Original Article

Colostral and mature breast milk protein compositional determinants in Qingdao, Wuhan and Hohhot: maternal food culture, vaginal delivery and neonatal gender

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Background and Objectives: Breast milk proteins are essential to infants as they provide nutrition and protection. This study evaluated multiple factors that might influence breast milk proteins to identify the determinants that lead to inter-individual and longitudinal differences. **Methods and Study Design:** Five major breast milk proteins (β-casein, α-lactalbumin, lactoferrin, serum albumin and κ-casein) from breast milk samples collected from 55 mothers in three cities (Hohhot, Wuhan and Qingdao) in China were analyzed using a validated ultra-performance liquid chromatography-mass spectrometry method. Various factors were statistically evaluated for their associations with breast milk proteins: mother's age, parity, delivery mode, infant gender and infant birthweight. **Results:** Although decreased in concentrations, the proportions of β-casein and α-lactalbumin increased from colostrum (33.8% and 26.8%) to mature milk (40.3% and 31.6%), respectively. Mothers of older age were found to produce a lower concentration of total protein. Compared with vaginal delivery, caesarean section was associated with lower concentrations of κ-casein, lactoferrin and β-casein in mature milk. Infant gender influenced breast milk proteins in colostrum: mothers who delivered a girl tended to produce more κ-casein, lactoferrin and total protein. Furthermore, regional differences were found, and mothers from Hohhot produced significantly higher concentrations of α-lactalbumin and lactoferrin than those from Qingdao and Wuhan. This regional difference might be linked to the different dietary patterns of these mothers among cities. **Conclusions:** Our study deepens the understanding of breast milk protein dynamics in Chinese population and provides evidence on potential determinants, which can serve as guidance for infant nutrition optimization.

Key Words: κ-casein; β-casein; lactoferrin; serum albumin; α-lactalbumin

INTRODUCTION

Breast milk is the only source of nutrition for infants before introducing complementary foods. Exclusive breastfeeding is recommended by the World Health Organization for the first 6 months, with continuation up to 2 years of age.[1] After lipids and carbohydrates, proteins are the third most abundant solid fraction in breast milk.[1] In general, breast milk proteins are divided into three groups, with micellar casein and aqueous whey protein being the major groups and milk fat globule membrane proteins comprising a minor percentage.[2,3] The five most abundant proteins in breast milk are β-casein, α-lactalbumin, lactoferrin, serum albumin and κ-casein, which represent approximately 85% of total breast milk proteins.[2] The protein composition of human breast milk is different from that of other mammals' milk, cow's milk for example, which is mostly the basis of infant formula.[4] Cow's milk mainly contains α-casein and β-lactoglobulin, with trace amounts of lactoferrin.[2] Therefore, research on breast milk proteins may help establish a guideline for improving infant formula composition and filling the gap between breast milk and infant formula.[3]

The benefits of breast milk proteins are two-fold: it fulfills the nutritional requirement of infants, and it protects infants from sickness through bioactive functions. β-Casein and α-lactalbumin contain well-balanced essential amino acids; they are thus nutritional sources for infants as well as sources of bioactive peptides after being digested in infants' gastrointestinal (GI) tract.[1,3,5,6] Peptides

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released from α-lactalbumin are antibacterial, immunostimulatory and bifidogenic.[4,6-8] β-Casein can be digested to form phosphopeptides, which aids with calcium absorption, and caseomorphins to benefit infants' sleep-wake patterns.[3,6,9] κ-Casein has antibacterial activity against pathogens due to its heavily glycosylated structure.[9,10] Lactoferrin has several benefits that have been extensively studied, such as its antibacterial activity, immunomodulation, cell proliferation and differentiation in the gut lumen, and influence on iron uptake.[11-18] Different from other protein fractions, lactoferrin is largely resistant to proteolytic enzymes in the infant's GI tract and might help with gut microbiota development.[19] Serum albumin provides amino acids to nourish newborns, but knowledge on its other functions is lacking. It is mostly believed to originate from the maternal blood circulation as a serum protein.[3]

The protein composition of breast milk is dynamic and varies widely due to various factors. Several cohort studies, either longitudinal or cross-sectional, have been performed to evaluate the changing pattern of breast milk proteins and identify the underlying influencing factors.[9,20-27] One largely studied factor is lactation stage, which has been reported to influence the protein composition in breast milk.[21,23,25,27] In general, the concentrations of lactoferrin and α-lactalbumin decrease over the lactation period, whereas the β-casein concentration first increases and then returns back to the original value.[3,9] To deepen the understanding of breast milk protein compositional changes and to obtain more solid evidence on its determinants, the current cohort study was performed in the Chinese population from three cities with characteristic regional differences. Five major breast milk proteins (β-casein, α-lactalbumin, lactoferrin, serum albumin and κ-casein) and total protein composition were analyzed and statistically evaluated to identify potential determinants: lactation stage, mother age, delivery mode, parity, infant gender and infant birthweight. Furthermore, a qualitative estimation of the dietary pattern of these mothers was conducted, which might provide insights into the influence of food culture on breast milk protein compositional changes.

METHODS

Cohort study

The breast milk proteins of Chinese mothers from three cities with characteristic regional differences were examined in the current cohort study. From each city (Qingdao, Wuhan and Hohhot), 20 lactating women were recruited to provide breast milk samples. Inclusion criteria were as follows: healthy women, 20-40 years of age, delivered their babies in a hospital and breast-fed them at least 6 weeks postpartum. Both colostrum (0-5 days postpartum) and mature milk (6 weeks postpartum) samples were collected once from each mother. All samples were collected from July to December 2016.

At the time of sample collection, questionnaires were filled out to collect general information of the mothers and infants (age of mother, delivery mode, parity and gender of infant). The questionnaires also collected information on the mothers' eating habits and 24-hour food recall.

The current study was approved by the Ethics Committee of the School of Public Health, Sun Yat-sen University, (Approval No.48 [2015]). Written informed consent was obtained from all participants.

Breast milk sample collection

Breast milk samples were collected at the hospital or through home visits in the morning (usually 9-11 a.m.). A breast pump was used on one breast of the mothers to sample at least 20 mL milk, which then was divided into two sterile containers. After collection, breast milk samples were temporarily stored at -20 °C in local freezers and subsequently transferred to -80 °C freezers in the laboratory for long-term storage.

