Lactoferrin – An Iron-Binding Antimicrobial Protein against *Escherichia coli* Infection

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Abstract

Escherichia coli (E. coli) are the most common aerobic gram-negative bacilli in a normal intestinal tract. They cause most of the intra-abdominal infections, wound infections associated with abdominal surgery, and septicemia. Most of these infections are of endogenous intestinal origin. Lactoferrin (LF) is an iron-binding glycoprotein found in milk and various external secretions. This protein has been found to have a number of biological functions, including antimicrobial, anti-cancer, antioxidant, and immunomodulatory effects. Partial degradation of LF by pepsin can give rise to peptides termed lactoferricin (LFcin) with more potent antimicrobial activity. LF and LFcin have been shown to inhibit the growth of a number of pathogenic bacteria (including *E. coli* and antibiotic-resistant strains), fungi, and even viruses in both *in vitro* and *in vivo* studies. We previously demonstrated that both recombinant porcine LF (pLF) produced from yeast and a synthetic 20-residue porcine LFcin peptide exhibit antimicrobial activity *in vitro*. In one of our recent studies, we performed pathogen challenges, including pathogenic *Escherichia coli, Staphylococcus aureus* and *Candida albicans,* of the digestive tract of a transgenic milk-fed animal model. The results showed that LF has broad spectrum antimicrobial activity in the digestive tract and protects the mucosa of the small intestine from injury. Our following study also revealed that pLF as a feedstuff additive enhances avian immunity, including antibody formation and cell-mediated immunity. All of these results suggest that LF could be a novel natural protein in the treatment and prevention of infections with *E. coli* or antibiotic-resistant bacteria strains.

Introduction

Escherichia coli (*E. coli*) strains are the most common aerobic gram-negative bacilli in the normal intestinal tract. The bacteria are present whenever there is fecal contamination, and their presence is regarded as an indicator of fecal pollution. The species includes a variety of strains, which range in virulence from commensal to lethal. In human, *E. coli* can induce a variety of intestinal and extraintestinal infections. *E. coli* is the most common cause of acute uncomplicated urinary tract infection (UTI) in the community and one of the common causes of nosocomial urinary tract infection, intra-abdominal infections, wound infections associated with abdominal surgery, and septicemia (Murray et al. *2009*).

In general, different strains are associated with different diseases. The diarrheagenic strains that cause gastrointestinal diseases fall into at least five groups: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC). The first three groups involve the small intestine and cause watery diarrhea in infants. The last two groups involve the large intestine and cause dysentery (Murray et al. *2009*). Uropathogenic strains that have the ability to adhere to bladder mucosa often originate from the colon and must travel to the urethral opening to gain admission to the urinary tract. The strains that cause invasive diseases, such as meningitis and septicemia, almost always have a capsule composed of a polysaccharide for antiphagocytosis (Murray et al. *2009*).

Normally, antibiotics are not used to treat diarrhea because the disease is often self-limiting. In suspected cases of EHEC, antibiotics are definitely not indicated because they may worsen disease (Salyers and Whitt *2002*). The therapy for extraintestinal *E. coli* infections does consist of antibiotics. However, antibiotics have

the disadvantage of adverse effects, safety problems during pregnancy and selective pressure that can induce the emergence of resistant strains (Camus et al. *2005*; Vincent *2003*). The rates of antibiotic resistance among *E. coli* strains have increased greatly over the past two decades, encouraging the search for novel antimicrobial strategies (Ref: 1, 2 from J Antibiotics 2009). Lactoferrin, one of the natural cationic host defense proteins that have both antimicrobial and immunomodulatory activities, is a novel agent for treatment and prophylaxis of the extraintestinal infections and even for shortening the course of diarrheagenic *E. coli* infections.

Pathogenesis of *E. coli* Infection

Pathogenic strains of *E. coli* differ from commensal organisms in that they possess certain virulence factors. Bacterial infection depends on the ability of the bacteria to adhere host cells, to escape from host immune response, to invade cells and tissues, or to produce toxins (Matthew et al. 2010, Nature Review Microbiology).

Adhesion is an essential and prerequisite step for the colonization of mucosal surfaces by microbial pathogens, and this colonization is required for the subsequent development of infection (Matthew et al. 2010, Nature Review Microbiology). Adherence of *E. coli* is mediated by plasmid-encoded adhesins. These proteins are expressed on the surface of the microorganisms, sometimes in the form of fimbriae or pili. The variable amino acids on the tip of the pili determine the binding specificity of bacteria. For example, the urogenic strains of *E. coli* express a specific P pilus that binds to cells lining the urinary tract and prevents the elimination of bacteria in voided urine. The ETEC strains adhere to the enterocytes via pili without invasion while EPEC strains adhere and efface the enterocytes with damages to the microvilli. Furthermore, the EICE strains have the capacity to induce endocytosis and invasion into the epithelial cells (Murray et al.2009).

