

Research Article

Profile of Twenty-Three Human Milk Oligosaccharides in Han Chinese Mothers throughout Postpartum 1 year

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Human milk oligosaccharides (HMOs) are multifunctional carbohydrates in breast milk, which are composed by a variety of structures. This study aimed to identify HMOs concentration profile, milk microbiota composition, and the associations with major maternal characteristics in Han Chinese mothers in the one-year lactation period. Seventeen healthy mothers aged from 28 to 36 years, who gave birth to healthy term infants, were recruited. Carbohydrates were detected using the MIRIS human milk analyzer (HMA), and twenty-three HMOs were quantified using ultra-performance liquid chromatography-triple quadrupole mass spectrometry (UPLC-MS). Results showed that carbohydrates were relatively stable, while total HMO concentrations ranged from 1.74 to 9.72 g/L and decreased gradually over lactation in breast milk. Based on the structure, seven sialylated HMOs concentration showed the significant decline ($p < 0.05$) after three months in lactation. In addition, the relationships between maternal factors, containing the lactation period, genetic status, delivery mode, parity, and milk microbiota profile, and the HMO composition in healthy women, which still need further investigations, were observed.

1. Introduction

Human breast milk is considered to be the only natural source food for infants, which contains a wide variety of nutritive fractions as carbohydrates, lipids, and proteins, which are suitable for infants with immature digestive and immune systems. The carbohydrates in human milk include not only lactose but also oligosaccharides, of which more than 150 structures have been described. Human milk oligosaccharides (HMOs) are the third most abundant component in human milk after lactose and fat and play a prominent role in infant nutrition [1]. Although not degradable by the infant's digestive system, HMOs could regulate the intestinal microbial flora, restrain against pathogenic bacteria, and promote the development of the immune system [2–5]. By way of exception, a few HMO with the terminal β 1,4-linked Gal may be cleaved off and absorbed to the systemic circulation [6, 7], indicating that

the potential effects extend to tissues and organs other than the intestine.

The structure of HMOs contains five monosaccharides: glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc), and N-acetylneuraminic acid (Neu5Ac); among of them, lactose (composed of Glc and Gal) could be elongated by β 1,3 linkages to lacto-N-biose or by β 1,6 linkages to GlcNAc and modified by fucose or sialic acid residues. So according to the modified moieties, they could be classified as neutral fucosylated HMO (about 35%–50% of the total HMOs content), neutral nonfucosylated HMO (about 42%–55% of the total HMOs content), and acidic HMO (about 12%–14% of the total HMOs content), with acidic ones commonly being present at a 10-fold lower concentration than total neutral HMOs [8, 9]. Beside the different sizes, structures, and functions of HMOs, its combination and amount present in human milk might be distinguished in four major types depending on the genetic

profile of the mother [10, 11]. Those are determined by the fucosyltransferases (FUTs) encoded by the secretor (Se) and Lewis (Le) genes in the mammary gland. FUT2 is encoded by Se, which could catalyze fucose residue to lactose or the elongated HMO chain in an α 1,2-linkage. On the other hand, FUT3 is the crucial enzyme in the Lewis blood group system, which determines the synthesis of α 1,4-fucosylated oligosaccharides [12, 13].

According to the complex data reported in the past 1960–2020, mainly from Europe and America, the amount of total HMOs was 17.7 ± 3.3 g/L at colostrum, 13.3 ± 6.5 g/L at transitional milk, and 11.3 ± 2.62 g/L at mature milk [14, 15]. Although the composition of total HMOs follows a basic rule and keeps relatively constant in the same mother during lactation, the distribution of HMOs in different individuals varies greatly, which might be influenced by geographical environments, genetics, and physiological status [16–19]. The research findings from Binia et al. [16] and Erney et al. [17] displayed significant differences in oligosaccharide profiles between different regions which might be attributed to evolutionary-driven genetic differences between the inhabitants of the different countries. In the same way, the research data on HMO profiles have been different from China to European and American countries [20–23]. Based on that, in the present study, 23 HMOs in human milk of Han Chinese from Hebei province were simultaneously quantified to fill up the limited data from China out of abundant geogen.

2. Materials and Methods

2.1. Sample. Seventeen healthy Han Chinese mothers who gave birth to healthy term infants were recruited in the Shijiazhuang region. The study was conducted according to the guidelines in the Declaration of Helsinki. Human milk collection was approved by the Medical Ethics Committee of Hebei Medical University, and written informed consent was obtained from all subjects participating in the study. All 24 samples were generally collected by an electric pump in the morning between 09:00 and 11:00 to avoid circadian influence. A single full breast was emptied, and an aliquot of 40 mL for mature milk was secured for characterization purposes. Each sample was distributed in 5 mL tubes and stored at -80°C until used.

