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Journal of Nutritional Biochemistry 41 (2016) 1–11

Journal of Nutritional Biochemistry

Longitudinal evolution of true protein, amino acids and bioactive proteins in breast milk: a developmental perspective☆

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Received 15 April 2016; accepted 7 June 2016

Abstract

The protein content of breast milk provides a foundation for estimating protein requirements of infants. Because it serves as a guideline for regulatory agencies issuing regulations for infant formula composition, it is critical that information on the protein content of breast milk is reliable. We have therefore carried out a meta-analysis of the protein and amino acid contents of breast milk and how they evolve during lactation. As several bioactive proteins are not completely digested in the infant and therefore represent "non-utilizable" protein, we evaluated the quantity, mechanism of action and digestive fate of several major breast milk proteins. A better knowledge of the development of the protein contents of breast milk and to what extent protein utilization changes with age of the infant will help improve understanding of protein needs in infancy. It is also essential when designing the composition of infant formulas, particularly when the formula uses a "staging" approach in which the composition of the formula is modified in stages to reflect changes in breast milk and changing requirements as the infant ages.

Keywords: Human milk; Breast milk; Protein; Lactation; Infant nutrition; Amino acids

1. Introduction

Breast milk is an excellent source of protein and the preferred source of nutrition for infants. Breast-fed infants experience fewer and shorter infections [1,2], exhibit different growth patterns [2], have different gut microflora [3], show better cognitive development [4] and even face differences in the risk of chronic diseases, such as obesity [5,6], Type 1 and Type 2 diabetes [7–8] and cardiovascular disease [8,9]. Although the composition of infant formulas has evolved with increasing knowledge of infant nutrition, differences in outcomes between breast-fed and formula-fed infants still persist [10]. Efforts to improve outcomes of formula-fed infants and the composition of infant formula are complicated by variability in breast milk nutrient content. Human milk and its key components, including proteins, change continuously over time [11,12]. Consequently, narrowing the gap between breast milk and infant formula requires a greater understanding how protein quality and quantity in human milk changes over time.

Milk proteins are classified into three groups: milk fat globule membrane (MFGM) proteins, caseins and whey proteins. MFGM proteins contribute only a small percentage of the true protein content of human milk [13], a percentage that is likely relatively stable over time [14]. The principal proteins in human milk are caseins and whey proteins, which include α-lactalbumin, lactoferrin and secretory immunoglobulin A (sIgA). Concentrations of both casein and whey change profoundly over the course of lactation. Early in lactation, the concentration of whey proteins is very high, while casein is virtually undetectable [15,16]. As infants age, casein synthesis and, consequently, casein concentrations increase, partially due to hormonal changes in the mother. Because the amino acid contents of whey proteins and casein differ, milk amino acid content also changes as infants mature.

The protein intake of breast-fed infants has been used as a model for infant protein requirements, given that breast milk is typically the only source of protein before complementary foods are introduced. Protein content in breast milk can be quantified by directly assessing the true protein content or quantifying the nitrogen content in breast milk. True protein can be calculated from the nitrogen content by subtracting nonprotein nitrogen from the total nitrogen and multiplying the difference by a conversion (Kjeldahl) factor [14].

☆ The work in this manuscript was sponsored by Nestlé (Vevey, Switzerland).

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http://dx.doi.org/10.1016/j.jnutbio.2016.06.001

Assessments of true protein content using these methods have reported concentrations of 14 to 16 g/l during early lactation, 8 to 10 g/l at 3 to 4 months and 7 to 8 g/l at 6 months [14,16,17]. However, true protein intake does not accurately reflect the amount of utilizable amino acids in infants because some breast milk proteins can be found intact in infant stool [18]. Lactoferrin and sIgA, for example, are found in relatively high amounts in feces from breast-fed infants.

These proteins — and many others — have important roles in breast milk beyond nutritional support. Bioactive proteins likely contribute to the numerous advantages of breast milk over infant formula. Bioactive proteins can have enzymatic activity, enhance nutrient absorption [19], stimulate growth [20], modulate the immune system [21] and assist in the defense against pathogens [21–24]. Key bioactive proteins in human milk include lysozyme, α-lactalbumin, κ-casein and β-casein, as well as lactoferrin and immunoglobulins, especially sIgA (Table 1) [10,14,24].

The extent to which true protein, amino acid and bioactive protein content in breast milk changes over time has been evaluated in a variety of studies using different methodologies. A single reference that analyzes true protein content, amino acid content and bioactive protein content, describes their changes throughout the course of lactation and considers the implications of these changes in infant development is needed and would be useful to efforts to improve infant nutrition.

To meet this need, we conducted a meta-analysis and literature review to evaluate changes in these protein parameters during infants' first year of life. In this meta-analysis and review, we compiled and analyzed data on protein and amino acid content in human milk available in the medical literature. We used this dataset to estimate the longitudinal evolution of total protein, amino acid and certain bioactive protein content in human milk from birth through 1 year. We also interpreted these changes in the context of the known and proposed biological functions of the evaluated proteins. It is hoped that this analysis will provide a reference dataset for changes in protein content over time, a dataset that can be used to improve our understanding of the protein and amino acid intake of breast-fed infants and to enhance the composition, staging and performance of infant formulas.

2. Methods

2.1. Literature search

To identify all published literature on protein content of breast milk, we performed literature searches using PubMed, Scopus, EMBASE and Google Scholar using the following keywords: *breast milk*, *human milk*, *protein*, *true protein*, *total protein nitrogen*, *protein nitrogen*, *bioactive proteins*, *whey to casein ratio*, *lactoferrin*, *α-lactalbumin*, *serum albumin*, *IgA*, *lysozyme*, *IgG*, *IgM* and *amino acid*. The most recent search was conducted in March 2015. Reference lists of the retrieved articles were also reviewed to identify references not found using electronic search methods. Only data from "normal" or "healthy" mothers who delivered healthy term infants were included in this meta-analysis. Studies evaluated mothers who consumed free-living diets; data from mothers consuming special diets were excluded. Selected studies provided sufficient information regarding geographic location, study design, sampling time and procedure, nature of sample, analytical methods and units. Other variables such as age, ethnicity, body weight, socioeconomic status and season were not considered. Milk could be obtained with mechanical, electrical and hand pumps or by manual expression. Samples were transported and stored in either liquid or freeze-dried form; defatted or whole milk was used for hydrolysis. Milk samples analyzed were taken from complete 24-h collections, the entire amount of milk from one or both breasts at one feeding or pooled or banked milk.

