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Original Article

The composition of the maternal breastmilk microbiota influences the microbiota network structure during early infancy

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Abstract *Background/purpose(s):* Human breastmilk (BM) is important for microbiome maturation in infants across different body sites. Streptococcus and Staphylococcus are considered universally predominant genera in the BM microbiota. However, whether the differential abundance of Streptococcus and Staphylococcus in BM can differentially affect microbiome maturation in infants remains unclear.
Methods: We recruited exclusively breastfeeding mothers from among the donors of the human milk bank established at National Cheng-Kung University Hospital. The donor mothers provided 35 BM samples at three months (3 M; before introducing children to complementary feeding) and 23 BM samples at six months (6 M; after introducing children to complementary

Abbreviations: CoNS, coagulase-negative *Staphylococcus*; BMI, body mass index; BM, breastmilk.

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feeding) postpartum. At both time points, samples from different body sites, including nasal swabs, oral swabs and stool, were collected from the mothers and their infants.
Results: Maternal BMI was inversely associated with coagulase-negative Staphylococcus (CoNS) abundance in breastmilk. *Staphylococcus caprae* representation in BM CoNS showed a negative correlation with Streptococcus abundance. Network analysis revealed that infants fed Staphylococcus-dominated BM had better gut and nasal microbiota networks than infants fed Streptococcus-abundant BM during early infancy.
Conclusion: Our work suggests that maternal metabolic status plays a crucial role in Staphylococcus/Streptococcus competition in BM, which in turn can impact the development of the infant microbiota. Our microbiota co-occurrence network analysis might serve as a helpful bioinformatic tool to monitor microbiota maturation during early infancy.

Introduction

The establishment of the gut microbiota in early human life is a critical milestone for overall health.[1] Several factors have been suggested to contribute to the formation of the core gut microbiota and influence the progression of stable compositions that have lasting effects on metabolism.[2] These factors include genetics, feeding method (breastfeeding or formula feeding), infections, and maternal health conditions. Breastmilk (BM) is considered the gold standard for infant nutrition, and the American Academy of Pediatrics recommends exclusive breastfeeding for approximately six months after birth due to the neurodevelopmental advantages that it confers.[3,4] BM also plays a role in shaping the early development of the infant microbiome by providing essential nutrients for early colonizing species and transferring crucial microbes to establish a proper microbial community structure.[5] The composition of the human BM microbiota is highly diverse and can be influenced by both infant and maternal factors.[6,7] The interactions and competition among different microbial species are thought to significantly impact the structure and stability of the microbiota.[8,9] Additionally, studies have found that the transmission of maternal microbes to infants during lactation may contribute to the risk of developing metabolic disorders in childhood.[10] However, beyond potential infection risks, the extent to which variations in the composition of breast milk microbiota affect the maturation of the infant gut microbiota remains largely unknown. Moreover, monitoring the maturation of gut microbiota poses significant challenges due to its dynamic nature and variations across individuals. Therefore, co-occurrence network analysis of microbial interactions and competition within an early established network may help elucidate the influence of maternal BM microbiota on the development of the infant gut microbiota.[11]

Entero-mammary transfer and retrograde translocation have been proposed to be the two major pathways for the establishment of the BM microbiota.[7,12] Although some studies suggest that certain maternal gut bacteria can access mammary glands,[13,14] recent evidence indicates that the infant oral microbiota may play a more vital role in influencing the composition of the BM microbiota.[7] The colonization of the infant oral cavity by oral-origin bacteria might occur before birth, as oral bacteria have been detected in the amniotic fluid in nearly 70% of pregnant women.[15] In addition, the placental microbiota is highly similar to the oral microbiota in pregnant women.[16] These findings support the observations that specific oral species, such as *Streptococcus*, *Fusobacterium*, and *Porphyromonas*, inoculate the infant oral cavity at a very early stage of infancy.[7,17,18] The three-way interaction between maternal oral, infant oral, and BM microbiota is critical for infant health during early life.[17]

Maternal body mass index (BMI) has been observed as an important factor affecting milk microbiota composition.[6,7] Across different studies, *Streptococcus* and *Staphylococcus* are considered universally predominant genera in BM.[19] Maternal BMI has been found to be associated with the abundance of *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Bifidobacterium* in BM.[20] The species shared between BM and the infant gut, such as *Streptococcus* spp. and *Veillonella*, were demonstrated to affect the development of the infant gut microbiome.[21] However, whether variations in the BM microbiota can differentially impact the hierarchical structure of microbiota across different body sites in infants remains unclear.

In this study, we explored the role of the BM microbiota in shaping early microbiome maturation in infants, investigating the influence of maternal BMI on BM composition, exploring the pathways and interactions involved in establishing the BM microbiota, and assessing the potential impact of the BM microbiota on the hierarchical structure of microbiota in infants. We found that maternal BMI is an important factor affecting the representation of *Staphylococcus caprae* in BM. Although the oral (infant)-mammary (mother) translocation of the infant's oral-origin bacteria to BM is a natural phenomenon during early infancy, we found that the abundance of *S. caprae* in BM was negatively correlated with the abundance of oral-related bacteria, including *Streptococcus* and *Rothia*. Furthermore, the BM microbiota type dominanted by *Staphylococcus* or *Streptococcus* differentially contributes to the establishment of the hierarchical structure of gut, nasal, and oral microbiota in infants. This study suggests that *Staphylococcus*/*Streptococcus* competition in the BM may play a critical role in influencing infant microbiota development. Moreover, the mother's metabolic status may significantly impact microbiome development in infants through specific BM microbiota changes.

Materials and methods

Study subjects and sample collection

This study was approved by the Ethics Committee of the National Cheng Kung Hospital (code B-ER-107-328), and was carried out at the Taiwan Southern Human Milk Bank (TSHMB) of National Cheng Kung University Hospital, the second human milk bank in Taiwan. The operation of TSHMB was described previously.[22,23] TSHMB recruits donors within three months postpartum. The donor qualification process requires multiple donations over time and negative serum virology for human immunodeficiency virus, hepatitis A/B/C virus, human T-lymphotropic virus type I & II, cytomegalovirus, and sexually transmitted diseases. Donor mothers who qualified for cooperation with TSHMB were prospectively invited to this study and subsequently provided consent to participate and structured questionnaires. Each mother provided one milk sample each at 3 and 6 months postpartum in a sterile milk container. Approximately 30—100 mL milk was collected by hand expression or vacuum pumping and immediately frozen at −20 °C. In addition, fecal, nasal, and oral samples were obtained at the same timepoint from the mothers and their infant dyads at postpartum or postnatal ages of 3 and 6 months. Collected samples were refrigerated immediately at home. Staff from the TSHMB visited the donors weekly to retrieve the frozen milk and other samples for return to the milk bank for analysis. Samples were stored at −80 °C until analysis.

16S rRNA gene sequencing and analysis

DNA was extracted from the BM microbiota by using a ZymoBIOMICS DNA kit (D4300, ZYMO RESEARCH). A reagent-only negative extraction control was included in each batch of extract. PCR amplification of 16S rRNA was performed on extracted DNA by using primers targeting the V3—V4 region (primer set: 341 F—805 R). The 16S rRNA amplicons were sequenced on an Illumina MiSeq platform and generated 300 bp paired-end reads. Raw data were obtained, and the barcode sequences were cleaned up. The sequencing data were analyzed using the QIIME 2 platform (version: 2021.4).[24] Paired-end sequences were demultiplexed by the q2-demux plugin, followed by denoising using DADA2[25] via the q2-dada2 plugin to ultimately generate amplicon sequence variants (ASVs). Taxonomic classification of ASVs was carried out using the classify-sklearn naïve Bayes taxonomy classifier in the q2-feature-classifier plugin[26] against the SILVA database (release 138). The species-level sequence assignments were verified by conducting NCBI blastn analysis with the following parameters: identity = 99% and E value = 1e-05. Taxonomic abundance, alpha diversity (Shannon index), and beta diversity (principal coordinate analysis (PCoA) of Bray—Curtis dissimilarity, weighted/unweighted UniFrac distance) were then calculated and plotted using the phyloseq,[27] ampvis2, and vegan (v2.5-7; http://CRAN.R-project.org/package=vegan) packages. Differences in alpha diversity between groups were tested by one-way ANOVA, followed by Tukey's honest significant difference test (Tukey's HSD) for between-group variance. Permutational multivariate analysis of variance (PERMANOVA) of beta diversity (999 permutations) was applied to evaluate the microbial community similarity across groups using the pairwise adonis function in the R package vegan. SourceTracker2[27] analysis was used to assess the similarities between maternal and infant microbiota. Genus-level enterotypes based on partitioning around medoids with Jensen−Shannon divergence were identified by previously described methods.[28] The Calinski−Harabasz (CH) index suggested the optimal number of clusters.