Measurement of breast milk proteins through ultra performance liquid chromatography-tandem mass spectrometry

Five major proteins (α-lactalbumin, lactoferrin, serum albumin, β-casein and κ-casein) in breast milk were analyzed using ultra-performance liquid chromatography-tandem triple quadrupole mass spectrometry (UPLC-MSMS). After protein digestion with trypsin, signature peptides and stable isotope-labeled internal standards were used for detection and quantification, with the multiple reaction monitoring (MRM) mode applied. This method has been evaluated and validated,[28] and it was adapted for the current analysis. All reagents used were of analytical purity or chromatographical purity, as required. Water purified using the Milli-Q system (Merck-Millipore, Burlington, MA, USA) was used.

In this study, 0.2 g of breast milk (accurate to 0.0001 g) was weighed and diluted with water to 10 mL in a volumetric flask. An aliquot of 10 μL diluted solution was mixed with 10 μL isotope internal standard, 10 μL dithiothreitol solution (15 mg/mL in water) and 845 μL water, followed by incubation in a water bath (80 °C for 30 min). After incubation, 10 μL iodoacetamide solution (54 mg/mL in water) was added to the solution, mixed, and incubated at room temperature in the dark for 30 min. For protein digestion, the mixture was added to 100 μL ammonium bicarbonate solution (39.6 mg/mL in water) and 10 μL trypsin solution (0.4 mg/mL in 1 mmol/L hydrogen chloride; enzyme activity of trypsin ≥3000 U); the reaction took place at 37 °C in a water bath. After a reaction time of 4 h, 5 μL formic acid was added and filtered through a 0.22 μm filter before sample injection for UPLC. Different concentrations of signature peptides were prepared to construct calibration curves for quantification. The same treatment procedures were applied to 10 μL of each calibration solution and to the breast milk samples. All samples were analyzed in duplicate, and the average values were used for statistical analysis.

Acquity Ultra Performance Liquid Chromatograph (Waters, Milford, MA, USA) equipped with a BEH 300 C18 column (100 x 2.1 mm, 1.7 μm; Waters) was used for separating the peptides. The temperatures of the column oven and sample tray were set at 40 ˚C and 10 ˚C, respectively. The injection volume of samples was 5 μL, and the flow rate was 0.3 mL/ min. Mobile phase A was 0.1% formic acid in water; mobile phase B was 0.1% formic acid in acetonitrile. The gradient elution profile was set as follows: 0-5 min, 3-32% B; 5-5.1 min, 32-100% B; 5.1-6.1 min, 100% B; 6.1-6.2 min, 100-3% B; 6.2-8 min, 3% B.

Xevo TQ-S Triple Quadrupole Mass Spectrometry (Waters) was used to acquire data on separated peptides, and was set as follows: ionization mode, ESI-positive; capillary voltage, 3.5 kV; desolvation temperature, 500 °C; desolvation gas flow rate, 800 L/ min; cone blowback gas flow rate, 150 L/ h; collision gas pressure, 3.0×10^{-3} mbar; and ion source temperature, 150 °C. The precursor and product ion and corresponding collision energy of signature peptide sequences for each breast milk protein are summarized in Table 1.

All samples were analysed within 24 hours after sample preparation.

To calculate the protein concentration in breast milk samples, the following equation was used:

$C = X \times M \times 10^{-7} \times 5000$,

where

C = protein concentration in breast milk (mg/100 mL);

X = concentrations of signature peptides in injection solution (nmol/L);

M = molar mass of proteins (g/mol);

10^{-7} = conversion factor, from nmol/L to mmol/100 mL;

5000 = dilution factor.

The total protein concentration in breast milk samples was determined using the Bradford method, which is a commonly used spectrophotometric method for protein quantification, based on the binding of the dye Coomassie Blue G250 to protein.[29]

Statistical analysis

Descriptive statistics were calculated for variables including general information on mothers and infants collected from questionnaires and for concentrations of the five major milk proteins. The data on parity, delivery mode and infant gender among three cities were evaluated using the Pearson's chi-squared test. The paired t-test was used to compare the means between colostrum and mature milk. One-way analysis of variance (ANOVA) was used to compare continuous data among different cities. If significant differences were found among cities, the post-hoc Duncan test was conducted for pair-wise comparison. Repeated-measures ANOVA was used to determine whether city would be a covariate of the difference between lactation stages. Pearson's correlation and linear regression between protein contents and mother's age were also conducted. The results were considered to be statistically significant if the p-value was less than 0.05. All analyses were conducted using SPSS Statistics Version 25 (IBM Corp., Armonk, NY, USA).

RESULTS

Chromatograms and calibration curves

A previously validated quantification method was adapted to determine the concentrations of five major breast milk proteins:[28] α-lactalbumin, lactoferrin, serum albumin, β-casein and κ-casein. Following trypsin digestion, breast milk samples were analyzed by ultra-performance liquid chromatography with tandem triple quadrupole mass spectrometry (UPLC-MSMS). Each protein was quantified based on corresponding calibration curves constructed from their signature peptides and isotope-labeled internal standards under multiple reaction monitoring (MRM) mode. Typical MRM chromatograms of these signature peptides and isotope-labeled internal standards are shown in Figure 1. Linear range, limit of quantification (LOQ) and linear correlation of calibration curves for the five milk proteins were evaluated (Table 2). All five curves showed good linearity with $R^2 > 0.99$, and the concentrations of the five proteins in breast milk samples in this current study were within the linear range.

Breast milk samples were analyzed in different batches from July to August 2018. For quality control of the analytical apparatus, one identical sample was included in all batches and was analyzed with each batch. The analytical results of this sample on different dates of analysis are given in Supplementary Table 1. The relative standard deviation (SD) of all five proteins among all batches (3.8% – 11%) showed reliable repeatability.

Characteristics of study subjects

In total, breast milk samples from 55 mothers were collected in the current study: 18 from Qingdao, 17 from Wuhan, and 20 from Hohhot (Table 3). The characteristics of all study subjects from the three cities and in general are summarized in Table 3. The mother's average age was highly significantly different among the three cities: Qingdao, 32 years old; Hohhot, 29 years old; Wuhan, 26 years old. Any difference found in terms of protein com-

Table 1. Precursor and product ion and corresponding collision energy of signature peptide sequences for each breast milk protein

Protein	Peptide	Precursor ion *(m/z)*	Product ion *(m/z)*	Collision energy *(eV)*
Serum albumin	LVTDLTK	394.7	248.1/577.3†	20/13
	LV*TDL*TK	401.7	248.1/584.2†	20/13
α-lactalbumin	CELSQLLK	495.7	290.0†/588.5	18/18
	CELSQL*L*K	502.7	290.0†/609.1	18/18
β-casein	VMPVLK	343.6	260.2/456.3†	12/10
	VMPV*L*K	350.2	267.7/469.3†	12/10
κ-casein	QYLPMSHPPTVVR	502.5	552.0/668.3†	15/22
	QYLPMSHPPTV*V*R	506.9	557.7/680.3†	15/22
Lactoferrin	VPSHAVVAR	312.6	326.6/419.0†	13/10
	VPSHAV*V*AR	312.5	332.6/424.3†	13/10

†This value was used for quantification.

