number of bedrooms, mean ± SD	2.35 ± 1.57	2.27 ± 1.48
	Pipe water supply inside the house, n (%)	227 (83.5%)	237 (86.2%)
	Sewer line inside the house, n (%)	233 (85.7%)	240 (87.3%)
	Electricity in the home, n (%)	260 (95.6%)	262 (95.3%)
	Refrigerator in home, n (%)	158 (58.1%)	145 (52.7%)
	Television in home, n (%)	260 (95.6%)	256 (93.1%)
	Cell phone, n (%)	202 (74.3%)	190 (69.1%)
	Chickens living inside the house, n (%)	78 (28.8%)	59 (21.5%)
Caregiver	Mother is the primary caregiver, n (%)	187 (67.8%)	205 (74.3%)
	Grandmother is the primary caregiver, n (%)	44 (15.9%)	45 (16.3%)
	Mother age in years, mean ± SD	28.18 ± 6.48	28.64 ± 6.57
	Mother works inside or outside the house, n (%)	107 (38.9%)	119 (43.5%)
	Daycare attendance, n (%)	7 (2.6%)	2 (0.7%)
Parents education	Mother did not complete high school, n (%)	60 (22.0%)	67 (24.4%)
	Mother completed high school, n (%)	134 (49.1%)	120 (43.6%)
	Father did not complete high school, n (%)	61 (23.4%)	54 (21.3%)
	Father completed high school, n (%)	134 (51.3%)	131 (51.8%)

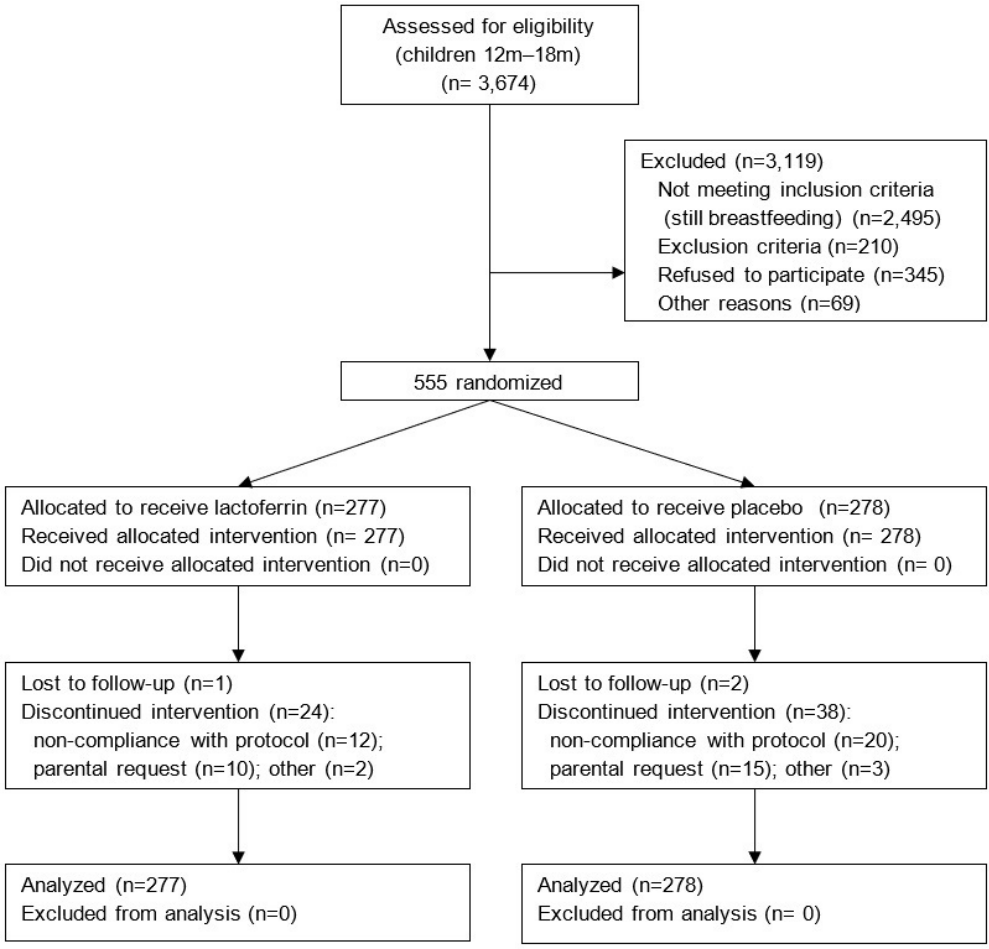

Figure 1. Study flow diagram. Enrollment: 3,674 children were assessed for eligibility. Allocation: 277 were randomized to lactoferrin and 278 to placebo; all received the allocated intervention. Follow-up: there were 25 and 40 drop-outs in the lactoferrin and placebo groups respectively. None were excluded from the analysis.

There were 91,446 child/days of observation: 46,545 bLF and 44,901 placebo. There were 65 drop outs (11.7%): 25 bLF (9.0%, 95% CI [5.9–13]) and 40 placebo (14.4%, 95% CI [10.5–19.1]), p=0.064 (Figure). The study compliance was: 98% for planned home visits, 90% for planned monthly clinic visits, and 92% for planned doses administered (Table 2).

Table 2. Follow- up of patients: study compliance, clinical characteristics of the diarrheal episodes and growth outcomes

		bLF	Placebo
Follow-up and compliance			
Home visits	Total days of observation	46,545	44,901
	Number of daily home visits	37,709	36,401
	Daily home visits, mean ± SD per child	168.03 ± 40.55	161.51 ± 48.77
	Compliance (actual /planned visits), mean ± SD	0.98 ± 0.10	0.98 ± 0.08
Medical visits	Number of planned monthly clinic visits	1,756	1,707
	Planned monthly clinic visits, mean ± SD per child	6.34 ± 1.50	6.14 ± 1.73
	Number of clinic visits for illness	734	724
	Sick visits, mean ± SD per child	2.65 ± 2.47	2.60 ± 2.36
	Compliance (actual/ planned monthly clinic visits), mean ± SD	0.91 ± 0.21	0.88 ± 0.25
Treatment	Total number of doses received	75,320	72,574
	Number of doses received, mean ± SD per child	271.91 ± 69.51	261.06 ± 81.57
	Compliance (doses received/planned), mean ± SD	0.94 ± 0.24	0.91 ± 0.28
Clinical characteristics of the diarrheal episodes			
Number of episodes		646	589
Duration in days	Median (minimum - maximum)	4 (1 – 28)	4 (1 – 62)
	1 – 3 days, n (%)	320 (49.5%)	266 (45.2%)
	4 – 6 days, n (%)	174 (26.9%)	171 (29.0%)
	7 – 13 days, n (%)	120 (18.6%)	116 (19.7%)
	14 – 20 days, n (%)	27 (4.2%)	22 (3.7%)
	≥ 21 days, n (%)	5 (0.8%)	14 (2.4%)
Loose stools	Mean ± SD per episode	13.3 ± 13.0	14.7 ± 15.0
	Mean ± SD per day during episode	3.0 ± 1.3	3.1 ± 1.5
	Maximum number per day, mean ± SD	4.6 ± 2.3	4.7 ± 2.3
Vomiting	Mean ± SD per episode	0.6 ± 1.9	0.5 ± 1.7
	Median per episode (minimum - maximum)	0 (0 – 21)	0 (0 – 24)
	Mean ± SD per day during episode	0.2 ± 0.5	0.1 ± 0.7
Blood in feces	Episodes with bloody stools, n (%)	27 (4.2%)	18 (3.1%)
Fever	Episodes with fever, n (%)	99 (15.3%)	89 (15.1%)
Dehydration	Moderate or severe (WHO), n (%)	6 (1.0%)	15 (2.6%)

		bLF	Placebo
Severity score	Moderate (MRV), n (%)	72 (11.1%)	71 (12.1%)
	Severe (MRV), n (%)	4 (0.6%)	5 (0.8%)
	Severe episode, by study definition, n (%)	208 (32.2%)	200 (34.0%)
Growth outcomes: WHO 2006 z scores			
HFA z	Initial	−0.60 ± 0.92	−0.56 ± 0.99
	1 month	−0.62 ± 0.92	−0.59 ± 0.95
	2 months	−0.67 ± 0.94	−0.58 ± 0.98
	3 months	−0.66 ± 0.91	−0.56 ± 0.96
	4 months	−0.64 ± 0.89	−0.53 ± 0.93
	5 months	−0.65 ± 0.89	−0.54 ± 0.96
	6 months	−0.57 ± 0.88	−0.48 ± 0.95
WFHz	Initial	0.29 ± 0.93	0.51 ± 0.92
	1 month	0.28 ± 0.93	0.52 ± 0.89
	2 months	0.26 ± 0.87	0.51 ± 0.85
	3 months	0.28 ± 0.91	0.61 ± 0.86
	4 months	0.28 ± 0.88	0.61 ± 0.86
	5 months	0.29 ± 0.87	0.60 ± 0.86
	6 months	0.25 ± 0.90	0.56 ± 0.89

MRV, Modified Ruuska-Vesikari score; HFAz, height-for-age z score (mean ± SD); WFHz, weight-for-height z score (mean ± SD)

1,235 diarrhea episodes occurred (646 bLF and 589 placebo), with an average duration of 5.04 ±4.79 days; 47.4% of episodes lasted ≤3 days and 5.5% were persistent; 33% of episodes were severe based on our study definition (Table 2). There was no difference in diarrhea incidence between groups; 5.4 vs. 5.2 episodes/child/year for bLF and placebo, respectively (p=0.375). However, there were small but significant differences in duration, longitudinal prevalence, dehydration and prevalence of loose stool, with less overall diarrhea burden with bLF (Table 3). This decrease in diarrhea severity was not associated with a decrease in ORS and/or antimicrobial usage.

We studied 915 diarrhea stool samples (74% of episodes had a sample collected). (Table 4). There were no differences in incidence, prevalence or clinical characteristics for any pathogen related to group assignment. There were no differences in prevalence of colonizing pathogens between diarrhea and control groups based on 2,734 stool samples collected in the absence of diarrhea (Table 4).

Anthropometric z-scores (Table 2) were tested in a linear mixed model regression having intercept, treatment group, time since start of supplementation and the product of both as fixed effect terms plus individual child intercept as random effect term. For HFA, significant differences (p=0.010) by group slope, but not intercept (p=0.525), were found. For WFH, significant differences by group intercept (p=0.002), but not slope (p=0.050), were found. Additional adjustment by

adding baseline anthropometry, age upon admission, day of year and completion status confirmed the treatment group slope significance in HFA (p<0.001) and dismissed any treatment significance in WFH. Modeling indicates that the bLF group had a slightly lower (0.12 z, 95%CI [0.07–0.17]) HFA than the placebo group at the end of treatment.

Table 3. Comparison of diarrhea outcomes between the bLF and placebo groups

	bLF (95% CI)	Placebo (95% CI)	Type*	Comparison (95% CI)	P
Incidence, episodes/child/year	5.43 (5.02 – 5.86)	5.15 (4.74 – 5.59)	R	1.05 (0.94 – 1.18)	0.375
Duration, days	4.76 (4.41 – 5.15)	5.34 (4.93 – 5.80)	I	0.89 (0.80 – 0.99)	0.046
Average prevalence, % of days with diarrhea	6.6 (6.4 – 6.8)	7.0 (6.8 – 7.2)	P	−0.4 (−0.7 – −0.1)	0.017
Episodes with moderate or severe dehydration, %	1.0 (0.4 – 2.1)	2.6 (1.5 – 4.3)	P	−1.6 (−3.3 – −0)	0.045
Episodes with severe diarrhea, %	32.2 (28.6 – 36.0)	34.0 (30.1 – 37.9)	P	−1.8 (−7.2 – 3.7)	0.545
Episode severity score (MVS), mean	4.89 (4.67 – 5.11)	5.05 (4.83 – 5.28)	M	−0.16 (−0.48 – 0.16)	0.625
Bloody diarrhea, days with blood/child/year	0.28 (0.19 – 0.38)	0.30 (0.21 – 0.42)	U	0.91 (0.56 – 1.49)	0.787
Total loose stools, loose stools /child/year	95.0 (91.3 – 95.0)	98.6 (95.0 – 98.6)	U	0.95 (0.93 – 0.98)	<0.001

R: estimation by incidence rate, comparison by bLF/placebo (P) rate ratio, p from Poisson test; I: estimation by duration as inverse of recovery rate, comparison by bLF/P rate ratio, p from Poisson test; P: estimation by binomial proportion, comparison by bLF-P difference of proportions, p from Fisher test; M: estimation by arithmetic mean, comparison by bLF-P difference of means, p unequal v Student test; U: estimation by rate, comparison by bLF/P rate ratio, p from Poisson test

During planed monthly outpatient clinic visits and sick visits there were no differences in the prevalence of common pediatric diagnosis between the bLF and placebo groups. Occurrence of possible allergic reactions, prevalence of skin allergy or eczema, allergic rhinitis, and bronchospasm were similar between groups as was use of antihistamines and anti-asthma medications. There were 17 severe adverse events (SAE), 9 bLF and 8 placebo. All SAE were hospitalizations for common pediatric illnesses; none were considered related to the intervention by the DSMB.

Table 4. Pathogens isolated from diarrheal and colonization samples

	Pathogen	DIARRHEA			COLONIZATION (healthy controls)		
		bLF N = 484 n (%)	Placebo N = 431 n (%)	Total N = 915 n (%)	bLF N = 1,396 n (%)	Placebo N = 1,338 n (%)	Total N = 2,734 n (%)
Bacteria	*Campylobacter*	61 (12.6)	36 (8.4)	97 (10.6)	107 (7.7)	109 (8.1)	216 (7.9)
	Shigella	24 (5.0)	36 (8.4)	60 (6.6)	12 (0.9)	18 (1.3%)	30 (1.1)
	Vibrio	2 (0.4)	0 (0)	2 (0.2)	3 (0.2)	3 (0.2)	6 (0.2)
	Other bacteria‡	4 (0.8)	6 (1.4)	10 (1.1)	16 (1.1)	22 (1.6)	38 (1.4)
	EPEC	57 (11.8)	47 (11.0)	104 (11.4)	138 (10.0)	169 (12.7)	307 (11.3)
	EAEC	42 (8.7)	34 (7.9)	76 (8.4)	128 (9.2)	124 (9.3)	252 (9.3)
	ETEC	37 (7.7)	26 (6.1)	63 (6.9)	55 (4.0)	59 (4.4)	114 (4.2)
	DAEC	14 (2.9)	13 (3.0)	27 (3.0)	28 (2.0)	21 (1.6)	49 (1.8)
	EIEC	5 (1.0)	2 (0.5)	7 (0.8)	8 (0.6)	12 (0.9)	20 (0.7)
	STEC	3 (0.6)	2 (0.5)	5 (0.5)	14 (1.0)	20 (1.5)	34 (1.3)
Virus	Rotavirus	11 (3.2)	15 (4.7)	26 (3.9)	ND	ND	ND
	Adenovirus	23 (3.6)	23 (3.8)	46 (3.7)	ND	ND	ND
	Norovirus*	141 (34.3)	134 (35.5)	275 (35.0)	ND	ND	ND
Parasites	*Giardia*	38 (7.9)	20 (4.6)	58 (6.3)	114 (8.2)	89 (6.7)	203 (7.4)
	Blastocystis	8 (1.7)	9 (2.2)	17 (1.9)	25 (1.8)	34 (2.5)	59 (2.2)
	Cryptosporidium	4 (0.9)	1 (0.2)	5 (0.6)	5 (0.4)	5 (0.4)	10 (0.4)
	Cyclospora	1 (0.2)	1 (0.2)	2 (0.2)	2 (0.1)	0 (0)	2 (0.1)
	Strongyloides	1 (0.2)	0 (0)	1 (0.1)	1 (0.1)	0 (0)	1 (0.0)
	Other parasites‡	18 (3.9)	11 (2.6)	29 (3.3)	55 (3.9)	69 (5.2)	124 (4.5)

ND, no data (viruses were not studied in the healthy controls samples).*Norovirus: 17.1% G-I, 82.9% G-II. †Other bacteria: Aeromonas, Plesiomonas, Salmonella.‡Other parasites: Chilomastix,, Endolimax, Entamoeba, Enterobius, Diphylobothrium, Hymenolepis, Trichuris, Isospora.

DISCUSSION

This study failed to achieve its primary objective of demonstrating decreased incidence of diarrheal disease with bLF as well as the secondary objective of demonstrating improved growth. However, measures of severity were positively affected although the benefit was small. The data suggest that chronic use of bLF such as is currently done in some infant formulas is unlikely to have a major impact on diarrhea in children. Lactoferrin without other breast milk factors may have limited value. Lactoferrin might have important benefits on immune or other functions, but its failure to improve growth does not support the concept that it is a major factor that could improve child health in this age group. Although adjusted analysis finds a clinically small difference in HFA, the authors do not unanimously agree on the interpretability, given the baseline WFH difference.

The small benefits noted in disease severity suggest that further studies ought to focus on lactoferrin as an adjunct to other measures aimed at management of acute or persistent diarrheal disease. A previous pediatric study of acute watery diarrhea showed that adding lysozyme and recombinant hLF expressed in rice to oral rehydration solution reduced the duration and recurrence of diarrhea.[26] Our findings are in concordance with previous smaller, less intensively monitored trials. A 12wk study of 298 Japanese children showed no difference in incidence of rotaviral gastroenteritis, but duration of episodes and frequency and duration of vomiting were decreased with bLF.[27] A study in 52 US infants receiving a bLF-enhanced formula for 12m found no differences in diarrhea incidence; however, there were significantly fewer lower respiratory tract illnesses in the bLF-fed compared with regular formula-fed infants.[28] We had conducted a prior pilot trial of bLF for 9m in 52 Peruvian children. The bLF group had less Giardia burden and better HFA z-score.[29] The current trial failed to confirm these preliminary findings.

The trial has some limitations. First, only one dose (1,000 mg/day), equivalent to the amount of LF in 100 mL of colostrum (10 mg/ml) or one liter of post colostral breast milk (1 mg/ml), was tested. This dose was chosen based on a preliminary pilot study of bLF for prevention of diarrhea.[29] However, LF dosing has been very variable in previous pediatric clinical trials. Other studies have used as low as 10mg/100mL of milk, 100 mg/day, or as high as 1,000 mg every 8 hours.[19] Therefore, although this dose seemed reasonable, it was potentially to low, especially for this age group. Obviously future studies should evaluate larger doses. Second, the intense observation, regular physician evaluations, and home health educational intervention may have modified risks so that the observed rates of diarrhea and growth may have been better than would have occurred in the absence of the trial. Third, the age range studied was narrow; bLF might have greater benefit in some other age range, such as neonates, because recent data has demonstrated an important effect of bLF on prevention of sepsis in preterm neonates.[18] Children in the second year of life may digest bLF so that it has less impact on enteric pathogens; however, the processing of LF in the gut is not fully defined. Fourth, enteropathogens in Peru are similar to those in most of the world but different from the organism in Japan, North America, and Europe. In such settings it is possible that bLF could have a role in immunologically naïve populations. The high frequency of exposure to enteropathogens in Peru[22] may have boosted pathogen specific immunity such that the effect of lactoferrin was lessened. A population with a lower frequency of infection might show a greater impact from bLF. Fifth, the use of bLF rather than hLF could be debated but we believe it is reasonable given that both have similar effects on diarrheal pathogens in multiple laboratory models.[30] HLF and bLF are well characterized. They have 691 and 689 amino acids, respectively; the sequence identity is 69%; and the 3-D structures are very similar.[31] Although differences in structural and biochemical properties exist, their bioactivity, as assessed in vitro or in animal models, is quite comparable.[15,16] BLF and hLF have been evaluated in several clinical trials in children with various objectives: iron metabolism, anemia, fecal flora, enteric infections, immunomodulation in HIV children and neonatal sepsis.[19] Although the efficacies have been variable in each trial (due to different study outcomes),

there are no data on the effectiveness of bLF vs. hLF for the same outcome measured in a pediatric clinical trial. However, based on the in vitro and animal studies and the available data in humans, both LF preparations appear to be comparable on their likely effects on enteric infections.

In summary, although this study makes it unlikely that bLF can have a major role in prevention of diarrhea in children in the second year of life, it leaves open the possibility that lactoferrin could have a role in younger infants or as an adjunct to other measures in treatment of diarrheal episodes, especially for the treatment of prolonged and persistent diarrhea, which are associated with malnutrition and impaired neurodevelopment.

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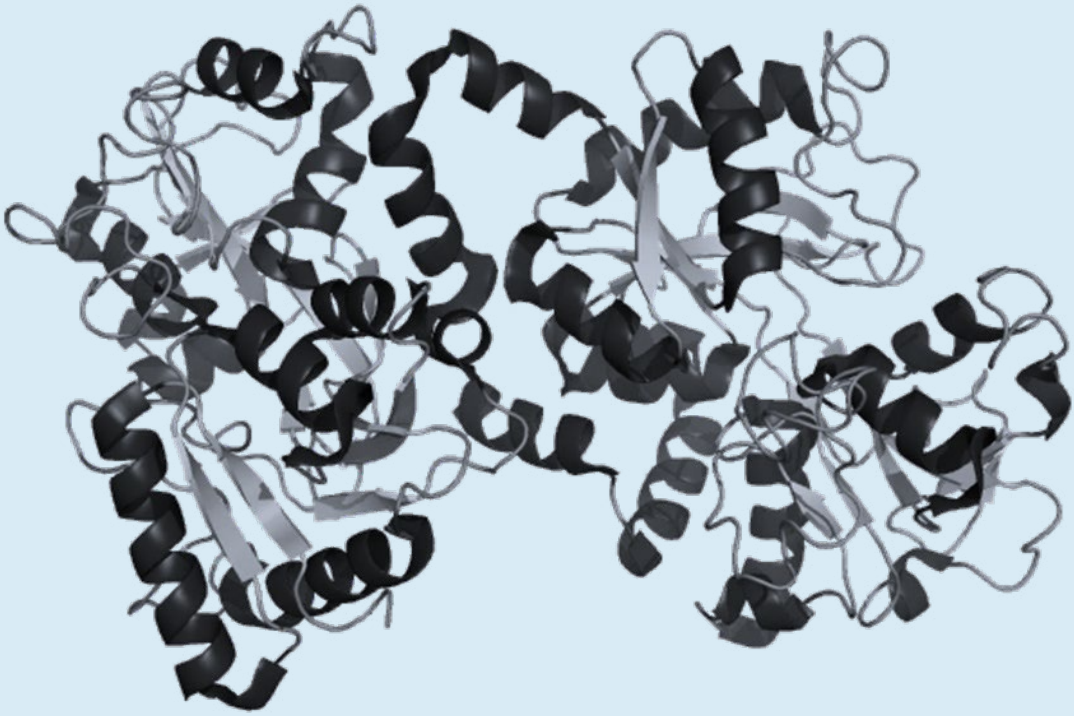

CHAPTER 2

Effect of bovine lactoferrin on prevention of neonatal infections in infants <2500g

RANDOMIZED CONTROLLED TRIAL OF LACTOFERRIN FOR PREVENTION OF SEPSIS IN PERUVIAN NEONATES LESS THAN 2500 G

Authors:
Theresa J. Ochoa, MD,*†‡ Jaime Zegarra, MD,†§ Luis Cam, MD,¶ Raul Llanos, MD,‖ Alonso Pezo, MD,* Karen Cruz, MD,* Alonso Zea-Vera, MD,* Cesar Cárcamo, MD, PhD,** Miguel Campos, MD, PhD,†† and Sicilia Bellomo, MD†§; for the NEOLACTO Research Group

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* Instituto de Medicina Tropical "Alexander von Humboldt", Universidad Peruana Cayetano Heredia, Lima, Peru
† Department of Pediatrics, Universidad Peruana Cayetano Heredia, Lima, Peru
‡ Center for Infectious Diseases, University of Texas School of Public Health, Houston, Texas
§ Hospital Nacional Cayetano Heredia, Lima, Peru
¶ Hospital Nacional Alberto Sabogal Sologuren, Lima, Peru
‖ Hospital Nacional Guillermo Almenara Irigoyen, Lima, Peru
**Department of Public Health, Universidad Peruana Cayetano Heredia, Lima, Peru
††Department of Mathematics, Universidad Peruana Cayetano Heredia, Lima, Peru.