Co-occurrence network analysis of a microbiota at the family level was conducted according to the Spearman correlation coefficients. Network centrality was analyzed by using the "Network Analyzer" plugin within Cytoscape software. The betweenness centrality of a node was determined by calculating the total number of shortest paths from all nodes to every other node that connects with the particular node.[29] The node degree was calculated as the number of linked neighbors to a node.

Statistical analysis

Differential abundance analysis was performed using analysis of compositions of microbiomes with bias correction (ANCOMBC package v 1.99.1; 10,000 iterations, with Benjamini—Hochberg correction to adjust for multiple hypothesis testing). MaAslin2 was used to determine multivariable associations via generalized linear regression between taxonomic relative abundance in the BM microbiota and maternal factors (BMI, age, and children number).[30] Pearson's correlation test was used to assess the relationships between the microbial communities and maternal BMI. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States2 (PICRUSt2) was adopted for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway prediction from 16S rRNA data. The DESeq2 package was used to detect KEGG pathways with differential abundance between BM type 1 and type 2 samples.

Results

Demographic characteristics of the mother—infant dyad participants

To investigate the impact of the BM microbiota on the establishment of infant microbiota across different body sites during early infancy, we recruited exclusively breastfeeding mothers among the donors of the human milk bank[22] and also invited their infants to participate. Only donors who tested negative for infection with various viruses and *Staphylococcus aureus* were enrolled in this study (Fig. 1A). A total of 35 donor mothers and 20 infants participated (Table S1). The donor mothers provided 35 BM samples at three months (3 M; before introducing children to complementary feeding) and 23 BM samples at six months (6 M; after introducing children to complementary feeding) postpartum. At both time points, samples across different body sites, including nasal swabs, oral swabs and stool, were collected from mothers and their infants (Fig. 1B). In total, we analyzed samples from 20

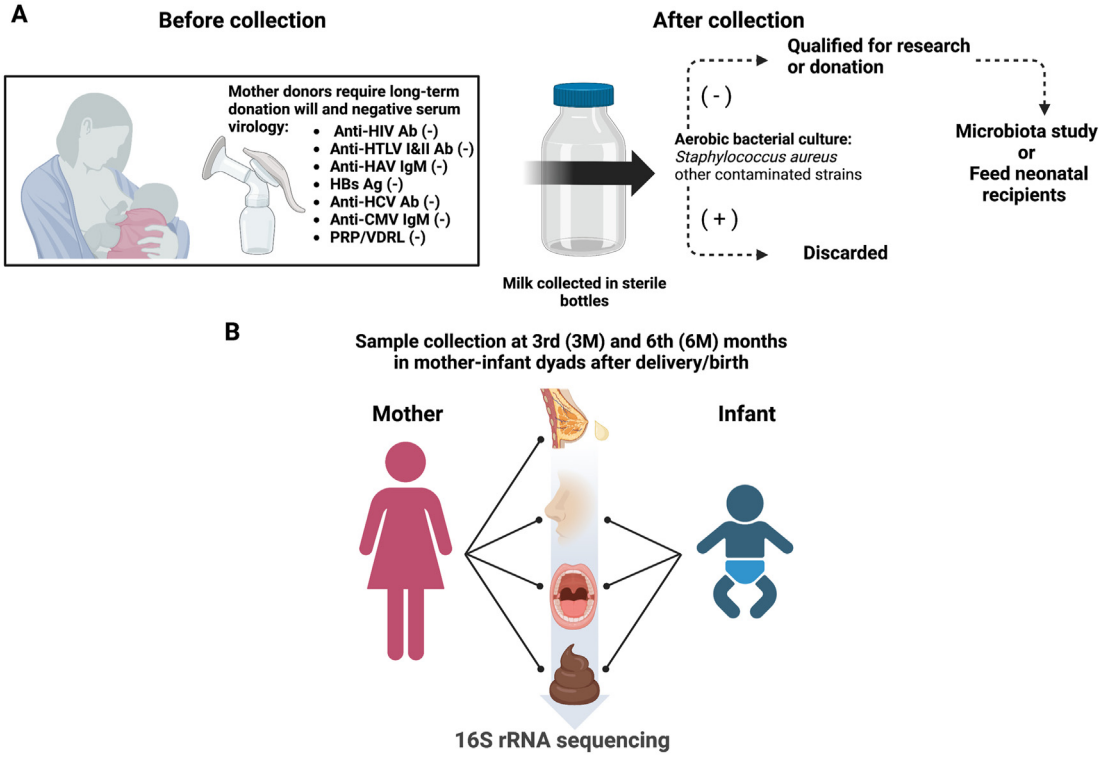

Figure 1. Schematic illustration of the enrollment criteria and sample collection strategy in this study. (A) Mother donors who exclusively fed their infants with breastmilk received education on how to express breastmilk with pumps and to collect the milk properly into sterile bottles. Mothers with long-term participation were qualified for donation after serum virology examination. Donor milk was disqualified for research or donation and discarded if it failed to pass bacterial culture tests for *Staphylococcus aureus* or other contaminant strains. Contaminant strains included gram-negative or gram-positive bacilli and Group B *Streptococcus*. (B) Samples collected from breastmilk, nasal, oral, and fecal specimens were obtained from the mothers and their infant dyads at the same time. Ab: antibody; IgM: immunoglobulin M; HIV: human immunodeficiency virus; HTLV Ab (I & II): human T-lymphotropic virus type I & II; HAV: hepatitis A virus; HBs Ag: hepatitis B surface antigen; HCV: hepatitis C virus; CMV: cytomegalovirus; PRP/VDRL: rapid plasma reagin/venereal disease research laboratory. This illustration was created by using BioRender.com.

mother–infant dyads at three months postpartum and 18 mother–infant dyads at six months postpartum. The number of samples analyzed by 16S rRNA sequencing and the abbreviated names of the samples are listed in Table S1.

Bacterial diversity of mother–infant dyads and their interactions across BM and multiple body sites

We found that the most dominant bacterial families were *Staphylococcaceae* in BM, *Bacteroidaceae* and *Lachnospiraceae* in maternal feces, *Corynebacteriaceae* in maternal nasal samples, *Streptococcaceae* and *Prevotellaceae* in maternal oral samples, *Bifidobacteriaceae* in infant fecal samples, *Corynebacteriaceae* in infant nasal samples and *Streptococcaceae* in infant oral samples (Fig. 2A). The Shannon index indicated that the mother fecal microbiome had the highest α-diversity of the microbiota across different sites in both mothers and infants (Fig. 2B). At the same collection sites between mothers and infants, the infants' nasal microbiota reached a similar α-diversity level as the mothers' nasal microbiota at 3 and 6 months after birth (Fig. 2B). This suggests that during early infancy, the infants' fecal and oral microbiota were still premature and had lower α-diversities than the mothers' microbiota (Fig. 2B). In contrast to the fecal and oral microbiota, the diversity of the infant's nasal microbiota was more synchronous with that of the mother's nasal microbiota.

