Infant formula and infant nutrition: bioactive proteins of human milk and implications for composition of infant formulas¹–³

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ABSTRACT

Human milk contains an abundance of biologically active components that are highly likely to contribute to the short- and long-term benefits of breastfeeding. Many of these components are proteins; this article describes some of these proteins, such as α-lactalbumin, lactoferrin, osteopontin, and milk fat globule membrane proteins. The possibility of adding their bovine counterparts to infant formula is discussed as well as the implications for infant health and development. An important consideration when adding bioactive proteins to infant formula is that the total protein content of formula needs to be reduced, because formula-fed infants have significantly higher concentrations of serum amino acids, insulin, and blood urea nitrogen than do breastfed infants. When reducing the protein content of formula, the amino acid composition of the formula protein becomes important because serum concentrations of the essential amino acids should not be lower than those in breastfed infants. Both the supply of essential amino acids and the bioactivities of milk proteins are dependent on their digestibility: some proteins act only in intact form, others act in the form of larger or small peptides formed during digestion, and some are completely digested and serve as a source of amino acids. The purity of the proteins or protein fractions, potential contaminants of the proteins (such as lipopolysaccharide), as well as the degree of heat processing used during their isolation also need to be considered. It is likely that there will be more bioactive components added to infant formulas in the near future, but guidelines on how to assess their bioactivities in vitro, in animal models, and in clinical studies need to be established. The extent of testing needed is likely going to depend on the degree of complexity of the components and their bioequivalence with the human compounds whose effects they are intended to mimic.  


INTRODUCTION

It is well recognized that several outcomes of breastfed infants are superior to those of formula-fed infants, both in the short and long term. Formula-fed infants have a different growth pattern, particularly gaining more body fat and weight from 3 to 6 mo and later, and have higher concentrations of serum amino acids, insulin, and blood urea nitrogen than do breastfed infants. They also have a different nutritional status and pronounced differences in gut microbiota. These variables may be associated with the higher risks of obesity (1, 2), type 1 and 2 diabetes (3, 4), and cardiovascular disease (5, 6) later in life, as documented in several reports and meta-analyses. Although the composition of infant formulas has changed frequently throughout the years with increasing knowledge of infant nutrition (eg, adjustment of whey: casein ratio, taurine fortification, and addition of DHA), the differences described above still persist.

An early goal for the composition of infant formula was to make it similar to that of human milk. However, with known differences in digestibility and bioavailability between nutrients in breast milk and infant formula, it was recognized that a better and more realistic goal was to attempt to make the performance of formula-fed infants more similar to that of breastfed infants. To achieve this, modifications of concentrations of nutrients as well as their sources (eg, phospholipids/marine oils, more mildly heat-treated whey protein concentrates, calcium salts, etc) need to be made. Furthermore, because human milk contains many bioactive components, alternative sources of such components need to be explored and possibly incorporated into infant formula. This is not an easy endeavor because the composition of infant formula is strictly regulated in many countries (7–10). Changes in nutrient composition require clinical trials in infants, which are costly and take considerable time. The addition of bioactive components requires not only that these are safe but that they also confer defined benefits to the recipient infants (11, 12). Furthermore, ingredient sources, their cost, and processing/ quality will also affect the feasibility of including them.

The addition of bioactive components should always be considered with caution, but depending on their nature, these require different levels of scrutiny. Compounds such as taurine and nucleotides, which would be added to formula in chemical forms identical to those in human milk, but in amounts higher than what is inherent to the formula ingredients, are likely of less concern and may only require growth and safety studies. Other components, such as α-lactalbumin, which is present in human milk, will need to be added in the bovine form. Human and bovine α-lactalbumin share a reasonable degree of similarity with regard to amino acid sequence and composition, and neither has any posttranslational modifications, making it likely that they have similar bioactivity (13). In this case, some in vitro

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bioassays (antimicrobial activity, immune assays, iron/zinc uptake, etc) followed by clinical studies with defined outcomes (infections, immune function, iron/zinc absorption/status, etc) related to the claimed bioactivity or bioactivities may be appropriate. Alternatively, a recombinant form of a human milk protein may be used. Although this requires further considerations (eg, impurities of the commercial source), the protein part in the case of α-lactalbumin is identical to the native human milk form. However, for glycoproteins, such as lactoferrin, the glycosylation is different. If the glycans are not involved in the bioactivities, it is highly likely that the bioactivity will be identical (14). Another group of proteins can be exemplified by bovine osteopontin, which has a different amino acid sequence and substantial posttranslational modifications (glycosylation, phosphorylation) than the human form. Because the posttranslational modifications have been implied to be involved in the bioactivities described for this protein, a combination of in vitro studies, animal models, and human trials may be required to assess outcomes (11).