2.2. Data extraction

Data were extracted from studies that reported true protein content, protein-bound amino acid content and bioactive proteins. Assessments of bioactive proteins included evaluations of whey-to-casein ratios and concentrations of lactoferrin, α-lactalbumin, serum albumin, sIgA, lysozyme, immunoglobulin G (IgG) or immunoglobulin M (IgM). Total, essential and nonessential amino acid content was evaluated in available studies. *Protein quality* was defined as the ratio of essential to nonessential amino acid concentrations. For protein content analysis, total nitrogen data were not considered relevant, and only true protein data obtained using Kjeldahl (total nitrogen — nonprotein nitrogen with a 6.25 conversion factor), Lowry, Biuret and bicinchoninic acid (BCA) kits were extracted. When other conversion factors (*i.e.*, 6.38) were used to estimate true protein content using the Kjeldahl method, the data were recalculated using 6.25 as conversion factor. Data summaries were prepared using the means from original reports converted into consistent units (g per 100 ml, mg per 100 ml or mg per ml). When sampling time was provided as ranges and not specific days, sampling time was calculated based on the average lactation day. Data were categorized by stage of lactation as follows: colostrum (0 to 5 days postpartum), 6 to 15, 16 to 30, 31 to 60, 61 to 90 and 91 to 360 days postpartum. Means, medians, 25th and 75th percentiles, minima, maxima and standard deviations were calculated and used to prepare summary tables and graphical plots. Linear regression of the true protein dataset was performed using R Version 3.0.1.

3. Results

Separate analyses were conducted for each evaluated endpoint. A total of 43 original articles published between 1953 and 2011 were included in at least one analysis.

3.1. True protein

In our evaluation of true protein content, we considered 34 original articles published between 1973 and 2011. Eight of these papers were excluded due to unreliable analytical methodologies, leaving 26

Table 1
Included studies on true protein content in human milk

First author	Year	Country	Study design
Allen [101]	1991	USA	Longitudinal
Andersson [102]	1983	USA	Longitudinal
Arnold [103]	1987	Australia	Longitudinal
Bauer [104]	2011	Germany	Longitudinal
Britton [105]	1986	USA	Longitudinal
Butte [106]	1984	USA	Longitudinal
Butte [107]	1984	USA	Longitudinal
Butte [108]	1990	USA	Cross-sectional
Dewey [109]	1983	USA	Longitudinal
Gross [110]	1980	USA	Longitudinal
Harzer [111]	1986	Germany	Longitudinal
Hibberd [112]	1982	UK and Germany	Longitudinal
Kunz [16]	1992	USA	Longitudinal
Lönnerdal [26]	1976	Sweden	Longitudinal
Marquis [32]	2003	Peru	Longitudinal
Mitoulas [113]	2002	Australia	Longitudinal
Montagne [114]	1999	France	Longitudinal
Nagasawa [115]	1973	Japan	Cross-sectional
Nagra [34]	1989	Pakistan	Longitudinal
Nommsen [116]	1991	USA	Cross-sectional
Ronayne de Ferrer [28]	2000	Argentina	Longitudinal
Saarela [11]	2005	Finland	Longitudinal
Sanchez-Pozo [27]	1986	Spain	Longitudinal
Sann [117]	1981	France	Longitudinal
Shehadeh [118]	2006	Israel	Cross-sectional
Stuff [119]	1989	USA	Longitudinal

articles for inclusion (Table 1). These 26 articles provided 130 data points during the first year after birth. Seventy percent of the data included in the analyses were collected less than 90 days after the birth of the infant.

Fig. 1 demonstrates that true protein content in human milk consistently declines over time. A linear regression was performed on the true protein dataset to better characterize the dynamic evolution of the true protein concentration over time. As the protein data exhibit a logarithmic decay, a linear regression model was fitted to the data and specified as true protein $= \beta_0 + \beta_1 * \text{month} + \beta_2 * \log(\text{month}) + \varepsilon$, in which $\beta_0 = 1.407$, $\beta_1 = 0.026$, $\beta_3 = -0.279$ and $\varepsilon \sim N(0,1)$. The percentage of variation explained by the model (adjusted R2) was 0.581. To take into account the variation of the data, the lower and upper confidence bands — represented as dashed lines — have been constructed using the same regression models but considering the lower and upper limits as input data, respectively. Then, the lower 95% CI limit has been displayed for the lower band, whereas the upper 95% CI limit has been used for the upper band. Median true protein content in milk expressed between 16 and 30 days after delivery was 24% lower compared with true protein in milk expressed 0 to 5 days after delivery (1.57 g/100 ml *vs.* 2.06 g/100 ml). True protein content continued to decrease throughout the first year but at substantially lower rates than those observed in the first weeks (Supplemental Fig. 1). By 90 to 360 days, true protein content in human breast milk was 47% lower compared to 0 to 5 days after delivery (1.10 g/100 ml). Fig. 2 also superimposes estimated protein requirements in infants over time as calculated by Dewey *et al.* [25]. Changes in true protein content closely parallel changes in infant protein requirements (Fig. 2).

3.2. Amino acids

Fourteen articles published between 1976 and 2009 that evaluated amino acid content were selected for analysis (Table 2). Total and essential amino acid content decreased over time (Supplemental Fig. 2). The largest decreases in amino acid content occurred between milk expressed 0 to 5 days after delivery and milk expressed 6 to 15 days after delivery, as shown in Table 3. In fact, total amino acid content in breast milk expressed 16 to 30 days after delivery was less than half of that observed in colostrum. Total, essential and nonessential amino acid content stabilized in samples collected after 2 weeks after delivery. Although changes in total, essential and nonessential amino acid content were observed, essential to total amino acids ratios were stable over time.

