ISSN 1722-6996 AIHTEI

# Agro FOOD industry hi-tech

Anno 16 • March/April 2005

Published by Tekno Scienze

European Journal of Nutraceuticals & Functional Foods

Dr. Narain Naidu

## The Future of Lactoferrin

2

Spedizione in a.p. - 45% art. 2 comma 20/b legge 662/96 Filiale di Milano

# Ultra-cleansing of lactoferrin: Nutraceutical implications

NARAIN NAIDU
Director, R&D
en-N-tech Research Laboratory, 981 Corporate Center Drive Suite #120, Pomona, California 91768, USA

## 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.*

## 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[1,2]. 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. Although, 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, mucins 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[2,3] (FIGURE-1).

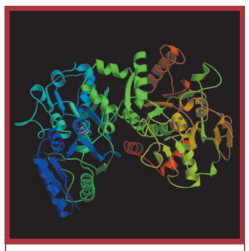


**Figure 1. Ribbon structure of bovine LF (drawn with RasMol ver.2.6 software). The N-terminus (Blue) and C-terminus (Red) regions constitute the two iron-binding lobes of the molecule.**

## 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 mucins; 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.

LF also co-exists with an array of molecules in different mucosal secretions with varying milieu conditions. However, molecular dysfunctionality or deficiency of LF levels in the body could cause several physiological disorders and predispose various infections[3-5]. Several studies have established that LF supplementation could provide exceptional health benefits and a powerful protection against several illnesses[1]. 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.

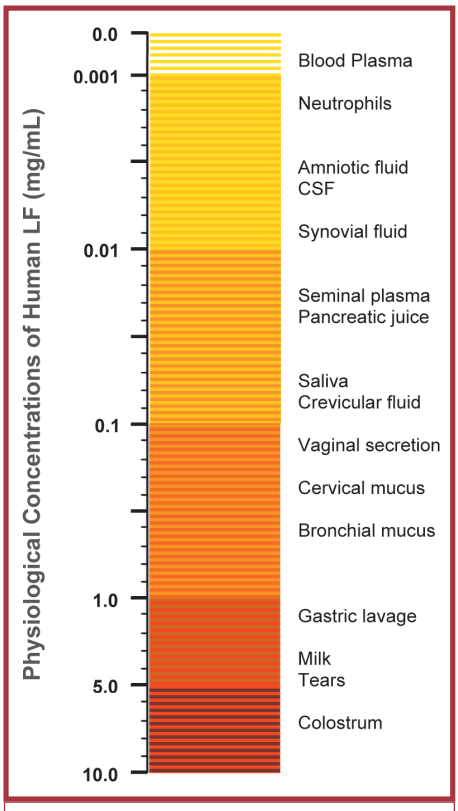


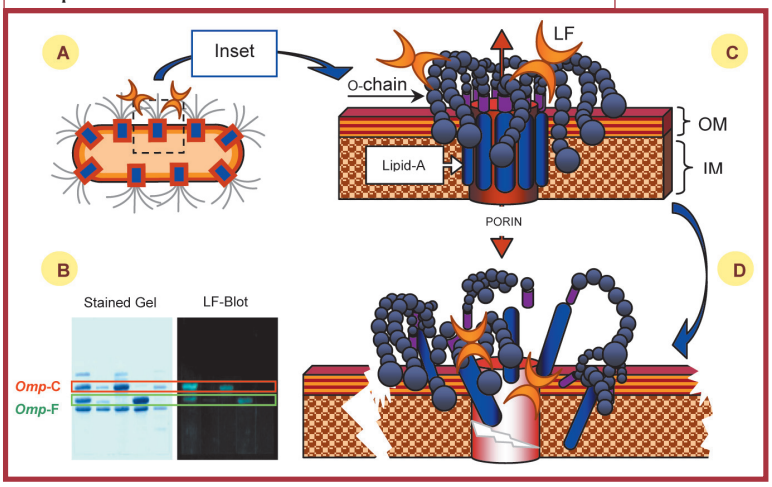

**Figure 2. Physiological distribution of LF in human body fluids and secretions (A schematic representation).**

### Pathogen elimination and gut protection

The antimicrobial functionality of LF is dependent on its protein conformation, metal-binding and milieu conditions[3]. Antimicrobial activity is enhanced when LF binds to the microbial cell surface[6]. Over the past 15 years, Naidu *et al.* have identified specific LF-binding microbial targets on different Gram-positive and Gram-negative bacterial pathogens[7-10]. 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[3,11]. Thus,

