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Original Article

Breastfeeding premature infants affects the microbiota composition of breast milk

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Abstract: Aim: To determine whether and how breast feeding of premature infants influences the human milk (HM) bacterial communities. Methods: HM samples before and after breastfeeding were collected from 40 preterm infant mothers at 24-36$^{6/7}$ weeks of gestational age in the neonatal intensive care unit of our hospital. Of these 40 babies, 11 at 24-27$^{6/7}$ weeks of gestational age and 12 at 28-31$^{6/7}$ weeks were grouped into an extremely premature (EPM) group and a very premature (VPM) group, respectively. In addition, 11 with a birth weight (BWT) of 1000 g ≤ BWT < 1500 g were classified as a very low birth weight (VLBW) group and 12 with BWT < 1000 g an extremely low birth weight (ELBW) group. Breast feeding and kangaroo mother care were given once a day for 7 days, from 14 to 21 days of age. The bacterial composition of HM was analyzed using high-throughput sequencing before and after feeding. Results: Linear discriminant analysis effect size of HM samples before and after feeding showed that *Bacillus*, *Prevotella* and *Fusobacterium* were significantly enriched in HM before breastfeeding ($P < 0.05$). Post-feeding HM for the EPM group showed significant enrichment in *Lactobacillales*, *Streptococcus*, *Desulfuromonadales*, *Ruminococcus*, *Geobacteraceae*, *Geobacter* and *Elizabethkingia_meningoseptica* ($P < 0.05$). *Bacillus* was significantly enriched in the HM for EPM group before feeding ($P < 0.05$). For mothers with VLBW infants, *Bacillus* was enriched before feeding, while *Lactobacillales* was predominant after feeding ($P < 0.05$). There was a moderate correlation between the diversity of HM bacteria and infant development and immune outcomes. Conclusion: Breastfeeding of preterm infants can significantly affect the bacterial diversity in HM.

Keywords: Human milk, breastfeeding, preterm infants, microbiota

Introduction

Intestinal microbial colonization in neonates plays an important role in shaping the immune system and developing the gastrointestinal tract [1-3]. This colonization is influenced by maternal factors from pregnancy through the early neonatal period, and it is affected by the mode of delivery and feeding methods [4-6]. Human milk (HM) is one of the most important sources of gut microbiota for infants, with its microbial composition influenced by various factors, such as environmental conditions, maternal diet, living habits, and the general health and weight of the mother [7]. Studies have shown that the HM microbiota may be derived from the bacteria in maternal intestinal tract [8] and skin [6], as well as the infants' oral bacteria [9, 10]. Thus, understanding the mechanisms and factors influencing the bacterial composition in HM is of great significance to the development of neonates and infants.

Preterm birth is associated with increased risks of mortality and morbidity in infants. Therefore, HM and breastfeeding are strongly recommended for preterm infants due to their recognized health benefits [11]. HM consumption is linked to lower rates of neonatal morbidities in preterm infants and improved long-term metabolic and neurocognitive outcomes [12]. HM microbiota is also essential in infants' intestinal microbiome development, providing protective effects against gut immaturity in preterm infants [13]. It has been well established that there are significant differences in the intestinal microbiome composition and quantity between preterm and full-term infants [14], potentially due to differences in HM and oral microbiota. Factors such as mechanical ventila-

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tion can affect the oral microbiota of preterm infants, and the changes in oral microbiota may in turn, influence the HM microbiota during breastfeeding [10]. However, there is limited research on the relationship between HM microbiota and breastfeeding in preterm infants.

In this study, we used high-throughput sequencing data to explore how breastfeeding affects HM microbiota and the how different gestational ages and birth weights (BWT) influence the diversity and composition of HM microbiota in preterm infants.

