Immune Health
The Future of Lactoferrin

Dr. Narain Naidu
Ultra-cleansing of lactoferrin: Nutraceutical implications

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INTRODUCTION
Emerging knowledge on diseases and the role of natural compounds in lowering the risk of such disease processes, research efforts for identification and development of medically important dietary supplements are all steadily increasing. Breast milk is the original delivery system for the transport of essential nutrients to the newborn. This natural system provides several bioactive ingredients for regular management of gastrointestinal functions including innate defense; scavenging of free radicals and toxins; gut maturation and repair; nutrient diffusion and transport across the mucosal barrier; and selective proliferation of probiotic microflora.

Accordingly, the consumption of cow’s milk has been an integral part of human civilization since antiquity, which provided remarkable benefits to mankind. Lactoferrin (LF) is an iron-binding glycoprotein present in milk and many exocrine secretions that bathe mucosal surfaces. The term ‘lacto-ferrin’ implies an iron-binding compound from milk, this molecule co-ordinately binds to other metal ions, eg., zinc, copper, and manganese, as well as present in divergent biological milieu including saliva, tears, seminal fluids, mucus and the secondary granules of neutrophils. LF has a multifunctional role in a variety of physiological pathways and is considered a major component of the mammalian innate defense. The ability of LF to bind two ferric ions with high affinity in co-operation with two bicarbonate ions is an essential characteristic that contributes to its major structure-functional properties.

MULTI-FUNCTIONAL GUT MANAGEMENT
LF is mainly present in the exocrine glands located mainly in the gateways of the digestive, respiratory and reproductive systems, to provide mucosal protection against invading microorganisms and toxic insults. It occurs in three different physiological pools: i) the secretory (exocrine) pool, ii) the circulatory pool and iii) the stationary (tissue-borne) pool. In the secretory pool, the normal levels of LF are reported at 1-2 mg/mL in breast milk, tears and gastric mucus; 0.1-1 mg/mL in vaginal, cervical and bronchial mucus; 0.01-0.1 mg/mL in seminal plasma, pancreatic juice, saliva and crevicular fluids; <0.01 mg/mL in plasma, cerebrospinal and synovial fluids. Neutrophils contain LF at about 0.01 mg/10^6 cells, which contributes to the plasma levels of LF in the circulatory pool. In the stationary pool, LF is localized in several tissues. Neoplastic cells have an increased iron requirement for the initiation and maintenance of DNA synthesis and for the cell multiplication. Therefore, in different types of malignancies, iron uptake is reported to be mediated by specific LF-binding receptors. The physiological distribution of LF in human body fluids and secretions is schematically depicted in FIGURE-2.

The ability of LF to bind ferric ions with high affinity in co-operation with two bicarbonate ions is an essential characteristic that contributes to its major structure-functional properties. LF co-exists with an array of molecules in different mucosal secretions with varying milieu conditions. However, molecular dysfunctions and deficiency of LF levels in the body could cause several physiological disorders and predispose various infections.

Several studies have established that LF supplementation could provide exceptional health benefits and a powerful protection against several illnesses. Recent advances in gene mapping, protein engineering and functional characterization technologies have elucidated the molecular mechanism(s) of LF-mediated multifunctional activities. Furthermore, investigators from laboratories around the world have validated the functional outcomes with LF supplements in randomized human trials and in vivo experimental models. The following section elaborates the physiological role for this unique milk protein in specific maintenance of gastrointestinal health.

Pathogen elimination and gut protection
The antimicrobial functionality of LF is dependent on its protein conformation, metal-binding and milieu conditions. Antimicrobial activity is enhanced when LF binds to the microbial cell surface. Over the past 15 years, Naidu et al. have identified specific LF-binding microbial targets on different Gram-positive and Gram-negative bacterial pathogens. The high-affinity interaction of LF with pore-forming outer-membrane proteins (OMPs) of Gram-negative enteric bacteria, including Escherichia coli, is critical for the antimicrobial outcome of LF. Thus, the antimicrobial functionality of LF is dependent on its protein conformation, metal-binding and milieu conditions.

ABSTRACT
Lactoferrin is a milk protein credited with an impressive list of multifunctional health benefits. Separation of LF from other ingredients of milk requires several complex steps of protein engineering. Despite high purity, proteins isolated from such processes may harbor microbial and endotoxin contaminants that could compromise LF functionality and applications in vivo. A novel treatment for contaminant reduction (TCR) to enhance the protein quality during commercial-scale LF production has been developed. LF-(TCR), based on this cutting-edge protein technology, contains ultra-clean LF that could offer the highest standards of microbiological quality and functional assurance for nutraceutical applications.

Figure 1. Ribbon structure of bovine LF (drawn with RasMol ver.2.6 software). The N-terminus (Blue) and C-terminus (Red) regions constitute the two iron-binding lobes of the molecule.

Figure 2. Physiological distribution of LF in human body fluids and secretions (A schematic representation).
It is known that the large intestine of breast-fed infants is colonized by a natural selection to enrich beneficial probiotic flora and affect the prebiotic effect by LF in the intestinal milieu is a phenomenon of selective evasive to iron-deprivation antimicrobial stasis by LF. This transport system. However, intestinal probiotic LAB are independent pathogens to generate ATP by cytochrome-dependent electron transport system. LF elicits microbial growth-inhibition by iron-deprivation stasis in acid bacteria (LAB).

Oral administration of LF supplemented milk has been shown to suppress proliferation of intestinal clostridium species and fecal counts in the stomach and inhibit pathogen establishment on the gut mucosa. As a result, a marked decline in the serum antibody titer against H. pylori could be achieved. Prophylactic and therapeutic effects of oral supplementation of LF against intractable stomatitis have also been reported.

**Probiotic proliferation and intestinal health**

The term ‘probiotic’, meaning ‘for life’, is derived from Greek. Naidu et al. (1999) have defined probiotics as “microbial-based dietary adjuncts that beneficially affect the host physiology by modulating mucosal and systemic immunity as well as improving nutritional and microbial balance in the intestinal tract”18. The term prebiotic was introduced by Gibson and Roberfroid (1995) and defined as “non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already established in the colon”19. Accordingly, probiotic intake could significantly modulate the intestinal microbiota, in particular, the beneficial probiotic lactic acid bacteria (LAB). LF elicits microbial growth-inhibition by iron-deprivation stasis mechanism. Iron is critical for many life forms including intestinal pathogens to generate ATP by cytochrome-dependent electron transport system. However, intestinal probiotic LAB are independent of cytochrome pathways for cellular energy synthesis, therefore are selectively evasive to iron-deprivation antimicrobial stasis by LF. This prebiotic effect by LF in the intestinal milieu is a phenomenon of natural selection to enrich beneficial probiotic flora and affect competitive exclusion of harmful pathogens by bacteriostasis. It is known that the large intestine of breast-fed infants is colonized predominantly by species of bifidobacteria, which have protective effects against enteric pathogens20. LF derived from human and bovine sources of mature milk could enhance growth of *Bifidobacterium infantis*, *B. breve* and *B. bifidum in vitro*, in a dose-dependent manner21. Feeding trials with LF supplemented (100 mg/mL infant formula were found to establish ‘bifidus flora’ in 50% of the babies at age three months. Certain peptide domains on LF have been identified to stimulate growth of bifidobacteria in vivo22,23.

**Elimination of endotoxins from the GI tract**

There is a continuous transfer of endotoxin from the intestinal lumen into the bloodstream. In healthy individuals, plasma inactivates the intestinal influx of endotoxin and protects internal organs from damage. However, any disturbances in gut permeability could increase endotoxin transfer into the bloodstream. Such massive influx could exhaust the ability of plasma to inactivate endotoxins and could ultimately lead to clinical endotoxemia24. Experimental evidence suggests that reactive oxygen species are important mediators of cellular injury during endotoxemia, either as a result of macrophage damage or by interfering with extracellular and intracellular regulatory processes. In addition, nitric oxide is thought to play a key role in the pathogenesis of endotoxemic shock. An important mechanism to prevent physiological endotoxemia is to reduce lipopolysaccharide (LPS) influx from the intestinal lumen. LF binds to lipid-A, the toxic moiety of LPS with high affinity and works as a therapeutic agent to neutralize effects of endotoxin25. LF could effectively reduce endotoxin influx into the bloodstream while toxins are still inside the intestinal lumen. In this process, however, LF is also depleted rapidly and may not be present in sufficient amounts to perform this function if endotoxin is continuously released in large quantities26.

A protective effect for LF against lethal shock induced by intravenously administered endotoxin has been reported. LF-mediated protection against endotoxin challenge correlates with both resistance to induction of hypothermia and an overall increase in wellness. In vitro studies with a flow cytometric measurement indicated that LF inhibits endotoxin binding to monocytes in a dose-dependent manner, which suggests that the mechanism of LF action *in vivo* could be due to the prevention of induction of monocyte/macrophage-derived inflammatory-toxic cytokines27.

**Anti-inflammatory activity in the gut**

The anti-inflammatory activity of LF is primarily associated with its ability to scavenge free iron. It is known that accumulation of iron in inflamed tissues could lead to catalytic production of highly toxic free radicals. During an inflammatory response, neutrophils migrate to the challenge site to release their LF containing acidic granules. This results in the creation of a strong acidic milieu at the inflamed tissue site to amplify iron-sequestering and detoxification capacities of LF. LF is also a key regulator of allergic cutaneous inflammation. LF synthesis in the epidermis of normal skin is elevated during allergic challenge as a protective mechanism28. Also, exogenous administration of LF could effectively inhibit allergen-induced cellular events of inflammation in a dose-dependent manner29. Besides modulating iron homeostasis during inflammation, there is mounting evidence that LF could directly regulate various inflammatory responses. This iron-independent mode of action is based on LF binding to bacterial LPS, which is a major pro-inflammatory mediator during bacterial infections and septic shock30. LF could play an important role in the modulation of gastric inflammation, since this protein is also expressed in the gastric mucosa of the stomach and interacts with receptors localized on gastric intestinal epithelial cells. Furthermore, the expression of LF is elevated in the feces of patients with inflammatory conditions including ulcerative colitis and Crohn’s disease. Several *in vivo* studies have shown that oral administration of LF could reduce gastritis induced by *Helicobacter* and protect gut mucosal integrity during endotoxemia31.
Modulation of mucosal immunity

Oral administration of LF (40 mg capsule/day) could enhance immune response in healthy human volunteers \((n=17)\). Human clinical trials showed a positive influence of LF consumption in primary activation of host defense. Healthy male volunteers \((n=10)\) fed with LF (2g/body/day for a week) showed an improvement in their serum neutrophil function including enhanced phagocytic activity and superoxide production. Furthermore, specific interaction of LF with alveolar macrophages, monocytes, Kupfer cells, liver endothelia, neutrophils, platelets, and T-lymphocytes emphasizes the role of LF in mucosal and cellular immunity.  

