



# Bioactive Proteins in Human Milk: Health, Nutrition, and Implications for Infant Formulas

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Breast milk confers many benefits to the newborn and developing infant. There is substantial support for better long-term outcomes, such as less obesity, diabetes, and cardiovascular disease, in breastfed compared with formula-fed infants. More short-term outcomes, such as incidence and duration of illness, nutrient status, and cognitive development during the first year of life also demonstrate benefits of breastfeeding. Several proteins in breast milk, including lactoferrin,  $\alpha$ -lactalbumin, milk fat globule membrane proteins, and osteopontin, have been shown to have bioactivities that range from involvement in the protection against infection to the acquisition of nutrients from breast milk. In some cases, bovine counterparts of these proteins exert similar bioactivities. It is possible by dairy technology to add protein fractions highly enriched in these proteins to infant formula. (*J Pediatr* 2016;173S:S4-9).

Infant formulas have undergone many modifications in previous decades so that the performance of formula-fed infants more closely resembles that of breastfed infants. These alterations, some of which are relatively recent, include: modifications of whey:casein; addition of taurine, nucleotides, docosahexanoic acid, and arachidonic acid; prebiotics (fructo-oligosaccharides/galacto-oligosaccharides); and lutein. Despite these modifications, it is clear that differences remain between breastfed infants and formula-fed infants with regard to both short-term (eg, illnesses, cognitive development) and long-term (eg, obesity, diabetes, cardiovascular disease) outcomes. It is, therefore, evident that further alterations in the composition of infant formula need to be considered.

Several components of breast milk have been shown to have bioactivities *in vitro*, but there is not yet enough evidence from clinical trials to incorporate them into infant formula. It also should be recognized that, with few exceptions, these components (complex oligosaccharides, proteins, and lipid components, such as gangliosides) are not commercially available.

Various bioactive proteins are the components with the most support from clinical trials to date.<sup>1</sup> It should be recognized that the proteins studied are largely of bovine origin and are not identical to their human counterparts. However, in many cases, the structure of the bovine milk proteins share a high degree of homology with the human milk proteins, and because *in vitro* studies have shown equivalence of the human and bovine proteins with regard to bioactivity(-ies), it is reasonable to study the effects that supplementation with these bovine proteins have on infants.

## Bioactive Proteins Involved in Health Outcomes

It is well recognized that breastfed infants have fewer infections than formula-fed infants. This is more pronounced in less developed countries but is significant in affluent countries as well.<sup>2</sup> Several breast milk proteins have been shown to be involved in protecting against infection (Figure 1).

### Lactoferrin

Breast milk was found early on to have bacteriostatic activity against *Escherichia coli*.<sup>3</sup> Investigators discovered that this activity was due to the presence of the protein, lactoferrin, an iron-binding protein that is, present in largely unsaturated form in breast milk. Its high-binding affinity for iron enables it to withhold iron from iron-requiring pathogens. Lactoferrin subsequently was found to be bactericidal, able to kill pathogens, such as *Vibrio cholera* and *Streptococcus mutans*.<sup>4</sup> Furthermore, lactoferrin can act in a synergistic fashion with lysozyme, another protein present in comparatively high concentration in breast milk, to kill Gram-negative bacteria, which are normally resistant to bactericidal action.<sup>5</sup> Lactoferrin, which is highly positively charged, can form a strong complex with bacterial lipopolysaccharide, which is negatively charged, and create holes in the outer membrane of Gram-negative bacteria. Following this attack, lysozyme penetrates the outer