Materials and methods

Subjects and samples

This study included 40 premature infants and their mothers who were hospitalized in the neonatal intensive care unit of Guangxi Zhuang Autonomous Region Maternal and Child Health Hospital from January 2019 to December 2020. Inclusion criteria: 1) Preterm infants with gestational age at 24-27$^{6/7}$ weeks and BWT less than 2500 g. 2) Infants born in the hospital, transferred to the neonatology department for treatment, and hospitalized for more than 3 weeks after birth. 3) Infants that had been liberated from the invasive ventilators. 4) Infants receiving proactive breastfeeding from their mothers. Exclusion criteria: 1) Infants with either congenital inherited metabolic diseases (e.g., cyanotic congenital heart disease) or developmental malformations (e.g., digestive tract malformations). 2) Infants undergoing surgical treatments during the study period. 3) Infants with unstable breathing or circulation requiring to be rescued by medical intervention. 4) Infants whose mothers had an infection during the perinatal period and needed systemic antibiotics. 5) Infants whose mothers were unable to breastfeed due to either maternal illness or medication.

Each preterm infant was cared for in the neonatal intensive care unit for the first two weeks post-delivery and initially fed with infant formula. From 14 to 21 days of age, they received kangaroo mother care (KMC) and breastfeeding once a day for 7 days. The room temperature for KMC was maintained at 26-28°C. Bathed mothers in loose clothing were seated in a leaning position on a sofa inclined at 60°. Each infant, wearing only a diaper, lay prone on the mother's chest to ensure maximum skin-to-skin contact. Breastfeeding sessions lasted for 0.5 hours each time. During breastfeeding, transcutaneous oxygen saturation and heart rate were monitored to ensure they remained within normal ranges. HM samples were collected from each mother twice: the first on day 14 post-delivery (before KMC and breastfeeding, preBF) and the second on day 21 (after KMC and breastfeeding, postBF). HM was collected through pumping, ensuring complete extraction each time. The milk was transported to the hospital in containers kept at 2-5°C. A 4 ml sample of the collected HM was placed in a disposable sterile tube and stored in a -80°C freezer within 2 hours of collection.

Methods

DNA extraction and high-throughput DNA sequencing: Guangzhou Magigene Biotechnology Co., Ltd. was commissioned to perform the bacterial DNA extraction and sequencing. The bacterial genomic DNA from the HM samples was extracted using the Bacterial DNA Extraction Mini Kit (Guangzhou Maibao Biotechnology Co., Ltd.). The quality of the bacterial DNA was visualized using 1% agarose gel electrophoresis, and its concentration and purity were measured using a NanoDrop spectrophotometer.

The genomic DNA served as a template to amplify the V4 region of the 16S rRNA gene using primers 515F/806R. Gene Tools Analysis Software (Version 4.03.05.0, SynGene) was used to compare the concentrations of the PCR products, which were then mixed according to the principle of equal quality. The EZNA Gel Extraction Kit was employed to recover the mixed PCR products. Target DNA fragments were recovered, and DNA libraries were constructed following the standard procedure for the NEB Next Ultra DNA Library Prep Kit (Illumina). Sequencing was conducted on the Illumina Nova 6000 high-throughput sequencing platform, with an average depth of 60,000 reads per sample.

Bioinformatics analysis: Fastp (https://github.com/OPenGene/fastp) was used for sliding window trimming of the paired-end raw reads. The raw reads were processed to remove primers and spliced using usearch-fastq_merge-

pairs (https://www.drive5.com/usearch). Fastp was then employed to process the raw tags to obtain clean tags. Then, the clean tags were clustered using UPARSE, generating operational taxonomic units (OTUs) [15]. Next, these OTUs were compared and annotated with the SILVA database [16] using usearch-sintax. A rarefaction curve was used to assess the adequacy of the sample size. Based on OTU data, the relative abundance of different groups at each taxonomic level (phylum, class, order, family, genus, species, etc.) was calculated using R software. Relative abundance distribution maps were generated for groups with a relative abundance above 0.01% and within the top 15 using the R package ggplot2. Chao1 and Simpson indices were calculated using usearch-alpha_div to describe community richness and diversity, respectively. For beta diversity analysis, the Bray-Curtis distance algorithm in the R package Vegan and principal coordinate analysis were used. A Linear discriminant analysis threshold of 3 was set, and the linear discriminant analysis effect size was used to identify differential abundance groups.