Gut maturation and mucosal repair

The gastrointestinal tract matures more rapidly in the newborn during suckling. Oral administration of LF, either at low (0.05 mg/g body wt/day) or high (1 mg/g body wt/day) dosages could function as an immune stimulating factor in the intestinal mucosa. This activation is dependent on LF binding to the intestinal epithelia. LF could also potentiate thymidine incorporation into crypt cell DNA in vivo. This trophic effect contributes to cell regeneration and tissue repair of intestinal mucosa in conditions such as gastroenteritis. Feeding of LF supplemented formula could increase hepatic protein synthesis in the newborn, which suggests an anabolic function for LF in neonates.

Intestinal iron absorption

Iron absorption from milk LF has received much attention in recent years, and contributed to the development of several infant formulas. LF plays an important role in the intestinal absorption of iron, zinc, copper, manganese and other essential trace elements. LF also protects the gut mucosa from excess uptake of heavy metal ions. Specific LF binding receptors in the human duodenal brush border are involved in the iron absorption. An intestinal LF receptor with a cellular density of 4.3 x 10^14 sites per milligram of solubilized human fetal intestinal brush-border membranes (IBBM) was identified. Increased iron absorption via this LF receptor from IBBM during the neonatal period has been reported.

Anti-tumor activity

Monocytes in the activated state could kill tumor cells and mediate antibody-dependent cell-mediated cytotoxicity. LF is shown to enhance natural killer (NK) activity of monocytes in a dose-dependent manner. LF strongly augments both NK and lymphokine-activated killer (LAK) cell cytotoxic functions. LF is an effective modulator of cell-mediated immune responses and serum cytotoxic factors at low dosages (<1 μg/mL); however, at higher concentrations the LF-mediated induction could lead to a positive or negative feedback according to the density and subsets of the immune cell population. Immuno-modulator effects of LF, particularly the NK and LAK functions, seem iron-independent, since the depletion of iron from LF with the chelator deferoxamine does not affect the cytotoxic augmentation capacity of LF. Discovery of specific LF receptors on macrophages, T and B-lymphocytes and leukemia cells establish the potential anti-tumor potential of LF.

LF SOURCE: QUALITY ASSURANCE

Oral administration of LF, and its role as a multifunctional system in the gastrointestinal tract, is clearly established in several in vivo studies. However, nutraceutical exploitation of LF as a prebiotic dietary supplement for human health application requires an innovative technology compatible with large-scale manufacturing practices. Such technology transfer should ensure the highest standards of product safety, quality assurance and delivery of an optimal dosage for an effective functional outcome. The following issues are critical for the development of LF as a prebiotic dietary supplement.

Microbiological quality

Contaminants in source material could compromise the human health applications of LF. Several factors including the origin of source material, protein separation and harvesting methods, manufacturing environment and storage conditions, all cumulatively contribute to the bioburden of LF protein. Accordingly, when used as a source material, whey or milk serum could carry through fermentative streptococci (Streptococcus thermophilus, in particular) and a medium with an acidic environment could selectively enrich several yeast and molds. Incidentally, these microbial populations are commonly known to proliferate and competitively limit the growth of several probiotics. LF derived directly from milk could minimize this problem; however, contamination of the milk pool (if any) from bovine mastitis source could introduce several Gram-positive cocci including Streptococcus uberis, Staphylococcus aureus and coagulase-negative staphylococci. On the other hand, environmental contaminants such as spore-forming Bacillus spp., Acinetobacter calcoaceticus, Klebsiella oxytoca, Pseudomonas spp., and coliforms including E. coli could gain entry into LF material through elution buffers, biofouled equipment, air ducts, etc. Similar, microbiological quality issues could exist for the GMO-derived and recombinant LF proteins from various expression systems such as rice, tobacco, yeast, cell cultures or transgenic animals. Therefore, elimination or significant reduction of such microbial contaminants is highly desirable for human health applications of commercial LF, in general, and for development of LF-based prebiotic dietary supplements.

Toxicological quality

The endotoxin content in the source material could adversely affect the prebiotic applications of LF. Lipopolysaccharides (LPS) in the Gram-negative bacterial OM typically consist of a hydrophobic domain known as lipid-A (or endotoxin), a non-repeating ‘core’ oligosaccharide, and a distal polysaccharide (or O-antigen). Endotoxins could stimulate the induction of cytokines and other mediators of inflammation, which in turn could trigger a broad range of adverse physiological responses. Gram-negative bacterial bioburden of milk or its derivatives in protein isolation, processing plant environment and conditions cumulatively contribute to endotoxin levels in an LF source material. Majde reviewed the potential reservoirs for endotoxin contamination during isolation of protein materials. Rylander has reviewed the occurrence of endotoxin levels in different environmental conditions and further pointed out the risks associated with non-bacterial endotoxins, particularly 1-3-B-glucan from mold cell walls. Thus, the microbiological keeping standards of chromatographic resins, sanitation practices of processing equipment, even more significantly the water quality used in LF isolation, could cumulatively contribute to the endotoxin levels of the isolated LF material and thereby could limit in vivo applications of commercial LF. Pre-existence of LF-endotoxin complexes reduce the potential of LF interaction with gut epithelia and diminish its ability to control intestinal influx of endotoxins.

Dosage

As a multifunctional protein, LF has a defining role in various physiological pathways; accordingly the bioactivity of LF is highly dependent on dosage and a compatible delivery system. In simple terms, regulatory proteins are like ‘traffic signals’, thus, at an optimal dose they work ‘positively’ in a ‘beneficial’ way by promoting a physiological function, while at other dosages (usually at high concentrations) could work ‘negatively’ with a ‘feedback inhibition’ by blocking body functions. In order to maintain an optimum body balance, LF is cleared by liver and spleen at a catabolic rate of 5.7 mg/day. LF dosage, therefore, is highly critical in the development of any dietary supplement formula. During such product design, estimation of average daily intake (ADI) values for LF in human dietary exposure plays a significant role. According to the United States Department of Agriculture (USDA) Continuing Survey of Food Intakes by Individuals (CSFII) data from 1994-96, the average intake of milk and milk products on both a gram per day (g/d) and gram per kilogram of body weight per day (g/kg body wt/d) are calculated. The CSFII 1994-96 data is based on dietary information from individuals of all ages. Considering that cow milk contains 0.1 to 0.2 mg/mL of LF, on an average, children 1 to 12 and teens 13 to 19 years consume about 396 and 377 g/d of milk, respectively. This is equivalent to 38 to 40 mg/d of LF. Adults (20+) consume less milk, i.e. ~240 g/d; their intake of LF is equal to about 24 mg/d. Thus, consumption of LF in the 90th percentile averages 73 mg/d for children, 75 mg/d for teens and 50 mg/d for adults.
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**Contamination monitoring of commercial LF**

The bioburden of different commercial LF preparations (whey-derived and milk-derived) was measured by standard assays according to the United States Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) Revision-A. The endotoxin contamination in commercial LF preparations was quantified by Limulus Ameobocyte Lysate (LAL) assay using a FDA approved QCL-1000 test kit (Cambrex Bioscience, Walkerville, MD). The analysis of APC (Efficacy of TCR process to reduce bioburden and neutralize endotoxins from commercial LF preparations (a schematic representation). Tier-1 consists of a surfactant phase to eliminate yeast, molds and Gram +ve bacterial contaminants; Tier-2 with antioxidant carbonium milieu removes the Gram -ve bioburden; and the final tier-3 polyphenolic phase effectively neutralizes the intrinsic endotoxin.

**Efficacy of TCR process**

The efficacy of TCR process to reduce bioburden and neutralize endotoxin from commercial LF was evaluated. Tests were performed with whey-derived LF with a high intrinsic load of microbial and endotoxin contaminants and the results were shown in Table 1. In the first tier, surfactant in the presence of LF has caused about 35% and 40% reduction in the APC and yeast/mold counts, respectively. However, in this tier the coliform counts and the endotoxin activity in LF remained unaffected. The surfactant and antioxidant combination with LF in the second tier has further resulted in >95% reduction in the APC and totally eliminated the coliforms as well as the yeast/mold populations. The endotoxin activity (measured as the function of TNF-α production) in LF was reduced by about 40%. In the final polyphenolic tier, the APC was markedly reduced by more than 99.9% and the endotoxin activity was greatly diminished almost to an undetectable level.

**Functional performance of LF-(TCR)**

LF-(TCR) is a result of an ultra-cleansing technology using all-natural food-grade decontaminants. The functional performance of LF-(TCR) has been evaluated with respect to its prebiotic effects on LAB, antioxidant activity and safety/toxicity by measuring apoptosis (if any) upon exposure to Caco2 cells. The functional activity of LF-(TCR) was compared with whey- and/or milk-derived LF preparations prior to their decontamination process.

**Prebiotic Activity**

The growth-multiplication of 18 different probiotic LAB (including 13 strains of Lactobacillus spp., 3 strains of Bifidobacterium spp., 1 strain each of Lactococcus spp., and Streptococcus spp.) was measured in the presence of LF-(TCR) and compared with untreated LF and control (without any LF exposure). Two different methods of bacterial growth measurements were used in evaluating the prebiotic activity of LF-(TCR).

**Method 1 - Growth impedance detection assay (GIDA):** Microbial metabolism causes electrical charge alterations in cultivation media due to breakdown of nutrients. A Bactometer® Microbial Monitoring System Model-128 (bioMerieux Vitek, Hazelwood, MO) was used to monitor the growth of probiotic LAB by measuring impedance signals (a function of both capacitance and conductance) in the cultivation media. GIDA was performed in 16-well modules; briefly, a volume of 0.5-mL double-strength Bactometer® broth (2x BB; general purpose culture medium for Bactometer®) was added to each well. A volume of 0.25-mL of LF-(TCR) sample followed by 0.25-mL of bacterial suspension (10² cells/mL) prepared in 0.9% saline was added to the wells.

