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Longitudinal Changes of Bioactive Proteins in Tibetan Human Milk and Their Association with Maternal and Infant Factors

Bingyan Li[a,b,c,1], Xiaomei Zhang[b,c,1], Yan Liu[b,c], Yaling Wang[b,c], Minghui Zhang[b,c], Junying Zhao[b,c], Lijun Chen[a,b,c,*]

[a] *Key Laboratory of Dairy Science, Ministry of Education, Food Science College, Northeast Agricultural University, Harbin, 150030, China.*

[b] *National Engineering Research Center of Dairy Health for Maternal and Child, Beijing Sanyuan Foods Co. Ltd., Beijing 100163, China.*

[c] *Beijing Engineering Research Center of Dairy, Beijing Technical Innovation Center of Human Milk Research, Beijing Sanyuan Foods Co. Ltd., Beijing 100163, China.*

ABSTRACT: Due to the unique and harsh geographical and climatic conditions at high altitudes, longitudinal studies on the composition of human milk (HM) in Tibet are important but scarce, particularly those investigating the dynamic changes of HM bioactive proteins. This study adopted a longitudinal study design to analyze the concentrations of immunoglobulin A (IgA), IgM, IgG, secretory IgA (SIgA), β-casein, κ-casein, and lactoferrin in HM from 33 Tibetan mothers at 2, 4, and 6 months after delivery using enzyme-linked immunosorbent assay. The influence of related maternal and infant factors on the contents was also analyzed. The results showed that the concentrations of SIgA and IgA were significantly higher at 6 months postpartum than those at 4 months postpartum (56.4 ± 6.3 mg/L vs. 46.4 ± 16.2 mg/L, P = 0.015; and 1769.8 ± 250.1 mg/L vs. 1220.4 ± 365.7 mg/L, P = 0.014, respectively), while other proteins showed no significant differences from 2 to 6 months. The increase of SIgA and IgA may be closely related to maternal immune adaptation and infants' exposure to the environment. The maternal immune system perceives pathogens in the environment and dynamically regulates the content of immunoglobulin in HM. Correlation analysis and generalized estimating equation analyses revealed that maternal occupation, maternal height, gestational weight gain, post-pregnancy body mass index, infant birth weight, and infant birth length influenced the levels of the seven HM bioactive proteins to varying degrees. These findings highlight the dynamic nature of HM bioactive proteins in Tibetan mothers and the impact of maternal and infant factors, offering insights for developing early nutrition strategies and infant formula tailored to the specific needs of Tibetan infants.

Keywords: Bioactive proteins, Infant factors, Maternal factors, Mature milk, Tibetan.

1. Introduction

Human milk (HM) is widely considered the best nutritional option for infants because of its rich content of essential nutrients that promote healthy growth and development[1]. The World Health Organization (WHO) recommends exclusive breastfeeding during the first 6 months of life, followed by breastfeeding plus complementary foods until 2 years of age or older[2, 3]. Breastfeeding not only supports infant growth and

[1] These authors have contributed equally to this work and share first authorship.
***Corresponding author**
chenlijun@sanyuan.com.cn (L. Chen).

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development but also has long-term advantages. Research indicates that it can reduce the incidence of cardiovascular diseases[4], protect against childhood obesity[5, 6], and decrease the risk of infectious and allergic diseases[7]. HM is rich in immunoglobulins, lactoferrin (LF), lysozyme (Lyz), and other immunological components that help prevent the invasion of pathogens and reduce the incidence of diarrhea and respiratory tract infections[8]. Additionally, breastfeeding not only strengthens the bond between mother and child but also promotes the infant's cognitive development and social adaptability, resulting in long-term benefits to mental health[9]. Breastfeeding also benefits the mother by lowering the risk of type 2 diabetes, hypertension, and metabolic syndrome, as well as improving postpartum recovery[10].

The nutrient composition in HM changes dynamically throughout lactation, particularly the protein composition, which differs significantly at various stages[11, 12]. Protein is essential for the development of an infant's body, providing not only the amino acids necessary for protein synthesis but also playing an important role in supporting the immune system and gut microbiota[13]. Proteomic studies have identified over 1577 proteins in HM[14]. HM protein is primarily composed of whey, casein, and trace milk fat globule membrane proteins[12, 15]. Casein mainly consists of β-casein, κ-casein, and $\alpha_{S\text{-}1}$-casein, while whey proteins predominantly contain α-lactalbumin, LF, and immunoglobulins[16]. β-casein enhances calcium absorption and is digested into bioactive peptides with immune-regulatory, antimicrobial, and hypotensive properties[17], whereas κ-casein has antioxidant and antimicrobial capabilities[18]. According to Jin et al. [19], LF participates in the body's immune response, promotes iron absorption in the gastrointestinal tract, and has several physiological functions, such as antioxidant, anticancer, anti-inflammatory, and antimicrobial properties. The main immunoglobulins in HM are immunoglobulin A (IgA), IgM, and IgG, with secretory IgA (SIgA) being the most abundant. SIgA specifically recognizes microorganisms in the digestive system and the mother's respiratory tract, neutralizes viruses, and inhibits the adhesion of pathogenic bacteria to host mucosa. IgA has been shown to enhance neonatal tolerance to microbial and food antigens and serves as an important link between adaptive and innate immunity[18]. IgM and IgG are involved in recognizing viral antigens and bacterial toxins, blocking viral invasion of host cells, and enhancing phagocytosis[20].

