Co-occurence of Secretory Immunoglobulin A-Coated Bacteria in Maternal Gut, Breast Milk, and Infant Gut in Humans

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Research

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Abstract

Background

Gut microbiota promote and maintain infant health. Vertical transmission of bacteria from the maternal gut through breast milk to an infant is an important source of microbial colonisation in human offspring. However, the causative active/culturable bacteria and mechanisms responsible for their mother-neonate vertical transfer via breastfeeding remain unclear. Secretory immunoglobulin A (sIgA) may mediate this vertical transmission; however, evidence supporting this hypothesis is required. In this study, we aimed to investigate whether sIgA-coated bacteria in the maternal intestine may migrate to breast milk and colonise the infant gut.

Results

Maternal faeces, breast milk, and neonatal faeces were collected from 19 mother-infant dyads during lactation stages specific to colostrum, transitional, and mature milk. sIgA-coated bacteria were enriched using magnetic-activated cell sorting, and live bacteria were cultured in lactic acid bacteria- and gut bacteria-specific medium. 16S ribosomal RNA gene amplicon sequencing showed that microbiota diversity in maternal faeces, breast milk, and infant faeces decreased sequentially from colostrum to transitional milk to mature milk. Significant beta diversity existed between sample types ($p < 0.05$). However, high similarity was found between sIgA-coated microbiota of the three types of samples at the mature milk stage. Source track analysis showed that sIgA-coated microbiota in breast milk and maternal gut are major contributors of sIgA-coated microbiota in infant gut. Genera with co-occurrence in sample types included *Bifidobacterium*, *Enterococcus*, *Streptococcus*, *Lactobacillus*, *Klebsiella*, *Escherichia-Shigella*, and an unclassified genus of Enterobacteriaceae. Shotgun sequencing of three dyads identified co-occurring species *Lactobacillus* and *Bifidobacterium*, including *Lactobacillus salivarius*, *Lactobacillus reuteri*, *Lactobacillus gasseri*, *Lactobacillus jonsonii*, *Lactobacillus oris*, *Bifidobacterium longum,* and *Bifidobacterium breve*.

Conclusions

Breastmilk and infant faeces samples showed unique microbial composition and diversity in the three lactation stages. The fractions of sIgA-coated microbiota in maternal faeces, breast milk, and infant gut showed similar bacterial abundance patterns. This study will facilitate development of strategies to adjust aberrant microbial establishment and reduce the risk of disease by providing essential information for effective probiotic administration to the neonate and/or breastfeeding mother.

Background

Gut microbiota play an important role in promoting and maintaining infant health [1]. During the lactation period after birth, infant intestinal microbiota are primarily shaped by breast milk bacterial colonisation [2]. A diverse community of bacterial species has been found in human milk in women in initial

pioneering studies. The origin of human milk bacteria remains largely unknown. Although the infant's oral cavity and maternal skin may provide microbes to milk, selected bacteria of the maternal digestive microbiota may access the mammary glands via oral and entero-mammary pathways involving interactions with immune cells. Although *Streptococcus* and *Staphylococcus* are predominant in human milk [3], a low abundance of gut-associated genera, including that of *Bacteroides*, *Clostridium*, *Faecalibacterium*, *Roseburia*, and *Bifidobacterium,* was repeatedly detected in breast milk [4]. However, the causative active/culturable bacterial species/strains and mechanisms responsible for mother-neonate vertical transfer of bacteria via breastfeeding remain unclear. Recent research has focused on shared bacterial species/strains between breast milk and infant faeces in mother-infant dyads using microbiological isolation and strain-level genotyping of bacterial isolates [5]. The findings confirmed that only limited isolated bacterial species were shared by a few mother-infant dyads, and were dominated by *Staphylococcus* (*Staphylococcus epidermidis*, *S. hominis)* and *Escherichia/ Shigella spp*, with lower levels of *Bifidobacterium* (*Bifidobacterium breve* and *B. longum*) and *Lactobacillus* (*Lactobacillus fermentum*, *L. gasseri*, *L. plantarum*, *L. reuteri*, *L. salivarius*, *and L. vaginalis*) [6]. Rare breast milk bacteria that occur at levels below the detection limit have been shown to colonise the gut in a breast milk microbiota-associated mouse model [7]. Metagenomic sequencing analysis identified the sharing of only *B. longum* and *Enterococcus faecalis* across maternal gut and breast milk [8]. Therefore, alternative methods are required to identify additional candidate bacteria that are potentially transferred from maternal breast milk to the infant gut.

