Lactoferrin in milk

Bo Lönnerdal

Department of Nutrition, University of California, Davis, CA 95616, USA

Introduction

Lactoferrin is a major component of breast milk protein, constituting 10-15% of the total content. Each molecule of lactoferrin is capable of binding two atoms of iron and it has been proposed that lactoferrin is involved in iron absorption in the newborn infant. It is known that iron status of exclusively breast-fed infants usually is satisfactory to 6-9 months of age, in spite of a low iron content in breast milk. As a significant part of iron in human milk is bound to lactoferrin, it has been hypothesized that lactoferrin could aid in the delivery of iron to specific sites on the brush border membrane of the small intestine. Such lactoferrin receptors have now been isolated and characterized and may indeed facilitate cellular uptake of iron. Since lactoferrin is unusually stable against proteolytic enzymes, surviving molecules may help the infant to accrue breast milk iron during the neonatal period. It is also possible that lactoferrin in milk may have other biological effects in the infant, such as inhibiting bacterial growth, stimulating mucosal growth and proliferation, as well as modulating immune function. Bovine lactoferrin can now be obtained commercially and be used in infant formulae, but clinical evaluations of its effects have so far been disappointing. Recombinant human lactoferrin may also soon become available, but the effects of this form of lactoferrin have not yet been evaluated. It is possible that human lactoferrin in the context of human milk may have important biological functions in breast-fed infants, but it may be difficult to mimic these functions by the use of bovine lactoferrin or recombinant human lactoferrin added to infant formulae.

Protein composition of human milk

The concentration of protein in human milk is lower than that of most species, possibly because of the comparatively slow growth rate of infants. In early lactation, breast milk contains 12-13 g protein/l, while mature milk has a protein concentration of 8-9 g/l [1]. Human milk contains less casein than most species do; only ~20% of total protein in early milk is casein, while mature milk contains ~40% casein [2]. The major whey proteins, i.e. soluble proteins, are lactoferrin, α-lactalbumin and secretory IgA [3]. Other protein components of whey are lysozyme, serum albumin, bile-salt stimulated lipase and amylase (Table I). There is a multitude of enzymes and binding proteins present in human milk at low concentrations; the physiological significance of these components is still largely unknown [4] (see Koldovsky, in this issue, pages 105-112).

Lactoferrin constitutes about 10-15% of the total protein content of human milk. Milk from early lactation can contain as much as 2-3 g/l of lactoferrin, while mature milk contains about

<table>
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<tr>
<th>Proteins</th>
<th>%</th>
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<tr>
<td>β-casein</td>
<td>20-30</td>
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<tr>
<td>κ-casein</td>
<td>&lt;5</td>
</tr>
<tr>
<td>α-casein</td>
<td>&lt;1</td>
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<tr>
<td><strong>Lactoferrin</strong></td>
<td><strong>10-15</strong></td>
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<tr>
<td>α-lactalbumin</td>
<td>10-15</td>
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<td>Secretory IgA</td>
<td>5-10</td>
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<td>IgG</td>
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<td>IgM</td>
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<td>Serum albumin</td>
<td>&lt;1</td>
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<tr>
<td>Lysozyme</td>
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<td>Bile-salt stimulated lipase</td>
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Maternal iron status or iron intake do not appear to affect the concentration of lactoferrin in milk [5], but it has been reported that lactoferrin levels may be elevated in milk from malnourished women [6]. Mastitis has been shown to increase lactoferrin concentrations in cow’s milk [7], but infection in general during lactation does not appear to affect human milk lactoferrin concentrations [8, 9].

The protein concentration of infant formulae is higher than that of human milk and is usually around 15-16 g/l. Lactoferrin is also a constituent of cow’s milk, but the concentration is considerably lower than in human milk, at about 0.01 g/l [10]. Thus, infant formulae based on cow’s milk will contain small quantities of bovine lactoferrin. It is now possible to obtain bovine lactoferrin commercially in large quantities so that infant formulae may be fortified with lactoferrin. A few companies have in fact started to fortify their products with bovine lactoferrin, but the concentrations used are much lower than that of lactoferrin in human milk. The effect(s) of these small quantities of bovine lactoferrin on infants is uncertain (see below).