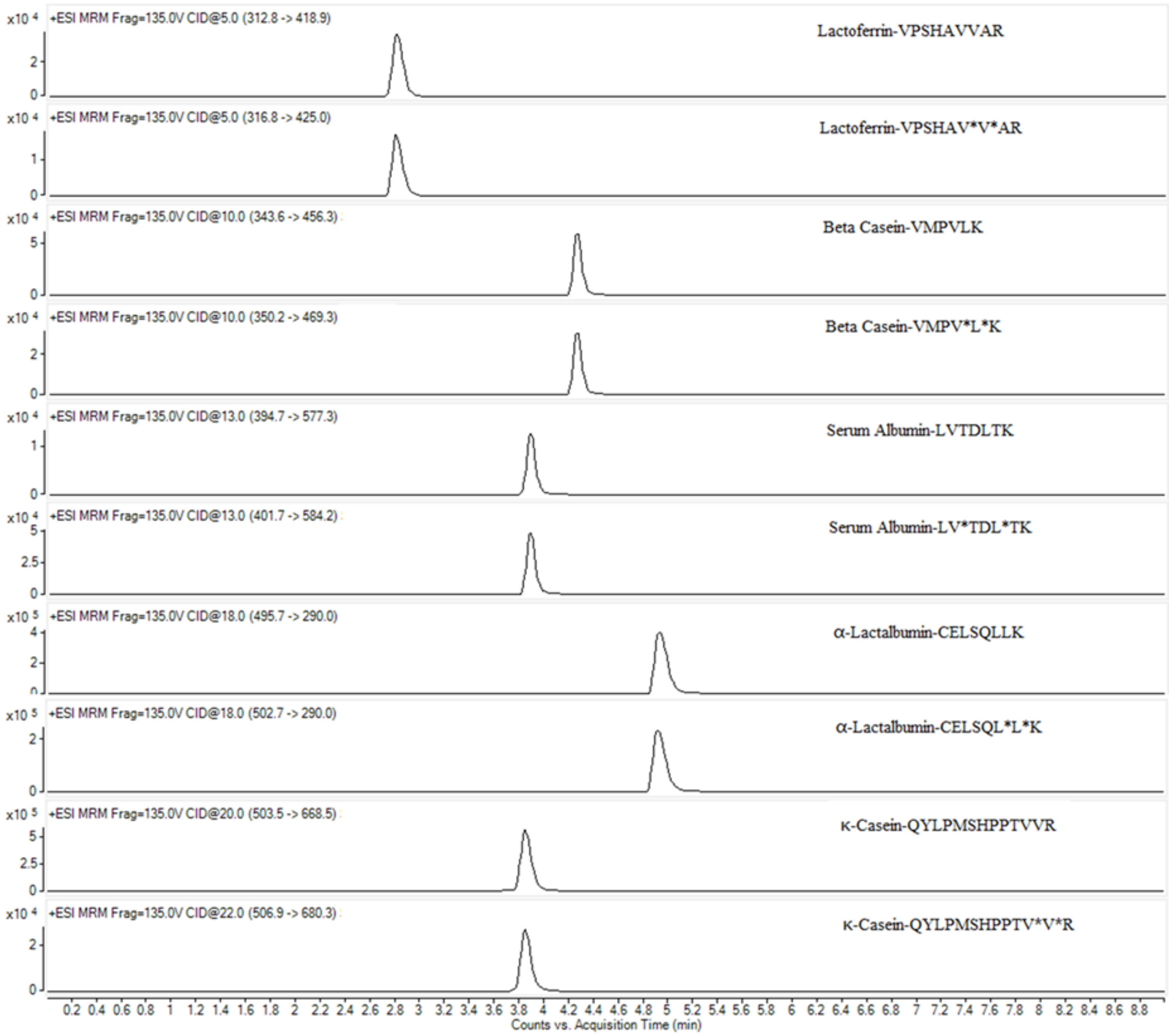

Figure 1. Multiple reaction monitoring (MRM) chromatograms of signature peptides and isotope-labeled peptides for breast milk proteins.

Table 2. Linear range, regression equation, correlation coefficients and limit of quantification (LOQ) of breast milk proteins

Protein	Linear range (μmol/L)	Linear regression equation	Linear correlation	LOQ (mg/100 g)
Serum albumin	0.75-15	y=0.380046*x-1.45301	0.995	0.5
α-lactalbumin	25-500	y=0.406754*x-1.76491	0.993	3.0
β-casein	15-300	y=1.03319*x+3.17537	0.995	3.0
κ-casein	4-80	y=0.291196*x-3.01858	0.995	0.5
Lactoferrin	1.6-16	y=1.06023*x-0.129415	0.999	3.0

Table 3. Characteristics of the study subjects from different cities and overall[†]

Characteristics	Qingdao	Wuhan	Hohhot	Overall	*p*-value
Actual sample size	18	17	20	55	
Mother's age (years), mean (SD)	32 (3)[a]	26 (4)[c]	29 (4)[b]	29 (4)	<0.001
Parity					
1, N (%)	3 (17)	8 (47)	7 (35)	18 (33)	0.065
2, N (%)	15 (83)	7 (41)	8 (40)	30 (55)	
Delivery mode					
Vaginal delivery, N (%)	4 (22)	9 (53)	9 (45)	22 (40)	0.051
Caesarean section, N (%)	14 (78)	6 (35)	7 (35)	27 (49)	
Infant's gender					
Male, N (%)	11 (61)	9 (53)	11 (55)	31 (56)	0.997
Female, N (%)	7 (39)	6 (35)	7 (35)	20 (36)	
Infant's birthweight (kg), mean (SD)	3.5 (0.6)	3.6 (0.6)	3.6 (0.4)	3.5 (0.5)	0.308

[†]Values with a different superscript letter were significantly different from each other in the same row. When two percentage values under the same term did not add up to 100%, the missing part was due to unknown data (some mothers did not provide certain information).