Fig. 3 illustrates changes in two essential amino acids, lysine and tryptophan, over time. Changes in lysine and tryptophan content paralleled changes in total amino acid and essential amino acid content, with substantial decreases between colostrum and milk collected between 16 and 30 days after delivery.

3.3. Bioactive proteins

Twelve articles published between 1972 and 2003 were identified and included in the analysis (Table 4) [16,17,26–35]. All studies did not evaluate all endpoints, and data on some endpoints were not available for all time points (*e.g.*, IgA, lysozyme, IgG and IgM).

3.3.1. Whey-to-casein ratio

Five studies evaluated concentrations of whey and casein and reported data that could be used to calculate whey-to-casein ratios [16,29,30,33,34]. Estimates of whey-to-casein ratios and their changes over time are presented in Table 5 and Supplemental Fig. 3. Whey-to-casein ratios were highest in breast milk collected in the first 5 days after delivery and declined over time. In the colostrum, the median whey-to-casein ratio was 89:11, a ratio that dropped to 65:35 in milk collected 6 to 15 days after delivery. At all subsequent time periods (days 16 through 360), the ratio stabilized to approximately 60:40 (ranging from 59:41 to 61:39).

3.3.2. Lactoferrin

Lactoferrin concentrations in breast milk in the first year after delivery were evaluated in nine studies [17,26–28,30–33,35]. As shown in Table 5 and Supplemental Fig. 4, the greatest concentrations of lactoferrin were observed in colostrum (5.05 mg/ml). Median lactoferrin concentrations declined to 3.30 mg/ml in milk expressed 6 to 15 days after delivery and continued to decrease over time. In milk collected 91 to 360 days after delivery, median lactoferrin concentrations were 1.44 mg/ml.

3.3.3. α-lactalbumin

Seven studies evaluated α-lactalbumin concentrations in breast milk in the first year after delivery [17,26,27,29,30,31,35]. Concentrations of α-lactalbumin were highest in colostrum (4.30 mg/ml), as shown in Table 5 and Supplemental Fig. 5. However, α-lactalbumin concentrations decreased more gradually than did lactoferrin, true protein content or amino acid content. In fact, α-lactalbumin concentrations in milk expressed 6 to 15 days after delivery were similar to those seen in colostrum (4.20 mg/ml). α-Lactalbumin concentrations began to decrease in samples collected 16 to 30 days (to 3.30 mg/ml) and continued to decrease over time to 2.6 mg/ml in samples collected between 91 to 360 days.

3.3.4. Secretory IgA (sIgA)

Three studies that evaluated sIgA concentrations were identified and included in the analysis [30–32]. Median sIgA concentrations decreased from 5.45 mg/ml in samples collected 0 to 5 days after delivery to 1.50 mg/ml in samples collected 6 to 15 days after delivery (Table 5 and Supplemental Fig. 6). sIgA concentrations in samples collected between 16 days and 90 days (the last time period during which sIgA data were available) ranged from 1.0 to 1.3 mg/ml.

3.3.5. IgG and IgM

IgG and IgM concentrations in breast milk were evaluated in two studies [17,26]. However, data on IgG or IgM content in the colostrum or in human milk collected between 31 and 60 days after delivery were not available in either study. IgG concentrations at the remaining time points (days 6 to 15, 16 to 30, 61 to 90 and 90 to 360) were low and ranged from 0.03 mg/ml at days 61 to 90 to 0.05 at days 6 to 15, as shown in Table 5 and Supplemental Fig. 7. IgM concentrations decreased from 0.12 in milk collected 6 to 15 days after delivery to 0.05 in milk collected 16 to 30 days after delivery.

3.3.6. Lysozyme

Four studies evaluated lysozyme concentrations in human milk and their changes over time [27,30–32]. No clear trends in lysozyme concentrations were apparent between samples collected 0 to 5 days after delivery and samples collected 61 to 90 days after delivery (Table 5 and Supplemental Fig. 8). Median concentrations of lysozyme were 0.32 mg/ml in colostrum, peaked at 1.10 mg/ml at 31 to 60 days and declined to 0.85 mg/ml at 61 to 90 days. No data on lysozyme concentrations were available after day 90.

3.3.7. Serum albumin

Five studies analyzed changes in serum albumin content over time [17,26,27,29,35]. Serum albumin levels appeared to slightly increase in the first 60 days after delivery (from 0.56 mg/ml in samples collected 0 to 5 days after delivery to 0.72 mg/ml in samples collected 31 to 60 days after delivery) and decrease thereafter (to 0.44 mg/ml in samples collected 91 to 360 days after delivery), as shown in Table 5 and Supplemental Fig. 9.