In contrast to the research from other locations, longitudinal studies on HM in China began relatively late, with most studies focusing on cross-sectional studies of HM nutrition. These studies primarily relied on retrospective data collection and have not sufficiently addressed dynamic changes in the nutritional composition of HM or the regional variability within China. Shigatse, Tibet, is located in the southwestern part of the Tibetan Plateau, with an average altitude of over 4,000 m. To adapt to the low-pressure, low-oxygen, and low-temperature environment at high altitude, the Tibetan people have formed unique physiological characteristics and dietary patterns through long-term genetic adaptation[21]. This unique climate characteristic and genetic adaptation mechanism can affect the physiological state of the mother (such as basal metabolic rate, fat mass, and body mass index), which in turn affects the composition of breast milk[22, 23]. Systematic reviews and meta-analyses have shown that long-term exposure to high altitudes alters physical function indicators as well as the composition of nutrients in breast milk[22]. Quinn et al. [23] showed that HM

composition is a key indicator of adaptive variation in response to high altitude environments. Although many studies have focused on the reproductive health and genetic adaptation mechanisms of high-altitude populations, research on the effects of biological variation on the composition of breast milk through chronological lactation stages is lacking[21-25].

This study examined the effects of altitude-related factors on the nutrient composition of HM and how maternal and infant characteristics contribute to these changes through a longitudinal cohort study of bioactive proteins in HM from Shigatse. This study fills the gap of previous longitudinal studies on HM composition in Chinese and global populations at different altitudes and provides an important basis for understanding the changing patterns of HM composition in high-altitude environments. The results of the study will not only help to develop personalized nutritional strategies and health management programs for Tibetan infants to improve their health but will also provide a reference for similar studies in other high-altitude regions in the future and promote the further development of HM protein research in high-altitude regions around the world.

2. Materials and Methods

2.1 Cohort study characteristics

This study recruited 50 mother-infant pairs from different areas of Shigatse, Tibet, between June 2018 and February 2019. Inclusion criteria included singleton pregnancy, vaginal deliveries, and healthy full-term newborns (gestational age ≥ 37 weeks). Exclusion criteria included pregnancy complications, such as preterm birth, gestational diabetes mellitus, hypertensive disorders of pregnancy, and congenital diseases. This study was sponsored by the National Engineering Center of Dairy for Maternal and Child Health and Beijing Sanyuan Food Co., Ltd., and was approved by the Ethics Committee of the Chinese Capital Institute of Pediatrics (approval number: SHERLL2014034). All procedures were performed in compliance with relevant laws and institutional guidelines. Written informed consent was obtained from all participants, and the privacy rights of the human subjects were observed.

Basic demographic and health information for mothers and infants was collected through questionnaires. This included maternal age, height, education, occupation, pre-pregnancy weight, pre-pregnancy BMI, post-pregnancy BMI, gestational weight gain, and infant characteristics such as gender, birth length, and birth weight. Maternal BMI was calculated as weight (kg) divided by height in m^2. In the longitudinal cohort, 33 mother-infant pairs completed three breast milk collections and provided complete baseline data. Of the remaining pairs, one infant had a cleft lip, two were preterm infants, and 14 had incomplete baseline information or breast milk collection.

2.2 HM collection

HM samples were collected at 2, 4, and 6 months postpartum. Participants were instructed to collect milk between 9 and 11 a.m. by expressing milk from one breast into a sterile bottle using an electric breast pump. The bottle was then inverted several times to manually mix the milk. Samples were stored at -20 ℃ for up to one week before being transferred to the laboratory via a cold chain and stored at -80 ℃ until analysis.

2.3 Analysis of HM protein

The levels of κ-casein, LF, β-casein, IgA, SIgA, IgM, and IgG in breast milk samples were determined using relevant commercial enzyme-linked immunosorbent assay (ELISA) kits (Cloud-Clone, Wuhan, China) following the manufacturer's instructions. HM samples were thawed and centrifuged (1000 × *g*, 20 min), and the skim milk under the fat layer was used for analysis. HM protein levels were determined based on a previously published study with modifications[26]. Breast milk protein concentrations are expressed as mg/L.

2.4 Statistical analysis

All data were entered into Excel, and statistical analysis was performed using SPSS 25.0 (IBM Co., Armonk, NY, USA). Data visualization was conducted using Origin 2021 (OriginLab Corporation, Northampton, MA, USA). The normality of data distribution was assessed using the Shapiro–Wilk test. Discrete variables are presented as numbers (n) and percentages (%), while continuous variables are medians and interquartile ranges [Q1; Q3]. The Kruskal–Wallis test was used to examine changes in bioactive proteins in HM at different time points during the lactation period of Tibetan mothers. Principal component analysis (PCA) was employed to evaluate the similarity of breast milk samples from different months, and hierarchical clustering was used to analyze the expression and classification of bioactive proteins. Spearman's correlation analysis assessed the correlation between maternal and infant factors and the bioactive proteins in HM. Based on the correlation analysis results, we introduced variables significantly correlated with the dependent variable into the generalized estimation equation (GEE). All p-values were two-sided, and statistical significance was set at $P < 0.05$.

3. Results and Discussion

3.1 Participant characteristics

The demographic characteristics of the study population are presented in Table 1. More than half of the participants had completed secondary school education, and the majority were employed in agriculture. The median maternal age was 27 years [24; 29]. All infants were delivered vaginally at full term. The median birth weight was 3000 g [2700; 3275], and the median birth length was 49 cm [48.0; 50.0]. Among the infants, 39.4% were boys and 60.6% were girls.

Table 1. Basic characteristics of the mothers and infants.