Bacterial toxins are classified as endotoxins and exotoxins. Endotoxin is a lipopolysaccharide (LPS) that is a component of the outer cell wall of Gram-negative bacteria. The activity of endotoxins depends on the lipid A component of LPS, which is released at cell lysis. LPS binds to the cell surface receptor CD14 and is delivered to Toll-like receptor 4 (TLR-4), which transmits signals that lead to release of pro-inflammatory cytokines, systemic inflammatory responses and even septic shock (Kawai and Akira 2007, Trends in Mol Med). Exotoxins are secreted proteins that cause cellular injury and disease. Some diarrheagenic strains can adhere to enterocytes, secret an enterotoxin, one kind of exotoxins, and cause gastroenteritis in children and travelers. The EHEC O157:H7 strains release Shigella-like toxins that cause damage to the colonic mucosa, and blood vessels in kidneys and elsewhere, leading to hemolysis and acute renal failure (Pennington 2010, Lancet).

Lactoferrin

Lactoferrin (LF) is an iron-binding glycoprotein found in milk and various external secretions such as saliva, tears, airway secretion, and the granules of neutrophils, implying an important role in innate immunity (Valenti and Antonini *2005*). The protein, which is approximately 80 kDa, comprises two homologous lobes with one iron-binding site in each lobe. Lactoferrin has a number of biological functions, including antimicrobial, anti-tumor, antioxidant, and immunomodulatory effects. Partial degradation of LF by pepsin in the stomach, can give rise to peptides termed lactoferricin with more potent antimicrobial activity. LF and lactoferricin have been shown to inhibit the growth of a number of pathogenic bacteria (including antibiotic-resistant strains), fungi, and even viruses in both *in vitro* and *in vivo* studies (Tomita et al. 2002; Valenti and Antonini 2005). In mouse experiments, oral administration of bovine LF reduced the number of bacterial infections in the

gastrointestinal tract (Tomita et al. 2002), while promoting the growth of bacteria with low iron requirements such as *Lactobacillus* and *Bifidobacteria*, which are generally believed to be beneficial to the host (Kim et al. 2002; Kim et al. *2004*). Oral administration of LF also reduced experimentally induced UTIs and endotoxin-induced septic shock (Haversen et al. *2000*; Lee et al. *1998*). Another important function related to infection is the immunomodulatory effect of LF, which consists of both immunotropic and anti-inflammatory effects (Actor et al. *2009*; Ando et al. *2010*). This article will concentrate on the antimicrobial and immunomodulatory activities of LF first and will then discuss the effects LF on *E. coli* and the proposed clinical applications of LF.

Mechanisms of Antimicrobial Activity of Lactoferrin

The antimicrobial activity of LF is well established. Originally, the bacteriostatic effect of intact LF was believed to be related to its ability to scavenge iron, the essential nutrient of microorganisms. Subsequent studies have shown that the highly cationic N-terminal regions of LF and its derived peptides, lactoferricin (LFcin17-30) and lactoferrampin (LFampin265-284), exert a potent bactericidal effect by interacting with the negatively charged elements in the bacterial membrane (Appelmelk et al. *1994*; Chen et al. *2006*; Zagulski et al. *1998*; Biometals2010 R1 & R2). These elements can be lipopolysaccharides (LPS) in Gram-negative bacteria, and they are assumed to be lipoteichoic or teichoic acid in Gram-positive bacteria (Gonzalez-Chavez et al. *2009*). The mechanism of the antifungal effect of LF is not clear at present. It is possible that its antifungal activity stems from its immunological effects (Kuipers et al. *1999*; Legrand et al. *2004*), or from its direct or indirect interaction with the cytoplasmic membrane (Viejo-Diaz et al. *2004*). In our previous report, the destructive effects of LF and LFcin on the cellular membranes of *E. coli*, *S.*

aureus, and *C. albicans* were directly observed under a scanning electron microscope (Chen et al. *2004*; Chen et al. *2006*). LF has also been revealed to have antiviral activity. The mechanisms behind this function have not clearly defined, but it is likely that LF prevents the adhesion of the pathogens to host cells (Gonzalez-Chavez et al. *2009*).

Lactoferrin also has a protease-like activity (Ling and Schryvers 2006,Bichem & Cell Biolo). In studies involving *E. coli*, LF was found to degrade and impair the function of the surface expressed virulence factors of *E. coli*, thereby decreasing their ability to adhere to or invade host cells (Ochoa et al. *2008*). Furthermore, LF has been found to prevent biofilm formation at sub-inhibitory concentrations by stimulating bacterial motility, which causes the bacteria to wander across the surface instead of forming cell clusters (Singh et al. *2002*).

Mechanisms of Immunomodulatory Effect of Lactoferrin

There are several studies demonstrating that LF has host-protecting properties, by its immunomodulatory action and its antimicrobial effect. Depending on the immune status of the host, LF can have an anti-inflammatory effect that downregulates the immune response and prevents septic shock and mortality (Kruzel et al. *2002*; Lee et al. *1998*; Zimecki et al. *2004*). LF also acts as a promoter of the immune system. LF promotes the maturation of T-cell precursors into competent T helper cells and stimulates the differentiation of immature B cells into functional antigen presentation cells (Actor et al. *2009*). Our recent studies on the effect of porcine LF (pLF) on avian immunity are in agreement with these findings. Recombinant pLF was used as a feedstuff additive in chickens vaccinated for the infectious bursal disease (IBD), a worldwide viral disease of chicken with high mortality. A high dose of pLF leads to significant increases in serum IgA, IgG and

IBD-specific antibodies. Porcine LF at either low or high doses enhances the expression of interferon-γ and IL-12, driving T lymphocytes toward Th1 rather than Th2 cells, and enhancing the immunity against microbial agents (Hung et al. *2010a*; Hung et al. *2010b*).

