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January 4, 2001

Linda S. Kahl, Ph.D.
Office of Premarket Approval (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street, S.W.
Washington, DC 20204

Dear Dr. Kahl:

This is to notify you that Farmland National Beef Packaging Company, L.P. (National Beef), claims that the use of the substance described in the enclosed dossier (provided in triplicate) is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because National Beef has determined such use to be Generally Recognized As Safe (GRAS) based on scientific procedures.

All required information is provided in the dossiers, including assurance that the data and information that are the basis for this GRAS determination will be sent to FDA upon request, or are available for the FDA's review and copying at reasonable times at the offices of National Beef or ENVIRON.

Sincerely,

James T. Heimbach, Ph.D., F.A.C.N.
Principal (As Agent for National Beef)

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GENERALLY RECOGNIZED AS SAFE (GRAS) NOTIFICATION FOR BOVINE LACTOFERRIN AS A COMPONENT OF A SPRAY TO PREVENT MICROBIAL CONTAMINATION OF BEEF PRODUCTS

Prepared for

Farmland National Beef Packaging Company, L.P
Liberal, Kansas

Prepared by

Environ International Corporation
Arlington, Virginia

January, 2001

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I. GRAS EXEMPTION CLAIM

This is to notify you that Farmland National Beef Packaging Company, L.P. (National Beef), claims that the use of the substance described below is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because National Beef has determined such use to be Generally Recognized As Safe (GRAS).

This determination and notification are in compliance with proposed Sec. 170.36 of Part 21 of the *Code of Federal Regulations* (21 CFR 170.36) as published in the *Federal Register*, Vol. 62, No. 74, p. 18936 *et seq.*, April 17, 1997.

A. Name and Address of Notifier

Farmland National Beef Packaging Company, L.P.
1501 East 8th Street
Liberal, Kansas 67905

Contact: Eric B. Hale, Vice President for Special Projects
Telephone: 801-694-4659
Fascimile: 801-281-0550

B. Name of GRAS Substance

Lactoferrin is the common or trade name for the iron-binding glycoprotein isolated from the bovine species. Published literature may also refer to lactoferrin as red milk protein, lactosiderophilin, ekkrinosiderophilin, or lactotransferrin (Brock, 1980; Naidu, 2000).

C. Intended Technical Effect

Bovine lactoferrin is known to be an effective natural microbial blocking agent (MBA). Antimicrobial sprays containing lactoferrin have been shown to prevent microbial contamination by a wide variety of microbes. The intended technical effect associated with this usage of lactoferrin is to provide an additional barrier to prevent microbial contamination of processed beef.

D. Intended Use and Consumer Exposure

Bovine lactoferrin is intended for use as a component of an antimicrobial spray product that will be applied to processed fresh beef. The water-based spray product will contain no more than 2% lactoferrin; the other components of the spray are food-grade materials approved for

direct addition to foods by the Food and Drug Administration (FDA) and regarded as appropriate for use on meat products by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA). Thus the current GRAS determination is for lactoferrin alone. Potential exposures to consumers may arise from residual levels of lactoferrin remaining on the beef products at the time of consumption, although the levels of residual lactoferrin will be relatively insignificant compared to the normal amount of lactoferrin present in the diet, primarily from milk and milk products but also from its natural occurrence in beef (see Section IV.A). The 90th percentile of intake of lactoferrin from the intended use by consumers who eat beef is estimated to be 9.1 mg/person/day. This is less than 20% of current 90th percentile intakes of lactoferrin from its natural occurrence in milk and milk products, which range from 75 mg/person/day for teenagers age 13-19 to 50 mg/person/day for adults age 20 or older.

Note also that bovine lactoferrin is GRAS for use as an ingredient in sports or functional foods and dietary supplements at an intake level up to and including 3.6 g/day (DMV International, GRN#42, received by FDA April 10, 2000), an exposure approximately 400 times higher than that resulting from the intended use.

E. Basis for GRAS Determination

National Beef's determination of the GRAS status of bovine lactoferrin for the use described is based on scientific procedures as specified in 21 CFR 170.30(b).

Bovine lactoferrin intake resulting from this intended use is safe, and is also GRAS under the Federal Food, Drug, and Cosmetic Act (FDCA), based on the views of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. This determination is based on the generally accepted safety of lactoferrin as established in the available scientific literature and on the potential exposures to the general population that are associated with the proposed use. Based on the long history of safe consumption of lactoferrin from dairy sources and dietary supplements and the lack of adverse findings in clinical trials at doses up to 3.6 g/day, an acceptable daily intake ("ADI") for bovine lactoferrin is less than or equal to 3.6 g/day for the general population (see Section IV.F).

The evaluation of the safety of lactoferrin under the intended conditions of use is accomplished by comparing the ADI to the estimated exposures to lactoferrin associated with the proposed use. The estimated daily intake ("EDI") of lactoferrin from the proposed use is 9.1 mg/person/day (see Section IV.B). Thus, the EDI for the proposed use is 0.25% (1/400) of the ADI, and the proposed use of lactoferrin as described in this document is GRAS.

F. Availability of Information

The data and information that serve as the basis for this GRAS determination will be sent to FDA upon request, or are available for the FDA's review and copying at reasonable times at the offices of the notifier as listed in Section A, above, or at the offices of ENVIRON International Corporation (4350 Fairfax Drive, Arlington, Virginia 22203).

II. DESCRIPTION OF SUBSTANCE

A. Physical and Chemical Composition

Bovine lactoferrin has a molecular weight of approximately 75 to 80-kDa (Table 1). The isoelectric point (pI) of lactoferrin measures 8.2 to 8.9 by the chromatofocusing method (Shimazaki et al. 1993) and 9.5 to 10 by the isoelectric focusing method (Yoshida and Xiuyun 1991). Desaturated lactoferrin (apo-form) has an absorbance of 12.7 when measured at 280 nm (Aisen & Leibman 1972). The absorbance of iron-saturated lactoferrin (holo-form) is 0.400 at 470 nm. Holo-lactoferrin is more resistant to thermal denaturation than desaturated apo-lactoferrin (Paulsson et al. 1993).

Bovine lactoferrin consist of a single polypeptide chain of about 708 amino acids (Goodman and Schanbacher 1991). The sequence has been determined by cDNA and protein sequencing (Mead and Tweedie 1990). The protein contains intramolecular disulfide bonds but is absent of free sulfhydryl groups. Lactoferrin is glycosylated at two different sites by N-linked glycans of the N-acetyllactosaminic type. These glycans are characterized by α-1,3-linked galactose residues in the terminal nonreducing position. Unlike human lactoferrin, bovine lactoferrin also contains glycans of the oligomannosidic type (Spik et al. 1988; Spik et al. 1994).

The polypeptide chain consists of two separate lobes (Figure 1) that are linked by a three-turn α-helix (Nuijens et al. 1996). These lobes, designated N- and C- terminal, are similar in amino acid sequence (Lönnerdal and Iyer 1995). The tertiary structure of this glycoprotein has two iron-binding sites, giving it the capability to bind two molecules of iron per molecule of protein. Carbohydrate analysis shows that each molecule of bovine lactoferrin contains 1 residue of terminal sialic acids, 10 to11 residues of N-acetylglucosamine, 5–6 residues of galactose and 15 to 16 residues of mannose (Castellino et al. 1970).

Table 1. Physical-Chemical Properties of Lactoferrin

Property	Bovine Lactoferrin	Reference
Molecular mass		
Sedimentation co-efficient	77,200 ± 1,300	Castellino et al. 1970
SDS-PAGE	76,000 ± 2,400	Querinjean et al. 1971
Iron titration	78,500	Aisen & Leibman 1972
Isoelectric		
Chromato focusing	8.2-8.9	Shimazaki et al. 1993
Isoelectric focusing	9.5-10.0	Yoshida and Xiuyun 1991
Absorption spectra		Aisen & Leibman 1972
Apo-form at 280 nm	12.7	
Holo-form at 470 nm	0.400	
Glycosylation	Low	Metz-Boutigue et al. 1984
Protease sensitivity	High	Brines & Brock 1983
IgA-complexes	Absent	Watanabe et al. 1984
Iron-binding		Aisen & Leibman 1972
Equilibrium dialysis (K1 x 10^{-4})	3.73	
Thermal denaturation		Paulsson et al. 1993
Apo-LF denaturation (Tmax: °C)	71 ± .3 & 90 ± .3	
Apo-LF enthalpy (ΔH_{cal}: J/g)	12 ± .4 & 2 ± .5	
Holo-LF denaturation(Tmax: °C)	65 ± .3 & 93 ± .3	
Holo-LF enthalpy (ΔH_{cal}: J/g)	2 ± 1 & 37 ± 1	

Based on Naidu 2000

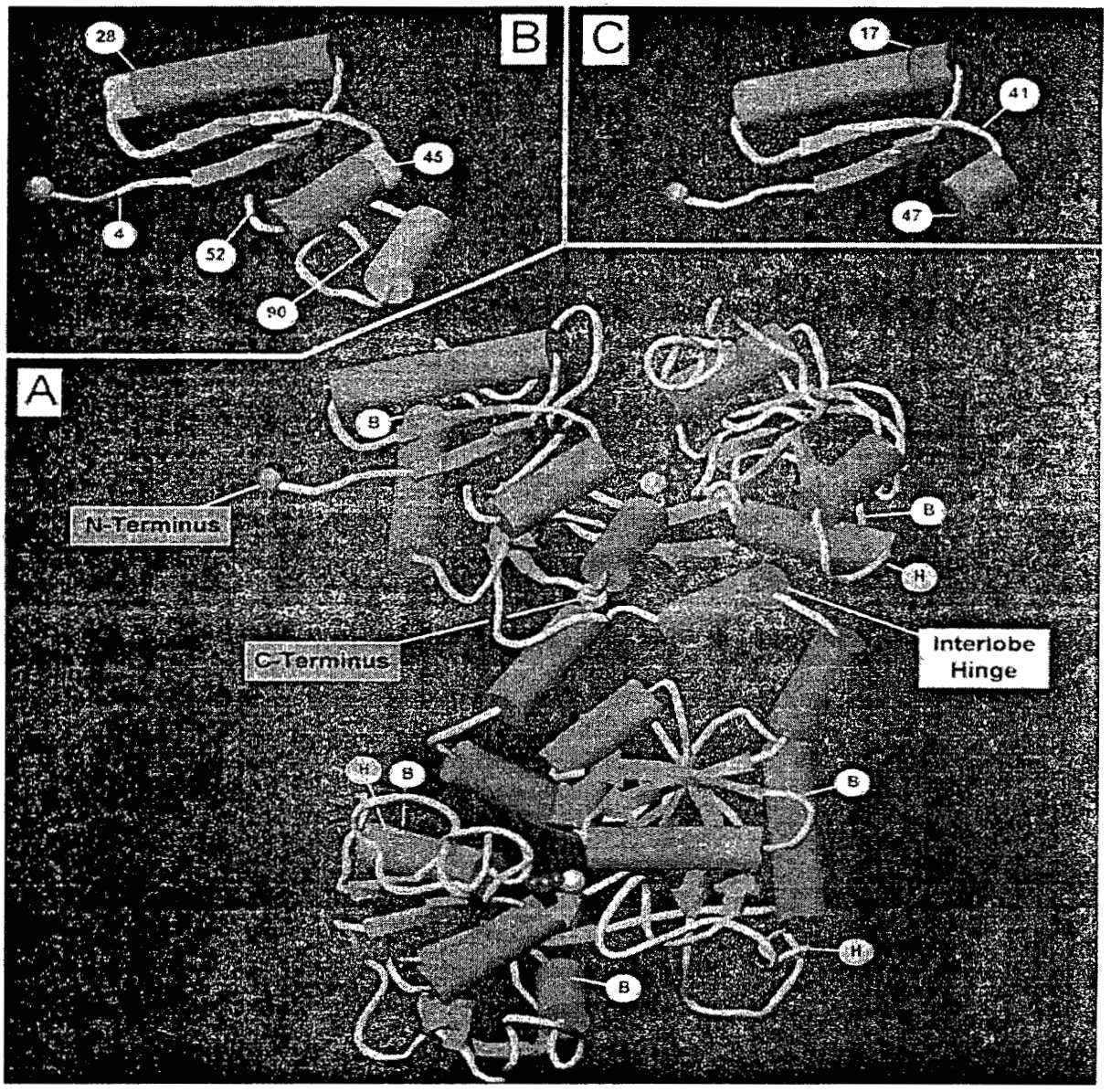

Figure 1. Diagram of the Linear Sequence & Three-Dimensional Structure of Human Lactoferrin
Secondary structure features are represented by cylinders (helices) and twisted arrows (beta-strands). The linear sequence diagram of lactoferrin shows the relative sequence location of the bactericidal peptides (lactoferricins H and B) and lymphocyte receptor binding regions, based on hLf sequence. The three-dimensional structure of human lactoferrin (A) shows iron (orange sphere) and coordinating anion (purple spheres) in the iron-binding site between the two domains (I & 11) within each lobe. The N-terminus is indicated by a blue sphere. The C-terminus (green sphere) and interlobe hinge helix (labeled) are shown for the full structure in (A). Alpha-helical structure is shown as red cylinders; P-strands as blue arrows. The glycosylation sites for human (H, gray) and bovine (B, white) lactoferrin are indicated as attached circles. Outsets highlight the N-terminal bioactive region showing: (B) the primary large (1-90) and the minimum (hLF 4-52, darker colors) lymphocyte receptor binding regions with the two putative receptor binding subregions (green) spanning residues 28-45, and (C) the bactericidal peptide regions of human (hLf 1-47, lactoferricin H) and bovine (bLf 17-41, lactoferricin B, shown in orange and green) lactoferrins superimposed onto the hLF structure. Diagram based on Nuijens et al. 1996.

B. Manufacturing Process

1. General Description of the Production Process

Bovine lactoferrin is extracted from cow's milk or milk derivatives such as whey. Raw milk is heated to 50°C, separated into skim milk and cream, and pasteurized at 70°C for at least 20 seconds. Whey or pasteurized skim milk is subjected to microfiltration in order to reduce microbial load and fat content. The filtrate is passed over a cation exchanger at a high velocity and high liquid load. Lactoferrin, which is adsorbed into the resin, is eluted and filtered through various systems before being spray dried. Figure 2 identifies each step in the production process. A detailed explanation of the process is disclosed in US Patent No. 5,596,082.

2. Processing Aids and Chemicals

Processing chemicals and aids used in the production of bovine lactoferrin are listed in Table 2. Processing chemicals consist of food grade sodium chloride, phosphoric acid (75%), disodium phosphate, and demineralized water. Processing chemicals comply with 21 CFR 182.1(a), 182.1073, and 182.6290, respectively. Processing aids are also FDA compliant (comply with 21 CFR 170.30(b), 177.2910 (a), 177.2240, and 177.2510) and include SP Sepharose Ion Exchange Media, microfilters and ultrafilters.