Mothers	
Education (n, %)	
Primary and below	14(42.4)
Junior high school	15 (45.5)
High school/technical secondary school and above	4 (12.1)
Maternal occupation (n, %)	
Non-agricultural	3 (9.1)
Agriculture	30 (90.9)
Maternal age (years)	27[24; 29]
Maternal height (m)	1.60 [1.58; 1.63]
MPPW (kg)	54.0 [52.0; 59.5]
MPPBMI (kg/m^2)	20.90 [20.35; 22.83]
MPBMI (kg/m^2)	21.87 [20.64; 23.34]

GWG (kg)	5 [4; 8]
Infants	
Infant gender (n, %)	
Male	13 (39.4)
Female	20 (60.6)
Birth length(cm)	49 [48; 50]
Birth weight(g)	3000 [2700; 3275]

Notes: Data are presented as numbers (percentages) or interquartile ranges. Abbreviations are defined as follows: MPPW (kg), maternal pre-pregnancy weight; MPPBMI (kg/m^2), maternal pre-pregnancy body mass index; MPBMI (kg/m^2), maternal post-pregnancy body mass index; and GWG (kg), gestational weight gain, which refers to the difference between postpartum and pre-pregnancy weight.

3.2 Characteristics of bioactive proteins in HM

This study determined total protein and true protein levels in breast milk at 2, 4, and 6 months postpartum. The results showed that there was no significant difference between each time point (measurement methods and data are shown in Supplementary Table S2), suggesting a relative stability in breast milk protein levels during this period. The study subsequently examined fluctuations in the levels of seven bioactive proteins in breast milk (IgA, IgM, IgG, SIgA, β-casein, κ-casein, and lactoferrin) at 2, 4, and 6 months postpartum.

The dynamic trends of the concentrations of the aforementioned seven HM bioactive proteins from 2–6 months postpartum are shown in Fig. 1. The concentrations of IgA and SIgA at 6 months postpartum were significantly higher than those observed at 4 months postpartum (IgA, $P = 0.014$; SIgA, $P = 0.015$). However, the levels of other bioactive proteins in breast milk showed no significant changes over this period. Specific values for the variations in HM protein concentration are shown in Supplementary Table S1. Notably, the concentrations of immunoglobulins (IgA, SIgA, IgM, and IgG) were lowest at 4 months postpartum.

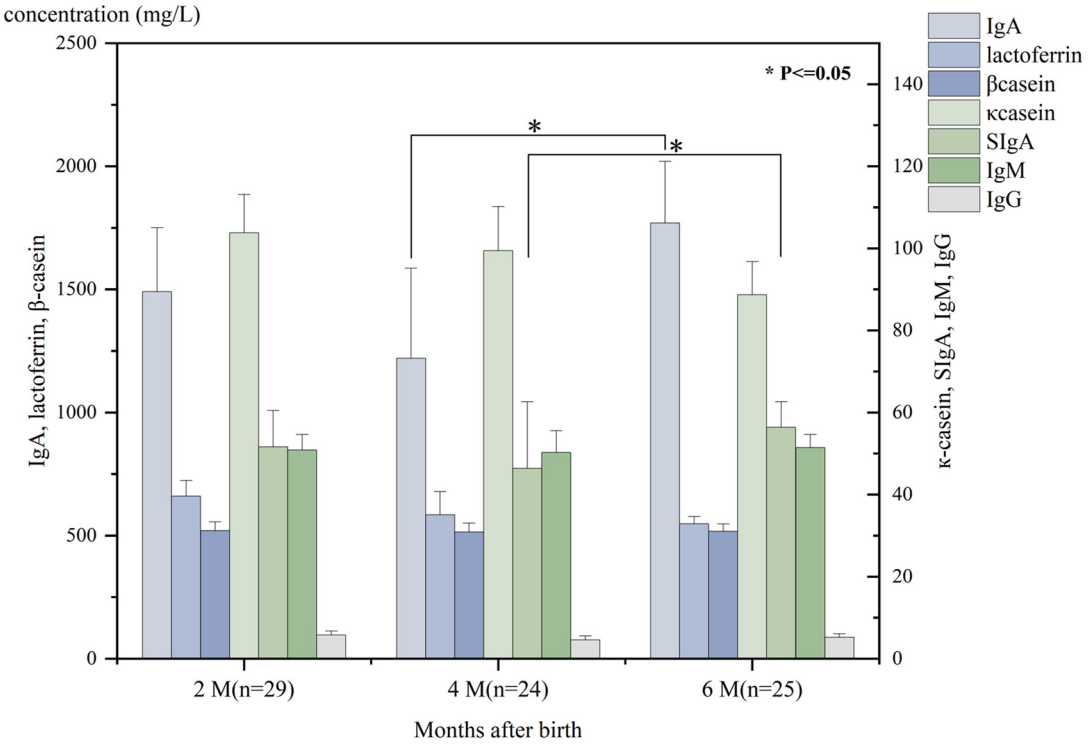

Figure 1. Trends in breast milk protein component in Tibetan mothers during the first 6 months postpartum. 2 M = 2 months, 4 M =4 months, 6 M =6 months. * $P < 0.05$.