To further investigate the compositional dissimilarities between each group, we performed β-diversity analyses on all samples. Based on the Bray–Curtis index, we found that the composition of the BM microbiota was more closely related to the composition of nasal and fecal microbiota than oral microbiota in infants (Fig. 2C). We further used the UniFrac distance metric to evaluate the similarity between groups (Fig. 2D and E). When compared with mothers' fecal microbiota, infants' fecal microbiota was significantly different at 3 and 6 months after birth (Fig. 3A–C). Similar results were observed for oral microbiota (Fig. 3D–F). These results suggest that a longer time is required for significant gut and oral microbiota maturation during infancy. For nasal microbiota, significant differences between the mother and infant groups were observed by Bray–Curtis (Fig. 3G) and weighted UniFrac distance (Fig. 3H) but not by unweighted UniFrac distance (Fig. 3I). This indicates that the early colonizing species

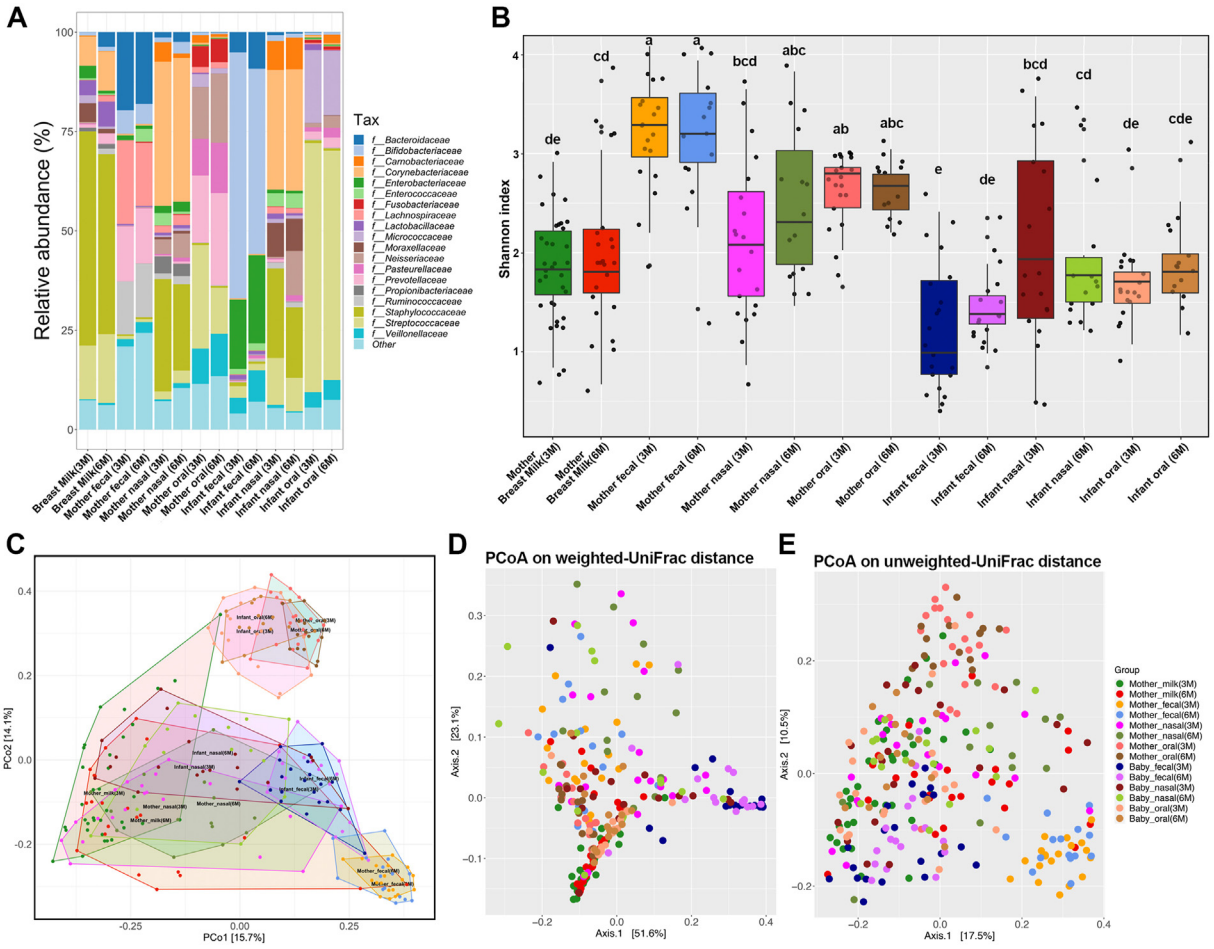

Figure 2. Microbial composition and diversity of maternal and infant microbiota across different body sites. (A) Relative abundance of the top 20 families in the microbiota of the indicated groups. (B) Shannon index of the indicated microbiota. Data are presented as the mean ± SEM. Statistical significance was determined by one-way ANOVA. Groups not sharing the same letter (a—e) were significantly different at $P < 0.05$. (C—E) PCoA plot of bacterial β-diversity based on the Bray—Curtis dissimilarity matrix (C), weighted UniFrac distance (D) and unweighted UniFrac distance (E). Samples are indicated as follows: Mother_milk (3 M) = breastmilk microbiota 3 months postdelivery, Mother_milk (6 M) = breastmilk microbiota 6 months postdelivery, Mother_fecal (3 M) = mother fecal microbiota 3 months postdelivery, Mother_fecal (6 M) = mother fecal microbiota 6 months postpartum, Mother_nasal (3 M) = mother nasal microbiota 3 months postdelivery, Mother_nasal (6 M) = mother nasal microbiota 6 months postdelivery, Mother_oral (3 M) = mother oral microbiota 3 months postdelivery, Mother_oral (6 M) = mother oral microbiota 6 months postdelivery, Infant_fecal (3 M) = fecal microbiota of 3-month-old infants, Infant_fecal (6 M) = fecal microbiota of 6-month-old infants, Infant_nasal (3 M) = nasal microbiota of 3-month-old infants, Infant_ nasal (6 M) = nasal microbiota of 6-month-old infants, Infant_oral (3 M) = oral microbiota of 3-month-old infants, Infant_oral (6 M) = oral microbiota of 6-month-old infants.

met the requirements for nasal microbiota maturation, although the compositional structure was still different from that of the adult microbiota.

The contribution of mother-origin bacteria to the infant microbiota

To examine whether the maternal microbiota was the primary source of microbial communities in infants, we utilized SourceTracker software to track the contribution of mothers' microbiota across different body sites in paired infants. Three months after birth, most infants' gut, nasal, and oral microbiota were most highly contributed by mothers' fecal (avg. 61.1%, Fig. 4A), nasal (avg. 68.4%, Fig. 4B), and oral (avg. 91%, Fig. 4C) microbiota, respectively. The average contributions of the BM microbiota to the infant gut, nasal and oral microbiota were 0.95% (Figs. 4A), 0.15% (Fig. 4B) and 0.06% (Fig. 4C), respectively. These results suggest that the early colonization (before three months after birth) of the maternal microbiota is critical for the taxonomic structure of the microbiota in the same body sites in infants.

Because BM microbiota contributed only a small proportion of the bacteria in infants' gut, nasal and oral microbiota, we further tested whether the specific bacteria in BM might differentially influence the ecological homeostasis of infants' microbiota across different body sites. By performing clustering analysis on the microbiota of the BM