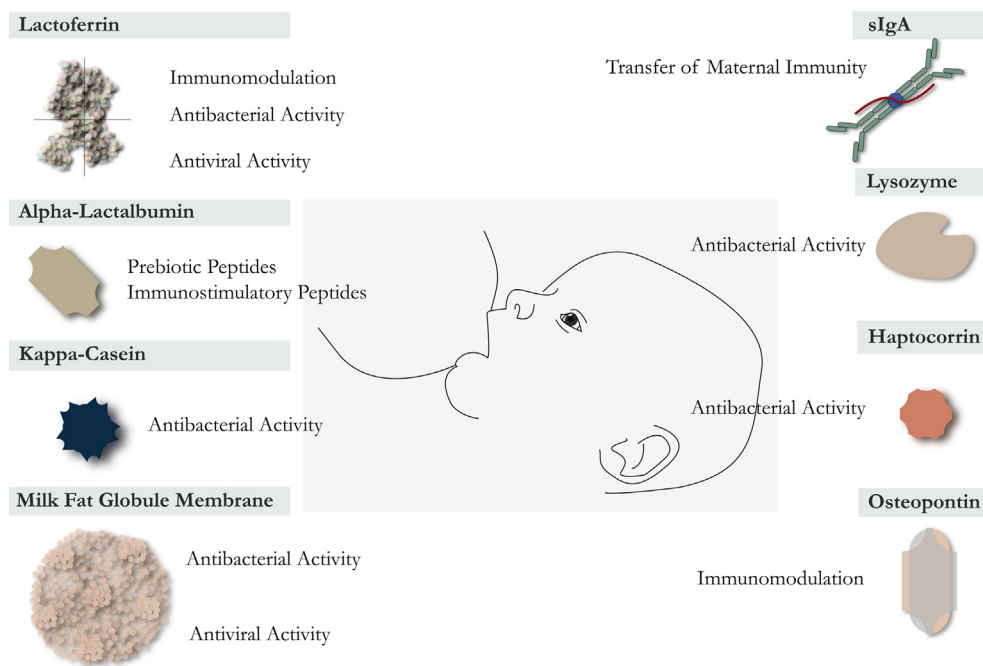
BSSL	Bile salt-stimulated lipase
CPP	Casein phosphopeptide
GMP	Glycomacropeptide
MFGM	Milk fat globule membrane
ORS	Oral rehydration solution
RCT	Randomized controlled trial
slgA	Secretory IgA
WHO	World Health Organization

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**Figure 1.** Bioactive proteins involved in health outcomes.

membrane, thereby obtaining access to and degrading the proteoglycan matrix, resulting in bactericidal action.

Lactoferrin also can modulate immune function.<sup>6</sup> This may occur through several mechanisms, one of which is mediated by an intestinal receptor for lactoferrin.<sup>7</sup> Lactoferrin has a structure that makes it comparatively resistant to digestion and intact lactoferrin is found in significant amounts in exclusively breastfed infants.<sup>8</sup> Following binding of lactoferrin to its receptor, the protein is internalized by an endocytotic process and subsequently binds to the nucleus.<sup>9</sup> Inside the nucleus, lactoferrin binds to specific sites on DNA and, thus, acts as a transcription factor.<sup>10</sup> Among the genes that are affected by lactoferrin are those for several cytokines (eg, interleukin-1 $\beta$  and transforming growth factor- $\beta$ ). Thus, lactoferrin can affect and modulate immune function in the infant, thereby affecting health outcomes.

A clinical study on bovine lactoferrin added to infant formula showed a reduction in upper respiratory disease in infants 6-12 months of age.<sup>11</sup> Manzoni et al<sup>12</sup> have shown a significant reduction in sepsis in premature infants given oral supplements of bovine lactoferrin. Further, Ochoa et al<sup>13</sup> showed that infants given bovine lactoferrin had significantly lower prevalence of *Giardia* species and better growth than infants not receiving the supplement.