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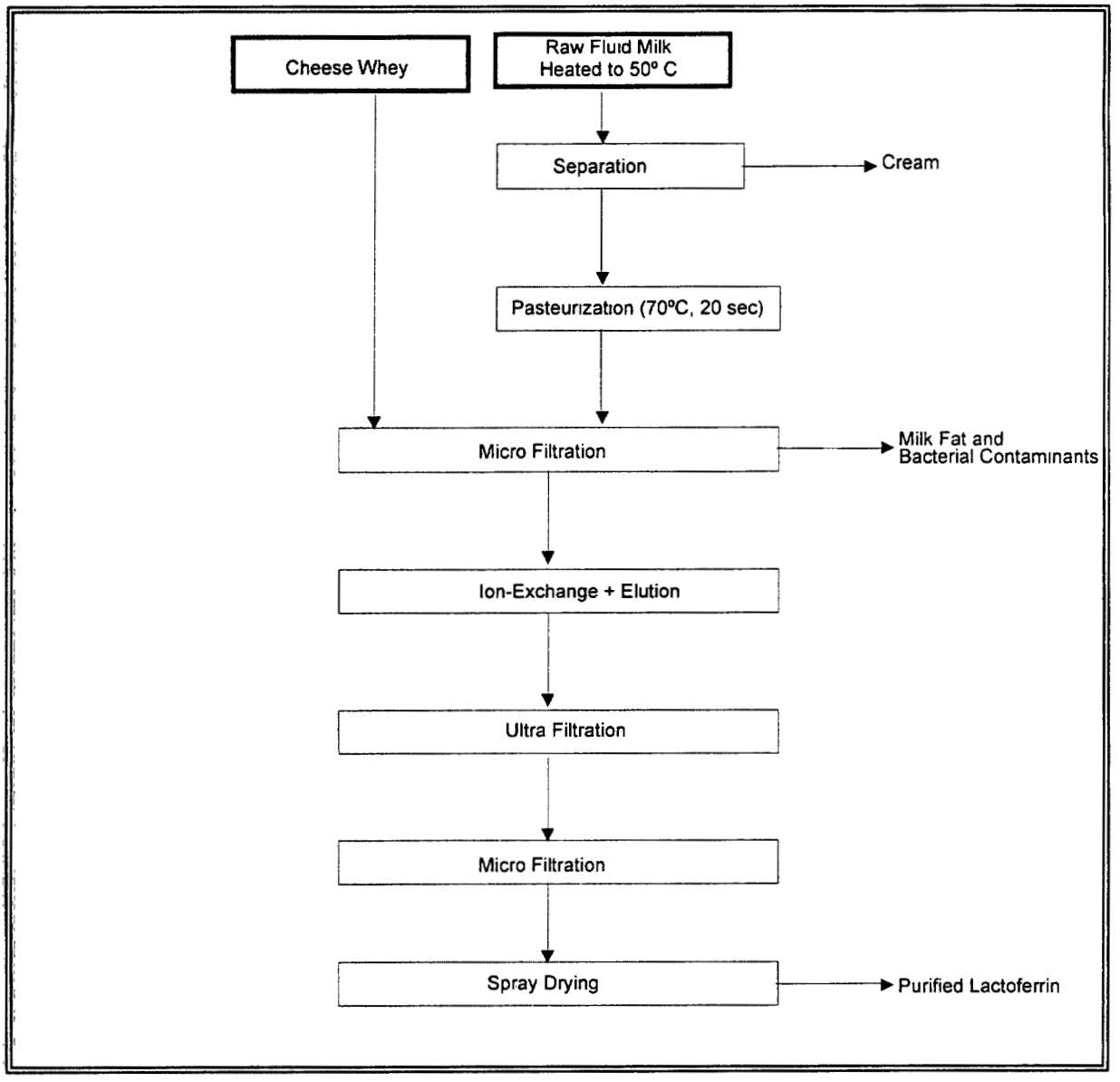

Figure 2. Diagram of the Production Process of Bovine Lactoferrin

Table 2. Processing Aids and Chemicals Used in the Production of Lactoferrin

Process Aid or Chemical	Manufacturer
Demineralized water	DMV International
Disodium phosphate	CFB – Chemische Fabrik Budenheim Rudolf A Oetker
Phosphoric acid (75%)	Caldic Nederland BV
Sodium chloride	Centrale Aankoop FNZ
SP Sepharose Ion Exchange Media	Pharmacia
Membralox Ceramic Membrane	SCT
HFK-131 (PES) Spiral Membrane	KOCH Membrane Systems
Durapore – PVDF	Millipore

3. Finished Product Specifications

The manufacturing specifications for bovine lactoferrin appear in Table 3. The product consists of 93.0% protein, 6% moisture and 1% ash. Lactoferrin makes up 95% of the protein content. Typical pH range of the finished product is between 5.5 to 6.5 with a water activity (a_w) of 0.2. Complete solubility is achieved when 2 g of lactoferrin is added to 100 ml of 20°C water. The iron binding capacity of the final product is ≥70%.

Table 3. Manufacturing Specifications for Bovine Lactoferrin

Chemical/Physical/Microbial Characteristics	Specifications
Protein	≥ 93.0%
• Lactoferrin	≥ 95.0%
Moisture	≤ 6.0%
Ash	≤ 1.0%
pH (2%, 20°C)	5.5-6.5
a_w – not measured on a routine basis	.2
Solubility	
• In water (2%, 20°C)	≥ 100%
• Transmittance, 2% sol., 600nm	≥ 80%
Iron Binding	
• Spectrophotometric method at 465 nm on solids	≥ 70%
Foreign matter (10g)	Absent
Heavy metals (as Pb)	≤ 1 mg/kg
Lead	<0.5 mg/kg
Arsenic	<1.0 mg/kg
Cadmium	<0.05 mg/kg
Mercury	<0.05 mg/kg
Copper	<2.0 mg/kg
PCB's [1]	<0.1 mg/kg
Pesticides [2]	<0.1 mg/kg
Antibiotics	<0.005 IU/ml
Alfatoxin MI	<0.5 mcg/kg
Radioactivity [3]	<5.0 Bq/kg
Standard plate count	≤ 1000/g
Enterobacteriaceae	≤ 10/g
Salmonella 2 x 1 g	Negative/50g
S. aureus	Negative
Total yeast and mold	≤ 10/g

[1] PCB 28, PCB 52, PCB 101, PCB 118, PCB 138, PCB 153, PCB 180

[2] Dieldrin, heptachlor epoxide, hexachlorobenzene, α-hexachlorocyclohexane, β-hexachlorocyclohexane, lindane, DDT-total

[3] Cs 134 + Cs 137

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4. Pathogenic Bacteria

Bovine lactoferrin was also analyzed for the presence of *Listeria monocytogenes*, *Clostridium perfringens*, *E. coli*, Brucellosis *and Yersinia enterocolytica* because they are potential contaminants of raw milk. No pathogenic bacteria were detected (Table 4).

Table 4. Pathogenic Bacteria Analysis of Bovine Lactoferrin

Microorganism	Result
Listeria monocytogenes	Not detectable
Clostridium perfringens	Not detectable
Escherichia coli	Not detectable
Brucellosis	Not detectable
Yersinia enterocolytica	Not detectable

III. INTENDED TECHNICAL EFFECT

Bovine lactoferrin is known to be an effective natural microbial blocking agent (MBA). Antimicrobial sprays containing lactoferrin have been shown to prevent microbial contamination by a wide variety of microbes. The intended technical effect associated with this usage of lactoferrin is to provide an additional barrier to prevent microbial contamination of processed beef.

A. Mechanism of Antimicrobial Action

The ability of lactoferrin to inhibit the growth of a wide variety of pathogenic microorganisms has been well documented (Kalfas et al. 1991; Naidu et al. 1991; Zagulski et al. 1989,1998; Bhimani et al. 1999). Both bovine and human lactoferrin appear to possess this property, as demonstrated by the enhanced survival rates of mice pretreated with either bovine or human lactoferrin prior to challenge with *E. coli* (Zagulski et al. 1989). Lactoferricin, a potent antimicrobial peptide produced by gastric cleavage of lactoferrin, was also found to enhance survival rates of mice challenged with *Toxoplasma gondii* infection (Isamida et al. 1998). A summary of the studies relevant to elucidating the mechanism of lactoferrin's antimicrobial effect both *in vitro* and *in vivo* is provided in Table 5.

The antimicrobial spectrum of lactoferrin includes stasis, cidal, cationic, phagocytic, and colonization/decolonization effects on susceptible microorganisms. Investigators have hypothesized that lactoferrin impedes iron utilization and causes bacteriostasis as a result of its iron-sequestering properties (Lönnerdal and Iyer 1995; Iyer and Lönnerdal 1993). However, the fact that some strains are resistant or unaffected by lactoferrin implies that bacteriostasis is not simply the result of iron sequestration (Bhimani et al. 1999). The selective interaction of lactoferrin with the microbial surface seems to play an essential role in regulating many of the antimicrobial events (Naidu and Arnold 1995; Alugupalli et al. 1995). A key event in this process is the binding of lactoferrin to specific outer membrane pore-forming proteins (porins) (Naidu and Bidlack 1998; Naidu et al. 1992, 1993; Naidu and Arnold 1994). Both human and bovine lactoferrin have been shown to bind to these proteins (Kishore et al. 1991). Bacterial susceptibility to lactoferrin is directly related to the magnitude of the lactoferrin-microbe interaction (Naidu et al. 1991). Certain strains show resistance to lactoferrin effects and do not demonstrate lactoferrin binding (Naidu et al. 1993). Although most bacteria express porins, the resistance of these bacteria was attributed to the shielding of the porin accessibility by the carbohydrate O-antigentic chains of lipopolysaccharide (LPS) (Naidu et al. 1993; Tigyi et al. 1992; Erdei et al. 1994).

Table 5. Antimicrobial Functions Of Lactoferrin

Study Design	Results	Comments	Reference
Human isolates of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Prevotella melaninogenica* from diseased periodontal pockets were examined for interaction with human lactoferrin in a standardized ^{125}I-labeled protein binding assay.	Strains of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Prevotella melaninogenica* interact with human lactoferrin in a standardized radioisotope-labeled protein binding assay. Nonspecific binding accounted for about 10, 75 and 60% of the total binding in P. *intermedia*, P. *melaninogenica* and P. *gingivalis*, respectively. Unlabeled human or bovine lactoferrin effectively displaced labeled human lactoferrin binding. All three species recognized both human and bovine lactoferrin to a comparable degree.	Lactoferrin is found in parotid saliva at about 10 µg/ml. As a secretory glycoprotein of polymorphonuclear leukocytes (PMN), a role of lactoferrin has been suggested in various host defense processes. Lactoferrin is bactericidal during exponential phase and bacteriostatic during the late stationary phase of bacterial growth. It may play an important role in host defense at the mucosal surfaces by causing deprivation of iron from bacteria. In addition to these properties, lactoferrin was shown in this study to bind to several strains of periodontis-associated bacteria. The antimicrobial effect of lactoferrin is related to its adsorption to the microbial surface. In such interactions, the metal-chelating property of lactoferrin enables damage to lipid bilayers in the outer cell membrane and the release of lipopolysaccharides.	Kalfas et al. 1991

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Study Design	Results	Comments	Reference
A total of 489 *Staphylococcus aureus* isolates from various clinical sources was examined for the occurrence of ^{125}I human lactoferrin binding.	Human lactoferrin binding was common among *S. aureus* strains associated with furunculosis (94.3%), toxic shock syndrome (94.3%), endocarditis (83.3%) and septicemia (82.8%) and other (nasal, vaginal or ocular) infections (96.1%). Furuncolosis-associated (low-invasive or localized) isolates were high-to-moderate binders of lactoferrin. In contrast, endocarditis-associated (high-invasive or systemic) isolates demonstrated low binding and did not bind lactoferrin at the above threshold level. *S. aureus* recognized human or bovine lactoferrin.	*S. aureus* is associated with human illnesses ranging from localized superficial infections such as folliculitis to severe systemic diseases such as fatal endocarditis and toxic shock syndrome. This pathogen requires iron for growth. During the early stages of pathogenesis, staphylococci confront inflammatory cell defense, mainly the polymorphonuclear leukocytes at mucosal surfaces. Among antibacterial compounds secreted by the PMNs is lactoferrin, an iron-binding protein. Lactoferrin deprives the invading pathogen of iron and provides an important defense at the host mucosal surface.	Naidu et al. 1991
A single dose of bovine lactoferrin was given intravenously (iv) to CFW mice 24 hours prior to the iv injection of the *E. coli* lethal dose (LD_{100}).	Bovine lactoferrin, when given iv, strongly accelerated the clearance rate of *E. coli* from the blood as well as its killing rate in the liver, lungs, spleen and kidney. Lactoferrin was much less effective in accelerating clearance of bacteria from the circulation when injected into animals subcutaneously. Unlike the case of clearing bacteria from the blood, however, subcutaneously injected lactoferrin efficiently stimulated killing of bacteria in internal organs similar to the case for the iv injected protein.	The results demonstrate that the protection afforded by lactoferrin against *E. coli* is primarily killing of the organisms. There is a dramatic acceleration of the clearing rate of liver *E. coli* from the blood.	Zagulski et al. 1998
A single intravenous dose of bovine lactoferrin was given to mice 24 hours before a challenge with a lethal dose of *E. coli*.	About 70% of mice pretreated with lactoferrin survived the challenge. The survival rates in control mice treated with *E. coli* alone and pretreated with bovine serum albumin were 4 and 8%, respectively. Human lactoferrin had almost the same protective effect as bovine lactoferrin.	This study demonstrated a protective role of iv administered lactoferrin in a systemic, nonspecific defense in mice during experimental infection with *E. coli*.	Zagulski et al. 1989

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Study Design	Results	Comments	Reference
Human and bovine lactoferrins were assessed *in vitro* and *in vivo* for their antibacterial effects on *Staphylococcus aureus*. Lactoferrin for feeding experiments was prepared by dissolving 2 grams of bovine lactoferrin in water or whole bovine milk and supplied to test animals ad libitum 1 day prior to infection. Animals were fed the experimental diet for a period of 14 days and sacrificed. Lactoferrin solutions were also prepared for injection and given to mice iv on day minus one. Mice were injected with *S. aureus* on day zero of the study and then sacrificed on day 14.	Lactoferrins showed weak *in vitro* antibacterial activity while Fe-saturated lactoferrins showed no activity. When lactoferrin was given to mice by iv injection, there was a 30-50% reduction in kidney infections and viable bacterial counts in the kidneys decreased 5-12 fold. Lactoferrins were effective when given 1 day prior to the bacterial challenge, after which there was no significant effect even at doses up to 5 mg. Apo- and Fe-saturated forms of human and bovine lactoferrins were all equally effective. Feeding mice with 2% bovine lactoferrin in drinking water also reduced the kidney infections by 40-60% and viable bacterial counts, 5-12 fold.	Antimicrobial action of lactoferrin has been attributed to the ability of lactoferrin to chelate Fe strongly, making this essential nutrient inaccessible to the invading pathogens. Evidence suggests, however, that the antimicrobial activity is more complex than simple Fe chelation. Lactoferrins have bactericidal activity that may involve direct interaction with the cell surface. Mechanisms of antibacterial effects may include blockade of bacterial carbohydrate metabolism, or destabilization of the bacterial cell surface. This study demonstrates the protective effects of lactoferrin feeding and injections *in vivo* against systemic kidney infections induced by *S. aureus* in mice.	Bhimani et al. 1999

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Study Design	Results	Comments	Reference
This study was aimed at elucidating the effect of lactoferrin-porin interaction on bacterial growth. For this purpose, the lactoferrin susceptibility patterns of different clinical isolates of *E. coli* with various magnitudes of lactoferrin binding capacity were examined. The effect of lactoferrin on bacterial growth was tested by measuring conductance changes in the cultivation media and was compared with the magnitude of ^{125}I labeled lactoferrin binding in 15 clinical isolates of *E. coli*. To illustrate the extent of lipopolysaccharide (LPS) involvement, various rough mutants of *S. typhimurium* with different lengths of polysaccharide moiety were tested for lactoferrin binding and susceptibility.	The binding property was inversely related to the change in bacterial metabolic rate and was directly related to the degree of bacteriostasis. The MIC is defined as the smallest amount of lactoferrin required to elicit a complete inhibition of bacterial growth at the time point when the metabolism of the control (bacteria grown without lactoferrin) reached stationary phase. The magnitude of lactoferrin-bacterium interaction showed no correlation with the MIC of lactoferrin. In certain strains, lactoferrin at supraoptimal levels reduced the bacteriostatic effect. Thus, the lactoferrin concentration in the growth media was critical for the antibacterial effect. The magnitude of lactoferrin binding to whole cells is inversely related to the length of the LPS polysaccharide moiety. Accordingly, the low-lactoferrin binding smooth parent was less susceptible to lactoferrin, and at corresponding conditions the growth of the LPS-deficient mutant was inhibited by 50%. Results of the study with *S. typhimurium* suggest that there is a correlation between lactoferrin binding to porins and the lactoferrin mediated antimicrobial effect.	Studies with clinical isolates of E. coli revealed that the concentration of lactoferrin in the growth media is a critical factor for the antibacterial effect. In addition, there is a correlation between lactoferrin binding to porins and the lactoferrin mediated antimicrobial effect. The polysaccharide moiety of lipopolysaccharides shielded porins from the lactoferrin interaction and concomitantly decreased the antibacterial effect.	Naidu et al. 1993