Previous studies have identified the major whey proteins in breast milk as α-lactalbumin, LF, osteopontin, immunoglobulins, Lyz, and growth factors, while casein proteins include κ-casein, β-casein, and $\alpha_{s\text{-}1}$ casein[27, 28]. This study examined κ-casein, β-casein, and several whey proteins. Among the major whey proteins, those with immune functions include immunoglobulins and LF. Recent studies indicate that infants have a high demand for immunoglobulins during the colostrum stage due to insufficient innate and acquired immunity, making them highly susceptible to pathogenic bacteria and other harmful factors. However, as the infant's immune system matures, the protective role of the mother's immune system diminishes, and the immunoglobulin content in breast milk decreases accordingly[29-31]. In this study, the concentrations of SIgA and IgA in the breast milk of Tibetan mothers initially decreased and then increased during the mature milk stage. SIgA and IgA can respond to various microbial antigens, preventing the invasion of pathogenic microorganisms in the infant's intestines and respiratory tracts[29]. Colostrum contains high levels of SIgA and IgA, which activate the infant's immune system to defend against foreign microorganisms. As the infant's immune system develops, SIgA and IgA levels in breast milk gradually decrease. However, in the mature milk stage, the rebound in the levels of SIgA and IgA may be related to the infant's exposure to exogenous antigens (such as complementary foods)[19], suggesting a potential immunomodulatory mechanism of mother and infant. No significant differences were observed in IgM and IgG levels during different lactation stages (IgM, $P = 0.284$; IgG, $P = 0.573$), consistent with previous studies[30, 32]. LF is an important immunobiologically active component of breast milk that protects infants from infections and helps regulate their inflammatory responses[33]. This study, along with other studies, observed a non-significant downward trend in LF levels in mature milk (LF, $P = 0.308$)[34, 35]. This trend may be attributed to the immaturity of the immune system of infants at birth, which necessitates higher levels of anti-inflammatory and antimicrobial active proteins. As their immune system matures, the LF levels in breast milk tend to decrease gradually[33].

Many studies have observed longitudinal changes in total casein levels, but few have examined different types of casein separately. In this study, we found a non-significant decreasing trend in κ-casein levels and no significant change in β-casein levels during the mature milk stage (κ-casein, $P = 0.543$; β-casein, $P = 0.976$), consistent with findings from other studies[32]. Additionally, the contents of some proteins in HM from Tibetan mothers were lower than the levels reported in the literature[36, 37]. Affolter et al.[38] reported that the protein content of HM proteins IgG and LF of lactating mothers at the mature milk stage (1–2, 2–4, and 4–8 months) in three cities of Beijing, Suzhou, and Guangzhou, China, was about 2–6 times higher than that of the Tibetan population during 2–6 months in this study. Liu et al. [39] reported on breast milk protein composition in Qingdao, Wuhan, and Hohhot, finding that average contents of κ-casein, β-casein, and LF in mature milk (6 weeks postpartum) were 23.1, 422.7, and 132.8 mg/100 mL, respectively, which were all higher than the values found in this study. One possible explanation is that HM protein levels vary across regions and ethnic groups in China[40]. Tibetans have developed unique genetic adaptations to extreme living conditions[40, 41]. Another explanation is dietary differences, as Tibetan mothers, during lactation, tend to follow a traditional

Tibetan diet, which includes zanba, buttered tea, and high-fat broth-based drinks. This diet is characterized by a higher carbohydrate intake and lower protein intake[42].

Differences in HM proteins are observed not only among individuals but also across different lactation stages[43]. Fig. 2 shows the percentages of the seven HM bioactive proteins at specific times. IgA, LF, and β-casein were the most abundant proteins at various times, collectively accounting for over 90% of the total relative proportion. Compared to 2 months postpartum, the proportion of IgA decreased by 3.27%, LF increased by 0.29%, and β-casein increased by 2.38% at 4 months postpartum. At 6 months postpartum, the proportion of IgA increased by 9.85%, while LF and β-casein proportions decreased by 5.15% and 3.39%, respectively, compared to 4 months postpartum.

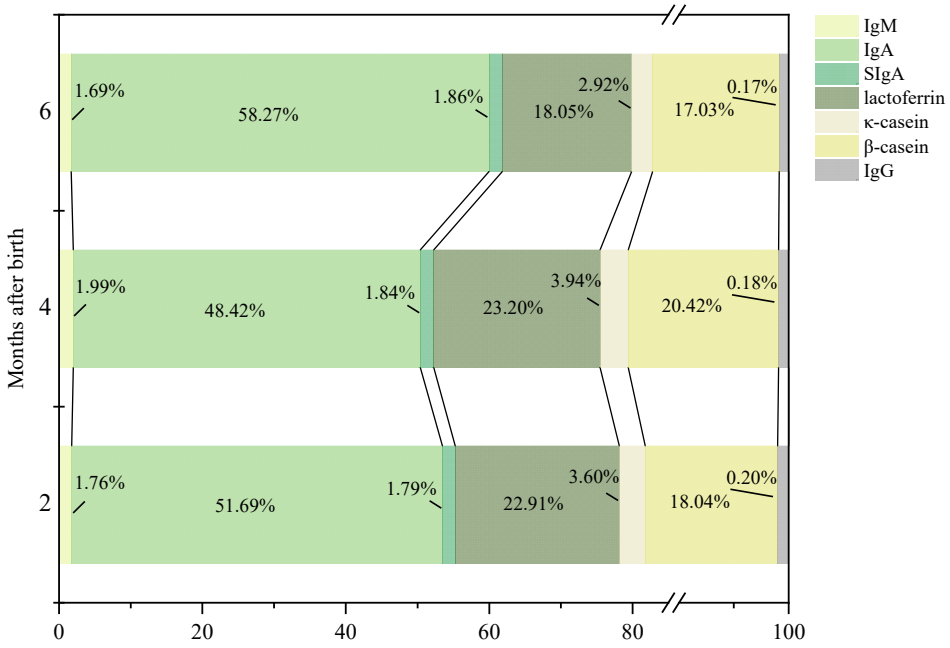

Figure 2. Comparison of the percentage concentration of bioactive proteins in human milk.