2.2. Carbohydrates and HMOs Analysis. Samples with volume of 3 mL were thawed by warming the milk to 40°C and then analyzed carbohydrates using the MIRIS human milk analyzer (HMA) (Miris, Sweden) after homogenization. At the same time, 1 mL of milk samples was centrifuged at 7000 rpm for 10 min at 4°C to remove lipid. Another centrifugation after adding ethanol was carried out to remove protein, and obtained supernatant was used for analysis by the ultra-performance liquid chromatography-triple quadrupole mass spectrometry (UPLC-MS) (Waters, MA, USA) system [24]. All used commercial chemicals were chromatographic grade and above for LC/MS requirements. The concentration of each oligosaccharide in collected milk

samples was determined according to the standard curve. Prior to this, the standards were divided into two groups, high content group (2'FL, 3FL, 3'SL, 6'SL, LNT, LNnT, LDFL, LNFP I, LNFP II, LNDFH I, and DSLNT) and low content group (LNFP III, LSTb, LSTc, LNnDFH I, LNnDFH II, LNDFH II, 3'SLNFP II+6'SLNFP VI, MFLNnH, MFLNH III, DFpLNnH, and DFLNHa), according to their content in the sample based on previous research data. The milk sample was injected twice for analysis, with $1\ \mu\text{L}$ standard in the high content group and $3\ \mu\text{L}$ standard in low one, respectively.

The human milks were distributed to their mother's secretor status and Lewis blood group phenotypes based on the previous report [24]. The levels of 2'FL, LNFP I, and LNFP II were assignment criteria, in which the sample of secretor mother with an active Se locus (Se^+) was abundant in 2'FL and LNFP I, besides the milk of Lewis positive status (Le^+) was plentiful in LNFP II. To sum up, human milk was assigned to their mother's Se^+Le^+ status using 2'FL, LNFP I, and LNFP II, while the first two oligosaccharides exclusively qualified the Se status, and LNFP II could be used as reference to the inactivation of FUT3 in the Se^-Le^- group.

2.3. Microbiota Analysis. The genomic DNA was extracted from human milk using an Invitrogen™ PureLink Microbiome DNA Purification Kit (Thermo Fisher Scientific, MA, USA). The samples were centrifuged at 13,000 rpm at 4°C for 20 min to remove fat, and the supernatant was stored for total DNA extraction. Samples were sequenced following amplification of V3-V4 hypervariable regions of the 16S rRNA gene on a MiSeq sequencing platform (Illumina, CA, USA) as previously described [25]. High-quality sequences no less than 97% were clustered into operational taxonomic units (OTUs), while the composition and abundance of taxonomy were mainly operated using QIIME and R packages.

2.4. Statistical Analysis. The concentrations of carbohydrates and HMOs were analyzed using SPSS v.22. For statistical analysis, Student's *t*-test for independent samples and one-way analysis of variance (ANOVA) were used. The significance level was set at *p* value of ≤ 0.05 . Spearman rank correlations were used to validate associations between HMOs concentrations and maternal secretor status and Lewis blood group phenotypes, lactation time, delivery mode, and parity. Microbiota composition was analyzed using R Studio v.1.4.1106, and raw OTU sequence counts were subjected to cumulative sum scaling normalisation to account for the differences in sequencing depth across the samples. In addition, taxa with less than 0.01% relative abundance across all samples were excluded.

3. Results

3.1. Characteristics of the Participants. The sociodemographic and demographic characteristics of all the participants are given in Table 1. The age of mothers ranged from 28 years to 36 years (30.63 ± 2.31 years). All of them were of the ethnic Han. There were nine of seventeen mothers who

TABLE 1: Characteristics of mothers.

	N	Mean	SD
Age (years)		30.63	2.31
BMI after delivery (kg/m ²)		24.11	2.18
Ethnicity			
Han	17		
Others	0		
Residence			
Urban district	9		
Suburban district	8		
Education level			
Senior high school	1		
College or university	15		
Masters	1		
Parity			
Primipara	9		
Pluripara	8		
Mode of delivery			
Vaginal	8		
Caesarean	9		
Smoking history	0		
Gestational age			
Full term (≥ 37 weeks)	17		
Preterm (< 37 weeks)	0		

had only one child. In total, eight mothers had vaginal delivery and others underwent caesarean delivery.