Properties of lactoferrin

Lactoferrin was first isolated from human milk in 1960 [11]. It is similar to transferrin in that it has a molecular weight of ~80,000 and binds two atoms of ferric iron together with either bicarbonate or carbonate as anions [12]. However, lactoferrin and transferrin are different gene products and there is no cross-reactivity of one antibody towards the other protein. Lactoferrin has an affinity constant for iron which is several orders of magnitude higher than that of transferrin and lactoferrin maintains its iron-binding capacity at a lower pH than does transferrin. The lactoferrin molecule contains two lobes, which are similar but not identical, and each lobe binds one atom of iron. Lactoferrin is a glycoprotein, containing one glycan per lobe and the bi- or tri-antennar structure contains terminal fucose residues [13]. The protein is unusually resistant against proteolysis, particularly in its iron-saturated form [14]. Studies in breastfed and formula-fed infants have shown that intact lactoferrin [or larger fragments thereof] can be found in significant quantities in the stool [15-17]. Thus, lactoferrin can survive digestion by pepsin and pancreatic enzymes in the infant gut and possibly perform biological functions in the gastrointestinal tract. An even higher proportion of lactoferrin is found in the feces of premature infants [18]. Intact lactoferrin also has been found in the urine of such infants, indicating that not only do some lactoferrin molecules survive digestion, but they also may be absorbed and excreted in intact form [19]. The extent to which this process occurs and its biological significance remain to be explored.

Recombinant human lactoferrin

With the advent of the techniques of genetic engineering it has become possible to produce recombinant human lactoferrin. The cDNA for human lactoferrin was cloned and the gene subsequently has been inserted into yeast [20], baby-hamster kidney (BHK) cells [21], Aspergillus [22] and cows [23]. The two latter systems are likely to produce large quantities of recombinant lactoferrin that may be used in infant formulae manufacture. There are, however, still many hurdles to overcome before this will occur. The stability of the transgene in transgenic cows and the long time needed for each generation are some of the problems encountered with this approach. Expression levels and potential antigenic contaminants from the fungi are potential problems with the Aspergillus system. Approval from authorities to use the recombinant lactoferrin in infant diets may also be difficult to obtain, as noted in some European countries. Finally, the effect of heat treatment of the formula on the biological properties of the recombinant lactoferrin needs to be evaluated carefully.

Biological functions of lactoferrin

Lactoferrin has been proposed to have several physiological functions (Table II). It was suggested early that apo-lactoferrin may exert a bacteriostatic function by withholding iron from iron-de-
manding bacteria [24]. As lactoferrin in human milk is only saturated with iron to about 1-4% [25], this might be a mechanism to help breast-fed infants resist infection. More recently, it was shown that a fragment of lactoferrin, named lactoferricin [26], may exert a direct bactericidal effect on some bacteria. Although both effects have been demonstrated clearly in vitro, there is limited evidence for these functions in vivo. Since lactoferrin binds a significant proportion of iron in human milk, it has also been hypothesized that lactoferrin may promote iron absorption in breast-fed infants [27]. The finding of intact lactoferrin in the stool of breast-fed infants even up to the age of four months [15] supported this idea. Although some digestion of lactoferrin certainly occurs, up to 10-15% of all lactoferrin appears to escape digestion. When calculating the iron-binding capacity of this amount of lactoferrin, it is more than adequate to deliver all of the iron in human milk. The ability of lactoferrin to partially escape digestion may be explained by the relatively high gastric pH of breast-fed infants, the comparatively low activity of gastric and pancreatic proteases and, particularly, the resistance of this molecule to proteolytic attack.