Table 4. Protein concentration in breast milk across different lactation stages and different cities, shown as means and standard deviations (SD)[†]

	Concentration in breast milk samples (mg/100 mL), mean (SD)					g/100 mL
	κ-Casein	Lactoferrin	Serum albumin	α-lactalbumin	β-casein	Total protein
Colostrum						
Overall	43.9 (16.5)	280.1 (82.4)	22.8 (7.00)	382 (69.3)	502 (123)	1.60 (0.28)
Qingdao	49.5 (21.8)	253^{a} (40.3)	20.6 (3.43)	367^{a} (60.3)	469 (92.2)	1.48 (0.22)
Wuhan	44.0 (12.7)	255^{a} (82.9)	24.8 (8.46)	355^{a} (57.5)	560 (113)	1.65 (0.23)
Hohhot	39.5 (13.6)	321^{b} (92.6)	22.9 (7.66)	416^{b} (73.5)	482 (140)	1.65 (0.33)
p-value (city)	0.236	0.023	0.279	0.026	0.095	0.155
Mature milk						
Overall	23.1 (8.08)	133 (65.3)	29.4 (12.0)	335 (78.9)	423 (95.7)	1.14 (0.25)
Qingdao	19.6 (6.49)	100^{a} (17.6)	29.5 (6.40)	299^{a} (61.5)	384 (73.6)	1.02^{a} (0.16)
Wuhan	23.9 (8.17)	121^{a} (54.9)	34.6 (18.4)	319^{a} (65.2)	447 (93.1)	1.19^{b} (0.26)
Hohhot	25.9 (8.53)	176^{b} (81.2)	24.6 (6.37)	384^{b} (83.6)	440 (109)	1.22^{b} (0.29)
p-value (city)	0.055	0.001	0.050	0.002	0.099	0.039
Overall	32.9 (16.4)	202 (104)	26.3 (10.4)	357 (77.9)	460 (116)	1.36 (0.35)
p-value (lactation stage)	<0.001	<0.001	<0.001	0.002	0.003	<0.001

[†]Values with a different superscript letter were significantly different from each other in the same sub-column.

position among the cities could be influenced by the difference in mother's age. No significant differences were observed in parity, delivery mode, infant gender and infant birthweight. The rate of caesarean section (C-section) delivery was quite high, with 49% overall, and it even reached 78% in Qingdao (possibly due to the mother's older age). However, this high rate of C-section rate was aligned with the rate reported in previous cohort studies performed in China.[21,30] China has one of the highest C-section rates worldwide, with approximately 50% babies delivered by C-section.[31]

Content of breast milk proteins

The concentrations of five major breast milk proteins, α-lactalbumin, lactoferrin, serum albumin, β-casein, κ-casein, and total protein compositions in the samples from mothers were determined in the current cohort study. Means and SDs for each protein and total protein are summarized in Table 4. Highly significant differences were observed in the concentrations of all five individual proteins as well as the total protein between colostrum and mature milk ($p<0.01$). As shown in Table 4, protein concentrations generally decreased from colostrum to mature milk. The concentration of κ-casein decreased from 43.9 to 23.1 mg/100 mL; lactoferrin from 280.1 to 132.8 mg/100 mL; α-lactalbumin from 382.4 to 334.7 mg/100 mL; β-casein from 501.8 to 422.7 mg/100 mL; and total protein from 1.60 to 1.14 g/100 mL. By contrast, the concentration of serum albumin increased from 22.8 mg/100 mL in colostrum to 29.4 mg/100mL in mature milk, showing a different pattern than that of the other proteins.

Protein concentrations among different cities were separately compared for colostrum and mature milk. At both lactation stages, significantly higher lactoferrin and α-lactalbumin concentrations were found in Hohhot than in Qingdao and Wuhan (Table 4). The lactoferrin concentration was 320.9 mg/100 mL in colostrum and 175.9 mg/100 mL in mature milk for mothers from Hohhot, followed by mothers from Wuhan with 255.0 and 121.3 mg/100 mL, and mothers from Qingdao showed the lowest at 252.9 and 100.1 mg/100 mL (colostrum and mature milk, respectively). Mothers from Hohhot showed α-lactalbumin concentrations of 415.8 mg/100 mL in colostrum and 384.2 mg/100 mL in mature milk, and mothers from Qingdao showed α-lactalbumin concentration of 366.8 and 299.4 mg/100 mL, and mothers from Wuhan showed α-lactalbumin concentrations of 355.2 and 318.8 mg/100 mL (colostrum and mature milk, respectively). The concentrations of the other three proteins (serum albumin, β-casein and κ-casein) in breast milk were not significantly different among Qingdao, Wuhan and Hohhot. When comparing the total protein concentrations among cities, values in the mature milk of mothers from Hohhot (1.22 g/100 mL) and Wuhan (1.19 g/100 mL) were significantly higher than those in the mature milk of mothers from Qingdao (1.02 g/100 mL). This might be related to the older average age of mothers from Qingdao compared to the age of those from the other two cities. However, this significant difference in total protein levels among cities was not found in colostrum.

The proportions of total protein in breast milk were calculated for each of the five major proteins and compared between colostrum and mature milk (Figure 2). Overall, β-casein and α-lactalbumin accounted for the highest proportions of total milk protein in both colostrum and mature milk, followed by lactoferrin, and the lowest proportion was found for κ-casein and serum albumin. When comparing their proportions between colostrum and mature milk, significant differences were found for all five proteins; however, not all proteins followed the same changing trends as their concentrations. The proportions of β-casein and α-lactalbumin increased from colostrum (median, 33.8% and 26.8% respectively) to mature milk (median, 40.3% and 31.6% respectively). The proportion of serum albumin also increased from colostrum to mature milk, whereas the other two proteins, κ-casein and lactoferrin, decreased, similar to their concentrations.