The entero-mammary pathway suggests that microbes located in the maternal gut translocate to the mammary glands, and upon milk consumption, colonise the infant gut [9]. Peyer's patches (PPs) in the small intestine and associated microfold cells represent primary sites for uptake and presentation of lumen bacteria [10]. Microfold cells can selectively uptake secretory immunoglobulin A (sIgA)-coated bacteria through specific immunoglobulin (Ig)A receptors that can be internalised by subepithelial dome PP dendritic cells (DCs) [11][12]. DCs can carry internalised bacteria to mesenteric lymph nodes and retain small numbers of live commensals for several days [13]. These commensals can be transported through the mucosal lymphatic circulation to other parts of the body, such as the mammary glands [14]. sIgA-coated bacteria in PPs that are closer to DCs suggest that transference to the mammary gland is easier than transference of bacteria to the intestinal lumen [15]. sIgA is also secreted into the breastmilk of lactating mothers and may support the establishment and maturation of the gut microbiota in early life by colonisation promotion [16]. Therefore, sIgA may mediate the vertical transmission of specific bacteria from mother to child. However, evidence supporting this hypothesis is required.

A mouse model revealed that sIgA level was highest on bacteria from the proximal small intestine and lowest in those from the colon, and sIgA coating was not homogenous, yet specifically enriched for some microbiota members [17]. Bacteria that demonstrated sIgA coating in the colon were typically also observed in the small intestine [17]. Commensal members of the facultative anaerobic γ-Proteobacteria or *Lactobacillus* are frequently motile bacteria with the ability to colonise in close proximity to the healthy epithelial surface and exhibit a high degree of sIgA coating [18].

The current study aimed to identify sIgA-bound bacterial genera in maternal faeces, breast milk, and infant faeces in mother-infant dyads in three stages of lactation. 16S rRNA sequencing and cluster analysis were used to verify whether bacterial transfer occurred in an sIgA-coated state. The findings of this study increases our understanding of neonatal gut development and provides future opportunities for adjusting aberrant microbial establishment and reducing the risk of disease by providing essential information for effective probiotic administration to the neonate and/or breastfeeding mother.

Results

Study sample characteristics

Information regarding basic anthropometrics and reproductive history of all subjects at enrolment is listed in Table 1. On average, women were 30 ± 6 years old, weighed 54 ± 8 kg prior to pregnancy, and had 1.3 ± 0.4 children. All infants were exclusively breastfed at 1 month. Most samples were collected from both mother and infant at each time point; however, we were unable to obtain all samples owing to their mishandling by study personnel. Ultimately, 19 paired samples were obtained, including maternal faeces, infant faeces, and breast milk from each of the colostrum, transition, and mature milk stages, and sIgA-coated microbiota of maternal faeces, mature milk, and infant faeces of mature milk stage, in addition to the absence of one maternal faeces, one colostrum, three mature milk, and one infant faecal sIgA-coated microbiota samples (Supplementary Table 1).

Sequencing summary

All samples were sequenced by bacterial 16S rRNA amplicon sequencing, resulting in a total of 184 high-quality metagenomes with average counts per sample of 21399. From these samples, three paired samples of maternal faeces, mature milk, and sIgA-coated microbiota samples of mature milk, infant, and maternal faeces were shotgun sequenced, yielding a total of 15 high-quality metagenomes, with an average of 5.34 (±0.13) bases per sample after quality control.

Alpha and beta diversity

As shown in Fig. 1, the bacterial diversity index (Shannon index) of maternal faeces was significantly higher than that of breast milk and infant faeces, except for transitional milk ($P < 0.05$). There was also a significant difference ($P = 0.02$) between infant faeces at the colostrum stage compared with that for the mature milk stage. However, no difference was found among milk from different lactation periods, and among sIgA-coated microbiota of different samples. For bacterial richness estimation (Chao1), there were significantly higher levels in colostrum ($P = 0.0.2$) and transitional milk ($P < 0.001$), and lower level in infant faeces of colostrum stage ($P = 0.02$) compared with maternal faeces, respectively. There were no differences between breast milk and infant faeces samples at different lactation periods and samples of sIgA-coated microbiota.