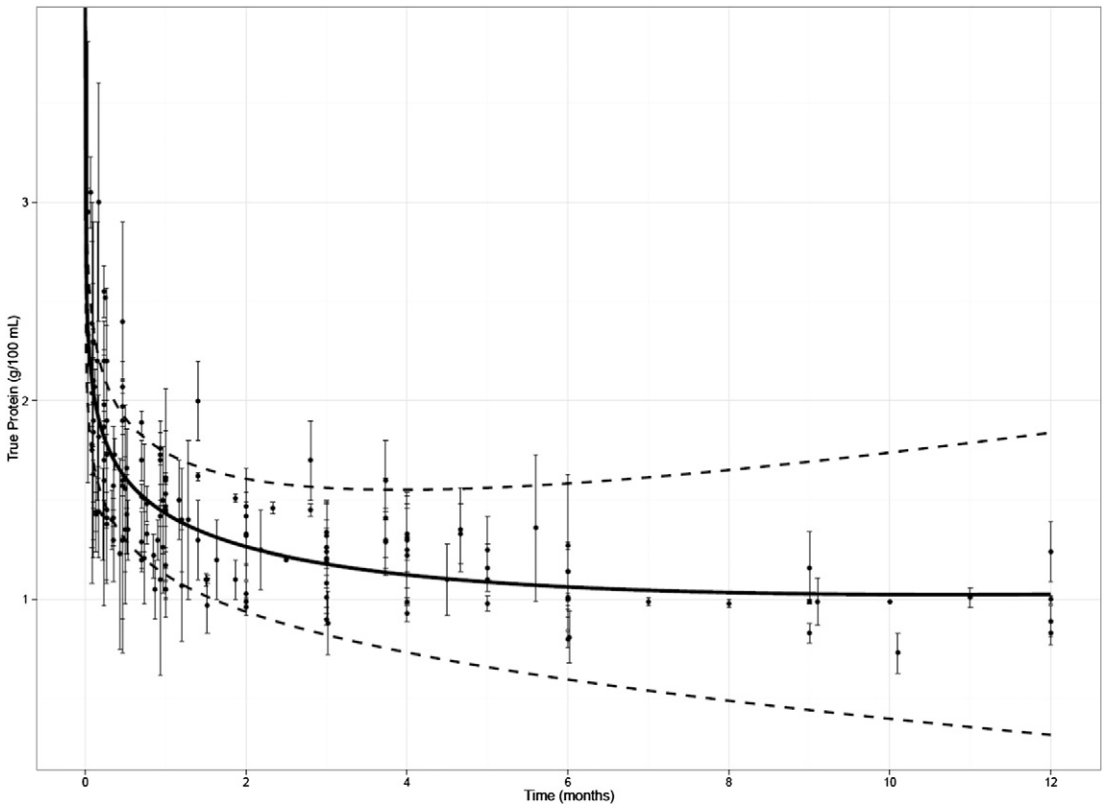

Fig. 1. Linear regression analysis (solid line) of the true protein dataset in g per 100 ml over the first year of lactation (lower and upper confidence bands are represented by the dashed lines). Data points correspond to mean values and error bars to +/− standard deviations.

4. Discussion

Quantifying the total (true) protein, amino acid and bioactive protein content of human milk at various stages of lactation may provide a useful guide to understanding the changing nature of protein requirements during an infants' first year of life. Results from this meta-analysis confirm that the protein content depends on the stage of lactation and time since delivery. The highest protein concentrations were seen in colostrum, as expected, with the greatest declines in true protein and most types of protein occurring in the first

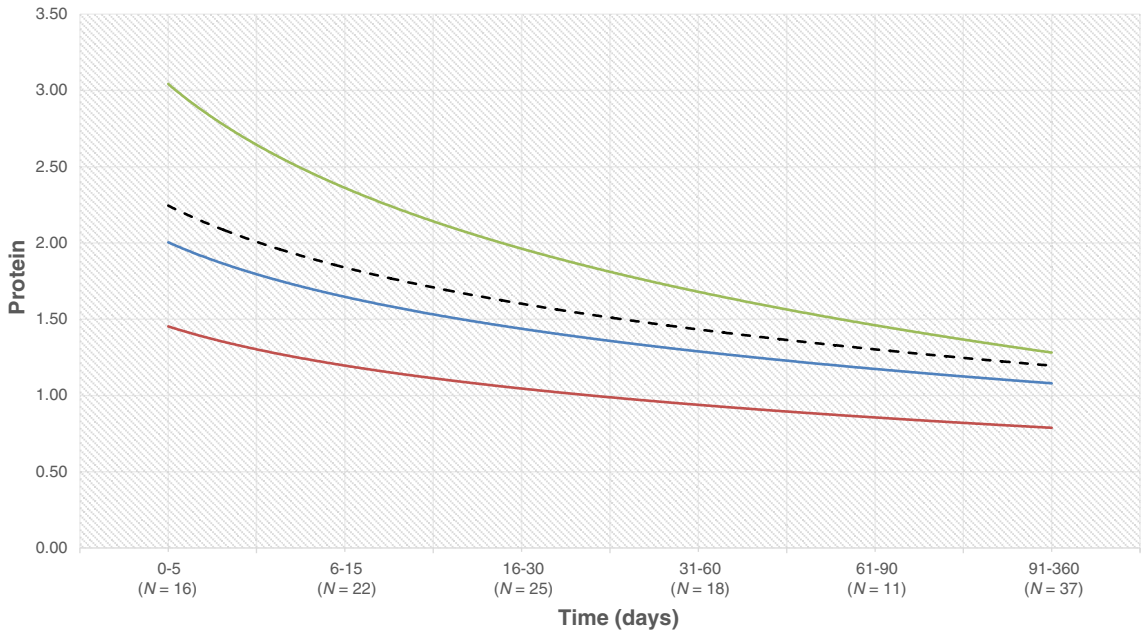

Fig. 2. Estimated evolution of infant protein requirements (in g per day and per kg of body weight; dashed black line) and true protein content in human milk (in g per 100 ml; minimum, solid red line; median solid light blue line and maximum solid green line). Logarithmic regressions were calculated from the human milk protein concentration dataset (present study) and from the dietary protein requirements dataset from Dewey *et al*., 1996.

Table 2
Included studies on protein bound amino acid content in human milk

First author	Year	Country	Study design
Britton [105]	1986	USA	Longitudinal
Chavalittamrong [120]	1981	Thailand	Cross-sectional
Darragh [121]	1998	New Zealand	One time point
Davis [122]	1994	USA	Not documented
Feng [123]	2009	Australia, Canada, Chile, China, Japan, Mexico, Philippines, UK, USA	Cross-sectional
Janas [124]	1986	USA	Longitudinal
Janas [125]	1987	USA	Longitudinal
Lauber [126]	1979	Ivory Cost	Longitudinal
Lönnerdal [26]	1976	Sweden	Longitudinal
Sarwar [127]	1996	Canada	One time point
Svanberg [128]	1977	Ethiopia and Sweden	Cross-sectional
Villalpando [129]	1998	Mexico	Cross-sectional
Wu [130]	2000	Taiwan	Cross-sectional
Yamawaki [131]	2005	Japan	Cross-sectional

month of delivery. For example, true protein content in samples collected ~60 days after delivery was nearly 40% less than that seen in colostrum. While protein content was greatest in colostrum, colostrum also exhibited the greatest variability in protein content. The variance in protein content decreased with age. Despite the reduction in protein over time, the nutritional value of protein in breast milk, as measured by the ratio of essential amino acids to total amino acids, appears to be consistent over time. These changes correlate well with the evolving needs of the growing infant.