Evaluation of possible influencing factors

In addition to the lactation stage, other possible influencing factors on breast milk protein compositions were

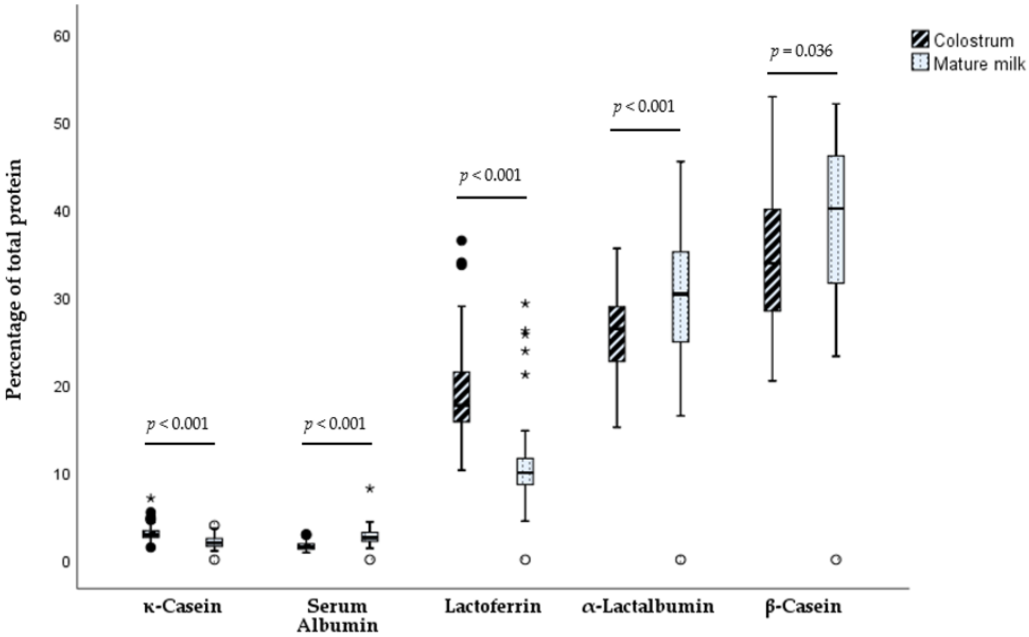

Figure 2. Comparison of breast milk protein proportions between colostrum and mature milk. Boxplots display minimum, first quartile, median, third quartile, maximum and outliers. Statistically significant p-values are shown in the graph.

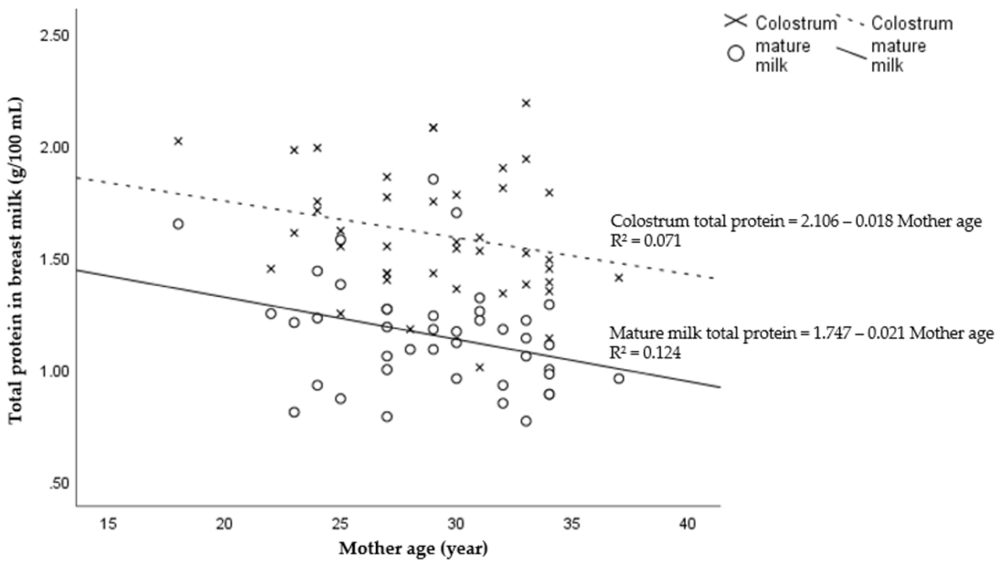

Figure 3. Linear regression of total protein content in colostrum and mature milk on mother's age, with *p*-values of 0.077 and 0.013, respectively.

evaluated, including mother's age, parity, delivery mode, infant gender and infant birthweight. Supplementary Table 2 summarizes the statistical results obtained from the Pearson's correlation test or t-test. Parity and infant birthweight were not found to be significantly influencing factors. Mother's age, delivery mode and infant gender seemed to be associated with certain breast milk protein parameters, and are further addressed in this section.

Mother's age

Mother's age was strongly correlated with the total protein concentration in mature milk, but not with individual proteins (Supplementary table 2). As shown in Figure 3, a negative relationship was found between mother's age and total protein in mature milk. The same negative association was also found between mother's age and total colostrum protein, although less strong. This result indicated that mothers of older age tended to have lower total protein concentrations in their breast milk, which might partly explain the lower total protein concentration found in the milk of mothers from Qingdao compared with those from the other two cities, as these mothers were the oldest.

Delivery mode

Almost half of the infants were delivered by C-section in this cohort study. Therefore, the possible influence of delivery mode on breast milk proteins was investigated, with the statistical results summarized in Supplementary Table 2 and visualized in Figure 4. Comparisons were made between mothers delivering infants by either vaginal delivery or C-section for determining their association