A stimulatory effect of lactoferrin on intestinal cell growth also has been proposed [28, 29]. However, these findings should be viewed with some caution as they were made in the rat, a species that does not have lactoferrin in its milk [10]. It is possible that lactoferrin has some mitogenic effect, but support for this in humans has not yet been obtained. Lactoferrin also is involved in immune function, particularly in phagocytic killing by macrophages, and is believed to be synthesized by neutrophils [30]. Part of the involvement of lactoferrin in immune function may be explained by the stimulatory effect of lactoferrin on cytokine release by cells [31]. Finally, it is known that lactoferrin binds avidly to DNA [19] and very recent findings of highly specific binding of lactoferrin to DNA sequences in the nucleus suggest that lactoferrin may act as a transcription factor and thus regulate cellular events [32]. Further research is needed to evaluate this potential function of lactoferrin.

### Lactoferrin receptors

The observation that lactoferrin is resistant to proteolysis led to the suggestion of specific receptors for lactoferrin in the small intestine. Such receptors may then facilitate the binding of lactoferrin to the mucosa and subsequently the delivery of iron to the intestinal cell. Studies by Cox et al. [33] showed that lactoferrin (and iron) bound to human adult intestinal tissue in a saturable and specific manner, supporting the notion of a lactoferrin receptor. Subsequent studies in the mouse demonstrated such a receptor and provided some biochemical data [34]. We first isolated the lactoferrin receptor from brush border membranes from infant rhesus monkey small intestine [35]. The receptor was found to be specific for human and rhesus lactoferrin, but did not bind bovine lactoferrin or human transferrin. Subsequent studies in the rhesus monkey showed that the receptor binds both iron- and manganese-saturated lactoferrin as well as apo-lactoferrin [36]. The receptor appears to be present at all ages, including the foetus, but the number of receptors per tissue weight appeared highest during infancy, i.e. the period when the intestine would be likely to be exposed to lactoferrin [35]. Studies in piglets showed no variation in receptor affinity or number of receptors during the infancy period and the receptor was present in similar quantities in all parts of the small intestine [37].

The lactoferrin receptor subsequently was purified from human foetal and infant small intestine by affinity chromatography and was found to have a molecular weight around 115,000 [38]. The lactoferrin receptor is highly specific for human lactoferrin, is glycosylated and has a subunit molecular weight of about 37,000. Deglycosylated lactoferrin binds to the receptor with similar

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**Table II: Proposed biological functions for lactoferrin.**

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<th>Function</th>
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<tr>
<td>Enhancer/facilitator of iron absorption</td>
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<tr>
<td>Bacteriostatic agent</td>
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<td>Bactericidal agent</td>
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<tr>
<td>Phagocytic killing</td>
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<tr>
<td>Growth factor</td>
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<tr>
<td>Immunostimulatory factor</td>
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<tr>
<td>Transcription factor (?)</td>
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It is evident that breast-fed infants utilize iron from their diet very effectively. This has been shown by a variety of methods in several populations and settings [39]. However, infants fed milk formula which has not been fortified with iron are frequently found to have iron deficiency or iron deficiency anaemia at 6 months of age [40, 41]. The lower iron status of infants fed unfortified formula as compared to breast-fed infants also supports a high bioavailability of iron from breast milk, as the iron content of formula generally is higher than that of human milk. Cow’s milk in itself only contains about 0.1-0.2 mg of iron/l, but mineral salts and other components added to formula increase the iron level to 0.7-1.0 mg/l.

Breast-fed infants have satisfactory iron status at 6 months of age as indicated by both haemoglobin (>105 g/l), mean corpuscular volume (MCV), transferrin saturation, and ferritin (>12 µg/l) values [42-44]. Infants fed iron-fortified formula have also been shown to have excellent iron status at 6, 9 and 12 months of age [44-46]. The level of iron fortification needed to assure satisfactory iron status is, however, a matter of disagreement. Infant formulae in the United States usually contain 12-14 mg of iron/l, while formulae in Europe contain 6-7 mg/l. We have evaluated recently the capacity of milk formula with lower levels of iron fortification to provide adequate amounts of iron to healthy term infants exclusively fed formula up to 6 months of age [44, 47]. Our first study showed that feeding a whey-predominant formula with 4 mg of iron/l resulted in satisfactory iron status at 6 months of age [44]. A subsequent study of the same formula with 2 mg of iron/l showed similar results, i.e. serum ferritin concentrations of all infants were higher than 12 µg/l [47].