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**Table 1. Efficacy of TCR process in ultra-cleansing of lactoferrin**

<table>
<thead>
<tr>
<th>Tier</th>
<th>TCR Process</th>
<th>CONTAMINANT ACTIVITY</th>
<th>LPS Activity (pp TNF-α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Starting quality</td>
<td>APC (CFU/g)</td>
<td>Coliforms (CFU/g)</td>
</tr>
<tr>
<td>I</td>
<td>Surfactant-phase</td>
<td>4650</td>
<td>92</td>
</tr>
<tr>
<td>II</td>
<td>Antioxidant-phase</td>
<td>260</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>Phytoenolic-phase</td>
<td>&lt;10</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure 4. Treatment for contaminant reduction (TCR) process utilizes a 3-tier ultra-cleansing system to eliminate bioburden and neutralize endotoxins from commercial LF preparations (a schematic representation). Tier-1 consists of a surfactant phase to eliminate yeast, molds and Gram +ve bacterial contaminants; Tier-2 with antioxidant carbonium milieu removes the Gram -ve bioburden; and the final tier-3 polyphenolic phase effectively neutralizes the intrinsic endotoxin.
Addition of 0.5-mL saline or bacterial suspension to module wells with 0.5-mL (2× BB) served as controls for sterility and growth, respectively. The inoculated modules (final volume: 1-mL) were incubated at 32°C, and impedance changes in the media was continuously monitored by the Bactometer at 6-min intervals for 48-h. Bacterial growth curves were graphically displayed as percent changes of impedance signals versus incubation time. The amount of time required to cause a series of significant deviation from baseline and impedance value was defined as the ‘detection time’ (DT). If the DT value of a test sample is lower than the control and test samples was considered to elicited a ‘prebiotic’ effect.

**Method 2 - Micro-scale optical density assay (MODA):**

This tubidometric assay has been used to measure microbial growth in vitro. The ability of LF-(T-GR) to inhibit microbial pathogens (‘antibiotic effect’) or to promote the growth of probiotic LAB (‘prebiotic effect’) can be measured by MODA. Briefly, 0.1 mL of sterile double strength (2×) deMann Rogosa Sharpe (MRS) broth was added to 96 wells of a sterile microtiter plate (Costar 3596, Corning, NY). A 0.05 mL volume of test solution was added to designated wells followed by inoculation with 0.05 mL microbial cell suspension containing ~10⁷ cells/mL (diluted from an optically pre-calibrated OD 1.0 at 600 nm) solution of 10⁸ cells/mL. After inoculation, the microplate was incubated at 37°C and the microbial growth was monitored at different time points as turbidity change in culture media by measuring OD at 600 nm using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA). Prior to OD measurement, contents of each microplate well was mixed for uniform suspension of microbial cells in the media. Wells containing broth without microbial inoculum served as the sterility control. Wells containing broth medium inoculated with bacteria, but without any test compound served as positive growth control. Based on the above working controls of sterility as well as growth, when a test strain proliferated typically under defined conditions of inoculation and incubation, the turbidity (OD) changes in the microbial growth media measured at 600 nm with the following criteria for MODA: * ‘Prebiotic effect’ is when an agent has enhanced the microbial proliferation compared to the growth control.

LF-(T-GR) was tested at 0.5% (w/v) concentration for prebiotic effect and compared with its milk-derived original source of LF. The average GIDA detection time for probiotic LAB test strains (n=18) was estimated at 15.7-h. This detection time was shortened by 4.5-h by LF-(T-GR) in comparison to 2.2-h by its original source of LF. According to MODA, the growth multiplication of probiotic LAB test strains (n=18) was enhanced by >100% with LF-(T-GR), which was at least twice as effective as the probiotic ability of LF (prebiotic counterpart), i.e. ~40% growth enhancement. Data enlisted in Table 2 with different LAB strains clearly indicate that LF-(T-GR) is a powerful prebiotic agent.

### Table 2. Prebiotic effects of lactoferrin-(T-GR)

<table>
<thead>
<tr>
<th>Probiotic (LAB) strain</th>
<th>GIDA - Detection Time (h)</th>
<th>MODA - Growth (%)</th>
<th>LF</th>
<th>LF-(T-GR)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bifidobacterium bifidum ATCC15696</strong></td>
<td>19.5</td>
<td>17.8</td>
<td>15.1</td>
<td>142%</td>
</tr>
<tr>
<td><strong>Bifidobacterium infantis ATCC15987</strong></td>
<td>21.3</td>
<td>19.4</td>
<td>17.2</td>
<td>149%</td>
</tr>
<tr>
<td><strong>Lactobacillus acidophilus ATCC84356</strong></td>
<td>12.6</td>
<td>11.0</td>
<td>9.4</td>
<td>148%</td>
</tr>
<tr>
<td><strong>Lactobacillus amylovorus ATCC83520</strong></td>
<td>17.9</td>
<td>17.0</td>
<td>15.1</td>
<td>148%</td>
</tr>
<tr>
<td><strong>Lactobacillus brevis ATCC14869</strong></td>
<td>18.2</td>
<td>15.2</td>
<td>14.0</td>
<td>129%</td>
</tr>
<tr>
<td><strong>Lactobacillus casei ATCC8806</strong></td>
<td>13.5</td>
<td>11.0</td>
<td>9.0</td>
<td>100%</td>
</tr>
<tr>
<td><strong>Lactobacillus crispatus ATCC33820</strong></td>
<td>14.0</td>
<td>13.1</td>
<td>10.4</td>
<td>118%</td>
</tr>
<tr>
<td><strong>Lactobacillus delbrueckii ATCC12315</strong></td>
<td>15.6</td>
<td>12.9</td>
<td>9.8</td>
<td>152%</td>
</tr>
<tr>
<td><strong>Lactobacillus fermentum ATCC14931</strong></td>
<td>16.2</td>
<td>14.0</td>
<td>11.2</td>
<td>150%</td>
</tr>
<tr>
<td><strong>Lactobacillus helveticus ATCC156009</strong></td>
<td>16.2</td>
<td>14.0</td>
<td>11.0</td>
<td>149%</td>
</tr>
<tr>
<td><strong>Lactobacillus paracasei ATCC25002</strong></td>
<td>14.0</td>
<td>10.8</td>
<td>9.9</td>
<td>163%</td>
</tr>
<tr>
<td><strong>Lactobacillus pentosus ATCC8041</strong></td>
<td>15.4</td>
<td>12.0</td>
<td>10.1</td>
<td>169%</td>
</tr>
<tr>
<td><strong>Lactobacillus plantarum ATCC14917</strong></td>
<td>15.5</td>
<td>12.0</td>
<td>10.1</td>
<td>171%</td>
</tr>
<tr>
<td><strong>Lactobacillus reuteri ATCC23272</strong></td>
<td>16.4</td>
<td>12.0</td>
<td>9.8</td>
<td>167%</td>
</tr>
<tr>
<td><strong>Lactobacillus rhamnosus ATCC74491</strong></td>
<td>13.6</td>
<td>11.7</td>
<td>9.0</td>
<td>131%</td>
</tr>
</tbody>
</table>

* Control (LAB inoculum in broth media without any LF) growth is considered as 100%.

### Figure 5. Antioxidant activity of LF-(T-GR) as measured by FRAP kinetic assay. LF-(T-GR) demonstrated a multi-fold superior antioxidant activity compared to its original source, the milk-LF.

![Figure 5](image_url)

tested a 0.1 mM concentration milk-LF showed an antioxidant activity (FRAP units) with an initial value of 60 with a gradual rise to 260 in 6-h and reached 583 in 24-h. Under similar test conditions, a 0.1 mM concentration LF-(T-GR) demonstrated an enhanced antioxidant activity (FRAP units) starting from 192 (3.2 x times higher than milk-LF) with an elevated value of 660 (2.5x times higher than milk-LF) in 6-h and peaked to 994 (1.7x times higher than milk-LF) in 24-h.

### Effects on Eucaryotic Cell Apoptosis

Effects of LF-(T-GR) on Caco2 (colon carcinoma cell line) undergoing apoptosis was tested and compared with the whey- and milk-derived LF proteins. The cell line was grown in Eagle’s minimal essential medium (EMEM supplemented with 1% non-essential amino acids and 10% fetal calf serum) in an 8-well tissue culture plate, for 72-h in a CO₂ incubator. After partial monolayers were obtained, each plate was washed twice with phosphate buffered saline (PBS, pH 7.2). A 2-mL volume of
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mounted in Vectashield® chambers were removed and slides were dark. After washing with PBS, cell 2% paraformaldehyde in PBS for 15 min at room temperature in the dark. Cells were washed with PBS, and fixed in Annexin V (Cell Signaling Inc., Beverly, MA) for 15 min at room 
three times and further incubated with binding buffer containing 5- µL of Annexin V (Cell Signaling Inc., Beverly, MA) for 15 min at room temperature in the dark. After washing with PBS, cell chambers were removed and slides were mounted in Vectashield® anti-fade solution containing 4', 6-diamidino-2-phenylindole (DAPI). Cells were viewed under Leica (Wetzlar, Germany) DMRA microscope with Plan-achromat x40/1.25 NA and x63/1.40 NA oil immersion objective lenses. Images were acquired with a SkyVision-2/VD5 digital CCD (12-bit, 1280 x 1024 pixel) camera in unbinned or 2 x 2-binned models into EasyFSHW software, saved as 16-bit monochrome, and merged as.

24-bit RGB TIFF images (Applied Spectral Imaging Inc., Carlsbad, CA). The images were processed using Adobe Photoshop 6.0. Cells positive to Annexin V staining indicated apoptosis. The number of positive cells was determined per a given field with various treatments and expressed as percentage of dead cells compared to untreated (control) cells.

In the presence of LF-TCR, Caco2 cells showed <1% apoptosis after 24-h incubation. Under similar tissue culture conditions, milk-derived LF and whey-derived LF elicited 35% and 53% apoptosis, respectively. Finally, the untreated Caco2 (control) cells demonstrated 5% apoptosis after 24 h. The results are depicted in FIGURE-6. These data indicated that LF-TCR protects intestinal cells from apoptosis, whereas commercial LF preparations (containing contaminants) could elicit cytotoxicity against Caco2 cells.