The two contrasting effects of LF, immunotropic vs. anti-inflammatory, have not been fully elucidated at present. Recent studies have implied that human LF may bind to LPS and CD-14 and then interfere with the formation of the CD14-LPS complex, resulting in the attenuation of the LPS/ CD-14/ TLR-4 signaling pathway that is essential for pathogenesis of sepsis. Human LF may stimulate the immune system by binding to CD-14 and then activating the TLR-4 mediated pathway while preventing overexpression of LPS-induced inflammation (Ando et al. *2010*).

Effect of Lactoferrin on Intestinal *E. coli* Infections

Diarrheal disease is still an important public health problem in developing countries. According to WHO estimates, diarrhea accounts for 18% of deaths in children before the age of 5 years, approximately 2 million deaths each year. *E. coli* is one the major causes of infant diarrhea in developing countries and also causes traveler's diarrhea in adults (Bryce et al. *2005*). A few outbreaks of EHEC disease, caused by *E. coli* O157:H7, have occurred in developed countries including the USA and Japan (Salyers and Whitt *2002*; Shin et al. *1998*). This strain can cause hemolytic uremic syndrome and even death (Cimolai et al. *1990*). Breast feeding is the most effective intervention for protecting children younger than 5 years of age against diarrhea and all causes of mortality. Lactoferrin is one of the main anti-infective components in the milk (Ochoa et al. *2008*).

In Vitro Studies

Many *in vitro* studies have revealed that LF inhibits adhesion of ETEC, EPEC, EHEC and EAEC to epithelial cells and intestinal mucosa (de Araujo and Giugliano *2001*; de Oliveira et al. *2001*; Giugliano et al. *1995*; Ochoa et al. *2003*). In addition to its bacteriostatic and bacteriocidal effects, LF has been found to exhibit a broad spectrum of anti-adhesion activity. The EHEC O157:H7 strain is susceptible to the antimicrobial effects of bovine LF and its derived peptides (Shin et al. *1998*) and its growth and attachment to intestinal epithial cells are also inhibited by LF (Yekta et al, 2010 Veter Med 55: 359-368). In the case of EPEC, both human and bovine LF block bacterial adhesion to cultured cells. At a concentration not affecting the growth of EPEC, LF blocks the attachment to and EPEC-induced actin polymerization in host cells. The mechanism of this inhibition comes from the ability of LF to cause degradation of the proteins of the type III secretory system that is necessary for bacterial contact and pore formation (Ochoa et al. *2003*). Bovine LF also inhibits EAEC biofilm formation, an important sequel of bacterial adhesion (Ochoa et al. *2006 Biochem Cell Biol 84(3): 369-376*). The combination treatment with antibiotics, such as cephalosporin and aminoglycoside, with LF or LFcin shows a synergistic effect against E. coli (Vorland 1999; Sanchez 1999; Chen PW 2004b)

We have previously demonstrated that both recombinant porcine LF (pLF) produced from yeast and a synthetic 20-residue porcine LFcin peptide exhibit antimicrobial activity on *E. coli* ATCC 25922, a surrogate of E. coli O157: H7, *in vitro* (Chen et al, 2004a; Chen et al. *2006*). Under the scanning electron microscopy (Fig. 1), the *E. coli* cells show either aggregative fragmentation (Fig. 1b) or display puncturing holes and membrane breakdown (Fig. 1c).

Animal Studies

Orally administration of LF has been found to suppress enteric infection of *E. coli*. In one study of ETEC strain, the bacteria counts of ETEC in various sections of

intestine is lower in the bLF treatment group than in the control group, implying the inhibitory effect of LF on the adherence of ETCE to the intestinal tract (Kawasaki et al, 2000). LF is able to reduce the endotoxemia and bacterial translocation to mesenteric lymph nodes and peritoneal cavity after enteral challenge of *E. coli* (Nebermann et al, 2001 Langenbeck's Arch Surg).

Our recent studies also revealed that probiotic *Lactobacillus casei* expressing human LF exhibit antibacterial activity on *E. coli* ATCC 25922 both *in vitro* and *in vivo* (Chen et al. *2010*). In another previous study, we demonstrated increased villous length in the small intestines of mouse pups fed pLF-enriched milk from transgenic females, compared with the length of villi in mice fed wild-type milk (Chen et al, 2007 Ref 14 from JID 2009). LF has been demonstrated to enhance the growth of intestinal cells both *in vitro* and in *vivo,* which may be mediated by binding to specific LF receptors (Ref 34 from JID 2009). The effect of LF on the intestinal cells may also be one of the mechanisms against enteric infections.