3.2. Carbohydrates and Total HMOs Concentrations. The carbohydrates and total HMOs concentrations in Han Chinese mature milk were investigated in eleven months during the lactation period. Measurable levels of carbohydrates ranged from 61.80 to 75.00 g/L over one year (Figure 1(a)). Total HMO concentrations in mother's milk, as a main sum of all individual HMOs, ranged from 1.74 to 9.72 g/L over lactation. The 23 HMOs identified in this study represent almost all of the oligosaccharides present in human milk. Human mature milk in first three months contained higher levels of total HMOs than the late lactation period from 4 months ($p < 0.05$). Fourteen oligosaccharides were classified in the neutral fucosylated HMO group, two in neutral the nonfucosylated HMO group, and seven in the acidic HMO group. On further analysis of the levels of these three groups (Figure 1(b)), only the acidic HMO group concentration showed the significant decline ($p < 0.05$) after three months in lactation; meanwhile, the level of the neutral fucosylated HMO group had a downtrend.

3.3. HMOs Concentration in Secretors versus Nonsecretors. In this study, twelve milk samples had the levels of 2'FL and LNFP I below the method LOQ (0.49 $\mu\text{g}/\text{mL}$ and 0.50 $\mu\text{g}/\text{mL}$, respectively), which were grouped in nonsecretor (Se^-) lacking a functional FUT2 enzyme. Then, compared to the Se^+ group, significantly higher concentrations of 3FL, LNT, LNFP II, LSTb, LNnDFH II, DSLNT, and 3'SLNFP II and 6'SLNFP VI were found in the Se^- group (Figure 2). In contrast, LDFT concentration in the Se^+ group was significantly abundant than Se^- one. Besides 2'FL and LNFP I,

levels of other 1,2-fucosylated HMOs such as LNnDFH I, LNDFH I, and DFLNHa, were undetectable in almost all of the Se^- samples. Furthermore, two samples in the Se^- group lacked LNFP II belonging to $\text{Se}^- \text{Le}^-$ status; the others were all assigned to Le^+ phenotype. Based on these described, their mothers' SeLe status was divided to the $\text{Se}^+ \text{Le}^+$ group ($n = 9$), $\text{Se}^- \text{Le}^+$ group ($n = 7$), and $\text{Se}^- \text{Le}^-$ group ($n = 1$).

3.4. Analysis of HMOs with Different Characteristics of Mothers. To further investigate the relations between individual HMOs and differential explanatory variables, which contains lactation time, birth parity, delivery mode, secretor status, and Lewis blood group phenotypes of mothers, the correlations were calculated with significant results visualized as a heat map in Figure 3. The heatmap clearly divided all HMOs into two clusters: one cluster with eight oligosaccharides was designated A, containing (α 1-2)-linked fucosylated, certain nonfucosylated and acidic HMOs (2'FL, LDFL, LNFP I, LNDFH I, LNnT, LNnDFH I, DFLNHa, and 3'SL), and fifteen in cluster B that contain devoid of (α 1-2)-linked fucosylated and the majority of acidic HMOs (3FL, LNFP II/III, LNnDFH II, LNDFH II, LNT, MFLNnH, MFLNH III, DFpLNnH, 6'SL, LSTb/c, DSLNT, 3'SLNFP II, and 6'SLNFP VI). Visible differences could be observed between the two clusters; the oligosaccharides in cluster B showed significant correlation with both the time after delivery and genetic type of mother, while cluster A only showed considerable relevance with genetic type (calculated values in Supplementary 1). This observation implies cross-correlation between the specific structures of complex glycans and status of lactation time and genetic type. In particular, there was an unexpected finding that three of 23 oligosaccharides (2'FL, MFLNH III, and LSTb) were also