**CONCLUSION**

Several LF-based dietary supplements are currently available in health food markets worldwide. A majority of such products are derived from partially isolated (enriched) LF fractions from colostrum or whey concentrates. Bulk isolation of LF directly from milk is limited and relatively an expensive process. High purity LF preparations commercially exist; however, products from such protein materials are cost-prohibitive and fall short of consumer acceptance without a valid functional assurance. Furthermore, the microbiological and toxicological quality issues compromise the in vivo performance standards of LF as a potent dietary supplement. To circumvent these issues, LF-TCR has been developed using a novel decontamination technology consisting of food-grade surfactants, antioxidants, and polyphenols. The compounds utilized in the multi-tier TCR process are also known to provide additional nutraceutical benefits [48-50]. The multi-functional in vitro performance of LF-TCR is summarized in TABLE-3. In conclusion, LF-TCR is a functionally enhanced prebiotic protein produced by an innovative process engineering technology.

**Table 3. Enhanced multi-functional performance of LF-TCR**

<table>
<thead>
<tr>
<th>FUNCTIONAL ACTIVITY</th>
<th>LF (whey)</th>
<th>LF (milk)</th>
<th>LF-(TCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ANTIOXIDANT ACTIVITY</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Antioxidant Status</td>
<td>&lt;1.0 µmol/mg</td>
<td>5.3 µmol/mg</td>
<td>72.9 µmol/mg</td>
</tr>
<tr>
<td>FRAP Value (at 24-h)</td>
<td>NT</td>
<td>583 mM</td>
<td>994 mM</td>
</tr>
<tr>
<td><strong>BACTERIOSTASIS ACTIVITY</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>15.4-h</td>
<td>26.2-h</td>
<td>&gt;48-h</td>
</tr>
<tr>
<td>Salmonella Typhimurium</td>
<td>12.5-h</td>
<td>22.9-h</td>
<td>&gt;48-h</td>
</tr>
<tr>
<td><strong>PREBIOTIC ACTIVITY</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillus spp. (n=13)</td>
<td>96%</td>
<td>147%</td>
<td>200%</td>
</tr>
<tr>
<td>Bifidobacterium spp. (n=3)</td>
<td>110%</td>
<td>146%</td>
<td>213%</td>
</tr>
<tr>
<td>Lactococcus lactis</td>
<td>105%</td>
<td>120%</td>
<td>186%</td>
</tr>
<tr>
<td>Streptococcus thermophilus</td>
<td>92%</td>
<td>116%</td>
<td>192%</td>
</tr>
</tbody>
</table>

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42. Naidu AS, Bidlack WR, Crecelius AT (2000) Flavonoids, In ‘Outstanding Researcher 1992’. He has authored over 100 publications; written chapters in over 30 reference volumes; received numerous national and international awards/recognitions including the ‘Young European Scientist 1989’ and ‘Outstanding Researcher 1992’. He is internationally acclaimed for ‘Lactoferrin’ and ‘Probiotic’ technology transfers and co-founder of four start-up ‘biotech’ companies in the USA. An elected fellow of several international societies such as the ISSVD, the Linnean Society of London and the Royal Society of Medicine; a member of 18 professional societies; and a scientific advisor to 14 multinational companies; Dr.Naidu is recipient of many prestigious international awards/recognition including the ‘Young European Scientist 1989’ and ‘Outstanding Researcher 1992’. He has authored over 100 publications; written chapters in over 30 reference volumes; and edited several books including the ‘Natural Food Antimicrobial Systems’ published by the CRC Press. He received Ph.D. in Medicine (1984) from the Osmania University, Hyderabad, India.
Lactoferrin Interaction with Salmonellae Potentiates Antibiotic Susceptibility in vitro

A. Satyanarayan Naidu and Roland R. Arnold

Interaction of lactoferrin (Lf) with the cell envelope (CE) and outer membrane (OM) of Salmonella typhimurium-type strain ATCC13311 was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western-blot analyses. The peroxidase-labeled bovine Lf (BLf) and human Lf both recognized a heat-modifiable protein with an estimated molecular mass of 38 kD in the OM. Simultaneous immunoblotting with an antiporin monoclonal antibody specific for a conserved porin domain in members of enterobacteriaceae confirmed that the Lf-binding protein is a porin. Such Lf-binding porin proteins (37–39 kD range) were readily detected in nine other common Salmonella species: S. dublin, S. panama, S. rostocc, S. abony, S. hartford, S. kentucky, S. pullorum, S. thompson, and S. virchow. The latter six species also demonstrated one to three weak Lf-reactive bands of low molecular weight in their CE. The antibiotic susceptibility of Salmonella in the presence of Lf was examined. A mixture containing sub-minimum inhibitory concentration (MIC) levels of Lf (MIC/4) and cefuroxime (MIC/2) inhibited the bacterial growth. Lf strongly potentiated the action of erythromycin (eightfold), whereas it increased the activity only by two-fold for ampicillin, ciprofloxacin, chloramphenicol, and rifampicin; similarly, these antibiotics also reduced the MIC of BLf by twofold in S. typhimurium. Such antimicrobial potentiation was not observed with BLf mixtures containing cefalexin, gentamycin, or polymyxin B against strain ATCC13311. BLf and cefuroxime also demonstrated potentiation of varying degrees (two to 16-fold) with nine other Salmonella species. These data established the binding of Lf to porins in salmonellae and a potentiation effect of Lf with certain antibiotics.

INTRODUCTION

Lactoferrin (Lf) is an iron-binding glycoprotein found in many exocrine secretions that bathe the mammalian mucosal surface (Masson et al., 1966). Lf may elicit bacteriostatic, bactericidal, and opsonic effects from susceptible microorganisms (Arnold et al., 1977; Lima and Kierszenbaum, 1985; Oram and Reiter, 1968; Rainard, 1986), and various mechanisms were proposed for such activities (Arnold et al., 1981 and 1982; Griffiths and Humphreys, 1977; Reiter et al., 1975; Stuart et al., 1984). The adsorption of Lf to a target organism seems to potentiate antimicrobial effects (Arnold et al., 1977; Naidu et al., 1993). Recent investigations revealed the involvement of specific proteins on microbial cell surfaces in Lf binding. From our laboratory, we demonstrated porins to be the major Lf-binding components in Escherichia coli, Shigella flexneri, and Aeromonas hydrophila (Erede et al., 1994; Gado et al., 1991; Kishore et al., 1991; Naidu et al., 1993; Tigy et al., 1992). These pore-forming proteins are a class of well-conserved molecules in members of enterobacteriaceae and other Gram-negative bacteria (Benz, 1988; Lugtenberg and van Alphen, 1983). Porins form diffusion channels and contribute to the barrier function of outer membrane (OM) against certain nutrients and antibiotics, and also serve as targets for certain bacteriocins and bacteriophages (Nikaido and Vaara, 1985).

Salmonellae are members of the enterobacteriaceae family and frequently cause gastrointestinal infections in humans and animals (Giannella, 1975). One of the major problems in the control of salmonellosis is multiple drug resistance of the bacterium (Cohen and Tauxe, 1986). Alteration or blockade of antibiotic diffusion through the bacterial OM is one
of the antibiotic resistance mechanisms (Nikaido, 1989). In *S. typhimurium*, the two major porins OmpF and OmpC are suggested to play a role in the penetration of certain β-lactams, quinolones, and aminoglycosides across the OM (Hirai et al., 1986; Jaffe et al., 1982; Nakae and Nakae, 1982). Synthetic chelators such as ethylenediaminetetraacetic acid (EDTA) are known to enhance the membrane diffusion of certain antibiotics (Mochizuki et al., 1988).

In this study, we established the following goals: a) to confirm porin involvement in Lf binding to salmonellae by simultaneously probing with a monoclonal antibody specific for a conserved porin domain (Pol) in different bacterial species of enterobacteriaceae (Henrikson and Maeland, 1991), and b) to examine the effect of Lf interaction on the susceptibility of selected *Salmonella* species to antibiotics.

**MATERIALS AND METHODS**

**Bacteria**

The *S. typhimurium*-type strain (ATCC13311) was obtained from the Culture Collection of the University of Göteborg [(CCUG), Sweden]. Nine other strains of *Salmonella* species (see Table 2) were kindly provided by Drs. E. Czirók and B. Lánya [Hungarian National Collection of Medical Bacteria (HNCMB), National Institute of Hygiene, Budapest, Hungary]. Bacteria were grown in special peptone yeast extract (SPYE) broth (Malthus Ltd., West Sussex, England) at 37°C for antimicrobial susceptibility tests.

**Antimicrobial Agents**

Human Lf (HLf; lot 63541) and bovine Lf (BLf; lot 62696) were purchased from US Biochemicals (Cleveland, OH, USA). Various antibiotics in this study were obtained from the following sources: ampicillin (lot RF665; Astra Läkemedel, Södertälje, Sweden), cefalexin (lot S1899M; Eli Lilly and Co., Indianapolis, IN, USA), polymyxin B sulfate (lot 41H0033; Sigma Chemical Company, St. Louis, MO, USA), chloramphenicol (lot 129C0128; Sigma), erythromycin (lot 74.056VC01; Abbott Scandinavia, Stockholm, Sweden), ciprofloxacin (lot 520124; Bayer Leverkusen, Germany), cefuroxime (CXM, lot B7526GC; Glaxo Operations UK, Poole, England), gentamycin (lot 91C1502; Schering Corporation USA, Kenilworth, NJ, USA), and rifampicin (AB Leo, Helisingborg, Sweden).

**Isolation of Bacterial Cell Envelope and Outer Membrane**

The cell envelope (CE) and OM of 10 selected species of *Salmonella* reference strains were isolated by detergent extraction (Schnaitman, 1971). Briefly, bacteria grown in nutrient broth (Oxoid, Basingstokes, England) were harvested at late exponential phase, washed three times in 20 mM Tris-buffered saline, pH 7.4, and were resuspended in 20 mM Tris-HCl, pH 7.4, containing 2 mM EDTA and 1 mM phenylmethylsulfonylfluoride. After sonic disruption three times for 2 min each under cooling, unbroken cells were removed by centrifugation (5300 g for 15 min). The supernate was recentrifuged (100,000 g for 1 h), and a pellet containing CE was obtained. After additional washing in the buffer, CE was solubilized in 50 mM Tris-HCl buffer, pH 7.4 (with 10 mM EDTA and 10 mM N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid) containing 1% sarkosyl or 2% sodium dodecyl sulfate (SDS) at room temperature for 1 h, with an end-to-end rotation. The insoluble fraction containing OM was recovered by centrifugation (100,000 g for 30 min).