This review discusses some of these examples in more detail, and because proteins are used as examples of bioactive components, the issue of protein digestibility will be considered.

**PROTEIN DIGESTIBILITY**

The extent of protein digestibility becomes a critical issue for both essential amino acid supply and the potential bioactivities of bovine-milk proteins. When lowering the protein concentration in infant formula, the first limiting amino acid becomes tryptophan (13). One approach that has been used to avoid this is to add free tryptophan (and possibly other amino acids that subsequently become limiting) to the formula (20). It is highly likely, however, that these added amino acids will be absorbed much faster than protein-bound amino acids, which need proteolysis to occur before uptake, and thus reach target tissues (liver, brain) more rapidly. The metabolic consequences of this have not yet been studied adequately. Another approach is to increase the proportion of α-lactalbumin in formula (21). α-Lactalbumin has a relatively high proportion of tryptophan and is commercially available as an enriched whey fraction. Clinical studies have shown that lowering the protein amount in infant formula coupled with an increased proportion of α-lactalbumin results in plasma tryptophan concentrations similar to those in breastfed infants (21, 22).

**ADDITION OF BIOACTIVE PROTEINS TO INFANT FORMULA**

Although reducing the protein content of infant formula is likely to be beneficial to formula-fed infants with regard to amino acid and insulin metabolism, it is unlikely that they will achieve any of the other benefits of breastfed infants. Breast milk contains a large variety of bioactive proteins (23), which are involved in the defense against infections, immune function, and development. Some of these proteins are discussed below in more detail, but these are only examples because human milk contains a multitude of proteins, as has recently been described by proteomics approaches (24–26). Some bovine-milk proteins are similar to human milk proteins, albeit not identical, and may provide some of the bioactivities of their human counterparts. Guidelines on how to assess their bioactivities in vitro, in animal models, and in clinical studies need to be established because any claims related to these bioactivities will need to be shown (11, 12).

**BIOACTIVITIES OF α-LACTALBUMIN**

The primary reason for adding α-lactalbumin to infant formula has so far been its relatively high content of tryptophan (described above) as well as lysine and cysteine (13). It should be recognized, however, that several peptides released from α-lactalbumin during digestion are likely to exert bioactivities. These peptides are likely to be formed in the upper part of the gastrointestinal tract (stomach, duodenum) but may exert functions during their passage through the more distal part of the small intestine (jejunum, ileum) as well as in the colon. Eventually, however, α-lactalbumin is likely to become digested and absorbed as small peptides and amino acids, because no intact α-lactalbumin is found in the stool of breastfed infants. However, some small peptides may resist digestion, and these are too small in size to be detected in the stool by immunologic methods.

An immunostimulatory tripeptide [Gly-Leu-Phe (GLF)] has been shown to be released by in vitro digestion of casein but also
from α-lactalbumin (27). This peptide (which is present in both human and bovine α-lactalbumin) has been shown to stimulate phagocytic activity of murine peritoneal macrophages in vitro and exerted a protective effect against Klebsiella pneumoniae infection in mice (28). It also stimulated phagocytosis of human macrophages, leading to bacterial killing (29). Other peptides have been shown to have prebiotic activity by stimulating the growth of bifidobacteria (30). Antibacterial peptides against Escherichia coli, K. pneumoniae, Staphylococcus aureus, Staphylococcus epidermis, Streptococci, and Candida albicans (31) have also been shown to be formed during in vitro digestion of α-lactalbumin. Although these observations have been made in vitro, it is possible that these small peptides are formed in the upper gut in vivo and then resist further digestion and exert their antibacterial activities in the lower gut (colon). This may explain our finding of an inhibitory effect of α-lactalbumin—supplemented infant formula on enteropathogenic E. coli-induced diarrhea in infant rhesus monkeys (32). The combined actions of prebiotic peptides stimulating a “beneficial” bacteria and antimicrobial peptides inhibiting the growth of pathogens may result in a microbiome (33), which may in itself affect immune function.