ABSTRACT

Background: Lactoferrin (LF) is a broad-spectrum antimicrobial and immunomodulatory milk glycoprotein.

Objective: To determine the effect of bovine LF on the prevention of the first episode of late-onset sepsis in Peruvian infants.

Methods: We conducted a pilot randomized placebo-controlled dou- ble blind study in infants with a birth weight (BW) less than 2500g in 3 neonatal units in Lima. Patients were randomized to receive bovine LF 200mg/kg/d or placebo for 4 weeks.

Results: One hundred and ninety neonates with a BW of 1591 ± 408 g and a gestational age of 32.1 ± 2.6 weeks were enrolled. Overall, 33 clinically defined first late-onset sepsis events occurred. The cumulative sepsis incidence in the LF group was 12/95 (12.6%) versus 21/95 (22.1%) in the pla- cebo group, and 20% (8/40) versus 37.5% (15/40) for infants less than or equal to 1500 g. The hazard ratio of LF, after adjustment by BW, was 0.507 (95% CI: 0.249–1.034). There were 4 episodes of culture-proven sepsis in the LF group versus 4 in the placebo group. Considering that children did not received the intervention until the start of oral or tube feeding, we ran a secondary exploratory analysis using time since the start of the treatment; in this model, LF achieved significance. There were no serious adverse events attributable to the intervention.

Conclusions: Overall sepsis occurred less frequently in the LF group than in the control group. Although the primary outcome did not reach statistical significance, the confidence interval is suggestive of an effect that justifies a larger trial.

Key Words: bovine, preterm, newborn, infant, late-onset sepsis

INTRODUCTION

Four million neonates die each year, mostly in low-income countries[1]. The 3 major causes of neonatal death are asphyxia, infection and complications of preterm birth, which account for 86% of deaths[1]. Globally almost one million newborns die because of infections (neonatal sepsis, pneumonia and meningitis)[1]. In developing countries, infection may be responsible for as many as 42% of deaths in the first week of life[2]. Up to 80% of newborn deaths are among low-birth weight (BW) babies, most of whom are preterm[1]. Multiple strategies have been designed to reduce infant mortality. Among these, breast-feeding is the most cost effective intervention for protection from infection and prevention of all causes of infant mortality[3].

Multiple strategies have been designed to reduce infant mortality. Among these, breastfeeding is the most cost-effective intervention for protection from infection and prevention of all causes of infant mortality[3]. Breast milk has a beneficial effect in term and preterm infants including improved cognitive and behavior skills, and decreased rates of infection[4-7]. The protective effects of human milk are thought to be due to multiple anti-infective, anti-inflammatory, and immunoregulatory factors[8,9]. We hypothesize that lactoferrin (LF) is the major milk factor responsible for decreased rates of infection due to its antimicrobial and immunomodulatory properties[10]. Recently, 472 very low-birth-weight infants (VLBW; birth weight <1500g) were randomly assigned to receive orally administered bovine LF, LF plus the probiotic *Lactobacillus rhamnosus* GG (LF+LGG), or placebo for 30 days[11]. The incidence of sepsis was significantly lower in the LF and LF+LGG groups compared with the placebo group (5.9% and 4.6% vs. 17.3%). Whether LF has an effect in higher risk populations in developing countries remains to be determined. Therefore, we conducted a hospital-based randomized placebo-controlled double blind study in 190 infants ≤2500g in Neonatal Units in Peru to determine whether bovine LF prevents the first episode of late-onset sepsis in neonatal setting from a low-income country.

METHODS

Study design

We conducted a randomized double blind placebo-controlled clinical trial in neonates, comparing daily supplementation with bovine LF versus placebo administered for four weeks.

Study population

We included neonates with a birth weight between 500 and 2500g born in or referred in the first 72 hours of life to the neonatal intermediate and intensive care units of one of the participating hospitals: Hospital Nacional Cayetano Heredia

(Cayetano), Hospital Nacional Guillermo Almenara Irigoyen (Almenara), and Hospital Nacional Alberto Sabogal Sologuren (Sabogal). We excluded neonates with underlying gastrointestinal problems that prevent oral intake, predisposing conditions that profoundly affect growth and development (chromosomal abnormalities, structural brain anomalies, etc.), family history of cow milk allergy, neonates that lived far from Lima, and neonates whose parents declined to participate. Consecutive patients who qualified for the study were approached by the attending neonatologist who explained the study and obtained written informed consent from both parents before the 72-hour cut-off.

Randomization

Patients were assigned a consecutive study number in the order they were enrolled. The numbers were previously randomly assigned to the intervention with fixed, equal allocation to each group, stratified by weight (500–1000g, 1001–1500g, 1501–2000g and 2001–2500g), and randomized with block size of 4. This randomization list was prepared by a third party (not the clinical investigators) and was known only by the research pharmacist who prepared the weekly treatment packages based on neonates' weight. Randomization occurred immediately after recruitment of each patient.

Intervention

Neonates received oral bovine LF (Tatua Co-operative Dairy Co, Ltd, Morrinsville, New Zealand) (200mg/kg/day in three divided doses each day) or placebo (maltodextrin, Montana S.A., Lima, Peru) (200mg/kg/day in three divided doses each day) for four weeks since the day of enrollment. The intervention product was composed of 97.1% bioactive protein of which 94.5% was LF, without additives. The iron saturation was 12%. Capsules containing LF or placebo were opened and mixed with whatever the neonates were taking orally or by tube at that time (breast milk, infant formula or dextrose); the intervention was given as soon as the patient started receiving any amount of enteral feedings. After discharge from the hospital, a research nurse visited the family weekly until the end of the first month of life. All children had a clinic visit at one and three months of chronological age.

Blinding

The physicians and study personnel were blinded to the treatment assignment throughout the study period. The data manager, statistician, and all investigators remained blinded to the group assignment until the end of the data analysis.

Study definitions

For this study, we determined the effect of LF on clinically-defined sepsis. We included both culture-proven sepsis and culture-negative clinical infection (probable or possible sepsis). Late-onset proven sepsis was defined by a positive blood culture and/or cerebrospinal fluid culture obtained after 72 hours of life in the

presence of clinical signs and symptoms of infection[12]. Culture-negative clinical infection or "probable sepsis" was defined by the presence of clinical signs and symptoms of infection (temperature instability, heart rate > 2SD above normal for age, respiratory rate >60 breaths/min plus grunting or desaturations, lethargy/altered mental status, glucose intolerance defined as plasma glucose > 10mmol/L, feed intolerance, blood pressure < 2SD normal for age, capillary refill > 3 sec, plasma lactate > 3 mmol/L) and at least two abnormal inflammatory variables[12]: leukocytosis (WBC count >34,000 $\times 10^9$/L), leukopenia (WBC count <5,000 $\times 10^9$/L), immature neutrophils >10%, immature-to-total neutrophil ratio (I/T)>0.2, thrombocytopenia <100,000 $\times 10^9$/L, and C-Reactive Protein (CRP) >10mg/dL or >2SD above normal. We have not measured procalcitonin, IL-6 or IL-8 or 16S PCR, which are other variables evaluated in Haque's criteria. "Possible sepsis" was defined by the presence of clinical signs and symptoms of infection and raised CRP with negative blood culture. An independent diagnostic board reviewed all sepsis episodes.

Outcome

The primary outcome was risk of first episode of late-onset clinically-defined sepsis (culture-proven sepsis and culture-negative clinical infection) within 4 weeks (28 days) from enrollment. Secondary outcomes were frequency of culture-proven sepsis, pathogen-specific late-onset sepsis; necrotizing enterocolitis (NEC), duration of hospitalization, overall mortality rate, infection-related mortality, frequency of adverse events, and treatment intolerance.

Sample size

The reduction in sepsis episodes with LF supplementation was 66% in the Italian study[11]. Based on data from local neonatal units, we expected 30% of sepsis episodes in the placebo group during the 4-week follow up. Assuming 5% drop outs, 95 children were needed in each group to detect a 60% reduction in the number of sepsis episodes (α 0.05; power 0.80). We, therefore, planned to recruit 190 neonates.

Data management and analysis

Data were collected and then double entered into databases using EpiInfo v3.4.5. Data entry formats had predefined ranges for acceptable values. Consistency checks were performed in STATA v8.0. Statistical analysis was performed in STATA and R v3.0.2. P values from the Student's t-test and Fisher's exact test are presented for the univariate analysis comparing baseline demographic characteristics, risk factors, medical and surgical complications, and weight during follow up. Unadjusted relative risks as cumulative incidence ratios with their corresponding confidence intervals and p values were used to compare sepsis outcomes. Birth weight-adjusted relative risks were calculated using Generalized Linear Models (GLM). Cox proportional hazards regression model was applied for survival analysis. The primary outcome variable was the occurrence of the first episode of confirmed, possible or probable late-onset-sepsis, and time was

counted as days from birth to that first episode or last day of follow-up, whichever happened first. The initial terms included were treatment group (TX), birth weight (BW in grams), hospital (HOS), peripartum maternal infection (dichotomous), age at start of follow-up, and the following interaction terms: TX:BW, TX:HOS, TX:BW:HOS, and BW:HOS. The model selection proceeded in four steps: initial set of terms, non-significant interaction terms removed, non-significant terms (except LF) removed and the final model, with removal of remaining non-significant terms.

Data Safety Monitoring Board (DSMB)

The DSMB, composed of an independent group of experts (neonatologists, pediatrician, epidemiologist, microbiologist), met every other month to review data for safety and study compliance. Any child experiencing a severe adverse event was referred to the DSMB for study continuation assessment.

Ethical and regulatory aspects

The study (www.clinicaltrials.gov:NCT01264536) was approved by the Institutional Review Board of Universidad Peruana Cayetano Heredia, University of Texas Health Science Center and by each of the three participating Hospitals. The study was also approved by the Peruvian Regulatory Institutions (INS and DIGEMID).

RESULTS

During the enrollment (January 31–August 6, 2011), 375 infants were assessed for eligibility, 185 were excluded (Figure 1). We enrolled 190 neonates; 80 (42.1%) had a birth weight <1500g. The gestational age was 32.1±2.6 weeks (26–38wks) and mean birth weight was 1591±408g. There were no significant baseline differences between groups in demographic and clinical characteristics or risk factors for late-onset sepsis (Table 1), except for peripartum maternal infections, which were more frequent in the placebo group, and more days of third and fourth-generation cephalosporin use in the placebo group. During the in-hospital follow up period nutritional characteristics were similar in both groups: approximately half of all days infants were fed only breast milk and approximately one third of days they were fed both infant formula and breast milk (Table 1). Additional information on the comparison between groups is presented in the supplementary data (Table S1).

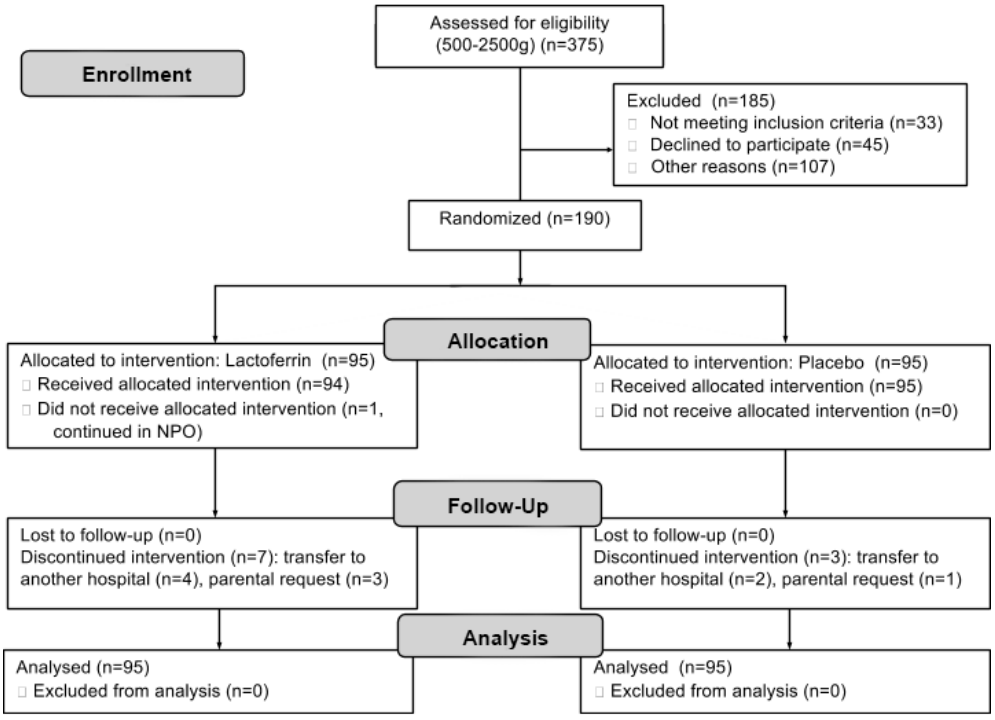

Figure 1. Study flow diagram. Enrollment: 375 neonates were assessed for eligibility. Allocation: 95 were randomized to LF and 95 to placebo; all received the allocated intervention. Follow-up: there were 7 and 3 drop-outs in the LF and placebo groups respectively. None were excluded from the analysis.

The intervention was administered completely per protocol in 82% of 3,244 child-days of observation. It was started on average at 4.0 ± 1.4 days of life. Some neonates started the treatment a few days after enrollment because the treating neonatologist considered them too sick to tolerate any amount of oral or tube feeding. The diluents used in the 7,796 doses administered were breast milk in 67%, infant formula in 32% and dextrose in 1%. Ten patients (5.3%) were withdrawn from the study due to patients return to a distant province or transfer to a different hospital (n=6), or parental request (n=4).

Table 1. Comparison between Lactoferrin and Placebo Groups

	Lactoferrin	Placebo	p
Demographic and clinical characteristics			
Peripartum maternal infection, n/total (%)	10/91 (11.0)	21/90 (23.3)	0.031
Use of antibiotics on last month of pregnancy, n/total (%)	19/90 (21.1)	25/92 (27.2)	0.389
Premature rupture of membranes, n/total (%)	23/90 (25.6)	25/94 (26.6)	1.000
Chorioamnionitis, n/total (%)	4/91 (4.4)	10/91 (11.0)	0.162
Cesarean delivery, n/total (%)	81/95 (85.3)	77/95 (81.1)	0.561
Birth weight in g, mean ± SD (range)	1582 ± 422 (770 – 2482)	1600 ± 395 (710 – 2470)	0.765
Gestational age in wks, mean ± SD (range)	32.2 ± 2.6 (26 – 38)	32.0 ± 2.6 (26 – 37)	0.742
Small for gestational age, n/total (%)	27/95 (28.4)	23/95 (24.2)	0.621
Neonatal resuscitation needed, n/l (%)	27/93 (29.0)	30/94 (31.9)	0.751
Birth weight group distribution, n/total (%)			1.000
500–1000 g	9/95 (9.5)	9/95 (9.5)	
1001–1500 g	31/95 (32.6)	31/95 (32.6)	
1501–2000 g	40/95 (42.1)	39/95 (41.1)	
2001–2500 g	15/95 (15.8)	16/95 (16.8)	
Risk factors for late-onset sepsis and nutritional characteristics (first month of life or until discharge or withdrawal) *			
Time of initiation of oral feeding, DOL, mean ± SD	3.1 ± 1.3	3.1 ± 1.6	0.964
Complete supplement administration in days, mean ± SD	12.5 ± 8.1	13.9 ± 8.0	0.234
Nutritional characteristics, n (%) of child-days			0.601
NPO	148 (9.6)	161 (9.5)	
Fed with only maternal milk	765 (49.5)	803 (47.3)	
Fed with only formula	154 (10.0)	185 (10.9)	
Fed with both formula and maternal milk	480 (31.0)	548 (32.3)	
Central venous catheters positioned in days, mean ± SD	4.9 ± 7.5	6.1 ± 8.6	0.294
Mechanical ventilation in days, mean ± SD	0.9 ± 3.3	1.2 ± 4.1	0.557
Medication, days treated, mean ± SD			
Antibiotics	4.9 ± 6.5	6.3 ± 7.5	0.168
Third/Fourth-generation cephalosporins	0.4 ± 1.8	1.4 ± 3.7	0.018

NICU, neonatal intensive care unit; DOL, day of life
*Lactoferrin: 1547 child-days; Placebo: 1697 child-days of observation.

Table S1. Supplementary information on the comparison between Lactoferrin and Placebo Groups

	Lactoferrin	Placebo	p
Demographic and clinical characteristics			
Maternal age in years, mean ± SD	30.7 ± 6. 9	30.0 ± 6.6	0.450
Obstetric complications, n/total (%)	87/95 (92.6)	88/94 (93.6)	0.782
Preeclampsia or eclampsia	18/95 (19.0)	28/95 (29.5)	0.127
Multiple pregnancy	23/95 (24.2)	15/95 (15.8)	0.204
Antenatal steroids, n/total (%)	57/94 (60.6)	56/89 (62.9)	0.763
Clear amniotic fluid, n/total (%)	80/86 (93.0)	78/91 (85.7)	0.147
Male sex, n/total (%)	42/95 (44.2)	50/95 (52.6)	0.310
APGAR Score at 5min, mean ± SD	8.4 ± 1.0	8.5 ± 1.1	0.842
Mother education, # of years, mean ± SD	11.5 ± 2.8	12.0 ± 2.3	0.193
Fathers education, # of years, mean ± SD	12.5 ± 2.5	12.0 ± 2.3	0.164
Total # of household members, mean ± SD (range)	4.5 ± 2.6 (2 – 15)	4.4 ± 2.7 (2 – 16)	0.798
Hospital enrollee distribution, n/total (%)			0.788
Cayetano	13/95 (13.7)	15/95 (15.8)	
Almenara	28/95 (29.5)	24/95 (25.3)	
Sabogal	54/95 (56.8)	56/95 (59.0)	
Risk factors for late-onset sepsis and nutritional characteristics (first month of life or until discharge or withdrawal)			
Duration of hospitalization in days, median (range)	16 (3 – 31)	20 (3 – 32)	0.324
Duration of stay in NICU in days, mean ± SD (range)	7.3 ± 9.2 (0 – 30)	7.9 ± 9.5 (0 – 29)	0.659
Use of TPN in days, mean ±SD	3.3 ± 5.4	3.0 ± 4.9	0.715
Use of catheters in days, mean ± SD	12.9 ± 9.4	14.8 ± 9.6	0.179
Umbilical catheter positioned	1.4 ± 3.2	1.4 ± 3.1	0.964
Assisted ventilation in days, mean ± SD	3.8 ± 7.1	4.6 ± 8.1	0.490
Supplemental O_2 (nasal canula, hood, CPAP)	3.1 ± 6.1	3.5 ± 6.6	0.641
Medication, days treated, mean ± SD			
H_2 Blockers	0.5 ± 1.8	0.5 ± 1.5	0.966
Vasopressors and inotropes	0.2 ± 0.7	0.4 ± 2.1	0.437

NICU, neonatal intensive care unit; TPN, total parenteral nutrition; *Lactoferrin: 1547 child-days; Placebo: 1697 child-days of observation.

Overall, there were 37 clinically-defined late-onset sepsis episodes during the 4 weeks after enrollment, 33 of them were first episodes, including 8 culture-proven (24.2%), 14 probable (42.4%) and 11 possible (33.3%), based on study definitions. Six of the 33 episodes occurred before starting the treatment intervention. The overall sepsis rate was 17.4% (33/190), and 28.8% (23/80) among infants with a birth weight less than 1500g.

Sepsis occurred less frequently in the LF group than in the control group. In the intention-to-treat analysis, the cumulative sepsis incidence in the LF group was 12/95 (12.6%) versus 21/95 (22.1%) in the placebo group (Table 2). For the VLBW neonates (<1500g) the sepsis rates in the LF group was 8/40 (20.0%) versus 15/40 (37.5%) in the placebo group, a 46% reduction in sepsis. The crude risk ratio (RR) between groups was 0.57(95% CI: 0.30–1.09). The RR adjusted for birth weight category was 0.57 (95% CI: 0.30–1.07) using GLM. Upon reviewing baseline and simple outcome tables, covariates were selected to be included in the adjusted analysis using Cox regression. The only significant term found in the Cox model was birth weight; the 95% confidence limits of the hazard ratio was 0.997–0.999, p<0.001. No statistically significant effect of lactoferrin upon hazard rate of first sepsis episode was detected. The hazard ratio of lactoferrin, after adjustment by birth weight, was 0.507 and the 95% confidence limits was between 0.249 and 1.034 (p=0.062). A non-adjusted Kaplan Meier plot illustrates constraints of the confidence limits (Figure 2). Of interest, after day 10 of intervention, there was 1 sepsis episodes in the LF group versus 6 in the placebo (Figure 2).

Table 2. Sepsis outcomes

Characteristics	Lactoferrin	Placebo	RR (95% CI)	p
Total late-onset sepsis episodes, n/total (%)	12/95 (12.6%)	21/95 (22.1%)	0.57 (0.30 – 1.09)	0.085
Possible and probable late-onset sepsis	8	17		
Confirmed late-onset sepsis	4	4		
Late-onset sepsis by birth weight group, n/total (%)			0.57 (0.30 – 1.07)	0.047*
501–1500g	8/40 (20%)	15/40 (37.5)	0.53 (0.26 – 1.12)	
15001–2500g	4/55 (7.3%)	6/55 (10.9%)	0.67 (0.20 – 2.23)	
Age (days) at onset of first late-onset sepsis episode, mean (range)	6.3 (3 – 12)	9.5 (4 – 25)		0.097
Mortality (prior to discharge), n/total (%)				
Overall (all causes)	7/95 (7.4%)	3/95 (3.1%)		0.330
Sepsis-related	4/95 (4.2%)	2 /95 (2.1%)		0.682

*p value and confidence interval from GLM, adjusting for weight group

Table S2. Medical and surgical conditions and growth measurements during follow up

Complication	Condition	Lactoferrin n/N (%)	Placebo n/N (%)	P
Metabolic	Jaundice	63/93 (67.7%)	66/95 (69.5%)	0.875
	Anemia	28/93 (30.1%)	19/95 (20.0%)	0.333
	Hypoglycemia	19/93 (20.4%)	22/95 (23.2%)	0.725
	Electrolyte disturbance	13/93 (14.0%)	17/95 (17.9%)	0.552
Pulmonary	Hyaline membrane disease or respiratory distress syndrome	22/93 (23.4%)	24/95 (25.3%)	0.866
	Pneumonia	6/93 (6.5%)	1/95 (1.1%)	0.063
	Bronchopulmonary dysplasia	4/93 (4.3%)	7/95 (7.4%)	0.537
	Pneumothorax	0/93 (0.0%)	4/95 (4.2%)	0.121
Cardiac	Symptomatic patent ductus arteriosus	4/93 (4.3%)	9/95 (9.5%)	0.250
	Congenital heart defect	6/93 (6.5%)	5/95 (5.3%)	0.766
Neurological	Intraventricular hemorrhage	10/93 (10.8%)	12/95 (12.6%)	0.821
	Hypoxic-ischemic encephalopathy	1/93 (1.1%)	1/95 (1.1%)	1.000
	Periventricular leukomalacia	0/93 (0.0%)	3/95 (3.2%)	0.246
	Meningitis	0/93 (0.0%)	1/95 (1.1%)	1.000
Other	Surgical procedures	5/93 (5.4%)	4/95 (4.2%)	0.746
	NEC	3/93 (3.2%)	2/95 (2.1%)	0.681
	ROP	8/93 (8.6%)	5/95 (5.3%)	0.403
Weight (g) at 1mo, mean ±SD (n)*	501-1000 g	1053 ±109 (4)	985 ±62 (6)	0.577
	1001-1500 g	1727 ±89 (22)	1695 ±56 (28)	0.754
	1501-2000 g	2342 ±50 (32)	2224 ±88 (28)	0.235
	2001-2500 g	2714 ±145 (11)	2841 ±93 (15)	0.446
Weight (g) at 3mo, mean ± SD (n)*	501-1000 g	2670 ±267 (4)	2654 ±285 (5)	0.969
	1001-1500 g	3666 ±138 (21)	4010 ±111 (24)	0.055
	1501-2000 g	4588 ±108 (31)	4551 ±100 (33)	0.802
	2001-2500 g	5033 ±339 (8)	5509 ±190 (12)	0.203

NEC, necrotizing enterocolitis; ROP, retinopathy of prematurity; * number of infants evaluated at 1 and 2 months of follow up

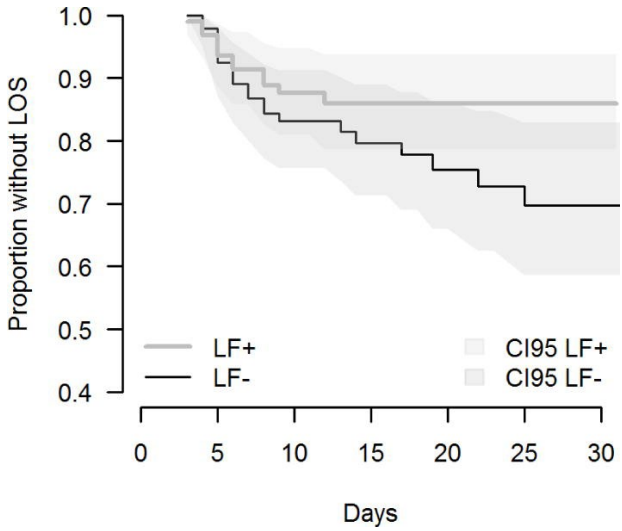

Figure 2. Kaplan-Meier survival estimates: time to event of late-onset sepsis by treatment group. Blue line: lactoferrin; red line: placebo; blue shadow: lactoferrin 95% confidence interval; red shadow: placebo 95% confidence intervals. LOS: Late-onset sepsis

There were 4 episodes of culture proven sepsis in the LF group (*Serratia* sp, *Enterobacter aerogenes*, *Klebsiella* sp, and coagulase-negative *Staphylococcus*), vs 4 in the placebo group (*Pseudomonas* sp, Group B *Streptococcus*, *Enterococcus faecalis*, and coagulase-negative *Staphylococcus*). All pathogens were isolated from blood cultures, none from CSF. There were no significant differences with respect to other secondary outcomes.
Considering that children did not receive treatment until the start of oral or tube feeding (which was later for VLBW), we ran a secondary exploratory model using time since the start of the treatment. In this model, LF achieved significance (p=0.03); the 95% confidence limits for the hazard ratio of LF, after adjustment by birth weight, is between 0.0003 and 0.665.