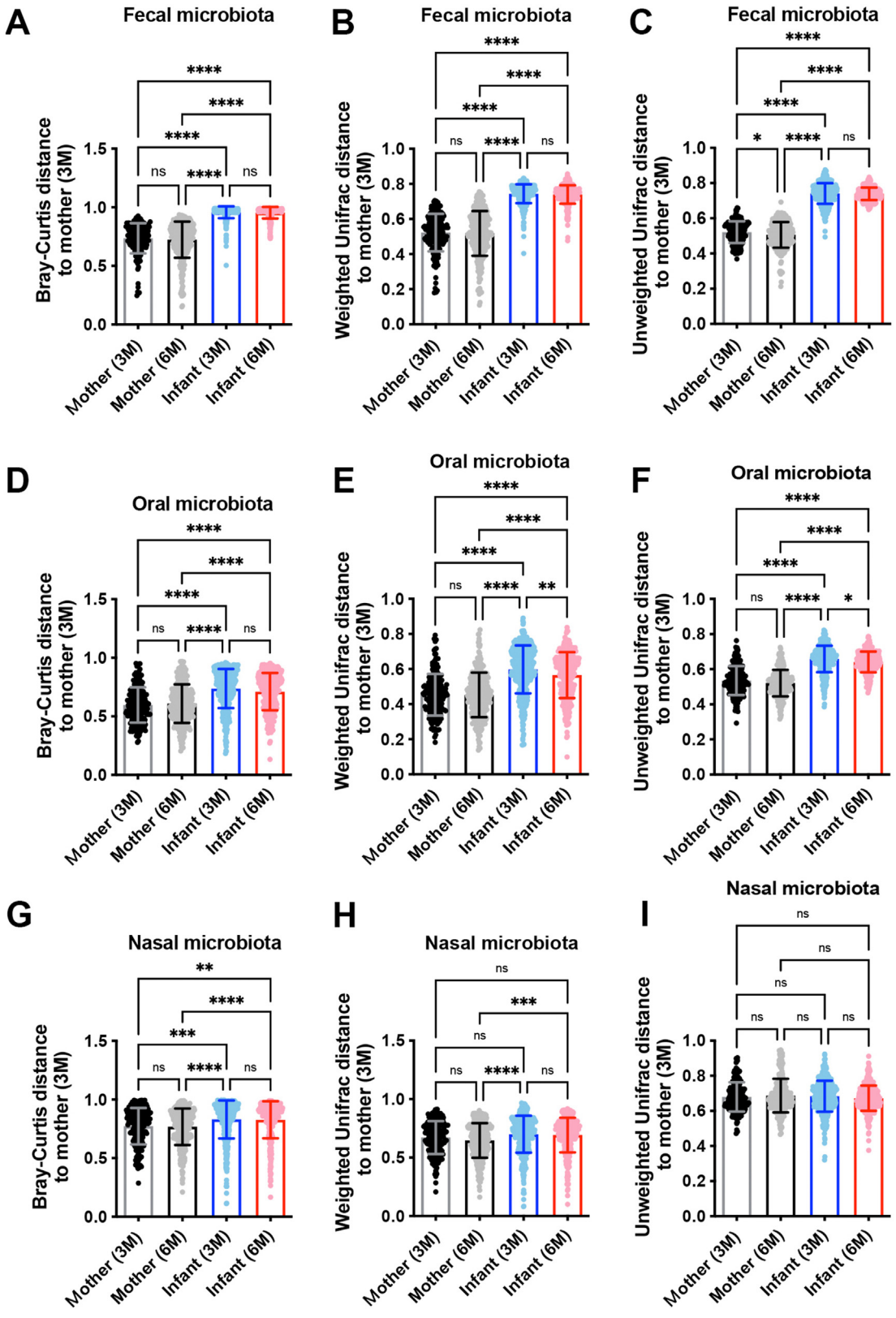

Figure 3. Similarity between maternal and infant microbiota of the same body site over time. Beta diversity among groups: (A, D, G) Box plots of beta-diversity distances based on Bray—Curtis dissimilarity, (B, E, H) weighted UniFrac distance and (C, F, I) unweighted UniFrac distance of fecal (A—C), oral (D—F) and nasal (G—I) microbiota of mother 3 months (3 M) and 6 months (6 M) postdelivery or infant 3 months (3 M) and 6 months (6 M) after birth. The y-axes represent the distance of each group compared to the mother group (3 M). The P values among all groups were estimated by using one-way ANOVA and Tukey's post hoc test. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

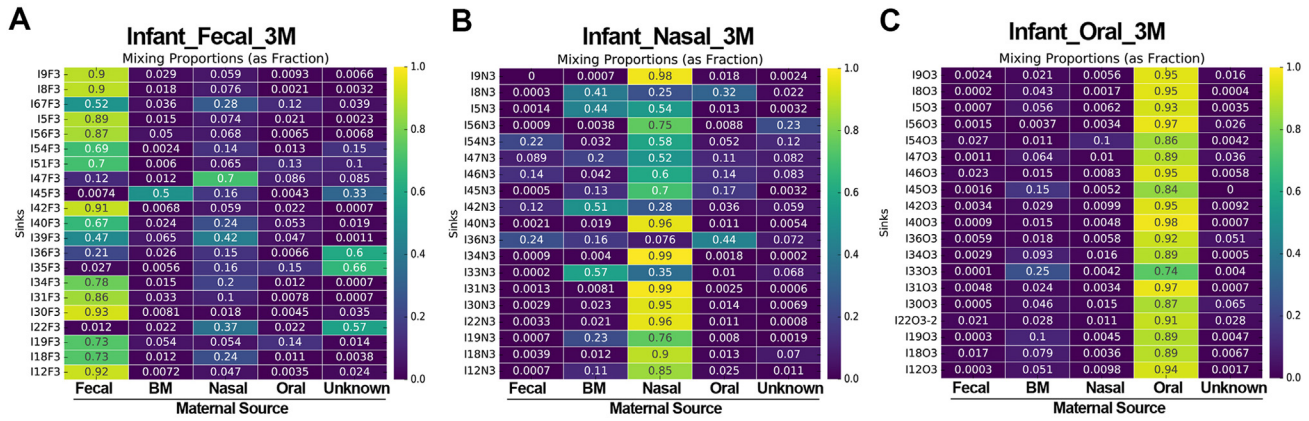

Figure 4. The source of infant microbiota across different body sites. SourceTracker was used to compare the infant (A) fecal, (B) nasal, and (C) oral microbiota 3 months (3 M) after birth with the mother fecal, BM, nasal, and oral microbiota.

(3 M) samples, we found that the BM microbiota could be clustered into two different types based on microbial composition (Fig. 5A). The maternal characteristics of the two types of the BM microbiota were compared, and the BMI cutoffs to define overweight ($\geq$23 kg/m^2) and obesity ($\geq$25 kg/m^2) were based on the International Obesity Task Force (IOTF) standard in South and East Asians (Table S2).[31]

The ordinations showed that the major microbial variables between type 1 and type 2 BM microbiota were *Staphylococcus*, *Acinetobacter*, *Streptococcus*, and *Rothia* (Fig. 5A). The type 1 BM microbiota was primarily dominated by *Staphylococcaceae* (average abundance = 35.8%) and *Streptococcaceae* (average abundance = 36%), and the type 2 BM microbiota was specifically dominated by *Staphylococcaceae* (average abundance = 64.6%) (Fig. 5B). By ANCOM-BC2 analysis, we confirmed that the genera *Streptococcus* and *Rothia* were significantly enriched in type 1 BM microbiota. We further used PICRUST2 analysis to understand the difference in microbiota functions between type 1 and type 2 microbiota (Fig. 5C). The results showed that the type 1 BM microbiota had enhanced functions associated with sugar metabolism, and the type 2 BM microbiota was primarily involved in the metabolism of fatty acids and amino acids (Fig. 5D).