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Study Design	Results	Comments	Reference
The ability of *Shigella flexneri* to interact with lactoferrin was examined with a ^{125}I-labeled protein binding assay.	The percent binding of human lactoferrin and bovine lactoferrin to 45 *S. flexneri* strains was 19 ± 3 and 21 ± 3, respectively. ^{125}I-labeled human and bovine lactoferrin binding to strain M90T reached an equilibrium within 2 hours. Unlabeled human and bovine lactoferrin displaced the ^{125}I-labeled human lactoferrin-bacteria interaction in a dose-dependent manner. The cell envelope and outer membrane preparations showed three distinct horseradish peroxidase-lactoferrin reactive bands of about 39, 22, and 16 kDa. The 39 kDa component was also reactive to a monoclonal antibody specific for porin proteins of members of the family *Enterobacteriaceae*. The lactoferrin binding protein pattern was similar with bovine or human lactoferrin.	The results of this study establish that lactoferrin binds to specific components in the bacterial outer membrane; the heat-modifiable, anti-porin-reactive, and lipopolysaccharide-associated properties suggest that the lactoferrin binding proteins are porins in *S. flexneri*.	Tigyi et al. 1992
The purpose of this study was to examine the presence of a specific human lactoferrin binding protein in *S. aureus* and to characterize the lactoferrin-staphylococcal interaction in *S. aureus* strain MAS-89 (associated with toxic shock syndrome). A ^{125}I human lactoferrin binding assay was used.	The binding of ^{125}I human lactoferrin to strain MAS-89 reached saturation in <90 minutes and was maximal between pH 4 and 9. Unlabelled human lactoferrin displaced ^{125}I human lactoferrin binding. A Scatchard plot was non-linear; this implied a low affinity and high affinity binding mechanism. The staphylococcal lactoferrin binding protein was partially susceptible to proteolytic enzymes or periodate treatment and was resistant to glycosidases. An active binding protein with an apparent M_r of c. 450 Kda was isolated from strain MAS-89 cell lysate by ion-exchange chromatography on Q-sepharose. In SDS-PAGE, the reduced binding protein was resolved into two components of 67 and 62 Kda. The two components demonstrated a positive reaction with human lactoferrin-horseradish peroxidase in a Western blot.	The data from this study establishes that there is a specific receptor for human lactoferrin in *S aureus*. This receptor-ligand interaction is distinct from the staphylococcal receptors binding to plasma or connective tissue proteins described previously.	Naidu et al. 1992

000022

Study Design	Results	Comments	Reference
The purpose of this study was to elucidate the interaction between lactoferrin and binding sites on *E. coli* using ^{125}I-labeled human lactoferrin.	A band with a mass of approximately 37 kDa, which was reactive with horseradish peroxidase-labeled Lf, was identified in the boiled cell envelope and outer membrane preparations of a lactoferrin binding *E. coli* strain (E34663) and a non-lactoferrin binding strain (HH45), by SDS-PAGE and Western blotting. Such a band was not detected in the unboiled native cell envelope and outer membrane preparations. The molecular mass and the property of heat modifiability suggested that the lactoferrin-binding proteins were porins. OmpF and OmpC were identified as the lactoferrin binding proteins by urea-SDS-PAGE and Western blotting and by ^{125}I-lactoferrin binding studies with intact bacteria.	The data from this study establishes that lactoferrin 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*, these pore forming proteins are shielded from lactoferrin interaction.	Erdei et al. 1994
The goal of this study was to confirm porin involvement in lactoferrin binding to Salmonella by simultaneously probing with a monoclonal antibody specific for a conserved porin domain (Pol) in different bacterial species of enterobacteriaceae and to examine the effect of lactoferrin interaction on the susceptibility of selected *Salmonella* species to antibiotics.	Interaction of both human and bovine lactoferrin with the cell envelope and outer membrane of *Salmonella typhiumurium* type strain ATCC13311 was confirmed to occur with a porin. Such lactoferrin binding porin proteins (37-39 kD range) were readily detected in nine other common Salmonella species. The antibiotic susceptibility of Salmonella in the presence of lactoferrin indicated that lactoferrin potentiates the effect of certain antibiotics.	Results further confirmed earlier reports of porin involvement in specific interaction of lactoferrin with bacterial members of the enterobacteriaceae family. Lactoferrin enhanced the susceptibility of bacteria for both types of antibiotics that cross the outer membrane either by a porin pathway or via a hydrophobic route. The affected antibiotics increased the antibacterial spectrum of bovine lactoferrin in an additive manner.	Naidu and Arnold 1994
The interaction of lactoferrin with *Actinobacilus actinomycetecomitans* was examined in a ^{125}I-labeled protein binding assay.	Binding of human and bovine lactoferrins reached maximum within 1 hour. Lactoferrin binding to the bacterium was pH dependent and reversible. Scatchard analysis indicated the existence of both high and low affinity binding sites. Bacteria in the exponential phase of growth showed higher binding than cells in the stationary phase. SDS-PAGE and Western blot analysis of the cell envelope as well as the outer membrane revealed lactoferrin reactive protein bands at 29 kDa and 16.5 kDa.	Data suggests a specific interaction of lactoferrin with outer membrane proteins of *Actinobacilus actinomycetecomitans*.	Alugupalli et al. 1995

000023

Study Design	Results	Comments	Reference
The interaction of lactoferrin with *Aeromonas hydrophila* was tested in a ^{125}I-labeled protein binding assay.	The mean percent binding values for human and bovine lactoferrin were 13.4 ± 2.0 and 17.5 ± 2.7 (mean + SEM), respectively. The human and bovine binding reached a complete saturation within 2 hours. Unlabeled human and bovine lactoferrin displaced ^{125}I-human lactoferrin binding in a dose-dependent manner and more effectively by the heterologous (1 μg for 50% inhibition) than the homologous (10 μg for 50% inhibition) ligand. Apo- and holo-forms of human and bovine lactoferrin inhibited more than 80%, while mucin caused approximately 50% inhibition of the human lactoferrin binding. Various other proteins (including transferring) or carbohydrates did not block the binding. Two binding proteins were identified in the boiled cell envelope preparation while the unboiled cell envelope demonstrated a short-ladder pattern at the top of the separating gel and a second band at approximately 60 kDa position These corresponded to porins.	Data establish a specific interaction of lactoferrin with *Aeromonas hydrophila* and the lactoferrin binding proteins seem to be porins.	Kishore et al. 1991
The protective effect of lactoferricin, a potent antimicrobial peptide produced by gastric pepsin cleavage of lactoferrin, against *Toxoplasma gondii* infection was examined in experimental murine toxoplasmosis. Mice were administered 5.0 mg lactoferricin orally and challenged with cysts of *T. gondii* at a dose level of LD_{90}.	All mice orally administered lactoferricin survived until the end of the experiment (35 days post challenge). Intraperitoneal administration of 0.1 mg of lactoferricin also prevented death in 100% of treated mice challenged with *T. gondii* cysts. In contrast, 80% of untreated mice died of acute toxoplasmosis within 14 days post challenge. In the mice treated perorally with lactoferricin, the number of cysts in the brain was significantly lower than that in untreated mice. Levels of interferon-r in the serum of infected mice treated perorally with lactoferricin showed a tendency to be lower than those in the infected mice without treatment.	Results demonstrate that oral administration of lactoferricin induces resistance to *T. gondii* infection in mice.	Isamida et al. 1998

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IV. INTENDED USE AND CONSUMER EXPOSURE

A. Historical Background Exposure

1. Human Lactoferrin

Human lactoferrin (hLF) is a naturally occurring protein that is found mainly in external secretions, such as breast milk, mucosal surfaces and in the secondary granules of the neutrophils (Chierici et al. 1994; Nuijens et al. 1996). Lactoferrin is present in human plasma at a concentration of about 0.2 to 1.5 mcg/ml (Rumke et al. 1975). It is synthesized by the epithelium and neutrophils and is released by these cells in response to inflammatory stimuli (Lönnerdale and Iyer 1995). Lactoferrin present in mother's milk may be involved in the host resistance mechanism of breast-fed infants (Bezkorovainy, 1977; Goldman and Smith 1973). It has been shown to inhibit the growth of a number of microorganisms (Chierici et al. 1994; Nuijens et al., 1996); play a role in intestinal iron uptake and excretion (Lönnerdale and Iyer 1995); and promote the growth of intestinal epithelial cells (Nuijens et al. 1996).

Johansson (1960) isolated lactoferrin from human breast milk and determined it was very similar to the serum iron-binding protein transferrin. The complete amino acid sequence (703 amino acid residues) of human lactoferrin was determined by Metz-Boutique (1984). The single polypeptide chain is folded into two lobes. Each lobe contains a single iron-binding site and a single glycosylation site. The protein contains 5 to 6% carbohydrate, mainly galactose, mannose, fructose, N-acetylglucosamine and sialic acid (Jenness 1979; Mazurier 1974).

a) Concentration in Human Milk

Human colostrum, which is expressed during the first 3 days postpartum, contains the highest concentration of lactoferrin, 3.1 to 0.58 mg/ml. Human milk also contains a relatively large amount of lactoferrin with a concentration ranging between 1 and 4.20 mg/ml (Table 6) with a mean of approximately 2.0 mg/ml. The changes in lactoferrin concentration during the different stages of lactation were determined by Lönnerdal et al. (1976b) and are presented in Table 7. As lactation period increases, the concentration of lactoferrin in the milk decreases.

Table 6. Concentration of Lactoferrin in Human Colostrum and Milk

Human Colostrum mg/ml	Human Milk mg/ml	Reference
10.58	1.74	Fransson and Lönnerdal 1980
3.3	1.67	Hambraeus et al. 1978
	1-2	Hennart et al. 1991
6.7	2.6	Hirai et al. 1990
	1.55	Lönnerdal et al. 1976a
	4.2	Masson and Heremans 1971
3.1		Mathur et al. 1990
	2.1	Nagasawa et al. 1972
	1.97-3.20	Pamblanco et al. 1986
	1-3	Rudloff and Kunz 1997
	> 2	Tomita M. 1999
	1.4-2.6	Velona et al. 1999

Table 7. Change in Lactoferrin Concentration (mg/ml) in Human Milk during Lactation

Months postpartum				Reference
0-0.5	0.5-1.5	1.5-3.5	3.5-6.5	
3.53	1.94	1.65	1.39	Lönnerdal et al. 1976b

b) Comparison of Human and Bovine Lactoferrin

The similarities in and differences between bovine and human lactoferrin have been studied extensively. The amino acid composition (mole %) of the two proteins is shown in Table 8. The nucleic acid sequence and the amino acid sequence of bovine lactoferrin are homologous (77% and 68%, respectively) with published sequences for human lactoferrin (Lönnerdal and Iyer 1995). The two also share extensive homology in disulfide bonding, the lack of free sulfhydryls, and in the secondary structure (Crichton 1990).

Baker et al. (1994) used high resolution crystallography to study the three-dimensional structure of various forms of lactoferrin. The most striking difference between human and bovine lactoferrin was in the relative orientation of the two lobes. If the N-lobes of the two lactoferrins were superimposed, one would have to rotate the C-lobes approximately 12° before they would line up.

The authors noted that only one of the four glycosylation sites on bovine lactoferrin was also found on human lactoferrin.

Wang et al. (1984) studied the chemical and immunochemical properties of human and bovine lactoferrin. Results showed that bovine lactoferrin could only be differentiated from human lactoferrin by immunochemical analysis. Bovine lactoferrin was shown to have an iron content approximately four-fold higher than that of human lactoferrin. Since lactoferrin bacteriostatic properties are inversely related to the extent of iron saturation, the authors concluded that bovine lactoferrin is less effective as an anti-infectious reagent than human lactoferrin.

Table 8. Amino Acid Composition of Bovine and Human Lactoferrin

Amino Acid	Bovine Lactoferrin mole %	Human Lactoferrin mole %
Lysine	7.75	6.44
Histidine	1.38	1.19
Arginine	5.82	5.49
Aspartic acid	9.72	10.84
Threonine	5.22	5.32
Serine	5.02	6.70
Glutamic acid	9.84	10.41
Proline	4.46	4.30
Glycine	7.43	7.71
Alanine	9.79	8.88
Half-cystine	3.84	4.49
Valine	6.64	7.28
Methionine	0.38	0.21
Isoleucine	2.62	2.78
Leucine	10.58	8.23
Tyrosine	3.46	2.79
Phenylalanine	4.32	3.96
Tryptophan	1.67	2.94

Source: Wang et al. 1984

2. Bovine Lactoferrin

Milk from cattle (*Bos taurus*) has been consumed by the human population for centuries. Bovine milk is composed of a complex mixture of lipids, proteins, carbohydrates, vitamins, and minerals. The average composition of milk consists of 86.6% water, 4.1% fat, 3.6% protein, 5.0% lactose, and 0.7% ash (Swaisgood et al 1985).

Milk protein is a combination of caseins and whey proteins. Their relative amounts are shown in Table 9. Caseins account for 79% (27 mg/ml) of the total milk proteins. The remaining 21% (7.1 mg/ml) is whey protein. Lactoferrin, which is a whey protein,

accounts for 0.3% (0.1 mg/ml) of the total protein in bovine milk (Barth and Behnke 1997).

Lactoferrin was first isolated from bovine milk by Groves in 1960. Since then several investigators have isolated and characterized this protein (Goodman and Schanbacher 1991; Magnuson et al. 1990; Mead and Tweedie 1990; Rejman et al. 1989). Bovine lactoferrin has been shown to inhibit the growth of a number of microorganisms including *Listeria monocytogenes* (Wakabayashi et al. 1992).

Table 9. Concentration of Proteins in Bovine Milk

Components	mg/ml Milk	%
Milk Proteins	34.1	100
• Caseins	27.0	79
• Whey Proteins	7.1	21
• Lactoferrin	0.1	0.3
[1] Adapted from Barth and Behnke 1997.		

a) Concentration in Bovine Milk and Milk Products

Just as in humans, the highest concentration of lactoferrin is found in the cow's colostrum (2 to 5 mg/ml) which is expressed the first few days postpartum (Table 10). Milk, by definition, is a lacteal secretion that is free of colostrum (21 CFR 182.6290). The average lactoferrin content of milk is approximately 0.1 mg/ml; which means an 8-oz glass of milk contains about 23 mg of lactoferrin.

Milk must be thermally processed by High Temperature Short Time (HTST) pasteurization or ultra-pasteurization (UP) in order to ensure consumer safety (FDA, 1997). Saito et al. (1994) showed that lactoferrin was easily denatured at ≥ pH 6 when heated at 80 to 120°C for 5 min. The time-temperature relation used in this experiment exceeds the guidelines for both HTST (72°C for 15 sec) and UP (138°C for 2 sec) treatments. Therefore the results are not applicable to the lactoferrin content in pasteurized milk.

Paulsson et al. (1993) looked at the thermal behavior of HSTS pasteurized and UP-treated bovine lactoferrin and iron-saturated lactoferrin. Pasteurized lactoferrin showed some signs of denaturation but iron-saturated lactoferrin was not affected by pasteurization. The UP-treatment denatured both lactoferrin and iron-saturated lactoferrin. The thermal denaturation (protein unfolding) is reversible upon cooling (Ruegg et al. 1977). Although pasteurization does not

denature lactoferrin, its effect on the biological properties of the protein has yet to be determined.