LF-mediated outer-membrane (OM) damage in Gram-negative bacteria and the LF-induced antibiotic potentiation by causing altered OM permeation are typical examples of such antimicrobial outcomes[12,13]. LF interaction with the microbial cell surface, OMPs in particular, has led to other antimicrobial mechanisms such as microbial adhesion-blockade to intestinal epithelia and specific detachment of pathogens from gut mucosa. Specific binding of LF could instantly collapse bacterial OM barrier function and leads to the shutdown of pathogen colonization factors and enterotoxin production [FIGURE-3].

**Figure 3. Mechanism of LF-mediated antimicrobial activity against Gram-negative enteric pathogens via the porin-mediated pathway. (A) LF interaction with bacterial outer membrane. (B) identification of porins Omp-C and Omp-F as specific LF-binding receptors on the bacterial outer membrane. (C) LF binding to porins. (D) LPS release and collapse of the bacterial outer membrane**




Oral administration of LF supplemented milk has been shown to suppress proliferation of intestinal clostridium species and fecal excretion of anaerobic pathogens[14]. Supplementation of milk in the diet with LF could also inhibit bacterial translocation, mainly with the members of the family *Enterobacteriaceae*, from the intestines to the mesenteric lymph nodes[15]. LF could block colonization of *Helicobacter pylori* and detach this pathogen from gastric epithelium *in vivo*[16]. Thus, oral administration of 1% LF for 3 to 4 weeks has been shown to decrease *H. pylori* 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* to an undetectable level could be achieved. Prophylactic and therapeutic effects of oral supplementation of LF against intractable stomatitis have also been reported[17].

## 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 adjuvants 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, prebiotic intake could significantly modulate the intestinal microflora, 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 pathogens[20]. 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 manner[21]. 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 vivo*[22,23].

## 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 endotoxemia[24]. Experimental evidence suggests that reactive oxygen species are important mediators of cellular injury during endotoxemia, either as a result of macromolecular 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 endotoxic 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 endotoxin[25]. 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 quantities[26].

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 cytokines[27].

## 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 allergen challenge as a protective mechanism[28]. Also, exogenous administration of LF could effectively inhibit allergen-induced cellular events of inflammation in a dose-dependent manner[29]. 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 shock[30].

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 endotoxemia[31].

### Modulation of mucosal immunity

Oral administration of LF (40 mg capsule/day) could enhance immune response in healthy human volunteers (*n=17*)[32]. Human clinical trials showed a positive influence of LF consumption in primary activation of host defense[33]. 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[2].

### 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/d) or high (1 mg/g body wt/d) dosages could function as an immune stimulating factor in the intestinal mucosa[34]. This activation is dependent on LF binding to the intestinal epithelia. LF could also potentiate thymidine incorporation into crypt cell DNA *in vivo*. This tropic effect contributes to cell regeneration and tissue repair of intestinal mucosa in conditions such as gastroenteritis[35]. Feeding of LF supplemented formula could increase hepatic protein synthesis in the newborn, which suggests an anabolic function for LF in neonates[36].

### 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[37]. 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[38]. An intestinal LF receptor with a cellular density of $4.3 \times 10^{14}$ 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[39,40].

### 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[41].

## 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[1]. 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)[42]. Endotoxins could stimulate the induction of cytokines and other mediators of inflammation, which in turn could trigger a broad range of adverse physiological responses[43].