BM types clustered by microbiota composition and its associations

We further verified the relative abundance of the *Staphylococcus*, *Streptococcus*, and *Rothia* genera in each type of BM. We found that the type 2 BM microbiota had a significantly higher proportion of coagulase-negative *Staphylococcus* (CoNS) than the type 1 BM microbiota (Fig. 6A). This difference was primarily contributed by *S. caprae* (Fig. 6B), and no difference was observed in *Staphylococcus epidermidis* (Fig. 6C), *Staphylococcus lugdunensis* (Fig. 6D), or *Staphylococcus warneri* (Fig. 6E). In contrast, the type 1 BM microbiota had a significantly higher proportion of *Streptococcus* (Fig. 6F) than the type 2 BM microbiota, which was primarily contributed by *Streptococcus mitis* (Fig. 6G). The oral-origin *Rothia* also had a significantly higher abundance in the type 1 BM microbiota. However, no significant difference in the well-known probiotic genera *Lactobacillus* (Fig. 6I) and *Bifidobacterium* (Fig. 6J) was detected between the two types of BM. To understand whether maternal health measures can affect the abundance of *Staphylococcus*, *Streptococcus* and *Rothia* genera in the BM, we selected three measures in the questionnaire for MaAsLin 2 analysis to determine the multivariable association between measures and microbiota features. The results showed that maternal BMI and age were negatively correlated with the abundance of *Staphylococcus* in BM three months after delivery (Fig. 6K). By Pearson correlation coefficient analysis, we confirmed that maternal BMI but not age had a significant negative correlation with the abundance of the *Staphylococcus* genus (Fig. 6L, Figs. S1A–S1D) and *S. caprae* (Fig. 6N) in the BM. However, neither maternal BMI nor maternal age affected the abundance of the *Streptococcus* genus or *S. mitis* species (Figs. S1E and S1F). This indicates that the lower maternal BMI was primarily associated with the representations of *S. caprae* but not *Streptococcus* species in BM. Therefore, we further considered that *S. caprae* might outcompete the *Streptococcus* genus in BM. By performing Pearson correlation coefficient analysis between *S. caprae* and *Streptococcus* or *Rothia* in BM (3 M) samples, we confirmed that *S. caprae* had dramatic negative correlations with *Streptococcus* and *Rothia* in BM (Fig. 6M and O). The BM microbiota has been proposed to be constructed by two pathways, namely, maternal entero-mammary translocation and retrograde transfer of the infant's oral microbiota.[6,21] Because *Streptococcus* and *Rothia* are oral-origin bacteria, our results suggest that maternal-origin bacteria, such as *S. caprae*, may play an important role in preventing the dominance of infant oral pathobionts in BM.

BM type influences the development of hierarchical structure in infant microbiota

Community hubs have been suggested to be critical for the function and stability of the human microbiome.[9,32] By co-occurrence (Spearman's rank correlation) analyses, we investigated whether different types of the BM microbiota can affect the establishment of community hubs in infant microbiota across various body sites. Here, we determined the community hubs by the nodes with high betweenness

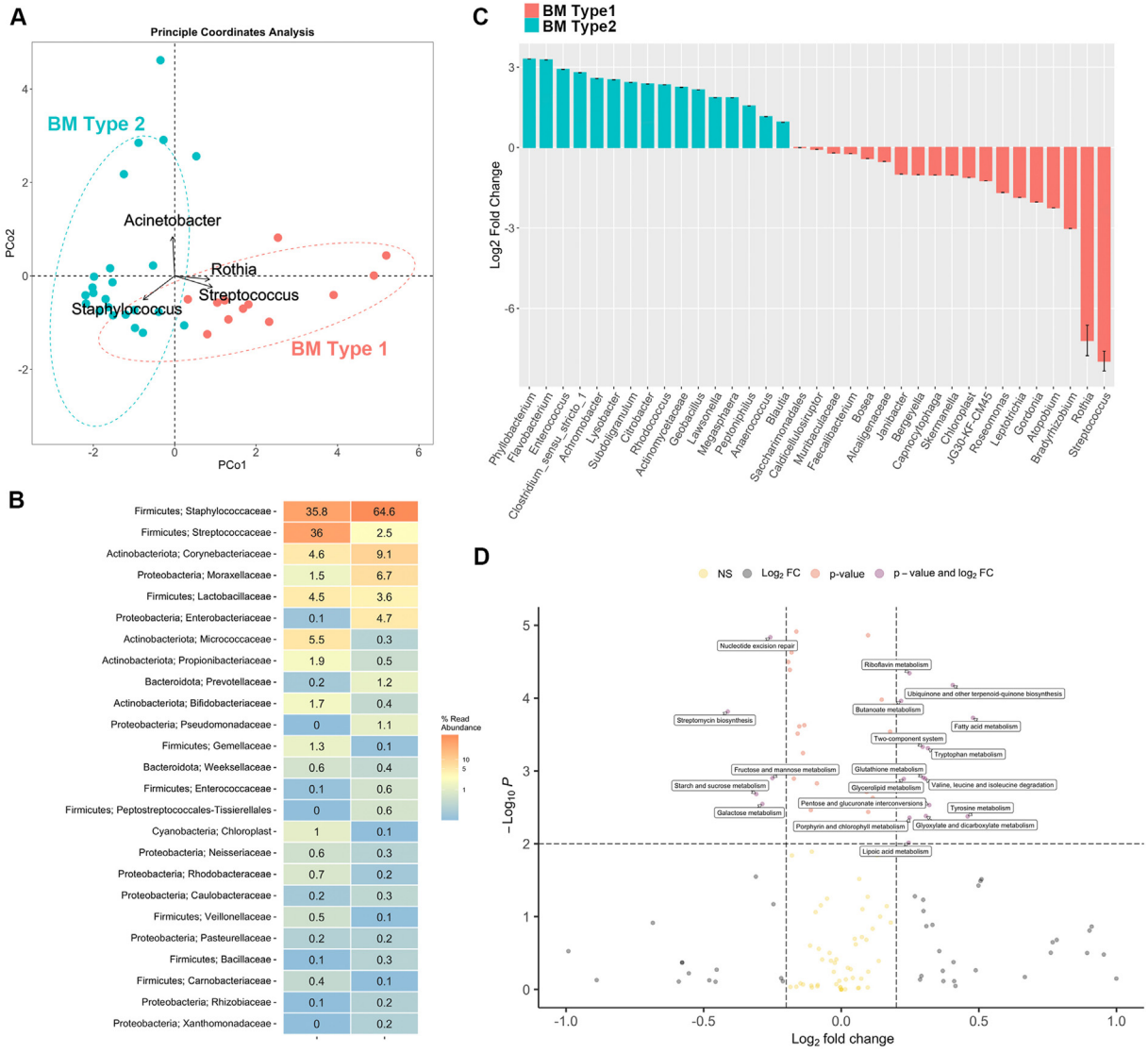

Figure 5. Clusters and changes in the BM microbiota. (A) Two BM microbiota types were identified by principal coordinate analysis (PCoA) and k-means clustering based on the Bray—Curtis distance matrix of the BM microbiota in the maternal subjects at 3 M postpartum. (B) Relative abundance of dominant bacterial genera in different BM enterotypes. (C) Comparisons of changes in the microbial abundance at the genus level between two BM enterotypes (BM Type 1 with *Streptococcus* enterotype, n = 12; BM Type 2 with *Staphylococcus* enterotype, n = 23) by ANCOM-BC test. All effect sizes with adjusted $P < 0.05$. (D) Volcano plot of enriched KEGG pathways identified by PICRUSt2 analysis. The horizontal dashed line is drawn at $P = 0.01$. Vertical dashed lines are added at $\log_2$-fold change (LFC) values of 0.2 and −0.2. The shrunken log2-fold change metric of DESeq2 was used. KEGG pathways with significant differential expression ($P < 0.01$ and LFC > 0.2 or LFC < −0.2) are colored in red. Sample names are as follows: BM Type 1 = Type 1 breastmilk 3 months after delivery, BM Type 2 = Type 2 breastmilk 3 months after delivery.

centralities representing the upper hierarchies in the microbial networks. When comparing the top 10 hubs in type 1 and type 2 BM microbiota, we found that the primary hubs in type 1 BM microbiota were oral-origin bacteria, including *Streptococcus*, *Corynebacterium*, and *Rothia* (Fig. 7A). In contrast, the type 2 BM microbiota had both oral- and gut-origin hubs, including *Porphyromonas*, *Bacillus*, and *Enterobacter* (Fig. 7B). Multiple oral-origin hubs were observed in the oral microbiota of infants receiving type 1 BM, including *Aggregatibacter*, *Fusobacterium*, *Rothia*, and *Streptococcus* (Fig. 7C). In contrast, the microbial hubs in the oral microbiota of infants receiving type 2 BM were primarily gut-origin hubs, including *Blautia*, *Clostridia*, *Bacteroides*, and *Escherichia* (Fig. 7D). The hierarchy structure in the nasal microbiota of infants receiving type 1 BM was not well established compared with that of infants receiving type 2 BM (Fig. 7E and F). Similar to the observations in oral microbiota, several gut-origin genera were observed in the nasal microbiota of infants receiving type 2 BM, including *Faecalibacterium*, *Lachnospiraceae*, *Escherichia*, and *Roseburia* (Fig. 7F). Consistent with this, the hierarchy structure was not well developed in the gut microbiota of infants receiving type 1 BM (Fig. 7G). However, infants receiving type 2 BM developed a well-