There are no known risks related to LF intake[13,14]. Over 300 preterm newborns had taken bovine LF in a previous study with no LF related side effects[11]. However, since there was the remote possibility of an allergic reaction to cow milk proteins, we closely monitored for possible signs (allergic rhinitis, diarrhea, vomiting and eczema). There were no signs of allergy or treatment intolerance in 99.7% of observed days; there were only three episodes of vomiting during the intervention period. The overall medical and surgical complications/conditions during follow up where similar between groups, as well as growth measurements at 1 and 3 months of age (Table S2). Fourteen patients (7.4%) were re-hospitalized during the first three months of life, primarily for bronchiolitis (n=7), probable sepsis (n=3), pneumonia (n=2), anemia (n=1) and complication of an inguinal hernia (n=1). There were 11 deaths, two after the first month of life. The overall case-fatality rate was 5.8% (11/190); 38.9% (7/18) in the 500–1000g birth weight group.

The DSMB had seven meetings to assess the safety of the intervention. None of the severe adverse events (14 re-hospitalizations and 11 deaths) were attributable to the intervention; all events were evaluated by the DSMB.

DISCUSSION

We were not able to demonstrate a statistically significant effect of LF on the rate of first late-onset sepsis episodes in infants with a birth weight < 2500g. However, the confidence limits for the hazard ratio of lactoferrin are suggestive of an effect that may be confirmed with a larger sample size. Although non-significant, there were less sepsis episodes in infants receiving LF, especially for VLBW neonates (46% reduction in sepsis). However, the study was not powered to detect significant difference in specific birth weight groups. Since many sepsis episodes occurred in the first week of life (Figure 2), and many of the infants had not received the intervention (LF or placebo) until the beginning of oral feeds, we have explored as a secondary analysis the effect of LF on sepsis using in the model time since the start of the treatment. Although in this model LF achieved significance, we do not consider it conclusive, since it was not the primary outcome in our trial design.

Our results are in concordance with Manzoni's study[11] and consistent with the potential for bovine LF to decrease infections in premature infants. The Italian study found a 66% reduction on sepsis episodes using LF in VLBW infants (RR 0.34, 95%CI: 0.17–0.70). However, there are some differences in study design that could explain some findings. First, we included infants with a birth weight up to 2500g; they included only infants <1500g. Second, we used a standardized dose by weight (200mg/kg/day); they used a fixed dose of 100mg/day for all infants. Third, we included as our main study outcome, not only culture-proven sepsis, but also clinical-defined sepsis (probable and possible). This study addressed some of the most important limitations in the Manzoni trial and presented important differences specific to resource-limited settings. Also, the LF we used was different from that used in Manzoni's trial, the variation in the additives and purity of the LF could affect the results. In a follow up study Mazoni found a significant reduction in the incidence of NEC in VLBW neonates with LF supplementation[15]. In our study we were not able to evaluate the effect on NEC due to the small sample size.

Despite its preliminary nature and small sample size, our study included larger birth weight group infants to investigate the effect of LF on this population. The overall neonatal mortality rate is low for late preterm infants; however, infections among these infants increase the risk of complications, prolong hospital stay and increase mortality[16]. In developing countries, many neonatal deaths occur in non-low-birth weight infants[2]. Thus, we enrolled neonates up to 2500g. We included high-risk babies, typical of those admitted in most hospitals in Latin America. However, neonates with a birth weight >1500g have less risk of sepsis, and obviously impacted the overall sepsis rates in the study.

In the Italian study, as noted both by the authors themselves and subsequent editorial critiques[17], the dose may have been inadequate for larger infants; no adjustments were made for birth weight. The protective effect of LF was clear for infants weighing <1000g, who received the higher dose per kilo. We standardize the dose by weight (200mg/kg/day), based on the dose effective in the smallest infants (500g) in Manzoni's study. Other ongoing LF trials, like the ELFIN study in the UK (Enteral LactoFerrin In Neonates; https://www.npeu.ox.ac.uk/elfin) and the LIFT study in Australia (Lactoferrin Infant Feeding Trial; http://research-data.ands.org.au/the-lactoferrin-infant-feeding-trial-lift) are also using a LF dose by weight (150mg/Kg/day).

This study has some limitations. First, the small sample size and power, due to the small number of high-risk VLBW infants. Second, the inclusion of culture-negative clinically-defined sepsis is not as precise as culture-proven infections as an outcome measurement. However, in developing countries, rates of clinically diagnosed neonatal sepsis are as high as 170/1000 live births whereas culture-confirmed sepsis are around 5.5/1000 live births due to limited laboratory capabilities[2]. Therefore, investigating the effect of LF on these clinically-defined sepsis episodes is of paramount importance in low and middle-income countries. For this trial we standardized sepsis definitions, using strict clinical and laboratory criteria, and evaluated each episode with an independent team of physicians. Third, we were not able to completely blind the study intervention. Both, LF and maltodextrin, were placed in capsules, which were then opened and diluted in breast milk or infant formula. However, the dilution of LF in milk still had a mild pink color. Thus, the nurses administering the intervention were not blinded; however, the physicians and the investigators that evaluated sepsis episodes as well as the statisticians remained blinded to treatment assignment throughout the study period. Fourth, in our study the treatment began when enteral feeding started. This is critically important and could explain the lack of significance. It is known from in vitro studies that LF (both bovine and human) is extremely active on the nascent enterocytes; it promotes cell proliferation in the first days of life[18, 19]. This is why many authors speculate that the anti-infectious role of LF relies strongly on its ability to interact with the immature enterocytes in the very first hours or days of life[20]. Nevertheless, despite these limitations, we suspect that the fundamental observation (LF decreases sepsis in neonates) is correct because it is consistent with experimental literature that has demonstrated LF's protective effect against infections.

LF protects against pathogens in multiple ways: it sequesters iron essential for bacterial growth; binds to lipopolysaccharide on the cell surface of Gram negative bacteria, disrupting the bacterial cell membrane; it has anti-lipoteichoic acid (against Gram positive organisms) and anti-Candida cell wall activities; LF peptide fragments have in vitro bactericidal properties[10]. LF impairs the ability of pathogens to adhere/invade mammalian cells, by binding to, or degrading, specific virulence proteins[21]. In addition to the direct antimicrobial effect, LF protects against infection due to its immunomodulatory properties[22, 23]. This wide range of LF beneficial properties is related to its functional structure[24].

In summary, this study shows feasibility of improving preterm infant health in developing countries by providing "added protection" with ingestion of a major milk protein. Within its preliminary nature, it has allowed more confident predictions of safety, cost, sepsis incidence in the target population, sample size power, and feasibility for extending it in resource-limited settings. Currently, we are conducting a larger trial on 414 neonates with a birth weight less than 2000g. In addition to confirming the suggested results of this preliminary study on sepsis prevention, we will follow infants up to 24 months of age to determine the effect of LF on growth and neurodevelopment (NICHD, R01-HD067694).

Given the high incidence and high morbidity and mortality of sepsis in preterm infants, efforts to reduce the rates of infection are among the most important interventions in neonatal care[25]. The use of LF as a broad-spectrum non-pathogen specific antimicrobial protective protein is an innovative approach that needs to be confirmed by multiple trials. If further studies confirm LF's protective role, they will profoundly affect clinical care of neonates both in developed and developing countries, serving as a cost-effective strategy to decrease infections and its long-term consequences on growth and development.

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FOOTNOTES

Clinical Trial registry name: Pilot Study: Lactoferrin for Prevention of Neonatal Sepsis (NEOLACTO), identifier: NCT01264536.

Conflict of Interest: The authors have no conflicts of interests to disclose, and declare that the study sponsors (Gates Foundation and NICHD) had no role in the study design; the collection, analysis, and interpretation of data; the writing of the report; or the decision to submit the manuscript for publication. Dr. Theresa J. Ochoa, corresponding author, wrote the first draft of the manuscript, and did not receive any form of payment for its production.

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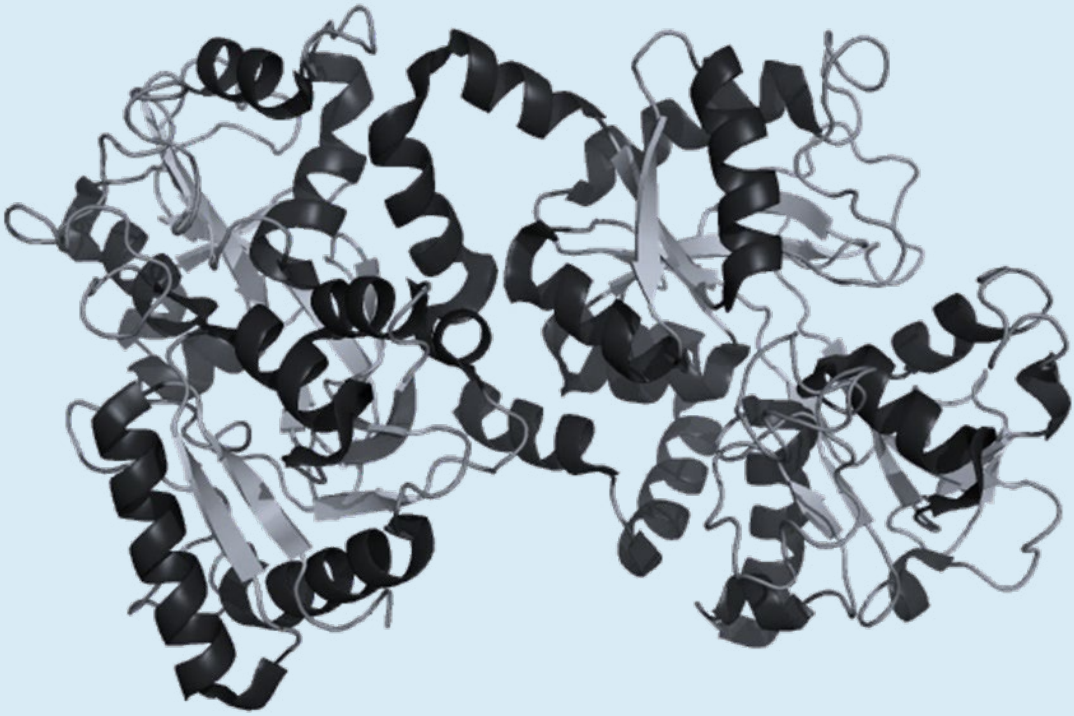

CHAPTER 3

Effect of bovine lactoferrin on prevention of late-onset sepsis and neurodevelopment improvement in preterm infants <2000g

RANDOMIZED CONTROLLED TRIAL OF BOVINE LACTOFERRIN FOR PREVENTION OF SEPSIS AND NEURODEVELOPMENT IMPAIRMENT IN INFANTS WEIGHING LESS THAN 2000 G.

Authors:
Theresa J. Ochoa MD[1,2,3], Jaime Zegarra MD[1,4], Sicilia Bellomo MD[1,4], Cesar P. Carcamo MD PhD[5], Luis Cam MD[6], Anne Castañeda MD[7], Aasith Villavicencio MD[1], Jorge Gonzales MD[1], Maria S. Rueda MD[1], Christie G. Turin MD[1], Alonso Zea-Vera MD[1], Daniel Guillen MD[1,4], Miguel Campos MD PhD[8], Linda Ewing-Cobbs PhD[9], and the NEOLACTO Research Group.

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[1]Department of Pediatrics, School of Medicine, Universidad Peruana Cayetano Heredia, Lima, Peru
[2]Instituto de Medicina Tropical "Alexander von Humboldt", Universidad Peruana Cayetano Heredia, Lima, Peru
[3]Center for Infectious Diseases, School of Public Health, University of Texas Health Science Center at Houston, Houston, Texas, United States
[4]Hospital Cayetano Heredia, Lima, Peru
[5]School of Public Health and Administration, Universidad Peruana Cayetano Heredia, Lima, Peru
[6]Hospital Nacional Alberto Sabogal, Lima, Peru
[7]Hospital Nacional Guillermo Almenara, Lima, Peru
[8]Department of Mathematics, School of Science and Philosophy, Universidad Peruana Cayetano Heredia, Lima, Peru
[9]Department of Pediatrics and Children's Learning Institute, School of Medicine, University of Texas Health Science Center at Houston, Houston, Texas, United States.

ABSTRACT

Objective
To determine the effect of bovine lactoferrin on prevention of late-onset sepsis (LOS) and neurodevelopment delay.

Study design
Randomized, double-blind, controlled trial in neonates with a birth weight of 500-2000 g in 3 neonatal units in Lima, Peru, comparing bovine lactoferrin 200 mg/kg/day with placebo administered for 8 weeks. The primary outcome was the first episode of culture-proven LOS or sepsis-associated death. Neurodevelopment delay was assessed by the Mullen Scales at 24 months corrected age.

Results
Of the 414 infants enrolled, 209 received bovine lactoferrin and 205 received placebo. LOS or sepsis-associated death occurred in 22 infants (10.5%) in the bovine lactoferrin group vs 30 (14.6%) in the placebo group; there was no difference after adjusting for hospital and birth weight; hazard ratio 0.73 (95% CI, 0.42-1.26). For infants with birth weights of <1500 g the hazard ratio was 0.69 (95% CI, 0.39-1.25). The mean age-adjusted normalized Mullen composite score at 24 months was 83.3 ± 13.6 in the bovine lactoferrin group vs 82.6 ± 13.1 in the placebo group. Growth outcomes and rehospitalization rates during the 2-year follow-up were similar in both groups, except for significantly less bronchiolitis in the bovine lactoferrin group (rate ratio, 0.34; 95% CI, 0.14-0.86).

Conclusions
Supplementation with bovine lactoferrin did not decrease the incidence of sepsis in infants with birth weights of <2000 g. Growth and neurodevelopment outcomes at 24 months of age were similar. Neonatal bovine lactoferrin supplementation had no adverse effects.

Trial registration
ClinicalTrials.gov: NCT01525316.

Keywords
Neonate; premature; milk; infection; mortality

Abbreviations
ABAS-II, Adaptive Behavior Assessment System, 2nd edition; Bailey-III, Bayley Scales of Infant and Toddler Development, 3rd edition; CoNS, Coagulase-negative staphylococci; DSMB, Data Safety Monitoring Board; LOS, Late-onset sepsis; Mullen, Mullen Scales of Early Learning; NICU, Neonatal intensive care unit

INTRODUCTION

Breast milk protects preterm infants from infections and improves cognitive development.[1-3] One of the multiple bioactive components in milk[4] is lactoferrin, a glycoprotein with antimicrobial, anti-inflammatory and immunomodulatory properties.[5] Lactoferrin has multiple mechanisms for protection against infection, including bacteriostatic effects (iron sequestration); disruption of bacterial cell membranes by binding lipopolysaccharide in gram-negative bacteria; binding pathogen–host cell receptors; inhibiting biofilm formation; modulating intestinal flora; promoting intestinal cell proliferation, differentiation and maturation; regulating immune response; and antioxidative effects.[6,7]

There is growing interest in clinical applications of lactoferrin, including protection against neonatal infections with published trials of bovine lactoferrin for protection against sepsis and necrotizing enterocolitis in preterm infants, including our pilot study.[8-18] However, most trials had small sample sizes. All trials followed infants until hospital discharge without information on long-term outcomes. It is postulated that preterm brain exposure to inflammatory mediators during infection contributes to brain injury and poor neurodevelopment.[19,20] We hypothesized that bovine lactoferrin would improve neurodevelopment by immunoregulation, decreasing infection-related inflammation.

This study aimed to determine the effect of bovine lactoferrin on prevention of late-onset sepsis (LOS) or sepsis-associated death in infants with a birth weight of <2000 g (aim 1) and the effect on neurodevelopment and growth at 24 months corrected age (aim 2).

METHODS

The study was a randomized, double-blind, placebo-controlled trial conducted in 3 neonatal intensive care units (NICUs) in Lima, Peru (Cayetano, Almenara, Sabogal). The study was approved by the ethics committee of Universidad Peruana Cayetano Heredia, University of Texas Health Science Center at Houston, each hospital, and Peruvian regulatory institutions (ClinicalTrials.gov: NCT01525316).

Infants were included if they weighed 500-2000 g at birth and were inborn or referred to the NICUs in the first 72 hours after birth. Infants were excluded if they had underlying gastrointestinal problems preventing enteral intake, predisposing conditions profoundly affecting growth and development, a family history of cow's milk allergy, or were unlikely to complete the study. Written informed consent was obtained from both parents.

The randomization list was performed with fixed, equal allocation to each group, in random blocks of 4, stratified by birth weight (500-1000, 1001-1500, and 1501-2000) and hospital. Infants were assigned a consecutive number in the order of enrollment after calling the central coordination office. Randomization occurred

immediately after recruitment. Bovine lactoferrin or placebo capsules were opened and dissolved in breast milk or formula for masking. Only the research nurse the knew treatment assignment; clinical and research staff and parents were blinded until the end of the study.

Enteral bovine lactoferrin (Friesland Campina, Amersfoort, the Netherlands) or placebo (maltodextrin) (Montana, Lima, Peru) 200 mg/kg/day was administered in 3 divided doses for 8 weeks (maximum of 600 mg/day). Capsules containing 100 or 200 mg of bovine lactoferrin or placebo were dissolved in breast milk or formula; 100 mg dissolved in a minimum volume of 4 mL (maximum bovine lactoferrin concentration of 25 mg/mL). The first dose was given on enrollment day or as soon as the infant tolerated enteral intake. The NICU nurse prepared and administered the intervention.

Hospital data were collected daily until discharge. Sepsis or meningitis evaluations were done per standard care at each hospital; in general, 2 sets of blood cultures were drawn for each episode of suspected sepsis and sent to the hospital microbiology laboratory and a centralized laboratory. Cultures were monitored for growth with automated systems (BACTEC and/or BacT/ALERT) and positive cultures were processed according to conventional techniques. The hospitals had no protocols for feeding, fluconazole prophylaxis, or antibiotic prophylaxis. All human milk was mother's own fresh expressed milk; no donor milk or probiotics were used. Breast milk (2-3 mL) was collected in the first 7 days of life (colostrum) and at 1 month to measure human lactoferrin using an ELISA (Assaypro, St. Charles, Missouri).

We followed infants for ≤24 months corrected age by phone every 2 weeks, using corrected age for evaluations. Pediatric evaluations were performed at 3, 6, 12, 18, and 24 months; auditory brainstem response examination at 37-44 weeks postmenstrual age or at hospital discharge; neurologic evaluations at 6, 12 and 24 months; and ophthalmologic evaluations at 24 months. Infants completed the Mullen Scales of Early Learning (Mullen) at 12, 18, and 24 months and the Bayley Scales of Infant and Toddler Development, 3rd edition (Bayley-III) at 24 months.21,22 The Mullen is a standardized assessment of 5 domains from 0 to 68 months: gross motor, fine motor, visual reception, receptive language, and expressive language. The Early Learning Composite is a standard score (100 ± 15) representing overall cognitive ability, derived from the latter subscales. The Mullen has favorable test–retest reliability for individual scales and excellent interrater reliability. Prior studies have shown that Mullen scores are significant predictors of later-developing intelligence and executive function scores.23-25 At 24 months, parents answered 2 questionnaires, the Adaptive Behavior Assessment System, 2nd edition (ABAS-II) and the Modified-Checklist for Autism in Toddlers, Revised.26,27

Culture-proven LOS was defined as clinical signs and symptoms of infection and ≥1 positive blood and/or cerebrospinal fluid cultures obtained at >72 hours of age. For coagulase-negative staphylococci (CoNS), we required 2 positive blood cultures or 1 positive blood culture plus a C-reactive protein of >10 mg/L.[28] Probable sepsis or culture-negative infection was defined by the presence of clinical signs and symptoms of infection plus ≥2 abnormal laboratory results or 1 CoNS-positive blood culture with ≥7 days of treatment with antistaphylococcal agents.[29] Each LOS episode was classified based on an algorithm and an expert blinded committee.[3]

Study outcomes

For aim 1, the primary study outcome was a composite outcome of the first culture-proven LOS or sepsis-associated death (deaths associated with probable sepsis). Secondary outcomes were the composite outcome in very low birth weight infants (<1500 g), pathogen-specific LOS, necrotizing enterocolitis (Bell stage ≥2), retinopathy of prematurity requiring surgery, intraventricular hemorrhage (grade III-IV), bronchopulmonary dysplasia (oxygen requirement for >28 days), serious infections before discharge, hospitalization duration, rehospitalization, overall mortality, infection-related mortality, and frequency of adverse events or intolerance.

For aim 2, the primary outcome was the mean age-adjusted normalized Mullen composite score at 24 months. Secondary outcomes were neurodevelopmental delay (Mullen composite score of ≤70, Bayley-III scores <85), delayed adaptive skills (ABAS-II general adaptive composite score of <70), neurodevelopmental impairment (Mullen composite score of ≤70, moderate to severe cerebral palsy, bilateral hearing impairment requiring amplification or bilateral blindness), and growth delay (height-for-age and weight-for- height Z-scores of <−2).[31,32] All study outcomes were prespecified in the protocol.

An independent Data Safety Monitoring Board (DSMB) monitored the study for safety and integrity. Serious adverse events were reported to the DSMB, institutional review boards, and regulatory institutions.

The quality and purity of the bovine lactoferrin sample used was analyzed and compared with bovine lactoferrin from our pilot study.[18] Samples were tested for purity and impurities using a Reversed Phase High-Performance Liquid Chromatography (Patheon-Pharmaceuticals, Cincinnati, Ohio); and for bacterial endotoxin (Nelson-Labs, Salt Lake City, Utah).

Statistical Analyses

Assuming 25% confirmed LOS episodes in the placebo group (based on the NICUs' statistics) and a 15% attrition rate, 207 children were needed in each group to detect a 45% decrease in the number of sepsis episodes ($\alpha = 0.05$; power = 0.80). For neurodevelopment, with this sample size and a 30% attrition rate for the 24-month follow-up, we estimated a power of 0.81 to detect a difference of 5 in the Mullen composite between the arms. Statistical analyses were adjusted for weight category and hospital. Two- sided tests at the .05 significance level were used. Cox regression was used to evaluate the primary outcome for aim 1 on an intention-to-treat analysis; time-at-risk started at day 3 and ended at day 56, at discharge, or with LOS, whichever came first. Secondary outcomes were evaluated using Cox regression analyses, incidence rate ratios, or prevalence ratios. For aim 2, linear regression was used for numerical variables and generalized linear models for binary variables. For the Mullen composite score aggregating data from the 12-, 18-, and 24-month evaluation, we used mixed effects multilevel regression, with random intercepts and slopes, and independent correlation. Post hoc analysis on human lactoferrin intake was performed using general linear models. Stata 8.2 was used (StataCorp, College Station, Texas).