Outcomes

The major outcomes were HM bacterial composition of preterm infants' mothers before and after breast feeding and HM microbiota diversity indicators, including the alpha and beta diversity of HM microbiota. The secondary outcomes were the development and immunity outcomes of preterm infants, including changes in weight, length, and the incidence of feeding intolerance before and after breastfeeding.

Statistical analysis

SPSS (version 25.0) was used for statistical analysis. Data were tested for normality using the Shapiro-Wilk test. Normally distributed clinical data were expressed as means ± standard deviations ($\bar{x} \pm s$), and independent samples t-test or One-Way Analysis of Variance (ANOVA) was used for comparisons between groups. Non-normally distributed data were expressed as the P_{50} (P_{25}-P_{75}), and the Mann-Whitney U test or Kruskal-Wallis H test was used for comparisons between groups. Enumerated data were expressed as percentages, and the comparisons between groups were performed by either the chi-square test or Fisher's exact tests. $P < 0.05$ was considered statistically significant.

Results

Clinical data of enrolled subjects

A total of 40 preterm infants and their mothers were selected to study the changes in HM bacterial composition before and after breastfeeding. Among the infants, 19 were naturally delivered and 21 were delivered via cesarean section. There were 20 male babies and 20 female babies, with BWT of 1368.80±467.00 g and gestational age of 30.65±3.61 weeks. HM samples were collected at day 14 (before breastfeeding) and day 21 (after breastfeeding). However, the bacterial DNA extracted from 27 HM samples (11 preBF samples and 16 postBF samples) were discarded because they did not meet the sequencing requirements.

Of these 40 babies, 11 at 24-27$^{6/7}$ weeks of gestational age and 12 at 28-31$^{6/7}$ weeks of gestational age were grouped into an extremely premature (EPM) group and a very premature (VPM) group, respectively. In addition, 11 babies with a 1000 g ≤ BWT < 1500 g were classified as a very low birth weight (VLBW) group and 12 with BWT < 1000 g as an extremely low birth weight (ELBW) group. The clinical manifestations and grouping of the selected infants are shown in **Table 1**.

Alpha diversity analysis

There were no statistically significant differences in microbial abundance and diversity between the HM samples before and after breastfeeding (as shown in **Table 2**).

Beta diversity analysis

The compositions of the HM microbial community before and after breastfeeding were compared using principal coordinate analysis. The corresponding points obtained from the HM samples were not well clustered and could not be distinguished, suggesting that the composition of the microbial community of the HM samples was similar before and after breastfeeding, as shown in **Figure 1**.

Composition of microbiota in HM

Ten bacterial phyla were detected in HM samples. *Proteobacteria*, *Firmicutes*, *Actinobacteria,* and *Bacteroidetes* accounted for more than 99% of the total bacteria found. At the

Table 1A. General clinical characteristics of the infants grouped by gestational age

Group	Birth weight (g, $\bar{x} \pm s$)	Before feeding		After feeding		Gender		Mode of delivery		Births	
		Length (cm)	Weight (g)	Length (cm)	Weight (g)	Male	Female	Cesarean section	Natural delivery	1st	Not 1st
EPM	866.36±173.80	30.95±2.07	1070.91±175.18	32.54±2.05	1172.27±173.04	6	5	5	6	7	4
VPM	1183.33±254.50	33.38±1.45	1380.42±251.63	35.04±1.20	1485.83±254.03	5	7	5	7	8	4
t/χ^2	3.512	3.276	3.446	3.600	3.484	-		-		-	
P	0.002*	0.004*	0.003*	0.002*	0.002*	0.684		1.000		1.000	

Note: * indicates significant differences. EPM = extremely premature infant gestational (age < 28 weeks); VPM = very premature infant (28 weeks ≤ gestational age < 32 weeks).