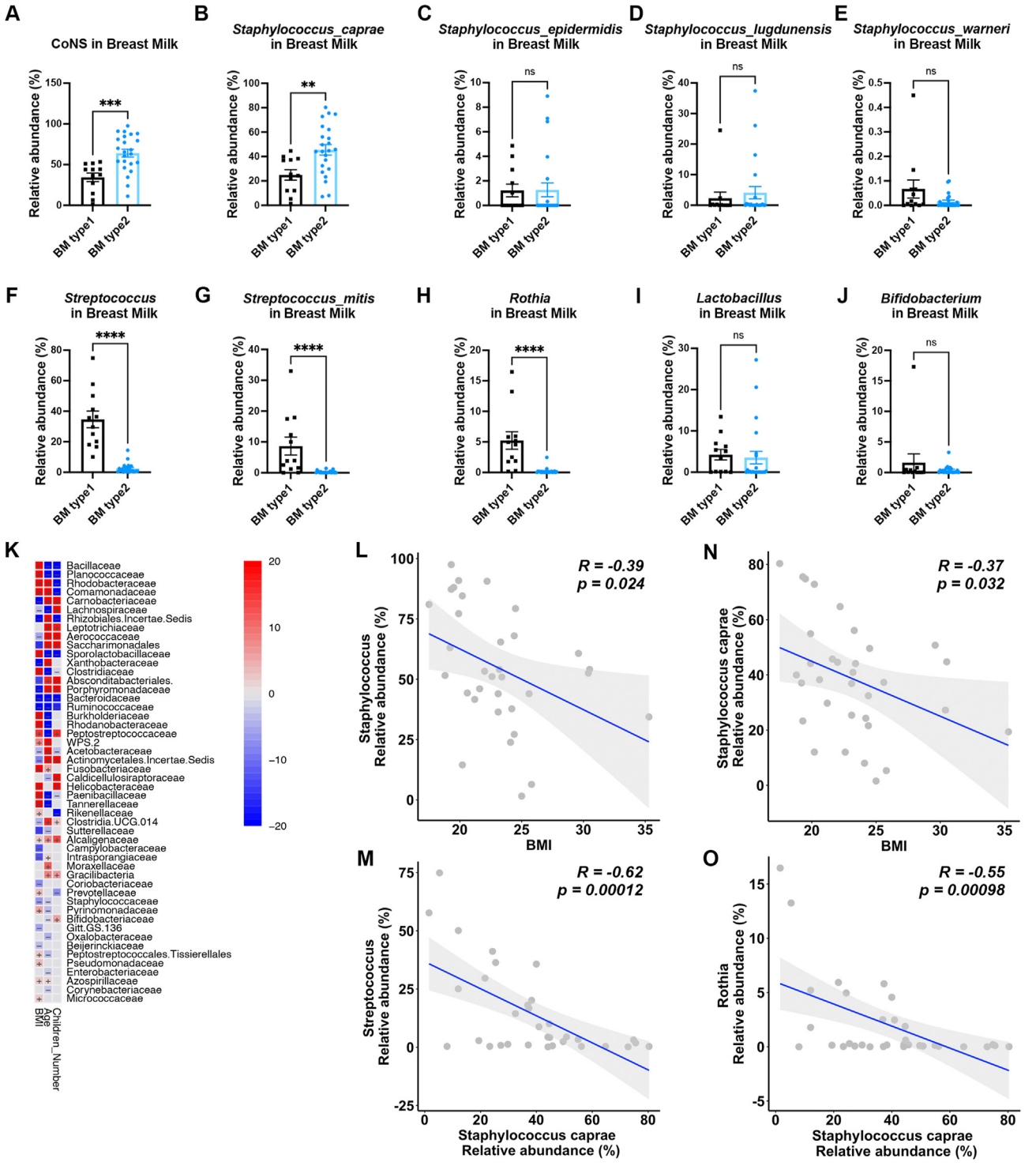

Figure 6. Maternal BMI is associated with the representations of *Staphylococcus* in the BM microbiota. (A–J) Composition of the BM microbiota in BM type 1 and type 2. The relative abundances of bacterial genera and species in BM type 1 and type 2 at 3 M postpartum. Data are presented as the mean ± SEM. ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ according to Mann–Whitney tests. (K) MaAsLin2 significant results (FDR < 0.25) and associations between maternal factors (BMI, age and number of children) and family-level gut microbiota composition of the mother at 3 M postpartum. Based on the normalized obtained significant results, the color scale bar shows a positive relationship (red) and a negative relationship (blue) between taxa and factors, ranging from the highest positive normalization (+20) to the lowest one (−20). Scatter plots of the linear regressions are shown for BMI significantly correlated with the genera *Staphylococcus* (L) and *Staphylococcus caprae* (N); *Staphylococcus caprae* significantly correlated with the genera *Streptococcus* (M) and *Rothia* (O), followed by calculation of Pearson's correlation coefficient.