### Lysozyme

In addition to acting together with lactoferrin to kill Gram-negative bacteria (as described above), lysozyme can independently kill Gram-positive bacteria by degrading its outer membrane. Lysozyme, like lactoferrin, is found intact

in the stool of both preterm and term infants<sup>8,14</sup> and may, therefore, exert antimicrobial activity in the gut of breastfed infants. Our group explored the possibility that recombinant forms of both human lactoferrin and human lysozyme have a beneficial effect in children hospitalized with acute diarrhea.<sup>15</sup> The 2 human milk proteins were added to the World Health Organization (WHO) rice-based oral rehydration solution (ORS), because both proteins were produced in rice, so that any contaminant in the proteins would be rice protein or starch. The hospital-based study was a double-blind randomized controlled trial (RCT). The other 2 groups received either regular WHO glucose-based ORS or the WHO rice-based ORS (without the recombinant proteins). All children were followed for 2 weeks. A significant reduction in diarrhea was found in the group receiving the human milk proteins, suggesting that they may serve a similar function in breastfed infants. Because of the study design, it is not known whether the activity was due to the individual components or the combination of the two.

### Secretory IgA

By linking 2 molecules of IgA together with a joining chain and a secretory component, the resulting secretory IgA (sIgA) becomes resistant to proteolytic degradation,<sup>16</sup> and intact sIgA is found in the stool of both preterm<sup>14</sup> and term<sup>8</sup> breastfed infants. Although the intestinal mucosa is capable of producing sIgA to some extent in all infants, the amount of sIgA in breastfed infants far outweighs that of formula-fed infants. Through the so-called enteromammary link, maternal immunity can

be transferred to the breastfed infant (ie, the infant receives antibodies against the same antigens to which the mother has been exposed).<sup>17</sup>

### K-casein

The human milk casein micelle is formed largely by  $\beta$ -casein and  $\kappa$ -casein, with small amounts of  $\alpha_{S1}$ -casein. K-casein is heavily glycosylated, containing ~40% carbohydrate as complex oligosaccharides. These oligosaccharides have structures similar to exposed glycans on mucosal surfaces, which often serve as binding sites for pathogens. K-casein, or more likely its immediate proteolytic fragment, glycomacropeptide (GMP), can serve as a “decoy” and bind to pathogens, thereby preventing attachment and infection. Our group has shown that human milk  $\kappa$ -casein can prevent attachment of *Helicobacter pylori* to human gastric mucosa,<sup>18</sup> possibly explaining the lower incidence of *H pylori* infection in breastfed infants. In a study on infant rhesus monkeys infected with enteropathogenic *E coli*, it was shown that bovine GMP added to infant formula reduced the severity of diarrhea and also altered the gut microflora of the infants to make it more similar to that of breastfed infants.<sup>19</sup> Adding GMP and  $\alpha$ -lactalbumin to the formula was even more effective in reducing diarrhea.

### Haptocorrin

The vitamin B<sub>12</sub>-binding protein, haptocorrin, is present in a relatively high concentration in breast milk. There is much more haptocorrin than vitamin B<sub>12</sub> on a molar basis in human milk and the protein is, therefore, present largely in an unsaturated (apo-) form. Haptocorrin (porcine) has been shown to be resistant against in vitro digestion and to have strong antibacterial activity against pathogenic *E coli* in vitro,<sup>20</sup> and may, therefore, serve a role in the defense against infection in breastfed infants. However, there are no commercially available quantities of haptocorrin available, and no clinical trials have been carried out to date. It is possible that haptocorrin may prevent bacterial growth by binding vitamin B<sub>12</sub> strongly, thereby depriving pathogens of an essential nutrient, but it is also possible that the glycans on the glycoprotein haptocorrin may act in a fashion similar to that of  $\kappa$ -casein (as described above).

### Milk Fat Globule Membrane Proteins (MFGM)

The triglycerides in milk fat are surrounded by 3 membranes containing a multitude of proteins.<sup>21</sup> Several of these MFGM proteins, such as lactadherin, butyrophilin, and MUC1, have been shown to have antibacterial and antiviral activity in vitro.<sup>22</sup> An observational study in Mexico found that a higher concentration of lactadherin in breast milk was correlated with a lower prevalence of rotavirus, supporting the concept of the antiviral activity of this protein.<sup>23</sup> Our group gave a bovine MFGM fraction or skim milk powder (control) in complementary food twice daily for