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Blotting with Peroxidase-Labeled Lactoferrin**

The CE and OM preparations were mixed with a sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue) and were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed in 1-mm-thick gels at 33 mA constant current for 70 min, in a discontinuous buffer system (Laemmli, 1970), using a Miniprotein II apparatus (Bio-Rad, Richmond, VA, USA). The samples were either boiled for 10 min or run without boiling. The stacking and separating gels contained 4% and 12.5% acrylamide, respectively. A volume of 20-µl sample containing 10-20 µg of protein was applied to each lane. Molecular weight standards (Pharmacia AB, Uppsala, Sweden) were used according to the manufacturer’s instructions. After electrophoresis, proteins in the gel were transferred to a nitrocellulose membrane (Sartorius, Göttingen, FRG) at 0.8 mA/cm² for 1 h, using Transblot-cell equipment (Bio-Rad). After we blocked the free sites with 1% Tween 20 for 15 min, the OM was probed with peroxidase-labeled HLf or BLf. A color reaction was developed with diaminobenzidine (0.025% wt/vol; Sigma) dissolved in 0.1 M sodium acetate buffer, pH 5.0, containing hydrogen peroxide (3 × 10⁻⁴% vol/vol), and the reaction was terminated by adding 5% sodium pyrosulfite (1%
wt/vol). Preincubation of blots with homologous unlabeled Lf abolished the peroxidase-labeled Lf interaction, excluding a possible direct binding of peroxidase to bacterial components.

**Immunoblotting With Antiporin Monoclonal Probe**

Monoclonal antibodies against a conserved Pol common to 10 different genera of the enterobacteriaceae, including salmonellae, were kindly provided by Prof. J.A. Mæland (Department of Microbiology, University of Trondheim, Norway). The boiled and unboiled CE preparations from *Salmonella* were run on SDS-PAGE and the proteins in the gel were transblotted to a nitrocellulose membrane as described. Free sites on the membrane were blocked for 30 min with 0.5 M saline containing 1% ovalbumin. Anti-Pol antibody (mAb F9-16) was mixed with the blocking solution at a working dilution of 1:5000, and incubated. After thorough washing with Tris-buffered saline, the membrane was incubated with peroxidase-labeled rabbit antibodies to mouse immunoglobulins (lot 059; Dakopatts A/S, Glostrup, Denmark) with gentle shaking at room temperature for 1 h. After a final washing step the membrane was developed with diaminobenzidine chromophore, as described. Strips incubated only with labeled second antibody were included as controls.

**Susceptibility Testing**

A serial twofold dilution of antibiotics and BLf were prepared in SPYE broth, and the minimum inhibitory concentration (MIC) values for both agents alone or in combination were determined by a checkerboard assay using flat-bottomed, sterile, 96-well microtiter plates (Nunc, Roskilde, Denmark). Each antibiotic dilution (in 50-μl vol) was serially added to wells in vertical rows starting from the left (lowest dilution) to the right side. Serial dilutions of BLf (in 50-μl vol) were added to wells in horizontal rows starting from the bottom (lowest dilution) to the top side. Finally, 50 μl of SPYE broth-grown bacteria (10^6 exponential-phase cells/ml optically adjusted to 600 nm with sterile SPYE broth) was added to each well. The final twofold dilutions for antibiotics and BLf were in the range of 25 μg to 0.1 ng and 800–12.5 μg, respectively, in a final volume of 150 μl/well. Plates were kept under constant shaking (Microshaker, Dynatech, England) at 37°C. At the time point when control cells reached stationary phase, the bacterial growth was measured optically at 540 nm using a Reader Microelisa system (Organon Technika, NV, Turnhout, Belgium). MIC determinations (highest dilution causing a complete inhibition of bacterial growth) were made at this time point, and also after a 24-h incubation. Each experiment was repeated at least five times.

**RESULTS**

**Demonstration of Porins as Lactoferrin-Binding Components**

The CE and OM of *S. typhimurium*-type strain ATCC13311 were examined for the presence of Lf-binding components by Western-blot analysis using horseradish peroxidase-labeled Lf (Figure 1). The boiled CE showed many protein bands in the electrophoresis; however, the corresponding blot indicated only a single, distinct, Lf-reactive band with an estimated molecular mass of 38 kD. This 38-kD protein band was absent in the native (unboiled) CE in SDS-PAGE, and its corresponding blot did not react with the peroxidase-labeled Lf. The Lf-binding protein was also found in the boiled, but not in the unboiled, preparations of sarkosyl-extracted OM and in the porin-enriched SDS-extracted OM. Finally, immunoblotting of CE proteins with anti-Pol monoclonal antibody showed a porin-reactive band at 38 kD only in the boiled preparation.

The presence of Lf-binding proteins was further investigated in nine species of *Salmonella* (Figure 2). The CE preparations of all nine reference strains showed Lf-reactive components as heat-modifiable OM proteins in the Western-blot. Three of the species showed a single Lf-reactive band, and six other species exhibited more than one Lf-reactive band (one major and one to three minor bands) in their CE. The molecular masses slightly differed among the major Lf-binding proteins; thus, a 38-kD protein was detected among six species: *S. abony, S. dublin, S. pullorum, S. kentucky, S. thompson,* and *S. virchow;* a 37-kD protein was found in *S. hartford* and *S. panama;* and a 39-kD protein was noticed only in *S. rostock.* These heat-modifiable, Lf-binding proteins were reactive against the anti-Pol monoclonal in immunoblotting (not shown) as demonstrated for *S. typhimurium* in Figure 1.

**Lactoferrin Effects on Antibiotic Susceptibility**

The growth of *S. typhimurium*-type strain ATCC13311 in the presence of BLf and/or cefuroxime was examined, and the MIC for BLf and cefuroxime was estimated to be 200 and 0.05 μg, respectively. BLf and cefuroxime in combination showed a potentiation and elicited an antimicrobial effect at concentrations lower than their individual MIC. The
antibacterial action of Blf was lower than their individual MIC. The antibacterial action of Blf was potentiated by fourfold (50 μg, MIC/4) and that of cefuroxime by twofold (0.025 μg, MIC/2). The bacterial growth kinetics was measured at these concentrations (Figure 3). The growth in control (without Blf or cefuroxime) reached stationary phase in 8 h. A similar growth pattern was obtained for Blf at MIC/4 concentration, and also a comparable curve with a slightly retarded multiplication was observed for cefuroxime at MIC/2 concentration. On the other hand, bacterial growth was completely inhibited by the mixture containing these concentrations of antimicrobial agents at 8-h (control growth at stationary phase) or 24-h incubation.

The inhibitory effect of mixtures containing Blf and various antibiotics on the growth of S. typhimurium strain ATCC13311 was examined (Table 1). Blf strongly potentiated the action of erythromycin (eightfold) and increased the activity only by twofold for ampicillin, ciprofloxacin, chloramphenicol, and rifampin; similarly, these antibiotics reduced the MIC of Blf by twofold. Such antimicrobial potentiation was not observed with Blf mixtures containing cefalexin, gentamycin, or polymyxin B against strain ATCC13311.

The inhibitory effect of Blf, cefuroxime, and their combination on the growth of nine Salmonella species was tested (Table 2). Both antimicrobial agents potentiated the inhibition; accordingly, the MIC of Blf and cefuroxime in the mixture was much lower (two- to 16-fold) than the individual MIC values. Notably, strains S. abony, S. virchow, and S. hartford were resistant to Blf (MIC >800 μg); however, in the presence of cefuroxime, bacteria demonstrated an increased susceptibility to Blf at above 16-fold, eightfold, and fourfold, respectively. On the other hand, Blf also potentiated the antibacterial action of cefuroxime eightfold against S. abony and S. virchow, and by fourfold against S. hartford.

DISCUSSION

The molecular mass and heat-modifiable and immunoreactive properties suggested that the Lf-binding proteins in the OM of strain ATCC13311 were porins. Lf-binding proteins with similar characteristics were also identified in nine different species of Salmonella reference strains. These data further confirmed our earlier report on the porin involvement in specific interaction of Lf with bacterial members of the enterobacteriaceae family (Erdei et al., 1994; Tigyi et al., 1992).

The size-exclusion property of porins contributes to a barrier function in the OM of the Gram-negative bacteria, against various hydrophilic antibiotics. On the other hand, the diffusion of many hydrophobic antibiotics through the OM could be mechanically prevented by the polysaccharide moiety of LPS (Bryan, 1984; Hancock, 1984; Nikaido, 1989). Specific mutations that either alter the porin content or shorten the polysaccharide moiety in lipopolysaccharides may breach these OM barrier mechanisms (Lugtenberg and van Alphen, 1983). Our observation that Lf binds to porins suggested that this specific mechanism of interaction might influence the antibiotic susceptibility in Salmonella.
Cefuroxime, a monoanionic β-lactam with a low diffusion rate through the porin (OmpF) channel (Yoshimura and Nikaido, 1985), was examined for BLF-induced antibiotic potentiation. BLF and cefuroxime demonstrated enhanced antimicrobial activity; thus the BLF susceptibility was potentiated by two- to 16-fold, whereas the antibiotic susceptibility was enhanced by two- to eightfold, in S. typhimurium and nine other species of Salmonella. Among the zwiterionic β-lactams, the diffusion rate of ampicillin through the OmpF porin was threefold lower than cephalaxin. BLF potentiated only the former antibiotic by twofold. Ciprofloxacin can cross the OM through OmpF channel (Hirai et al., 1986), and BLF caused a twofold increase in the antimicrobial activity of this hydrophilic quinolone. Rifampicin and erythromycin cross the OM by a hydrophobic route (Bryan, 1984), and the activity of these antibiotics was also potentiated by BLF. Finally, the spectrum of aminoglycoside gentamycin was unaffected by BLF. Therefore, regardless of the route of entry or hydrophobicity of the antibiotic, BLF demonstrated increased antimicrobial activity. Possibly, the interaction of LF might have altered the porin channel and contributed to an increased diffusion of antibiotics that cross the OM via this pathway.