RESULTS

Enrollment occurred from May 2012 to July 2014; of 905 infants within the birth weight range, 414 were enrolled and randomized, 209 allocated to receive bovine lactoferrin and 205 placebo (Figure 1). Mean birth weight and gestational age were 1380 ± 365 g and 30.8 ± 3.0 weeks, respectively; 256 were very low birth weight infants. Four infants were born at term. There were 97 infants (23.4%) from Cayetano, 137 (33.1%) from Almenara, and 180 (43.5%) from Sabogal Hospital. Baseline characteristics and risk factors for LOS were comparable between the groups (Table 1). Treatment compliance was similar; 82.3% of 16 852 prescribed bovine lactoferrin doses were administered completely per protocol vs 83.5% of 15 880 placebo doses. The diluents used were fresh mother's milk (57.7% of the doses), infant formula (42.2%), and dextrose (0.1%).

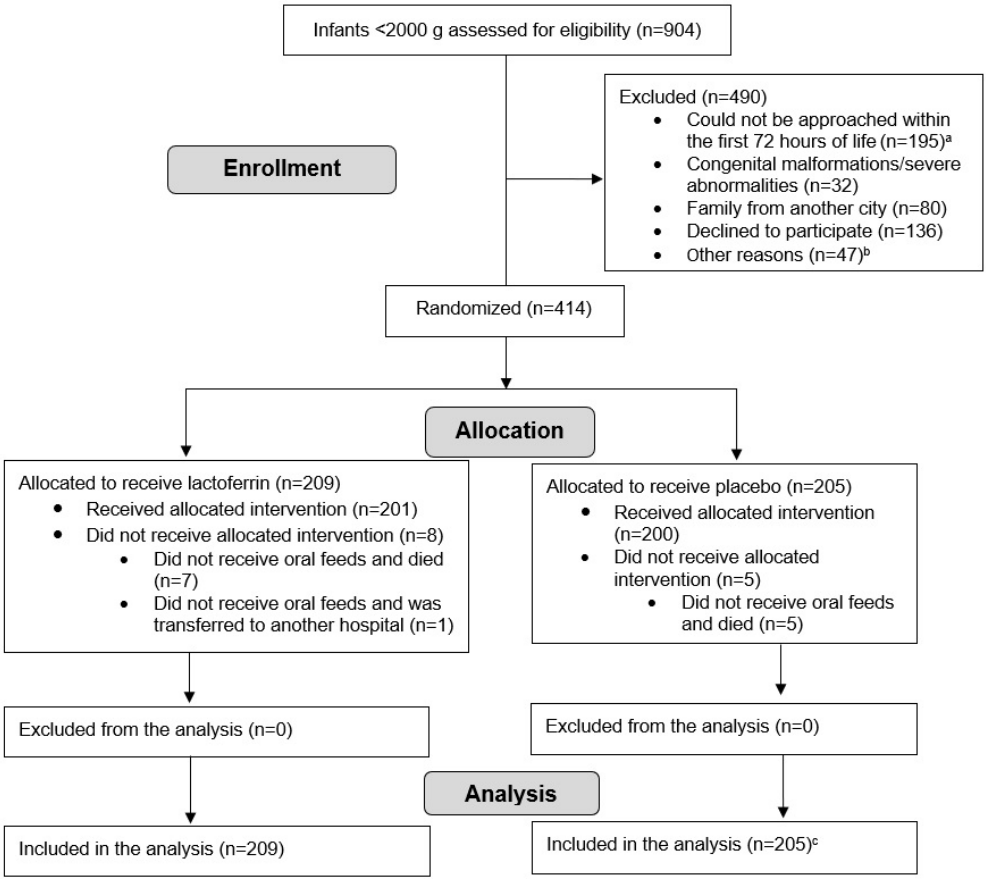

Figure 1. Consort diagram Lactoferrin vs Placebo.
[a] Infants that died during the first 72 hours of life (n=75), were discharged (n=17), or whose parents were not available for enrollment during that period (n=103). [b] Infants of single mothers (n=8), adolescent mothers (n=13), mothers in the UCI (n=10), father outside the city (n=5), father absent (n=10), father participating in the trial (n=1).[c] This analysis includes a patient that was diagnosed after randomization with esophageal atresia, a condition that prevented oral intake, an exclusion criterion.

Table 1. Baseline demographic and clinical characteristics

Characteristic	Lactoferrin (n=209) No. (%)	Placebo (n=205) No. (%)
Pregnancy and delivery		
Maternal age, mean (SD), y	29.8 (6.4)	29.4 (6.5)
Single mother[a]	16 (8.0)	14 (7.0)
Mother, higher education[b]	96 (47.5)	88 (44.4)
Monthly family income, mean (SD), US dollars	567 (492)	536 (430)
ATB use in the last month of pregnancy	41 (20.9)	39 (20.4)
Multiple pregnancy	45 (21.6)	47 (22.9)
Preeclampsia/eclampsia	62 (29.8)	60 (29.3)
Peripartum infection	54 (28.6)	50 (26.2)
Peripartum fever	18 (9.3)	16 (8.2)
Chorioamnionitis (clinical or suspected diagnosis)	63 (30.9)	58 (28.6)
Premature rupture of membranes	76 (36.9)	58 (29.3)
Antenatal steroids[c]	152 (82.6)	141 (75.0)
Cesarean delivery	168 (80.8)	164 (80.0)
Antibiotics during labor	68 (38.2)	52 (28.9)
Neonatal		
Gestational age at birth, mean (SD), weeks	30.8 (2.8)	30.8 (3.2)
Birth weight, mean (SD), g	1382 (371)	1378 (353)
Small for gestational age	57 (27.3)	59 (28.8)
Male sex	114 (54.5)	116 (56.6)
5-min Apgar score, mean (SD)	8.5 (1.1)	8.3 (1.2)
Neonatal resuscitation needed	117 (56.3)	119 (58.0)
Early-onset sepsis Post randomization risk factors for LOS until discharge or death, mean (SD)	90 (43.1)	92 (44.9)
Hospitalization in NICU, days	14.8 (18.7)	16.2 (18.1)
CVC use, days[d]	12.2 (17.0)	14.2 (16.5)
Mechanical ventilation, days	3.9 (10.2)	4.0 (10.4)
CPAP use, days	3.4 (7.4)	3.5 (5.9)
Treatment with antibiotics, days	10.3 (14.8)	11.3 (15.0)
Use of steroids, days	0.1 (1.4)	0.1 (0.9)
Use of H2-receptor antagonists, days	0.6 (2.1)	0.4 (1.5)
TPN use, days	9.2 (13.7)	10.9 (13.4)
Age at establishment of full enteral feeding, days	6.8 (8.8)	7.2 (9.9)

Abbreviations: SD, standard deviation; LOS, late-onset sepsis; NICU, neonatal intensive care unit; CVC, central venous catheters; CPAP, continuous positive airway pressure; TPN, total parenteral nutrition. [a] Unmarried or without a partner. [b] Higher education defined as post-secondary education including college, university and/or institutes of technology. [c] At least one dose administered. [d] Central inserted, peripheral inserted central catheter (PICC) and umbilical catheter.

During the 8 weeks of the intervention, there were 97 culture-proven or probable sepsis episodes (43 in lactoferrin vs 54 in placebo). The mean day of onset of first culture-proven LOS was 15.2 ± 10.4 in the bovine lactoferrin group vs 15.0 ± 12.1 in placebo. Among 67 positive blood cultures, there were fewer gram-negative bacteria, CoNS, and Candida isolates in the bovine lactoferrin group. However, there was no significant effect of bovine lactoferrin on the primary composite outcome adjusting for clustering within hospital and birth weight (hazard ratio [HR], 0.73; 95% CI, 0.42-1.26; P = .26) (Table 2). Among very low birth weight infants, the primary outcome occurred in 19 infants (14.6%) in the bovine lactoferrin group vs 27 (21.4%) in placebo (HR, 0.69; 95% CI, 0.39-1.25; P = .22).

Table 2. Late-onset sepsis (LOS) and secondary outcomes

Outcomes[a]	Lactoferrin (n=209)	Placebo (n=205)	
Sepsis outcomes	**No. (%)**	**No. (%)**	**Hazard Ratio (95% CI)**
Primary composite outcome, No. (%)	22 (10.5)	30 (14.6)	0.73 (0.42 – 1.26)
First culture-proven LOS, No. (%)	17 (8.1)	22 (10.7)	
Sepsis-related deaths, No. (%)	5 (2.6)	8 (4.4)	
Primary composite outcome by birth weight group, n/N (%)			
500g - 1000g	7/39 (17.9)	15/38 (39.5)	0.45 (0.18 – 1.10)[b]
1001g - 1500g	12/91 (13.6)	12/88 (13.8)	0.99 (0.45 – 2.21)
1501g - 2000g	3/79 (3.8)	3/79(3.8)	1.04 (0.21 – 5.16)
First culture-proven or probable LOS, No. (%)	34 (16.3)	44 (21.5)	0.76 (0.48 – 1.19)
All culture-proven and probable LOS[c], No. (%)	43 (43.1)	54 (53.6)	0.82 (0.55 – 1.23)
Isolated pathogens (all culture-proven and probable sepsis), No.	29	38	
Gram negative bacteria[d]	8	12	
Gram positive bacteria (excluding CoNS)[e]	5	3	
CoNS	15	19	
Candida	1	4	
Secondary outcomes	**No. (IR/10⁴)[f]**	**No. (IR/10⁴)[f]**	**Rate ratio (95% CI)**
NEC Bell's stage ≥2	5 (0.5)	11 (1.1)	0.46 (0.16 – 1.31)
ROP requiring surgery	8 (8.4)	11 (11.4)	0.74 (0.30 – 1.83)
Intraventricular hemorrhage III-IV (3-4week)[c]	3 (1.9)	7 (4.6)	0.42 (0.17 – 1.02)
Periventricular leukomalacia (3-4weeks)[c]	2 (1.3)	4 (2.6)	0.48 (0.23 – 1.01)
Bronchopulmonary dysplasia[c]	25 (12.0)	26 (12.7)	0.94 (0.85 – 1.05)
Serious infections prior to discharge			
Pneumonia	24 (40.3)	25 (40.1)	1.01 (0.58 – 1.78)
Meningitis / encephalitis	4 (6.4)	6 (9.5)	0.64 (0.18 – 2.27)
UTI	7 (11.2)	4 (6.3)	0.50 (0.05 – 5.52)

Outcomes[a]	Lactoferrin (n=209)	Placebo (n=205)	
Conditions requiring re-hospitalization			
Pneumonia	20 (1.6)	21 (1.6)	1.00 (0.54 – 1.84)
Meningitis/encephalitis	1 (0.1)	2 (0.2)	0.51 (0.05 – 5.62)
UTI	1 (0.1)	2 (0.2)	0.50 (0.55 – 5.52)
Bronchiolitis	6 (0.5)	18 (1.4)	0.34 (0.14 – 0.86)[g]
Wheezing	7 (0.6)	3 (0.2)	2.49 (0.64 – 9.64)
Pertussis-like syndrome	3 (0.2)	3 (0.2)	1.04 (0.21 – 5.15)
Mortality			
Mortality in the first 8 weeks	29 (29.8)	24 (24.5)	1.23 (0.72 – 2.11)
In-hospital mortality	30 (48.1)	26 (40.7)	1.13 (0.67 – 1.91)
Overall 24-month mortality	37 (2.6)	29 (2.1)	1.32 (0.81 – 2.15)
Infection-associated mortality[h]	17 (1.3)	16 (1.2)	1.13 (0.57 – 2.24)

Abbreviations: LOS, late-onset sepsis; CoNS, coagulase-negative staphylococci; NEC; necrotizing enterocolitis; ROP, retinopathy of prematurity; UTI, urinary tract infection.[a] Adjusted for hospital and weight category (≤1000 g, 1001-1500 g, 1501-2000 g), considering follow up from day 3 to day 56 or death for all neonatal outcomes for all infants.[b] p=0.08[c] Cumulative incidences/100 cases and cumulative incidence ratio (95% CI).[d] *Klebsiella* (4 and 3), *E. coli* (3 and 3), *Enterobacter* (1 and 2), *Pseudomona* (0 and 2), *Acinetobacter* (0 and 1) and *Empedobacter* (0 and 1) among BLF and placebo groups respectively. [e] *Enterococcus* (3 and 1), *Staphylococcus aureus* (1 and 2) and *Streptococcus* (1 and 0) among BLF and placebo groups respectively.[f] IR/10^4, Incidence rate per 10,000 child-days. [g] p=0.02 [h] Mortality associated with sepsis and pneumonia, excluding early-onset sepsis. No infant died of meningitis/encephalitis, UTI or diarrhea.

There were no significant differences in secondary outcomes of aim 1, except for less rehospitalization for bronchiolitis in the bovine lactoferrin group (rate ratio, 0.34; 95% CI, 0.14-0.86; P = .02) (Table 2). The length of hospitalization was 31.8 ± 25.6 days in the bovine lactoferrin group vs 33.2 ± 22.4 days in the placebo group. Mortality rates were similar between the groups (Table 3). Overall mortality was high among extremely low birth weight infants (<1000 g), namely, 59.0% (23/39) in the bovine lactoferrin group vs 52.6% (20/38) in placebo. Sepsis-related mortality was also high: 20.5% (8/39) in bovine lactoferrin vs 28.9% (11/38) in placebo. The mortality rates for extremely low-birth-weight infants reported are similar to rates in the same NICUs and others in Lima.

For aim 2, 152 infants (72.7%) completed the 24-month follow-up in the bovine lactoferrin group (20 dropped out, 37 died) vs 158 infants (77.1%) in placebo (18 dropped out, 29 died). The 24-month drop-out rate was 9.2% (38/414). Follow-up was completed in October 2016. Table 3 shows demographic information comparing infants lost to follow-up with those who were seen for the developmental follow-up at 24 months. A total of 899 Mullen tests were performed (Figure 2). The Mullen, Bayley-III, and ABAS-II results at 24 months were similar among groups (Table 4). Neurodevelopmental delay in very low birth weight infants was 18.8% (15/80) in the bovine lactoferrin group vs 21.2% (18/85) in the placebo group (Table 5). Growth outcomes were comparable during the 2-year follow-up (Figure 3).

Table 3. Demographic characteristics of infants that completed the 24-month evaluation and infants lost to follow-up

	Mullen at 24 months	Dead	Lost to follow-up	Cerebral palsy	Other[a]	Total
Lactoferrin						
N	143	37	20	5	4	209
Birth weight, g, mean (SD)	1462 (323)	978 (325)	1572 (274)	1118 (228)	1655 (185)	1382 (371)
Gestational age at birth, weeks, mean (SD)	31.4 (2.4)	28.2 (2.5)	31.8 (2.8)	27.8 (2.7)	31.0 (3.3)	30.8 (2.8)
Male sex, n (%)	79 (55.2)	19 (51.4)	9 (45.0)	3 (60.0)	4 (100.0)	114 (54.6)
SGA, n (%)	37 (25.9)	14 (37.8)	5 (25.0)	0 (0.0)	1 (25.0)	57 (27.3)
Placebo						
N	149	29	18	6	3	205
Birth weight, g, mean (SD)	1432 (309)	947 (248)	1661 (313)	1214 (295)	1475 (117)	1378 (353)
Gestational age at birth, weeks, mean (SD)	31.2 (2.8)	27.6 (2.8)	32.8 (3.1)	29.8 (4.1)	31.3 (1.5)	30.8 (3.2)
Male sex, n (%)	87 (58.4)	12 (41.4)	11 (61.1)	3 (50.0)	3 (100.0)	116 (56.6)
SGA, n (%)	46 (30.9)	5 (17.2)	6 (33.3)	1 (16.7)	1 (33.3)	59 (28.8)

Abbreviations: SD, standard deviation; SGA, small for gestational age
[a] Other: Completed the 24-month follow up, but did not completed the Mullen test

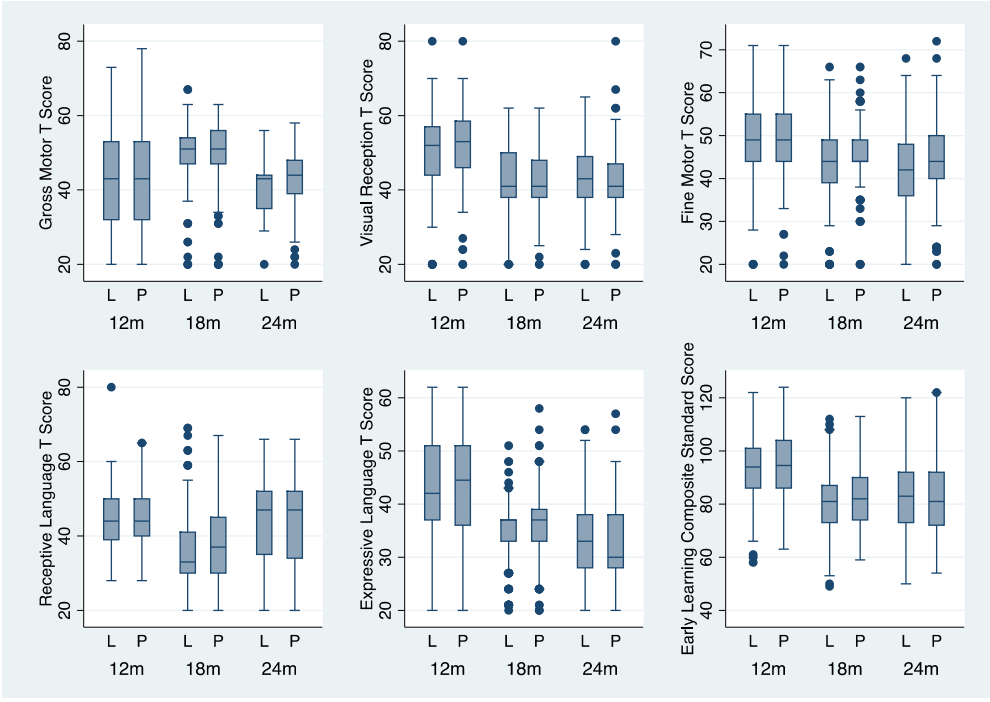

Figure 2. Mullen Scores at 12, 18 and 24 months corrected age among infants supplemented with lactoferrin (L) or placebo (P) in the neonatal period. A total of 299 tests were performed at 12 months, 299 at 18 months and 301 at 24 months corrected age; 438 in the lactoferrin group and 461 in the placebo.

Table 4. Neurodevelopmental and growth outcomes at 24 months corrected age

Outcome[a]	Lactoferrin	Placebo	
Mullen, mean (SD)	**(n=143)**	**(n=149)**	**Adjusted difference (95% CI)**
Composite	83.3 (13.6)	82.6 (13.1)	0.62 (-3.07 – 4.31)
Gross Motor	41.5 (7.0)	42.5 (7.9)	-1.04 (-3.38 – 1.31)
Visual Reception	43.2 (9.4)	42.6 (9.2)	0.66 (-1.88 – 3.20)
Fine Motor	43.2 (10.3)	43.8 (9.9)	-0.53 (-1.91 – 0.84)
Receptive Language	44.2 (11.5)	43.9 (12.2)	0.23 (-2.83 – 3.29)
Expressive Language	33.8 (7.7)	32.9 (7.4)	0.97 (-1.48 – 3.42)
Composite combined (12, 18 and 24 months)[b], mean	84.1	85.2	-1.34 (-3.40 – 0.73)
Bayley-III, mean (SD)	**(n=112)**	**(n=112)**	**Adjusted difference (95% CI)**
Cognitive	94.0 (8.3)	94.7 (6.9)	-0.76 (-2.11 – 0.59)
Language	86.9 (9.6)	85.7 (9.0)	1.26 (-1.74 – 4.24)
Motor	95.4 (10.7)	96.3 (10.9)	-0.91 (-2.02 – 0.21)
Social emotional score	110.7 (20.4)	111.7 (17.5)	-1.00 (-5.66 – 3.66)
Neurodevelopmental delay	**n/N (%)**	**n/N (%)**	**Relative risk (95% CI)**
Mullen Composite ≤70	22/143 (15.4)	30/149 (20.1)	0.76 (0.44 – 1.32)
Bayley-III Cognitive <85	8/112 (7.1)	5/112 (4.5)	1.60 (0.68 – 3.75)
Bayley-III Language <85	40/110 (36.4)	46/110 (41.8)	0.87 (0.57 – 1.31)
Bayley-III Motor <85	12/110 (10.9)	12/110 (10.9)	1.00 (0.46 – 2.16)
ABAS-II, GAC <70	42/111 (37.8)	39/112 (34.8)	1.09 (0.84 – 1.41)
M-CHAT-R, Score ≥3[c]	16/126 (12.7)	12/125 (9.6)	1.32 (0.69 – 2.54)
Neurodevelopmental impairment (NDI)[d]	31/147 (21.1)	39/153 (25.5)	0.83 (0.56 – 1.22)
Growth outcomes	**n/N (%)**	**n/N (%)**	**Relative risk (95% CI)**
Weight-for-length Z-score ≤-2(wasting)	4/117 (3.4)	6/125 (4.1)	0.71 (0.24 – 2.14)
Length-for-age Z-score ≤-2(stunting)	15/117 (12.8)	15/125 (12.0)	1.07 (0.54 – 2.11)
Weight-for-age Z-score ≤-2(underweight)	9/120 (7.5)	13/126 (10.3)	0.73 (0.29 – 1.85)
Head circumference Z-score ≤-2 (microcephaly)	7/103 (6.8)	7/113 (6.2)	1.10 (0.56 – 2.15)

Abbreviations: Mullen, Mullen Scales of Early Learning; SD, standard deviation; Bayley-III, Bayley Scales of Infant and Toddler Development Third Edition ; ABAS-II, Adaptive Behavior Assessment System Second Edition; GAC, general adaptive composite; M-CHAT-R, Modified Checklist for Autism in Toddlers-Revised; NDI, neurodevelopmental impairment.

[a] Adjusted for hospital and weight category (≤1000 g, 1001-1500 g, 1501-2000 g), excluding patients with cerebral palsy (5, BLF; 7, placebo). Corrected age is reported for all outcomes. [b] Generalized estimating equations [c] Medium to high risk for autism spectrum disorder (ASD). [d] NDI (composite outcome): defined as the presence of any of the following: Early Learning Composite Mullen Score ≤ 70, moderate to severe cerebral palsy, bilateral hearing impairment requiring amplification or bilateral blindness.

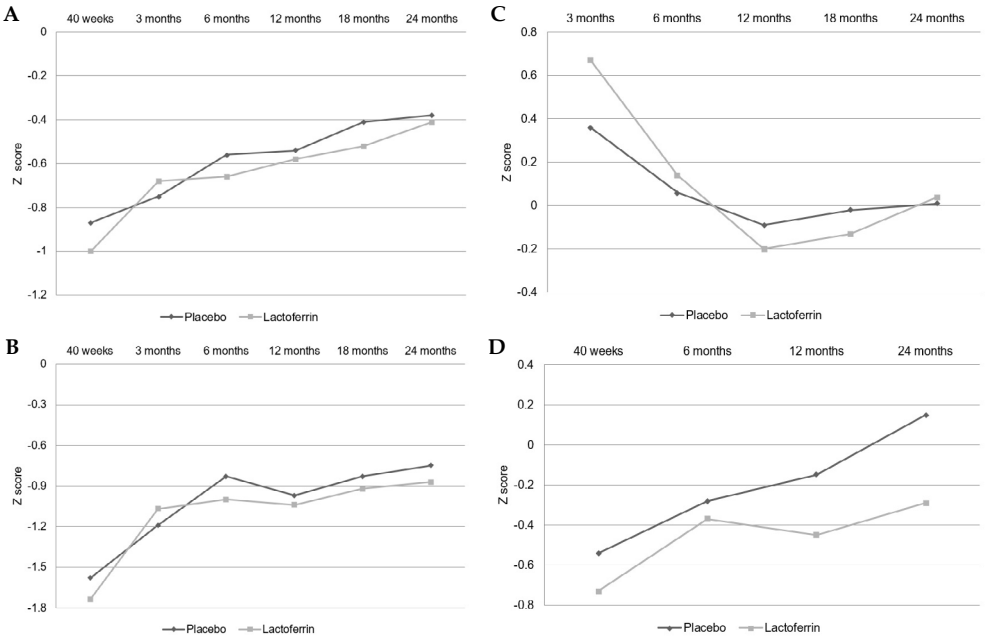

Figure 3. Comparison of growth measurements from 40 weeks to 24 months corrected age. A. Weight-for-age Z-scores (mean); B. Length-for-age Z scores (mean); C. Weight-for-length Z-scores (mean); D. Head circumference Z-scores (mean).