The observation that infants fed human milk, which contains 0.2-0.4 mg of iron/l [25], had better iron status at 6 months of age than infants fed formula with 0.7 mg of iron/l can be used as indirect evidence to support the notion that iron bioavailability is considerably higher from breast milk than from milk formula. However, radioisotope studies also support a high bioavailability of human milk iron. Garby and Sjölin showed early that iron absorption is high in breast-fed infants and that absorption is highest during early infancy [48]. Schultz and Smith showed that iron absorption from cow’s milk is substantially lower than from human milk [49]. By using the extrinsic tag method, Saarinen et al. demonstrated that iron absorption from human milk is around 50%, while it is substantially lower (~20%) from milk formula [50]. Although the method of extrinsic labeling may not be entirely valid for human milk [27, 51], there is no doubt that some component(s) of human milk leads to high iron absorption, while some component of cow’s milk (and formula) causes lower iron absorption [27]. Therefore, several studies have attempted to evaluate the relative influence of different constituents of human milk and formula on iron absorption.

**Effect of bovine lactoferrin on iron absorption**

The potential effect of lactoferrin on iron absorption and/or iron status has been evaluated in several animal models. As bovine lactoferrin was the first lactoferrin source that was likely to be available in large quantities, most studies have used bovine lactoferrin. The effect of adding bovine lactoferrin to milk formula was evaluated in suckling piglets [52]. This animal model was chosen since sow’s milk has relatively high levels of lactoferrin and the newborn piglet has a high requirement for dietary iron. Piglets were fed a “piglet formula” (based on the composition of sow’s milk) with iron added in the form of bovine lactoferrin or as ferrous sulfate. The formulae were radiolabeled with $^{59}$Fe and the piglets were fed the formula in a fasting state. Radioisotope incorporation in red blood cells and plasma appearance were monitored following dosing. No significant differences were found in net iron uptake between the two forms of iron, although iron uptake into red blood cells and plasma appeared somewhat more rapid from lactoferrin than from ferrous sulfate.

The mouse also has been used as an animal model for studying iron absorption from lactoferrin,
Iron status of mice fed a milk diet supplemented with iron as bovine lactoferrin was similar to that of mice fed iron as ferrous chloride when the mice had been depleted of iron prior to the supplementation. In contrast, milk supplemented with ferric chloride resulted in better iron status than lactoferrin-supplemented milk when iron status was satisfactory. This may suggest that the role of lactoferrin in iron absorption may depend on the iron status of the individual.

Iron absorption from infant formula supplemented with bovine lactoferrin was evaluated in newborn infants by Fairweather-Tait et al. [54]. Seven days old term infants were fed formula labeled with a stable isotope during a three day balance period. The experimental formula contained 2.85 g of lactoferrin/l, while the control formula contained no extra lactoferrin. Iron absorption was similar from the two formulas, 46% vs 44%, respectively [54]. Iron balance studies on formula fortified with bovine lactoferrin also have been performed. Term infants, 3-17 weeks old, were used in this study and the balances were performed during three days. Formula with either 1 g of bovine lactoferrin/l or no lactoferrin added were used, and the iron concentration of these products were 1.1 and 0.77 g/l, respectively. Iron retention from the lactoferrin-supplemented formula was 36% and from the control formula 28%; this difference was not significant. Iron retention from human milk was found to be 47% [55].