Table 10. Concentration of Lactoferrin in Cow's Colostrum and Milk

Cow's Colostrum mg/ml	Cow's Milk mg/ml	Reference
	0.1	Barth and Behnke 1997
	Traces	Gordon and Kalan 1974
	0.02-0.2	Masson and Heremans 1971
2-5	0.02-0.2	Reiter 1978
	Traces	Rudloff and Kunz 1997
	0.02-0.2	Tomita 1999
2-5	0.1-0.3	Welty et al. 1975

Using food intake data from the 1994-96 United States Department of Agriculture's (USDA) Continuing Survey of Food Intakes by Individuals (CSFII), ENVIRON determined the average intake of milk and milk products on both a gram per day (g/d) and gram per kilogram body weight per day (g/kg bw/d) basis (Table 11). The CSFII 1994-96 is a two-nonconsecutive-day database of dietary information from individuals of all ages that was collected between January 1994 and January 1997 through in-person interviews using 24-hour recalls. The CSFII 1994-96 sample was a stratified, multistage area probability sample. Sampling weights provided by USDA compensate for variable probabilities of selection, differential response rates, and possible deficiencies in the sampling frame. Weighted data were used to calculate mean intakes and 90th percentiles that are listed in the Table 11. The survey database also includes data on nutrients for each food reported.

On average, children 1 to12 and teens 13 to 19 years old consume approximately 396 and 377 g milk/day, respectively. This provides a mean intake of 38 to 40 mg lactoferrin/day. Adults (20+ years old) consume less milk, a mean of 240 g/d; thus their mean intake of lactoferrin is equal to about 24 mg/day.

Consumption of lactoferrin for milk consumers in the 90th percentile of intake averages 73 mg/d for children, 75 mg/d for teens and 50 mg/d for adults.

Table 11. Estimated Daily Intakes of Lactoferrin from Milk and Milk Products[1]

Population Group	Dairy Products		Protein in Dairy Products		Lactoferrin in Dairy Products	
	Mean 2 Day Average Intake per User	90th Percentile 2 Day Average Intake per User	Mean 2 Day Average Intake per User	90th Percentile 2 Day Average Intake per User	Mean 2 Day Average Intake per User[2]	90th Percentile 2 Day Average Intake per User
Children (1-12 yr.)	395.6 g/d (18.8 g/kg bw/d)	731.1 g/d (37.6 g/kg bw/d)	13.1 g/d (0.6 g/kg bw/d)	24.2 g/d (1.248 g/kg bw/d)	40 mg/d (1.9 mg/kg bw/d)	73 mg/d (3.8 mg/kg bw/d)
Teens (13-19 yr.)	377.1 g/d (6.2 g/kg bw/d)	746.8 g/d (12.4 g/kg bw/d)	12.5 g/d (0.2 g/kg bw/d)	25.1 g/d (0.417 g/kg bw/d)	38 mg/d (0.6 mg/kg bw/d)	75 mg/d (1.2 mg/kg bw/d)
Adults (20 yr. +)	240.3 g/d (3.3 g/kg bw/d)	500.4 g/d (7.1 g/kg bw/d)	8.0 g/d (0.1 g/kg bw/d)	16.9 g/d (0.238 g/kg bw/d)	24 mg/d (0.3 mg/kg bw/d)	50 mg/d (.7 mg/kg bw/d)

[1]Estimates reflect consumption of milk and milk products which includes all fluid and flavored milks, reconstituted milk, milkshakes, ice cream, puddings, cream and whipped cream.
Analyses include individuals who provided dietary recalls for two days and reported a body weight.
With the exception of the number of users, all results are based on weighted data; estimates calculated with WesVar Complex Samples 3.0 (SPSS, 1998).
Data source: 1994-96 Continuing Survey of Food Intakes by Individuals (USDA 2000).
[2] Calculations based on 0.1 mg lactoferrin per g of milk or 0.3% of protein consumption (Barth and Behnke 1997).

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b) Dietary Supplements

Lactoferrin is also currently marketed in dietary supplement form (products listed in Table 12) with dose recommendations ranging from 10 to 1200 mg/day.

Table 12. Concentration and Dosages of Lactoferrin in Dietary Supplements[1]

Product	Manufacturer, Country	Lactoferrin Concentration	Recommended Dosage	Total Daily Intake
Intestinal Flora Factors	Ethical Nutrients, USA	NR	8-12 tablets/day	
Jarro-Dophilus + Lactoferrin	Jarrow Formulas, Canada	200 mg/capsule	1-3 capsules/day	200-600 mg
Lactoferrin	GNC Corp., USA	50 mg/tablet	1-2 tablets/day	50 mg
Lactoferrin	Jarrow Formulas, Canada	250 mg/capsule	1 capsule/day	250 mg
Nutri Elle	Tonipharm Labs., France	10 mg/ capsule	1 capsule/day	10 mg
Nutri Femme	Tonipharm Labs., France	20 mg/capsule	1 capsule/day	20 mg
Nutrinatal	Tonipharm Labs., France	50 mg/capsule	1 capsule/day	50 mg
Oenobiol	Oenobiol Labs., France	356 mg/ tablet	NR	
Prime Colostrum, Lactoferrin, Pectin	Lactoferrin Products Co., USA	10 mg/lozenger	1 lozenger/day	10 mg
Sentosa Fe	Sentosa, Taiwan	100 mg/tablet	1-2 tablets/day	100-200 mg
Ultra Immune	Lactoferrin Inc. USA	50 mg/tablet	1 tablet/day	50 mg
Uzer Ferrine	Pharmafood, The Netherlands	100 mg/tablet	6-12 capsules/day	600-1200 mg

[1] Data retrieved from the World Wide Web.

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B. Estimated Exposure to Bovine lactoferrin from Proposed Use

1. Application Rates and Concentration of Lactoferrin in Beef:

The spray formulation (maximum 2% lactoferrin) is applied a maximum of three times to beef during processing: to carcasses, to subprimals, and to finished cuts. The application rates for these uses are as follows:

1. Beef carcass (average weight = 250-320 kg): 50 ml formulation
2. Subprimal (average weight = 9-10 kg): 5 ml formulation
3. Finished cut (average weight = 0.4-0.5 kg): 1 ml formulation

The maximum concentrations of formulation per kg beef occur with the smallest weights of beef, and thus the maximum concentrations of formulation are as follows:

1. Beef carcass: 0.20 ml formulation/kg beef
2. Subprimal: 0.56 ml formulation/kg beef
3. Finished cut: 2.50 ml formulation/kg beef

The maximum concentration of formulation in consumer-ready beef, assuming that a given finished cut was treated at all three levels of processing and that 100% of the formulation remains in the beef, is the sum of the above three concentrations or 3.26 ml formulation/kg beef.

The maximum concentration of lactoferrin in the formulation is 2.0% by weight, or 20.0 mg lactoferrin/ml formulation. Thus the maximum concentration of lactoferrin in a finished cut of beef is 65.2 mg lactoferrin/kg beef, or 0.0652 mg lactoferrin/g beef. (While a small amount of lactoferrin is naturally present in beef due to residual blood (Hayden 1978), this contributes only insignificantly to lactoferrin intake.)

2. Estimated Daily Intake of Lactoferrin From Proposed Use

Consumer consumption of beef and intake of lactoferrin from beef were estimated based on data from the 1994-96 USDA Continuing Survey of Food Intakes by Individuals (CSFII) and its Supplemental Children's Survey (CSFII 1998) as provided on CD-ROM (USDA, 2000). In order to estimate consumption of beef, which is frequently consumed in mixed dishes such as cheeseburgers or stews, the Food Commodity Intake Database, Version 2.1 (EPA, 2000) was used to determine the amount of beef present in all survey food codes.

Nearly 90% of consumers age 2 years or older reported consumption of at least one food containing beef as an ingredient. The mean daily consumption of beef among these beef consumers was 62.7 g and the 90th percentile of consumption was 139.2 g (Table 13). Since these intake data include consumers who may have eaten foods that contain only small amounts of beef, thus reducing the average intake per user, consumption was estimated for only those

who ate foods containing beef as a primary ingredient, such as a steak or a roast. While this restriction reduced the proportion of the population regarded as consumers of beef to only 43%, the mean and 90th percentile intakes were lower than those estimating using all beef consumption (Table 13). Thus, the higher numbers were used to estimate potential intake of lactoferrin from the proposed use.

Since the maximum concentration of lactoferrin in beef is 0.0652 mg lactoferrin/g beef, as shown in Table 13 the estimated mean lactoferrin intake is 4.1 mg/person/day and the 90th percentile of intake is 9.1 mg lactoferrin/person/day.

Table 13. Estimated Daily Intake of Lactoferrin from Proposed Use

TABLE 10

Population Group	All Beef Products[1]		Primary Cuts of Beef		Lactoferrin from Beef	
	Mean 2 Day Average Intake per User	90th Percentile 2 Day Average Intake per User	Mean 2 Day Average Intake per User	90th Percentile 2 Day Average Intake per User	Mean 2 Day Average Intake per User[2]	90th Percentile 2 Day Average Intake per User[2]
U.S. population age 2+ years	62.7 g	139.2 g	57.0 g	121.1 g	4.1 mg	9.1 mg

[1] Includes beef meat, fat, byproducts, and dried.
Analyses include individuals who provided dietary recalls for two days.
All results are based on weighted data; estimates calculated with WesVar Complex Samples 3.0 (SPSS, 1998).
Data source: 1994-96, 1998 Continuing Survey of Food Intakes by Individuals (USDA 2000).
[2] Calculations based on 0.0652 mg lactoferrin per g of beef and on consumption of all beef products.

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V. SAFETY ASSESSMENT

A. Fate of Lactoferrin in the GI Tract

After gastric emptying, digestion products are subjected to the action of pancreatic enzymes and subsequent membrane peptidase hydrolysis and absorption along the small intestine. Proteins, according to both their structure and the nature of the chyme, are degraded differently by gastric and pancreatic proteases into peptides and amino acids and taken up by the intestinal mucosa, where brush border membrane peptidases and transport systems are responsible for the ultimate transfer of amino acids to the blood. Study of the overall digestion and absorption process of milk proteins show that gastric emptying appears to be the major mechanism controlling the absorption kinetics of nitrogen from milk (Tomé and Debabbi 1998).

Some dietary proteins, however, may elude the luminal hydrolytic process and reach the intestinal mucosa in significant amounts, where they are subsequently absorbed through different mechanisms. Transcytosis of intact protein was detected for both β-lactoglobulin, α-lactalbumin, prolactin and lactoferrin but not for casein (Tomé and Debabbi 1998). The fact that lactoferrin is only slowly degraded, as demonstrated in the piglet (Schmitz et al. 1989); baby monkey (Lindberg et al. 1997), and human newborn (Lindberg et al. 1998; Davidson and Lönnerdal 1987, 1985; Spik et al. 1982), is of particular interest from a physiological perspective. Very limited proteolysis in the stomach might actually release fragments of lactoferrin that have more potent bactericidal activity than the native molecule (Hamosh 1998).

A review of the studies examining lactoferrin fate in the gastrointestinal tract is presented in Table 14. Studies in human infants demonstrate that considerable quantities of intact lactoferrin are excreted by the breast-fed infant. Higher amounts are excreted during the early weeks of life and the quantity excreted is progressively smaller during successive weekly measurements (Davidson and Lönnerdal 1987, 1985). During the first weeks of life, approximately 2 to 6% of the lactoferrin consumed by the infant is excreted in the feces per day, however, by 4 months, less than 2% of the lactoferrin intake is excreted. Increased proteolysis of the lactoferrin takes place as the infant ages. The lactoferrin excreted in the feces was demonstrated to be intact (Davidson and Lönnerdal 1987). The large amounts of lactoferrin excreted in the feces intact in infants, along with the finding in this study that no other major whey proteins are detected in the feces, reinforces the fact that lactoferrin is protected against degradation. This is consistent with its purported physiologic role as an antimicrobial agent in the digestive system of the infant. Two studies of lactoferrin excretion in infants fed formula containing supplemental lactoferrin (2.8 g/L and 285 mg/L) (Balmer et al. 1989; Fairweather-Tait et al. 1987) indicated that as expected, babies fed formula containing bovine lactoferrin excreted significantly more

lactoferrin in their stools than babies fed the basic formula. The levels excreted, however, were significantly lower than levels excreted from breast-fed babies. Most of the lactoferrin from the formula appeared to have been broken down.

Intact lactoferrin has also been found in the urine of human milk-fed preterm infants. Because serum levels of lactoferrin in both preterm and term infants do not vary with diet (i.e., human milk vs. bovine milk based formula), Hutchens et al. (1991) conducted a study to determine the origin of the lactoferrin in urine. In this study, it was determined that nearly all of the intact lactoferrin in the urine is of maternal origin. The physiologic significance of absorbed, undegraded lactoferrin has not yet been determined. In a similar study by Goldman et al. (1990), two groups of very low birth weight infants were investigated; one group received cow's milk formula and the second received fortified human milk. Lactoferrin was not found in excreta from infants fed cow's milk formula; however, intact and fragmented forms of lactoferrin were found in stools and concentrated urine of each infant who received human milk. The close resemblance between the lactoferrin fragments in the stools and urine suggest that the urinary lactoferrin fragments originated in the GI tract.

In contrast to the findings of Balmer et al. (1989) and Fairweather-Tait et al. (1987), a comparison of the fate of bovine lactoferrin and human lactoferrin fed to healthy babies age 3 days to 2 months showed that bovine lactoferrin is less degraded than the human forms and total excretion per day of the intact protein was greater (Spik et al. 1982). The iron saturated form was more resistant to degradation. Quantitative determination of human copro-lactoferrin from birth through a period of up to 3 weeks showed that the daily elimination decreased from 25 to 5 mg. Amount of copro-lactoferrin corresponding to the endogenous secretion (biosynthesis by the intestinal tract) was calculated to be from 0.5 to 1 mg/day.

The mean plasma level of lactoferrin in adults is 122 ± 40 μg/L; in term infants up to the age of 21 days, mean lactoferrin level is 385 ± 113 μg/L. The difference in the means between adults and infants is statistically significant. There were no significant differences between the means of breast-fed and formula-fed infants. No significant differences in plasma levels were found between adults and infants at 15 weeks of age (Scott 1989).

The plasma lactoferrin concentration in adults is derived from neutrophils. Lactoferrin derived from breast milk, given to adults, is rapidly cleared from the circulation, being avidly sequestered by the liver and spleen. The sequestered lactoferrin is promptly catabolized. The iron, however, is not excreted in the urine, but rather retained and slowly transferred to the bone marrow, where it is subsequently incorporated into developing erythrocytes (Bennett and Kokocinski 1979).