Gram-negative bacterial bioburden of milk or its derivatives used in protein isolation, processing plant environment and conditions cumulatively contribute to endotoxin levels in an LF source material. Majde[44] has reviewed the potential reservoirs for endotoxin contamination during isolation of protein materials. Rylander[45] 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-β-D-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[46]. 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 90$^{th}$ percentile averages 73 mg/d for children, 75 mg/d for teens and 50 mg/d for adults.

## TREATMENT FOR CONTAMINANT REDUCTION (TCR)

Based on the types and levels of contaminants, as well as the microbiological quality assurances implemented with cGMPs in the commercial-scale manufacturing of LF, a multi-tier TCR process has been developed using natural substrates as decontaminant agents (FIGURE-4). This novel technology could systematically extend the scope of TCR process in combination with defined synergistic compositions, to enhance multifunctional properties of LF, thereby creating a powerful physiological system to deliver probiotic LAB and other bioactive compounds. The TCR process could be used as a stand alone technology or integrated with different lab-scale, pilot-scale or commercial-scale technologies practiced in the isolation and purification of LF.

The TCR process includes the creation of a surfactant tier analogous to the physiological gastric detergents to selectively disrupt the cell membranes of contaminant microorganisms. Natural and/or food-grade surfactants for use in the present invention include plant-derived saponins, food-grade polysorbates and bile salts. The decontamination process also utilizes carbonate or bicarbonate anions at specific ratios in combination with natural antioxidants such as vitamins A, C or E to enhance the anion-dependent LF bioactivity. For the purpose of restoring the anion-dependent bioactivity of commercial LF, methods related to generating carbonated aqueous systems or anaerobic encapsulations are also suitable. Finally, the ultra-cleansing technology uses effective and permissible amounts of food-grade polyphenols to neutralize endotoxin contaminants in commercial LF preparations. Polyphenols of particular use for this purpose include oleoresins, aquaresins, oleuropeins, terpenes, flavonoids and biliproteins.

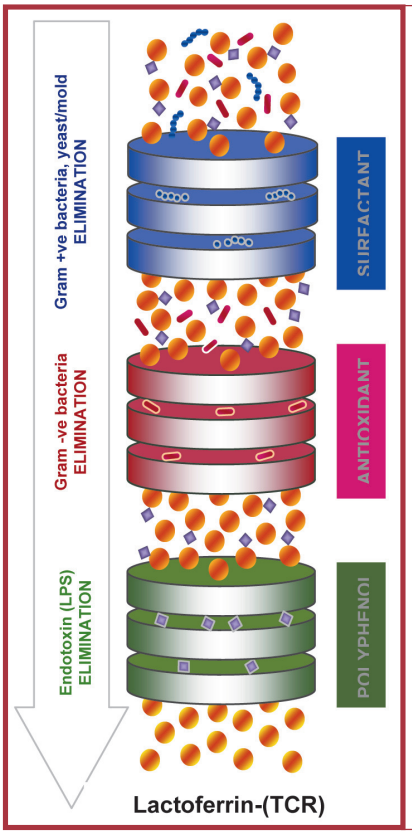



Figure 4. Treatment for contaminant reduction (TCR) process utilizes a 3-tier ultra-cleansing system to eliminate bioburden and neutralize endotoxins from commercial LF preparations (a schematic representation). Tier-I consists of a surfactant phase to eliminate yeast, molds and Gram +ve bacterial contaminants; Tier-2 with antioxidant carbonium milieu removes the Gram -ve bioburden; and the final tier-3 polyphenolic phase effectively neutralizes the intrinsic endotoxin.

### 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 Amoebocyte Lysate (LAL) assay using a FDA approved QCL-1000 test kit (Cambrex Bioscience, Walkerville, MD). The analysis of commercial samples revealed that whey-derived LF harbored more bioburden than the milk-derived LF. A significant portion of aerobic plate counts (APC) of whey-derived LF were identified as Gram-positive microorganisms. The whey-derived LF contained more mold contaminants such as *Penicillium* spp. and *Aspergillus* spp., while milk-derived LF showed contamination with species of yeast.