Table 1B. General clinical characteristics of the infants grouped by birth weight

Group	Gestational age (w, $\bar{x} \pm s$)	Before feeding		After feeding		Gender		Mode of delivery		Births	
		Length (cm)	Weight (g)	Length (cm)	Weight (g)	Male	Female	Cesarean section	Natural delivery	1st	Not 1st
ELBW	27.32±2.55	30.63±1.55	1038.75±133.32	32.42±1.76	1142.92±128.97	7	5	5	7	8	4
VLBW	29.28±2.81	33.95±0.96	1443.64±204.17	35.41±0.92	1546.36±213.64	4	7	5	6	7	4
t/χ^2	-1.782	6.110	5.577	5.050	5.423	-		-		-	
P	0.089	< 0.001*	< 0.001*	< 0.001*	< 0.001*	0.414		1.000		1.000	

Note: * indicates significant differences. ELBW = extremely low birth weight infant (birth weight < 1000 g); VLBW = very low birth weight infant (1000 g ≤ birthweight < 1500 g).

Table 2A. Comparison of Alpha diversity of microbe in HM before and after feeding

	preBF (n=29)	postBF (n=24)	*t*	*P*
Chao1 Index	81.50 (68.40~104.00)	80.60 (68.20~91.95)	-0.322	0.748
Simpson Index	0.429 (0.332~0.533)	0.490 (0.450~0.633)	-1.608	0.108

Table 2B. Comparison of Alpha diversity of microbe in HM before and after feeding by gestational age group

	EPM-preBF (n=9)	EPM-postBF (n=8)	VPM-preBF (n=11)	VPM-postBF (n=12)	χ^2	*P*
Chao1 Index	73.30 (70.00~74.60)	76.65 (66.85~84.05)	72.65 (63.20~83.80)	74.75 (67.80~93.10)	0.877	0.831
Simpson Index	0.475 (0.388~0.608)	0.432 (0.356~0.547)	0.458 (0.333~0.518)	0.510 (0.460~0.633)	1.627	0.653

Note: EPM = extremely premature infant gestational (age < 28 weeks); VPM = very premature infant (28 weeks ≤ gestational age < 32 weeks); preBF = before breastfeeding; postBF = after breastfeeding.

Table 2C. Comparison of Alpha diversity of microbe in HM before and after feeding by birth weight group

	ELBW-preBF (n=9)	ELBW-postBF (n=11)	VLBW-preBF (n=10)	VLBW-postBF (n=9)	χ^2	*P*
Chao1 Index	73.30 (64.10~74.60)	80.00 (66.85~90.65)	75.25 (66.50~83.80)	75.00 (69.30~81.10)	1.140	0.767
Simpson Index	0.488 (0.388~0.537)	0.498 (0.421~0.660)	0.433 (0.333~0.718)	0.485 (0.442~0.563)	0.653	0.884

Note: ELBW = extremely low birth weight infant (birth weight < 1000 g); VLBW = very low birth weight infant (1000 g ≤ birthweight < 1500 g); preBF = before breastfeeding; postBF = after breastfeeding.

genus level, *Acinetobacter, Staphylococcus, Ralstonia, Pseudomonas, Streptococcus, Stenotrophomonas, Bacillus, Corynebacterium,* and *Enterococcus* constituted the majority of the bacterial communities, accounting for over 84% (as shown in **Figure 2**).

Difference in microbial composition in HM before versus after breastfeeding

In all selected subjects, *Bacillus*, *Prevotella,* and *Fusobacterium* were significantly enriched before feeding compared to after feeding. When grouping by gestational age, the EPM group exhibited significant enrichment in *Luteimonas* before feeding, and in *Lactobacillales*, *Streptococcus, Elizabethkingia*, *Desulfuromonadales*, *Geobacteraceae*, *Geobacter,* and *Ruminococcus* after feeding. In the VPM group, *Bacillus* and *Rhizobium* were enriched in HM before feeding. In the BWT groups, the VLBW group showed significant enrichment in *Bacillus* and *Bacillaceae* in the HM samples before feeding, and in *Lactobacillus* after feeding (as shown in **Figure 3**).