Table 14. Fate of Ingested Lactoferrin in the Body

Study Design	Results	Conclusions	Reference
Fecal samples were collected from exclusively breast-fed term infants and milk samples were collected from mothers. The first collection was taken soon after return from the hospital, within the first week of age. Thereafter, samples were collected once a week for the first month of life, then once a month for as long as the infant was exclusively breast-fed. Soluble proteins in the feces were extracted and analyzed for total protein, nitrogen, lactoferrin, secretory IgA, serum albumin and lysozyme.	Considerable quantities of lactoferrin were excreted by the breast-fed infant, with higher amounts excreted during the early weeks of life and the quantity excreted getting progressively smaller at successive weekly measurements. Quantities excreted for the three infants ranged from: 153 - 24 mg/24 hours (infant #1 from weeks 1 - 16); 46 - 12 mg/24 hours (infant #2 from weeks 3 - 22) and 70 - 9 mg/24 hours (infant #3 from weeks 2 - 12). This corresponded to concentrations of lactoferrin in the milk which were highest during the first weeks of life and decreased in concentration during later weeks. Gel filtration chromatography demonstrated the excreted lactoferrin to be intact. Fragments of these proteins were not detectable by immunoelectrophoresis following gel filtration. (Serum albumin, a major whey protein which can be assumed to be completely utilized by the infant, was undetectable in all of the fecal extracts.) Using milk consumption by the infant along with concentration of the specific proteins in the milk, 24 hour intakes of lactoferrin were calculated. Intakes by infants #1 and #2 ranged from 2,461 – 1,456 mg/24 hours (weeks 1 - 16) and 3,537 - 1,829 mg/24 hours (weeks 1 - 12), respectively. The percent of lactoferrin ingested that was excreted in the feces ranged from 6.2% - 1.3% in infant #1 and 2.0 - 0.4% in infant #2 during these same time periods.	This study examined the normal protein excretion from full-term exclusively breast-fed infants. Of the protein excreted by the infants, lactoferrin and IgA make up a significant proportion. Fecal lactoferrin is detectable throughout the study. Lactoferrin is believed to provide additional protection in the infant gut against several microorganisms. In its iron-saturated form, it is quite resistant to proteolysis. During the first weeks of life, approximately 2 - 6% of the lactoferrin consumed by the infant is excreted per day, however by 4 months, less than 2% of the lactoferrin intake is excreted. Increased lactoferrin proteolysis takes place as the infant ages. The large amounts of lactoferrin and IgA excreted intact by the infant, along with the fact that no other major whey proteins is detected in the feces reinforces the fact that lactoferrin and secretory IgA are protected against degradation, which is consistent with their physiological roles in the infants' digestive system.	Davidson and Lönnerdal (1987)

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Study Design	Results	Conclusions	Reference
The origin of intact (78-kD) lactoferrin found in the urine of human milk-fed preterm infants was investigated using human milk containing proteins enriched with [^{13}C]leucine and [$^{15}N_2$]lysine or [$^{2}H_4$]lysine. Labeled milk was collected from each mother, pooled, fortified with a lyophilized human milk fraction, and fed to her preterm infant by continuous orogastric infusion for a period of 48 hours. Urine was collected from each infant for 96 hours.	Mass spectral analyses indicated that the isotopic enrichment of the purified urinary lactoferrin was 87 to 100% of that in the labeled human milk lactoferrin. Similar results were obtained for the isolated DNA binding lactoferrin fragments. The ratios of isotopically labeled leucine to lysine in the purified milk lactoferrins and urinary lactoferrins were similar for each mother/infant pair. Isotopically labeled lysine, added to the milk as free amino acid, was not incorporated into the purified urinary lactoferrin. These results demonstrate that undegraded lactoferrin of maternal origin is absorbed by the gut and excreted intact in the urine of preterm infants; nearly all the urinary lactoferrin was of maternal origin.	Serum levels of lactoferrin in both preterm and term infants reportedly do not vary with diet (i.e., human milk vs. bovine milk based formula). As a result, the origin of biologically significant quantities of lactoferrin in the urine of preterm infants fed human milk has been the subject of debate. Because fecal excretion of lactoferrin accounts for only 1 - 6% of the dietary lactoferrin in preterm infants, it has been suggested that much of the remaining lactoferrin is digested. This study demonstrated that nearly all of the intact lactoferrin in the urine of premature infants fed human milk is of maternal origin. The physiologic significance to preterm infants of absorbed, undegraded lactoferrin remains to be established. It uptake may proceed by nonspecific macromolecular absorption or by way of association with specific lactoferrin receptors.	Hutchens et al. 1991
Forty healthy babies aged 3 days to 2 months were fed on 6 different kinds of regimes: Group I, 15 babies were completely breast fed; Group II, 5 babies were completely bottle fed on a commercial concentrated and homogenized cow's milk; Group III, 5 babies were bottle fed on this formula for 2 days and then bottle fed on cow's milk with added 200 mg of partially iron-	Quantitative determination of human copro-lactoferrin from birth through a period of up to 3 weeks showed that the daily elimination decreased from 35 to 5 mg. The amount of copro-lactoferrin corresponding to the endogenous secretion (biosynthesis by the intestinal tract) was calculated to be from 0.5 to 1 mg/day. When a cow's milk diet was supplemented by partially or completely iron-saturated human or bovine lactoferrin and fed to the babies, the amounts of copro-lactoferrin excreted depended upon the origin and iron saturation of the lactoferrin. Total excretion (mg/24 hours) of lactoferrin in the stools of infants receiving the cow's milk supplemented by: human lactoferrin, ranges from 8 to 20 for	Data from this study showed that unhydrolyzed lactoferrin may be found in fecal extracts of babies. The fact that bovine lactoferrin is less degraded than the human form suggests that it may also have a powerful bacteriostatic effect in the infant gut and cold be used to supplement artificial formula.	Spik et al. 1982

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Study Design	Results	Conclusions	Reference
saturated lactoferrin at each of the 5 feeds/day for 3 days and again bottle fed on cow's milk for 2 days; Groups IV, V and VI, 5 babies in each group, were treated the same as Group III, except that the cow's milk was enriched by human iron-saturated lactoferrin for group IV, by bovine partially iron-saturated lactoferrin for Group V and by bovine iron-saturated lactoferrin for group VI. Lactoferrin was characterized in the fecal extracts.	the native lactoferrin and 21 to 37 for the saturated lactoferrin; and bovine lactoferrin, ranges from 62 to 220 for the native lactoferrin and 120 to 364 for the saturated lactoferrin. The iron saturated forms appeared to be more resistant to proteolytic degradation. Fingerprints of native human and bovine lactoferrins hydrolyzed *in vitro* by infant's gastric or duodenal secretions showed that both proteins were not extensively digested.		
Blood was obtained from 23 infants between 24 and 36 weeks of age. Blood was obtained within 24 hours of birth. Plasma lactoferrin measurements were made.	Plasma lactoferrin concentrations ranged from below to above those found in term infants. Plasma lactoferrin ranged from approximately 50 - 900 μg/L. Analysis of increase in lactoferrin concentration (> 200 μg/L in less than 48 hours) with clinical judgment of infection, indicated that there was a substantial degree of agreement. Infection was correlated with increased lactoferrin concentration.	The process of phagocytosis by circulating neutrophils has been shown to involve the release of several proteins, one of which is lactoferrin. Lactoferrin concentrations in preterm babies may rise transiently, such increases often being associated with clinical signs of infection.	Scott 1989
Two groups of very low birth weight infants were investigated; one group received cow's milk formula and the second received fortified human milk prepared daily by adding pasteurized, lyophilized mature donor milk to fresh maternal milk. Stools and urine were collected continuously during the 96 hour balance periods at 2.5 and 5 weeks of age.	Lactoferrin was not found by Western blotting in excreta from infants fed cow's milk formula. In contrast, intact and fragmented forms of lactoferrin were detected in stools and concentrated urine of each infant who received human milk. Only intact lactoferrin was detected in the fortified human milk preparation, whereas many types of lactoferrin fragments were present in the stools and urine. The approximate molecular weight of the most prominent fragments were 44, 38, 34 and 32 kD. However, the stools also displayed lower molecular weight fragments that were not found in urines of those infants. These fragments were similar in size to those produced *in vitro* by limited digestion of apo-lactoferrin with trypsin.	Fragments of lactoferrin in stools of very low birth weight infants fed human milk appeared to be produced by *in vivo* proteolysis. The close resemblance between the lactoferrin fragments in the stools and urine suggest that the urinary lactoferrin fragments originated in the GI tract.	Goldman et al. 1990
Plasma lactoferrin was measured using a non-isotopic enzyme	Venous blood specimens were obtained from 21 adults. Mean adult plasma lactoferrin value was 122 ± 40 μg/L (range of 42 -	The infant plasma lactoferrin concentrations remained	Scott 1989

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Study Design	Results	Conclusions	Reference
immunoassay in normal full term infants up to 15 weeks of age and in normal adults. Values of plasma lactoferrin were also compared in exclusively breast-fed infants with those receiving feeding formulas.	202). Capillary specimens from 13 adults gave a mean plasma lactoferrin value of 107 ± 73 µg/L. For infants, 175 capillary blood specimens were obtained. Mean plasma lactoferrin from term infants up to the age of 21 days was 385± 113 µg/L, which was significantly higher than that found in adult plasma. Plasma lactoferrin values at 7, 11 and 15 weeks gave means of 267 ± 176 µg/L, 269 ± 163 µg/Land 176 ± 165 µg/L, respectively. Means at 7 and 11 weeks were significantly higher than in adults, however no significant difference was found at 15 weeks. There were no significant differences between the means of breast-fed and formula-fed groups.	significantly higher than the adult up until 15 weeks. However, the concentration of lactoferrin in cow's milk feeding formula is low (20 - 200 mg/L) compared to human milk (approximately 1 g/L in mature milk), however there was no effect of feeding type on plasma lactoferrin levels.	
Fecal samples from breast-fed infants and milk samples were collected for 24 or 48 hours.	Milk from donor #1 contained 1.3 mg/ml lactoferrin. Fecal samples from this mother's infant contained 14 mg lactoferrin/24 hours at 15 weeks. Milk from donor #2 contained 1.8 mg/ml lactoferrin. Fecal samples from this mother's infant contained 8.0 mg lactoferrin/24 hours at 15 weeks. Lactoferrin constituted a fairly large proportion of fecal protein (32% for infant #1 and 19% for infant #2).	Lactoferrin is found in high concentration in breast milk compared to cow's milk and infant formula. It is thought to act as a bacteriostatic agent through the GI tract of the infant. This proposed role necessitates the persistence of the protein through at least part of the GI tract of the infant.	Davidson and Lönnerdal 1985
Formula was given to babies for fourteen days. Formulas used were (a) no added iron and no added lactoferrin (n=20); (b) no iron but added lactoferrin (n=18); (c) added iron and lactoferrin (n=20). Lactoferrin was added at 2.8 g/L formula. Anthropometric measurements were made on day 1. Feces were collected on days 4, 11 and 14. Specimens collected on days 4 and 14 were used to measure the bacterial flora and the 24 hour specimen collected on day 11 was used to estimate fecal lactoferrin concentration. A blood specimen was taken on day 14.	Lactoferrin excretion in the feces was greater in the lactoferrin fed babies than in those fed the basic formula, however, observed excretion was far below that expected. Reports of lactoferrin excretion in breast fed babies are in the range of 5.7 to 23.6 mg/day. The babies in this study excreted a mean of 4.5 and 3.67 mg/day for the lactoferrin and lactoferrin plus iron groups.	The addition of lactoferrin had little effect upon the fecal microflora and did not move the pattern of the fecal flora in the direction of the breast fed baby. There was less lactoferrin excreted in the feces than anticipated; it is possible that some of the lactoferrin had been broken down or degraded higher in the gut.	Balmer et al. 1989
Twenty-nine babies were fed	The mean bovine lactoferrin concentration in the 24 hour stool	As expected, the babies fed the	Fairweather-Tait

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Study Design	Results	Conclusions	Reference
from birth either a basic infant formula or the same formula with added bovine lactoferrin (285 mg/L). On the 7th day after birth the babies were given a single dose of ^{58}Fe-labeled bovine lactoferrin or ^{58}Fe-labeled ferric chloride plus ascorbic acid. Iron retention was estimated. Fecal bovine lactoferrin was estimated in a 24-hour stool collection 11 days after birth.	collected on day 11 after birth was 0.55 ± 0.65 mg in infants fed the basic formula and 4.50 ± 1.52 mg in those fed the lactoferrin fortified formula from birth. The babies fed bovine lactoferrin excreted statistically significantly more lactoferrin in their stools than babies fed the basic formula; however, most of the lactoferrin appeared to have been broken down. When a similar assay was performed on stools from 11 day old breast-fed babies, the lactoferrin levels were 8.32 ± 4.28 mg/24 hours, which was significantly higher than babies receiving the lactoferrin formula.	formula containing added bovine lactoferrin excreted significantly more lactoferrin in their stools than babies fed the basic formula; however, most of the lactoferrin from the basic plus lactoferrin formula appeared to have been broken down.	et al. 1987
Proteins in human milk in human milk fortifiers (Presemp and Enfamil), in preterm formulas (Similac Special Care and Enfalac) and whey protein concentrates with varying degrees of denaturation were digested by duodenal juice from healthy preterm infants, from a 3 year old child, and from adults. Digestion was studied *in vitro*.	Casein was the protein most rapidly degraded in all products. Human and bovine whey proteins were more slowly digested; as much as 68% of human lactoferrin was still immunoreactive after 40 minutes of digestion. Bands of bovine and human milk proteins showed a similar pattern, whether the digestion was performed with juice from the 3-year old or adult.	Results show that whey proteins are slowly hydrolyzed whereas casein is hydrolyzed at a much faster rate.	Lindberg et al. 1998
The turnover of ^{125}I-labelled lactoferrin, obtained from breast milk and administered iv, was measured in 10 adults. Simultaneous organ radioactivity counting was performed. Ferrokinetic studies were performed in three adults after the	Lactoferrin was rapidly eliminated from the plasma with a mean fractional catabolic rate of 5.7/day. Assuming the plasma lactoferrin to be in a steady state, the mean synthetic rate was 16.8 mg/day. Of the administered ^{125}I label, 99% was recovered in the urine, as free iodine, within the first 24 hours. In the ^{59}Fe studies, no appreciable activity was found in the urine. Organ radioactivity counting showed that lactoferrin was rapidly taken up by the liver and spleen. the rapid excretion of free ^{125}I	Lactoferrin is found in many human secretions and in neutrophils. The concentration of lactoferrin in human plasma is about 0.2 - 1.5 μg/ml. The plasma lactoferrin concentration is derived from neutrophils; this theory is derived from the correlation of	Bennett and Kokocinski 1979

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Study Design	Results	Conclusions	Reference
iv injection of [^{59}Fe]lactoferrin.	suggests catabolism at these sites. In the ^{59}Fe studies, the radioactivity persisted in the liver and spleen for several weeks and was slowly transferred to the bone marrow before appearing in the circulating erythrocytes.	plasma values of lactoferrin with the total neutrophil pool. Lactoferrin is rapidly cleared from the circulation, being avidly sequestered by the liver and spleen. The sequestered lactoferrin is promptly catabolized. The iron, however, is not excreted in the urine, but rather retained and slowly transferred to the bone marrow, where it is subsequently incorporated into developing erythrocytes. These results are based on lactoferrin derived from breast milk and not neutrophils.	

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Study Design	Results	Conclusions	Reference
The rhesus monkey (preterm and infant) was used as an animal model to study the luminal phase of the hydrolysis of proteins in human milk, human milk fortifier, and preterm formula at 6 weeks and 7 months of age.	In six-week old monkeys, the trypsin and elastase activities in the duodenal contents decreased postprandially by 50%, whereas they increased in the 7-month old monkeys. In the preterm and 6 week old monkeys, hydrolysis of human and bovine whey proteins was slow, and in 6-week old monkeys, 30-50% of the proteins could still be detected immunochemically in duodenal contents after 60 minutes. At seven months of age, no or small amounts of lactoferrin could be detected in duodenal contents after 15 minutes.	The low capacity to digest whey proteins in suckling monkeys may depend upon an immaturity of the exocrine pancreas to respond to secretogogues. In the fasting state, the trypsin concentration in duodenal juice from 6-week and 7-month old monkeys is comparable to those found in human infants and children, whereas the elastase activities in 6-week old monkeys are somewhat higher and in 7-month old monkeys, much higher, than in human infants. In conclusion, there is an age-dependent capacity to hydrolyze bovine and human whey proteins in the monkey gut lumen which possibly depends upon an immaturity of the exocrine pancreas of suckling rhesus monkeys to respond to secretogogues.	Lindberg et al. 1997

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Study Design	Results	Conclusions	Reference
The degradation of bovine lactoferrin was examined in six adult minipigs and three piglets, 21 days old. They were fed 1% by weight lactoferrin in a test meal of 180 g dry matter. Six hours postprandially, the animals were sacrificed and digesta were collected as completely as possible from the different segments of the intestine. The suckling piglets consumed 75 mg lactoferrin in 50 ml fresh sow's milk and intestinal samples were obtained 3 hours postprandially.	Less than 0.25% of the lactoferrin consumed was detected in total digesta of adult pigs. In piglets, however, there was a considerable amount of lactoferrin the distal part of the small intestine (39.2 mg found in the ileum).	Supplementation of the diet of the human newborn with bovine lactoferrin has been reported to result in up to 20% undegraded lactoferrin in the feces. In piglets, a higher amount (52%) was found in the prececal segment of the intestine. This may be the result of a species difference. In addition, it can be expected that a certain percentage of lactoferrin is degraded in the hindgut by microbial activity, which was excluded in this experiment.	Schmitz et al. 1989

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B. Animal Toxicology Studies

The safety of bovine lactoferrin similar in specifications to the bovine lactoferrin which is the subject of this GRAS determination was assessed in an acute toxicity study, a 4-week oral toxicity study, a thirteen-week oral toxicity study, and an Ames assay.