The endotoxin levels of both commercial LF preparations reflected their coliform and Gram negative bacterial loads.

The median endotoxin levels in whey-derived LF were about 20 times higher than in the milk-derived LF. The endotoxin contaminants in both LF preparations were biologically active and induced TNF-$\alpha$ production in stimulated monocytes.

### 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-$\alpha$ 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.

Briefly, the TCR process consisting of a three tier system with surfactant, antioxidant and polyphenolic phases effectively eliminated the microbial contaminants in a sequentially progressive manner to provide an ultra-clean LF preparation. Furthermore, a high degree of detoxification of intrinsic pyrogens in commercial LF preparations was also accomplished.

Table I. Efficacy of TCR process in ultra-cleansing of lactoferrin

| Tier | TCR Process | CONTAMINANT ACTIVITY | | | |
|---|---|---|---|---|---|
| | | APC (CFU/g) | Coliforms (CFU/g) | Yeast & Mold (CFU/g) | LPS Activity (pg TNF/g ) |
| 0 | Starting quality | 7200 | 96 | 25 | 5400 |
| I | Surfactant-phase | 4650 | 92 | 15 | 5375 |
| II | Antioxidant-phase | 260 | 0 | 0 | 3160 |
| III | Phytophenol-phase | <10 | 0 | 0 | <1 |

### 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 ($10^4$ cells/mL) prepared in 0.9% saline was added to the wells.

Addition of 0.5-mL saline or bacterial suspension to module wells with 0.5-mL (2x 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 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 elicit 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-(TCR) 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 (2x) 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 ~$10^5$ cells/mL [diluted from an optically pre-calibrated (OD 1.0 at 600 nm) solution of $10^9$ 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-(TCR) was tested at 0.5% (w/v) concentration for prebiotic activity 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-(TCR) 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-(TCR), which was at least twice as effective as the prebiotic ability of its LF (untreated) counterpart, i.e. ~40% growth enhancement. Data enlisted in TABLE-2 with different LAB strains clearly indicate that LF-(TCR) is a powerful prebiotic agent.

Table 2. Prebiotic effects of lactoferrin-(TCR)

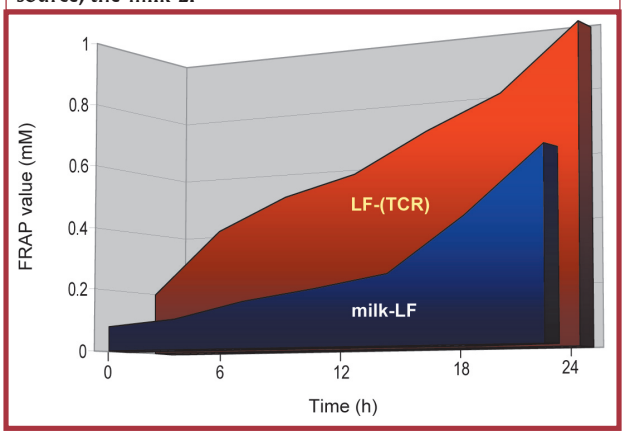
| Probiotic (LAB) strain | PREBIOTIC ACTIVITY | | | | |
|---|---|---|---|---|---|
| | GIDA - Detection Time (h) | | | MODA - Growth (%)* | |
| | Control | LF | LF-(TCR) | LF | LF-(TCR) |
| *Bifidobacterium bifidum* ATCC15696 | 19.5 | 17.8 | 15.1 | 142% | 210% |
| *Bifidobacterium infantis* ATCC15697 | 21.3 | 19.4 | 17.0 | 150% | 225% |
| *Bifidobacterium longum* ATCC15707 | 19.0 | 16.9 | 14.5 | 148% | 205% |
| *Lactobacillus acidophilus* ATCC4356 | 12.6 | 11.0 | 8.7 | 132% | 189% |
| *Lactobacillus amylovorus* ATCC33620 | 17.9 | 17.0 | 15.1 | 121% | 165% |
| *Lactobacillus brevis* ATCC14869 | 18.2 | 15.2 | 14.0 | 155% | 196% |
| *Lactobacillus casei* ATCC393 | 13.5 | 11.8 | 9.0 | 129% | 203% |
| *Lactobacillus crispatus* ATCC33820 | 14.0 | 13.1 | 10.4 | 118% | 182% |
| *Lactobacillus delbrueckii* ATCC12315 | 15.6 | 12.9 | 9.8 | 152% | 233% |
| *Lactobacillus fermentum* ATCC14931 | 16.2 | 14.0 | 11.2 | 150% | 215% |
| *Lactobacillus helvaticus* ATCC15009 | 16.2 | 14.1 | 11.0 | 148% | 220% |
| *Lactobacillus paracasei* ATCC25302 | 14.0 | 10.8 | 9.9 | 163% | 195% |
| *Lactobacillus pentosus* ATCC8041 | 15.4 | 12.0 | 10.1 | 168% | 199% |
| *Lactobacillus plantarum* ATCC14917 | 15.5 | 12.0 | 10.1 | 171% | 200% |
| *Lactobacillus reuteri* ATCC23272 | 15.4 | 12.0 | 9.8 | 167% | 201% |
| *Lactobacillus rhamnosus* ATCC7469 | 13.5 | 11.7 | 9.0 | 131% | 204% |
| *Lactococcus lactis* ATCC11454 | 12.6 | 11.3 | 8.9 | 120% | 186% |
| *Strep. thermophilus* ATCC19987 | 12.0 | 11.1 | 8.1 | 116% | 192% |