Effects of Lactoferrin on Extraintestinal *E. coli* Infections and Sepsis

Effect on UTI, Meningitis and Systemic Infections

In vitro studies have demonstrated that LF or LFcin enhance the activity of antibiotics against E. coli strains isolated from cases of human UTI and bovine mastitis (Sanchez 1999; Longhi 2009 J of Antibiotics). Orally administration of LF or derived peptides is effective in reducing *E. coli* infection at a remote site such as the urinary tract (Haversen et al. *2000*). Uropathogenic *E. coli* O6:K5 was inoculated into the mice urinary bladder to induce an experimental UTI. The number of bacteria in the urinary tract was significantly reduced by the LF treatment. Urinalysis revealed that hLF was excreted into the urine 2 hours after feeding. Intact human LF of

maternal origin has been detected in the urine of preterm infants fed human milk (Hutchens et al. *1991*). These studies imply that LF may remain intact throughout the GI tract and be absorbed into the blood when the integrity of the GI tract is compromised in some medical conditions (Legrand et al. *2004*). Another report demonstrated that oral administration of recombinant hLF protects neonate rats against systemic infection by *E. coli* O18:K1:H7, a strain that can induce meningitis, from a challenge in the gastrointestinal tract. Neonate rats pretreated with hLF have less bacteremia and lower disease severity scores than those not pretreated. The *in vitro* studies in this report also suggested that hLF may act with other natural peptides, such as lysozyme, or may prime macrophages to kill *E. coli* in vivo (Edde et al. *2001*).

Effects on Septic Shock

Gram-negative sepsis results from overwhelming systemic inflammation that is caused by excessive release of proinflammatory cytokines, which are induced in response to bacterial endotoxin (LPS). A number of immunotherapies, including anti-LPS antibodies and anti-tumor necrosis factor (TNF) antibodies, which block the action of endotoxin, have been proposed as potential therapies for sepsis but have failed in clinical studies (Baumgartner et al. *1985*; Fisher et al. *1996*; Ziegler et al. *1982*). Lactoferrin has been found to have protective effects against septic shock induced by endotoxin or bacterial challenge by *E. coli* (Edde et al. *2001*; Lee et al. *1998*). Oral administration of LF significantly improves survival in neonatal piglets that are later challenged with intravenous *E. coli* endotoxin. The *in vitro* studies of the same report also demonstrated that LPS binding to monocytes/macrophages is inhibited by LF in a dose-dependent fashion (Lee et al. *1998*). Overall, the proposed mechanisms against *E. coli* of LF are illustrated in Fig 2.

Clinical Studies of Antimicrobial Activity of Lactoferrin

A few studies of bovine LF supplementation have been conducted to determine its effects on fecal flora, hepatitis C, Helicobacter pylori and anemia (Kaito et al. *2007*; Nappi et al. *2009*; Zullo et al. *2007*). No evident adverse effect has been reported related to the clinical use of bovine LF. However, studies that address the effect on diarrhea and sepsis caused by *E .coli* are rare.

One multicenter, randomized, double-blind control trial enrolled 472 Italian very low birth weight neonates and revealed that bovine LF supplementation, alone or in combination with probiotic Lactobacillus rhamnosus GG reduces the incidence of later-onset sepsis. The antimicrobial effects of LF cover various pathogens, including Gram-negative bacteria, Gram-positive bacteria and invasive fungi (Manzoni et al. *2009*). Another randomized, double-blind control study, which enrolled 140 Peruvian children with acute diarrhea and dehydration, revealed that addition of recombinant human LF and lysozyme to an oral rehydration solution reduces the duration and the relapse of diarrhea. Bacteria, especially *E. coli*, are the predominant pathogens isolated in the stools of the children. This result supports the antimicrobial effects of combined treatment with LF and lysozyme but fails to discriminate the relative potency of LF and lysozyme (Zavaleta et al. *2007*). A few clinical studies have demonstrated that LF reduces the severity of vomiting and diarrhea during rotavirus infection, reduces the incidence of lower respiratory tract illness, decreases *Giardia lamblia* colonization, and results in better growth of the affected children (King et al. *2007*; Ochoa et al. *2008*).

Clinical Application

Prevention against E. coli Infection and Nosocomial Infection

Based on the studies mentioned above, LF supplementation is a novel strategy for preventing *E. coli* infections, such as EHEC (O157:H7) infection, diarrheal diseases, and nosocomial infections due to *E. coli*. Preventing nosocomial infections and limiting the emergence of antibiotic-resistant strains of pathogens are two important issues in critical care medicine. It has been estimated that 50%-60% of the nosocomial infection that occur each year in USA are caused by antibiotic-resistant strains (Jones *2001*). Pathogen colonization of the nasopharynx and oropharynx predisposes patients to the development of nosocomial pneumonia, and bacterial overgrowth in the digestive tract may result in bacterial translocation and sepsis. Selective decontamination of the digestive tract (SDD), which aims to eradicate colonization of pathogens from digestive tract, is one of the strategies used to reduce ventilator associated pneumonia and sepsis in critically ill patients (Bonten and Grundmann *2003*; de Jonge et al. *2003*; Pittet et al. *2001*).