Beta diversity analysis is presented using principal coordinate analysis (PCoA) of the Brary–Curtis distance matrices (Fig. 2). For the colostrum stage sample, PC1, PC2, and PC3 explained 17.09%, 13.97%, and 10.38% of the between-sample variation, respectively, and all different sample types showed high differences (Anosim R ranged from 0.456–0.893, P = 0.001) (Fig. 2A). At the transitional milk stage, the difference between infant faeces and breast milk decreased (R = 0.339, P = 0.001) (Fig. 2B). However, at the mature stage, there was a high similarity between the breast milk and sIgA-coated microbiota in the breast (R = 0.133) (Fig. 2C). For all sIgA-coated microbiota samples, infant faeces showed high similarity with mother faeces (R = 0.188) and mature milk (R = 0.17). To a lesser extent, due to PC3 contribution (10.32%), maternal faeces showed relatively high similarity to breast milk (R = 0.395) (Fig. 2D).

Potential contribution of the maternal gut and breast milk to infant gut bacterial communities

Using Feast software for microbial source tracking, we estimated likely contributions to infant faecal bacterial communities (sink) using rarefied taxon read counts of operational taxonomic units (OTUs) from milk (source), maternal faecal (as a proxy of the maternal colonic bacteria; source), and sIgA-coated maternal faeces microbiota (source), at three lactation periods. For sIgA-coated microbiota, we also studied contributions of the maternal gut (source) and breast milk (source) to the infant gut (sink).

The contribution of maternal faeces to infant faeces microbiota increased from the colostrum to mature milk stage. At the colostrum stage, source proportions were 20%–44% in 22% of mother/infant pairs (Fig. 3A) and changed to a 18%–67% contribution in 50% of mother/infant dyads in the transitional milk stage (Fig. 3B). The contribution increased to 25%–78% at the mature milk stage (Fig. 3C). Breast milk showed a relatively stable contribution during the three stages, ranging from 12 to 86% in 22% of mother/infant dyads. It is evident that sIgA-coated microbiota of breast milk and maternal gut are the major contributors of sIgA-coated microbiota in infant gut, 14~93% of source proportion in approximately 94 mother/infant dyads (Fig. 3D).

Co-occurrence of specific genera between different sIgA-coated samples.

The unweighted pair-group method with arithmetic means (UPGMA) analysis of sIgA-coated microbiota resulted in three typical clusters (Fig. 4A). The majority of samples of maternal faeces/breast milk, maternal faeces/infant faeces, or breast/infant faeces pairs had similar microbial patterns belonging to cluster G1, accounting for 68% of sample pairs. Clusters G2 and G3, G1 and G2 are specific samples of maternal and infant faeces, respectively. The abundance distribution of the 30 dominant genera among the three types of samples was displayed in a species abundance heatmap (Fig. 4B). The heatmap revealed several genera that exhibited co-occurrence between samples, including *Bifidobacterium*, *Enterococcus*, *Streptococcus*, *Lactobacillus*, *Klebsiella*, *Escherichia-Shigella* and an unclassified genus of Enterobacteriaceae. In addition, *Staphylococcus* is the dominant bacteria in breast milk and infant faeces; however, it occurs at a very low level in maternal faeces.

We defined core milk microbiota genera present in at least 90% of individuals with a minimum mean relative abundance of 0.01 % [19], as shown in Fig. 3C. Colostrum is rich in core bacteria and 11 genera.

In the transitional stage, there were seven co-occurrence core genera with colostrum including *Streptococcus*, *Bifidobacterium*, *Escherichia-Shigella*, the three typically core genera of maternal faeces, and *Staphylococcus*, *Klebsiella*, *Acinetobacter*, and *Lactobacillus*. However, only *Staphylococcus*, *Klebsiella*, and *Acinetobacter* remained in the mature stage. *Streptococcus*, *Bifidobacterium*, *Staphylococcus* and *Escherichia-Shigella* because core genus of infant gut of corresponding lactation period. At the mature milk stage, infant gut and sIgA-coated microbiota in the mature milk stage, the intestinal flora of infants, and the sIgA-coated microbiota share similar five core bacteria. Among them, *Streptococcus*, *Bifidobacterium*, and *Klebsiella* are also core genera of sIgA-coated breast milk microbiota. The most prominent feature was that the mother's gut contained 11 specific core bacteria, suggesting that the infant's gut microbiota is much simpler than that of the adult. Meanwhile, only *Escherichia-Shigella* and *Enterococcus* were in sIgA-binding bacteria of maternal faeces.