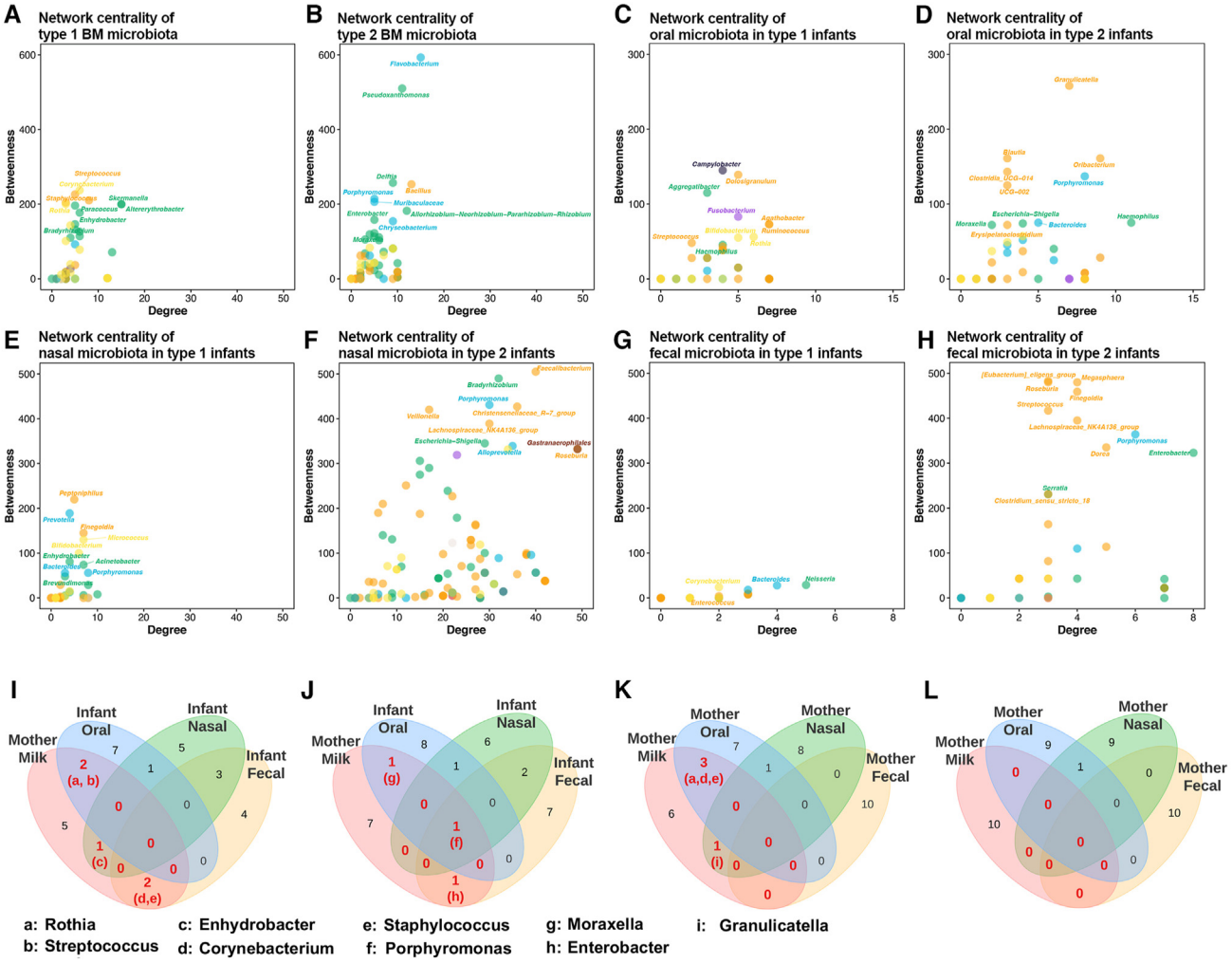

Figure 7. Shared microbial hubs between mother and infant across different body sites. (A–H) Co-occurrence networks at the genus level of the microbiota of mother and infant body sites at 3 M postpartum and 3 M after birth, respectively. Scatter plot of betweenness centrality and degree centrality of BM Type 1 (A), BM Type 1 (B), infant oral microbiota from BM Type 1 (C) and BM Type 2 (D), infant nasal microbiota from BM Type 1 (E) and BM Type 2 (F), and infant fecal microbiota from BM Type 1 (G) and BM Type 2 (H). The co-occurrence analyses were performed based on Spearman's correlation coefficient. Each dot represents a genus and is colored by class. Hub (Top10) genera were selected by the nodes with high betweenness centralities in each group. (I) Venn diagram showing the number of overlapping genera among the BM Type 1 (3 M) and paired infant (3 M) body sites. (J) Overlapping genera among the BM Type 2 (3 M) and paired infant (3 M) body sites. (K) Overlapping genera among BM Type 1 and maternal body sites at 3 M after delivery. (L) Overlapping genera among BM Type 2 and maternal body sites at 3 M after delivery.

structured network with gut-origin hubs (Fig. 7H). These results demonstrate that the type 1 BM microbiota was closely related to the infant's oral microbiota and shared similar microbial hubs, such as *Streptococcus* and *Rothia* (Fig. 7A and C). Unlike type 1 BM microbiota, type 2 BM microbiota was primarily dominated by gut-origin hubs that help establish gut-associated hubs in infants' oral, nasal, and gut microbiota. In contrast, the infants fed with type 1 BM microbiota did not develop nasal and gut microbiota with a good hierarchical structure.

By comparing the top ten hubs (based on betweenness centrality) in both the mother's and infant's nasal, oral, and fecal microbiota, we found that the type 1 BM microbiota shared two hubs with the infant oral microbiota (*Rothia* and *Streptococcus*), one hub with the infant nasal microbiota (*Enhydrobacter*), and two hubs with the infant gut microbiota (*Corynebacterium* and *Staphylococcus*) (Fig. 7I). The type 2 BM microbiota shared a core hub, *Porphyromonas*, with all oral, nasal and gut microbiota in infants. In addition to *Porphyromonas*, the type 2 BM microbiota shared additional hubs with the infant oral microbiota (*Moraxella*) and with the infant gut microbiota (*Enterobacter*) (Fig. 7J). When comparing the top ten hubs between the BM microbiota and mother's microbiota across different body sites, the type 1 BM microbiota shared three hubs with the mothers' oral microbiota (*Rothia*, *Streptococcus* and *Staphylococcus*), one hub with the mothers' nasal microbiota (*Granulicatella*) and no hub with the mothers' gut microbiota (Fig. 7K). Intriguingly, the type 2 BM microbiota shared no hub with all oral, nasal, and gut microbiota in mothers (Fig. 7L). Taken together, our results suggest that the BM microbiota may play an important role

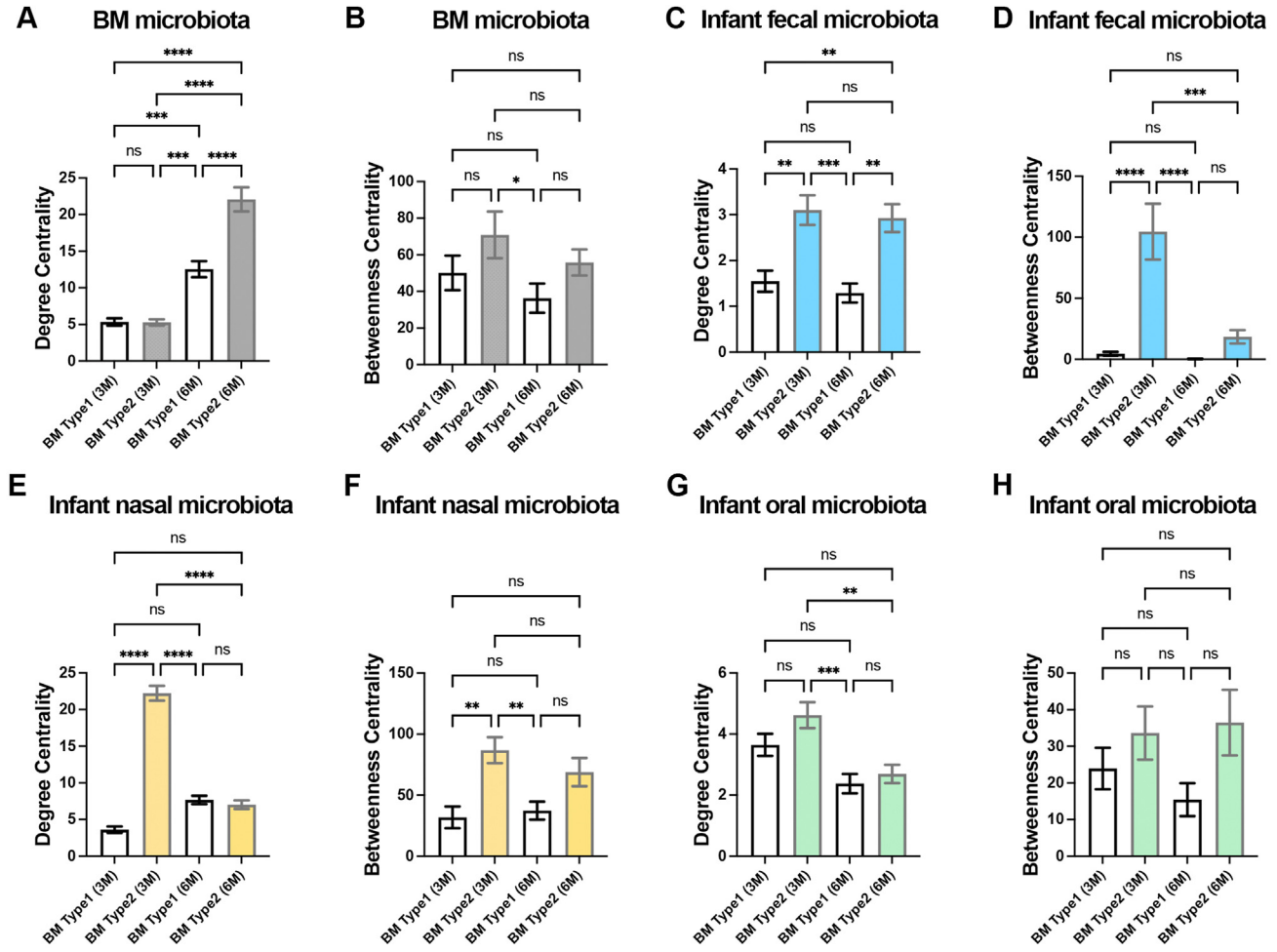

Figure 8. The BM microbiota is critical for the establishment of network centralities in the infant gut and nasal microbiota. Box plots showing the degree centrality in the BM (A), infant fecal (C), infant nasal (E) and infant oral (G) microbiota networks at 3 M and 6 M postpartum. Betweenness centrality in the BM (B), infant fecal (D), infant nasal (F) and infant oral (H) networks 3 M and 6 M postpartum. Data are presented as the mean ± SEM. ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ according to one-way ANOVA and Tukey's post hoc test.

in supporting the establishment of specific microbial hubs in the infant microbiota. Moreover, because we noticed that the type 1 BM, type 1 mother oral, and type 1 infants' oral microbiota all had *Rothia* and *Streptococcus* in their top 10 hubs, the early colonization of mothers' oral hubs in infants' oral cavities might be critical for the formation of type 1 BM microbiota. Furthermore, the dominance of oral-origin hubs may negatively influence microbiota development in infants across different body sites.