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

## Antioxidant Activity

Ferric reducing/antioxidant power (FRAP) assay as described by Benzie & Strain[47] with minor modifications has been used to measure the antioxidant activity of LF-(TCR). The FRAP reagent was prepared by mixing 40 mL of 0.3 M acetate buffer (pH 3.6), 4 mL of 20 mM ferric chloride, and 4 mL of 10 mM TPTZ [2,4,6-Tris(2-pyridyl)-s-triazine]. Serial dilutions (0.1 to 1.0 mM) of 6-OH-2,5,7,8-tetramethyl chroman-2-carboxylic acid (CAS 53188-07-1) were used as FRAP standards. All reagents were brought to 37°C prior to the assay. FRAP assay was performed in a 96-well microplate by mixing 20 μL of DI water, 10 μL of LF-(TCR) sample, and 150 μL of FRAP reagent. In combination studies 10 μL of DI water and 20 μl of LF-(TCR) were mixed with 150 μL of FRAP reagent. After instant incubation at 37°C for 5 min (for ascorbic acid) and for a time lapse of 5 min to 24 h [for LF-(TCR) and milk-derived LF] the absorbance of reaction mixtures was measured at 593 nm (Spectramax 340PC). Test compounds were given antioxidant (FRAP) scores compared to the FRAP value of ascorbic acid.
The FRAP reaction kinetics (measured as the rate of increase in absorbance of reaction mixtures at 593 nm) of LF-(TCR) was compared with its source material, the milk-LF (FIGURE-5). When

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




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-(TCR) 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-(TCR) 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 $CO_2$ incubator. After partial monolayers were obtained, each plate was washed twice with phosphate buffered saline (PBS, pH 7.2). A 2-mL volume of

LF-(TCR) or other test solutions diluted in EMEM were added to each well containing approximately $10^5$ Caco2 cells. The proliferation of Caco2 cells and the cytomorphological changes in the monolayer was examined after 24-h and 48-h incubation with the following two methods: i) Annexin V label, an early marker for dying cells, and ii) Tunel assay, a late marker for dying cells in which the reagent binds to fragmented DNA. Apoptosis was also induced with 1 μM staurosporine treatment for 30 min followed by washing with tissue culture medium. Cells were rinsed with binding buffer 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. Cells were washed with PBS, and fixed in 2% paraformaldehyde in PBS 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-apochromat x40/1.25 NA and x63/1.40 NA oil immersion objective lenses. Images were acquired with a SkyVision-2/VDS digital CCD (12-bit, 1280 x 1024 pixel) camera in unbinned or 2 x 2-binned models into EasyFISH 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.