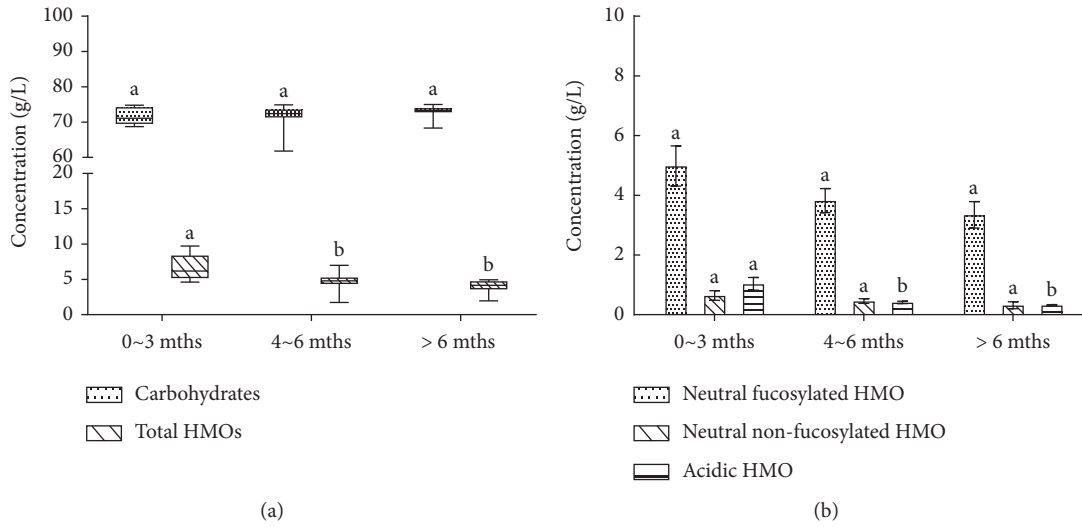


FIGURE 1: (a) Concentrations of carbohydrates and total HMOs in Han Chinese mature milk over lactation. Error bars indicate the min and max values, respectively. (b) Concentrations of HMOs grouped by structure in Han Chinese mature milk over lactation. Error bars indicate the standard deviation. Different alphabet letters indicate different carbohydrates and total HMOs concentrations between different groups in lactation (one-way ANOVA, $p < 0.05$).