The antibiotic-induced BLF potentiation in Salmonella raises new questions on the mechanisms of LF action. Accumulating evidence indicates that LF might affect certain cellular metabolic pathways, involving mechanisms that are independent of the iron deprivation of bacteria (Arnold et al., 1982; Peterson and Alderete, 1984). Recently, we have demonstrated a correlation between the magnitude of cellular LF binding and the alteration of metabolic rate in E. coli (Naidu et al., 1993). Whether the antibiotic has exposed other cellular targets than porins for LF activity and caused such an inhibitory potentiation is currently under investigation.

In conclusion, we have demonstrated the interaction of LF with outer-membrane porins in S. typhimurium and nine other Salmonella species. LF enhanced the susceptibility of bacteria for both types of antibiotics that cross OM either by a porin pathway or via a hydrophobic route. The affected antibiotics increased the antibacterial spectrum of BLF in an additive manner.

This work was supported by US Public Health Service Grant NIH RO1-DE06869.
TABLE 1  Minimum Inhibitory Concentration Determinations for Antibiotics in the Absence or Presence of Bovine Lactoferrin Against Salmonella typhimurium Strain ATCC13311

<table>
<thead>
<tr>
<th>Antibiotic (µg MIC)</th>
<th>MIC of the Combination (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antibiotic (fold)*</td>
</tr>
<tr>
<td>Cefuroxime (0.05)</td>
<td>0.025 (2)</td>
</tr>
<tr>
<td>Ampicillin (0.025)</td>
<td>0.0125 (2)</td>
</tr>
<tr>
<td>Cefalexin (0.4)</td>
<td>0.4 (0)</td>
</tr>
<tr>
<td>Ciprofloxacin (0.003)</td>
<td>0.0015 (2)</td>
</tr>
<tr>
<td>Gentamycin (0.2)</td>
<td>0.2 (0)</td>
</tr>
<tr>
<td>Erythromycin (3.2)</td>
<td>0.4 (8)</td>
</tr>
<tr>
<td>Chloramphenicol (0.1)</td>
<td>0.05 (2)</td>
</tr>
<tr>
<td>Rifampicin (0.8)</td>
<td>0.4 (2)</td>
</tr>
<tr>
<td>Polymyxin B (0.05)</td>
<td>0.05 (0)</td>
</tr>
</tbody>
</table>

*Decrease in the minimum inhibitory concentration (MIC) (or the potentiation effect) is shown in parenthesis in twofold multiples. The MIC of bovine lactoferrin (BLf) was estimated to be 200 µg (see Figure 3).

TABLE 2  Minimum Inhibitory Concentration Determinations for Bovine Lactoferrin, Cefuroxime, and the Combination Against Nine Reference Strains of Salmonella Species

<table>
<thead>
<tr>
<th>Salmonella Species</th>
<th>MIC of BLf (µg)</th>
<th>MIC of CXM (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alone</td>
<td>+ CXM (fold)*</td>
</tr>
<tr>
<td>S. abortus (NCTC6017)</td>
<td>&gt;800</td>
<td>50 (&gt;16)</td>
</tr>
<tr>
<td>S. dublin (NCTC9676)</td>
<td>200</td>
<td>100 (2)</td>
</tr>
<tr>
<td>S. hartford (HNCBH10063)</td>
<td>&gt;800</td>
<td>200 (&gt;4)</td>
</tr>
<tr>
<td>S. kentucky (NCTC5799)</td>
<td>200</td>
<td>25 (8)</td>
</tr>
<tr>
<td>S. panama (NCTC5774)</td>
<td>100</td>
<td>50 (2)</td>
</tr>
<tr>
<td>S. pullorum (NCTC5776)</td>
<td>200</td>
<td>100 (2)</td>
</tr>
<tr>
<td>S. rostoch (NCTC5767)</td>
<td>200</td>
<td>50 (4)</td>
</tr>
<tr>
<td>S. thompson (NCTC5740)</td>
<td>100</td>
<td>50 (2)</td>
</tr>
<tr>
<td>S. virchow (NCTC5742)</td>
<td>&gt;800</td>
<td>100 (&gt;8)</td>
</tr>
</tbody>
</table>

*Decrease in the minimum inhibitory concentration (MIC) (or the potentiation effect) is shown in parenthesis in twofold multiples. BLf, bovine lactoferrin; and CXM, cefuroxime.

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Lactoferrin

Interactions and Biological Functions

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Chapter 17

Influence of Lactoferrin on Host–Microbe Interactions

A. Satyanarayan Naidu and Roland R. Arnold

Summary

The antimicrobial spectrum of lactoferrin (Lf) includes statistic, cidal, cationic, phagocytic, and colonization/decolonization effects on susceptible microorganisms. The selective interaction of Lf with microbial surface seems to play an essential role in regulating many of these events. Lf bound to specific outer membrane (OM) pore-forming proteins (porins) of various enteric bacteria. The magnitude of Lf–microbe interaction showed a direct relationship with the bacterial susceptibility to Lf. Although most bacteria expressed porins, certain strains showed resistance to Lf effects and did not demonstrate specific Lf binding. This Lf “resistance” was attributed to the shielding of porin accessibility by the carbonylate O-antigenic chains of lipopolysaccharide (LPS). Mutants with progressive deletions in O-side-chain and core polysaccharide demonstrated increased Lf binding and progressive susceptibility to Lf effects. Lf interaction with bacterial surface affected the porin channels in the OM and potentiated the diffusion of antibiotics. Lf also affected the intestinal colonization of Escherichia coli. Lf inhibited the expression of various bacterial fimbriae and also blocked bacterial interaction with host subepithelial matrix components, such as fibronectin, fibrinogen, various collagens, and laminin. Our studies suggested that specific interaction with cell target involved

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the anion-binding ligands of Lf, followed by coordinate binding and oxidation of Fe$^{2+}$ to Fe$^{3+}$ on bacterial surface. This metal transition in the presence of suitable oxygen species seems to fulfill the necessary components of Haber-Weiss reaction and promote a targeted radical generation, with iron (Fe) serving as the Lf prosthetic group on the bacterial surface.

**Introduction**

Mucosal surface is the primary target for many infections. A variety of antimicrobial systems in mucosal secretions establish a potent innate host defense against invading pathogens, among which the Lf system contributes a wide spectrum of antimicrobial activities with a role in various cellular pathways of antigen recognition and processing. The high concentrations of Lf in colostrum and milk (2–7 mg/mL) has been suggested to account for the protection of breast-fed infants against intestinal illnesses (Bullen et al., 1972).

In the human body, Lf occurs in two major reservoirs, a stationary pool covered on the mucosal surfaces and a circulatory pool stored in the polymorphonuclear leukocytes (PMNLs). Lf accounts for ~11.5% of the total secretory proteins of the bronchial glands. In human tears, Lf is synthesized in the lacrimal glands (1–3 mg/mL). In the male reproductive tract, Lf is found in the prostate, seminal vesicles, and seminal plasma (0.2–1.0 mg/mL). In the female reproductive tract, Lf has been detected in the cervical mucus and endometrium of the secretory uterus. In the PMNLs, Lf is associated with the secondary (specific) granules (15 μg/10$^6$ cells) and is released isochromously with other lysosomal proteins into the plasma during phagocytosis. During inflammation and toxemia, interleukin 1 (IL-1) may mediate multiple aspects of acute-phase response and induce the exocytosis of PMNLs to release intracellular granule contents, including Lf. Thus, the plasma Lf levels significantly rise in various pathological conditions (van Snick et al., 1974; Gutfreund et al., 1988). Lf has an important role in various cellular pathways of antigen recognition, attenuation, and processing. During this cascade of events, the interactions of Lf with specific targets could be decisive in the microbial pathogenesis.
Lf-Microbe Interaction (Microbial Advantage)

**Fe Acquisition**

Certain microorganisms produce siderophores to compete with Lf and other Fe-binding compounds. These bacteria subsequently acquire the siderophore-bound Fe through a receptor-mediated pathway. Fe is acquired in most *E. coli* through the production of enterochelin and/or aerobactin, siderophores that demonstrate higher affinity for Fe than Lf or transferrin (Brock et al., 1983). On the other hand, human pathogens, such as Neisseria, do not produce siderophores to facilitate Fe acquisition, but these bacteria are capable of utilizing Fe from human Lf. Specific Lf-binding proteins and the importance of these receptors in Fe scavenging have been reported (Schryvers and Morris, 1988).

**Bacterial Adhesion**

Specific Lf-binding receptors are reported on the human brush border epithelia, with a suggested role in intestinal Fe regulation (Cox et al., 1979; Davidson and Lönnerrdal, 1988). The brush border also serves as a target site for colonization of *E. coli*, as well as many other intestinal commensals and pathogens. Thus, the Lf interaction may result in the bridging of certain microorganisms with the intestinal mucosa favoring microbial attachment and colonization.

**Shielding from Host Recognition**

As the sperm-coating antigen, Lf has been suggested to mask the spermatozoa as a foreign antigen in the female reproductive tract during fertilization (Broer et al., 1977). Recent studies have also suggested a homology between Lf and major histocompatibility complex (MHC) antigen. In a similar fashion, intracellular pathogens, such as Mycobacteria and *Listeria monocytogenes*, may utilize Lf interaction for antigen masking to accomplish an effective dormancy and invasion of macrophages.

Lf-Microbe Interaction (Host Advantage)

**Microbial Elimination**

Iron sequestration by Lf prevents or suppresses microbial growth in mucosal secretions and tissue fluids. This nutritional immunity is favored at alkaline pH conditions. Lf could also
extinguish the susceptible microbes by oxidative killing mechanisms, both extracellular (i.e., periodontal pockets) and intracellular (i.e., PMNLs, macrophages) milieu under acidic conditions.

Selective Colonization

The climax intestinal flora of the infant is attained by successive stages. The inoculation phase is controlled mainly by the composition of the environmental flora. These bacteria multiply in the intestine under the influence of the diet, inherent bacterial interactions, and individual gut environment. Supplementation of infant formula with bovine Lf has suggested an enrichment of bifidus flora in the gut (Roberts et al., 1992). Antimicrobial properties of Lf, such as the defimbriation of specific colonization factors, may favor selective establishment of enteric flora.