1. Acute Toxicity Study In Rats

Nishimura (1991) evaluated the acute toxicity of bovine lactoferrin in rats. Male and female Crj:CD (SD) SPF rats were exposed to single oral doses of 1,000 or 2,000 mg/kg bovine lactoferrin (MONL-01) or bovine iron-saturated lactoferrin (MONL-02) via stomach intubation. Control animals received vehicle alone (2,000 mg/kg water). Animals were observed for mortality, clinical signs, and any changes in general condition during a 14-day observation period following administration. Body weights were measured prior to the study and periodically throughout the observation period. After 14 days, the animals were sacrificed and the organs examined macroscopically for any abnormalities.

Exposure to 1,000 or 2,000 mg/kg MONL-01 and MONL-02 resulted in no deaths or abnormal clinical signs or effects on the general condition of the animals. Lactoferrin had no effect on body weight. There were no significant differences in body weights throughout the study period in treated animals compared to controls. No abnormal gross pathological findings were observed in any organ in the cranial, thoracic, and abdominal cavities.

Lactoferrin exhibited low acute oral toxicity. A single oral dose of 1,000 or 2,000 mg/kg bovine lactoferrin or bovine iron-saturated lactoferrin resulted in no adverse effects or deaths. Based on these results, the lethal dose of lactoferrin exceeds 2,000 mg/kg.

2. Four-Week Oral Toxicity Study in Rats

The safety of bovine lactoferrin was assessed in a 4-week oral toxicity study in rats (Nishimura 1997). Four-week old male and female Sprague-Dawley rats were exposed by oral intubation to 200, 600, or 2,000 mg/kg/day bovine lactoferrin once daily for 4 weeks (28 days). Animals in the control group received water by the same route of administration. Animals were observed daily for any changes in appearance or behavior. Body weight and food consumption was measured prior to the start of treatment and twice weekly every 3 or 4 days prior to dosing. Ophthalmology, urinalysis, hematology, and blood chemistry analyses were conducted in week 4 or at necropsy. Animals in each group were sacrificed on day 29 and observed for external abnormalities. Absolute and

relative weights were determined for all organs and tissues in the cephalic, thoracic and abdominal cavities. Organs and tissues of animals in the control group and high-dose group were examined histopathologically.

There were no deaths or changes in the general condition, behavior or appearance of the animals due to administration. Body weight and food consumption were similar in all groups throughout the study; no significant differences were observed between groups. No changes in males or females or significant differences between test and control groups were observed in urinalysis (pH, protein, ketone body, glucose, occult blood, bilirubin, urobilinogen, color, urinary sediments, 24-hour urine volume, osmolarity, sodium, potassium, chloride, or water intake); hematology (red cell count, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte ratio, platelet count, white blood cell count, differential leukocyte count, prothrombin time, activated partial thromboplastin time, or fibrinogen), or blood chemistry parameters (GOT, GPT, LDH, ALP, total cholesterol, triglycerides, phospholipids, total bilirubin, glucose, blood urea nitrogen, creatinine, sodium, potassium, chloride, calcium, inorganic phosphorus, total protein, A/G ratio or protein fractions).

Absolute and relative body weights did not change significantly throughout the treatment period or differ significantly between groups. Unilateral and/or bilateral persistence of the hyaloid artery in the eye was observed in at least one animal in each group, including the control group. This effect was not considered treatment-related or abnormal because it occurs naturally during the development of the eyeball and disappears with growth. Gross pathological findings observed included excoriation in the neck of 2 males and 1 female in the 200 mg/kg/day group and 1 male in the 600 mg/kg/day group, pneumatosis-like enlargement of the lung in 1 male in the 200 mg/kg/day group, a dark-red spot in the lung of 2 males in the 2,000 mg/kg/day group, and dark-red spots in the glandular stomach in 1 male and 1 female in the control group and 1 female in the 600 mg/kg/day group. Corresponding macroscopic findings were mild ulcer, mild overinflation of the lung, and slight erosion of the stomach, respectively. One 1 female administered 600 mg/kg/day had a fracture of the incisors. These effects are considered incidental since they were not dose-related or consistently observed among the animals. A few microscopic changes were observed in male and female animals that were not considered to be related to treatment. These were cellular infiltration and focal hemorrhage of the lung; erosion in the glandular stomach; cellular infiltration of the cecum; microgranuloma in the liver; ectopic thymus; tubular basophilia, eosinophilic body in tubular epithelium, and cellular infiltration in interstitium of the kidney; degeneration and necrosis of spermatocyte; decrease of sperm in the epididymus

duct; fibrosis in the muscle layer of the esophagus; hyperplasia of ductal epithelium in the sublingual gland; and disarrangement of the retina. These changes, which were slight-to-mild in severity and occurred sporadically in one or two animals, were considered incidental.

Administration of 200, 600, and 2,000 mg/kg/day bovine lactoferrin to male and female rats resulted in no deaths or treatment-related changes in body weight, food consumption, organ weight, ophthalmology, hematology, blood chemistry, urinalysis, or gross pathology and histology examinations. Therefore, the no observed adverse effect level (NOAEL) of bovine lactoferrin was estimated to be in excess of 2,000 mg/kg/day.

3. Thirteen-Week Oral Toxicity Study in Rats

As part of a safety assessment of bovine lactoferrin, a 13-week oral toxicity study in rats was conducted (Nishimura 2000). Male and female Sprague-Dawley rats, 4 weeks of age, were exposed to 200, 600, and 2,000 mg/kg/day bovine lactoferrin by oral intubation once daily, seven days a week for 13 weeks. Control animals received vehicle alone. Animals were examined daily for changes in appearance, nutrition condition, or behavior. Body weight and food consumption measurements were taken prior to the start of treatment and twice weekly every 3 or 4 days prior to dosing. Ophthalmology examination and urinalysis (including water consumption), hematology (mean corpuscular volume, red cell count, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte ratio, platelet count, white blood cell count, differential leukocyte count, prothrombin time, activated partial thromboplastin time, or fibrinogen), and blood chemistry determinations (GOT, GPT, LDH, ALP, total cholesterol, triglycerides, phospholipids, total bilirubin, glucose, blood urea nitrogen, creatinine, sodium, potassium, chloride, calcium, inorganic phosphorus, total protein, A/G ratio or protein fractions) were measured in week 13 (urinalysis was also done at week 6). Animals were sacrificed after 91 days and observed for external abnormalities. Absolute and relative weights were determined for all organs and tissues in the cephalic, thoracic, and abdominal cavities. Histopathological examinations were conducted of all organs and tissues in the control and high-dosed animals, the pancreas of males at all dose levels, and in any animal that died or exhibited macroscopic lesions.

There were two deaths during the treatment period. One male in the 200 mg/kg/day group died at week 10 without any overt clinical signs of disease. Examination revealed perforation in the esophagus and hydrothorax with food. The death was attributed to an error in intubation. One female in the 2,000 mg/kg/day group exhibited swelling of the subcutis at week 12 and died at week 13. At necropsy, enlargement of the lymph nodes,

thymus, spleen, and liver and white spots in the kidney were observed. Upon histologic examination, malignant lymphoma and tumor cells in the brain, harderian gland, heart, lung, pituitary, adrenal, ileum, cecum, ovary, uterus and bone marrow were observed. Slight extramedullary hematopoiesis was seen in the adrenal gland. Death of the animal was attributed to the presence of malignant lymphoma.

No abnormal clinical signs due to administration of bovine lactoferrin were seen in surviving animals. However, a few clinical signs deemed incidental because of their sporadic occurrence were observed. These were subcutaneous mass in the axillary mass accompanied by hemorrhage and paleness of skin in 1 male in the control group, fracture of incisors in 1 female in the control group, and excoriation in the neck in 1 female in the 200 and 600 mg/kg/day groups and 2 females in the 2,000 mg/kg/day group. One female in the 2,000 mg/kg/day group had decreased spontaneous movement and oligopnea. Perforation in the esophagus, hydrothorax, and dark-reddening of the lung was observed at necropsy suggesting that the effects were due to an error in administering the compound.

No significant differences were observed in body weight or food consumption between groups during the treatment period. No ophthalmologic abnormalities were observed in any animal. Hematology and blood chemistry determinations were not significantly different between the study groups. At week 6, no significant changes in urinalysis parameters were observed in animals administered 200 mg/kg/day; at week 13, significant increases in urine volume and daily excretion of sodium, potassium, and chloride in males occurred. These changes, however, were not dose-related. In the 600 mg/kg/day group, significant decreases in daily excretion of sodium were observed in males at week 6; however, no significant changes were observed in any urinalysis parameter at this dose at week 13. In the 2,000 mg/kg/day group, pH and daily excretion of sodium were reduced in males at week 6. At week 13, the reduction in pH was seen in male and females and there was no change observed in sodium excretion. The change in urinary pH is not thought to be of toxicological significance since there was only a slight degree of change and there were no significant changes in any other urinary parameters such as volume and content of electrolytes. In addition, there were no corresponding histological findings in the kidney or blood chemistry values.

No changes in absolute or relative organ weights were observed in males or females administered 200 or 600 mg/kg/day. At the highest dose administered, significant decreases in absolute and relative thyroid weights compared to controls were observed in females ($p<0.05$). These changes were within the normal histological background range. In addition, no corresponding findings were observed upon histopathological examination.

Gross pathology findings in a few animals and histopathologic changes in several organs were observed. These findings were not consistently observed among animals and were considered incidental. No histopathological findings were considered to be treatment related, but were judged incidental in view of their occurrence and the nature of the lesions. Slight or mild islet fibrosis of islet acinar cells were observed in 3 males in the control group and 6 males each in the 200, 600, and 2,000 mg/kg/day groups. Although the incidence and severity of the finding in each treated group was slightly higher than that of the controls, these findings were not considered to be treatment-related. No morphological differences in the fibrosis of islets between the control and treated groups were observed and the distribution of lesions in animals in the treated groups were limited to the same small section of tissue as in the control animals.

Bovine lactoferrin administered by oral intubation to rats for 13 weeks did not result in significant treatment-related changes in the appearance, general condition, body weight, food consumption, ophthalmology, hematology, blood chemistry, gross pathology, or histopathology of the animals. Thus, under the conditions of this study, the no-observed-adverse-effect level (NOAEL) of bovine lactoferrin was estimated to be in excess of 2,000 mg/kg/day.

4. Ames Assay

The genotoxic potential of bovine lactoferrin was examined in the reverse mutation assay (Kawai and Tanaka 1997). The assay was performed using *Salmonella typhimurium* strains TA100, TA1535, TA98, and TA1537 and *Escherichia coli* strain WP2*uvr*A, with and without metabolic activation. Metabolic activation was provided by an Aroclor-induced rat liver microsome fraction (S9 mix). The tester strains were exposed to lactoferrin via the preincubation method. Lactoferrin was tested at six concentrations: 160, 320, 630, 1,250, 2,500, and 5,000 mcg per plate, based on the results of a dose range finding test. Physiological saline was used as the vehicle control. In the absence of metabolic activation, the positive controls were 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide for tester strains TA100, TA98, and WP2*uvr*A, sodium azide for tester strain TA1535, and 9-aminoacridine for tester strain TA1537. In the presence of metabolic activation, the positive controls were benzo(a)pyrene for tester strains TA100, TA98, and TA1537 and 9-aminoacridine for tester strains TA1535 and WP2*uvr*A. Lactoferrin was evaluated in duplicate for each concentration in the presence or absence of metabolic activation.

No precipitation or crystallization was observed at any lactoferrin concentration. The positive controls for the tester strains yielded the expected number of revertants per plate of at least a 2-fold increase in the number of revertants relative to the vehicle control. For

the test article to be classified as producing a positive result, there had to be a proportional increase in the number of revertant colonies relative to the increase in the concentration of lactoferrin and the ratio of the number of revertant colonies to that of the control group had to be at least 2.0. For all concentrations of lactoferrin in all tester strains, with or without metabolic activation, the ratio of the number of revertant colonies to that of the control group was 1.4 or lower. A second assay produced similar results.

Lactoferrin did not cause a positive response with any of the tester strains in the presence or absence of S9 activation at concentrations up to 5,000 mcg/plate. Thus, under the conditions of this study, lactoferrin was not found to be genotoxic in the *Salmonella typhimurium* reverse mutation assay or *Escherichia coli*/mammalian microsome reverse mutation assay.

C. Allergenicity

Allergic reactions to foods and dietary components of food are common. While the degree of sensitivity to food allergens varies from individual to individual, even trace amounts of an offending food allergen may trigger a reaction. Symptoms can include hives, vomiting, diarrhea, and respiratory distress. In some cases, just one bite of food can bring on anaphylaxis, and in extreme cases, it can cause death. Cow's milk, egg white, peanuts, shellfish, and soybeans are among the known food allergens. There are various proteins in milk known to be food allergens, including β-lactoglobulin, α-lactalbumin, caseins, bovine immunoglobulins, and bovine serum albumin. IgE antibodies to several other minor milk proteins--including lactoferrin--have been identified in a few patients (Baldo 1984). Lactoferrin protein has also been identified as eliciting allergic reactions in several animal models tested. Individuals, especially children, who are allergic to cow's milk should be aware of the allergenicity to lactoferrin. The following studies demonstrate the potential of lactoferrin to induce allergic reaction.

Host et al. (1992) analyzed specific IgE to bovine milk proteins including lactoferrin by crossed radioimunoelectrophoresis (CRIE). The distribution of elevated specific IgE antibodies to several cow milk proteins showed a high frequency of antibodies to bovine se-albumin and less frequency to IgG, alfa-lactoalbumin and lactoferrin in the serum samples from infants with cow milk allergy. At 12 months of age the frequency of IgE to lactoferrin increased in infants with IgE mediated cows milk allergy from 0 in 18 and 17 (cord blood and 6 months of age, respectively) to 5 in 20 infants following a challenge with bovine milk.

Atkinson and Miller (1994) used serum from Brown Norway (BN) rats fed various levels of skimmed milk (SSM) to profile the milk protein antibody responses to the antigens present in SSM. The rats produced both IgG and reaginic antigen-specific response against lactoferrin. The reaginic response to lactoferrin was similar to alpha-casein and higher than that produced against bovine serum albumin, the lactoglobulins, and β- or κ-casein.

Miller et al. (1998) investigated the allergenicity of lactoferrin in studies using Brown Norway (BN) rats. Using parenteral sensitization regimens, the authors reported the highest dose that did not induce reaginic antibody production for lactoferrin (0.01 mcg), ovalbumin (0.1 mcg) and bovine serum albumin (1 mcg). Therefore, the comparative allergenic potential of these proteins was lactoferrin>ovalbumin>bovine serum albumin. Semiskimmed milk (SSM) was found to be less allergenic than ovalbumin as the total dose of antigen required to induce sensitization to SSM was 20-fold greater than that required for ovalbumin.

Miller et al. (1998) also demonstrated that BN rats sensitized parenterally with 500 mcg SSM on days 0 and 7 developed reaginic IgE responses to a range of milk proteins, including lactoferrin. Under these conditions, lactoferrin was found to be as allergenic as the caseins and β-lactoglobulin in the BN rat.

Debbabi et al. (1998) investigated the nature of the immune responses induced by repeated oral administration of bovine milk lactoferrin in mice and detected anti-lactoferrin IgA and IgG in the intestinal fluid and serum of mice fed lactoferrin daily for four weeks. Total immunoglobulins were also higher in the intestinal fluid in lactoferrin-fed mice than in the control group. Debbabi et al. suggest that lactoferrin could act as an immunostimulating factor on the mucosal immune system and that activation of the mucosal immune system is dependent on the ability of lactoferrin to bind to the intestinal mucosa.

D. Human Exposure

Bovine lactoferrin has been consumed by humans for thousands of years as a naturally occurring protein found primarily in milk and in low concentrations in residual plasma in beef products. Consumption of bovine lactoferrin through dairy sources has been estimated to be 73, 75, and 50 mg/day at the 90th percentile of intake for children, teens and adults, respectively. In addition to dairy sources, lactoferrin is currently marketed in the U.S. as a dietary supplement. Consumption of lactoferrin from this source ranges from 10 to 1,200 mg/day.