6 months to 6-month-old infants in an RCT in Peru.<sup>24</sup> A significant reduction in the incidence of diarrhea in the infants receiving MFGM was observed, particularly bloody diarrhea, again supporting the anti-infectious activity of the MFGM fraction. In a recent RCT in Sweden, the same bovine MFGM fraction was added to infant formula, and infants were fed this formula from 6 weeks to 6 months of age, then followed up to 1 year of age.<sup>25</sup> Infants fed regular formula served as a control and a reference group of breastfed infants was included. A significant reduction in illness in the infants fed the MFGM-supplemented formula was found compared with infants fed regular formula, and there was no difference with the breastfed infants. The difference was especially pronounced for otitis media, which is known to be less common in breastfed infants. Prescriptions of antipyretics also were significantly less for the MFGM-supplemented infants, and there was again no difference with the breastfed infants. Thus, addition of MFGM to infant formula had significant health benefits for infants. These observations support a role of human milk MFGM in the defense against infection in breastfed infants.

### Osteopontin

The protein osteopontin has received little attention in infant nutrition and health to date. This protein, which was first discovered in bone, is involved in the regulation of the immune system, where it serves several functions.<sup>26</sup> The concentration of osteopontin in breast milk is comparatively high (~140 mg/L), and it is considerably lower in cow's milk and lower still in infant formula. Bovine osteopontin has several structural features similar to those of human milk osteopontin, particularly with regard to binding motifs.<sup>27,28</sup> A bovine milk osteopontin enriched fraction has become commercially available, and our group conducted a RCT on infants from 1-6 months of age in Shanghai, China.<sup>29</sup> Infants were fed regular formula or formula supplemented with osteopontin at 2 different levels (50% or 100% of that found in human milk). Infants fed formula supplemented with osteopontin had significantly lower serum concentrations of the proinflammatory cytokine transforming growth factor- $\alpha$  compared with infants fed regular formula. Several other cytokines also were affected, making the cytokine profile more similar to that of breastfed infants. Immune cell distribution also was affected, resulting in an immune profile that was more similar to breastfed infants (West CE et al, personal communication). Days of illness were significantly lower in the infants fed osteopontin-supplemented formula compared with those fed regular formula, and there was no difference in the number of days of illness compared with breastfed infants. No marked dose-dependency was observed, suggesting that the lower level used may be sufficient for these effects. Taken together, these results strongly suggest that osteopontin affects immune function in infants, conferring health benefits.

## Bioactive Proteins Involved in Nutrient Utilization

Nutrient utilization from breast milk is known to be high and concentrations of nutrients in breast milk are used for setting minimum concentrations of nutrients in infant formula. Several proteins in breast milk have been shown to facilitate the uptake of nutrients present in breast milk (Figure 2).