Correlation between the diversity of HM bacteria and infant outcomes

Spearman correlation analysis showed moderate correlations between the alpha diversity of HM bacteria and the changes in infant length, weight, and feeding intolerance before and after breastfeeding, as shown in **Table 3**.

Discussion

HM is recognized as the best natural food for newborns. It not only provides nutrients and bioactive substances for newborns in appropriate proportions, but also acts as a carrier to transport bacteria from the mother to the newborns, affecting the colonization of intestinal bacteria and immune function. There are two hypotheses about the origin of microbes in HM. The endogenous pathway refers to the transfer of maternal intestinal microorganisms into the blood/lymphatic system via dendritic cells and macrophages to the mammary gland [17]. The exogenous route suggests that the microorganisms in HM originate from the mother's skin as well as the baby's skin and oral cavity [9]. As KMC is applied in more countries and regions, it raises the possibility of early microbial exchange between mother and baby. In this study, we compared the microbiological composition of HM of 40 mothers with preterm babies before and after breastfeeding.

Our results showed that there was no significant difference in the microbial diversity of HM before and after breastfeeding. At the phylum level, the composition of HM bacteria in each group included *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes*, which ac-

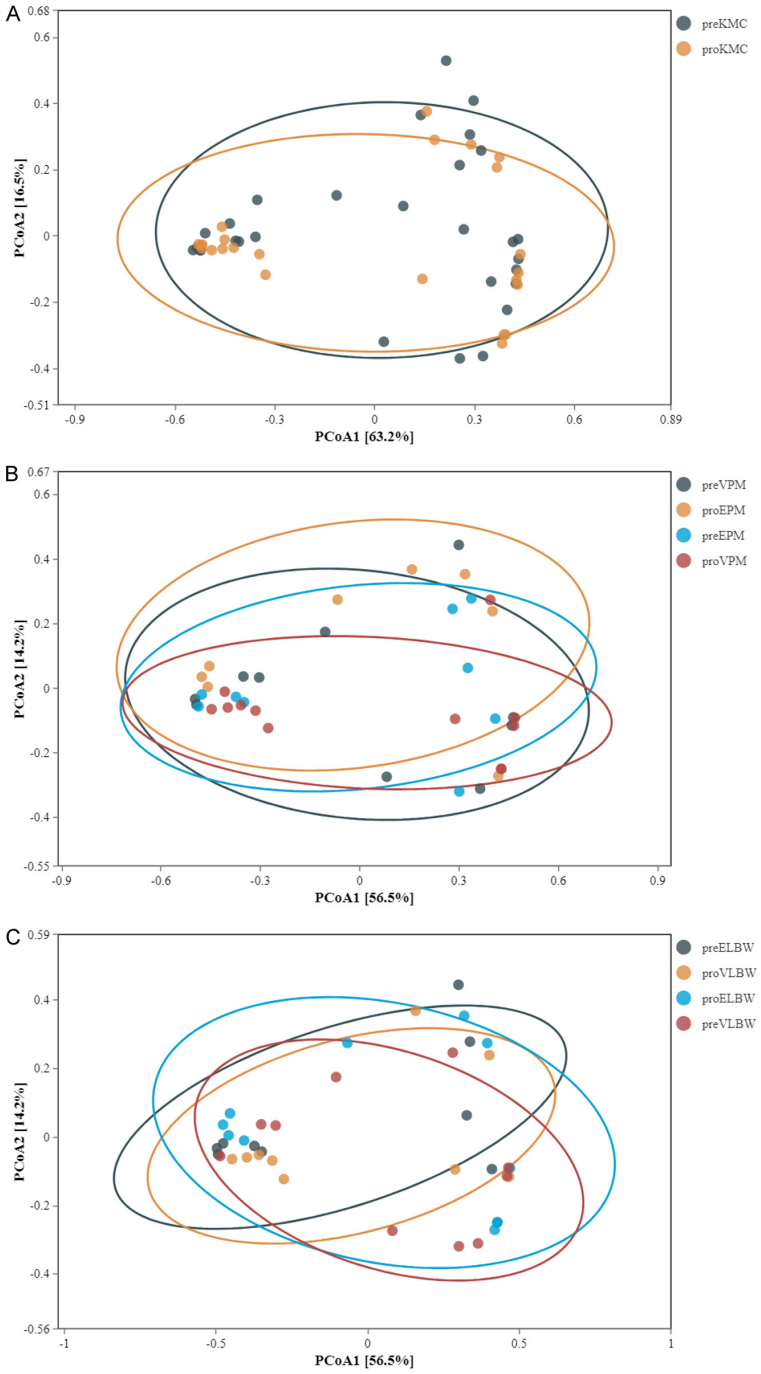

Figure 1. Beta diversity of HM bacterial composition before and after breastfeeding under the conditions of no grouping (A), grouping by gestational age (B), and grouping by birth weight (C), respectively.