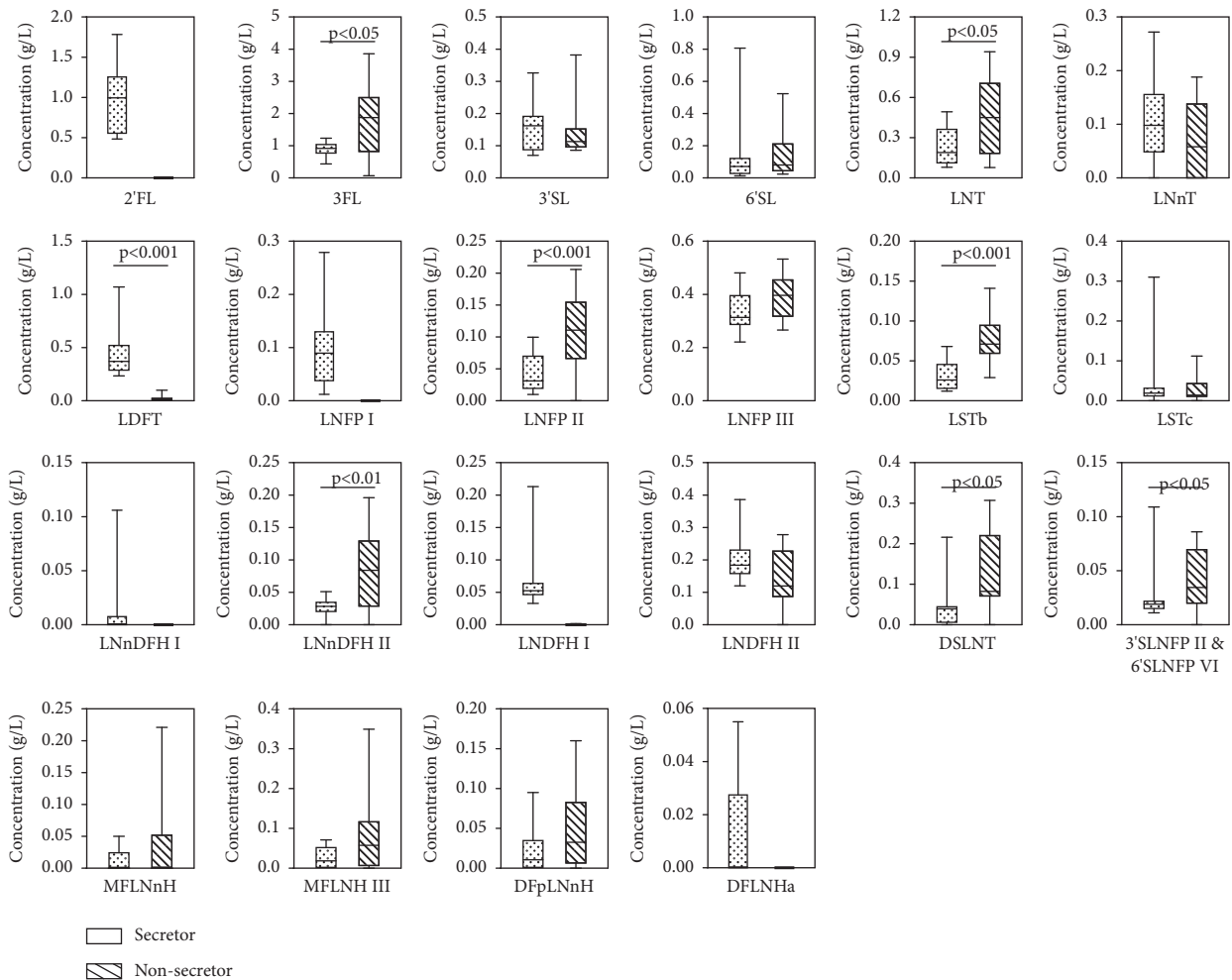


FIGURE 2: HMOs concentrations in both the secretor group ($n = 12$) and nonsecretor group ($n = 12$) in mature milk during lactation. There were no 2'FL, LNFP (I), LNnDFH (I), LNDFH I, and DFLNHa in almost all of the nonsecretor samples. Levels of 3FL, LNT, LNFP II, LSTb, LNnDFH II, DSLNT, 3'SLNFP II and 6' SLNFP VI, and LDFT show a significant difference between the secretor group and nonsecretor group. Error bars indicate the min and max values.

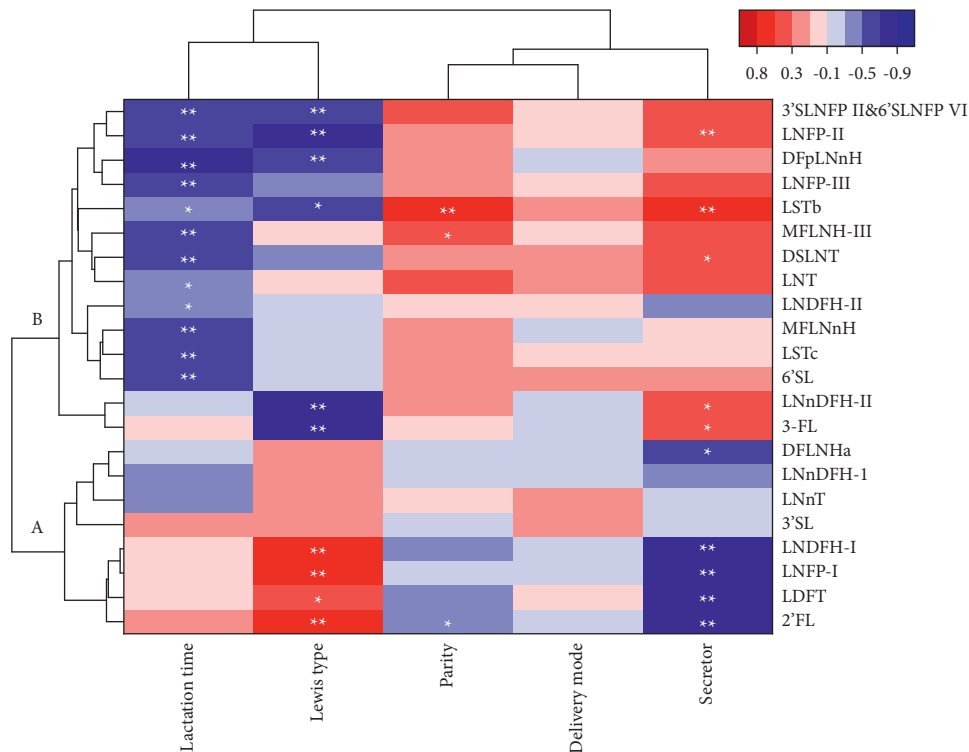


FIGURE 3: Heat map of relative relation between the levels of HMOs composition and differential explanatory variables with respect to lactation time, birth parity, delivery mode, secretor, and Lewis status of mothers. The colors range from blue to red represents the correlation coefficient from -1 to $+1$. * $P < 0.05$; ** $P < 0.01$.

closely related to the maternal birth parity, which is highly worth of further analysis.

3.5. Analysis of Microbiota with Different Characteristics of Mothers. In this section, milk microbiome composition and abundance were examined based on the same factors mention (Figure 4), including lactation time, birth parity, delivery mode, and secretor status of mothers. In total, 42 phyla were identified from all samples, of which, *Proteobacteria* ($85.42\% \pm 13.08\%$, range $43.99\%–98.99\%$) followed by *Firmicutes* ($7.31\% \pm 11.12\%$, range $0.03\%–50.17\%$) and *Bacteroidetes* ($5.30\% \pm 6.74\%$, range $0.14\%–32.38\%$) dominated the milk microbiota. At the phylum level, *Proteobacteria* ($p < 0.01$) and *Actinobacteria* ($p < 0.01$) revealed the significant differences in milk from mothers with delivery mode between vaginal and caesarean; the same goes for *Cyanobacteria* ($p < 0.05$) in different secretor status. On further analysis based on these findings, significant differences were also observed in genus level with respect to delivery mode, including *Cupriavidus* ($p < 0.01$), *Acinetobacter* ($p < 0.05$), *Stenotrophomonas* ($p < 0.05$), and *Brevibacterium* ($p < 0.05$). The constrained redundancy analysis (RDA) was used to visualize and investigate potential intricate connections between milk community composition and the multiple variables, exhibiting that the lactation time, birth parity, delivery mode, and secretor status of mothers playing the strong effects of the microbiota.