The long-term effects of feeding infant formula supplemented with bovine lactoferrin was evaluated by Chierici et al. [56]. Term infants were fed from birth to 5 months of age either formula with 0.1 or 1.0 g/l of lactoferrin or the same formula without lactoferrin added. The iron concentration of the three formulas were 0.73, 0.98 and 0.70 mg/l, respectively. Iron status as assessed by serum ferritin concentrations at 5 months of age had group means of 18, 25 and 20 µg/l, respectively. Although the ferritin values were slightly higher for the group fed formula with the higher concentration of lactoferrin, it should be noted that the iron concentration of this product also was slightly higher. As the formula was fed exclusively for five months, it is feasible that the slightly higher iron concentration, and not lactoferrin, was the reason for the somewhat higher ferritin concentrations. Bovine lactoferrin also has been used to substitute part of the iron in infant formula [44]. Term infants were fed formula exclusively from six weeks to six months of age. The formula contained ferrous sulfate at 4 or 7 mg of iron/l or bovine lactoferrin providing 1.4 mg of iron and ferrous sulfate providing 2.6 mg of iron/l. At six months of age, infants in all groups had similar haemoglobin values and no infant had iron deficiency as judged by serum ferritin (<12 µg/l). There were no significant differences between groups fed the two iron levels or between the groups fed lactoferrin-iron and ferrous sulfate versus ferrous sulfate alone.

It is possible that the lack of positive effect of lactoferrin on iron absorption and iron status found in these studies can be explained by the inability of bovine lactoferrin to bind to the intestinal lactoferrin receptor (see above). Instead of binding to the receptors, lactoferrin may be digested in the small intestine and iron released for absorption. In the high ascorbic acid environment created by the formula, this iron is likely to be in the ferrous form and consequently be absorbed to an extent similar to that of ferrous sulfate.

The newborn rhesus monkey is an excellent model for the human infant. Its nutrient requirements and the development of its gastrointestinal function are very similar to human infants. In addition, rhesus monkey milk has a composition which closely resembles that of human milk [57]. In fact, lactoferrin is the major iron-binding protein of rhesus milk and the isolated protein was found to be almost identical to human lactoferrin with regard to biochemical properties [58]. Close similarity was demonstrated by the binding of the human lactoferrin antibody to rhesus lactoferrin. Similar to the human lactoferrin receptor, bovine lactoferrin was not found to bind to the rhesus lactoferrin receptor. We therefore used the infant rhesus monkey to evaluate the effect of human lactoferrin as well as bovine lactoferrin on iron absorption [59]. Care was taken to assure proper labeling of all iron-binding ligands with the
radioisotope and that a realistic meal size was used. Fasted animals were then fed the radiolabeled meals consisting of human milk or whey-predominant formula with or without ferrous sulfate fortification or bovine or human lactoferrin. Iron retention ($^{59}$Fe) was monitored in a whole body counter. In order to correct for large interindividual variations in iron absorption, a reference dose of $^{55}$Fe in a ferrous ascorbate solution was given to each animal. No significant differences in iron absorption were found between groups. It is possible that the bovine lactoferrin was ineffective because its lack of binding to the lactoferrin receptor and that the human lactoferrin had been somewhat altered during the isolation procedure and therefore did not behave like normally in vivo.

Obtaining large quantities of isolated human lactoferrin for clinical studies on human infants has been difficult. We therefore used an alternative approach to evaluate the effect of human lactoferrin on iron absorption in human infants [60]. In this study, healthy term infants were given human milk or human milk without lactoferrin. Iron absorption was followed by using a stable isotope of iron and iron incorporation into red blood cells. Lactoferrin was removed by first gently removing fat and casein from human milk by centrifugation and then the lactoferrin was separated from the whey fraction by affinity chromatography. The lactoferrin-free whey was added back to the fat and the casein and the fractions were then stirred gently to homogeneity. Intact human milk and lactoferrin-free human milk were then isotopically labeled. All infants were exclusively breast-fed and each infant was fed his/her own mother’s milk. Iron absorption was found to be slightly higher from the lactoferrin-free milk than from intact breast milk. However, caution must be used when evaluating these results. In order to feed sufficient quantities of the stable isotope to allow detection, relatively large volumes of milk were needed and, consequently, larger infants. Therefore, all infants except one were older than four months of age. We have found earlier that by this age most of the lactoferrin in human milk is digested and it is therefore possible that the amount of intact lactoferrin that survived and reached the receptors was very small. It is interesting to note that in the only infant that was younger than four months, iron absorption was higher from intact breast milk than from the lactoferrin-free milk. Unfortunately, the total number of infants studied was low. It should also be cautioned that even if we attempted to remove the lactoferrin by gentle methods, the milk had been processed, possibly altering its properties and therefore, possibly, the effect on iron absorption. Finally, the role of homeostasis on iron absorption must be considered carefully. It is possible that long-term iron status has more pronounced effects on iron absorption [and erythrocyte incorporation of iron] than dietary components during a short-term study. However, evaluation of iron status at this age is very difficult. Thus, the relative importance of these two factors is complicated to assess with any degree of certainty.