In one of our recent studies, we performed pathogen challenges, including pathogenic *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Candida albicans* (ATCC 14053), through the gastrointestinal tract of a transgenic milk-fed mouse animal model. Compared with the control group, the group fed pLF-enriched milk had significant improvement in weight gain; less severity of illness; lower bacterial load in the intestinal tract, blood and liver; healthier microvilli in the small intestine; and healthier alveoli in the lungs. Lactoferrin, which is effective against most pathogens while leaving the anaerobic flora undisturbed, could be an ideal regimen for SDD (Yen et al. *2009*).

Combination Therapy with Antibiotics for E. coli infection and Sepsis

Lactoferrin is considered to be a host defense protein involved in protection against a wide variety of microbial infections including many resistant strains, and in

prevention against the systemic inflammatory response induced by endotoxins. LF and LFcin have been used in combination with antibiotics to treat *E. coli* including the resistant strains, *Helicobacter pylori* and hepatitis C infection (Ref: J Antibiotics 2009+ Ref 11,12, 13; Kaito et al. *2007*; Zullo et al. *2007*), and in these studies, adding LF/LFcin has produced positive results. This combination therapy is proposed to be an important strategy for treating *E. coli* infections, especially those due to resistant strains and those with a fulminant course.

Discovery of many immunosuppressive agents has led to great progress in the treatment of cancers and autoimmune diseases. Oral administration of LF has been found to restore humoral immune response in immunocompromised mice after cyclophosphamide treatment (Artym et al. *2003*). LF may be used as an adjuvant therapy aimed at reconstruction of B and T cells or in combination therapy with antibiotics when the immunocompromised patients have infectious diseases.

Limitation of the Antimicrobial Activity of Lactoferrin

An *in vitro* study showed that recombinant human LF (rhLF) alone did not kill *E. coli*. Conversely, human milk lysozyme slightly reduced the number of *E. coli*, whereas a combination of rhLF and lysozyme had evident bacteriocidal effect (Edde et al. *2001*). A clinical trial in Peruvian children also added rhLF and lysozyme to an oral dehydration solution at the same time (Zavaleta et al. *2007*). Our previous study also revealed that the natural immune defense mechanisms, perhaps secretory IgA and lysozyme, can overcome a pathogen challenge from the gastrointestinal tract. LF is one of the natural host defense protein that can act synergistically with other defense peptides or proteins to protect the host against pathogens (Yen et al. *2009*). In addition, it is known that pathogens have developed countermeasures to resist the defense

peptides/proteins, such as reducing the net negative charge of the bacterial membranes or the development of a capsule polysaccharide (CPS) to avoid the attack of the peptides/proteins (Campos et al. *2004*; Llobet et al. *2008*). The concept of combination therapy with LF and other natural defense peptides/proteins or therapeutic antibiotics is emerging as a new strategy to prevent or treat bacterial infection.

Conclusion

Lactoferrin is one of the natural host defense proteins that have multiple physiological functions. It has board-spectrum antimicrobial activity against Gram positive bacteria, Gram negative bacteria (including *E. coli*), and even antibiotic-resistant strains. The antimicrobial mechanisms include bacteriostatic, bactericidal, and anti-adhesion effects. It also plays an important role in host immunity with immunotropic and anti-inflammatory effects that protect the host against LPS-induced shock. Based on the growing body of evidence of LF's action against *E. coli*, LF is a novel natural host defense protein with potential utility in the prophylaxis and treatment of *E. coli* infection.

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References

Actor JK, Hwang SA, Kruzel ML (2009) Lactoferrin as a natural immune modulator. Curr Pharm Des 15:1956-1973

Ando K, Hasegawa K, Shindo K, Furusawa T, Fujino T, Kikugawa K, Nakano H, Takeuchi O, Akira S, Akiyama T, Gohda J, Inoue J, Hayakawa M (2010) Human lactoferrin activates NF-kappaB through the Toll-like receptor 4 pathway while it interferes with the lipopolysaccharide-stimulated TLR4 signaling. FEBS J 277:2051-2066

Appelmelk BJ, An YQ, Geerts M, Thijs BG, de Boer HA, MacLaren DM, de Graaff J, Nuijens JH (1994) Lactoferrin is a lipid A-binding protein. Infect Immun 62:2628-2632

Artym J, Zimecki M, Paprocka M, Kruzel ML (2003) Orally administered lactoferrin restores humoral immune response in immunocompromised mice. Immunol Lett 89:9-15

Baumgartner JD, Glauser MP, McCutchan JA, Ziegler EJ, van Melle G, Klauber MR, Vogt M, Muehlen E, Luethy R, Chiolero R (1985) Prevention of gram-negative shock and death in surgical patients by antibody to endotoxin core glycolipid. Lancet 2:59-63

Bonten MJ, Grundmann H (2003) Selective digestive decontamination and antibiotic resistance: a balancing act. Crit Care Med 31:2239-2240

Bryce J, Boschi-Pinto C, Shibuya K, Black RE (2005) WHO estimates of the causes of death in children. Lancet 365:1147-1152