Abundance correlations between twenty-three HMOs and major milk microbiota were also visualized in a

heatmap, as shown in Figure 5, and the distinctive correlations were observed. Interestingly, the oligosaccharides dependent on $(\alpha 1-2)$ -linked fucosylation, containing 2'FL, LDFT, LNFP I, LNDFH I, and DFLNHa clustered into the same branch, were negatively correlated with *Firmicutes* and positively correlated with almost other phyla. In milk microbiota, only *Proteobacteria* was clustered into branch B, which showed the positive relationship with $(\alpha 1-2)$ -linked fucosylated HMOs and negative with some of other fucosylated HMOs, such as 3FL, LNFP II, LNnDFH II, LNDFH II, 3'SLNFP II, 6'SLNFP VI, and DFpLNnH. Apart from that, abundance levels between these oligosaccharides also displayed different correlations (data in Supplementary 2).

4. Discussion

The research data in our study showed that concentrations of HMOs are highly dynamical between mothers and evaluate five fixed and modifiable factors associated with variation. Total HMO content and variation were consistent with previous results showing the decline over the course of lactation, especially the sialylated oligosaccharides, which was inconformity with Azad et al. [19]. However, 3'SL concentration was consistently higher during lactation as same as they found and might be related to the requirements for growth, immunity, and neurodevelopment in infancy. Between maternal Se^+ and Se^- genes, the levels of most oligosaccharides are in good agreement with those of Wang et al. and Wu et al. [22, 26] who observed the similar presence in other Chinese human milk. To date, the