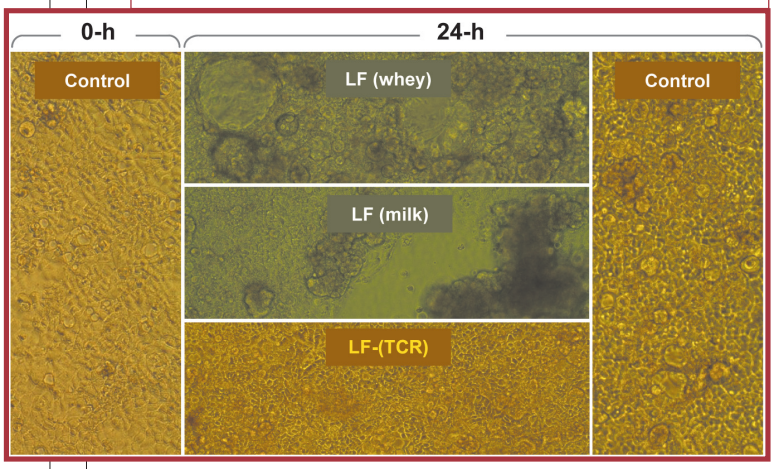
## CONCLUSION

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)**

| FUNCTIONAL ACTIVITY | LF (whey) | LF (milk) | LF-(TCR) |
|---|---|---|---|
| **ANTIOXIDANT ACTIVITY** | | | |
| • Total Antioxidant Status | <1.0 μ.mol/mg | 5.3 μ.mol/mg | **72.9 μ.mol/mg** |
| • FRAP Value (at 24-h) | NT | 583 mM | **994 mM** |
| **BACTERIOSTASIS ACTIVITY** | | | |
| • *Escherichia coli* | 15.4-h | 26.2-h | **>48-h** |
| • *Salmonella* Typhimurium | 12.5-h | 22.9-h | **>48-h** |
| **PREBIOTIC ACTIVITY** | | | |
| • *Lactobacillus* spp. (n=13) | 96% | 147% | **200%** |
| • *Bifidobacterium* spp. (n=3) | 110% | 146% | **213%** |
| • *Lactococcus lactis* | 105% | 120% | **186%** |
| • *Streptococcus thermophilus* | 92% | 116% | **192%** |

**Figure 6. Apoptosis of Caco2 (colon carcinoma) cell lines after 24-h of exposure to different LF preparations. LF-(TCR) showed <1% apoptosis compared to whey-derived LF (53%) and milk-derived LF (35%). Tissue culture without any LF exposure served as control.**