We also measured the average degree or betweenness centrality of different microbiota networks in infants 3 and 6 months after birth to understand the impacts of type 1 and type 2 BM microbiota on the hierarchical structure of the infant microbiota. A significant difference in degree centrality between type 1 and type 2 BM microbiota was observed in samples collected six months after delivery but not in samples collected three months after delivery (Fig. 8A). This may be explained by the cosuccession of BM and infant oral microbiota that occurred during this interval. The type 2 BM microbiota also showed higher betweenness centralities than the type 1 BM microbiota at both three and six months after delivery (Fig. 8B). At three months after birth, both the degree and betweenness centralities of the fecal and nasal microbiota in infants were significantly affected by the type of the BM microbiota (Fig. 8C—F), suggesting that the BM microbiota plays a critical role in regulating the community structure of fecal and nasal microbiota in infants. However, the type 2 BM-mediated induction of degree and betweenness centralities in the infant gut and nasal six months after birth were compromised (Fig. 8C—F). This might be due to the effects of complementary feeding and other environmental factors that regulate microbiota maturation at this stage. For oral microbiota in infants, the type 2 BM microbiota also showed a trend toward increased degree and betweenness centralities, although the changes were not statistically significant (Fig. 8G and H). Taken together, the results in this study suggest that the composition of the BM microbiota has a significant impact on the development of hierarchical structure in infant microbiota across different body sites.

Discussion

BM is a complex fluid that provides a range of nutrients and bioactive compounds that support infant growth and development. One critical component of BM is the microbiota, which is a diverse community of bacteria that can have a significant impact on the establishment and composition of the infant's microbiota across different body sites.[6] However, BM can also introduce potentially harmful bacteria into the infant's microbiota, especially if the mother has an infection or is taking antibiotics.[33] In this study, we carefully excluded participants with potential infection issues to specifically investigate the impact of BM commensals on microbiota maturation in infants. Although no pathogen was identified in the BM samples from any enrolled mothers, our results demonstrate that the compositional variations of the BM microbiota may differentially affect the structural maturation of the infant's microbiota. This finding will be further applied to improve the screening criteria of donor milk in our BM bank to enrich the development of preterm infants. Moreover, the microbiota type of the BM may be essential to assure the quality of unpasteurized donor human milk.

Staphylococcus and *Streptococcus* are two genera of bacteria that are commonly found in human BM.[19] While some species of these bacteria, such as *S. aureus* and *Streptococcus agalactiae*,[34,35] are pathogenic and can cause infections, most of them are considered to be commensal and to have a symbiotic relationship with the host. However, the infants receiving *Streptococci*-abundant BM showed compromised microbial networks in the nasal, oral, and gut microbiota when compared with the infants receiving *Staphylococci*-dominant BM in this study. We further confirmed that the primary contributor to *Streptococcus*-abundant BM is *S. mitis*. This suggests that the overloading of the BM with oral-origin bacteria may negatively influence the maturation of the infant's microbiota. Furthermore, good oral hygiene habits for both mothers and infants during the lactation period might help promote a good composition of the BM microbiota.

Intriguingly, we showed that the representation of *S. caprae* had significantly negative correlations with the abundance of the *Streptococcus* genus in BM (3 M) samples. *S. caprae* is a catalase-positive, coagulase-negative *Staphylococcus* that was first described in 1983 in milk samples taken from healthy goats, and is known to colonize the skin and mammary glands of goats.[36] It was later found to colonize healthy human skin, nails, and nasal mucosa.[37,38] Rarely, this commensal organism can become pathogenic in humans, with bone and joints being the most common sites of infection.[37,39] *S. caprae* has been found to inhibit *agr*-mediated quorum sensing by all classes of *S. aureus* and successfully competed against *S. aureus* in colonization and infection models, suggesting that it may have beneficial, protective properties as a human commensal.[40] Nakatsuji et al. recently showed that *Staphylococcus hominis*, another commensal CoNS species, can protect atopic dermatitis patients against *S. aureus* colonization.[41] This exciting advancement demonstrates the successful translation of a commensal's protective properties into an effective treatment, and it highlights the significance of investigating the contribution of normal skin flora to human health. To our knowledge, this is the first study describing the role of *S. caprae* in human BM and demonstrating that it confers a competitive advantage to limit the outgrowth of oral-origin organisms in BM. Further studies are warranted to characterize the inhibitory activity of *S. caprae* and investigate its impact on *S. aureus* and *S. mitis* colonization and pathogenesis.

Numerous studies have explored the connection between maternal BMI and BM microbiota, and have demonstrated that maternal BMI can have an impact on the abundance and diversity of specific bacterial taxa in BM.[6,7] However, contradictory findings have emerged[42,43] suggesting the involvement of complex factors. Hormone profiles, breastfeeding practices, dietary patterns, and metabolic changes associated with obesity may contribute to this association.[44,45] Hence, this study specifically focused on mother—infant pairs to enhance our understanding of the interplay between maternal and infant microbiota across different body sites.

The impact of high-BMI-associated bacteria on infant health remains inconclusive. While some studies have reported an increased abundance of *Staphylococcus* and a decreased abundance of *Bifidobacterium* in the BM of mothers with higher BMI,[45,46] our findings indicate an inverse association between maternal BMI and the abundance of *Staphylococcus* genera, primarily attributed to *S. caprae* in the BM microbiota. Notably, the presence of *S. caprae* exhibits a significant negative correlation with oral-origin species in BM, suggesting that the interplay between maternal BM microbiota and the infant's oral microbiota plays a crucial role in orchestrating the composition of the BM microbiota. This implies that specific maternal species may be critical in regulating the dominance of oral-origin bacteria, which is important for infant microbiota maturation across different body sites. Based on our findings, a lower maternal BMI is linked to an increased representation of *S. caprae*, thereby maintaining a lower abundance of oral-origin bacteria in BM and promoting an observable hierarchical structure of nasal and gut microbiota in infants. However, the detailed mechanisms that connect maternal BMI and BM microbiota are not fully understood. Further research is necessary to elucidate the cause-and-effect relationship and its implications.

All of the enrolled mothers were donors recruited from the milk bank, and were well educated by milk bank staff on how to avoid the contamination of BM. The strict enrollment criteria excluded cases with positive donor serum virology or BM culture of *S. aureus*. This enabled us to investigate the commensal interactions between mothers and infants specifically. The infants of the dyads were exclusively fed BM during the study period. Hence, BM can be representative of the sole food source of infants in their early life (3 M). The long-term influence of BM type on infants' physical status and susceptibility to diseases will be further investigated in a follow-up study.

In conclusion, we demonstrated that maternal BMI is significantly correlated with the abundance of *S. caprae* in their BM, which contributes to the construction of microbiota type. Our results suggest that the microbial composition of BM contribute significantly to the establishment of the

microbiota hierarchy in infants. The alterations in the BM microbiota composition associated with higher maternal BMI may have implications for infant health.

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Author contributions

JWR, YJC, YHT, CYC and YCL designed, performed, and evaluated the sample collection, questionnaire, and experiments; JWR, YCL and PCC performed bioinformatic analyses; PJT and YJY provided resources; JWR, CYC and YCL wrote the manuscript; JWR, CYC and YCL supervised the entire study.

Data availability

The 16S rRNA sequencing data have been deposited in NCBI under the following BioProject ID: PRJNA910662. This study does not report the original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Declaration of competing interest

All authors have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2023.07.005.