Campos MA, Vargas MA, Regueiro V, Llompart CM, Alberti S, Bengoechea JA (2004) Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. Infect Immun 72:7107-7114

Camus C, Bellissant E, Sebille V, Perrotin D, Garo B, Legras A, Renault A, Le Corre P, Donnio PY, Gacouin A, Gohda J, Inoue J, Hayakawa M (2005) Prevention of acquired infections in intubated patients with the combination of two decontamination regimens. Crit Care Med 33:307-314

Chen HL, Lai YW, Chen CS, Chu TW, Lin W, Yen CC, Lin MF, Tu MY, Chen CM (2010) Probiotic Lactobacillus casei expressing human lactoferrin elevates antibacterial activity in the gastrointestinal tract. Biometals 23:543-554

Chen HL, Lai YW, Yen CC, Lin YY, Lu CY, Yang SH, Tsai TC, Lin YJ, Lin CW, Chen CM (2004) Production of recombinant porcine lactoferrin exhibiting antibacterial activity in methylotrophic yeast, Pichia pastoris. J Mol Microbiol Biotechnol 8:141-149

Chen HL, Yen CC, Lu CY, Yu CH, Chen CM (2006) Synthetic porcine lactoferricin with a 20-residue peptide exhibits antimicrobial activity against Escherichia coli, Staphylococcus aureus, and Candida albicans. J Agric Food Chem 54:3277-3282

Cimolai N, Carter JE, Morrison BJ, Anderson JD (1990) Risk factors for the progression of Escherichia coli O157:H7 enteritis to hemolytic-uremic syndrome. J Pediatr 116:589-592

de Araujo AN, Giugliano LG (2001) Lactoferrin and free secretory component of human milk inhibit the adhesion of enteropathogenic Escherichia coli to HeLa cells. BMC Microbiol 1:25

de Jonge E, Schultz MJ, Spanjaard L, Bossuyt PM, Vroom MB, Dankert J, Kesecioglu J (2003) Effects of selective decontamination of digestive tract on mortality and acquisition of resistant bacteria in intensive care: a randomised controlled trial. Lancet 362:1011-1016

de Oliveira IR, de Araujo AN, Bao SN, Giugliano LG (2001) Binding of lactoferrin and free secretory component to enterotoxigenic Escherichia coli. FEMS Microbiol Lett 203:29-33

Edde L, Hipolito RB, Hwang FF, Headon DR, Shalwitz RA, Sherman MP (2001) Lactoferrin protects neonatal rats from gut-related systemic infection. Am J Physiol Gastrointest Liver Physiol 281:G1140-1150

Fisher CJ Jr, Agosti JM, Opal SM, Lowry SF, Balk RA, Sadoff JC, Abraham E, Schein RM, Benjamin E (1996) Treatment of septic shock with the tumor necrosis factor receptor:Fc fusion protein. The Soluble TNF Receptor Sepsis Study Group. N Engl J Med 334:1697-1702

Giugliano LG, Ribeiro ST, Vainstein MH, Ulhoa CJ (1995) Free secretory component and lactoferrin of human milk inhibit the adhesion of enterotoxigenic Escherichia coli. J Med Microbiol 42:3-9

Gonzalez-Chavez SA, Arevalo-Gallegos S, Rascon-Cruz Q (2009) Lactoferrin: structure, function and applications. Int J Antimicrob Agents 33:e301-308

Haversen LA, Engberg I, Baltzer L, Dolphin G, Hanson LA, Mattsby-Baltzer I (2000) Human lactoferrin and peptides derived from a surface-exposed helical region reduce experimental Escherichia coli urinary tract infection in mice. Infect Immun 68:5816-5823

Hung CM, Wu SC, Yen CC, Lin MF, Lai YW, Tung YT, Chen HL, Chen CM (2010a) Porcine lactoferrin as feedstuff additive elevates avian immunity and potentiates vaccination. Biometals 23:579-587

Hung CM, Yeh CC, Chen HL, Lai CW, Kuo MF, Yeh MH, Lin W, Tu MY, Cheng HC, Chen CM (2010b) Porcine lactoferrin administration enhances peripheral

lymphocyte proliferation and assists infectious bursal disease vaccination in native chickens. Vaccine 28:2895-2902

Hutchens TW, Henry JF, Yip TT (1991) Structurally intact (78-kDa) forms of maternal lactoferrin purified from urine of preterm infants fed human milk: identification of a trypsin-like proteolytic cleavage event in vivo that does not result in fragment dissociation. Proc Natl Acad Sci USA 88:2994-2998

Jones RN (2001) Resistance patterns among nosocomial pathogens: trends over the past few years. Chest 119:397S-404S

Kaito M, Iwasa M, Fujita N, Kobayashi Y, Kojima Y, Ikoma J, Imoto I, Adachi Y, Hamano H, Yamauchi K (2007) Effect of lactoferrin in patients with chronic hepatitis C: combination therapy with interferon and ribavirin. J Gastroenterol Hepatol 22:1894-1897