The infants' nutritional characteristics were similar in both groups, except for more mother's milk intake in the placebo group (Table 6); a higher percentage of child-days of observation in which infants received only mother's milk (32.6% vs 38.0%; P < .001). In addition, we found high levels of human lactoferrin in breast milk in both arms (Table 6). We explored the amount of human lactoferrin consumed by the infants and found a nonsignificant higher mean human milk lactoferrin intake within the first week of life in the placebo group (Table 6). Post hoc analyses adjusting for human lactoferrin intake showed no statistically significant differences for the primary composite outcome (HR, 0.87; 95% CI, 0.46-1.63). No differences between groups were found adjusting for breast milk consumption in milliliters per kilogram either (HR, 0.67; 95% CI, 0.39-1.18)

Adverse Events

Signs or symptoms of allergic reactions or intolerance were closely monitored. Among 12 745 child-days of observation, vomiting was present in 0.2% vs 0.1% in the bovine lactoferrin and placebo groups, respectively, a >2-cm increase in abdominal circumference in 0.3% vs 0.8%, and diarrhea in 0.1% in both. No infant was diagnosed with cow's milk allergy. There were 141 rehospitalizations (67 in the bovine lactoferrin group; 74 placebo) and 66 deaths (37 in the bovine lactoferrin group; 29 placebo); the DSMB evaluated all serious adverse events, and none was found attributable to the intervention.

Overall the purity of the two BLF lots was similar at 95.3% and 94.8% as measured by RP-HPLC. The percentage of label claim was slightly less for one BLF lot (89.0% vs. 97.5%) but still very close to the industry standard 90-110% of label claim. Bacterial endotoxin was <0.500EU/mg for both lots.

Table 5. Neurodevelopmental outcomes at 24 months corrected age in VLBW infants

Outcome	Lactoferrin	Placebo	
Mullen[a], mean (SD)	**(n=80)**	**(n=85)**	**Adjusted difference (95% CI)**
Composite	83.5 (14.4)	81.8 (12.3)	1.59 (-7.12 – 10.30)
Gross Motor	41.6 (7.0)	41.7 (7.6)	-0.09 (-2.20 – 2.01)
Visual Reception	43.7 (9.3)	42.0 (8.7)	1.68 (-4.25 – 7.61)
Fine Motor	42.0 (10.1)	42.8 (9.1)	-0.80 (-4.43 – 2.83)
Receptive Language	44.7 (11.9)	43.5 (12.2)	1.23 (-5.20 – 7.66)
Expressive Language	34.3 (7.7)	33.2 (7.3)	1.13 (-5.67 – 7.92)
Composite combined (12, 18 and 24 months)[b], mean	84.1	85.2	-1.34 (-3.40 – 0.73)
Bayley[a], mean (SD)	**(n=63)**	**(n=66)**	**Adjusted difference (95% CI)**
Cognitive	94.3 (9.2)	94.5 (6.8)	-1.22 (-2.87 – 0.43)
Language	86.0 (10.6)	85.6 (10.0)	0.37 (-5.78 – 6.52)
Motor	95.4 (12.5)	96.9 (11.3)	-1.44 (-2.89 – 0.01)
Social emotional score	113.2 (18.4)	111.7 (18.1)	1.45 (-0.78 – 3.67)
Neurodevelopmental delay[a]	**n/N (%)**	**n/N (%)**	**Relative risk (95% CI)**
Mullen Composite ≤70	15/80 (18.8)	18/85 (21.2)	0.89 (0.40 – 1.95)
Bayley Cognitive <85	6/63 (9.5)	4/66 (6.1)	1.57 (0.54 – 4.61)
Bayley Language <85	27/61 (44.3)	25/65 (38.5)	1.15 (0.72 – 1.84)
Bayley Motor <85	8/62 (12.9)	7/65 (10.8)	1.20 (0.84 – 1.71)
ABAS-II, GAC <70	24/59 (40.7)	21/64 (32.9)	1.24 (0.86 – 1.80)
M-CHAT-R, Score ≥3[c]	9/72 (12.5)	8/72 (11.1)	1.13 (0.81 – 1.56)

Abbreviations: Mullen, Mullen Scales of Early Learning (MSEL); SD, standard deviation; Bayley-III, Bayley Scales of Infant and Toddler Development Third Edition (BSID-III); ABAS-II, Adaptive Behavior Assessment System Second Edition; GAC, general adaptive composite; M-CHAT-R, Modified Checklist for Autism in Toddlers-Revised; a Adjusted for hospital and weight category (≤1000 g, 1001-1500 g, 1501-2000 g), excluding patients with cerebral palsy (5, LF; 7, placebo). Corrected age is reported for all outcomes. b Generalized estimating equations. c Medium to high risk for autism spectrum disorder (ASD).

Table 6. Nutritional characteristics and human milk lactoferrin intake

Characteristic	Lactoferrin (n=209) mean (SD)	Placebo (n=205) mean (SD)
Age of enteral feeding initiation in days	2.2 (3.1)	2.1 (3.3)
Age of full enteral feeding establishment in days	6.8 (8.8)	7.2 (9.9)
Daily mother´s milk consumption in cc/kg during complete hospitalization	50.2 (56.0)	54.4 (54.8)
Percentage of daily mother´s milk consumption during complete hospitalization	53.7 (42.0)	60.0 (40.7)
Cumulative mother´s milk intake entire hospitalization in L, mean	2.0	2.3
Number of days with at least 1 direct oral take of breast milk	5.5 (7.2)	5.8 (6.7)
Direct daily oral takes of breast milk	2.2 (0.9)	2.3 (0.9)
	6284 child-days No. (%)	6461 child-days No. (%)
NPO	613 (9.9)	648 (10.2)
Use of TPN	1931 (30.7)	2231 (34.6)
Fed only with mother´s milk	2019 (32.6)	2414 (38.0)[a]
Fed only with artificial formula	1132 (18.3)	793 (12.5)
Fed both with breast milk and artificial formula	2421 (39.1)	2490 (39.2)
Breast milk human lactoferrin (hLF) concentration and intake	mean (SD) [n][b]	mean (SD) [n][b]
hLF concentration in colostrum, mg/mL	14.3 (7.3) [145]	15.6 (8.6) [132]
hLF concentration at 1 month, mg/mL	9.9 (6.1) [130]	10.8 (6.5) [129]
hLF intake from colostrum, mg/day	266 (267) [141]	317 (363) [126]
hLF intake at one month, mg/day	554 (571) [57]	663 (676) [64]

Abbreviations: SD, standard deviation; NPO, nothing per mouth; TPN, total parenteral nutrition; hLF, breast milk human lactoferrin.
[a] $p<0.001$ [b] [n], number of infants with hLF determination in their mother´s milk sample.

DISCUSSION

This study found that bovine lactoferrin supplementation in low birth weight infants had no significant effect on LOS, sepsis-associated death, neurodevelopment, and growth outcomes. A nonsignificant 55% decrease in the risk for the composite outcome (LOS and sepsis-associated death) was observed in infants with a birth weight of <1000 g (extremely low-birth-weight), similar to Manzoni's studies in Italy; they found significantly lower incidence of LOS mainly among extremely low-birth-weight infants.[10,11] The ELFIN study found no significant effect in the subgroup analysis by gestational age.[17] Other previous trials have not analyzed bovine lactoferrin effect by birth weight category, owing to their small sample size.[13-16,18] Extremely low-birth-weight infants are the most vulnerable population and most likely the

ones that will benefit from this intervention.[33] In the first days after birth, small infants receive minimal amounts of human milk; therefore, bovine lactoferrin supplementation may be critical during this period when they need additional protection.

Fewer pathogens were isolated from all culture-proven and probable sepsis episodes in the lactoferrin group, including fewer Gram-negative bacteria and Candida species (9 vs 16 isolates). Bovine lactoferrin may target pathogens in the gut, by modulating the intestinal microbiome and preventing translocation of bacteria from the gut.[7] In contrast, it is unlikely that bovine lactoferrin can target CoNS, predominantly originating from the skin.

Our study differs from previous trials in several aspects. We used a bovine lactoferrin dose based on the infant's weight (200 mg/kg/day), whereas previous studies used a fixed dose (100, 200, 300 mg/day).[10-13,15,16] The study by Kaur et al, our pilot study (200 mg/kg/day), and the ELFIN study (150 mg/kg/day) used weight-based dosing.[14,17,18] The dose chosen was the effective dose in the smallest infants (500 g) in the study by Manzoni et al.[10] Moreover, we administered bovine lactoferrin 3 times daily for 8 weeks to mimic the effect of breast milk lactoferrin, administered continuously with each feeding, during the period at risk; most studies used bovine lactoferrin once daily for 4 weeks or until discharge. All trials including ours used bovine lactoferrin, except for the study by Sherman et al, which used recombinant human lactoferrin.[15] Lactoferrin purified from human and bovine milk have similar structural and biochemical properties; their bioactivity, assessed in vitro and in animal models, is comparable, but not identical.[5]

A contribution of our study to the body of knowledge of bovine lactoferrin in neonates is the neurodevelopmental and long-term follow-up. If lactoferrin becomes a standard of care, it needs to be demonstrated to be safe. Although we were not able to prove our hypothesis that bovine lactoferrin improves neurodevelopment in preterm neonates, safety was demonstrated; outcomes for infants in the bovine lactoferrin group were similar to those in the placebo group for neurodevelopmental delays and overall neurodevelopmental impairments. In both the bovine lactoferrin and placebo groups, Mullen expressive language subscale scores were lower than scores for gross motor, visual reception, fine motor, and receptive language, as previously described for extremely low birth weight infants.[34] Neonatal infections in very preterm infants are associated with worse neurodevelopment including higher incidence of cerebral palsy, representing an economic burden for families and society.[20,33,35-37]

We found significantly fewer rehospitalizations for bronchiolitis in the bovine lactoferrin group. This was in line with a previous study; children receiving a bovine lactoferrin-enhanced formula had significantly fewer lower respiratory tract illnesses in the first year (0.5 vs 1.5 episodes/year).[38] This finding is worth exploring given the high burden of respiratory infections in preterm populations, especially in extremely low birth weight infants, who have the highest rate of respiratory hospital readmissions in early childhood.[39]

Some prior trials showed significantly decreased rates of sepsis but others, including our pilot study and the ELFIN trial (2203 participants) had negative results, as did the current study.[16-18] There are several explanations for this discordant outcome. First, this study was underpowered; the overall number of sepsis episodes in both arms was lower than expected. For our sample size calculation, we estimated a 25% sepsis rate in the placebo group; the final LOS rate was only 10.7%, mainly because infants >1500 g contributed few sepsis episodes, with a sepsis rate of <4%. The discrepancy between the prestudy LOS rates and the actual ones, probably related to our more strict definition of LOS owing to CoNS. Second, the infants had higher human lactoferrin intake from colostrum and breast milk than prior studies.[40,41] Therefore, the high lactoferrin levels in human milk and the high breast milk consumption overall could have diluted the effect of bovine lactoferrin. Third, another possible explanation for the lack of effect is the quality and purity of the bovine lactoferrin. However, both bovine lactoferrin preparations were similar, with optimal purity and no bacterial endotoxin. This analysis is critical, because there are many commercial bovine lactoferrin preparations with potentially different degrees of denaturation and purity and no standard guidelines for the quality of products in clinical studies.[42]

This study has some limitations. First, we had a small number of enrolled very low birth weight infants. Initially, we did not plan a quota for each birth weight category; however, in the middle of the study, reviewing the sepsis rates by birth weight category (blinded to the treatment allocation), we decided to stop enrolling infants >1500 g to increase the power of the study; nevertheless, the number of very low birth weight infants (n = 256) enrolled at completion of the sample size (n = 414), was not sufficient. Second, the clinical evaluation of suspected sepsis episodes was done according to standard care of each hospital; therefore, the appropriateness and timing of blood cultures and antibiotic use varied between centers.[43] However, the analysis of the main study outcome was performed adjusting for this variable. Third, the Mullen test is not validated in preterm infants or our population. With the company's approval (Pearson, San Antonio, Texas), we translated the test from English to Spanish and back-translated it to English, but have not done a validation study. However, the Bayley-III, validated in Spanish, showed similar results.

In summary, supplementation with bovine lactoferrin did not decrease the incidence of sepsis in infants with birth weights <2000 g, but the use of bovine lactoferrin as a broad-spectrum antimicrobial protective protein may have potential effect in infants weighing <1000 g on LOS that needs to be confirmed in future trials. One additional large ongoing study, the LIFT trial (Australia)[44] will provide further evidence on bovine lactoferrin effectiveness on sepsis, mortality and neurodevelopment.

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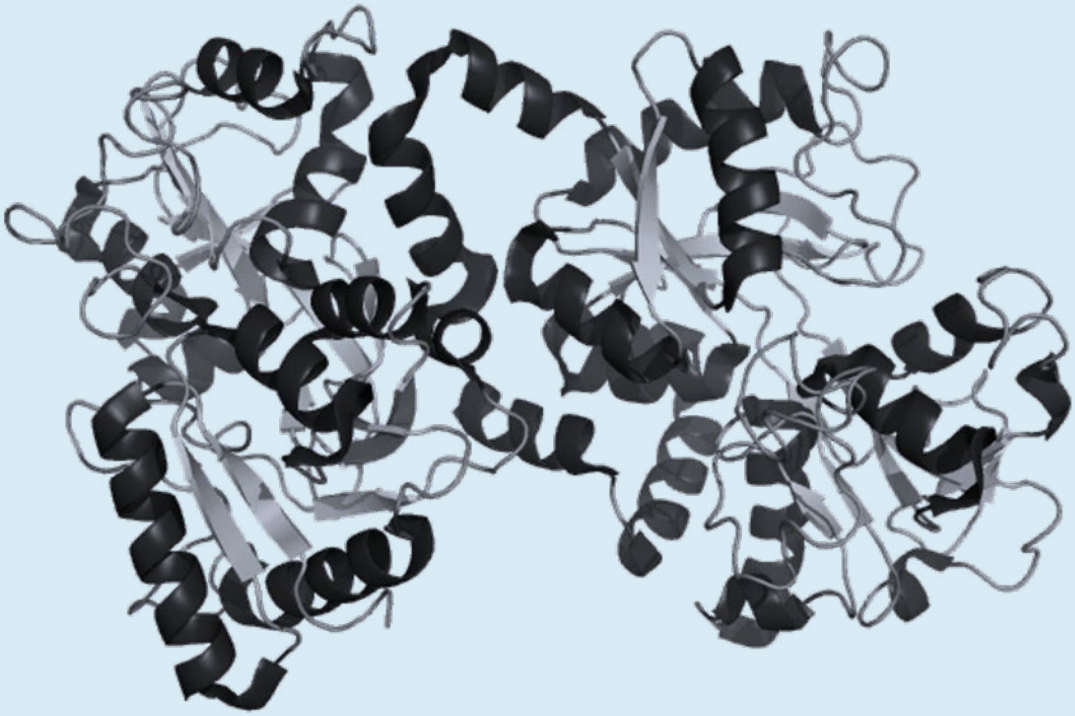

CHAPTER 4

Effect of bovine lactoferrin on prevention of late-onset sepsis in infants <1500g

EFFECT OF BOVINE LACTOFERRIN ON PREVENTION OF LATE-ONSET SEPSIS IN INFANTS <1500G: A POOLED ANALYSIS OF INDIVIDUAL PATIENT DATA FROM TWO RANDOMIZED CONTROLLED TRIALS.

Authors:
Theresa Ochoa[1,2,3], Sebastian Loli[4], Karina Mendoza[4], Cesar Carcamo[4], Sicilia Bellomo[1,5], Luis Cam[6], Anne Castaneda[7], Miguel Campos[8], Jan Jacobs[9,10], Veerle Cossey[11] and Jaime Zegarra[1,5]

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[1] Facultad de Medicina, Universidad Peruana Cayetano Heredia, Lima, Peru
[2] Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru
[3] School of Public Health, University of Texas Health Science Center at Houston, USA
[4] Facultad de Salud Pública y Administración, Universidad Peruana Cayetano Heredia, Lima, Peru,
[5] Hospital Cayetano Heredia, Lima, Peru
[6] Hospital Nacional Alberto Sabogal, Lima, Peru
[7] Hospital Nacional Guillermo Almenara, Lima, Peru
[8] Facultad de Ciencias y Filosofia, Universidad Peruana Cayetano Heredia, Lima, Peru
[9] Department of Microbiology and Immunology, KU Leuven, Belgium
[10]Department of Clinical Scienses, , Institute of Tropical Medicine, Antwerp, Belgium
[11]Department of Development and Regeneration, KU Leuven, Belgium.

ABSTRACT

Background. We previously conducted two randomized, double-blind, placebo-controlled trials of bovine lactoferrin (bLF) for prevention of late-onset sepsis (LOS) in Peruvian infants <2500g (Study 1) and <2000g (Study 2). The aim of this study was to determine the effect of bLF supplementation on prevention of culture-proven or probable LOS in infants with a birth weight <1500g from both studies, and to determine the bLF effect depending on human milk intake.

Methods. We included neonates <1500g followed during the first 4 weeks of life. Both trial designs had similar inclusion and exclusion criteria; same bLF dose (200mgkg/day) and same control. We used multivariate Cox regression models to estimate the effect of bLF on the risk of development of the composite outcome, adjusting for covariates. Time-at-risk started at day 3 and ended at day 28.

Results. We included 335 neonates, 80 from study 1, and 255 from study 2. The mean birth weight was 1162g ±244g, 27.5% were <1000g, and 52.2% were male. There were 33 first episodes of LOS in the bLF group and 48 in the control group (19.5% vs 28.9%). bLF had a protective effect on the risk of development LOS, Hazard Ratio (HR) 0.645 (%95CI: 0.41-0.99, p=0.047); particularly among infants <1000g, HR 0.44 (%95CI: 0.21-0.91, p=0.027) and among infants with low human milk intake, HR 0.62 (%95CI: 0.41-0.95, p=0.027), adjusting by birth weight and sex.

Conclusion. bLF supplementation protects against LOS in infants <1500g, especially among infants not receiving human milk.

INTRODUCTION

Lactoferrin (LF) is a bioactive protein present in milk and other secretions; it has anti-infective, anti-inflammatory, and immune-modulating properties that are relevant in the premature infant that is at risk of infection, inflammation and oxidative stress injuries.[1] This protection early in life may be related to LF effect on modulating bacterial growth in the gastrointestinal track, promoting intestinal cell proliferation, differentiation and maturation, and regulating the host immune response to infection.[1,2]

Over the last decade there has been a large interest in studying the effects of LF preventing neonatal sepsis and promoting neurodevelopment.[1,3,4] To date, there are ten published clinical studies evaluating the effect of the supplementation of bovine LF (bLF) on the prevention of sepsis and NEC[5-16] with conflicting results. There are several reasons why these bLF supplementation trials may have inconsistent results. Some possibilities are differences in the population enrolled, the quality and bioactivity of the bLF used, the bLF dose used, the outcome determinations and the presence of other confounding variables not adequately measured.

Our research group has conducted two randomized, double blind, placebo controlled trials to evaluate the effect of daily supplementation of bLF on the prevention of neonatal late-onset sepsis (LOS) in newborns. The first was a pilot study in 190 neonates with a birthweight <2500 g[12] and the second in 414 neonates <2000 g.[17] Although there were less sepsis episodes in the bLF groups, the difference was not statistically significant in any of the studies. One of the main limitations of these studies is that we have not enrolled a large number of very low birth weight (VLBW) infants (<1500g), which is the target population of most other clinical studies of bLF supplementation, as it is the population at higher risk for infection and death. Since both trials were very similar in the study design and intervention, we propose a pooled analysis of individual patient data from these two trials. The aim of the study was to determine the effect of bLF in VLBW infants on the prevention of culture-proven and probable late-onset sepsis (LOS) during the first 4 weeks of life, and among extremely low birth weight (ELBW) infants (<1000g). The second aim was to determine the effect of bLF in different human milk intake groups.

METHODS

We performed a pooled analysis of individual patient data from two trials: Study 1: "*Pilot study: lactoferrin for the prevention of neonatal sepsis"* (NCT01264536), and Study 2: "*Lactoferrin for the prevention of sepsis in infants*" (NCT 01525316) (NEOLACTO trial). Briefly, both were randomized double blind placebo controlled trials to evaluate the effect of oral supplementation of bLF (200 mg/kg/day) in newborns with birthweight between 500 - 2500g (Study 1) and 500 - 2000g (Study 2), born in or referred to the Neonatal Intensive Care Units (NICU) of 3 hospitals in Lima, and enrolled during the first 3 days of life. Newborns were excluded

if they had congenital malformations which were severe or incompatible with life, could not complete the follow-up (parents who lived outside of Lima), had a family history of allergies to milk proteins, or had non-consenting parents. In Study 1 newborns were randomized to receive bLF or placebo (maltodextrin), with groups stratified by birth weight; in Study 2 newborns were randomized to receive bLF or placebo (maltodextrin), with groups stratified by birth weight and hospital. They received the intervention 3 times per day for 4 weeks (Study 1) or for 8 weeks (Study 2). Data on factors associated with sepsis were recorded daily during hospitalization as well as on maternal milk and formula consumption. Other health related outcomes were recorded for up to 3 months of age (Study 1) or 24 months of age (Study 2) but were not included in this study. The main outcome for Study 1 was the first episode of clinically defined LOS (culture-proven sepsis and culture-negative clinical infection), and for Study 2 LOS confirmed by grown blood culture or death associated with probable sepsis. The main study characteristics of both trials are summarized in Table 1.

Study definitions for the clinical trials were: culture-proven LOS defined as clinical signs and symptoms of infection and one or more grown blood and/or cerebrospinal fluid cultures obtained after 72 hours of life. For coagulase-negative staphylococci (CoNS) two grown blood cultures or one grown blood culture plus an elevated C-reactive protein were required. Probable sepsis or culture-negative clinical infection was defined by the presence of clinical signs and symptoms of infection plus at least two abnormal laboratory results; [18] or one CoNS grown blood culture with ≥ 7 days of treatment with an anti-staphylococcal agent. Each LOS episode was classified based on a study algorithm[19] and the judgement of an expert panel.

For the first aim, we determined the effect of bLF supplementation on the prevention of culture-proven LOS or probable sepsis, as a composite outcome, during the first 4 weeks of life. Therefore, the follow-up period and events after week 4 from study 2 were not included in the analysis. For the second aim, since exclusive breastfeeding (human milk only with no other nutrients, supplements, or liquids) is not very common in the NICUs, we used the definition from Lawrence & Lawrence[20] of high partial breastfeeding (nearly all feedings are human milk, >80%); thus, for this study we classified infants in two human milk intake groups: high (≥80% of days with human milk feeding) and low (<80% of days with human milk feeding). The volume of maternal milk was measured directly, since mothers manually extracted the milk and a known volume was fed to the neonate in a syringe directly into the mouth or via a nasogastric tube. In these populations donor milk was not used. For this analysis we did not include the volume from direct feeding from the breast, since this volume was not quantified; however, in these premature infants, direct breastfeeding was a rare event in the first weeks of life.