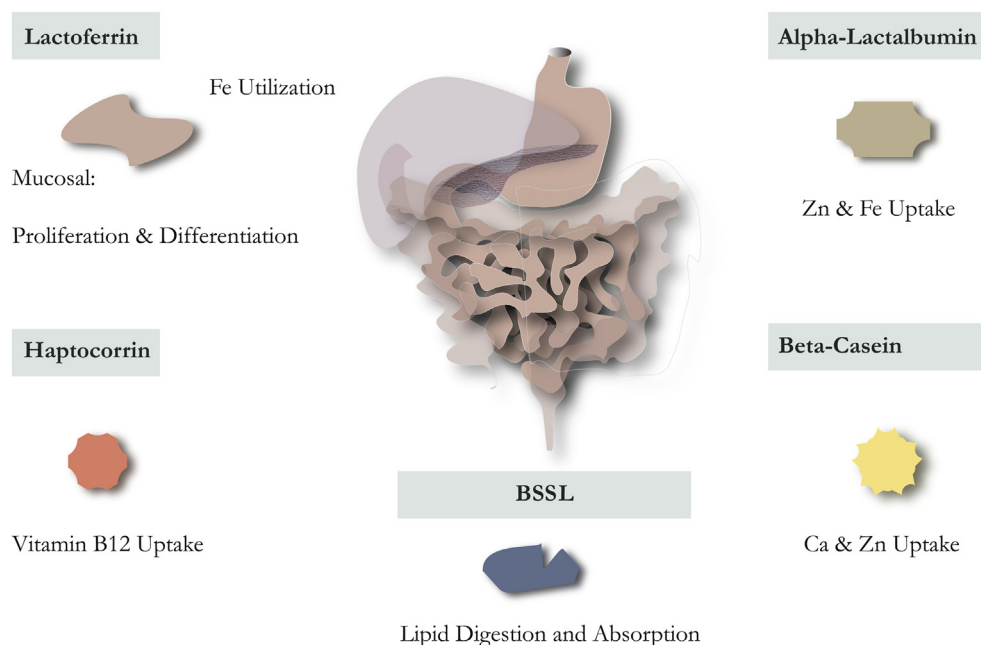
### Lactoferrin

The iron content of breast milk is comparatively low and a major part of it is bound to lactoferrin.<sup>30</sup> It has, therefore, been suggested that lactoferrin is involved in iron uptake from breast milk. Because intact lactoferrin has been found in the stools of exclusively breastfed infants (see above), it was hypothesized that there may be a specific receptor for lactoferrin in the small intestine, facilitating the uptake of iron by the breastfed infant. A lactoferrin receptor was isolated and characterized<sup>31</sup> and subsequently cloned and overexpressed in Caco-2 cells.<sup>7</sup> Lactoferrin uptake was significantly enhanced in cells transfected with the lactoferrin receptor, compared with mock-transfected cells. Iron uptake also was significantly enhanced, although there was no significant effect of iron transport across the cell monolayer. This, however, is likely due to the fact that cell iron homeostasis is strong and iron export out of the cell is dictated by systemic iron needs, which was not possible to regulate in the cell model used. Taken together, the results showed that lactoferrin facilitated the first step of the iron absorption process (ie, the import of iron across the apical membrane, which is the limiting step in iron acquisition by the body). Lactoferrin also has been found to stimulate

epithelial cell proliferation and differentiation.<sup>10,32</sup> High concentrations of lactoferrin (as found in breast milk) were shown to significantly increase cell proliferation, whereas lower concentrations increased cell differentiation.<sup>32</sup> This makes biological sense because early in life the gut lumen of the breastfed infant will have a high concentration of lactoferrin both attributable to a high concentration in milk and limited proteolytic degradation, resulting in high cellular proliferation. Later in life, the concentration will be much lower, attributable to a lower concentration of lactoferrin in the milk, and more efficient digestion of the protein, resulting in increased differentiation. Although these observations need confirmation in vivo, it is well known that the intestinal mucosa of breastfed infants is more developed than that of formula-fed infants. Increased mucosal development caused by lactoferrin may, therefore, increase the mucosal surface and not only enhance the uptake of iron but also of other nutrients.

### $\alpha$ -Lactalbumin

During digestion, the protein  $\alpha$ -lactalbumin forms peptides, some of which have biological activities.<sup>1</sup>  $\alpha$ -Lactalbumin has a specific binding site for calcium and another binding site for essential trace elements, such as iron and zinc.<sup>33</sup> Human and bovine  $\alpha$ -lactalbumin share a large degree of structural homology, and their amino acid sequences are similar. The uptake of iron was shown to be enhanced in infant rhesus monkeys fed infant formula with added bovine  $\alpha$ -lactalbumin compared with regular formula.<sup>34</sup> Further, improved indicators of iron status were found in Swedish infants fed a formula enriched in bovine  $\alpha$ -lactalbumin, when compared with infants fed regular formula.<sup>35</sup> Although



**Figure 2.** Bioactive proteins involved in nutrient utilization.

the mechanism behind these observations has not been elucidated, it is likely that peptides from  $\alpha$ -lactalbumin formed during digestion retain their binding capacity for trace elements and that the uptake of these essential micronutrients by the mucosal cell is facilitated.