counted for more than 99% of the total bacteria. This was similar to the results of Huang et al. [18]. At the genus level, the core bacterial genera before breastfeeding were *Acinetobacter* (36.70%), *Staphylococcus* (25.51%), *Ralstonia* (15.01%), *Pseudomonas* (3.46%), *Bacillus* (2.20%), *Streptococcus* (1.23%), *Stenotrophomonas* (0.91%), *Enterococcus* (0.69%), and *Corynebacterium* (0.65%). The core bacterial genera after breastfeeding were *Acinetobacter* (41.93%), *Staphylococcus* (26.00%), *Ralstonia* (13.19%), *Streptococcus* (6.41%), *Pseudomonas* (4.17%), *Stenotrophomonas* (1.96%), *Corynebacterium* (0.97%), *Enterococcus* (0.59%), and *Bacillus* (0.05%). This is consistent with the results of Hunt et al. [19]. *Serratia*, *Corynebacte-rium*, *Propionate Bacillus*, *Sphingomonas* and *Rhizobium* were also detected in HM.

William et al. reported that the most common aerobic bacteria in HM were *Streptococcus* and *Staphylococcus*, while the most prominent aerobic bacteria in the areolas of breastfeeding mothers and infants' mouths were also *Streptococcus* and *Staphylococcus* [17, 20]. Sakwinska et al. [21] studied the bacterial composition in HM of Chinese nursing mothers using 16S rRNA sequencing and compared HM collected aseptically with that collected by standard procedures. The microbiota of the HM collected aseptically by hand predominately contained *Streptococcus* and *Staphylococcus*, while that collected by the standard procedure using a breast pump was dominated by *Acinetobacter spp*. Although some previous studies found *Lactobacillus* and *Bifidobacterium* to be the major genera of HM [7, 22], in the study by Sakwinska et al., these bacteria only existed in low amounts in some specimens. Other studies have also found minimal concentrations of *Lactobacillus* and *Bifidobacterium* in HM [23], with their abundance during aseptic collection being lower than that collected by the standard procedure, which is consistent with this study.