## REFERENCES


1. Naidu AS (2000) Lactoferrin - Natural multifunctional antimicrobial. Boca Raton, CRC Press.
2. Hanson LA (1988) Biology of human milk. Nestlé Nutrition Workshop Series, Vol.15. New York, Raven Press.
3. Naidu AS, Arnold RR (1995) Influence of lactoferrin on host-microbe interactions. In 'Lactoferrin interactions and biological Functions, eds. TW Hutchens, B Lonnerdal, pp.259-75. Totowa, NJ, Humana Press.
4. Naidu AS, Bidlack WR (1998) Milk lactoferrin - Natural microbial blocking agent for food safety. *Environ Nutr Interact* 2:35-50.
5. Tabak L *et al.* (1978) Changes in lactoferrin and other proteins in a case of recurrent parotitis. *J Oral Pathol* 1:97-9.
6. Naidu SS *et al.* (1993) Relationship between antibacterial activity and porin binding of lactoferrin in Escherichia coli and Salmonella typhimurium. *Antimicrob Agents Chemother* 37:240-5.
7. Naidu AS *et al.* (1990) Bovine lactoferrin binding to six species of coagulase-negative staphylococci isolated from bovine intramammary infections. *J Clin Microbiol* 28:2312-9.
8. Naidu AS *et al.* (1991) Human lactoferrin binding in clinical isolates of Staphylococcus aureus. *J Med Microbiol* 34:323-8.
9. Naidu AS *et al.* (1992) Identification of human lactoferrin-binding protein in Staphylococcus aureus. *J Med Microbiol* 36:177-83.
10. Naidu SS *et al.* (1991) Specific binding of lactoferrin to *Escherichia coli* isolated from human intestinal infections. *APMIS* 99:1142-50.
11. Erdei J, Forsgren A, Naidu AS (1994) Lactoferrin binds to porins OmpF and OmpC in Escherichia coli. *Infect Immun* 62:1236-40.
12. Naidu AS, Arnold RR (1994) Lactoferrin interaction with salmonellae potentiates antibiotic susceptibility in vitro. *Diagn Microbiol Infect Dis* 20:69-75.
13. Ellison RT, Giehl TJ, LaForce FM (1988) Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. *Infect Immun* 56:2774-81.
14. Teraguchi S *et al.* (1995) Bacteriostatic effect of orally administered bovine lactoferrin on proliferation of Clostridium species in the gut of mice fed bovine milk. *Appl Environ Microbiol* 61:501-6.
15. Teraguchi S *et al.* (1995) Orally administered bovine lactoferrin inhibits bacterial translocation in mice fed bovine milk. *Appl Environ Microbiol* 61:4131-4.
16. Wada T *et al.* (1999) The therapeutic effect of bovine lactoferrin in the host infected with Helicobacter pylori. *Scand J Gastroenterol* 34:238-43.
17. Sato N *et al.* (1999) Lactoferrin inhibits Bacillus cereus growth