Table 1. Study characteristics of the two previous RCTs of bovine lactoferrin (bLF) for prevention of sepsis

Characteristic	Description	Study 1[12]	Study 2[17]
Population	Birth weight for inclusion	500 – 2500g	500 – 2000g
	Total number of infants	190	414
	Number of infants by BW category		
	500 – 1000 g	18	77
	1000 – 1500 g	62	179
	1500 – 2000 g	79	158
	2000 – 2500 g	31	0
	Total number of VLBW infants	80	256
Intervention	bLF used	Tatua, New Zealand	FrieslandCampina, The Netherlands
	bLF dose	200mg/kg/day, divided three times a day	200mg/kg/day, divided three times a day
	Duration of treatment intervention	4 weeks	8 weeks
	Duration of follow up	3 months	24 months
Randomization	Type of randomization	Equal allocation in each group, randomized complete blocks of size 4, with stratification by 4 BW categories.	Equal allocation in each group, randomized complete blocks of size 4, with stratification by 3 BW categories and 3 hospitals
	Randomized to bLF	95/190 (50%)	209/414 (50.5%)
	Randomized to bLF among VLBW	40/80 (50%)	130/256 (50.8%)
Control	Placebo	Maltodextrin	Maltodextrin
	Placebo dose	200mg/kg/day, divided three times a day	200mg/kg/day, divided three times a day
Outcome	Primary outcome	First episode of LOS clinically defined (culture-proven sepsis and culture-negative clinical infection)	First episode of LOS confirmed by positive culture or death associated with probable sepsis (composite outcome)

BW, birth weight; VLBW, very low birth weight; bLF, bovine lactoferrin

We carried out a pooled analysis of the studies mentioned above, in order to determine the effect of bLF supplementation on prevention of culture-proven late-onset sepsis or probable sepsis (composite outcome). We used anonymized data from both trials. We fitted multivariate Cox regression models to estimate the effect of bLF on the risk of development of the composite outcome, adjusted by hospital, and stratified by study and birthweight. Time-at-risk started at day 3 and ended at day 28. We assessed the potential heterogeneity of treatment effect between studies by the introduction of a treatment by study interaction term to the Cox regression model. We checked the assumptions for proportional hazard; Kaplan-Meier curves were plotted after the final model. We performed sub-analysis for each category of birthweight, fitting Cox regression models stratified by study (we could not adjust for hospital because of too low numbers).

Both trials were approved by the institutional ethical committee of Universidad Peruana Cayetano Heredia, University of Texas Health Science Center at Houston and by each of the participating hospitals. The studies were approved and registered at the Peruvian National Institute of Health (INS). The current study was approved by the institutional ethical committee of Universidad Peruana Cayetano Heredia.

RESULTS

Of the 336 eligible infants from both studies, we included 80 from study 1and 255 from Study 2 (one infant from Study 2 was discharged on day 2 of life before starting the intervention); 169 (50.5%) were randomized to bLF (Figure 1). Follow-up was 6198 child-days, without interval censored data. The mean birth weight was 1162g ± 244g, 27.5% were <1000g, and 52.2% were male. The baseline demographic and clinical characteristics of the enrolled infants are presented in Table 2.

During the first 4 weeks of life there were 81 first LOS episodes, including 33 culture confirmed and 48 probable sepsis. The main pathogens isolated were CoNS (9), *Klebsiella* (5), *Candida* (5*), Enterococcus* (4) and *Escherichia coli (3)* (see Table 3). The proportion of sepsis was higher among ELBW infants in comparison with infants >1000g (35.9% vs 19.8%, p=0.002). The adjusted Hazard Ratio (HR) of LOS for ELBW infants was 2.35 (%95CI: 2.06-2.69, p<0.001)

We found less LOS episodes in the bLF group (19.5% vs 28.9%, p=0.045). bLF had a protective effect on the risk of development LOS; the HR was 0.64 (%95CI: 0.41-0.99, p=0.047) adjusting for birth weight and sex (Table 3 and Figure 2). bLF protective effect was particularly significant among the ELBW infant group; adjusted HR 0.44 (%95CI: 0.21-0.91, p=0.027).

In relation to the nutritional characteristics of the enrolled infants, of the 7580 child-days of total follow-up, 48.7% received only human milk, 26.2% mixed feedings (human milk and infant formula), 10% received only infant formula and 14.6% received nothing by mouth (NPO). For purpose of estimating the effect based on the human milk intake group, we classified 163 infants (48.7%) in the low human

milk intake and 172 (51.3%) in the high intake group. bLF had a protective effect on the risk of development LOS among infants with low human milk intake; HR 0.50 (%95CI: 0.27-0.94, p=0.032) stratified by study and birthweight (Table 3).

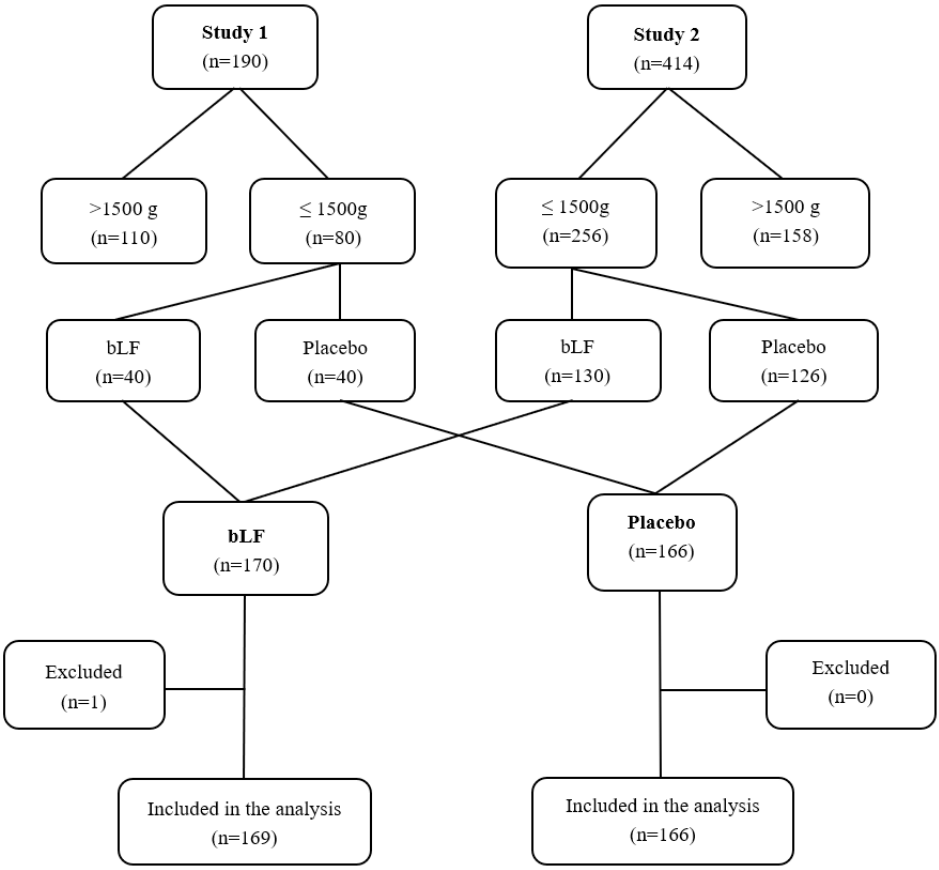

Figure 1. Flowchart of infants enrolled in both studies and in the pooled analysis

Table 2. Baseline demographic and clinical characteristics of infants included in the pooled analysis

Characteristic	Lactoferrin (n=169) n (%)	Placebo (n=166) n (%)	p-value
Pregnancy and delivery			
Maternal age, mean (SD), years	30.1 (6.2)	29.8 (6.5)	0.666
Multiple pregnancy	36 (21.3)	29 (17.5)	0.375
Preeclampsia/eclampsia	45 (26.6)	47 (28.3)	0.730
Peripartum infection	42 (27.1)	43 (28.1)	0.843
Chorioamnionitis (clinical or suspected diagnosis)	42 (25.5)	38 (23.2)	0.629
Premature rupture of membranes	58 (34.9)	49 (30.6)	0.407
Antenatal steroids[a]	120 (79.0)	112 (74.7)	0.378
Cesarean delivery	133 (78.7)	135 (81.3)	0.548
Antibiotics during labor	47 (33.1)	46 (31.1)	0.713
Neonatal			
Gestational age at birth, mean (SD), weeks	29.5 (2.6)	29.2 (2.7)	0.413
Birth weight, mean (SD), g[b]	1155.7 (244.9)	1171.5 (241.9)	0.519
Birth group distribution			
500g – 1000g	46 (27.2)	45 (27.1)	0.982
1001g – 1500g	123 (72.8)	121 (72.9)	
Small for gestational age	55 (32.5)	46 (27.7)	0.335
Male sex	86 (50.9)	89 (53.6)	0.617
Post randomization risk factors for LOS until discharge or death, mean (SD)[b]			
Hospitalization in NICU, days	14.5 (9.3)	15.7 (9.3)	0.162
CVC use, days[c]	13.2 (9.1)	14.6 (8.9)	0.195
Mechanical ventilation, days	3.7 (6.5)	4.1 (7.4)	0.856
CPAP use, days	3.9 (6.2)	4.3 (5.9)	0.066
Treatment with antibiotics, days	9.2 (7.7)	10.5 (8.3)	0.190
TPN use, days	9.7 (7.5)	10.9 (7.9)	0.182

Abbreviations: SD, standard deviation; LOS, late-onset sepsis; NICU, neonatal intensive care unit; CVC, central venous catheters; CPAP, continuous positive airway pressure; TPN, total parenteral nutrition.
[a] At least one dose administered. [b] Mann-Whitney test
[c] Central inserted, peripheral inserted central catheter (PICC) and umbilical catheter.

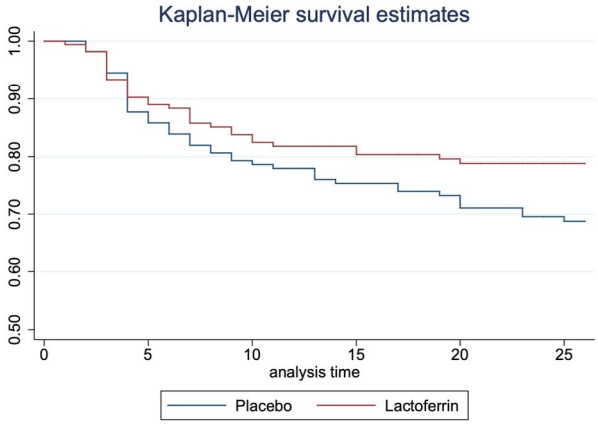

Figure 2. Kaplan–Meier survival estimates: time-to-event of late-onset sepsis (LOS) by treatment group. Red line: lactoferrin; blue line: placebo.

Table 3. Effect of bovine lactoferrin on late-onset sepsis (LOS) by birth weight and human milk intake categories

Outcomes	Lactoferrin n/N (%)	Placebo n/N (%)	Adjusted Hazard Ratio (95% CI)	p-value
Sepsis outcomes				
Primary outcome, LOS	33/169 (19.5)	48/166 (28.9)	0.64 (0.41-0.99)[a]	0.047
First culture-proven sepsis, n	13/169 (7.7)	20/166 (12.1)		
First probable sepsis, n	21/169 (12.4)	28/166 (16.9)		
LOS by birth weight group				
500g - 1000g	11/47 (23.4)	22/45 (48.9)	0.44 (0.21-0.91)[a]	0.027
1001g - 1500g	22/122 (18.0)	26/121 (21.5)	0.80 (0.45-1.42)[a]	0.445
LOS by human milk intake				
Low intake (< 80%)			0.62 (0.41-0.95)[a]	0.027
High intake (≥ 80%)			0.59 (0.24-1.45)[a]	0.253
Isolated pathogens (first culture-proven sepsis), n	13	20		
Gram negative bacteria[b]	5	7		
Gram positive bacteria (excluding CoNS)[c]	3	4		
CoNS[d]	4	5		
Candida	1	4		

Abbreviations: LOS, late-onset sepsis; CoNS, coagulase-negative staphylococci; NEC; necrotizing enterocolitis.

[a] Adjusted by hospital and stratified by study and birthweight, considering follow up from day 3 to day 28.

[b] Gram negatives: *Klebsiella* (4 and 1), *E. coli* (1 and 2), *Pseudomona* (0 and 2), *Enterobacter* (0 and 1), and *Empedobacter* (0 and 1) among lactoferin and placebo groups respectively.

[c] Gram positives: *Enterococcus* (2 and 2), *Staphylococcus aureus* (0and 2) and *Streptococcus* (1 and 0) among lactoferrin and placebo groups respectively.

[d] All CoNS culture confirmed episodes had 2 positive cultures per episode.

DISCUSSION

In this pooled analysis of individual patient data from our two previous trials we found a significant protective effect of bLF on the risk of development LOS in VLBW infants. This protection was significant especially in the ELBW infants and in those receiving less amounts of maternal milk. The main strength of this pooled analysis is that the included trials were very similar, not only on the design, the bLF dose, the timing of initiation of the intervention, the data collection forms, the study definitions and outcomes, but also on the population characteristics: both trials were conducted in the same hospitals with no important changes in pathogen epidemiology of LOS, infection control practices and other NICU practices over the two periods. Enrollment in study1 was from January to August 2011, and in study 2 from May 2012 to July 2014. Thus, both trials were a continuum.

There have been three recent meta-analysis of LF supplementation for prevention of LOS in preterm neonates. The first Cochrane meta-analysis from Pammi & Suresh in 2017[21] included 6 trials; the second from He et al in 2018[22] included in addition three new studies conducted in China (not published in PubMed)[13-15]; and the third from Razak & Hussain in 2019[23] analyzed 10 trials including the recent large ELFIN study from the UK[16] which showed no effect of LF in reducing sepsis. The latter (most recently updated) meta-analysis involved 3679 infants and despite including the ELFIN study concluded that there is low to moderate quality of evidence that suggests that enteral LF supplementation reduces the risk of LOS. However, as mentioned by the authors, one must be cautious when interpreting these results given the low certainty in the evidence from risk of bias among previous trials and the statistical heterogeneity.[23]

The effect of bLF on ELBW infants is particularly relevant, since those infants have the highest risk for infection and other complications. Currently there is a big interest in nutritional interventions to reduce morbidity and mortality in very preterm infants. Several clinical trials suggest that LOS, NEC and death can be influenced by the choice of food or some food components.[24] However, only donor milk and multiple-strain probiotics have been introduced into routine clinical care in some countries. [24] Neonatologist are reluctant to change clinical practice, since the evidence from clinical trials and meta-analysis need to be robust and applicable to local practice. Based on the initial trials from Manzoni,[5,6] some NICUs in Italy and New Zealand are already using bLF for very preterm infants. In a retrospective cohort study Meyer & Alexander reported the routine use of *Lactobacillus* GG and bLF from 2011 to 2015.[25]

There are several important clinical and methodological differences among trials reported in the literature. Among the most relevant are the differences in nutritional characteristics, since the intake of human milk is associated with less morbidity and mortality in preterm infants,[26] due to its multiple bioactive and protective components. In a recent secondary data analysis of the NEOLACTO trial, we found that the consumption of higher amounts of mother´s own milk

in the first days of life is associated with less infections, NEC and death in the first 8 weeks of life.[27] Moreover, infants who developed LOS, NEC or death had significantly less median daily human-LF intake than those who did not develop an event (≈ 90 vs. 330 mg/kg/day respectively, p<0.0001).[27] A recent study by Manzoni et al[28] evaluated the effect of bLF supplementation from two previous clinical trials (the ELFIN[16] and the original Manzoni´s trials[5-7]) and compared the effect among infants receiving only formula and mixed feeds. Their results suggest that bLF supplementation may have a benefit among infants not receiving mother´s own milk, and that probably there is no advantage of giving extra LF to infants already receiving good quantities of mother´s own milk. The findings of the present analysis are in line with the conclusion of their analysis. Indeed, we found a significant protective effect of bLF supplementation among infants receiving low human milk intake, but not among infants in the high milk intake group, who are indeed receiving already higher amounts of human LF and other protective factors from their mothers´ own milk.

This study has some limitations. First, we included in the outcome both culture-confirmed and clinical-defined LOS. A stricter outcome would have been only culture-confirmed sepsis; however, in many countries, especially in low- and middle-income countries, this is a limitation (low positivity of cultures). Moreover, the ELFIN[16] study also had as the primary outcome microbiologically confirmed or clinically suspected late-onset infection, with very similar criteria as our studies. Second, we did not perform a formal meta-analysis of individual participant data; however, since we designed and conducted both trials and knew that both studies were very similar, we only corrected for the potential heterogeneity of treatment effect between studies by the introduction of a treatment by study interaction term to the Cox regression model. Third, for the human milk intake analysis, ideally we should have calculated the percentage of human milk intake in relation to the total amount of oral intake (in ml); however, for study 1, we had only registered the type of intake, not the quantity. Nevertheless, we used a very stringent definition for high human milk intake (>80% of all days with human milk intake). Despite these limitations, the current study provides evidence that supports the use of bLF supplementation for prevention of infections in neonates with the highest risk of infection: the ELBW and the ones receiving insufficient amounts of human milk. Future research should focus on confirming these findings in other high risk settings.

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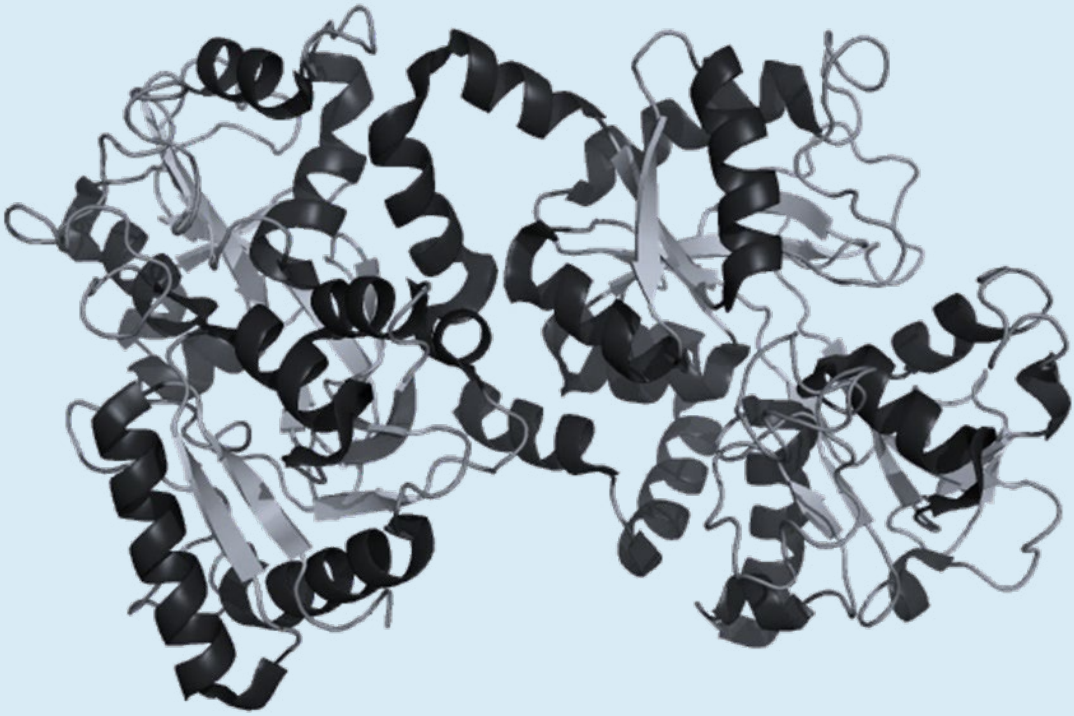

CHAPTER 5

Effect of breast milk lactoferrin on late-onset sepsis, NEC or death

IS MOTHER´S OWN MILK LACTOFERRIN INTAKE ASSOCIATED WITH LESS NEONATAL SEPSIS, NEC OR DEATH?

Authors:
Theresa J. Ochoa[1,2,3*], MD, Karina Mendoza[4], MSc, Cesar Carcamo[4], MD, PhD, Jaime Zegarra[1], MD, Sicilia Bellomo[1], MD, Jan Jacobs[5,6], MD, PhD, and Veerle Cossey[7], MD, PhD.

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[1]Facultad de Medicina, Universidad Peruana Cayetano Heredia, Lima, Peru
[2]School of Public Health, University of Texas Health Science Center at Houston, USA
[3]Doctoral School of Biomedical Sciences, KU Leuven, Belgium
[4]Facultad de Salud Pública y Administración, Universidad Peruana Cayetano Heredia, Lima, Peru;
[5]Department of Microbiology and Immunology, KU Leuven, Belgium
[6]Department of Clinical Science, Institute of Tropical Medicine, Antwerp, Belgium
[7]Department of Development and Regeneration, KU Leuven, Belgium.

ABSTRACT

Introduction. Lactoferrin (LF) is a protective protein present in milk with anti-infective and immune-modulating properties.

Objectives. To determine the association of maternal-LF intake and mother´s own milk intake in the first 10 days of life on the prevention of late-onset sepsis (LOS), necrotizing enterocolitis (NEC) or death in the first 8 weeks of life in newborns with a birth weight < 2000g.

Methods. Retrospective cohort, with the exposure being the consumption of mother´s own LF and mother´s own milk in the first 10 days of life and the outcome being LOS, NEC or death during days 11 and 56 of life, analyzed by Cox regression.

Results. 299 infants were enrolled, including 240 with human-LF intake information. The average daily human-LF intake over days 4-10 of life was 283 mg/kg/day (IQR 114-606 mg/kg/day). The hazard ratio (HR) of own mother´s milk LF intake ≥ 100mg/kg/day in days 4-10 for LOS, NEC or death was 0.297 (95% CI 0.156-0.568, p<0.001); the adjusted HR was 0.752 (95%CI 0.301-1.877, p=0.541). The adjusted HR of mother´s own milk cumulative intake (days 4-10) of 54-344 ml/kg (25-75quartiles) for LOS, NEC or death was 0.414 (95% CI 0.196-0.873, p=0.02). Infants who developed an event (LOS, NEC or death) had significantly less median daily human-LF intake than those that did not (89 vs. 334 mg/kg/day respectively, p<0.0001).

Conclusion. Consumption of higher amounts of mother´s own milk in the first days of life is associated with less infections, NEC and death. Early human milk intake should be strongly encouraged in all newborns.

INTRODUCTION

Breastmilk provides protection against infections and necrotizing enterocolitis (NEC) and improves neurodevelopment, due to its multiple bioactive components.[1-3] Among these is lactoferrin (LF), a glycoprotein with anti-infective and immune-modulating effects.[4-6] There is currently high interest in studying the effects of bovine-LF supplementation on the prevention of neonatal sepsis and NEC.[7-16]

Our research group has recently conducted a randomized trial to evaluate the effect of daily supplementation of bovine-LF on the prevention of neonatal late-onset sepsis (LOS) in newborns with a birth weight <2000g. The study failed to demonstrate a significant effect on the reduction of sepsis.[17] As part of the trial, we obtained breastmilk samples to measure human-LF concentrations.[18]

Previous studies have demonstrated that the consumption of colostrum and maternal breastmilk offers protection against sepsis in very low birth weight (VLBW) infants.[19-20] However, there is a gap in knowledge of the true effects of the consumption of human-LF on the protection against infections and death in premature newborns. The aims of this study were to determine the effect of mother´s own milk (MOM) lactoferrin (human-LF) intake (aim 1) and MOM intake (aim 2) in the first 10 days of life on the prevention of neonatal LOS, NEC, or neonatal death in the first 8 weeks of life in newborns born with birth weight <2000 g and in the subgroup of VLBW infants.

METHODS

This is a secondary analysis of the data from the NEOLACTO trial (NCT 01525316).[17] Briefly, this was a randomized double blind placebo controlled study to evaluate the effect of oral supplementation of bovine-LF in 414 newborns enrolled in the first 3 days of life in Lima. We requested a breastmilk sample in the first 6 days of life (colostrum) and between 7 and 14 days (transitional milk) to measure the concentration of human-LF using a commercial ELISA kit (Assaypro, St.Charles, MO, USA).[18] Study definitions: culture-proven LOS, clinical signs and symptoms of infection and a positive blood culture obtained after 72h of life; probable sepsis or culture-negative infection, clinical signs and symptoms of infection plus at least two abnormal laboratory results; each LOS episode was classified based on a study algorithm.[22] . For NEC we included NEC, Bell´s stage ≥ 2.[1]

To measure the effect of maternal-LF intake on the prevention of sepsis we performed a retrospective cohort study. The exposure was the cumulative consumption of maternal-LF (mg/kg) during days 4-10 of life measured by the daily consumption of MOM (ml/kg) multiplied by the concentration of LF in the colostrum and early transitional milk of each mother, which was taken as a homogenous value for the first 10 days of life. No donor milk was used in these hospitals. The volume of maternal milk was measured directly; mothers manually extracted the milk and the corresponding volume was given to the neonate in a syringe directly

to the mouth or via a nasogastric tube. The outcome was the first episode of LOS (culture confirmed or probable sepsis), NEC, or death between day 11 to 56 of life.