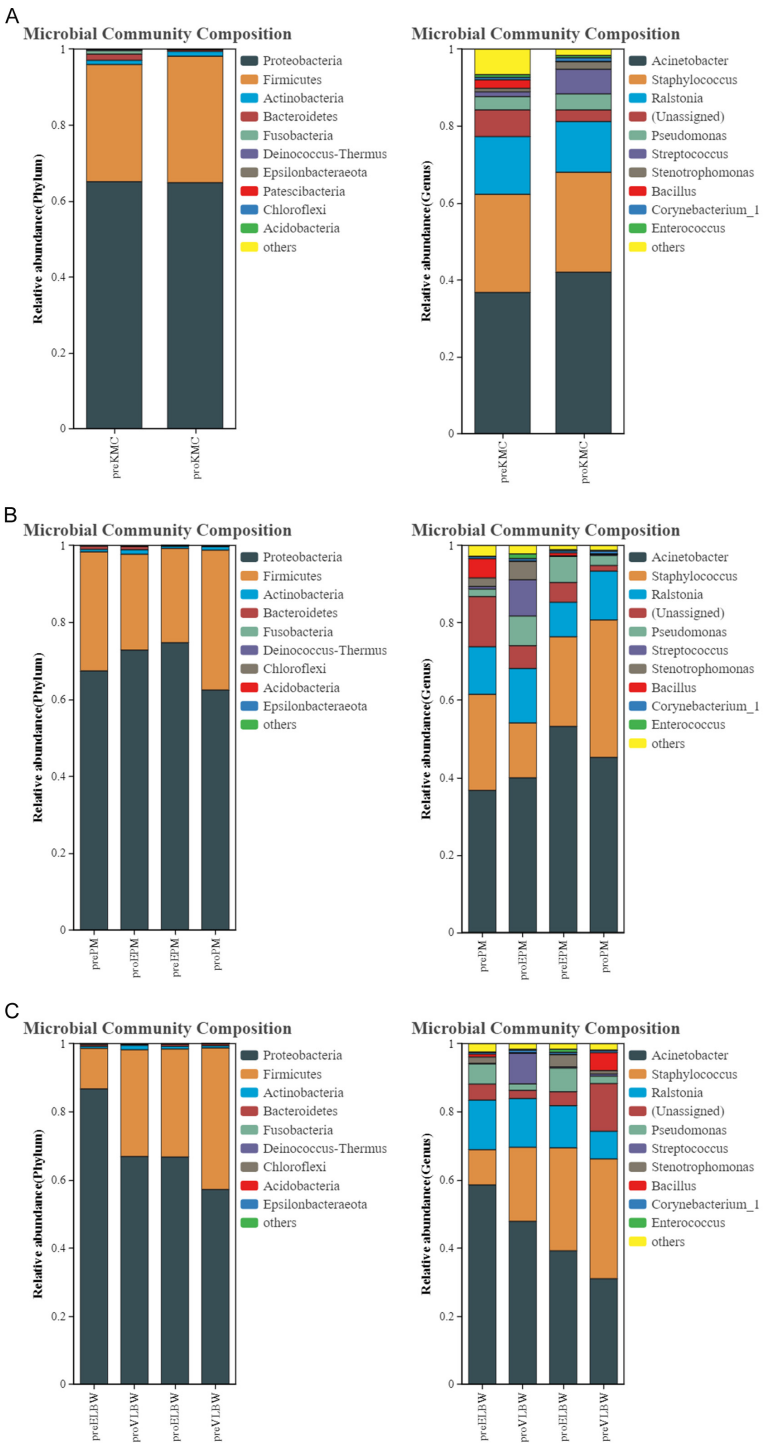

Figure 2. HM bacterial composition before and after breastfeeding under the conditions of no grouping (A), grouping by gestational age (B), and grouping by birth weight (C), respectively.

In our study, the abundance of *Lactobacillus* in HM of EPM and VLBW groups was higher after breastfeeding than before.

Enterococcus is an important part of human intestinal bacteria and one of the first bacterial genera to colonize the infant intestinal tract. It is also an opportunistic pathogen of nosocomial infection in neonates. Most of the potential virulence determinants were found to be absent in all strains of *Enterococcus faecium* isolated from HM by Reviriego et al. [24]. This suggests that the milk of healthy mothers may be the source of nonpathogenic *Enterococcus faecium* isolates in newborns.