Results of recent meta-analyses and systematic reviews of breast milk nutrient content are consistent with our findings [12,36]. A systematic review and meta-analysis comparing the nutrient content of preterm and term human milk reported higher true protein content in preterm milk than in term milk, although these differences had dissipated by postnatal day 3 and were no longer apparent by week 10 to 12. In both preterm and term milk, colostrum had the highest protein content. Protein content in colostrum was nearly twice as high as that seen in milk collected 3 to 4 weeks after delivery, a pattern that was observed in preterm and term milk.

Similarly, a systematic review of the longitudinal changes in amino acid profiles in term and preterm human milk reported that total amino acid content was highest in the colostrum, declined in the first 2 months of lactation and then remained stable [36]. The authors did note that this pattern did not hold for all free amino acids when they were individually analyzed. This review also noted significant regional differences for certain amino acids, indicating the importance of including samples from a variety of geographic regions. Our analyses included data from more than 20 different countries across the globe.

4.1. Changes in bioactive proteins

It has been suggested that the higher protein content of colostrum and early breast milk may represent nondigestible bioactive proteins [12,37,38]. While changes in true protein and amino acid content of human milk have been described in several publications [12,36], less is known about longitudinal changes in bioactive proteins. Many proteins in human milk have demonstrated roles beyond nutrition, providing enzymatic activity, enhancing nutrient absorption, stimulating growth, modulating the immune system and defending against pathogens (Table 6). Whey proteins, such as lactoferrin, α-lactalbumin, immunoglobulins and lysozyme, and caseins are among the most thoroughly characterized.

Table 3
Median values of the total, essential, nonessential and essential to total amino acids (AA) ratio in human milk

Time (days)	Total AA	Essential AA	Nonessential AA	Essential to total AA ratio
	mg per 100 ml			%
0–5	2240.3	893.4	1346.9	39.9
6–15	1623.2	687.9	935.2	41.5
16–30	1111.0	491.0	620.0	44.2
31–60	1143.0	487.5	655.5	43.6
61–90	1026.0	436.0	590.0	40.1
91–360	1008.1	423.9	584.2	42.7

4.1.1. Whey-to-casein ratio

The whey-to-casein ratio significantly affects the bioactivity of milk proteins. For example, early milk contains a very high proportion of whey proteins, especially lactoferrin and sIgA, which may be particularly important for immunity and protection against infection during the newborn period, whereas caseins become more predominant in later lactation, possibly providing bioactivities that are more important in later infancy.

Several studies have demonstrated that whey-to-casein ratios vary significantly over the course of lactation [15,16]. Early in lactation, whey concentrations are high, and casein is virtually undetectable. At the start of lactation, whey-to-casein ratios of approximately 80:20 have been reported [16,24]. Our analysis confirmed these findings but revealed an even greater disparity between whey and casein concentrations early in lactation. In our study, the median whey-to-casein ratio was nearly 90:10 in colostrum and dropped to 65:35 by week 2. Whey protein contains lactoferrin, α-lactalbumin and immunoglobulins, all of which promote immunomodulation, a critical function in newborn infants with an immature immune system. These findings reinforce the need for lower casein levels in formulas developed for newborn infants.

4.1.2. Caseins

Key caseins in human milk include β-casein and κ-casein. The αs1 casein subunit is present in very low concentrations in human milk, unlike in cow's milk. When β-casein is digested, smaller casein phosphopeptides and caseomorphins are formed [24]. Negatively charged casein phosphopeptides can chelate Ca^{2+} and may facilitate calcium absorption. Although Teucher *et al.* have shown that bovine caseinphosphopeptides do not enhance calcium absorption in adults [39], the presence of such peptides in infants may help to keep calcium in solution and thereby improve net calcium absorption. The presence of casein phosphopeptides in human milk may explain, in part, the more effective uptake of calcium from breast milk than from formula [39]. Caseomorphins have structures similar to opioid peptides [40] and may thus affect infant sleep–wake patterns and psychomotor development [41]. β-casein may also exhibit antimicrobial activity towards *Haemophilus influenza* [42] and *streptococci* [43,44].

κ-casein inhibits bacterial adhesion, including the adhesion of *Heliobacter pylori* [45]. In fact, *H. pylori* is less common in breast-fed than in formula-fed infants [24]. This may result from the structural similarity between the glycans of κ-casein and the surface-exposed carbohydrates of cells in the mucosa of the gastrointestinal tract, suggesting that these glycans may act as soluble "decoys" for pathogens [45]. Studies also indicate that caseins may exhibit immunomodulatory activity by regulating chemotaxis and ameliorating inflammation [46–48].

4.1.3. Lactoferrin

Among the whey proteins, lactoferrin is a dominant component; it constitutes 20% of true protein in breast milk. Lactoferrin is a protein with multiple functions and a structure that makes it remarkably resistant to proteolytic enzymes and, thus, difficult to digest. In newborns and even in infants up to 4 months of age, intact lactoferrin can be found in infant stool, suggesting that lactoferrin survives and may be active in the small intestine [18,24]. Undigested lactoferrin can bind to specific lactoferrin receptors on the surface of epithelial cells