Bacteriostatic and bactericidal effects of lactoferrin

It was hypothesized early that lactoferrin may inhibit iron utilization by bacteria and cause bacteriostasis as a result of its iron-sequestering properties [24]. Bovine lactoferrin in the iron-unsaturated form was shown to have bacteriostatic activity against Escherichia coli. However, a few strains were resistant or unaffected, indicating that mechanisms other than simple iron withholding may be involved in the antimicrobial action of lactoferrin. For example, lactoferrin has been shown to cause release of lipopolysaccharide (LPS) from cell walls of Gram-negative bacteria [61]. More recently, it was shown that lactoferrin binds to porins, a group of molecules common in E. coli, thus causing permeability changes [62].

Breast-fed infants are more resistant to intestinal infections than those fed formula, which may in part be due to the presence of lactoferrin [24]. Bacteriostatic effects of lactoferrin and human milk were demonstrated that could be abolished by the addition of iron. Moreover, gut microflora of the breast-fed infant differs considerably from that of the formula-fed infant in that the former is predominantly comprised of bifidobacteria, lactobacilli, and staphylococci, whereas the latter contains enterococci, coliforms, and bacteroides [63].
Supplementation of infant formula with bovine lactoferrin, however, did not influence gut microbiota [63, 64], which may indicate that lactoferrin may act in conjunction with other factors in breast milk, e.g. secretory IgA, lysozyme, citrate and bicarbonate.

A lactoferrin domain with bactericidal activity was isolated recently and described in bovine and human lactoferrin [26]. This peptide showed a marked growth-inhibitory effect on *E. coli* and is different from the iron-binding region [65].

**Lactoferrin as a growth factor**

Milk, and particularly colostrum, has been shown to stimulate proliferation of the small intestine [66]. Lactoferrin is present in the milk of most mammals and was suggested to be a possible growth factor for the intestinal mucosa when Nichols et al. reported that thymidine incorporation into DNA of rat crypt cells was enhanced in the presence of human lactoferrin [28]. This effect does not appear to be dependent on the presence of iron in human lactoferrin [67]. Most of the lactoferrin in human milk is unsaturated with iron [25], which indirectly supports the above theory.

Various cell lines also have been used to study the growth stimulation of lactoferrin. However, no consensus has been reached. In cells such as mouse embryo cells and neonatal rat hepatocytes, only iron-saturated lactoferrin, both human and bovine, was shown to be growth stimulatory [12]. Studies on a human adenocarcinoma cell line (HT-29) in a defined serum-free medium showed that lactoferrin could not substitute for transferrin or allow cell proliferation(s) [68]. In studies on bovine mammary epithelial cells, lactoferrin has been shown to be growth inhibitory [69]. Further studies clearly are needed to evaluate the effects of lactoferrin on cell growth and proliferation.

**Conclusions**

A multitude of biological functions can be demonstrated for lactoferrin in laboratory experiments in cells or animal models. Further, carefully designed, clinical studies are needed to evaluate whether lactoferrin exerts these functions in human subjects. Before such clear evidence has been obtained, it will be difficult to justify the use of lactoferrin in food products.

**References**

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