In the EPM group, the *Elizabeth meningosepticum* was more abundant in HM after feeding than before. *Elizabeth meningosepticum* is a non-fermenting gram-negative bacterium that widely exists in nature and is an opportunistic pathogen found in ventilator pipelines, various catheters, and water supply systems in hospitals. Preterm infants have poor immune function and are more susceptible to infection than term infants [25]. *Elizabeth meningosepticum* was detected in BM26 (2691 sequences) from the VPM-preBF group and BM38 (4446 sequences) from the EPM-postBF group, and was also present in another 13 HM samples, each containing 1-5 sequences. *Elizabeth meningosepticum* was cultured from the infant sputum corresponding to the BM38 sample.

In summary, it is possible that the bacteria are transferred and colonized from the infant to the mother's breast through feeding, thus detected in HM. *Stenotrophomonas*, *Pseudomonas*, and *Rhizobium*, which grow in soil and

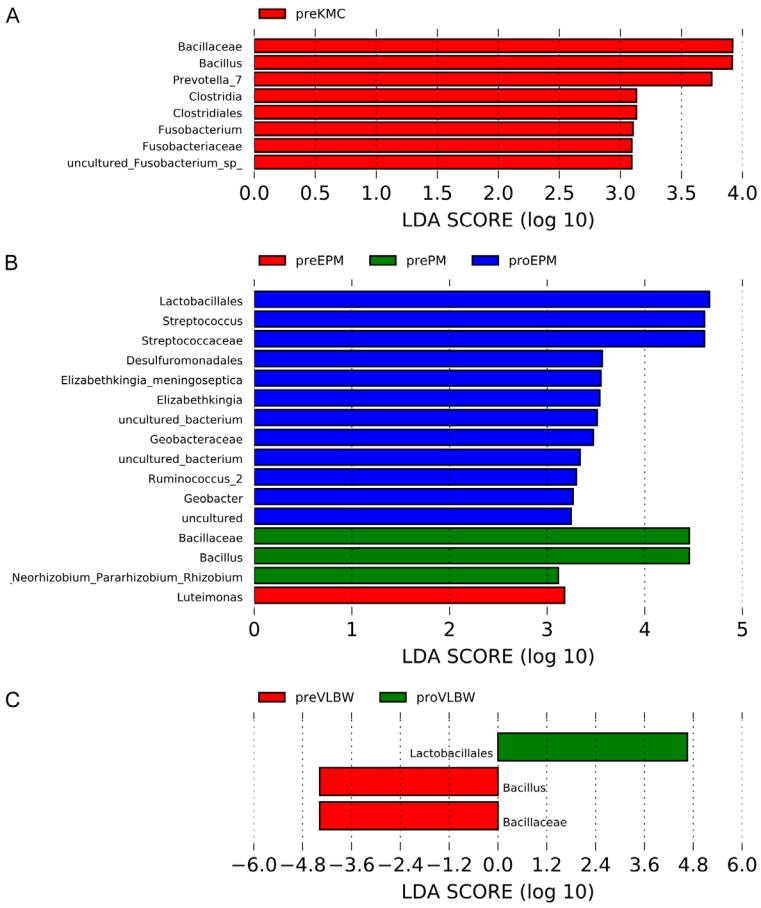

Figure 3. LEfSe analysis results of HM bacterial composition before and after breastfeeding under the conditions of no grouping (A), grouping by gestational age (B), and grouping by birth weight (C), respectively.

Table 3. Correlation between the diversity of human milk bacteria and infant outcomes

	Chao1 Index			Simpson Index		
	Length	Weight	FI	Length	Weight	FI
r	0.322	0.486	0.670	0.359	0.541	0.635
P	0.056	0.029	0.001	0.045	0.002	0.001

Note: FI: feeding intolerance.

water, have been detected in HM in multiple previous studies, as well as in this study, suggesting possible contamination during sampling and storage of HM [19, 26].

The limitation of this study include that the skin and oral microbiota of infants were not tested. Also, there was no direct evidence suggesting that breastfeeding through KMC could alter HM microbiota, which needs to be verified in further research. In addition, the possibility that infants' microbiota can affect the mothers' immune status as well as the composition and content of antibacterial active substances in HM also needs to be further investigated.

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Disclosure of conflict of interest

None.

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