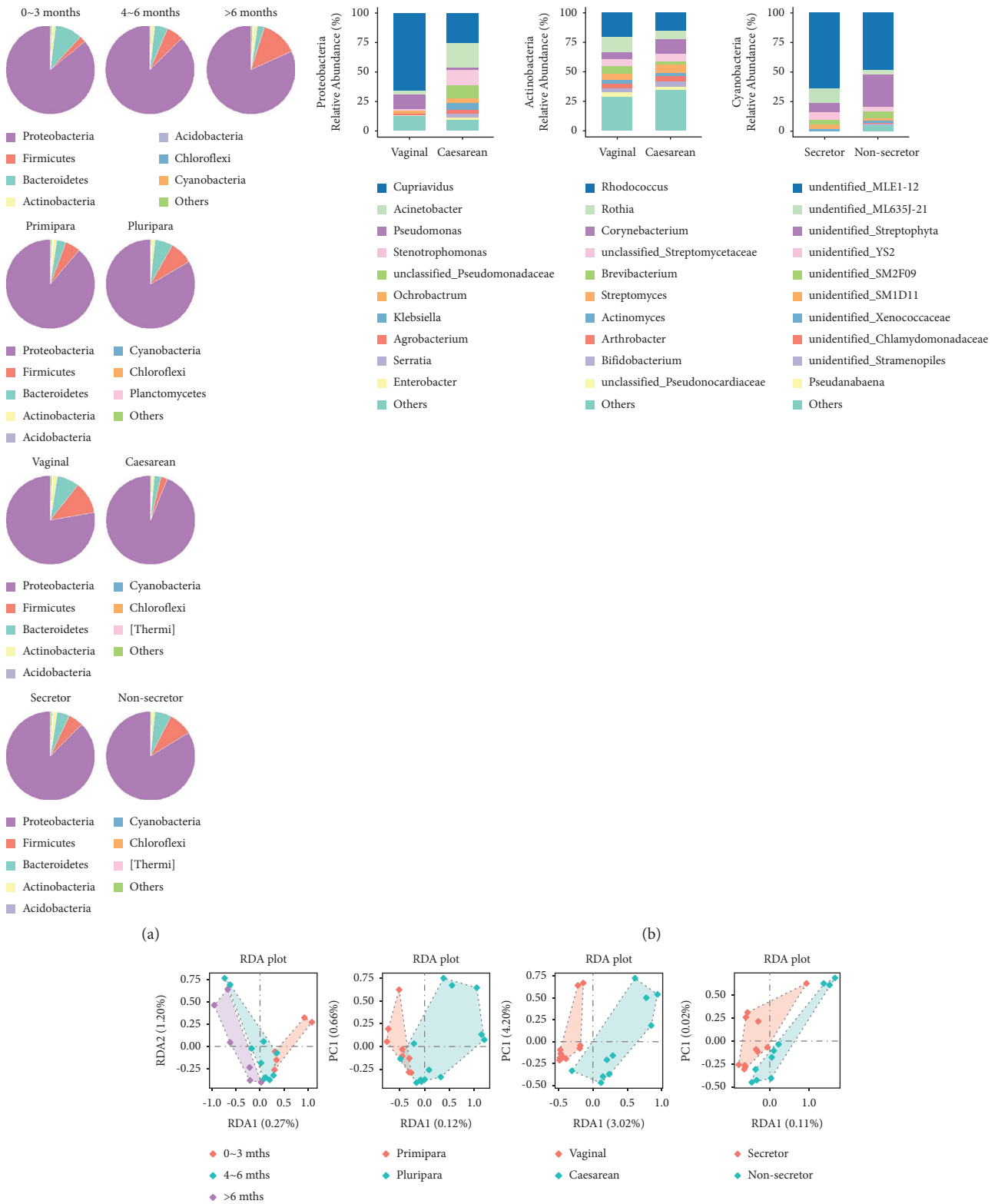


FIGURE 4: (a) Pie charts showing the relative abundance of the human milk microbiota at phylum level in four factors, including lactation time, birth parity, delivery mode, and secretor status of mothers. (b) Histogram showing distribution of genus level in three phyla, which contains *Proteobacteria*, *Actinobacteria*, and *Cyanobacteria*, in delivery mode and secretor status of mothers. (c) RDA showing all samples separate based on lactation time, birth parity, delivery mode, and secretor status of mothers (with total OUT values).