Kawasaki Y, Tazume S, Shimizu K, Matsuzawa H, Dosako S, Isoda H, Tsukiji M, Fujimura R, Muranaka Y, Isihida H. (2000) Inhibitory effects of bovine lactoferrin on the adherence of enterotoxigenic Escherichia coli to host cells. Biosci Biotechnol Biochem 64:348-354

Kim WS, Ohashi M, Tanaka T, Kumura H, Kim GY, Kwon IK, Goh JS, Shimazaki K (2004) Growth-promoting effects of lactoferrin on L. acidophilus and Bifidobacterium spp. Biometals 17:279-283

King JC Jr, Cummings GE, Guo N, Trivedi L, Readmond BX, Keane V, Feigelman S, de Waard R (2007) A double-blind, placebo-controlled, pilot study of bovine lactoferrin supplementation in bottle-fed infants. J Pediatr Gastroenterol Nutr 44:245-251

Kruzel ML, Harari Y, Mailman D, Actor JK, Zimecki M (2002) Differential effects of prophylactic, concurrent and therapeutic lactoferrin treatment on LPS-induced inflammatory responses in mice. Clin Exp Immunol 130:25-31

Kuipers ME, de Vries HG, Eikelboom MC, Meijer DK, Swart PJ (1999) Synergistic fungistatic effects of lactoferrin in combination with antifungal drugs against clinical Candida isolates. Antimicrob Agents Chemother 43:2635-2641

Lee WJ, Farmer JL, Hilty M, Kim YB (1998) The protective effects of lactoferrin feeding against endotoxin lethal shock in germfree piglets. Infect Immun 66:1421-1426

Legrand D, Vigie K, Said EA, Elass E, Masson M, Slomianny MC, Carpentier M, Briand, JP, Mazurier J, Hovanessian AG (2004) Surface nucleolin participates in both the binding and endocytosis of lactoferrin in target cells. Eur J Biochem 271:303-317

Llobet E, Tomas JM, Bengoechea JA (2008) Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. Microbiology 154:3877-3886

Manzoni P, Rinaldi M, Cattani S, Pugni L, Romeo MG., Messner H, Stolfi I, Decembrino L, Laforgia N, Vagnarelli F et al (2009) Bovine lactoferrin supplementation for prevention of late-onset sepsis in very low-birth-weight neonates: a randomized trial. JAMA 302:1421-1428

Murray PR, Rosenthal KS, Pfaller MA (2009) Enterobacteriaceae. In: Medical microbiology, 6th edn. Mosby Elsevier, Philadephia, pp 301-315

Nappi C, Tommaselli GA, Morra I, Massaro M, Formisano C, Di Carlo C (2009) Efficacy and tolerability of oral bovine lactoferrin compared to ferrous sulfate in pregnant women with iron deficiency anemia: a prospective controlled randomized study. Acta Obstet Gynecol Scand 88:1031-1035

Ochoa TJ, Chea-Woo E, Campos M, Pecho I, Prada A, McMahon RJ, Cleary TG (2008) Impact of lactoferrin supplementation on growth and prevalence of Giardia colonization in children. Clin Infect Dis 46:1881-1883

Ochoa TJ, Noguera-Obenza M, Ebel F, Guzman CA, Gomez HF, Cleary TG (2003) Lactoferrin impairs type III secretory system function in enteropathogenic Escherichia coli. Infect Immun 71:5149-5155

Pittet D, Eggimann P, Rubinovitch B (2001) Prevention of ventilator-associated pneumonia by oral decontamination: just another SDD study? Am J Respir Crit Care Med 164:338-339

Salyers AA, Whitt DD (2002) Diarrheagenic *Escherichia coli* strains. In: Bacterial pathogenesis: A molecular approach. 2nd edn. ASM press, Washington, DC, pp 407-421

Shin K, Yamauchi K, Teraguchi S, Hayasawa H, Tomita M, Otsuka Y, Yamazaki S (1998) Antibacterial activity of bovine lactoferrin and its peptides against enterohaemorrhagic Escherichia coli O157:H7. Lett Appl Microbiol 26:407-411

Singh PK, Parsek MR, Greenberg EP, Welsh MJ (2002) A component of innate immunity prevents bacterial biofilm development. Nature 417:552-555

Tomita M, Wakabayashi H, Yamauchi K, Teraguchi S, Hayasawa H (2002) Bovine lactoferrin and lactoferricin derived from milk: production and applications. Biochem Cell Biol 80:109-112

Valenti P, Antonini G (2005) Lactoferrin: an important host defence against microbial and viral attack. Cell Mol Life Sci 62:2576-2587

Viejo-Diaz M, Andres MT, Fierro JF (2004) Modulation of in vitro fungicidal activity of human lactoferrin against Candida albicans by extracellular cation concentration and target cell metabolic activity. Antimicrob Agents Chemother 48:1242-1248

Vincent JL (2003) Selective digestive decontamination: for everyone, everywhere? Lancet 362:1006-1007

Yen CC, Lin CY, Chong KY, Tsai TC, Shen CJ, Lin MF, Su CY, Chen HL, Chen CM (2009) Lactoferrin as a natural regimen for selective decontamination of the digestive tract: recombinant porcine lactoferrin expressed in the milk of transgenic mice protects neonates from pathogenic challenge in the gastrointestinal tract. J Infect Dis 199:590-598