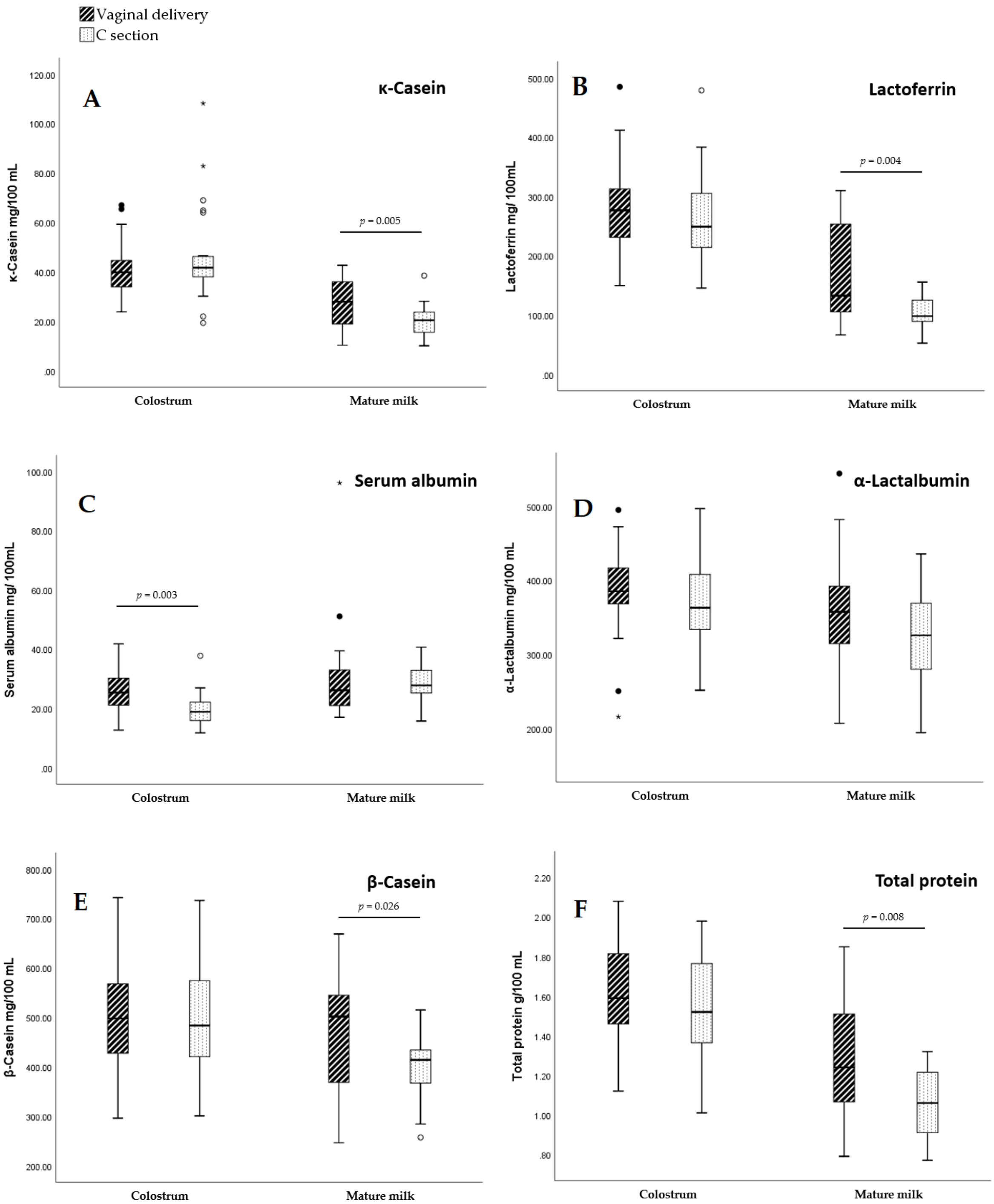

Figure 4. Comparison of breast milk protein concentrations between mothers delivering infants in different modes: vaginal delivery or caesarean section (C-section): A, κ-casein; B, lactoferrin; C, serum albumin; D, α-lactalbumin; E, β-casein; F, total protein. The comparisons were performed for colostrum and mature milk separately. Boxplots display minimum, first quartile, median, third quartile, maximum and outliers. Statistically significant *p*-values are shown in the graph.

with proteins in colostrum and mature milk separately. Three proteins, namely κ-casein, lactoferrin and β-casein, as well as the total protein, were significantly lower in the mature milk of mothers with C-section delivery than those with vaginal delivery (Figure 4); however, this difference was not found in their colostrum. By contrast, serum albumin was found to be significantly lower in the colostrum of mothers with C-section mothers, but not in mature milk. In general, the delivery mode seemed to affect breast milk protein compositions at a later stage of lactation. Mothers who delivered infants by C-section seemed to produce lower protein concentrations in breast milk.

Infant gender

When comparing the breast milk protein compositions of mothers who delivered either a boy or a girl, significant differences were found only in colostrum for two proteins, namely κ-casein and lactoferrin, as well as the total protein content (Figure 5). In the colostrum of mothers who delivered a girl, higher concentrations of these proteins were found compared with those who delivered a boy.

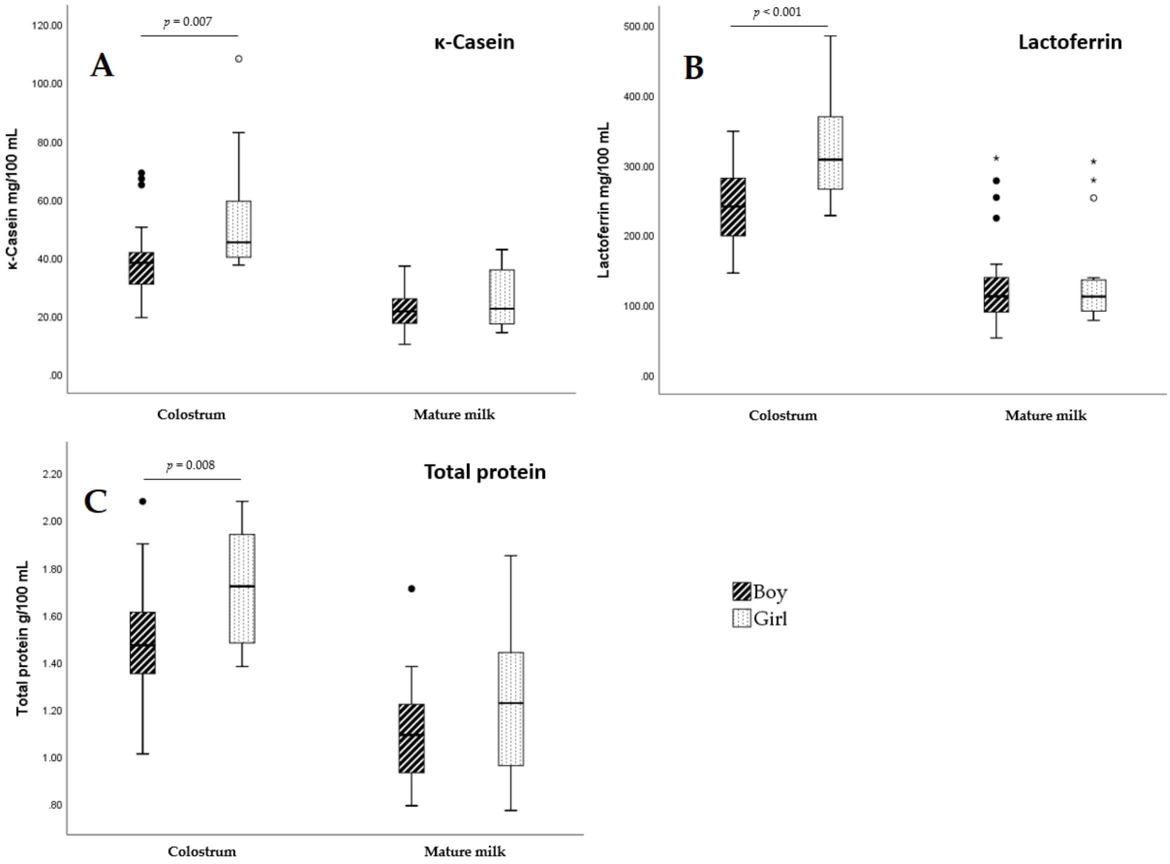

Figure 5. Comparison of breast milk protein concentrations between mothers who gave birth to a boy or a girl: A, κ-casein; B, lactoferrin; C, total protein. The comparisons were performed for colostrum and mature milk separately. Boxplots display minimum, first quartile, median, third quartile, maximum and outliers. Statistically significant p-values are shown in the graph. For the p-values of serum albumin, α-lactalbumin and β-casein, please refer to Supplementary table 2.