Furthermore, the ratio of whey proteins to casein decreases continuously during lactation, stabilizing at approximately 60:40[37]. However, this conclusion could not be drawn in the present study because we did not systematically analyze the entire HM proteome but rather focused on selected proteins. The level of β-casein was significantly higher than that of κ-casein. IgA and SIgA are the major immunoglobulins in the HM immunoglobulinome, with relatively low proportions of IgM and IgG[44, 45]. During the lactation period between colostrum day 3 and week 24, the percentages of κ-casein, LF, IgA, and IgM decreased, whereas that of β-casein increased, and IgG remained essentially unchanged[30]. In contrast, in the present study, protein abundance remained essentially unchanged, except for the proportions of IgA, β-casein, and LF, which showed partial changes during lactation (2–6 months), all of which were < 10%. These differences in bioactive protein levels between studies may be due to variations in the lactation stage[30, 31, 46].

According to the findings of this study, Tibetan breast milk had "stable total protein levels with significant changes in bioactive proteins" during the research period. This is consistent with lactation characteristics; that is, the total protein content of breast milk gradually stabilizes in the late lactation phase, but the composition of breast milk will continue to change to satisfy the needs of infant growth and

development[12, 47]. Previous studies have reported an initial decrease in the concentration of HM proteins (LF, SIgA, IgG, IgA, and Lyz) from colostrum to mature milk, followed by a steady increase during late lactation or weaning[48-51]. These studies typically had longer sample collection periods, analyzing longitudinal changes in milk protein concentrations over 1 to 2 years postpartum. However, differences in the population characteristics, geographic regions, and sample sizes across studies led to varying results regarding the dynamics of HM protein concentrations.

This study found a statistically significant 10 mg/L increase in breast milk SIgA levels between 2 and 6 months postpartum, with an effect size analysis indicating a moderate effect. Previous research has established that breast milk SIgA helps to maintain infant intestinal homeostasis by modulating the infant gut microbiota. Xi et al. [52], in a longitudinal study of a Chinese mother-infant cohort, found that breast milk SIgA at 42 days postpartum promotes colonization of the beneficial gut bacterium *Veillonella parvula* in infants while simultaneously suppressing long-term colonization by the harmful bacterium *Erysipelatoclostridium ramosum*. The physiological effects of breast milk SIgA are not only determined by concentration; its role in regulating the microbiota and inhibiting pathogenic bacteria is more in line with the developmental needs of the infant gut[53]. As a result, the physiological significance of such variations in SIgA levels can be evaluated in future studies using applicable controlled animal or clinical models.

3.3 Discrepancy analyses of bioactive proteins in HM at different lactation stages

PCA and hierarchical clustering were used to assess differences of the seven bioactive proteins in breast milk from Tibetan mothers at 2, 4, and 6 months postpartum. As shown in Fig. 3A, the total variance contribution rate of the first (PC1) and second (PC2) principal components was 68.5%, of which PC1 explained 49.2% and PC2 19.3%. Few outliers were observed outside the 95% confidence ellipse interval at all postpartum time points, confirming the reliability of the PCA. Further examination of the principal component characteristics showed that PC1 was characterized by LF, SIgA, IgA, and IgM, all of which had a positive correlation. PC2 was characterized by β-casein, κ-casein, and IgG, and all except κ-casein showed a negative correlation. The sample distribution shows that the composition of bioactive proteins in HM at 4 months postpartum differs from that at 2 and 6 months postpartum. The 4-month postpartum samples show partial separation from the other two groups; however, the 2- and 6-month HM samples show no significant separation. This phenomenon directly confirms the unique bioactive protein composition in HM at 4 months postpartum. Meanwhile, the compositions of HM samples from the three time points partially overlap, indicating a certain stability in the overall composition of the seven bioactive proteins, which corresponds to the fact that the total protein content of breast milk did not differ significantly from 2 to 6 months postpartum (Supplementary Table S2). It also suggests that the changes in the composition of bioactive proteins in HM at 4 months postpartum are particular temporal variations within a stable general framework.