Zagulski T, Lipinski P, Zagulska A, Jarzabek Z (1998) Antibacterial system generated by lactoferrin in mice in vivo is primarily a killing system. Int J Exp Pathol 79:117-123

Zavaleta N, Figueroa D, Rivera J, Sanchez J, Alfaro S, Lonnerdal B (2007) Efficacy of rice-based oral rehydration solution containing recombinant human lactoferrin and lysozyme in Peruvian children with acute diarrhea. J Pediatr Gastroenterol Nutr 44:258-264

Ziegler EJ, McCutchan JA, Fierer J, Glauser MP, Sadoff JC, Douglas H, Braude AI (1982) Treatment of gram-negative bacteremia and shock with human antiserum to a mutant Escherichia coli. N Engl J Med 307:1225-1230

Zimecki M, Artym J, Chodaczek G, Kocieba M, Kruzel ML (2004) Protective effects of lactoferrin in Escherichia coli-induced bacteremia in mice: relationship to reduced serum TNF alpha level and increased turnover of neutrophils. Inflamm Res 53:292-296

Zullo A, De Francesco V, Scaccianoce G, Manes G, Efrati C, Hassan C, Maconi G, Piglionica D, Cannaviello C, Panella C, Morini S, Ierardi E (2007) Helicobacter pylori eradication with either quadruple regimen with lactoferrin or levofloxacin-based triple therapy: a multicentre study. Dig Liver Dis 39:806-810

Figure legends

Fig. 1. Pathogenic mechanisms of extraintestinal *Escherichia coli* and the multifunctional properties of lactoferrin against *E. coli* infection. Lactoferin exhibits bacteriocidal and bacteriostatic effects before *E. coli* entrance into the epithelia cells of gut. Lactoferin also displays anti-CD14-TRL4 signaling and immunotropic effects after *E. coli* entrance into the epithelia cells of gut. LPS: lipopolysaccharides; LF: lactoferrin.

Fig. 2. *In vitro* antimicrobial activity assays of bovine LF by scanning electron microscopy (SEM). *E. coli* was cultured to logarithmic phase and resuspended in 2% PBW buffer for further 2h incubation without adding hLF protein as a control group (**a**). In the test group, *E. coli* was cultured in the same condition but adding with 1 mg/ml LF purified from cow milk for further 2 hours (**b**) or 4 hours (**c**). The samples were observed under 30,000X magnifications with the JSM-6300 mode of scanning electron microscope.

Figure 1.

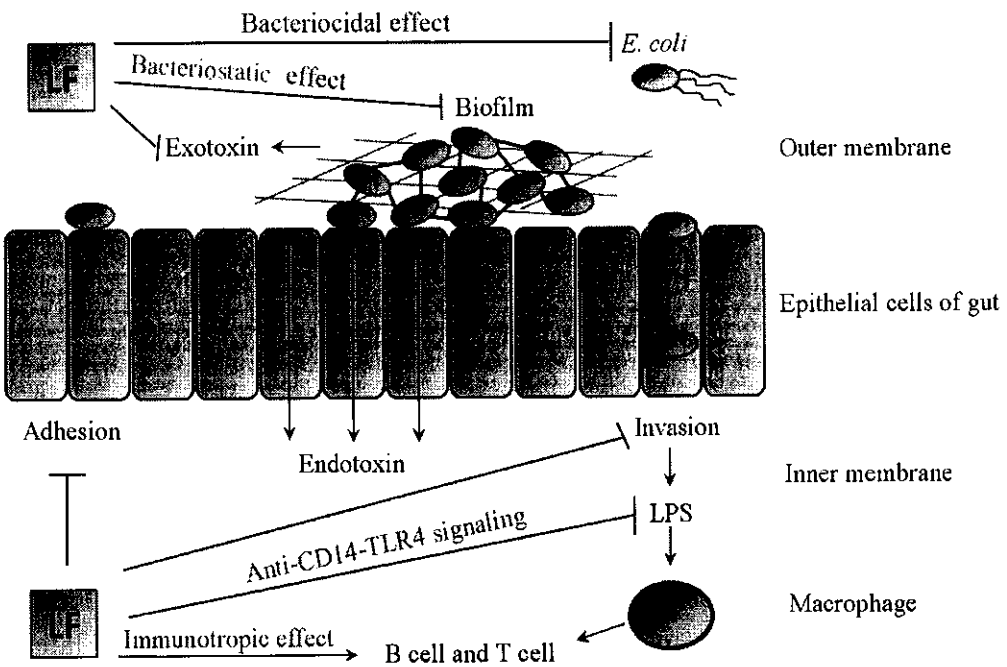
Bacteriocidal effect
E. coli
LF
Bacteriostatic effect
Biofilm
Exotoxin
Outer membrane
Epithelial cells of gut
Adhesion
Endotoxin
Invasion
Inner membrane
LPS
Anti-CD14-TLR4 signaling
LF
Macrophage
Immunotropic effect
B cell and T cell

Figure 2