Different genera and families between sample types

As lactation progressed, *Streptococcus* abundance in breast milk gradually decreased, while *Enterococcus* and *Bifidobacterium* gradually increased. *Staphylococcus* maintains a considerable superior abundance. *Enterococcus* and *Escherichia-Shigella* increased in mature milk at the cost of reduced *Lactobacillus* (Fig. S1). Edge analysis identified several families significantly decreased in abundance in mature milk compared with those in colostrum, including *Ruminococcaceae*, *Corynebacteriaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, and *Microboccaceae* (Fig. 4).

In infant faeces, *Klebsiella* and *Escherichia_ Shigella* abundance increased and decreased gradually, respectively, during the lactation stages. The abundance of adult-specific families, Lachnospiraceae and Bacteroidaceae, significantly increased in mature milk compared with that in colostrum (Fig. 4). In sIgA-coated microbiota, *Lactobacillus* and *Bifidobacterium* became gradually enriched from maternal faeces to breast milk to infant (Fig. S1). Lactobacillaceae in breast milk and infant faeces significantly increased compared with maternal faeces (Fig. 5).

Identification of co-occurrence species by shotgun sequencing

According to the principal component analysis of shotgun sequenced results in Fig, 6A, there was a significant difference between breast milk, maternal faeces, and sIgA-coated microbiota. Three different sIgA-coated microbiota showed no separation. In contrast with 16S amplicon sequencing, two additional co-occurrence genera were identified by shotgun sequencing, *Clostridium* and *Gardneralla* (Fig, 6B). Among the classified species, *B. longum* was the dominant co-occurrence, followed by *Bifidobacterium breve*. Co-occurrences *of Lactobacillus* in sIgA-coated bacteria are primarily *L. salivarius*, *L. reuteri*, *Lactobacillus gasseri*, *Lactobacillus jonsonii*, and *Lactobacillus oris*. Among them, *L. reuteri* and *L. gasseri* were the dominant species in sIgA-coated bacteria in the infant gut.

Kyoto Encyclopedia of Genes and Genomes (KEGG) functional categories shared in metagenomes of sample dyads of sIgA-coated microbiota

Eight KEGG pathways (level 4) shared between shotgun sequencing samples were identified (Fig. 7). Two pathways were associated with energy metabolism, including sucrose-6-phosphatase, which is involved in starch and sucrose metabolism (K07024) and the LacI family transcriptional regulator involved in maltosaccharide utilisation (K02529). Four pathways were associated with the survival of bacteria in an ever-changing and hostile environment. Among them, the putative ABC transport system permease protein (K02004) is involved in negative regulation of biofilm formation. The putative ABC transport system ATP-binding protein (K02003) can facilitate the acquisition of essential compounds from the extracellular environment. The ATP-binding cassette, subfamily B (K06147), and ABC-2 type transport system ATP-binding protein ABC importers (K01990) evolved the use of multiple mechanisms to transport nutrients across the membrane that aid survival in an ever-changing and hostile environment. Another two enzyme that is widely used in bacteria is the putative transposase K07497 and the ABC-2 type transport system permease protein (K01992).

Discussion

This study compared the membership and composition of microbiomes from maternal milk and faeces, and infant faeces from the same mother-infant dyad at three time points during the lactation period. Meanwhile, the composition of sIgA-coated bacteria in matched samples of maternal faeces, breast milk, and infant faeces at the mature milk stage were also studied. Samples from different niches showed distinct microbiota structure and core microbiota, as has been shown previously for other subsets of mother-infant pairs [20][21][22]. The present study provides novel insight into the transmission of bacteria from maternal to infant gut through breast milk. Our findings provide evidence that sIgA-coated bacteria are shared and potentially transferred from mothers to infants through breastmilk.

In our study, the Shannon index of colostrum was lower than that of maternal faeces; and Caho1 was higher than that of maternal faeces, indicating that colostrum contained a variety of bacteria with low abundance; however, the dominant bacteria were relatively simple. During each lactation period, the alpha diversity of breast milk microbiota was higher than that of the corresponding infant faeces, consistent with other reports [23]. We observed an increase in the Shannon index from the colostrum to mature stage in the infant faecal microbiome, as reported previously [20] [24]. This might be a result of continuous seeding of breast milk bacteria and/or interactions with resident gut microbes, leading to successional community shifts. The beta diversity of the total microbiota does not support the hypothesis that the milk microbiome would most closely resemble that of infant faeces, which concurs with a previous study [22].