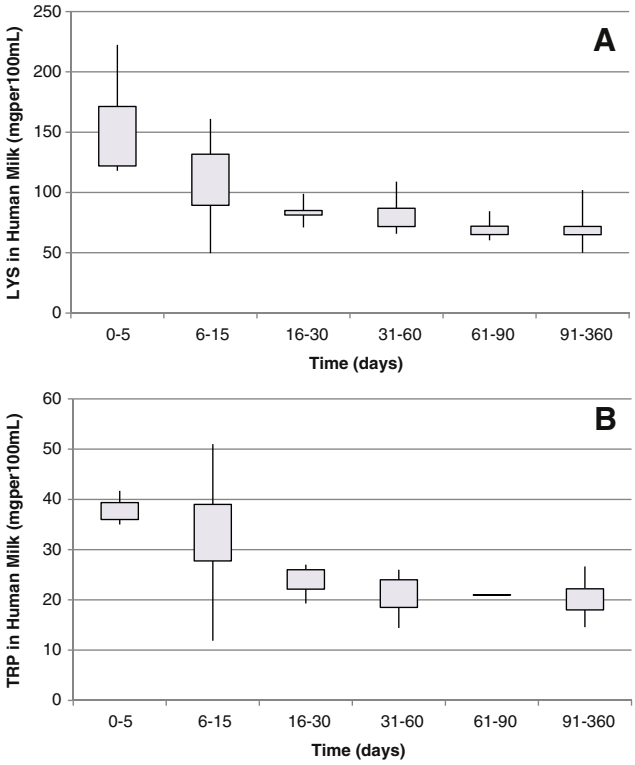

Fig. 3. Boxplots displaying the longitudinal evolution of (A) lysine and (B) tryptophan content in human milk (mg per 100 ml).

and be internalized through endocytosis [10,49,50]. Once inside the cell, lactoferrin enters the nucleus and binds to specific promoter sites, acting as a transcription factor and thereby regulating the expression of many genes including several cytokines [51]. This may help explain why and how lactoferrin can influence so many diverse activities [10].

Lactoferrin is known to facilitate the uptake of iron into cells. However, early studies on infant formulas supplemented with bovine lactoferrin largely showed that lactoferrin supplementation had no impact on iron absorption or iron status [14,52,53]. These findings may be explained by the fact that commercial bovine lactoferrin at that time was contaminated with lipopolysaccharaide (LPS), which has a very high affinity to lactoferrin and may interfere with its bioactivities. A more recent clinical trial, although limited in size, showed that feeding infant formula with bovine lactoferrin at a concentration similar to that of lactoferrin in human milk resulted in improved iron status and significantly less upper respiratory illness compared to regular formula without lactoferrin supplementation [54].

Lactoferrin is both bactericidal and bacteriostatic in that it limits the growth of several pathogens and kills others. The iron-free form of lactoferrin, its most common form in breast milk, can kill *Streptococcus mutans*, *Streptococcus pneumoniae*, *Escherichia coli*, *Vibrio cholera*, *Pseudomonas aeruginosa* and *Candida albicans* [55]. The bacteriostatic effects of lactoferrin result, in part, from its ability to withhold iron from bacteria that require it for growth. It also exhibits antibacterial, antivirus, antifungal and antiprotozoan activities that are likely independent of iron chelation. For example, it has been suggested that lactoferrin disrupts bacterial cell membranes and blocks cell–virus interactions [44,56,57].

Lactoferrin is also an effective modulator of inflammatory and immune responses. Evidence suggests that it increases the number and activity of T lymphocytes, B lymphocytes and natural killer cells, accelerates B and T cell maturation, and increases the expression of cellular receptors [44,58].

These preclinical observations are reinforced by the finding that administration of bovine lactoferrin to very low-birth weight infants protects against late-onset sepsis and necrotizing enterocolitis (NEC) arising from Gram-negative, Gram-positive and invasive fungal infections [59–62].

Results from our meta-analysis indicated that reductions in lactoferrin concentrations parallel reductions in true protein content over time. The highest lactoferrin levels were noted between days 1 and 3 (5.05 mg/ml), decreasing to 3.30 mg/ml in milk expressed at 6 to 15 days and to 1.44 mg/ml in milk collected at 91 to 360 days. These findings are consistent with previous research [63–66]. In some studies, lactoferrin concentrations peak at 7 mg/ml in colostrum and decrease to 1 or 2 mg/ml in mature milk [63–65,67]. A recent systematic review reported that lactoferrin concentrations were highest during early lactation (4.91 g/l in milk<28 days lactation) and rapidly declined to essentially constant levels after 1 month of lactation (2.1 g/l) [68]. The decrease in milk lactoferrin during lactation together with the increased capacity of the newborn infant to digest lactoferrin [18] will gradually lead to lower concentrations of intact lactoferrin in the gut lumen. This may result in a changing role for lactoferrin during infancy in that higher concentrations of lactoferrin promote cell proliferation, whereas lower concentrations stimulate cell differentiation [51,69], which corresponds with the development of the infant gut.

Table 4
Included studies on bioactive proteins in human milk

First author	Year	Country	Study esign	Protein analyzed
Lönnerdal [17]	1976	Sweden and Ethiopia	Longitudinal	Lactoferrin, α-lactalbumin, serum albumin, IgG, IgM
Lönnerdal [26]	1976	Sweden	Longitudinal	Lactoferrin, α-lactalbumin, serum albumin, IgG, IgM
Sanchez-Pozo [27]	1986	Spain	Cross-sectional	Lactoferrin, α-lactalbumin, serum albumin, lysozyme
Ronayne de Ferrer [28]	2000	Argentina	Longitudinal	Lactoferrin
Nagasawa [29]	1972	Japan	Not documented	α-lactalbumin, serum albumin
Montagne [30,31]	2000	France	Cross-sectional	Whey, caseins, lactoferrin, α-lactalbumin, serum albumin, lysozyme, IgA
Marquis [32]	2003	Peru	Longitudinal	Lactoferrin, lysozyme, IgA
Nagasawa [33]	1972	Japan	Not documented	Whey, caseins, lactoferrin
Nagra [34]	1989	Pakistan	Longitudinal	Whey, caseins
Kunz and Lönnerdal [16]	1992	USA	Longitudinal	Whey, caseins
Sanchez-Pozo 1987 [35]	1987	Spain	Exclusive	Lactoferrin, α-lactalbumin, serum albumin

4.1.4. α-lactalbumin

α-Lactalbumin is a digestible whey protein that comprises 25% to 35% of the true protein in human breast milk [70–71]. It has several

Table 5
Median values of whey to casein ratio, lactoferrin, α-lactalbumin, serum albumin, IgG, IgM, IgA and lysozyme in human milk (data in mg per ml)

Time (days)	Whey-to-casein ratio	Lactoferrin	α-Lactalbumin	Serum albumin	IgG	IgM	sIgA	Lysozyme
0–5	89:11	5.05	4.30	0.35	–*	–	5.45	0.32
6–15	65:35	3.30	4.20	0.62	0.05	0.12	1.50	0.30
16–30	59:41	2.31	3.30	0.67	0.05	0.05	1.10	0.28
31–60	61:39	1.95	3.10	0.69	–	–	1.00	1.10
61–90	61:39	1.89	2.84	0.45	0.03	0.03	1.30	0.85
91–360	60:40	1.44	2.62	0.37	0.04	0.03	–	–

* Not reported.