However, this difference disappeared at a later stage of lactation. The effect of infant gender on breast milk proteins seemed to occur at an early stage of lactation.

DISCUSSION

Breast milk proteins in the samples of 55 Chinese mothers from three cities with characteristic regional differences were studied in the current cohort study. By using ultra-performance liquid chromatography with tandem triple quadrupole mass spectrometry (UPLC-MSMS) under the multiple reaction monitoring (MRM) mode, major breast milk proteins were analyzed for all mothers. The five proteins were β-casein, α-lactalbumin, lactoferrin, serum albumin and κ-casein, and their quantification was based on calibration curves built based on corresponding signature peptides and isotope-labeled internal standards. All calibration curves showed good linearity and covered the quantification range of breast milk samples. The total protein content in breast milk was also determined for all samples through the Bradford method, which is well validated. Overall, the average concentrations of β-casein, α-lactalbumin, lactoferrin, serum albumin and κ-casein in breast milk samples were 459.8, 357.1, 202.0, 26.3 and 32.9 mg/100 mL, respectively, and that of total protein was 1.36 g/100 mL. The concentration range of each protein obtained in this study was well aligned with that in the literature.[2,3,20,21,32]

The lactation stage had obvious effect on the breast milk proteins, both in terms of concentration and proportion of total protein. All major breast milk proteins and total protein concentrations decreased from colostrum to mature milk, with the exception of serum albumin, which showed an increase. Considering their corresponding proportions of the total protein with lactation duration, β-casein, α-lactalbumin and serum albumin increased, whereas lactoferrin and κ-casein decreased. The decrease in the concentration with the elongation of lactation period has previously been reported for lactoferrin, α-lactalbumin, κ-casein and total protein.[1,3,20,21,24,26] A systematic review on the breast milk composition of Chinese women reported a decrease in protein content from colostrum (1.64±0.32 g/100 mL) to mature milk (1.22±0.12 g/100 mL),[33] which is well aligned with the values found in the present study (Table 4). However, the β-casein concentration was believed to first slightly increase before returning back to the starting value,[1,9] and serum albumin stayed relatively stable.[21] In general, lactating mothers produce a larger volume of milk to fulfill infants' growing daily intake of breast milk.[34] Therefore, lower concentrations of these proteins in milk from a later stage of lactation do not necessarily lead to a decreased amount of protein consumed by infants. The serum albumin concentration change was different from those of other major proteins with the lactation period, indicating that it might have a different pathway of regulation or synthesis. Development of infants is suggested to be in stages with different emphasis on the functions of breast milk. Immune protection seems more vital at an earlier stage when

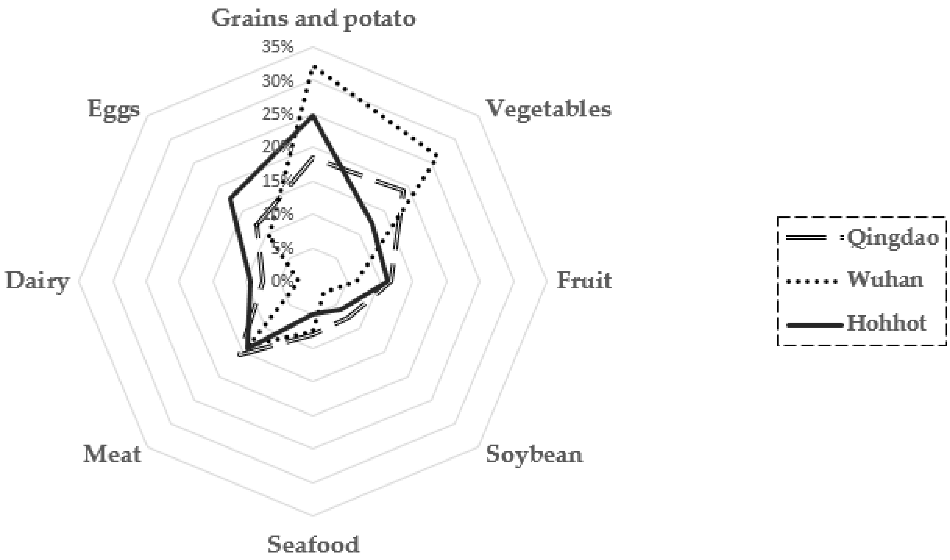

Figure 6. This spider plot shows a qualitative comparison on dietary patterns of mothers from the three cities. The values are based on a rough estimation of average proportion per food category, as consumed by lactating mothers one day before breast milk sampling. The detailed values are given in Supplementary Table 3.

newborns are just delivered from a relatively sterile uterus to external environment.[26,35] With the growth of infants, their immune system gradually develops, and the requirement shifts more to nutritional support.[28] β-Casein and α-lactalbumin are key nutritional proteins for infants, as they contain abundant indispensable amino acids that are easily digested;[25] whereas lactoferrin and κ-casein protect newborns, given their antibacterial and immunomodulatory functions.[5] Our findings that β-casein and α-lactalbumin are up-regulated in terms of proportion of total protein, whereas lactoferrin and κ-casein are down-regulated with a longer lactation period, adequately support this suggestion.

Major breast milk protein compositions seemed to be related to mother's age, delivery mode, infant gender and lactation stage. Similar to a previous finding,[2] mothers of older age tended to produce milk with lower total protein composition in mature milk, and also in colostrum, although to a lesser extent. This partly explained the lower total protein concentration in breast milk samples collected from mothers in Qingdao than in breast milk samples collected from mothers in Wuhan and Hohhot, as mothers recruited from Qingdao were the oldest among the three cities. The C-section delivery rate is quite high in China compared with that in other countries;[31] the rate was found to be associated with lower concentrations of κ-casein, lactoferrin and β-casein at a later stage of lactation, and lower serum albumin at an earlier stage in the current study. This finding differed from that in the literature, which revealed hardly any influence of delivery mode on breast milk proteins.[21,25] Another study found higher colostrum protein concentration in mothers with vaginal delivery than in those with C-section, which was suspected to be the result of hormonal activity caused by labor pain and uterine contractions.[36] C-section was also reported to lead to the late onset of lactation;[37] however, the actual underlying mechanism needs further study. Nevertheless, it is proved again that the regulation of serum albumin in breast milk follows a different pattern than that of other proteins. Different from the delivery mode, infant gender influenced breast milk protein in the colostrum, but not mature milk. We found higher κ-casein and lactoferrin concentrations in mothers who delivered a girl than a boy. However, these results were not in agreement with previous results, as some found no difference in the lactoferrin concentration,[24,25] and others reported a higher protein content in breast milk when infants were male.[38,39] Although there is not yet any agreement on the influence of infant gender, its stronger effect on colostrum might be related to hormonal differences in pregnant women caused by the gender of the fetus.