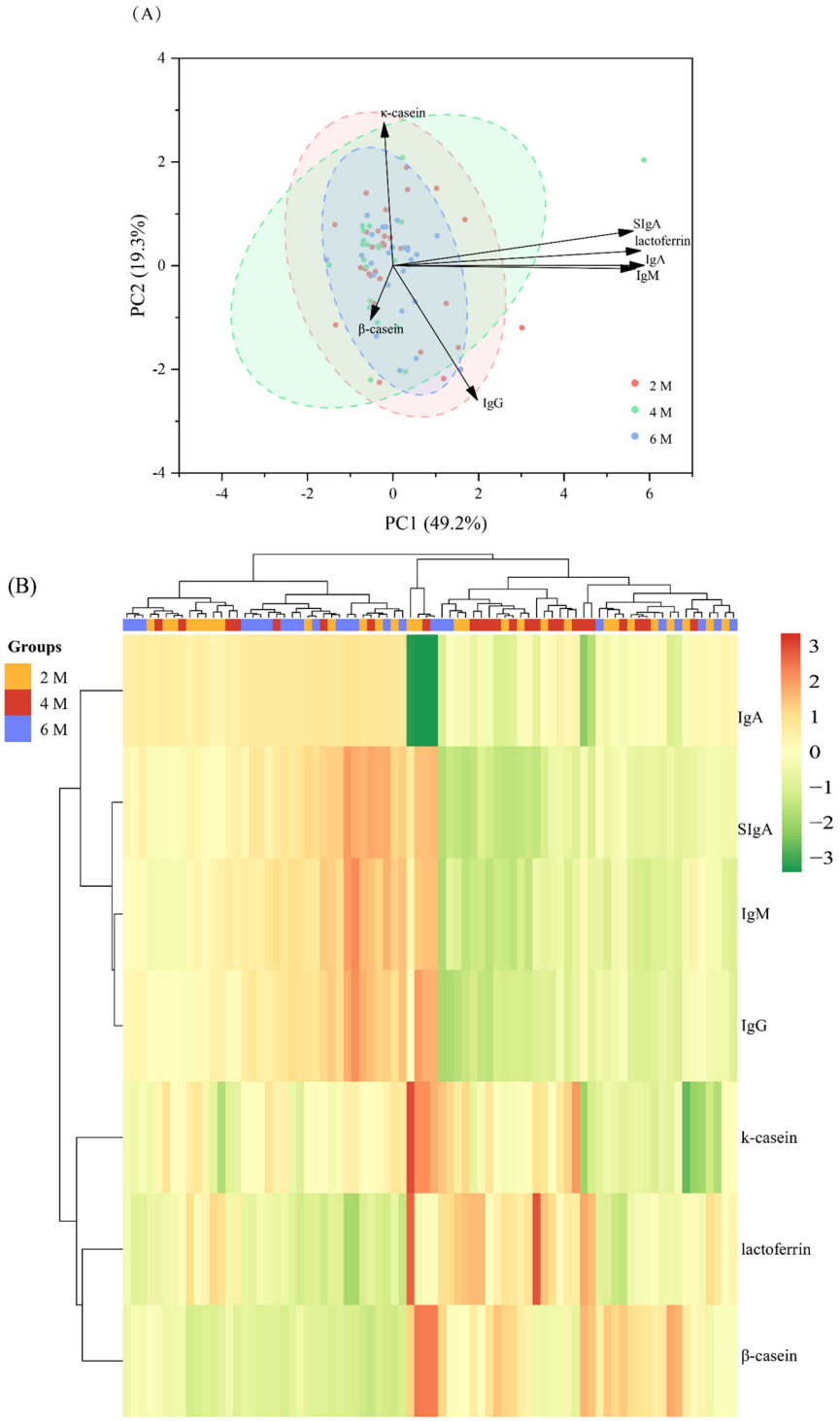

Figure 3. Principal component analysis (A) and hierarchical cluster analysis (B) of the bioactive protein composition in Tibetan human milk at 2, 4, and 6 months postpartum. 2 M = 2 months, 4 M =4 months, 6 M =6 months.

Hierarchical clustering was used to enhance visualization and verify this difference. Figure 3B shows that the seven bioactive proteins of HM were divided into two main clusters; one cluster consists of HM immunoglobulins (SIgA, IgA, IgG, and IgM), while the other includes β-casein, κ-casein, and LF. The results of the hierarchical clustering generally corresponded to the protein trends presented in Fig. 1, with immunoglobulins showing a consistent expression trend from 2 to 6 months of lactation, high levels at 2 and 6 months postpartum, and a significant decrease at 4 months. The expression of κ-casein and LF showed a general decreasing trend, while the expression of β-casein remained relatively constant. While most breast

milk samples from 4 months formed a distinct sub-cluster, samples from 2 and 6 months were intermixed, reflecting no significant difference in clustering patterns across these times.

The concentration of bioactive proteins in breast milk often fluctuates with the progression of lactation [31, 48]. The differential analysis of seven bioactive proteins in HM at 2, 4, and 6 months revealed stage-specific differences in bioactive proteins at 4 months postpartum, demonstrating that HM continues to be a dynamic source of these proteins throughout the mature milk stage. SIgA and IgA levels in breast milk were considerably reduced at 4 months postpartum compared to those at 6 months. This finding demonstrates that HM is a dynamic source of bioactive proteins for infants from 2 to 6 months postpartum. Furthermore, our findings highlight the critical importance of sample collection timing in longitudinal breast milk cohort studies, as significant stage-specific changes in bioactive proteins persist even in the mature milk phase. However, the precise regulatory mechanisms underlying these changes are still not well understood.

Currently, research on longitudinal changes in breast milk proteins during lactation mainly focuses on colostrum, transitional milk, and mature milk[40, 54, 55], with limited studies examining changes in breast milk composition during late lactation. Most studies have demonstrated that the protein profile of colostrum differs significantly from that of transitional and mature milk[40, 56, 57]. The composition of breast milk is dynamic and evolves with the duration of lactation[18]. After birth, the immune system of the infant is underdeveloped. Colostrum supplies essential antibodies to protect the baby from pathogens. As lactation progresses, the protein content in transitional milk gradually decreases while fat and lactose levels increase to meet the infant's growing nutritional needs. By the mature milk stage, the nutrient composition stabilizes, with protein proportions appropriately balanced[55]. This study examined the bioactive protein fractions in mature breast milk and found that bioactive protein composition in the breast milk varied at three different time points.

3.4 Influence of maternal and infant factors on the seven bioactive proteins in HM

As shown in Fig. 4, the Spearman correlation analysis showed that, except for maternal education, MPPW, MPP BMI, and infant gender, the seven bioactive proteins in the HM of Tibetan mothers were associated with other maternal and infant factors. κ-casein was negatively correlated with maternal occupation ($P = 0.001$), maternal height ($P = 0.027$), infant birth weight ($P = 0.002$), and infant birth length ($P = 0.033$). β-casein was negatively correlated with maternal age ($P = 0.025$) and positively correlated with GWG ($P = 0.016$). IgA levels were positively correlated with MPBMI ($P = 0.006$) and infant birth length ($P = 0.016$). LF levels were negatively correlated with maternal occupation ($P = 0.006$) and positively correlated with infant birth length ($P = 0.009$). IgG levels were positively correlated with maternal height ($P < 0.001$), GWG ($P = 0.022$), infant birth weight ($P < 0.001$), and infant birth length ($P < 0.001$). SIgA and IgM levels were negatively and positively correlated with infant birth weight ($P = 0.023$) and infant birth length ($P < 0.001$), respectively.