α-Lactalbumin is a calcium-binding protein, but one of its binding sites can also bind iron and zinc (34), albeit with relatively low affinity. Peptides from α-lactalbumin binding iron and/or zinc have received little attention, but studies in infant rhesus monkeys (35) and in human infants (22) suggest a stimulating effect of α-lactalbumin on iron and zinc absorption and status.

Clinical studies on formulas with added α-lactalbumin have been conducted, but they have largely focused on growth, acceptability, and plasma amino acids. Future studies should include outcomes related to the proposed bioactivities of α-lactalbumin, among them immune function (morbidity), trace element status (iron, zinc), and gut microflora.

**BIOACTIVITIES OF LACTOFERRIN**

Lactoferrin is a major protein in human milk, comprising 15–20% of the total protein content. It is well known as a multifunctional protein; lactoferrin has antibacterial and antiviral activity, is involved in immune function and iron uptake by intestinal cells, and is anti-inflammatory (36). The reason lactoferrin can exert all of these functions is that it is remarkably resistant to proteolytic enzymes. Although some or most of lactoferrin in human milk is digested, at least in part, intact lactoferrin is found in significant quantities in the stool of breastfed infants (16). Undigested lactoferrin can bind to specific lactoferrin receptors covering the surface of intestinal epithelial cells and be internalized via an endocytic mechanism (37). Once inside the cell, lactoferrin will localize to the nucleus (Figure 1) and bind to specific promoter sites, acting as a transcription factor (38). Thus, expression of cytokines (eg, IL-1) and growth factors [eg, transforming growth factor (TGF) β] is upregulated. This event most likely explains why lactoferrin can exert so many and diverse bioactivities (eg, immune function, cell proliferation/differentiation).

Lactoferrin can also exert bioactivities that are not mediated by the binding to its receptor. Intact lactoferrin can have bacteriostatic effects by withholding iron from iron-requiring bacteria,
TGF-β and IL-1) similar to those of human lactoferrin (41). This shows that bioactivities may be impeded by contaminants and that the purity of the added bioactive component also may be an issue. Although “contaminants” of bovine-milk protein fractions added to formula in most cases just consist of other milk proteins, which are not likely to affect the bioactive components more than the proteins already in the formula, components such as lipopolysaccharide may accumulate in the dairy fraction during processing. The degree of heat processing of the bioactive component may also affect its biological effects. In the past, heat processing of dairy components was more extensive, and it is possible that thermal changes in protein structure (denaturation) and/or lipopolysaccharide contamination may explain why studies more than a decade ago failed to show any effect of lactoferrin. An indication that this may be true is that recent clinical studies on bovine lactoferrin showed positive outcomes (42, 43).

**BIOACTIVITIES OF OSTEOPONTIN**

Osteopontin was first described in bone but is also found in a relatively high concentration in human milk (44). It is a heavily glycosylated and phosphorylated acidic protein and contains an RGD (Arg-Gly-Asp; integrin-binding) cell attachment sequence (45, 46). Osteopontin is a multifunctional protein involved in many physiologic processes, including immune activation, wound healing, angiogenesis, and bone remodeling (47). It is also present in cow milk but at a much lower concentration (~1:10). The primary structure of human and bovine osteopontin is relatively similar, but the extent of the glycosylation and phosphorylation, as well as the localization of the posttranslational modifications, is different. It is thus possible that their bioactivities differ.

When comparing the effects of human and bovine osteopontin on gene expression in human intestinal cells (Caco-2), it is apparent that they both affect the transcription of some genes, and to a similar extent, but also that they each affect the expression of many genes quite differently. When subjecting recombinant human osteopontin to the same gene expression study (microarray analysis), other sets of genes than those affected by native human osteopontin were affected, showing that the type of glycosylation modifies gene expression (48).