Antigen Processing

Lf may regulate the primary antibody responses in B-cells and coordinate various mononuclear cell functions (Horwitz et al., 1984). Monocytes in activated state have the capacity to kill tumor cells and also mediate the antibody-dependent cell-mediated cytotoxicity. Lf markedly affected adherent monocyte toxicity, but had no effect on nonadherent lymphocytes (T) and natural killer cells (Bennett and Davis, 1981). Apo- and Fe-saturated Lfs enhance the adherent cell cytotoxicity against K562-erythro leukemia cell line and hepatoma cell lines. Lf could also inhibit prostaglandin synthesis, which can inhibit interferon enhancement of natural killer activity (Horwitz et al., 1984).

Antimicrobial Effects of Lf

Lf is capable of eliciting antimicrobial effects by more than one molecular mechanism, and it is important to distinguish the type of effect being examined. It is evident that distinct antimicrobial mechanisms involve different regions of the Lf molecule.

Stasis Effect

Lf could bind reversibly two Fe$^{3+}$ ions with a high affinity ($K_a = 10^{30}$ L/mol), in cooperation with two HCO$_3^-$ ions (Masson and Heremans, 1968). Thus, the Lf-mediated inhibition of bacterial growth was believed to be a result of Fe limitation in the
milieu caused by this protein (Oram and Reiter, 1968). This hypothesis gained support from two in vitro observations, i.e., Fe-saturated Lf was ineffective in certain bacteria and addition of exogenous Fe reversed the inhibition. However, other studies have indicated a more complex mechanism of Lf-mediated antimicrobial action than simple Fe deprivation.

**Cidal Effect**

Our laboratory was the first to demonstrate a direct bacterial killing property of Lf on a variety of bacteria. This effect was irreversible with the addition of exogenous Fe and required adsorption of Lf to the target cell surface (Arnold et al., 1977, 1981, 1982). Our further investigations elucidated that culture conditions, such as temperature, pH, and osmolarity, influence the bactericidal effect and that bacterial metabolic phase is a critical factor. Thus, cells during exponential growth phase are highly susceptible to cidal activity. Furthermore, hydrophobic drugs that cross bacterial outer membrane become more effective in the presence of Lf (Ellison et al., 1988). These data suggested that Lf may affect bacterial membrane and may have more potential during active cell division.

**Cationic Effect**

Fragments isolated from proteolytic digests of both human and bovine Lfs demonstrate microbicidal activity against a variety of microorganisms (Bellamy et al., 1992; Yamauchi et al., 1993). These arginine- and lysine-rich peptide fragments are associated with the surface of the N-lobe away from the Fe-binding cleft. These cationic basic peptides demonstrate nonspecific charge interactions with a variety of surfaces, including glycosaminoglycans, and easily dissociate in the presence of salt.

**Phagocytic Effect**

In the presence of Lf, peritoneal macrophages and monocytes demonstrate an increased uptake and killing of intracellular parasites, such as *Trypanosoma cruzi* and *Listeria monocytogenes* (Lima and Kierszenbaum, 1987). Lf seems to bridge the parasite and phagocytes by binding specific targets on either surface. The presence of Fe is required for the Lf molecule to stimulate intracellular killing by macrophages, but not to enhance the uptake of microorganisms.
Antiadhesive Effect

Nonimmunoglobulin fractions of human milk inhibit enterotoxin binding and adhesion of enterotoxigenic E. coli to mammalian cells (Holmgren et al., 1981). Furthermore, fucose-containing glycoconjugates from human Lf could block Shigella flexneri adherence to guinea pig intestinal epithelia (Izhar et al., 1982). Lf could also inhibit the adsorption of Streptococcus mutans to hydroxyapatite and possibly affect deposition of this bacterium on tooth surfaces. The basic character of Lf molecule may alter the surface charge of bio-surfaces (Boxer et al., 1982) and may interfere in the electrostatic adhesive forces of microorganisms. We have recently found that Lf may inhibit specific fimbrial appendages, essential for bacterial adhesion and colonization.

Microbial Factors Affecting the Interaction

Proteases

Lf is readily cleaved by Porphyromonas gingivalis (a periodontitis pathogen) in vitro, and the fragments of apo-Lf behave as an intact molecule for several hours after exposure as determined by nonreducing SDS-PAGE, in comparison to the relatively rapid digestion of Fe-saturated Lf. Interestingly, this proteolytic digestion results in an approx 40-kDa half molecule similar to that obtained with pepsin. This unique proteolytic mechanism seems to be a virulence trait of P. gingivalis, and this pathogen seems to utilize Lf and various other serum/mucosal proteins as nutrients (Liu and Arnold, unpublished). The cholera vibros were also reported to produce an HA-protease that cleaves apo- and Fe-saturated forms of Lf; this characteristic of Vibrio cholerae was suggested as a pathomechanism for counteracting Fe sequestration by Lf.

LPS

A majority of fresh clinical isolates of E. coli and Salmonellae demonstrate resistance to Lf, and repeated in vitro cultivation in laboratory media renders these strains susceptible to Lf (Naidu et al., 1991, 1993). Later studies with well-
defined isogenic mutants and constructs established that this resistance to Lf was mainly owing to a shielding effect of the carbohydrate moiety of LPS on bacterial surface (Gadó et al., 1991). In analogy, LPS polysaccharide is involved in the blockade of porin interaction with certain colicins and bacteriophages. *Salmonella typhimurium* could also demonstrate an increased susceptibility to magainin 2 (a small cationic antimicrobial peptide) with a gradual decrease in the length of the LPS polysaccharide moiety.

**Capsular Polysaccharide**

Lf-susceptible, avirulent strains of *Streptococcus pneumoniae* after animal passages recover virulence character and become resistant to Lf. This phenomenon was concomitant with an increase in capsular material and interference with the Lf target sites (Arnold et al., 1981).

**Host Factors Affecting the Interaction**

**Citrate**

At high concentrations in bovine colostrum and milk, citrate can be utilized by a number of bacteria to sequester Fe, reducing the antibacterial capacity of Lf. However, during inflammatory conditions or involution, Lf increases and citrate levels decline. These changes correspond to a dramatic decrease of the citrate:Lf molar ratio and a potential increase of antibacterial properties of the secretion (Bishop et al., 1976). Furthermore, variations in the citrate:bicarbonate molar ratios in the biological fluids influence the Fe-binding status of the Lf molecule and affect antimicrobial activities.

**Gastric Digestion**

Although gastrointestinal survival of Lf appears likely, at least limited proteolysis probably also occurs. Trypsin digestion of diferric human Lf produces two major fragments corresponding to the N-lobe (30 kDa) and C-lobe (50 kDa) (Legrand et al., 1986). Trypsin digestion does not affect Fe-binding or bacteriostatic properties of Lf. The N-lobe of the molecule is pepsin-sensitive, whereas the C-lobe remains intact after pepsin digestion (Line et al., 1976). However, certain fragments of Lf
generated by pepsin hydrolysis could gain access to porins in resistant strains with smooth LPS and elicit antibacterial effects. A peptic fragment from Lf without Fe-binding capacity was recently identified as a potent bactericidal domain (Bellamy et al., 1992).

**Molecular Coexistence**

The most important interaction of Lf is its autoaggregation, i.e., the tetramerization in the presence of Ca\(^{2+}\). This occurs even at relatively low calcium levels (<10 mM). In human milk, 70% of its content of Lf is tetrameric (Hekman, 1971). However, the tetrameric Lf still retains the bacteriostatic properties. Lf binds to both slgA and the secretory component, and these associations inhibit the bacteriostatic effect. The complex of these two basic proteins turns out to be negatively charged. Lf and lysozyme form a very specific complex containing 1 mol of Lf and 2 mol of lysozyme. Lysozyme has a specific carbohydrate-binding site on each lobe of the Lf molecule. Lf also binds 2 mol of \(\alpha\)-lactalbumin, which enhances its bacteriostatic effect. In the presence of free fibronectin (e.g., in tears), a complex of Lf–fibronectin is formed, which has opsonizing activity. Different forms of Lf seem to appear at distinct stages of certain infections. Lf is polymerized in saliva during acute parotitis. However, apparent dimers and monomers appear during recovery when the inflammation gradually subsides. Similarly, bovine Lf trimers appear in milk during acute stages of bovine mastitis, whereas dimers and eventually monomers emerge as predominant forms during the healing process.

**Molecular Orientation**

Lf could exist in immobilized forms on various substrates. The N-terminus of Lf molecule seems to be involved in charge/affinity interactions with mucosal components heparan sulfate, dermatan sulfate (Zou et al., 1992), and cellular component DNA (Bennett et al., 1986; Hutchens et al., 1989). Furthermore, various eukaryotic cells demonstrate specific receptors for Lf interaction. Such immobilization processes could grossly alter the molecular conformation as well as the N- and C-lobe orientation of Lf. These conditions may further define the abilities of Lf–microbe interactions.
Lf-Binding Proteins in Enteric Bacteria

Colicin Analogy

Certain *E. coli* strains produce colicins, a class of bacteriocins. Colicins are known to kill other sensitive *E. coli* and related bacteria by adsorbing to specific receptors in the outer membrane. Some of these receptors perform key functions, such as selective uptake of various nutrients, adsorption of bacteriophages, and may also demonstrate porin activity, e.g., colicin A binds to outer membrane protein (Omp)F (see review by Konisky, 1982). Adsorption to bacterial surface is an essential step for the colicin action. The O-antigenic chains of LPS in *E. coli* may shield the receptors and inhibit colicin adsorption. Accordingly, most colicin-insensitive strains of *E. coli* become colicin-sensitive by partial removal of LPS. We have found that an Lf low-binding, virulent *E. coli* strain tolerant to a wide range of colicins, when modified into an Lf high-binding, avirulent strain, also showed an increase in colicin susceptibility. Furthermore, three colicin-insensitive *E. coli* strains that do not bind to human Lf, when converted to colicin-sensitive, demonstrated a high HLf-binding capacity. Thus, a strong correlation exists between human Lf binding and colicin sensitivity in *E. coli*. The analogies in the shielding and the exposure of bacterial surface for HLf and colicin interactions suggested a similar recognition mechanism for the two antimicrobial systems in the *E. coli* outer membrane (Gadó et al., 1991).