Table 6
Bioactive proteins in breast milk and their mechanisms of action

Bioactive protein	Mechanism of action	References
Lactoferrin	Bacteriostasis, bactericidal activity, immunomodulatory activity, cell proliferation and differentiation, iron uptake	[10,14,44,55,58]
Lysozyme	Antibacterial activity; degradation of cell wall glycans	[23,92]
Secretory IgA	Transfer of maternal immunity; antibodies against bacteria and viruses	[83,85]
Bile–salt stimulated lipase	Hydrolysis of triglycerides; fatty acid absorption	[132]
Milk fat globule membrane proteins	Antibacterial and antiviral activities	[133–135]
α-lactalbumin	Prebiotic activity; immunostimulatory; enhancing trace mineral (Fe, Zn) absorption; antibacterial function	[10,73,74,77,78,79,81,82]
β-casein	Opioid activity; enhancing calcium absorption	[24,40,41]
K-casein	Antibacterial activity by acting as structural analogs	[24,45]
Osteopontin	Immunomodulatory activity	[136,137]

physiological functions in the developing infant beyond its role as a well-balanced source of essential amino acids. For example, α-lactalbumin is a calcium-binding protein that can also bind iron and zinc with lower affinity [10,72]. Current evidence suggests that α-lactalbumin has a stimulating effect on the absorption of these minerals [10,73–74]. α-Lactalbumin has also been shown to inhibit the growth of several potential pathogens both *in vitro* [75–77] and *in vivo* [78].

Interestingly, several of the functions associated with α-lactalbumin may actually be attributable to peptides released during its digestion [10]. Although these peptides are likely formed in the upper gastrointestinal tract, they may exert their functions as they pass through the distal part of the small intestine and the colon. Several of these peptides, which appear to be transiently formed, have been shown to have antibacterial and immunostimulatory properties [10,14,79–81]. In one study, three polypeptide fragments from α-lactalbumin exerted antimicrobial activity against *E. coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Streptococci* and *C. albicans* [82]. Certain peptides arising from the digestion of α-lactalbumin have also been shown to encourage the growth of bifidobacteria, a species that dominates the gut of breast-fed infants but is less prevalent in formula-fed infants.

Longitudinal changes in α-lactalbumin over the course of lactation are not well characterized in the literature. In our study, changes in α-lactalbumin concentrations appear to reflect the decreases in true protein content over time. For example, concentrations of α-lactalbumin were highest in the colostrum (4.30 mg/ml) but decreased more gradually compared to concentrations of lactoferrin, true protein content, or amino acid content. In fact, α-lactalbumin concentrations in milk expressed 6 to 15 days after delivery (4.20 mg/ml) were similar to those seen in colostrum, but began to decrease in samples collected 16 to 30 days (to 3.30 mg/ml) and continued to decline over time. The impact of changing α-lactalbumin concentrations in the developing infant requires additional study.

4.1.5. Secretory IgA

Several immunoglobulins found in serum are also found in human milk, including sIgA, IgG and IgM. sIgA is quantitatively the most prominent immunoglobulin, though, accounting for 90% of total immunoglobulins in human milk [24]. sIgA consists of a dimer of IgA linked with a secretory component and a joining chain [14,83]. Unlike other types of IgA, sIgA is not easily degraded by the proteolytic enzymes in the infant gut [18,84]. As a result, maternal immunity against several general pathogens can be transferred through the breast milk *via* sIgA, mediated by the enteromammary immune pathway. This process boosts the immunity of the infant through the acquired immunity of the mother [83,85].

When sIgA specifically binds to the antigen of a pathogen, the binding renders the pathogen less infective. In fact, sIgA antibodies against numerous bacterial pathogens (*e.g.*, *E. coli*, *V. cholera*, *H. influenza*, *S. pneumoniaie*, *Clostridium difficile* and *Salmonella*), viruses (rotavirus, cytomegalovirus, HIV, influenza, respiratory syncytial virus) and yeasts (*C. albicans*) have been found in breast milk, demonstrating the breadth of this line of immune defense [14,83].

Studies also suggest that the broad spectrum and high diversity of sIgA antibodies may contribute to the proper development of the breastfed infant's mucosal immune system. SIgA polyreactive auto-antibodies with broad specificity have been described in colostrum, and these may exert antiinflammatory and tissue-protective activities [86]. In addition, a recent study in mice lacking milk SIgA and/or endogenous mucosal SIgA found that lack of early exposure to sIgA in milk affected the gut microflora, which in turn resulted in a pattern of epithelial cell gene expression that differed from that seen in mice not exposed to milk sIgA, including genes associated with intestinal inflammatory diseases in humans [87].

While sIgA is present at relatively high quantities throughout lactation, its levels are highest in colostrum [44,67,88]. In one 12-week study, the highest sIgA level occurred at day 3. This level decreased rapidly in the first 4 weeks and more gradually in the remainder of the study [88]. This temporal trend makes intuitive sense given the infant's developing immune system and its increasing ability to mount an effective immune response against pathogens.

Results from our meta-analysis quantify these trends in more detail. In our analysis, median sIgA concentrations decreased from 5.45 mg/ml at days 0 to 5 to 1.50 mg/ml 6 to 15 days after delivery. Concentrations of sIgA remained at or below this level throughout the first year of lactation.