Statistical analysis. We performed a multivariate Cox regression model to estimate the effect of consumption of human-LF (cumulative consumption in mg/kg) in the first 10 days of life on the risk of development of LOS, NEC, or death. The analysis was adjusted by (1) breast milk consumption (cumulative intake in ml/kg during the days of exposure), (2) the percentage of breast milk consumption in relation to the total milk intake (breast milk + infant formula), and (3) human LF intake (cumulative breast milk intake in ml/kg during the days of exposure multiplied by the concentration of LF in colostrum [days 1-6] and early transitional milk [days 7-10]). The time to event was calculated from day 11 of life until discharge, day 56 of life (in the absence of an event), or the occurrence of the event. As we excluded all events prior to the completion of the exposure period, we excluded all infants with an event or discharge prior to day 11. Potentially confounding variables, supplementation of bovine-LF (yes/no), gestational age (weeks), birthweight (grams), gender, hospital (1, 2 or 3) and age of milk sample collection were evaluated and added to the model one by one. In order to visualize the effect we created a Kaplan-Meier survival curve. We analyzed the average daily human-LF intake in two groups based on the quartiles. For aim 2, the analysis was adjusted by the percentage of human milk consumption, and adjusted for the same confounding variables. For the Kaplan-Meier survival curve, we analyzed human milk intake in three groups, based on the quartiles.

RESULTS

Of the 414 newborns enrolled in the trial, we excluded 115 infants because they were discharged or had an event before day 11. The total number of eligible infants for the analysis was 299 (Supplemental Table 1). The mean birth weight of enrolled infants was 1410±308g, with a gestational age of 31±2.7weeks; 61.2% were VLBW, 81.3% were born by C-section and 50.5% were randomized to bovine-LF supplementation. Among the VLBW infants the mean birth weight was 1214±216g and the mean gestational age was 29.8±2.6weeks. Information on MOM LF was available in 324 infants (277 colostrum samples and 47 early transitional milk); after exclusions, 240 infants were included in the analysis of human-LF intake (Supplemental Table1). There have been 41 events during the outcome evaluation period (days 11-56 of life), including 15 culture confirmed sepsis episodes (5 CoNS, 3 *E.coli*, 2 *Klebsiella*, 2 *Enterococcus*, 2 Candida, 1 *S. aureus*).

The mean human-LF concentration in colostrum and transitional milk was 14.4 ± 8.1 mg/ml. The concentrations of human-LF varied over time (Figure 1). For infants <2000g the average daily human-LF intake over days 4-10 of life was 283mg/kg/day, interquartile range (IQR) 114-606mg/kg/day. The intake of more than 100mg/kg/day of human-LF (equivalent to the first quartile approximately) over the exposure period was associated with less episodes of LOS, NEC or death, as observed in the Kaplan Meier survival curves (Figure 2A), with a protective crude hazard ratio (HR). (Table 1). For VLBW infants the median daily human-LF intake over days 4-10 of life was 178mg/kg/day, IQR 74-391mg/kg/

day. The adjusted HRs were not significant (Table 1). For extremely low birth weight infants (ELBW, <1000g) the median daily human-LF intake over days 4-10 of life was 66 mg/kg/day, IQR 20-120 mg/kg/day. The adjusted HRs were not significant. We performed a secondary analysis including only the LF values from days 4-10 (12.7± 7.0 mg/ml), and applied this mean value to all infants (n=299). With this analysis the adjusted HR of MOM LF intake ≥100mg/kg/day for LOS, NEC or death was 0.412 (95% CI 0.191-0.888, p=0.024).

Supplemental Table 1. Number of infants enrolled and events by birth weight category and analysis

Analysis	Birth Weight	Event period	Discharged home[a]	Culture confirmed LOS[b]	Probable LOS[c]	NEC[d]	Death[e]	Total excluded[f]	Total included
Own mother´s milk intake	<2000g (n=414)	First 10 days of life	40	18	27	6	24	115	299
		11-56 days of life	-	15	12	3	11	-	41 (events)
	<1500g (n=256)	First 10 days of life	4	15	25	5	24	73	183
		11-56 days of life	-	12	12	3	9	-	36 (events)
	<1000g (n=71)	First 10 days of life	0	7	12	4	15	38	33
		11-56 days of life	-	3	4	1	6	-	14 (events)
Own mother´s milk LF intake	<2000g (n=324)	First 10 days of life	30	16	21	5	12	84	240
		11-56 days of life	-	15	9	3	10	-	37 (events)
	<1500g (n=191)	First 10 days of life	1	13	20	4	12	50	141
		11-56 days of life	-	11	9	3	8	-	31 (events)
	<1000g (n=53)	First 10 days of life	0	7	10	3	8	28	25
		11-56 days of life	-	3	2	1	5	-	11 (events)

Total infants excluded: f= a + b + c + d + e; LOS, late onset sepsis; NEC, necrotizing enterocolitis

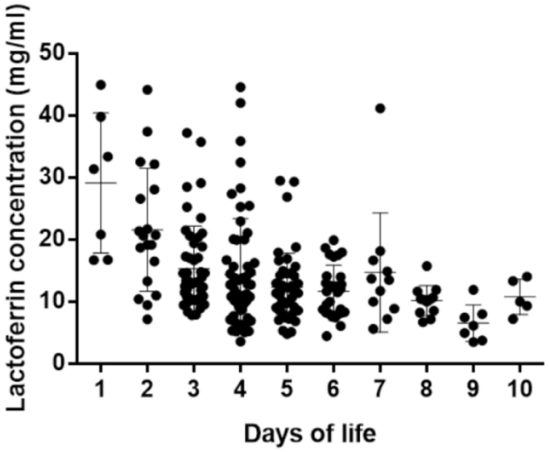

Figure 1. Concentration of human lactoferrin in the first 10 days of life in 240 mothers of infants <2000g.

For infants <2000g the median cumulative intake of MOM (aim 2) over days 4-10 of life was 154ml/kg (IQR 54-344ml/kg). The percentage of human milk consumption was 77±32%. The intake of more MOM over the exposure period was associated with less episodes of LOS, NEC or death as observed in the Kaplan Meier survival curves by quartiles (Figure 2B). The adjusted HR of MOM cumulative intake (days 4-10) of 54-344 ml/kg (25-75 quartiles) for LOS, NEC and death was 0.414 (95% CI 0.196-0.873, p=0.02) (Table 2). If we include in the outcome only culture confirmed sepsis, the HR was not significant. For VLBW infants the median cumulative intake of MOM over days 4-10 of life was 92ml/kg, IQR38-202 ml/kg. The percentage of human milk consumption was 84±28%, and the adjusted HR of MOM cumulative intake was also protective (p=0.034) (Table 2)

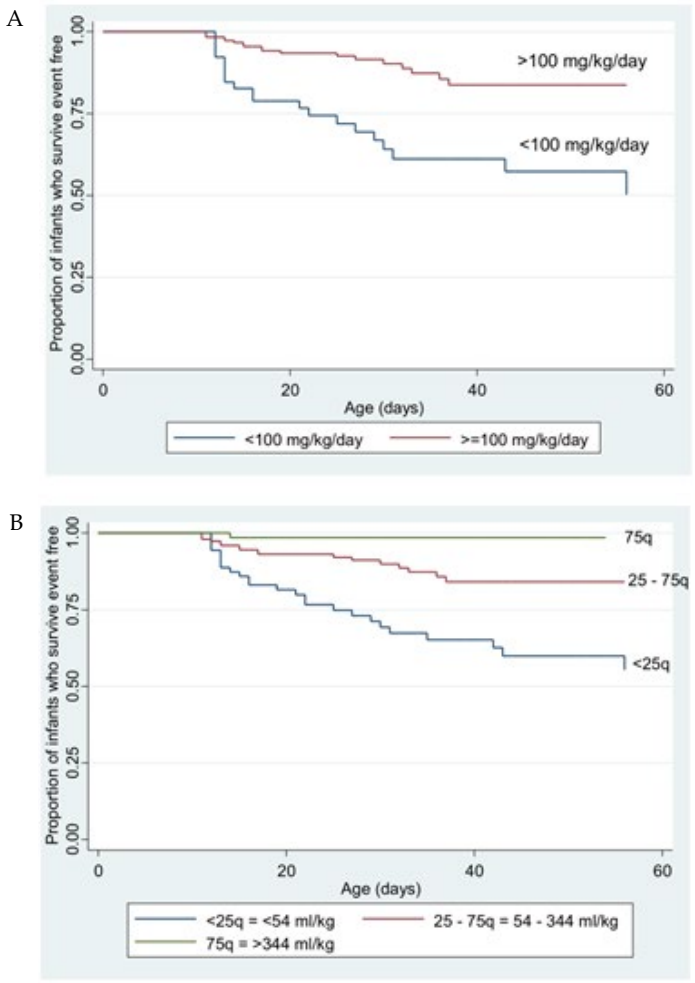

Figure 2. Survival curves for LOS, NEC and death by (A) the amount of mother´s milk lactoferrin intake over days 4-10 of life. The line represent the average daily lactoferrin intake; blue line < 100 mg/kg/day, red line ≥ 100 mg/kg/day; and (B) the amount of mother´s own milk intake over days 4-10 of life. The line represent the accumulative milk intake separated by quartiles: blue line <25 quartile (< 54 ml/kg), red line 25-75 quartile (54-344 ml/kg) and green line ≥ 75 quartile (≥ 344 ml/kg).

Table 1. Effect of own mother's lactoferrin intake on the prevention of LOS, NEC or death

Birth Weight group	< 2000 g (N=240)*				< 1500 g (N=141)**			
Variables	Crude HR (95% CI)	p	Adjusted HR (95% CI)	p	Crude HR (95% CI)	p	Adjusted HR (95% CI)	p
Average own mother´s milk LF intake								
< 100 mg/kg/day	reference		reference		reference		reference	
≥ 100 mg/kg/day	0.297 (0.156 - 0.568)	<0.001	0.752 (0.301 - 1.877)	0.541	0.319 (0.156 - 0.650)	0.002	0.582 (0.211 - 1.606)	0.296
Age of milk sample collection, days	0.288 (0.148 – 0.561)	<0.001	1.045 (0.887 – 1.232)	0.598	0.318 (0.153 – 0.660)	0.002	1.054 (0.889 – 1.249)	0.549
Cumulative own mother´s milk (ml/kg)	0.994 (0.991 – 0.998)	0.001	0.998 (0.994 - 1.002)	0.340	0.995 (0.992 – 0.999)	0.019	1.000 (0.996 - 1.005)	0.930
% human milk	1.008 (0.993 - 1.022)	0.316	1.012 (0.998 - 1.027)	0.089	1.013 (0.992 - 1.035)	0.214	1.019 (0.999 - 1.039)	0.070
Gestational age, weeks	0.798 (0.700 - 0.910)	0.001	0.905 (0.778 - 1.052)	0.193	0.797 (0.680 - 0.933)	0.005	0.899 (0.752 - 1.075)	0.241
Birth weight, >1000 g	reference		reference		reference		reference	
Birth weight, ≤ 1000 g	2.585 (1.256 - 5.319)	0.010	1.036 (0.420 - 2.554)	0.939	2.320 (1.102 - 4.882)	0.027	1.094 (0.442 - 2.709)	0.847
Female	reference		reference		reference		reference	
Male	1.551 (0.779 - 3.092)	0.211	1.979 (0.949 – 4.126)	0.069	1.534 (0.735 -3.202)	0.255	1.745 (0.785 - 3.880)	0.172
Trial intervention, placebo	reference		reference		reference		reference	
Trial intervention, bovine-LF	1.264 (0.655 - 2.436)	0.485	1.442 (0.709 – 2.931)	0.313	1.298 (0.630 - 2.674)	0.480	1.631 (0.745 - 3.574)	0.221
Hospital 1	reference		reference		reference		reference	
Hospital 2	0.785 (0.221 - 2.785)	0.708	0.425 (0.113 - 1.598)	0.205	0.702 (0.198 - 2.491)	0.584	0.449 (0.113 - 1.784)	0.256
Hospital 3	8.566 (2.965 - 24.746)	<0.001	4.942 (1.526 – 16.001)	0.008	5.853 (1.981 - 17.298)	0.001	4.306 (1.190 - 15.587)	0.026

LOS, late onset sepsis; NEC, necrotizing enterocolitis; LF, lactoferrin; *37 events; **31 events

Table 2. Effect of own mother's milk intake on the prevention of LOS, NEC or death

	< 2000 g (N=299)*				< 1500 g (N=183)**			
Variables	**Crude HR (95% CI)**	**p**	**Adjusted HR (95% CI)**	**p**	**Crude HR (95% CI)**	**p**	**Adjusted HR (95% CI)**	**p**
Cumulative own mother´s milk intake								
< 25q (ml/kg)	reference		reference		reference		reference	
25 – 75q (ml/kg)	0.325 (0.172 - 0.612)	0.001	0.414 (0.196 - 0.873)	0.020	0.306 (0.150 - 0.624)	0.001	0.427 (0.194 - 0.939)	0.034
> 75q (ml/kg)	0.060 (0.008 – 0.446)	0.006	0.140 (0.017 - 1.184)	0.071	0.268 (0.099 - 0.725)	0.009	0.865 (0.233 - 3.213)	0.828
% human milk	1.006 (0.993 - 1.020)	0.348	1.011 (0.998 - 1.025)	0.088	1.011 (0.994 - 1.028)	0.205	1.015 (0.999 - 1.032)	0.074
Gestational age, weeks	0.809 (0.715 - 0.916)	0.001	0.965 (0.832 - 1.119)	0.639	0.822 (0.713 - 0.946)	0.006	0.965 (0.819 - 1.136)	0.666
Birth weight, >1000 g	reference		reference		reference		reference	
Birth weight, ≤ 1000 g	2.883 (1.487 - 5.591)	0.002	1.596 (0.756 - 3.372)	0.220	2.559 (1.298 - 5.042)	0.007	1.922 (0.886 - 4.169)	0.098
Female	reference		reference		reference		reference	
Male	1.558 (0.817 - 2.972)	0.179	2.118 (1.077 - 4.166)	0.030	1.42 (0.726 - 2.775)	0.305	1.643 (0.819 - 3.294)	0.162
Trial intervention, placebo	reference		reference		reference		reference	
Trial intervention, bovine-LF	1.424 (0.765 - 2.651)	0.265	1.482 (0.776 - 2.831)	0.234	1.343 (0.692 - 2.607)	0.383	1.485 (0.741 - 2.974)	0.265
Hospital 1	reference		reference		reference		reference	
Hospital 2	0.976 (0.327 - 2.912)	0.965	0.509 (0.163 - 1.586)	0.244	0.869 (0.291 - 2.594)	0.801	0.751 (0.225 - 2.510)	0.641
Hospital 3	6.258 (2.400 - 16.317)	<0.001	3.548 (1.294 - 9.727)	0.014	4.508 (1.696 - 11.978)	0.003	4.380 (1.343 - 14.290)	0.014

LOS, late onset sepsis; NEC, necrotizing enterocolitis; LF, lactoferrin; *41 events; **36 events

When comparing the clinical and nutritional characteristics of the infants who developed an event (LOS, NEC or death) or not, the infants who developed an event had significantly lower gestational age and birth weight and less intake of MOM during the exposure period 42 vs. 176ml/kg (Table 3). The infants that developed an event had less average daily human-LF intake during the exposure period compared to the infants without an event, 89 vs. 334mg/kg/day in infants <2000g ($p<0.0001$) and 77 vs. 184mg/kg/day in VLBW infants ($p=0.003$) (Table 3 and Figure 3).

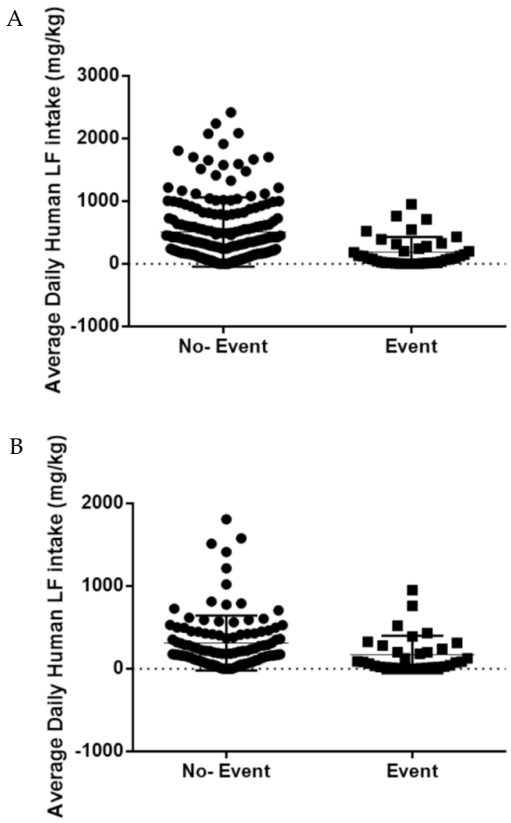

Figure 3. Average daily human lactoferrin intake in mg/kg/day over days 4-10 among infants that developed an event (LOS, NEC or death) or not, after day 10 of life. Figure 3A is for infants <2000g, n=240 (37 cases or events: LOS, NEC or death and 203 controls: no event). Figure 3B is for infants <1500g, n=141 (31 cases or events: LOS, NEC or death and 110 controls: no event)

Table 3. Clinical and nutritional characteristics in the first 10 days of life in infants with and without LOS, NEC or death

	All infants (< 2000g)		VLBW infants (<1500g)		ELBW infants (<1000g)	
	LOS, NEC or Death (n= 41)	**No - Event (n=258)**	**LOS, NEC or Death (n=36)**	**No - Event (n=147)**	**LOS, NEC or Death (n=14)**	**No - Event (n=19)**
Clinical characteristics						
Gender, female, n (%)	14 (34.2)	115 (44.7)	14 (39.0)	71 (48.3)	5 (35.7)	9 (47.4)
Birth weight in g, mean ± SD	1169 ± 305	1448 ± 291	1090 ± 228	1245 ± 202	851 ± 98.0	842.7 ± 98.6
Gestational age in weeks, mean ± SD	29 ± 2.4	31 ± 2.6	29 ± 2.2	30 ± 2.5	27.4 ± 2.3	27.7 ± 2.5
Small for gestational age, n (%)	7 (17.1)	71 (27.5)	7 (19.4)	41 (27.9)	4 (28.6)	6 (31.6)
Born by C-section, n (%)	33 (80.5)	210 (82.0)	28 (77.8)	120 (81.6)	8 (57.1)	11 (57.9)
Hospital, n (%)						
Hospital 1	5 (12.2)	66 (25.6)	5 (14.0)	36 (24.5)	1 (7.14)	3 (15.8)
Hospital 2	9 (22.0)	132 (51.2)	9 (25.0)	80 (54.4)	7 (50.0)	12 (63.2)
Hospital 3	27 (65.9)	60 (23.3)	22 (61.1)	31 (21.1)	6 (42.9)	4 (21.1)
Randomized to bovine LF, n (%)	24 (58.5)	127 (49.2)	21 (58.3)	75 (51.0)	9 (64.3)	10 (52.7)
Nutritional characteristics (days 4-10)						
Cumulative intake of MOM in ml/kg, median (IQR)	42 (11–146)	176 (69–373)	39 (10–139)	106 (51–230)	30 (12–42)	44 (19–78)
% of human milk consumption, mean ± SD	87.9 ± 28.5	75.3 ± 32.0	91.9 ± 24.0	82.6 ± 29.1	99.1 ± 3.5	91.9 ± 25.4
Human LF concentration in mg/mL, mean ± SD	15.28 ± 7.8*	14.2 ± 8.2*	13.9 ± 6.8†	13.3 ± 7.4†	15.6 ± 7.3§	15.8 ± 10.6§
Cumulative human LF intake in mg/kg, median (IQR)	621 (116-1971)*	2335 (1051-4946)*	542 (111–1694)†	1285 (714–2936)†	312 (111–621)§	531 (320–1225)§
Average daily human LF intake in mg/kg, median (IQR)	89 (17–282)*	334 (150–707)*	77 (16–242)†	184 (102– 419)†	45 (16–89)§	76 (46– 175)§

LOS, late onset sepsis; NEC, necrotizing enterocolitis; LF, lactoferrin; MOM, mother´s own milk; *n=240 infants (37 events and 203 no- events); †n=141 infants (31 events and 110 no- events), §n=25 infants (11 events and 14 no- events)

DISCUSSION

This study demonstrates that the consumption of human milk in the first days of life protects against infections and death in the first 8 weeks of life in infants <2000g and in VLBW infants. Neonates with higher human-LF intake were less likely to have an event than neonates with lower intake. The daily human-LF intake depends on the concentration of LF in milk and the amount of human milk intake. In our study the concentration of LF in milk was around 14 mg/ml (days 1-10 of life) and around 13 mg/ml (days 4-10 of life), which is higher than what is considered the average in the literature (6-10mg/mL in colostrum).[23] The amount of human milk intake is critical since there are many other factors present that can account for the protective effect, including antibodies, oligosaccharides, lysozyme, mucins, among others.[3]

Trend et al[21] in a small case-control study in newborns <32 weeks found that infants with LOS consumed lower quantities of human-LF, measured on days 7 and 21 of life, in comparison with newborns who did not develop LOS. However, this analysis did not consider the consumption of breastmilk prior to the development of sepsis; they measured consumption in general. Nevertheless, these results are in the same line of our findings: infants that develop sepsis have lower intake of human-LF.

To our knowledge, this is the first study to explore human-LF intake and its relation with LOS, NEC and death. This protection early in life may be related to LF effect on (1) modulation of bacterial growth in the gastrointestinal tract; (2) promotion of intestinal cell proliferation, differentiation and maturation, which may decrease intestinal permeability and prevent bacterial translocation from the gut to the bloodstream; and (3) regulation of the host immune response. These protective effects, as demonstrated in vitro and in animal models using bovine- and human-LF, have been extensively reviewed[5,6] and are much more relevant in the premature infant who is at risk of infection, inflammation and oxidative stress injuries.[6]

In this study we demonstrated that human milk intake as a whole protects against infections and death in first 8 weeks of life. Several previous studies have shown similar results. Furman et al[24] demonstrated that a daily threshold of at least 50 ml/kg of maternal milk through week 4 of life was needed to decrease the rates of LOS in VLBW infants. In a prospective cohort study in 175 VLBW infants Patel et al[20] demonstrated that the intake of at least 25ml/kg/day over the first 28 days of life was significantly associated with a decrease in sepsis. However, both studies, as recognized in their limitations, have not calculated the human milk intake before the onset of sepsis, or excluded the infants with sepsis episodes during a certain time of human milk exposure, to avoid the potential effect of reverse causality. A well-designed study by Corpeleijn et al[19] demonstrated that the consumption of colostrum in the first 5 days of life and higher percentages (>50%) of MOM intake over days 6-10 of life protected against sepsis, NEC, and/or death in

the first 60 days of life in VLBW infants. The relevance of this study and ours is that both demonstrated that the consumption of MOM early in life has a protective effect against infection and death for a prolonged period, up to two months of life. Our study adds the information on LF intake. Several research studies have suggested that the early postnatal period (first 14 days of life) is a critical period for feeding human milk to decrease the risk of sepsis and other morbidities, including NEC[1,25] and even re-hospitalizations over the first year of life.[26]

In our study we found that VLBW infants who did not develop an event had a daily human-LF intake of around 200 mg/kg in the first 10 days of life. This information is relevant to extrapolate the ideal dosing of bovine-LF supplementation in the current clinical trials, which is not clearly defined. The ELFIN [15] study in the UK used a dose of 150mg/kg of bovine-LF, with no significant protective effect against LOS, suggesting that probably a higher dose is needed in top of the amount that neonates are already receiving from mother´s own milk. A recent study by Manzoni et al[27] evaluated the effect of bovine-LF supplementation from two previous clinical trials and compared the effect among infants receiving only formula and mixed feeds. This study suggested that bovine-LF supplementation may have a benefit among infants who did not receive MOM, and that probably there is no advantage of giving more LF to infants who are already receiving good quantities of MOM.