Porin OMPs

Certain strains of *E. coli* (bacterial whole cells) demonstrate specific interaction with $^{125}$I-labeled Lf (Naidu et al., 1991). An ∼37-kDa protein reactive with horseradish peroxidase (HRPO)-labeled Lf was identified in the boiled cell envelope and OM preparation of an Lf-binding *E. coli* strain E34663 and an Lf-non-binding strain HH45, by SDS-PAGE/Western blot. Such a band was not detected in the unboiled native cell envelope and OM preparations. The molecular mass and the heat-modifiable property suggested that the Lf-binding proteins are porins. The native trimeric form of porin OmpF isolated from *E. coli* strain B6 and its dissociated monomeric form both reacted with HRPO-labeled Lf and with MAb specific for OmpF. Further-
more, using *E. coli* constructs with defined porin phenotypes, OmpF and OmpC were identified as the Lf-binding proteins by urea-SDS-PAGE/Western blot, and by $^{125}$I-Lf-binding studies with intact bacteria. These data establish that Lf binds to porins, a class of well-conserved molecules common in *E. coli* and many other gram-negative bacteria. However, in certain strains of *E. coli*, Shigellae, and Salmonellae, these pore-forming proteins are shielded from Lf interaction (Tigyi, 1992; Erdei et al., 1994; Naidu and Arnold, 1994).

**Mechanisms Studied in Relation to Porin Binding**

**Antibacterial Effects**

The Lf-binding property of *E. coli* showed an inverse relation to the changes in bacterial metabolic rate ($r$, 0.91) and a direct relation to the degree of bacteriostasis ($r$, 0.79). The magnitude of Lf–bacteria interaction showed no correlation with the minimal inhibitory concentration of Lf. In certain strains, at supraoptimal levels, Lf has reduced the bacteriostatic effect. Thus, Lf concentration in the growth media seemed to be critical for the antibacterial effect. The cell envelopes of *S. typhimurium* 395MS with smooth LPS and its five isogenic R-mutants revealed 38-kDa porin proteins as peroxidase-labeled Lf-reactive components in the SDS-PAGE and Western blot analysis. However, in the whole-cell binding assay, the parent 395MS demonstrated a very low interaction with $^{125}$I-Lf. On the other hand, Lf interaction gradually increased corresponding to the decrease in LPS polysaccharide moiety in the isogenic R-mutants. Conductance measurement studies revealed that the low Lf-binding strain 395MS with smooth LPS was less susceptible to Lf, whereas the high Lf-binding mutant Rd was more susceptible to Lf. These data suggested a correlation between the Lf binding to porins and the Lf-mediated antimicrobial effect. The polysaccharide moiety of LPS shielded porins from the Lf interaction, and concomitantly decreased the antibacterial effect (Naidu et al., 1993).

**Membrane Permeation**

The size-exclusion property of porins contribute to a barrier function in the OM of gram-negative bacteria, against various hydrophilic antibiotics. On the other hand, the diffusion of
many hydrophobic antibiotics through the OM could be mechanically prevented by the polysaccharide moiety of LPS. Specific mutations that either alter the porin content or shorten the polysaccharide moiety in LPS may breach these OM barrier mechanisms. Our observation that Lf binds to porins prompted a possibility that this specific interaction mechanism might influence the antibiotic susceptibility in gram negative bacteria. Cefuroxime (CXM) is a monoanionic β-lactam with a low diffusion rate through the porin (OmpF) channel, and therefore was selected for Lf-induced antibiotic potentiation studies. Lf and CXM demonstrated a cooperative potentiation. Thus, the Lf activity was potentiated by 2–16-fold, whereas CXM sensitivity was enhanced by two- to eightfold, in S. typhimurium and nine other species of salmonella. Among the zwitterionic β-lactams, the diffusion rate of ampicillin through the OmpF porin was threefold lower than cephalaxin. Lf has potentiated only the former antibiotic by twofold. Ciprofloxacin could cross the OM through OmpF channel, and Lf caused a twofold increase in the antimicrobial activity of this hydrophilic quinolone. Rifampicin and erythromycin cross the OM by a hydrophobic route, and the activity of these antibiotics was also potentiated by Lf. Finally, the spectrum of aminoglycoside gentamycin was unaffected by Lf. Therefore, regardless of the route of entry or hydrophobicity of the antibiotic, Lf demonstrated a synergistic antimicrobial activity. Possibly, the interaction of Lf altered the porin channel and contributed to an increased diffusion of antibiotics that cross OM by this pathway (Naidu and Arnold, 1994).

Effects on Bacteria–Matrix Protein Interactions

Various mammalian subepithelial matrix proteins, such as fibronectin, fibrinogen, collagen type-I, collagen type-IV, and laminin, serve as eukaryotic cell targets for bacteria during their host mucosal adhesion and colonization. Electrostatic and van der Waal forces are important in bacterial–eukaryotic cell interactions. Lf binding to neutrophils alters cellular surface charge and cause leukocyte aggregation on endothelium during inflammatory conditions (Boxer et al., 1982). Similar influence of Lf on surface charge of either bacteria or matrix proteins could also affect the interactions. Bovine Lf caused a dose-dependent
inhibition of $^{125}$I-labeled matrix protein interactions with a porcine enterotoxigenic E. coli strain ETEC263. Bovine Lf strongly inhibited the interactions of bacteria with the connective tissue protein collagen type-1 and the basement membrane protein laminin. Unlabeled Lf also caused an effective concentration-dependent displacement of E. coli interactions with collagen type I and laminin. The bacterial interaction with fibronectin, fibrinogen, and collagen type IV was only slightly dissociated and a 50% effect was not achieved at the Lf concentrations tested (Naidu, Arnold, and Naidu, unpublished).

**Effects on Bacterial Fimbriation**

Fimbriae (or pili) are slender appendages on the cell surface of E. coli and many gram-negative bacteria. Specific types of fimbriae may either serve as virulence determinants for pathogenic E. coli during infection or as an anchorage for commensal E. coli in the colonization of the normal intestinal tract. Type 1 pilus has been suggested to play an important role in the mucosal colonization of E. coli. In vivo factors may suppress the expression of type 1 pili in E. coli. Milk seems to contain such inhibitory factors, since only colostrum-deprived, but not replete newborn piglets demonstrate intestinal colonization with type 1 piliated enterotoxigenic E. coli. Lf suppressed the fimbriaton colonization factor antigens (CFA) I and II types in ETEC strain and type 1 fimbria in EPEC strain F18 within 5 h. However, Lf did not affect the P fimbria in E. coli (Naidu, Czirok, Arnold, and Naidu, unpublished).

**Effects on Bacterial Colonization In Vivo**

The influence of Lf on the colonization of E. coli in the mouse intestine was examined. Streptomycin-treated mice were challenged with strain F18 by gastric intubation, and bacterial excretion was estimated as CFU/g feces. The excretion of strain F18 in feces reached a steady state ($10^8$ CFU/g) within 7 d, independent of challenge (dosage: $8 \times 10^8$ or $10^9$ CFU). Oral administration of bovine Lf (in 20% sucrose solution) caused 1- to >3-log reductions in CFU/g feces with high and low dosages of strain F18. The bacterial multiplication in vivo was markedly affected during the early 24 h of infection, reflecting >3-log lower number of bacteria in the feces ($2 \times 10^3$ CFU/g) than the
control group. Postinfective oral administration of Lf seems ineffective on the intestinal colonization of strain F18 in mouse (Czirok, Naidu, Herpay, and Naidu, unpublished). In analogy, preinfective iv administration of Lf seems to protect mice against lethal doses of *E. coli*, whereas postinfective treatment was ineffective (Zagulski et al., 1989).

**Lf Structure Relation to Antimicrobial Functions**

**Heparin-Binding Character**

It seems evident that the distinct killing mechanisms involve different regions of the Lf molecule. Lactoferricins H (human) and B (bovine) generated by pepsin hydrolysis of Lf were reported as the bactericidal domains of the molecule (Bellamy et al., 1992). These arginine- and lysine-rich basic peptides are structurally associated with the surface of the N-lobe away from the coordinate Fe-binding cleft (Anderson et al., 1990), and the intact molecule would therefore be expected to elicit cidal effects in apo- or Fe-saturated forms. In addition to the lack of killing with the Fe-saturated Lf, lactoferricin did not comply with a number of parameters defining the bactericidal effect. The heparin void volume fragment (C-lobe lacking lactoferricin equivalent sequence) is capable of potent bactericidal activity. The IgA–Lf complex also lacks heparin binding is also capable of Lf-dependent killing. The stoichiometric inhibition of killing with selected carboxyl anions, the salt-independent cidal activity, the reduction in killing by anaerobiosis, the potentiation of cidal spectrum by SCN, and the reduced pH are all difficult to explain based on a lactoferricin mechanism of killing.

**Oxidant Activity**

Lf has been reported to mediate the formation of oxygen radicals and may contribute to the hydrolysis of nucleic acids. Lf interacts with DNA via surface-exposed histidyl residues, and Lf-bound Cu(II) can cause extensive degradation of DNA and RNA molecules. Also, the copper-binding sites serve as centers for repeated production of OH radicals via a Fenton-type Haber-Weiss reaction (Zhao and Hutchens, 1994). This activity is consistent with our original hypothesis that Lf killing is mediated by targeted radical generation at a susceptible
bacterial site. These studies suggested that specific binding to a bacterial target involved the anion-binding ligands ofLf, followed by coordinate binding and oxidation of bacterial surface Fe$^{2+}$ to Fe$^{3+}$. This metal transition in the presence of suitable oxygen species would fulfill the necessary components of the Haber-Weiss reaction, and thus, may possibly generate radicals in close proximity to susceptible targets. Our observations indicate that Lf killing is competitively inhibited by suitable carboxyl anions (but not by Fe ions) at a molar stoichiometry. These studies also indicated that anaerobiosis in the absence of CO$_2$ prevented Lf-mediated killing. Consistent with the hypothesis of targeted radical generation, radical scavengers were ineffective at blocking Lf killing. In retrospect, our earlier observations that S. mutans grown under conditions of Fe limitation were resistant to the bactericidal effects of Lf, when compared to Fe-replete, carbohydrate-limited, or carbohydrate-replete bacteria, were consistent with bacterial-associated Fe serving as the Lf prosthetic group in the generation of oxygen radicals.

Conclusions

The conformational changes and thereby the exposure of specific molecular domains are critical factors in defining the multifunction characters of Lf and its host-microbe interactions. Antimicrobial effects of Lf, in particular, are strongly dependent on the physical environment (i.e., pH, temperature, osmolarity) and preferentially require a specific binding target on microbial surface. In enteric bacteria, the pore-forming proteins in the outer membrane seem to fulfill this requisite.

References


Host–Microbe Interactions


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