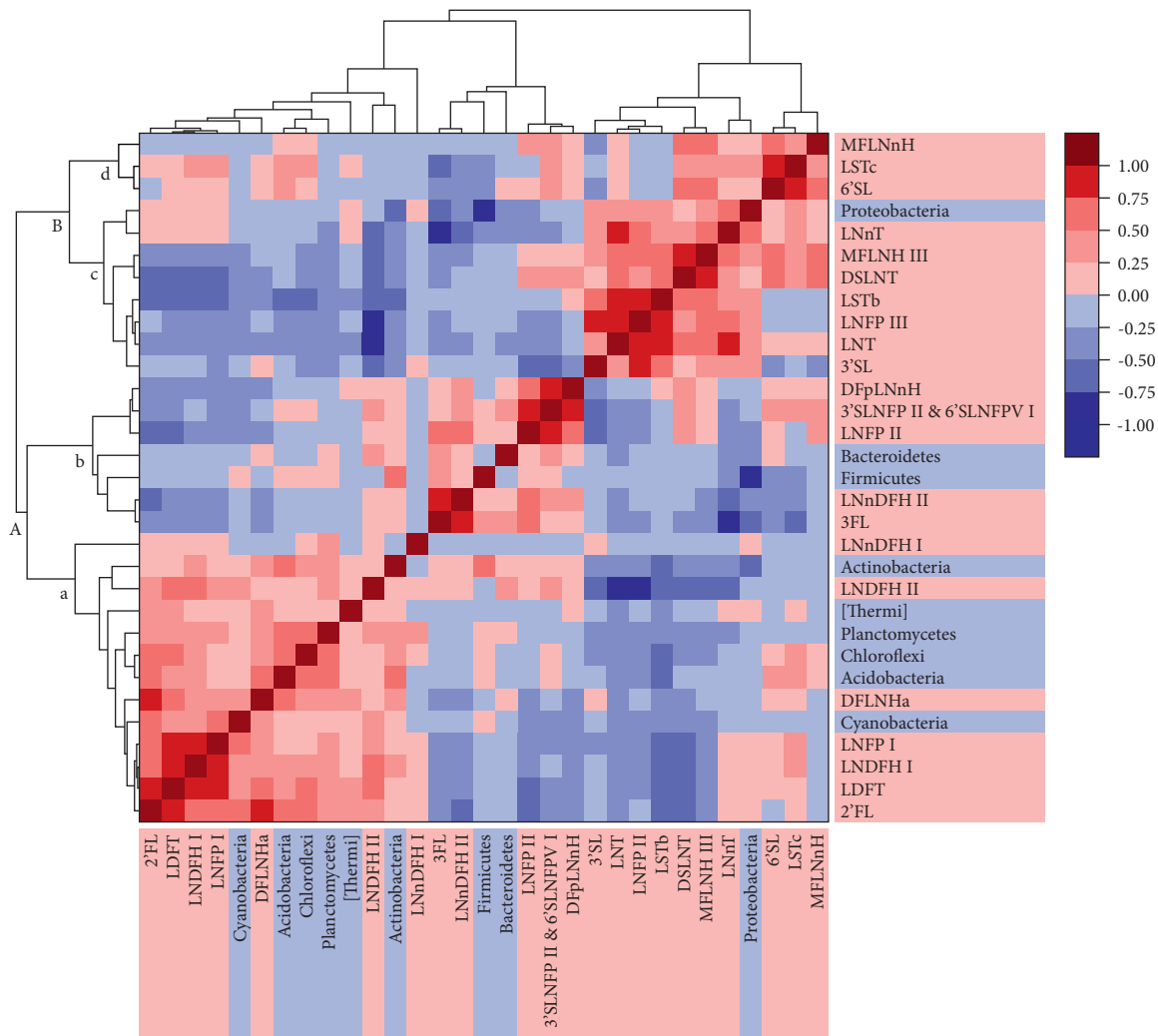


FIGURE 5: Inter and intracorrelations between HMOs and the phylum level of milk microbiota by the way of clustering heat map based on Pearson correlation. The colors range from blue to red representing the correlation coefficient from -1 to +1.

proportion of secretor status and Lewis blood group phenotypes varies among different ethnic populations, such as the Se⁺ group varies at 87–98% in Latin Americans [27], 73–91% in both Europeans and North Americans [18, 19, 21], 65–81% in Africans [28], and 77–82% in Chinese mothers [20, 22, 26], approximately.

Although the structures of HMOs were mainly dependent to maternal genetics, Se and Le genes, other factors such as lactation time and parity even cause different oligosaccharides profiles in these data and delivery mode exclusivity. Interestingly, in the multivariable analysis, parity was seemingly associated with the levels of three of twenty-three HMOs, which have few studies aiming at their association, without agreement. Except no associations mentioned by Elwakiel et al. [21], Samuel et al. [18] observed higher levels of LNnT in primiparous mothers, and in contrast, Azad et al. and Tonon et al. [19, 29] found higher LNT and LNnT and lower 3FL concentrations in multiparous mothers with or without secretor status. In Chinese mothers, Wang et al. [26] found a negative correlation between parity and other HMOs in colostrum, containing LDFT, LNFP I, LNFP IV,

LNDFH I, LNDFH II, and DfPpLNH I. Whereas, in this study, we only observed the potential relationships in both parity and 2'FL, LSTb, and MFLNH III, which were also mentioned by Ferreira and her colleagues [27]. In addition to this, some studies also observed the association between parity and the macronutrient content in human milk, such as lipid, protein, and carbohydrate, although the data remain inconsistent [30–32]. The hypothesis was supposed that there might be structural or functional changes in the mammary gland related to successive pregnancies, which may lead to changes in human milk nutrient contents [33, 34].

Moreover, milk microbiota and its potential interactions with HMOs and other maternal factors were analyzed. Compared with lactation time and birth parity, delivery mode and secretor status were identified to be significantly associated with the dynamic changes of microbiota. *Bifidobacterium* was predominant in the intestinal tract of breastfed infants via vertically transferred from mothers as previously reported [35, 36], while the abundance amount in human milk samples in our study was relatively few, which

was consistent with the findings from Moossavi et al. [37]. They supposed that bifidobacteria are presumably more prevalent in the early stages, and the origin of bacteria in milk is unclear. It was also known that HMOs could be utilized by some bacteria, for instance, *Bifidobacterium*, *Bacteroides*, and *Lactobacillus* [38, 39]. In our data, the correlations were relatively different between HMOs and microbiota phylum in both positive and negative directions. *Firmicutes* and *Bacteroidetes* were negatively correlated with the most of (α 1-2)-linked fucosylated HMOs suggesting the different HMOs consumption characteristics of these bacteria. However, it is indeed unknown whether these HMOs could be metabolized by milk microbiota due to the large amounts of lactose was the more accessible carbon source. Notably, the data of Han Chinese milk in this study was a small group, which may limit conclusions. It still needs further investigation for filling up more meaningful data to try to understand the biological relevance of these maternal factors.

5. Conclusions

In conclusion, our study supplemented the regional data on twenty-three kinds of HMOs composition and milk microbiota profile from Han Chinese, as well as the associations of different nonmodifiable and modifiable factors and the concentrations of specific HMOs. The results showed decreased levels of total HMOs during the prolonged lactation period, ranged from 1.74 to 9.72 g/L, especially acidic ones. In addition, more maternal factors including genetics, parity, and microbiota have relationship with individual HMOs profile which need further anticipated research studies.

Data Availability

The data used to support the findings of this study are included within the article and are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplementary Table 1. Values of the relevance relationship between HMOs and five maternal factors. Supplementary Table 2. Values of abundance levels in both HMOs and milk microbiota. (*Supplementary Materials*)

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