E. Human Clinical Trials

Bovine lactoferrin was ingested by both infants and adults in numerous clinical trials that were designed to examine effects on either iron absorption or modulation of microflora or infection. In studies of infants, dose levels used ranged from 1.4 mg/day (0.3 mg/kg/day) to 2.9 g/day (1.0 g/kg/day) and study durations were from 11 days to 5 months. In studies of adults, dose levels used ranged from 100 mg/day (1.7 mg/kg/day) to 3.6 g/day (60 mg/kg/day) and study durations varied from a single dose in one study to 8 weeks. These studies are summarized in Table 15.

These studies were not designed specifically to look for issues of tolerance to ingestion of lactoferrin; nevertheless, no adverse health effects as a result of lactoferrin intake are reported in

any of the studies. There are also no indications of subjects discontinuing due to adverse reactions. These data are also helpful, from the standpoint of assessing safety and tolerance to infants, due to the number of studies that were conducted using long durations. Of the seven studies looking at effects of lactoferrin in infants, four were conducted using supplemental lactoferrin feeding for periods of 3-5 months, the time during which infants are utilizing formula as a sole source of nutrition. In the studies of adult subjects, not only healthy individuals, but subjects with leukemia, chronic hepatitis and tinea pedis were given bovine lactoferrin without any reported adverse effects.

Table 15. Human Studies of Lactoferrin

Study Design	Objective	Results	Reference
Iron balance studies were performed in 16 term infants from their 3rd until their 17th week of life. Seven infants were fed an adapted infant formula supplemented with bovine lactoferrin (100 mg/100 ml) and nine received the same formula without lactoferrin. Assuming a mean intake of formula of 1,031 ml/day or 194 ml/kg/day for infants 0-4 months old and a bodyweight of 5.3 kg; the dose of ingested lactoferrin was 1.0 g/day or 194 mg/kg/day.	Iron balance studies were comprised of five periods with an interval of 3 to 4 weeks, each consisting of three 24-hour collections of milk and stool samples.	Iron retention in the lactoferrin supplemented group was 63 µg/kg/day and in the unsupplemented group it was 43 µg/kg/day. Mean percentage retention of iron in the supplemented group was 36%, in the unsupplemented group 28%. Although iron availability was slightly better in the supplemented group, this difference was not significant. A further comparison with previous work on ingestion of human milk indicated that although iron bioavailability from human milk was significantly better than both formula studies; the absolute iron retention in the breast fed infants (32 µg/kg/day) was lower than in the formula groups. No adverse health effects of lactoferrin consumption were reported.	Schulz-Lell et al. 1991
Infant formula supplemented with various amounts of bovine lactoferrin (10 or 100 mg lactoferrin/100 ml formula) was given to two groups (n=14 for each group) of infants for 150 days starting from birth. These infants were compared with infants receiving unsupplemented formula (n=14) and breast-fed babies (n=10). Assuming a mean intake of formula of 1,031 ml/day or 194 ml/kg/day for infants 0-4 months old and a bodyweight of 5.3 kg; the dose of ingested lactoferrin was 1.0 g/day or 194 mg/kg/day at a concentration of 100 mg/100 ml and 0.1 g/day or 19.4 mg/kg/day at a concentration of 10 mg/100 ml.	Effects of the diets on levels of hemoglobin, hematocrit, serum iron, ferritin and zinc were examined for the 150-day study period.	At birth, concentrations of iron, hemoglobin, hematocrit, and zinc were comparable in all four feeding groups. During the first three months of life, hemoglobin, hematocrit and iron serum levels were not substantially different in the four feeding groups. No difference in serum zinc values was demonstrated between groups at any time during the study period. Serum ferritin was measured to provide a way to calculate the amount of total body iron stores. Serum ferritin levels increased from day 0 to 7 in all groups and persisted at these levels at day 30. The levels then decreased (day 90 and 150). At day 30, ferritin was significantly higher in the human milk group compared to all the formula-fed groups. At day 90, significantly higher ferritin values were observed in the human milk group compared to the unsupplemented formula group, but these levels were not significantly different than those from the lactoferrin supplemented group. At day 150, lactoferrin (100 mg/100 ml) supplemented formula-fed infants had serum ferritin levels significantly higher than the unsupplemented formula group. No adverse health effects of lactoferrin consumption were reported.	Chierici et al. 1992

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Study Design	Objective	Results	Reference
Formula was given to babies for fourteen days. Formulas used were (a) no added iron and no added lactoferrin (n=20); (b) no iron but added lactoferrin (n=18); (c) added iron and lactoferrin (n=20). Lactoferrin was added at 2.8 g/L formula. Anthropometric measurements were made on day 1. Feces were collected on days 4, 11 and 14. Specimens collected on days 4 and 14 were used to measure the bacterial flora and the 24-hour specimen collected on day 11 was used to estimate fecal lactoferrin concentration. A blood specimen was taken on day 14. Babies weighed approximately 3 kg. Using this weight, and the default value of mean intake of formula of 1,031 ml/day, intake of lactoferrin was approximately 2.9 g/day or 1.0 g/kg/day.	The objective of the study was to determine the effects of lactoferrin on the fecal flora.	The addition of lactoferrin had little effect on the fecal microflora and did not move the pattern of the fecal flora in the direction of the breast-fed baby. Lactoferrin excretion in the feces was greater in the lactoferrin-fed babies than in those fed the basic formula, however, observed excretion was far below that expected. Reports of lactoferrin excretion in breast-fed babies are in the range of 5.7 to 23.6 mg/day. The babies in this study excreted a mean of 4.5 and 3.67 mg/day for the lactoferrin and lactoferrin plus iron groups. No adverse health effects of lactoferrin consumption were reported.	Balmer et al. 1989
Four infant feeding groups were studied. Fifty-one infants finished the study; an additional four infants provided two samples during the study period. The subjects were fed the following diets: group A, breast-feeding (n=12); group B, formula feeding (n=14) infants; group C, experimental formula supplemented with 10 mg/100 ml lactoferrin (n=15); and group D, experimental formula supplemented with 100 mg/100 ml lactoferrin (n=14). Study duration was 3 months. Assuming a mean intake of formula of 1,031 ml/day or 194 ml/kg/day for	The objective of the study was to investigate the development of the fecal flora in infants given lactoferrin supplemented formula.	Breast-fed infants developed a flora rich in Bifidobacteria. Facultative anaerobes were ubiquitous, but in relatively small numbers within the diet group. Other obligate anaerobes, such as *Clostridium* sp. and *Bacteroides* sp. were rarely isolated. Standard formula produced a flora rich in bifidobacteria, but the growth of facultative organisms was not suppressed by this diet. *Clostridium* sp. and *Bacteroides* sp. were more common in this feeding group. The addition of 10 mg lactoferrin/100 ml formula produced a flora similar to that of the standard formula fed babies. Lactoferrin at 100 mg/100 ml was able to establish a "bifid flora" in half of the babies given this formula, but only at age three months. *Clostridium* sp. and *Bacteroides* sp. were common isolates from babies receiving both the lactoferrin diets. No adverse health effects of lactoferrin consumption were	Roberts et al. 1992

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Study Design	Objective	Results	Reference
infants 0-4 months old and a bodyweight of 5.3 kg; the dose of ingested lactoferrin was 1.0 g/day or 194 mg/kg/day at a concentration of 100 mg/100 ml and 0.1 g/day or 19.4 mg/kg/day at a concentration of 10 mg/100 ml.		reported. The formula was supplemented with lactoferrin at levels that are relatively low compared with human milk (200-600 mg/100 ml milk). This may have contributed to the lack of effect of lactoferrin on the fecal flora. In addition, the fact that the lactoferrin was 20% saturated with iron may have negated or weakened its effect.	
Iron absorption was measured from human milk in eight breast-fed infants with the native level of lactoferrin present and after removal of greater than 97% of the lactoferrin. The infants ranged in age from 2-10 months (mean age = 5 mo.). The study had a randomized, cross-over design and Fe absorption was measured by stable isotope technique, using ^{58}Fe as a label, with Fe absorption measured by analysis of the incorporation of ^{58}Fe into RBCs 14 days after administration.	The study was designed to investigate the influence of lactoferrin on iron absorption from human milk in infants.	Total volume of labeled milk varied between 700-1,000 g/administration, but was held constant between administrations for each infant. Absorption of Fe was found to be significantly higher when lactoferrin-free breast milk was fed, as compared to breast milk. The mean absorption was 11.8% for breast milk and 19.8% for lactoferrin-free breast milk. In the youngest infant studied (2 months old), Fe absorption was higher from human milk than from the lactoferrin-free milk. No adverse events were reported.	Davidsson et al., 1994
Twenty-nine babies were fed from birth either a basic infant formula or the same formula with added bovine lactoferrin (285 mg/L). On the 7th day after birth the babies were given a single dose of ^{58}Fe-labeled bovine lactoferrin or ^{58}Fe-labeled ferric chloride plus ascorbic acid. Iron retention was estimated by measuring the unabsorbed ^{58}Fe excreted in the feces during the 3 days following administration. Fecal bovine lactoferrin was estimated in a 24-hour stool collection 11 days after birth. Mean body weight was 3.1 kg. Assuming a formula consumption of 1,031 ml/day, the lactoferrin dose	The study was conducted to investigate the effect of lactoferrin on iron absorption in infants. The extent of lactoferrin broken down by digestive enzymes and microorganisms was also investigated.	Mean percent Fe retention from ferric chloride was 44.4 % and 46.2 % from lactoferrin. There was no significant difference between the two iron sources, nor did the previous formula (basic vs basic plus lactoferrin) have any effect on iron retention from either ferric chloride or lactoferrin. As expected, the babies fed the formula containing added bovine lactoferrin excreted significantly more lactoferrin in their stools than babies fed the basic formula; however, most of the lactoferrin from the basic plus lactoferrin formula appeared to have been broken down. No adverse events were reported.	Fairweather-Tait et al, 1987

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Study Design	Objective	Results	Reference
ingested was approximately 294 mg/day or 95 mg/kg/day.			
Six groups of healthy, term infants 6 wks of age were fed breast milk or a cow's milk-based formula (with varying amounts of iron, selenium, copper, and in one group lactoferrin) until the infants were 6 months old. Groups B and C were fed 4 mg/L iron, 10 µg/L selenium. The iron source for Group B was $FeSO_4$ while Group C received 1.4 mg/L as bovine lactoferrin (35% of iron) and 2.6 mg/L as $FeSO_4$. There were 10 infants per group. Hematological indices analyzed included hemoglobin, serum iron, transferrin, serum ferritin and serum transferrin receptor). Also measured were BUN, height, weight, serum copper, ceruloplasmin, serum zinc, and α-macroglobulin. Assuming a mean intake of formula of 1,031 ml/day or 194 ml/kg/day for infants 0-4 months old and a bodyweight of 5.3 kg; the dose of ingested lactoferrin was 1.4 mg/day or 0.3 mg/kg/day at a concentration of 1.4 mg/L.	This study investigated the effect of different levels of iron, copper, and selenium fortification of infant formula on the mineral status of infants. Specifically, selenium, iron, zinc, and copper status were evaluated.	BUN values were significantly lower in breast-fed infants than in formula-fed infants at 6 weeks of age. At 6 months, these differences were smaller but still significant. There were no significant differences in birth weight, weight or height at 6 weeks or 6 months of age among the groups. No significant differences were observed in the hematological parameters when comparing Group C receiving lactoferrin with the group receiving the same amount of iron (4 mg/l) as ferrous sulfate (Group B). There was no significant effect of copper supplementation of formula on plasma copper or ceruloplasmin levels when comparing infants fed a formula without supplementary copper and the identical formula with 0.4 mg/l of supplemental copper. Supplementation with selenium as selenite at a level of 10 µg/l resulted in a similar selenium status in formula-fed infants as that of breast-fed infants. Plasma zinc levels were not significantly different between groups. No adverse events from the intake of lactoferrin were reported.	Lönnerdal & Hernell, 1994
Ten healthy male volunteers were given bovine lactoferrin (2 g/person/day or 33 mg/kg/day assuming a 60 kg body weight) for 4 weeks. Blood samples were drawn before, during and after administration of lactoferrin.	Phagocytic activity and superoxide production activity of polymorphonuclear leukocytes (PMN) were evaluated. Expression levels of CD11b, CD16 and CD56 molecules on leukocytes were quantified using flow cytometry.	The phagocytic activity or superoxide production activity of PMN or the proportions of CD11b+, CD16+ and CD56+ in lymphocytes were increased during the period of lactoferrin administration. The effects on the immune system were different in individual cases. Lactoferrin may influence primary activation of the host defense system. No adverse health effects of lactoferrin consumption were reported.	Yamauchi et al. 1998

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Study Design	Objective	Results	Reference
Five neutropenic patients receiving chemotherapy for acute myelogenous leukemia were given lactoferrin (4 x 200 mg/day, for a total of 800 mg/day or 13 mg/kg/day assuming a body weight of 60 kg, p.o., days 1 through 10 of neutropenia). Nine patients who fulfilled the admission criteria but received no prophylaxis except amphotericin B tablets for the prevention of oral candidiasis served as controls.	To determine if lactoferrin, after enteral administration, is able to prevent, postpone or attenuate enterogenic infection in patients with chemotherapy-induced neutropenia.	Lactoferrin treatment did not significantly prolong the infection-free interval (4.4 ± 2.6 days versus 3 ± 1.9 days in controls). It did, however, significantly shorten the duration of the first infection (3.4 ± 2.7 days versus 7.7 ± 3.5 days in controls). Bacteremias with *Enterobacteriaceae* were observed in only two of five lactoferrin-treated patients compared to six of nine controls. No adverse health effects of lactoferrin consumption were reported.	Trümpler et al. 1989
Eleven patients with chronic hepatitis C received an 8-week course of bovine lactoferrin (1.8 or 3.6 g/day corresponding to 30 or 60 mg/kg/day, respectively, assuming a body weight of 60 kg).	The objective of this study was to test the hypothesis that lactoferrin inhibits hepatitis C virus (HCV) viremia in patients with chronic hepatitis C.	At the end of lactoferrin treatment a decrease in serum alanine transaminase and HCV RNA concentrations was apparent in 3 (75%) of 4 patients with low pretreatment serum concentrations of HCV RNA. However, 7 patients with high pretreatment concentrations showed no significant changes in these indices.	Tanaka et al. 1999
Either 600 or 2,000 mg lactoferrin/day (10 or 33 mg/kg/day, assuming a body weight of 60 kg) was administered orally for 8 weeks to 37 adults who were judged to have mild or moderate tinea pedis. Controls received a placebo.	The study was conducted to evaluate the effectiveness of lactoferrin on the treatment of tinea pedis.	In the analysis of all subjects, dermatological symptoms scores in all groups decreased, and the differences were not statistically significant comparing all three groups. However, in the analysis limited to subjects with moderate vesicular or interdigital tinea pedis, dermatological symptoms scores in the lactoferrin treated groups decreased significantly as compared to the placebo group. There were no adverse events for any of the thirty-seven subjects and no subject withdrew because of an adverse event.	Yamauchi et al. unpublished
Eight healthy iron-sufficient adult subjects were fed a single dose of 100 ml of a test milk (human milk, simulated human milk, simulated human milk containing 100 mg lactoferrin per 100 ml milk (total dose of 100 mg lactoferrin or 1.7 mg/kg assuming a body weight of 60 kg), and two commercial infant formulas). Feedings were conducted at intervals over two weeks. The iron content of	The study was designed to compare the absorption of iron from several proprietary formulas with that from human milk in an attempt to identify the factor, or factors, responsible for the enhanced iron availability of human milk.	The percent iron incorporation was greatest from the human milk (15.4%) compared to simulated human milk (9%), simulated human milk with added lactoferrin (4.7%), SMA (3.08%), and Similac (2.6%). Actual iron incorporation was greatest in the iron-fortified formulas (approx 0.038 mg/100 ml) followed by human milk (0.010 mg/100 ml), and simulated human milk (0.006 mg/100ml). The addition of lactoferrin to the simulated human milk increased actual iron incorporation to 0.010 mg/100 ml. No adverse events were reported.	McMillan et al. 1977

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Study Design	Objective	Results	Reference
each of these milks was 66, 70, 210, 1250, and 1,250 ug/100 ml milk, respectively. Blood samples were obtained 14 days following sample ingestion, just prior to ingestion of the next milk sample to be studied. Incorporation of iron into RBCs was determined.			