Bovine osteopontin is now commercially available in large quantities, and we therefore studied the effects of osteopontin added to infant formula on the intestinal transcriptome in infant rhesus monkeys (49). The concentration of osteopontin in human milk is ~130 mg/L compared with 10 mg/L in infant formula, and the infant monkeys were fed exclusively infant formula without or with 125 mg osteopontin/L or exclusively breastfed from birth to 3 mo of age. At this time, jejunal samples were obtained and mRNA extracted. Microarray analyses showed a large number of genes that were differentially expressed between formula-fed and breastfed infants. The addition of osteopontin reduced the difference in gene expression relative to breastfed infants by >5-fold. Pathways differing between infants fed regular formula and formula with osteopontin were related to development, galactose metabolism, cytoskeleton remodeling, and immune response. Thus, osteopontin added to formula shifted overall gene expression differences toward a profile more similar to that in breastfed infants.

**BIOACTIVITIES OF THE MILK FAT GLOBULE MEMBRANE FRACTION**

The proteins in human milk largely consist of caseins and whey proteins. However, a minor part of the proteins reside in the lipid fraction, as an integral part of the membrane surrounding the fat globules (triglycerides) (50). This is a diverse group of proteins (25), including mucin (MUC1), lactadherin, and lactoferrin; and several of them have been shown to have bioactivities, primarily as antimicrobial agents. The milk fat globule membrane (MFGM) also contains sphingomyelin, gangliosides, sialic acid, and cholesterol—components that are involved in development of brain myelination and function (51–54). Until recently, all infant formulas were lacking the MFGM because this fraction is lost during regular dairy processing.

Several proteins in the MFGM have been shown to exert inhibitory activities against various pathogens, and a whey protein concentrate enriched in MFGM may therefore help to protect against diarrhea of both bacterial and viral origin (55). A bovine-milk fraction containing MUC1 has been shown to inhibit hemagglutination of *Vibrio cholera* and *E. coli* (56). Furthermore, mucin purified from MFGM was shown to decrease the adherence of *Yersinia enterocolitica* to intestinal membranes (57). The MFGM fraction has also been found to inhibit rotavirus in vitro (58). Sphingolipids, particularly gangliosides, have been shown to inhibit enterotoxins both in vitro and in vivo (59). In addition, infant formula with added sphingolipids (gangliosides) has been shown to reduce *E. coli* counts in infant feces and to increase beneficial bifidobacteria (60).

The MFGM fraction is now commercially available and can thus possibly be added to infant formula. The components of the bovine MFGM are similar to those in human milk; and because in vitro tests have shown bioactivities (eg, antibacterial and antiviral activities, immune function), clinical trials on the MFGM are needed to explore effects on relevant outcomes in infants. A randomized controlled study in Peru in which 6- to 8-mo-old infants were fed a complementary food with bovine MFGM twice daily for 6 mo showed a decreased prevalence of diarrhea, particularly bloody diarrhea (61). A variety of pathogens were shown to be the cause of the diarrhea episodes in these infants, and it is likely that the various components of the MFGM shown to have antibacterial and antiviral effects in vitro had similar effects in older infants (6–12 mo old). No adverse effects were observed.

In a recent randomized controlled trial in Sweden, young infants were fed infant formula with MFGM or standard formula from 6 wk to 6 mo of age (62). Breastfed infants were also followed as a reference group. The primary outcome was cognitive, motor, and verbal function at 12 mo measured with Bayley III. Cognitive scores were significantly higher in the group fed formula with MFGM than in the standard-formula group, and there was no difference in cognitive scores between the MFGM group and the breastfed group. This suggests that supplementation with bovine MFGM of infant formula provides formula-fed infants components that support cognitive development.

**CONCLUSIONS**

Although efforts have been made to make nutrition-related outcomes of formula-fed infants more similar to those of breastfed infants, there has been limited progress with regard to...
bioactive components that can affect short- and long-term outcomes. Several bovine-milk proteins are similar to their human milk counterparts but may be present in lower concentrations. Enriched bioactive milk proteins need to be evaluated for their ability to exert functions in the formula-fed infant, and whether these functions will have long-term benefits.

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REFERENCES