4.1.6. IgG and IgM

IgG and IgM are found in small concentrations in human colostrum and milk [89]. However, these immunoglobulins are not always detected in colostrum samples, and only two studies that assessed their concentrations in human milk were suitable for inclusion in our analysis [17,26]. Neither study evaluated IgG or IgM levels in colostrum nor were we able to detect any clear trend in the concentrations of either immunoglobulin in more mature milk over time. A study by Gao *et al.* did report that IgM levels decreased and IgG levels increased over the course of lactation, but this study did not evaluate the levels of these immunoglobulins in colostrum [90].

The presence of IgG in human milk helps to counteract the infant's deficiencies in opsonization and antibody-mediated cytotoxicity [89]. However, the newborn is fully capable of producing IgM in response to infection. This finding may explain why IgM concentrations in colostrum are low and progressively decrease in more mature milk over time. It has been hypothesized that the higher levels of sIgA in colostrum and transitional milk and the lower levels of IgG in more mature milk suggest a transformation in the immunological function of human milk as infants age [90]. Early in lactation, the high levels of sIgA in colostrum and transitional milk support the direct killing of pathogens, while the increased levels of IgG in more mature milk support the development of the infant's own immunity as lactation progresses. This hypothesis is supported by the finding that IgG levels in newborns are comparable to those of their mothers due to the transport of IgG across the placenta. Moreover, IgG production in infants is delayed until about 6 months of birth. The late production of IgG by infants and the catabolism of maternal IgG suggest that there

may be a transient deficiency in IgG levels in infants during the first year [91], and the increasing supply of IgG in breast milk may attempt to compensate for this deficiency [90]. Additional research is needed to explore this hypothesis.

4.1.7. Lysozyme

Lysozyme is another major component of the whey fraction in human milk. Lysozyme is an enzyme capable of degrading the outer cell wall of gram-positive bacteria [14,92]. An *in vitro* study using electron microscopy also demonstrated that lysozyme can act synergistically with lactoferrin to kill gram-negative bacteria [14,23]. Lactoferrin first binds to the lipopolysaccharides of the outer cell membrane of these bacteria, creating holes in the membrane. Lysozyme can then enter and degrade the glycomatrix of these bacteria through these holes, killing the pathogen [23]. A clinical trial in which recombinant human lysozyme and human lactoferrin were given in oral rehydration solution (ORS) to children hospitalized with acute diarrhea may support these *in vitro* observations [93]. The prevalence and duration of diarrhea as well as relapse rate were significantly reduced by these two human milk proteins as compared to regular ORS. However, a weakness of the study was that neither protein was studied separately, meaning that either human lysozyme or human lactoferrin alone may have caused the effect. *In vitro* data also indicate that lysozyme may inhibit the growth of HIV *in vitro* [94], although the mechanism for its antiviral activity is unknown.

Our findings confirm that lysozyme concentrations in breast milk vary by duration of lactation. However, the temporal trend in lysozyme concentrations contrasted with the trends we observed for other bioactive proteins. Concentrations of lysozyme were lowest in colostrum and increased through the first month of lactation, peaking at 31 to 60 days and declining thereafter. These results are not entirely consistent with previous research. In one study, lysozyme concentrations were higher in colostrum, decreased in milk collected in the first month after delivery and peaked between days 57 and 84 [95]. In a second study, lysozyme concentrations were low in the colostrum and progressively increased during the course of lactation [89]. Due to these discrepancies, additional research on this protein may be warranted.

4.1.8. Serum albumin

Serum albumin is a major serum protein also present in human milk. Because its properties in human milk are similar to those in blood, it is thought that it may not be synthesized by the mammary gland [14]. Instead, it is believed to be transferred from maternal circulation. While serum albumin does serve as a source of amino acids for the breastfed infant, whether it has other physiologic functions in human milk is unclear. In blood, serum albumin binds many ligands, including fatty acids, trace elements, calcium and other molecules. Similarly, in milk, serum albumin has been associated with zinc, copper and thyroxine [96,97]. However, it is unlikely that serum albumin plays a major role as a nutrient binder or a source of nutrients for infants because its associations with these ligands are weak and binding to these ligands would not persist in the infant gut [98].

Our findings are in line with previous research demonstrating that serum albumin concentrations are approximately 0.4 to 1.0 mg/ml in the colostrum [17,26,27,98–100]. While previous evidence has shown that serum albumin concentrations remain relatively constant throughout lactation [17,26,27,98–100], our results show that concentrations slightly increase from the colostrum through day 60 and then decline over time. Additional research is needed to further clarify the role of serum albumin in human milk and its concentrations in human milk over the course of lactation.

4.2. Limitations

Our analysis has several limitations. First, it is limited by the availability of results from the individual studies. Not all endpoints were analyzed in all studies, and there were large variations in endpoints, populations, geographic regions, timing, design and methods of milk collection, data collection and data analysis. The variation in the timing of milk collection was also considerable, complicating the ability to create a precise characterization of changes in the individual proteins over time. Moreover, sample sizes in some studies were small, a limitation that may increase the variability of the findings.

5. Conclusion

Human milk contains a wide array of proteins with biological activities ranging from antimicrobial protection to immunomodulation and the facilitation of nutrient absorption. The proteins in human milk also provide adequate amounts of essential amino acids to support the growth of maturing infants. This highly adapted system likely is responsible for providing many of the advantages of human milk compared to infant formula. Results from our meta-analysis represent a useful dataset for the evaluation of protein quantity and quality in efforts to narrow the nutritional and immunological gap between breast milk and currently available breast milk substitutes.

Disclosure statement

Peter Erdmann and Frédéric Destaillats are employees of Nestlé Nutrition in La Tour-de-Peilz, Switzerland. Sagar K. Thakkar and Julien Sauser are employees of the Nestlé Research Center in Lausanne, Switzerland.

Role of the funding source

This research was funded by Nestlé Nutrition.

Acknowledgments

The authors would like to thank Nicole Cooper for providing medical writing assistance, a service funded by Nestlé. Authors would like to thank Dr. Francesca Giuffrida for her expert opinion related to the analytical methods for true protein determination in breast milk.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2016.06.001.

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