Differences in protein concentrations in breast milk were found among the three cities: mothers from Hohhot showed more α-lactalbumin and lactoferrin than did mothers from Qingdao and Wuhan. The study subjects from these cities were evaluated in terms of several characteristics, with mother's age being the only factor that was significantly different. However, the difference in mother's age among the three cities could not explain why mothers from Hohhot produced higher concentrations of α-lactalbumin and lactoferrin. Qingdao is a coastal city in east China; Wuhan is a typical inland city along the Yangtze River; Hohhot is an inland city located in a grassland area and is at the national border between China and Mongolia. A previous cohort study in China showed different lactoferrin concentrations in breast milk with different maternal ethnicities.[24] Hohhot has a population with a more mixed ethnicity than the other two cities, which might partly explain the difference observed in the current study. In addition to genetic differences, nutrition and the dietary pattern could be other factors worth investigating. All mothers in this study were asked to fill out a 24-h food recall questionnaire at the time of breast milk sampling. However, only food names were provided by the mothers, without the quantity consumed. Therefore, no quantitative results or statistical analysis could be used to evaluate the influence of diet. Inspired by literature,[40] we made a qualitative comparison of dietary patterns among the three cities. Figure 6 is based on a rough estimation of the average proportion per food category, as calculated from the frequency of appearance on the food list of individual mothers. In the dietary pattern of mothers from Hohhot, dairy and eggs seemed to account for a higher proportion than that from Qingdao and Wuhan (Figure 6). Dairy and egg are good sources of nutritive proteins providing essential amino acids, for instance tryptophan, as a building block of breast milk proteins.[41,42] Mothers from Wuhan consumed a higher percentage of grains, potatoes and vegetables than those from the other two cities, and mothers from Qingdao seemed to have an intermediate diet. However, the dietary pattern data shown here are only an indication and no definite conclusion should be drawn. More well-designed surveys on diet and nutritional status of lactating Chinese mothers are required in the future.

Some limitations of the current study were the relatively small sample size and the lack of data on mothers' body mass index or gestational age of infants. This information could also contribute to our understanding of major breast milk proteins. Furthermore, it should be realized that the determinants reported in the current manuscript could have influenced other macronutrient components in the breast milk, in addition to proteins, as indicated by the literature.[30,33] It is known from the literature that microRNAs and exosomes are important in understanding lactational physiology, considering their potential effects on protein production,[43] although not included in the present study. The strengths of this study include its longitudinal design, which allowed breast milk at different lactation stages from the same mothers to be studied, and the fact that a validated analytical method was used that yielded reliable protein data. Various factors were evaluated in terms of their influence on breast milk protein, and promising results were reported. The physiological mechanisms underlying these findings are worth further investigation to optimize personalized infant nutrition.

Conclusion

In this cohort study of 55 Chinese mothers from three cities, the major breast milk proteins and total protein content were analyzed in breast milk. Lactation stage, mother's age, delivery mode and infant gender were found to be associated with differences in breast milk protein compositions, although to different extents and patterns. Regional difference also existed among cities, which still requires further research. In summary, our study deepens the understanding of breast milk protein dynamics in the Chinese population and provides more evidence and guidance on infant nutrition optimization.

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AUTHOR DISCLOSURES

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Supplementary table 1. Analytical results of one identical sample on different dates of analysis, for quality control of the analytical apparatus. Means and relative standard deviations (RSD%) are also given in the table

Date of analysis	Analytical result (mg/100 g)					g/100 g
(YYYY/MM/DD)	κ-Casein	Lactoferrin	Serum albumin	α-Lactalbumin	β-Casein	Total protein
20180712	15.20	55.00	22.44	297.96	363.02	0.89
20180713	17.08	62.75	21.61	312.57	398.08	0.95
20180716	13.83	52.34	22.84	320.48	439.00	0.96
20180717	17.08	63.06	24.74	322.81	416.32	0.97
20180718	16.32	62.38	22.50	384.17	443.50	0.90
20180719	19.38	60.55	24.87	380.70	447.00	0.99
20180801	16.21	64.03	22.27	374.60	402.25	0.95
20180817	14.23	59.96	22.22	306.96	395.06	0.97
Mean	16.17	60.01	22.94	337.53	413.03	0.95
RSD%	11.0	7.0	5.3	10.6	7.0	3.8

Supplementary table 2. Summary of *p*-values obtained from Pearson's correlation test or t-test

	Concentration in breast milk samples (mg/100 mL), mean (SD)					g/100 mL
	κ-Casein	Lactoferrin	Serum albumin	α-Lactalbumin	β-Casein	Total protein
Colostrum						
Mother's age	0.873	0.982	0.720	0.808	0.091	0.077
Parity	0.724	0.559	0.528	0.825	0.132	0.076
Delivery mode	0.322	0.369	0.003	0.506	0.682	0.195
Infant gender	0.007	<0.001	0.052	0.266	0.272	0.008
Infant birthweight	0.879	0.429	0.862	0.090	0.253	0.306
Mature milk						
Mother's age	0.207	0.479	0.779	0.164	0.071	0.013
Parity	0.734	0.816	0.374	0.305	0.350	0.172
Delivery mode	0.005	0.004	0.638	0.093	0.026	0.008
Infant gender	0.248	0.743	0.286	0.271	0.173	0.117
Infant birthweight	0.990	0.891	0.865	0.335	0.132	0.907

Supplementary table 3. Qualitative comparison of dietary patterns of mothers from three cities. The values in this table are rough estimations of average proportion per food category, as consumed by lactating mothers one day before breast milk sampling

Food category	Qingdao	Wuhan	Hohhot
Grains and potato	19%	32%	25%
Vegetables	19%	26%	12%
Fruit	12%	7%	11%
Soybean	8%	2%	6%
Seafood	8%	8%	5%
Meat	16%	13%	14%
Dairy	8%	2%	9%
Eggs	12%	9%	17%