### Haptocorrin

As described previously, haptocorrin is present in human milk relatively unsaturated with vitamin B<sub>12</sub>. However, virtually all vitamin B<sub>12</sub> in breast milk is bound to haptocorrin, and it is possible that this binding protein is involved in the absorption of vitamin B<sub>12</sub> from breast milk. This was investigated using human intestinal Caco-2 cells in culture and haptocorrin isolated from breast milk.<sup>36</sup> The uptake of radiolabeled vitamin B<sub>12</sub> was increased by human haptocorrin, suggesting that haptocorrin may facilitate the absorption of vitamin B<sub>12</sub> in young infants, a period when the production of intrinsic factor is immature. There is no commercial source of haptocorrin allowing clinical trials, and there have been no studies on vitamin B<sub>12</sub> absorption from haptocorrin in infants to date.

### Bile Salt-Stimulated Lipase

Breast milk is unusually high in the active enzyme, bile salt-stimulated lipase (BSSL). This enzyme catalyzes the digestion of triglycerides in human milk fat and is unique in that it also breaks the bond of palmitic acid and glycerol in the sn-2 position, which is common in human milk fat, but many other lipases cannot.<sup>37</sup> This suggested a mechanism for why lipid digestion is so efficient in breastfed infants. Andersson et al<sup>38</sup> demonstrated this in a crossover design study in preterm infants who were fed unpasteurized or pasteurized breast milk, in which the BSSL was inactivated. After 1 week of feeding, fecal fat loss was significantly lower in the infants fed unpasteurized breast milk and their weight gain and knee-heel length were significantly higher than in the infants fed pasteurized breast milk, strongly supporting a role for BSSL in lipid digestion and utilization. There is no BSSL in cow's milk, but recombinant human BSSL has been produced in human cells in culture. Phase 2 clinical studies on preterm infants fed preterm formula with or without added recombinant BSSL have shown promising results,<sup>39</sup> but further studies are needed. Because of concerns over the widespread use of genetically modified organisms, the use of recombinant human milk proteins such as lactoferrin and BSSL will require ethical review.

### $\beta$ -casein

The phosphorylated protein,  $\beta$ -casein, forms a multitude of phosphopeptides during digestion. Several of these phosphopeptides have been shown to bind divalent cations, such as calcium and zinc, and it has been suggested that they facilitate the absorption of these nutrients. Experiments in animal models have shown enhancement of calcium absorption by bovine casein phosphopeptides (CPPs),<sup>40</sup> but human studies to date have been equivocal.<sup>41</sup> The reason for this is not known, but it is possible that the structures

of human and bovine  $\beta$ -casein, which are similar, but not identical, result in different binding affinities for these cations, which may affect absorption. Our group has shown that purified human milk CPPs formed in vitro greatly facilitated the uptake of calcium by human intestinal Caco-2 cells (Rudloff and Lönnerdal, personal communication), but there have been no clinical studies on CPPs in human infants to date. It also should be recognized that the primary role of these CPPs may be to keep calcium and zinc in solution,<sup>42</sup> which would have positive effects on their uptake and utilization.

### Conclusions

It is evident that breastfed infants have better health outcomes than formula-fed infants and that nutrient utilization from breast milk is very efficient. Evidence for specific human milk bioactive proteins being involved in these outcomes is accumulating, but clinical trials showing this are difficult to perform because of a lack of large quantities of these proteins. Instead, clinical trials using bovine milk proteins having equivalent bioactivities will need to serve as proof-of-concept. Studies to date suggest that several such bovine milk protein fractions may improve outcomes of formula-fed infants. ■

### Author Disclosures

The author received an honorarium to serve as a co-chair of the Mead Johnson Pediatric Institute Bioactive Expert Panel to write a manuscript and serve as a co-guest editor for this supplement. The sponsor had no involvement in preparing the manuscript, and the author is entirely and exclusively responsible for its content. The author has received honoraria from Mead Johnson Nutrition, Arla Foods, Hero, Albion, Valio, Humana, Biostime, Nestle, and Nestle Nutrition Institute.

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