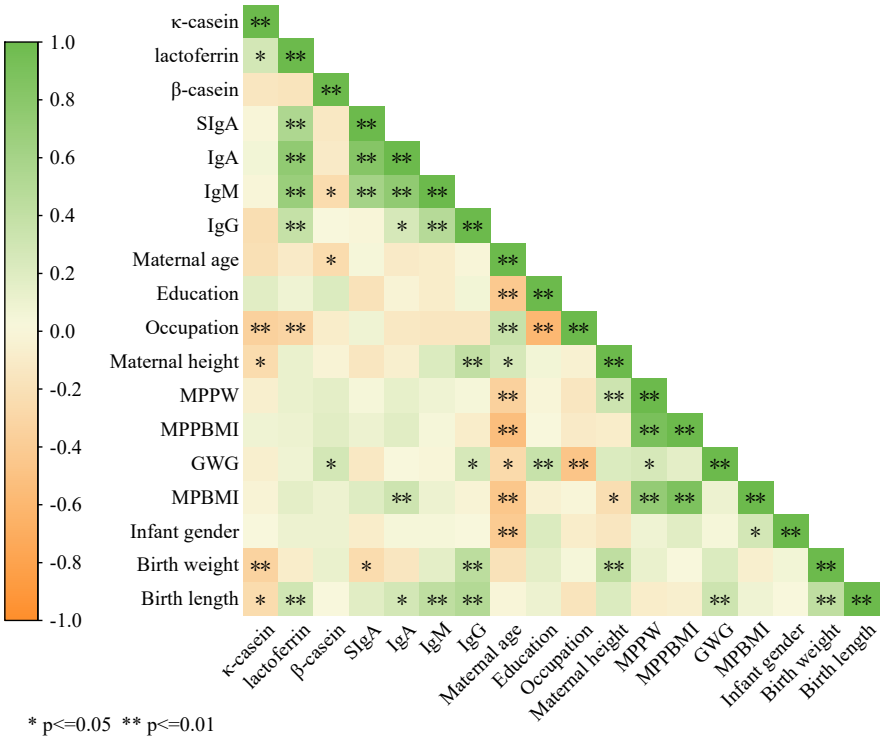

Figure 4. Correlation between maternal and infant factors and bioactive proteins in human milk. * p≤0.05， ** p≤0.01. The color intensity in each square is proportional to the correlation coefficient, which was calculated using Spearman correlation. Abbreviation: MPPW (kg), maternal pre-pregnancy weight; MPPBMI (kg/m^2), maternal pre-pregnancy body mass index; MPBMI (kg/m^2), maternal post-pregnancy body mass index; GWG (kg), gestational weight gain, defined as the difference between postpartum weight and pre-pregnancy weight.

GEE is used for further multifactorial analyses. The variables with $p \leq 0.05$ in the correlation analysis were selected as candidate parameters. The variance inflation factor (VIF) was used to assess collinearity, and all VIF values were less than 5. In the correlation analyses, no significant correlation was found between infant gender and the seven bioactive proteins of HM. However, according to relevant literature, infant gender is an important factor influencing the concentration of bioactive proteins; therefore, it was still included in the GEE model. The candidate parameters entered into the GEE model were maternal occupation, infant gender, maternal age, maternal height, GWG, MPBMI, infant birth weight, and infant birth length.

After controlling for relevant covariates, maternal occupation, height, GWG, MPBMI, infant birth weight, and birth length remained the main factors influencing bioactive proteins in the HM of Tibetan mothers. The results of the GEEs are shown in Table 2. Based on Spearman correlation analysis and GEE double validation, this study screened and explored the key maternal and infant factors significantly related to breast milk protein content.

Table 2. Generalized estimating equation analysis.

		κ-casein		lactoferrin		β-casein		SIgA		IgA		IgM		IgG	
		β	p	β	p	β	p	β	p	β	p	β	p	β	p
Maternal occupation	Agriculture	-77.854	**<0.001***	-189.503	**0.016***	51.554	0.473	7.706	0.429	183.629	0.6	-12.281	0.079	-0.729	0.682
	Non-agricultural	Reference		Reference		Reference		Reference		Reference		Reference		Reference	
Infant gender	Female	-5.061	0.634	145.717	0.073	13.476	0.829	7.363	0.645	341.464	0.311	4.224	0.281	0.669	0.552
	Male	Reference		Reference		Reference		Reference		Reference		Reference		Reference	
Maternal age		-2.185	0.052	1.069	0.894	-4.918	0.491	0.269	0.87	-19.268	0.55	-0.146	0.79	0.158	0.3
Maternal height		-146.384	0.441	2993.315	**<0.001***	-174.932	0.84	254.824	0.07	9122.318	**0.011***	208.033	**<0.001***	45.205	**0.005***
GWG		-6.51	0.117	-35.048	**0.014***	26.757	0.106	-5.536	**<0.001***	-88.323	0.163	-3.943	**0.018***	0.162	0.673
MPBMI		-3.331	0.408	19.136	0.213	-5.014	0.75	4.128	0.165	160.89	**0.03***	2.871	**0.022***	0.546	0.074
Birth weight		-0.016	0.085	-0.186	**0.033***	-0.012	0.882	-0.037	**0.038***	-0.891	**0.013***	-0.005	0.283	0.002	0.216
Birth length		-1.265	0.392	19.729	**0.014***	-4.636	0.585	2.351	**0.028***	101.663	**<0.001***	1.807	**0.006***	0.43	**0.007***

Notes: MPBMI, maternal post-pregnancy body mass index; GWG, gestational weight gain, defined as the difference between postpartum and pre-pregnancy weight. * $P < 0.05$.