and heme analogs recover its growth. *Biol Pharm Bull* 22:197-9.
18. Naidu AS, Bidlack WR, Clemens RA (1999) Probiotic spectra of lactic acid bacteria (LAB). *Crit Rev Food Sci Nutr* 39:13-126.
19. Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota - introducing the concept of prebiotics. *J Nutr* 125:1401-12.
20. Petschow BW *et al.* (1999) Ability of lactoferrin to promote the growth of Bifidobacterium spp. in vitro is independent of receptor binding capacity and iron saturation level. *J Med Microbiol* 48:541-9.
21. Liepke C *et al.* (2002) Human milk provides peptides highly stimulatingthe growth of bifidobacteria. *Eur J Biochem* 269:712-8.
22. Lonnerdal B (2003) Nutritional and physiologic significance of human milk proteins. *Am J Clin Nutr* 77:1537S-43S.
23. Roberts AK *et al.* (1992) Supplementation of an adapted formula with bovine lactoferrin – effects on the infant faecal flora. *Acta Paediatr* 81:119-24.
24. Opal SM (2002) The clinical relevance of endotoxin in human sepsis - a critical analysis. *J Endotoxin Res* 8:473-6.
25. Appelmelk BJ *et al.* (1994) Lactoferrin is a lipid-A binding protein. *Infect Immun* 62:2628-32.
26. Caccavo D *et al.* (2002) Antimicrobial and immuno-regulatory functions of lactoferrin and its potential therapeutic applications. *J Endotoxin Res* 8:403-17.
27. Lee WJ *et al.* (1998) The protective effects of lactoferrin feeding against endotoxin lethal shock in germfree piglets. *Infect Immun* 66:1421-6.
28. Zweiman B *et al.* (1990) Release of lactoferrin and elastase in human allergic skin reactions. *J Immunol* 144:3953-60.
29. Cumberbatch M *et al.* (2000) Regulation of epidermal Langerhans cell migration by lactoferrin. *Immunology* 100:21-8.
30. Miyazawa K *et al.* (1991) Lactoferrin-lipopolysaccharide interactions. Effect on lactoferrin binding to monocyte/ macrophage-differentiated HL-60 cells. *J Immunol* 146:723-9.
31. Di Mario F *et al.* (2003) Use of bovine lactoferrin for Helicobacter pylori eradication. *Dig Liver Dis* 35:706-10.
32. Zimecki *et al.* (1998) Immuno-regulatory effectsof a nutritional preparation containing bovine lactoferrin taken orally by healthy individuals. *Arch Immunol Ther Exp Warsz* 46:231-40.
33. Yamauchi *et al.* (1998) Effects of orally administered bovine lactoferrin on the immune system of healthy volunteers. *Adv Exp Med Biol* 443:261-5.
34. Debbabi H *et al.* (1998) Bovine lactoferrin induces both mucosal and system immune responses in mice. *J Dairy Res* 65:283-93.
35. Nichols BL *et al.* (1990) Iron is not required in the lactoferrin stimulation of thymidine incorporation into the DNA of rat crypt enterocytes. *Pediatr Res* 27:525-8.
36. Burrin DG *et al.* (1996) Orally administered lactoferrin increases hepatic protein synthesis in formula-fed newborn pigs. *Pediatr Res* 40:72-6.
37. Lonnerdal B (1989) Trace element absorption in infants as a foundation to setting upper limits for trace elements in infant formulas. *J Nutr Suppl* 119:1839-44.
38. Cox *et al.* (1979) Iron-binding proteins and influx of iron across the duodenal brush border. Evidence for specific lactotransferrin receptors in the human small intestine. *Biochem Biophys Acta* 588:120-8.
39. Kawakami H, Lonnerdal B (1991) Isolation and function of a receptor for human lactoferrin in human fetal intestinal brush-border membranes. *Am J Physiol* 261:G841-6.
40. Rosa G, Trugo NM (1994) Iron uptake from lactoferrin by intestinal brush-border membrane vesicles of human neonates. *Braz J Med Biol Res* 27:1527-31.
41. Shau *et al.* (1992) Modulation of natural killer and lymphokine-activated killer cell cytotoxicity by lactoferrin. *J Leukoc Biol* 51:343-9.
42. Erridge C *et al.* (2002) Structure and function of lipopolysaccharides. *Microbes Infect* 4:837-51.
43. Raetz CR, Whitfield C (2002) Lipopolysaccharide endotoxins. *Annu Rev Biochem* 71:635-700.
44. Majde JA (1993) Microbial cell-wall contaminants in peptides - a potential source of physiological artifacts. *Peptides* 14:629-32.
45. Rylander R (2002) Endotoxin in the environment - exposure and effects. *J Endotoxin Res* 8:241-52.
46. Bennett RM, Kokocinski T (1979) Lactoferrin turnover in man. *Clin Sci* 57:453-60.
47. Benzie IF, Strain JJ (1999) Ferric reducing (antioxidant) power as a measure of antioxidant capacity: the FRAP assay. In: 'Methods in Enzymology: Oxidants and Antioxidants', ed. L Packer, pp15-27. Orlando: Academic Press.
48. Davidson PM, Naidu AS (2000) Polyphenols, In 'Natural Food Antimicrobial Systems', ed. AS Naidu, pp. 265-94. Boca Raton: CRC Press.
49. Chan MM *et al.* (1995) Effects of three dietary phytochemicals from tea, rosemary and turmeric on inflammation-induced nitrite production. *Cancer Lett* 96:23-9.
50. Naidu AS, Bidlack WR, Crecelius AT (2000) Flavonoids, In 'Natural Food Antimicrobial Systems', ed. AS Naidu, pp. 325-48. Boca Raton: CRC Press.

## ABOUT THE AUTHOR

**Dr. A.S. 'Narain' Naidu**, is CEO, en-N-tech Inc.; Professor/Director, Center for Antimicrobial Research at the California State Polytechnic University, Pomona, California, USA. 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/recognitions 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.