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F. Determination of Acceptable Daily Intake (ADI) for Bovine Lactoferrin

An extensive database, consisting of both animal and human exposure and safety data, is available for determination of the safety of bovine lactoferrin used as an barrier to microbial contamination in processed beef. These data were used to determine the ADI for the use of bovine lactoferrin as described in this document.

Bovine lactoferrin has been consumed by humans for thousands of years as a naturally occurring protein found primarily in milk. Consumption of bovine lactoferrin through dairy sources has been estimated to be 73, 75, and 50 mg/day at the 90th percentile of intake for children, teens and adults, respectively. In addition to dairy sources, lactoferrin is currently marketed in the U.S. as a dietary supplement. Consumption of lactoferrin from this source ranges from 10 to 1200 mg/day.

Infants who are breast-fed consume human lactoferrin, which is a natural constituent of human milk. Human milk contains from 1 to 4.2 mg lactoferrin/ml, resulting in a daily consumption of 1 to 4.2 g lactoferrin/day, assuming a daily consumption of 1 liter. As a comparison, infants who consume infant formula derived from cow protein consume up to 245 mg/day of bovine lactoferrin, derived from the whey protein component. There are several infant formulas sold outside the U.S. that contain supplemental lactoferrin. The supplemental lactoferrin adds up to 107 mg/day in addition to the maximum of 245 mg/day derived from whey protein. Therefore, infants consuming these formulas may consume up to 352 mg/day bovine lactoferrin.

A great deal is known about the fate of ingested human and bovine lactoferrin in the body. Unlike many dietary proteins, lactoferrin is only slowly degraded in the gut, as demonstrated in the piglet, baby monkey, and human newborn. Studies in human infants demonstrate that considerable quantities of intact human lactoferrin are excreted by the breast-fed infant. Higher amounts are excreted during the early weeks of life and the quantity excreted is progressively smaller during successive weekly measurements. During the first weeks of life, approximately 2 to 6% of the lactoferrin consumed by the infant is excreted in the feces per day; however, by 4 months, less than 2% of the lactoferrin intake is excreted. Increased proteolysis of the lactoferrin takes place as the infant ages. The lactoferrin excreted in the feces is intact. Compared with other whey proteins, the relatively large amounts of lactoferrin excreted intact in the feces from infants, along with the finding that no other major whey proteins are detected in the feces, reinforces the fact that lactoferrin is protected from degradation.

Two studies of lactoferrin excretion in infants fed formula containing supplemental bovine lactoferrin indicated that, as expected, babies fed formula containing bovine lactoferrin excreted significantly more lactoferrin in their stools than babies fed the basic formula. The levels excreted, however, were significantly lower than levels excreted from breast fed babies. Most of the lactoferrin from the formula appeared to have been broken down.

In contrast to these findings, however, a third study of the fate of bovine lactoferrin and human lactoferrin fed to healthy babies aged 3 days to 2 months showed that bovine lactoferrin was less degraded than the human forms and total excretion per day of the intact protein was greater. Quantitative determination of human copro-lactoferrin from birth through a period of up to 3 weeks showed that the daily elimination decreased from 25 to 5 mg. The amount of copro-lactoferrin corresponding to the endogenous secretion (biosynthesis by the intestinal tract) was calculated to be from 0.5 to 1 mg/day.

Intact lactoferrin has also been found in the urine of human milk-fed preterm infants. Because serum levels of lactoferrin in both preterm and term infants do not vary with diet (i.e., human milk vs. bovine milk based formula), studies were conducted to determine the origin of the lactoferrin in urine. It was determined that nearly all of the intact lactoferrin in the urine is of maternal origin

The mean plasma level of lactoferrin in adults is 122 $\pm$ 40 mcg/L; in term infants up to the age of 21 days, the mean lactoferrin level is 385 $\pm$ 113 mcg/L. The difference in the means between adults and infants is statistically significant. There were no significant differences between the means of breast fed and formula fed infants. No significant differences in plasma levels were found between adults and infants at 15 weeks of age.

The plasma lactoferrin concentration in adults is derived from neutrophils. Lactoferrin derived from breast milk given to adults is rapidly cleared from the circulation, being avidly sequestered by the liver and spleen. The sequestered lactoferrin is promptly catabolized. The iron, however, is not excreted in the urine, but rather retained and slowly transferred to the bone marrow, where it is subsequently incorporated into developing erythrocytes.

The safety of bovine lactoferrin similar in specifications to the bovine lactoferrin which is the subject of this GRAS determination was assessed in an acute toxicity study, a 4-week oral toxicity study, a thirteen-week oral toxicity study, and an Ames assay.

Lactoferrin exhibited low acute oral toxicity. A single oral dose of 1,000 or 2,000 mg/kg bovine lactoferrin or bovine iron-saturated lactoferrin resulted in no adverse effects or deaths. Based on these results, the lethal dose of lactoferrin exceeds 2,000 mg/kg.

In the 4-week toxicity study, male and female Sprague-Dawley rats were exposed by oral intubation to 200, 600, or 2,000 mg/kg/day bovine lactoferrin once daily for 4 weeks (28 days). Administration of 200, 600, and 2,000 mg/kg/day bovine lactoferrin to male and female rats resulted in no deaths or treatment-related changes in body weight, food consumption, organ weight, ophthalmology, hematology, blood chemistry, urinalysis, or gross pathology and histology examinations. Therefore, the no-observed-adverse-effect (NOAEL) level of bovine lactoferrin was estimated to be in excess of 2,000 mg/kg/day.

In a thirteen-week toxicity test, male and female Sprague-Dawley rats, 4 weeks of age, were exposed to 200, 600, or 2,000 mg/kg/day bovine lactoferrin by oral intubation once daily, seven

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days a week for 13 weeks. Bovine lactoferrin administered by oral intubation to rats for 13 weeks did not result in significant test material related changes in the appearance, general condition, body weight, food consumption, ophthalmology, hematology, blood chemistry, gross pathology, or histology of the animals. Thus, under the conditions of this study, the NOAEL for bovine lactoferrin was estimated to be in excess of 2,000 mg/kg/day.

Lactoferrin was examined for mutagenic potential in a reverse mutation assay. It did not cause a positive response in any of the tester strains in the presence or absence of S9 activation at concentrations up to 5,000 mcg/plate. Thus, under the conditions of the study, lactoferrin was not found to be genotoxic in the *Salmonella typhimurium* reverse mutation assay or the *Escherichia coli*/mammalian microsome reverse mutation assay.

Humans have been exposed to bovine lactoferrin in numerous clinical trials that were designed to look at effects on either iron absorption or modulation of microflora or infection. In studies of infants, dose levels used ranged from 1.4 mg/day (0.3 mg/kg/day) to 2.9 g/day (1.0 g/kg/day) and study durations were from 11 days to 5 months. In studies of adults, dose levels used ranged from 100 mg/day (1.7 mg/kg/day) to 3.6 g/day (60 mg/kg/day) and study durations varied from a single dose in one study to 8 weeks. Despite the fact that the studies were not designed specifically to look for issues of tolerance to ingestion of lactoferrin, no adverse health effects as a result of lactoferrin intake were reported in any of the studies. There are also no indications of subjects discontinuing treatment due to adverse reactions.

In conclusion, animal studies indicate that there are no adverse effects related to lactoferrin consumption at levels up to 2,000 mg/kg/day given for up to 13 weeks. *In vitro* studies provide no evidence of mutagenic potential.

Background exposure to lactoferrin by children, teen and adults, from dairy products ranges from 50 to 75 mg/day at the 90th percentile of consumption and from dietary supplements ranges from 10 to 1,200 mg/day.

Human clinical trials have exposed infants to lactoferrin doses of up to 2.9 g/day and adults to doses up to 3.6 g/day. Human clinical data indicate that bovine lactoferrin intakes in adults at levels of 3.6 g/day and below and in infants at levels of 2.9 g/day and below are well tolerated and without reported adverse health effects.

Thus, the ADI for bovine lactoferrin can be derived from the long history of safe consumption of lactoferrin from dairy sources and dietary supplements and the lack of adverse findings in clinical trials at doses up to 3.6 g/day. The ADI is therefore less than or equal to 3.6 g/day for the general population with the exception of infants under 1 year.

VI. GRAS DETERMINATION

A. Regulatory Framework

The regulatory framework for establishing whether a substance can be considered GRAS in accordance with Section 201(s) of the Federal Food, Drug, and Cosmetic Act is set forth under 21 CFR 70.30. This regulation states that general recognition of safety may be based on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. Determination of GRAS status may be made either: (1) through scientific procedures (170.30(b)); or (2) through experience based on common use in food in the case of a substance used in food prior to January 1, 1958 (170.30(c)).

A scientific procedures GRAS determination (under 170.30(b)) requires the same quantity and quality of scientific evidence as is required to obtain approval of the substance as a food additive. Moreover, in addition to requiring scientific evidence of safety (as with a food additive), a GRAS determination also requires that this scientific evidence of safety be generally known and accepted. This so-called common knowledge element of a GRAS determination includes two facets: (1) the data and information relied upon to establish the scientific element of safety must be generally available; and (2) there must be a basis to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

The criteria outlined above for a GRAS determination based on scientific procedures were applied as described below in an assessment of whether bovine lactoferrin, employed as a barrier to microbial contamination in processed beef, is GRAS. Once bovine lactoferrin is determined to be GRAS for its intended use, it is permitted to be used for that purpose because it is not (by definition) a food additive, and therefore does not require promulgation of a specific food additive regulation under 21 CFR prior to marketing.

B. Safety of Bovine Lactoferrin

The determination of safety is based upon a critical review of both toxicological and clinical safety studies as well as supporting information derived from a history of exposure. The information reviewed included background information (i.e., composition and uses), consumer exposure data, and biological studies in both animals and humans.

Human exposure to lactoferrin and data from human and animal studies indicate that bovine lactoferrin intake at levels of up to 3.6 g/day in the general population is well tolerated and without reported adverse health effects. It can thus be concluded that the ADI for supplemental intake of bovine lactoferrin is up to 3.6 g/day. The EDI from the proposed use of bovine lactoferrin is 9.1 mg/day. This EDI is approximately 0.25% (1/400) of the ADI and, therefore, the consumption of bovine lactoferrin from the proposed uses is concluded to be safe.

C. GRAS Determination

Based on the total background information, studies, and conclusions presented in this document, the use of bovine lactoferrin as a component not to exceed 2% of a spray to be used on processed beef at a maximum concentration of 3.26 ml spray/kg beef, i.e., 65.2 mg lactoferrin/kg beef, as a barrier to microbial contamination is generally recognized as safe. The potential exposures to the general population associated with this use will result in exposures that are well below an acceptable fraction of the ADI.

Determination of the GRAS status of bovine lactoferrin as a food ingredient has been made through the deliberation of experts, Dr. Vasilios Frankos, Dr. Claire Kruger, Dr. James Heimbach, and Dr. Barry Hooberman, scientists qualified by training and experience to evaluate the safety of food ingredients. These experts have carefully reviewed all the available data on the metabolism and short-term and long-term toxicity of bovine lactoferrin, human exposure and tolerance to bovine lactoferrin, and human clinical safety studies evaluating safety of administered intakes of bovine lactoferrin and have concluded that:

> There is no evidence in the available information on bovine lactoferrin that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when it is used at levels that are now current or that might reasonably be expected from the proposed application. Bovine lactoferrin is GRAS for use as a component of spray products to be used as barriers to microbial contamination of processed beef..

It is their opinion that other qualified and competent scientists reviewing the same publicly available data would reach the same scientific conclusion. It is noted that bovine lactoferrin has already been determined to be GRAS for use as an ingredient in sports or functional foods and dietary supplements at an intake level up to and including 3.6 g/day (DMV International, GRN#42, received by FDA April 10, 2000).

Therefore, bovine lactoferrin, to be used as a component at 2% or less of spray products applied to beef at a maximum concentration of 3.26 ml spray/kg beef (or 65.2 mg lactoferrin/kg beef) is GRAS. Because bovine lactoferrin is GRAS for its intended use, it is excluded from the definition of a food additive, and thus may be marketed for this use without the need to promulgate a specific food additive regulation under 21 CFR.

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SUBMISSION END

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August 1, 2001

Judith L. Kidwell
Consumer Safety Officer
Food and Drug Administration
Office of Premarket Approval
Division of Petition Control
Direct Additives Branch, HFS-215
200 C Street, SW
Washington, DC 20204

REFERENCE: GRN No. 67, Milk-Derived Lactoferrin

Dear Judy:

As we agreed at our last meeting with FDA on May 22, we have planned appropriate labeling to assure that consumers of beef products that have been treated with the subject lactoferrin spray are made aware of this fact. On July 17 Eric Hale of Farmland National Beef and I met with officials of the Labeling and Additives Policy Division of the USDA Food Safety and Inspection Service (USDA/FSIS) to review our labeling plans and to seek their advice on how best to assure that this goal will be accomplished.

Proposed Label Content
All beef products treated with lactoferrin will bear one of the following statements in a prominent position on the package:

- Treated with lactoferrin from milk
- Treated with [brandname] lactoferrin from milk
- Treated with lactoferrin, a milk protein
- Treated with [brandname] lactoferrin, a milk protein

It is possible that the statement above may be followed by a phrase such as "for your protection" or "for your added safety." This added wording, if used, will comply fully with USDA labeling regulations and will not interfere with the visibility or clarity of the disclosure of the presence of lactoferrin from milk.

Assuring that Labeling Will Follow the Product
Beef products treated with lactoferrin are regarded by Farmland National Beef as "value-added" products that will command a premium price in the market. Consequently, there is an economic incentive for retailers purchasing such product to display the required labeling; it will not be a matter of a forced disclosure that retailers would wish to avoid.

Much of the beef product treated with lactoferrin is produced as "case-ready beef." This means that the product is shipped directly by Farmland to the retailer in its finished form, which includes the final package with the final consumer label. The fresh beef industry is moving rapidly in this

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direction, and thus an increasing proportion of treated product will be packaged and labeled in-house with no further intervention by the retailer.

Most of the remainder of the product treated with lactoferrin is sold directly to the retailer in a pre-finished form which may require additional cutting, trimming, and portioning. The retailer is responsible for the final packaging of the product for sale to consumers. The product shipped from Farmland to the retailer contains a complete label on the outside of the shipping carton and the retailer receives accompanying instructions regarding labeling of the retail product. Every package shipped from Farmland contains production information and can be tracked all the way through to the individual retailer.

A small percentage of the Farmland product is shipped to other processors for further processing into products such as hot dogs and sausages. All of the products shipped from Farmland contain the appropriate label with instructions to the processor regarding consumer labeling. The products that are eventually produced by these processors contain standard ingredient labeling to which the lactoferrin label will be added.

USDA/FSIS Experience and Capability to Assure Proper Labeling
The USDA/FSIS officials with whom we met indicated that they have no concern with the ability of the agency to assure that the labeling will follow the product as it moves through commerce and that the appropriate label will appear on the product actually presented to consumers. They cited their previous experience with citric acid labeling as an example of a successful program of this type.

In summary, Farmland will label beef products treated with lactoferrin spray in a way that provides full disclosure to consumers of the presence of a milk protein. Systems are already in place to assure that this labeling will be carried forward and will appear on packages presented at retail. Finally, the regulatory agency with oversight responsibility for meat products, USDA/FSIS, has indicated complete confidence that existing regulatory procedures are adequate to assure that required labeling will appear on products offered to consumers.

Sincerely

James T. Heimbach, Ph.D., F.A.C.N.
Principal

cc. Robert C. Post, Ph.D., USDA/FSIS/Labeling and Additives Policy Division