As previously mentioned, the anthropometric characteristics of mothers and infant births are related to the bioactive protein components in HM. After accounting for other variables in the GEE model, we found that maternal occupation influenced κ-casein and LF levels in breast milk. Mothers with non-agricultural occupations had higher levels than mothers with agricultural occupations. This disparity may be associated with occupation-related dietary differences. Non-agricultural working mothers, in particular, have a higher level of education and family income, and they are more likely to have a balanced diet (see Supplementary Table S3 for detailed dietary data across occupational groups). Dietary nutritional status regulates the synthesis and secretion of bioactive proteins in breast milk[58, 59]. In the present study, no significant association was found between MPPW and MPPBMI and HM protein; however, a significant positive correlation was observed between MPBMI and IgA levels, which is consistent with the findings of previous studies[60]. Liang et al. [61] found that the effect of maternal BMI on HM protein content may be mediated through fat mass rather than directly. The underlying mechanism may be related to increased oxidative stress due to maternal fat accumulation during pregnancy, which subsequently triggers metabolic changes and affects HM composition[62, 63]. Furthermore, the present study found that maternal height showed a significant positive correlation with IgG content, which differed from the results of the study on perinatal factors and HM composition by Hascoët et al. [64]. The results of the existing studies indicate that some interactions exist between maternal height and HM protein composition, but the exact mechanism of action is still unclear.

Some studies have found no association between the longitudinal changes in total HM protein content or individual protein components and infant birth weight or birth length[46, 65]. In contrast, our study found that some HM protein components remained associated with infant birth anthropometric characteristics despite confounding factors. For example, SIgA was negatively correlated with birth weight, with higher birth weight associated with lower SIgA levels in breast milk. Additionally, IgA, IgG, IgM, and LF levels were positively correlated with birth length; longer birth lengths were associated with higher levels of these proteins in breast milk. Hahn et al. reported that fat content, moisture content, and calories in breast milk correlate with infant birth length and birth weight[66], while Galante et al. [65] reported a positive correlation between adiponectin levels in breast milk and infant birth weight. Further studies are needed to explore the association between breast milk protein levels and infant growth outcomes. HM protein levels are not influenced by maternal age when multiple factors are considered. However, Rigourd et al. [67] found that higher maternal age was associated with lower breast milk protein concentrations in a multivariate analysis. This study found no correlation between HM protein levels and infant gender, which aligns with previous studies[32, 68, 69]. However, infant gender affects HM protein content, particularly in the early stages of lactation, with this effect disappearing in later stages[39]. Therefore, the impact of maternal age and infant gender on breast milk protein remains controversial, and further research is needed to investigate the underlying mechanisms behind the association between milk protein and these factors[45, 54].

Previous studies have shown that the protein composition of breast milk is affected by different maternal and infant factors; however, their results are inconsistent. In low-altitude areas, infant gender, gestational age,

maternal occupation, education level, and maternal BMI are important influencing factors of breast milk protein content[39, 46, 54, 70, 71], whereas infant birth length and weight are not significantly associated with breast milk protein content[46, 47]. In contrast, studies at high altitudes are limited, and the conclusions are mixed. For example, Schafrank et al. [25] showed that maternal BMI, waist-to-hip ratio, and infant age significantly affect the protein composition of breast milk in a study of a population in the Peruvian Andes, while Quinn et al. [23] found no correlation between maternal and infant factors and breast milk protein in a high-altitude population in the Nubri Valley of Nepal. This study found that the birth length and weight of infants have a significant impact on the composition of breast milk protein through the analysis of the population in Tibet. This finding may reflect the different regulatory effects of the altitude environment on the relationship between maternal and infant factors and breast milk composition. Future studies are needed to further explore the mechanisms by which maternal and infant factors affect the composition of breast milk in different high-altitude environments.

While this study provides valuable insights into the dynamic changes of certain bioactive proteins in breast milk of Tibetan mothers, some limitations must be acknowledged. First, the limited sample size may restrict the generalizability of the findings. Additionally, the focus on Tibetan mothers may not represent all ethnic groups in China or beyond. The observational design precludes the establishment of causal relationships, and further controlled mechanical research is required to confirm the observed associations. This study failed to consider the potential seasonal variation and altitudinal heterogeneity of the Shigatse region, which may have implications for the measurement of HM protein. These limitations highlight the need for larger, more diverse cohorts and longitudinal studies to better understand the factors influencing breast milk protein levels across regions and ethnic groups.

4. Conclusions

This study investigated the dynamic changes in seven key bioactive proteins in HM from 2 to 6 months of lactation, as well as the influence of maternal and infant factors. SIgA and IgA levels showed significant fluctuations, while other proteins exhibited minor variability. Maternal occupation, height, MPBMI, and infant birth weight and length were identified as key influencing factors. These findings emphasize the interplay between maternal and infant characteristics and milk composition, offering a valuable reference for the understanding of lactation dynamics among Tibetan mothers. Future research should aim to increase sample sizes, investigate other parameters, and study diverse populations to further elucidate the nutritional composition of HM.

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Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have influenced the work reported in this study.

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