This study has some limitations. First, we have not measured LF concentrations in milk daily; only one measurement per mother was used as a homogeneous value for the daily calculations of LF intake. Since LF concentrations are high in colostrum and then decrease over time (Figure 1), we have probably overestimated the amount of LF intake in some infants and underestimated in others. To control for this, we have included in the analysis the date of milk sample collection. Second, we used a non-randomized design for the exposure of interest (high dose of human-LF intake); therefore, the observed associations may have arisen because group differences in variables not measured or not correctly measured. It is possible, for example, that sicker infants –even without LOS/NEC, may have received less MOM in the first 10 days, and will also had a higher subsequent risk of LOS/NEC/death. We have not adjusted by a measure of illness severity in the first 10 days of life.

Despite these limitations, our study has several strengths and important implications. The main strengths are the number of infants enrolled in the study and the exclusion from the analysis of all infants with an event during the exposure period. For clinicians, the main implication is that the finding that feeding higher amounts of human LF is associated with less infections and death, highlights the importance of promoting MOM intake in the early postnatal period, especially for infants at risk in the neonatal units. For researchers, the protective doses of human-LF intake reported in this study may aid in calculating the best dose for future clinical trial of bovine-LF supplementation or other related research.

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STATEMENTS

Statement of Ethics
The study was approved by the institutional ethical committee of Universidad Peruana Cayetano Heredia. We used data without personal identifiers, only codes.
Disclosure Statement
The authors have no potential conflicts of interest to declare.
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Discussion and future perspectives

This PhD thesis reports the results of five studies on the effect of lactoferrin on pediatric infections (Table 1).

Table 1. General characteristics of the study design of each chapter

Chapter/ Ref.	Type of study	Topic	Population	N	Interven-tion	Control	Outcome
1	RCT	Diarrhea	Children 12-24m	555	bLF	Placebo	Diarrhea incidence
2	Pilot RCT	Neonatal sepsis	Neonates < 2500g	190	bLF	Placebo	Clinical LOS
3	RCT	Neonatal sepsis & neuro-Develop-ment	Neonates < 2000g	414	bLF	Placebo	Culture-confirmed LOS or death
4	Pooled analysis of 2 RCTs	Neonatal sepsis	Neonates < 1500g	335	bLF	Placebo	Clinical LOS
5	Cohort study	Neonatal sepsis	Neonates < 2000g	299	Exposure: High hLF and MOM intake	Low hLF and MOM intake	LOS, NEC or death

RCT, randomized controlled trial; bLF, bovine lactoferrin; hLF, human lactoferrin; MOM, mother´s own milk; LOS, late-onset sepsis; NEC, necrotizing enterocolitis.

The major findings of this thesis are summarized in Table 2.

Putting all this information together we found that in general, although not statistical significant in all cases, there were less infections or less severe infections in the lactoferrin patient group, for all outcomes measured; thus, we can conclude that our hypothesis (lacoferrin given as an oral supplement to infants in resource-limited settings will improve their health by mimicking its protective roles in breast milk, decreasing the incidence and severity of common pediatric infections probably due to its antimicrobial and immunomodulatory properties) was partially confirmed. It is clear that in order to achieve this aim it is necessary to refine some aspects of the design of the trials.

In the case of diarrhea, the results of our trial are consistent with the findings from Egashira in Japan, Zavaleta in Peru and Chen in China[6-8], showing that the supplementation of bovine lactoferrin decreases the duration and severity of the diarrhea episodes; however, does not prevent illness. There are many potential reasons why lactoferrin did not decrease diarrhea incidence, including the dose and type of lactoferrin used and the age of the children enrolled. Probably the latter is the most important factor. We speculate that older children in source-limited settings already had multiple exposures to pathogens early in life and have built-up some degree of immunity; therefore, adding lactoferrin at this age has

no major benefit for prevention. On the contrary, younger infants with a more immature immune system could benefit from lactoferrin supplementation and protection from enteric pathogens in the gut.

Table 2. Main results of each chapter

Chapter	Main results
1	• Bovine lactoferrin supplementation in infants 12-24 months of age did not prevent diarrhea. • There was a significant reduction on the duration and severity of the diarrhea episodes, although not clinically relevant.
2	• Bovine lactoferrin supplementation in infants <2500g did not prevent culture-confirmed or clinical-defined late-onset sepsis in the first month of life. • In a secondary exploratory model using time since the start of the treatment, lactoferrin supplementation was protective.
3	• Bovine lactoferrin supplementation in infants <2000g did not reduce culture-confirmed late-onset sepsis or sepsis-associated deaths in the first 2 months. • Bovine lactoferrin supplementation did not protect from neurological impairment measured by the Mullen Scales at 24 months of age. • As a secondary outcome infants supplemented with bovine lactoferrin had significant less re-hospitalization due to bronchiolitis in the 2 years of follow-up.
4	• Bovine lactoferrin supplementation in infants <1500g (VLBW) reduced culture-confirmed or clinical-defined late-onset sepsis in the first month of life. • This effect was statistically significant especially among infants <1000g (ELBW) and infants not receiving sufficient amount of human milk.
5	• The intake of higher amounts of mother´s own milk in the first days of life protected against culture-confirmed or clinical-defined late-onset sepsis, NEC or death in infants <2000g in the first 8 weeks of life. • In the adjusted analysis the intake of higher amounts of human lactoferrin was associated with less sepsis, NEC or death, however did not reach statistical significance. • Infants who developed LOS, NEC or died had significant less daily human lactoferrin intake than those that did not develop an event.

LOS, late-onset sepsis; NEC, necrotizing enterocolitis.

Based on these results the next ideal studies to demonstrate lactoferrin effect on enteric infections, could be: (1) a RCT to determine the effect of bovine lactoferrin for prevention of diarrhea in infants 4-6 month of age when they stop exclusive breastfeeding and start to get exposure to pathogens; this age (6-12months) has the highest incidence of diarrhea and the major impact on the nutritional status of the child. (2) a RCT to determine the effect of bovine lactoferrin as an adjunct therapy for the treatment of moderate to severe diarrhea in children < 3 years of age in resource-limited setting with high burden of diarrhea. Currently, in addition to low osmolarity oral rehydration solutions, which is the corner stone of diarrhea treatment in children, other adjunct therapies include use of zinc,

probiotics, racecadotril, antiemetics among others.[9] Of those, only zinc is recommended by WHO and UNICEF for all children >6 months of age living in countries with zinc deficiency. However, recent meta-analysis has concluded that zinc decreases diarrhea duration only in half a day[10]; therefore, there is still room for other interventions in order to decrease illness duration and its consequences on growth and development at this critical age.

In relation to neonatal infections and lactoferrin, this is a hot topic in neonatal care, and there is still no definitive answer. There are 10 published studies[2,11-21] and 3 meta-analysis.[22-24] Most studies have shown a reduction on the rate of culture-confirmed late-onset sepsis (LOS) (see Table 7 in the introduction), except for our two studies[2,3] and the ELFIN study.[21] The ELFIN study (enteral lactoferrin to prevent infection for very preterm infants) was conducted in the UK in 2203 infants <32 weeks of gestational age who were randomized to receive bovine lactoferrin (150mg/kg/day) or placebo (sucrose) once a day until 34 weeks postmenstrual age. The main study outcome was culture-confirmed or clinical suspected LOS. The study found no difference in the composite outcome (29% in the lactoferrin group vs. 31% in the control), with an adjusted risk ratio of 0.95 (95%CI 0.86-1.04). Most of the other studies, except the Manzoni trial[11] and our NEOLACTO trial[3], included relatively small number of infants (<200 in each of the other 8 trials). Thus, it is possible that the small trials had some methodological weaknesses and biases, overestimating the effect size.[24]

There are other factors related to the population, the intervention and the outcome that can explain the contradictory results. First, we will discuss the factors related to the **population.**

(1) Main factors are the birth weight and gestational age or the enrolled infants. Except for our pilot study[2] that included infants <2500g, and the study in India[18] and our NEOLACTO trial[3], that included infants <2000g, all studies enrolled infants <1500g or <33 weeks gestational age. It is clear that the smallest infants have the highest risk of infection and this is the population that potentially will benefit the most from the intervention, due to the immaturity of their immune system and the need for additional protection. For this reason we performed the secondary analysis, pooling data from our two trials and including only VLBW infants.[4] Although the pooled analysis had inherent methodological limitations, we were able to demonstrate a protective effect of lactoferrin on LOS in VLBW infants, similar to the rest of the literature.

(2) The location where the study is conducted is also critical. Each NICU has its own epidemiology of LOS, not only related to the infection control practices, prophylactic interventions, standards for performing on-time blood cultures and starting antibiotic, but also related to the pathogen distribution. For example, in high resource countries, the most common pathogens associated with LOS are CoNS, GBS (Group B *Streptococcus)*, *E. coli* and S. *aureus*[25]; however, in limited-resources countries, Gram negatives bacteria (mainly *Klebsiella*, *E. coli*, *Pseudomonas*, *Enterobacter)*, are responsible for 60% of all hospital infections in the

neonatal period.[26] Therefore, we speculate that based on lactoferrin´s mechanism of action, the effect of lactoferrin most likely will be on pathogens translocated from the gut (Gram negative bacteria and Candida), more than line-associated infections and other Gram positive organisms. To highlight this point and as a possible explanation of the different results, when comparing the two larger trials (Manzoni and ELFIN) they had different pathogen distribution. In the Manzoni study in Italy 10.1% of neonates in the control group had Gram negative infections and 5.4% had Candida[11]; on the contrary, the ELFIN study in the UK had 3.6% of Gram negative infections and only 0.2% of Candida.[21] In our pooled analysis 4.2% of infants had Gram negative bacteria and 2.4% had Candida.[4]

(3) Finally, the feeding practices of each unit could also influence the results, associated to the clear benefits of breast milk early in life. In the ELFIN study 26.4% (291/1101) of the infants in the control group received only breast milk vs. 22.0% (37/168) in the Manzoni trial. Thus, it is possible that bovine lactoferrin supplementation will have better results among infants receiving less amounts of breast milk. [4,27]

In relation to the **intervention**, this is also a very critical factor.

(1) The doses of lactoferrin varied widely among studies; from a fixed dose of 100mg/day to a maximum dose of 300mg/kg/day. All studies, except ours, have administered the lactoferrin only once a day; we administered three times a day, trying to mimic its protection from breast milk. Up to now, it is not clear which is the best lactoferrin dose. Kaufman in the US has performed a safety and tolerability study in 30 VLBW infants randomized to 100, 200 or 300 mg/kg/day of bovine lactoferrin. He found that the intervention was safe[28], and detected bovine lactoferrin levels in plasma and urine, and high levels in saliva, with all three doses.[29] In our analysis of mother´s own milk intake among VLBW infants the median daily human-lactoferrin intake over days 4-10 of life was ≈180mg/kg/day (IQR 70-390mg/kg/day)[5], suggesting that this should be the minimal protective amount.

(2) The timing of the lactoferrin administration is also important, since early administration may protect by promoting cell proliferation and maturation. In the Manzoni study, lactoferrin was administered on the third day of life, whereas in the ELFIN study, when infants reached >12ml/kg of enteral feeding; in the NEOLACTO trial we started as soon as the infant tolerated enteral intake (2 ± 3 days). Thus, there is important variability.

(3) Lastly, the type of lactoferrin use is also critical. There are variations related to protein concentration, iron saturation, type of purification process, type of pasteurization, among others. The quality control of commercial lactoferrin is standardized through Novel Food in the EU and GRAS in the USA. These regulations require more than 95% lactoferrin purity. However, some commercially available samples have issues with purity, contamination with LPS and / or angiogenin, and degradation of samples.[30] More critical is the lack of consensus

on the criteria to measure lactoferrin bioactivity, which is fundamental, since it is a functional food. As an example, the Manzoni trials used bovine lactoferrin from Dicofarm, which contains lactoferrin with FOS (fructo-oligossacharide). It is unknown if the addition of this simple sugar could be responsible in part for the major benefits found in the study.[22-24]

In relation to the **outcome**, most studies focused on culture-confirmed LOS, however some trials also included clinically-defined infection. Since there is a lack of definition of neonatal sepsis with clear endpoint for clinical trials, and concerns with the accuracy of cultures on this population[31], the investigators from the ELFIN study, for example, have used a "pragmatic approach" including both definitions and adding clinical, laboratory and treatment criteria. Nevertheless, the current meta-analysis mainly focuses on culture-confirmed LOS.

After this analysis of the main heterogeneities, strength and limitations of the published studies, and base on the most recently updated meta-analysis[24], which despite including the ELFIN study concluded that bovine lactoferrin supplementation does reduce the risk of LOS. I agree with the conclusion that additional research is needed to improve the certainty in the evidence. An ideal design would be a multi-center RCT with infants <1500 g, assessing the effect of several lactoferrin doses (100, 200 and 300 mg/kg/day), and using the same commercial available lactoferrin, same control groups and same outcome definition, including both culture-confirmed and clinically-defined sepsis with the same clinical, laboratory and treatment criteria. Ideally this trial should be conducted in countries with the highest burden on neonatal infections, where the potential benefit is expected to have the largest impact.

Finally, in relation to our analysis of mother´s own lactoferrin intake, probably this is the most important contribution for neonatal care. Although the study in Chapter 5 had some limitations because it was a secondary data analysis, this is the study assessing the largest sample size quantifying the concentration of lactoferrin in milk and the daily intake in preterm neonates. The Chapter 5 study however was not able to demonstrate that the intake of higher amounts of human lactoferrin was associated with less sepsis, NEC or death, probably related to the sample size; however, infants who had infections or died had significantly less intake of human lactoferrin in comparison with infants without an event. For VLBW infants, the average daily human lactoferrin intake in the first ten days of life was ~80 mg/kg/day among infants that later developed an event versus ~180 mg/kg/day among infants without an event.[5] This information is relevant for estimating the adequate doses of bovine lactoferrin supplementation in future clinical studies, but more important for convincing neonatologist, pediatricians and mothers of the relevance of human milk consumption. As mentioned in a recent meta-analysis of human milk intake for protection against sepsis and NEC, any volume of human milk is better than preterm formula, and the higher the dose of human milk the greater the protection.[32] Thus, as stated by Walker, "breast milk is the gold standard for protective nutrients".[33] At the end, no single milk component expected to have the same benefit as human milk as a whole.

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Summary

Diarrhea and neonatal infections represent an important cause of morbidity and mortality in children in resource-limited countries. Among infants who survive, many suffer with long-term consequences in growth and development. Therefore, there is an urgent need to implement preventive strategic to decrease the risk and burden of infections in vulnerable populations.

One of the most important and cost-effective interventions to reduce infant mortality is exclusive breastfeeding. Lactoferrin, a milk protein, is one of the major factors responsible for these protective effects due to its antimicrobial and immunomodulatory properties.

Multiple previous studies have demonstrated the potential role of lactoferrin in preventing infections in young children. However, there is gap in knowledge about the clinical applications of lactoferrin in specific pediatric infections and patient groups. This thesis focuses on the effect of lactoferrin on enteric infections (Chapter 1) and on neonatal infections (Chapters 2-5).

The general hypothesis is that lactoferrin given as an oral supplement to infants in resource-limited settings will improve their health by mimicking its protective roles in breast milk, decreasing the incidence and severity of common pediatric infections due to its bioactive properties.

In a randomized controlled trial (RCT) in 555 previous weaned infants 12-24 months of age from peri-urban communities of Lima, we evaluated the effect of bovine lactoferrin supplementation on prevention of diarrhea (**Chapter 1**). We found that bovine lactoferrin did not prevent diarrhea in this age group; however, there was a significant reduction on the duration and severity of the diarrhea episodes, although not clinically relevant.

In a pilot RCT in 190 low birth weight infants (<2500g) from neonatal intensive care units (NICUs) in Lima we evaluated the effect of bovine lactoferrin supplementation on prevention of culture-confirmed or clinical-defined late-onset sepsis in the first month of life (**Chapter 2**). We found that lactoferrin did not prevent late-onset sepsis; however, in a secondary exploratory model using time since the start of the treatment, we found lactoferrin supplementation to be protective.

In a second RCT (NEOLACTO trial) in 414 infants with a birth weight <2000g from three NICUs in Lima we evaluated the effect of bovine lactoferrin supplementation on prevention of culture-confirmed late-onset sepsis or sepsis-associated deaths in the first 2 months of life and prevention of neurodevelopment impairment measured by the Mullen Scales at 24 months of age (**Chapter 3**). We found that bovine lactoferrin did not prevent late-onset sepsis and did not protected from neurological impairment; however, as a secondary outcome we found less re-hospitalization due to bronchiolitis in the 2 years of follow-up in the lactoferrin group.

We performed a pooled analysis of individual patient data of the 2 previous neonatal trials (Chapters 2 and 3) to evaluate the effect of bovine lactoferrin supplementation on prevention of culture-confirmed or clinical-defined late-onset sepsis in the first month of life in 335 infants <1500g (**Chapter 4**). We found that lactoferrin prevents late-onset sepsis in this birthweight group. This effect is significant especially among ELBW infants (<1000g) and infants not receiving sufficient amount of human milk.

In a secondary analysis of the NEOLACTO trial in 299 infants <2000g we evaluated the effect of mother´s own milk lactoferrin intake in the first days of life on protection against culture-confirmed or clinical-defined late-onset sepsis, NEC or death in the first 8 weeks of life (**Chapter 5**). We found that the intake of higher amounts of mother´s own milk in the first days of life protected against late-onset sepsis, NEC or death; however, in the adjusted analysis human lactoferrin did not reached statistical significance. Infants who developed sepsis, NEC or died had significant less daily human lactoferrin intake than those that did not develop an event.

Putting all this information together we found that in general, although not statistically significant in all cases, there were less infections or less severe infections in the lactoferrin patient group, for all outcomes measured. Thus, in order to confirm our hypothesis it is necessary to refine some aspects of the design of future trials. Ideally these trials should be conducted in countries with the highest burden of diarrhea and neonatal infections, where the potential benefit is expected to have the largest impact.

Acknowledgement, Personal Contribution and Conflict of Interest statements

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I would like to thank all the co-authors of the publications / chapters of this PhD thesis and my research team at Cayetano University, for their support and work in these studies. Special thanks to:

- Chapter 1: Dr Thomas Cleary, my mentor, for the study idea, helping writing the protocol, getting funding and supervising the study. Dr Elsa Chea, pediatrician and Principal Investigator at the study site, for her dedicated work conducting the trial. Dr. Nelly Baiocchi, pediatrician, for the nutritional evaluation of enrolled infants. Dr Miguel Campos, statistician, for data management and data analysis. The study coordinator, Mrs. Gladys Valdiviezo, for coordinating the community-based trial, particularly supervising the nurses and Community Health Workers.

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PERSONAL CONTRIBUTION

I have been the Principal Investigator (PI) of all studies, except the first diarrhea trial (Chapter 1). Dr Thomas Cleary, my mentor, was the PI of this NIH-funded study; I was the co-investigator and the overall study coordinator in Peru.
The first three chapters were randomized clinical trials (RCT) and were conducted with several co-investigators mainly from Cayetano University and a large research team (physicians, nurses, study coordinators, Community Health Worker, lab technicians, clerks, administrators).

I have been responable for conducting all studies. I wrote the protocols, informed consents and designed the Case Report Forms (CRF) of all five chapters (including the first trial). I was reposable for getting ethics and IRB approbal, approbal from the clinical trial regulatory agencies in Peru and getting funding (Gates Foundation, Chapter 2, and NIH, Chapter 3). I have coordinated all aspects of the trials: importation of lactoferrin and preparation of small containers/capsules, quality control, meetings with the Data Safety Monitoring Board (DSMB), Severe Adverse Reports (SAE), regulatory agency inspections, budget management, data management, writing the scientific and financial reports; I have supervised the members of the research team responsible for each of these tasks. I have planned and discussed the data analysis and results with the statisticians and other co-authors, wrote the first draft of the manuscripts and the final submissions to the journals.

I have not performed the data analysis myself for any of the Chapters. The co-authors that performed the data analysis are acknowledged in the previous section.

As a summary, my role in the different components of the five chapters of my PhD thesis is listed in Table 1.

Table 1. Role of PhD candidate

Taxonomy category*	Description or role*	Chapter				
		1	2	3	4	5
Study conception	Ideas; formulation of research question; statement of hypothesis	S	+	+	+	+
Methodology	Development or design of methodology; creation of models.	S	S	S	S	S
Computation	Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms.	-	-	-	-	-
Formal analysis	Application of statistical, mathematical or other formal techniques to analyze study data.	-	-	-	-	-
Investigation: performed the experiments	Conducting the research and investigation process, specifically performing the experiments.	S	S	S	NA	NA
Investigation: Data / evidence collection	Conducting the research and investigation process, specifically data/evidence collection.	S	S	S	S	S
Resources	Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation or other analysis tools.	S	S	S	S	S
Data curation	Management activities to annotate (produce metadata) and maintain research data for initial use and later re-use.	S	S	S	S	S
Writing/manuscript preparation: initial draft	Preparation, creation and/or presentation of the published work, specifically writing the initial draft.	+	+	+	+	+
Writing/manuscript: critical review, commentary or revision	Preparation, creation and/or presentation of the published work, specifically critical review, commentary or revision.	S	S	S	S	S
Writing/manuscript: visualization/data presentation	Preparation, creation and/or presentation of the published work, specifically visualization/data	+	+	+	+	+
Supervision	Responsibility for supervising research; project orchestration; principal investigator or other lead stakeholder.	S	+	+	+	+
Project administration	Coordination or management of research activities leading to this publication.	S	+	+	+	+
Funding acquisition	Acquisition of the financial support for the project leading	S	+	+	NA	NA

*Taxonomy categories and Description role, proposed by Liz Allen *et al.* Nature. 2014; 508(7496):312-3.
+ Denotes role of the PhD candidate; S denotes shared role of the PhD candidate; - denotes role of co-researchers, NA not applicable.

CONFLICT OF INTEREST STATEMENT

All the co-authors of the publications / chapters of this PhD thesis and myself have no potential conflicts of interest to disclose.

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About the author

Theresa J. Ochoa was born in Cusco, Peru in 1968. She studied Biology (1991), Medicine (1997) and Pediatric Residency (2001) at Cayetano Heredia University (UPCH) in Lima. Then she did her Pediatric Infectious Diseases training at the University of Texas Health Science Center at Houston, USA (2004). After completing her fellowship, she worked for one year as a post-doctoral fellow at Baylor Medical School in Houston before moving back to Peru in 2005.

Currently, she is Associate Professor of Epidemiology at the University of Texas School of Public Health (since 2012) and Associate Professor of Pediatrics at UPCH (since 2015). Since 2019 Dr. Ochoa is the Director of the Tropical Medicine Institute at UPCH were she was Head of the Enteric Diseases and Nutrition Laboratory (2006-2017), Head of the Pediatric Infectious Diseases Laboratory (since 2011) and Head of the Research Office (2014-2018). At Cayetano Medical School she was Head of the Research, Science and Technology Unit (2016-2018).

Her areas of research focus on pediatric diarrhea, pneumonia, neonatal infections, protective factors of breast milk and malnutrition in children, important challenges in pediatric global health. Her main work is in diarrheal pathogens and the protective factors of breast milk. Her early research was on the mechanisms of action of lactoferrin on diarrheagenic *Escherichia coli*, *Shigella* and *Salmonella.* More recently she conducted four clinical trials of lactoferrin on pediatric diarrhea and neonatal sepsis, and two large studies in nasopharyngeal carriers and in invasive pneumococcal disease in children. Based on this expertise Dr. Ochoa is a Member of the Advisory Committee for the National Immunizations Program of the Peruvian Ministry of Health.

In addition to her experience as a leader of clinical trials in pediatrics, epidemiology studies, molecular diagnosis of infectious agents, and research related to the mechanisms of action of lactoferrin, Dr. Ochoa teaches Pediatrics, Infectious Diseases and Tropical Medicine to students and Pediatric Residents at Cayetano University.

Her long-term objectives are to find cost-effective interventions to decrease the burden of infectious diseases and improve outcomes in pediatric populations in developing countries.

She has 136 peer-reviewed publications in international journals (H-index 27). Her complete list of published work is in MyBibliography:
https://bit.ly/2VzGM76

Theresa Ochoa is married to Julio Huby. They have two teenagers, José Ignacio and Illary.

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