The aim of our study was to discover both anaerobic and aerobic, active/culturable, transmissible bacteria that pass from mother to infant via breastfeeding. sIgA-coated bacteria were collected in an anaerobic environment during all steps. Multiple magnetic bead enrichments were performed to verify the purity of sIgA-coated bacteria. The original study focused on sIgA-coated bacteria using bacterial flow cytometry, magnetic-activated cell sorting (MACS), and 16S rRNA gene sequencing (IgA-Seq) to identify IgA-bound bacteria [25]. However, it is possible that some anaerobic bacteria died during bacterial flow

cytometry analysis. Our study used only MACS to analyse the composition of sIgA-coated bacteria in mature milk, corresponding neonatal faeces, and maternal faeces. Live bacteria were enriched by lactic acid bacteria and gut microbiota medium (GMM) [26], which enabled the analysis of active bacteria.

Feast-based microbial source tracker analysis showed an increased contribution of maternal faeces and breast milk to infant faeces microbiota from the colostrum to mature milk stage. During the colostrum stage, a large number of bacterial sources were labelled “unknown”. The source of these “unknown” bacteria may be the vagina, skin, and intrauterine environment such as the placenta and amniotic fluid [27]. There was a high contribution of sIgA-coated bacteria in breast milk or maternal faeces to that of infant faeces. This partially supports the hypothesis that sIgA might mediate vertical transmission of specific bacteria from the maternal gut to the infant gut through breast feeding. Recent studies revealed that sIgA coats a “diverse but defined subset of the microbiota” in the gut [28][29]. This may enhance the colonisation of these bacteria by promoting adhesion and/or nutrient utilisation [16]. These sIgA-coated bacteria may be recognised by sIgA receptors present in the microfold cell in follicular epithelium of Peyer’s patch and dendritic cells [11][12], which may mediate their transport by dendritic cells to the mammary gland [13]. High levels of sIgA are also found in breast milk, with concentrations up to 15 mg/mL in colostrum and ~1 mg/mL in mature milk, representing >90% of milk antibodies [30], providing the breastfed infant 0.5~1 g/day. Infants are completely reliant on sIgA owing to their immature immune system. Early exposure to passive sIgA in breast milk has lasting beneficial effects by regulating gene expression in intestinal epithelial cells in offspring throughout life [31]. IgA-producing plasma cells in the mammary gland originate from the maternal gut [32]. sIgA specificity in breastmilk is therefore dictated by maternal exposure to commensal bacteria in the maternal gut. This may allow sIgA to coat the maternal gut-derived bacteria in breast milk and help them colonise the infant gut.

We also found that maternal faeces, breastmilk, and infant faeces shared several species of sIgA-coated bacteria including *Bifidobacterium*, *Enterococcus*, *Streptococcus*, *Lactobacillus*, *Klebsiella*, *Escherichia-Shigella*. We previously found that *Bifidobacterium*, *Enterococcus*, *and Lactobacillus* also belonged to the dominant genus in sIgA-coated microbiota of healthy late pregnant women [33]. In addition, although *Staphylococcus* is the shared bacterium of sIgA-coated microbiota in breast milk and baby faeces, it is not the dominant bacterium of in maternal faeces, which indicates that the species origin is the skin.

However, the shared bacteria of mother and infant samples were not identical to the core bacteria of the corresponding group. This is because no bacteria were commonly shared in all dyads, potentially reflecting inter-individual variability of milk microbiota profiles, consistent with recent research [34]. Nevertheless, increased sharing with prolonged and direct breastfeeding, and strong positive correlations between the milk and gut relative abundances of shared bacteria provide evidence that some degree of bacterial seeding is plausible.

B. longum and *B. breve* were dominant co-occurring species in sIgA-coated microbiota. A previous study based on real-time polymerase chain reaction (RT-PCR) showed that *B. longum* was the most commonly found species in breast milk [35]. Some strains persisted in the infant gut while co-existing with the other

predominant bifidobacterial species [36]. It was found that the secretory component of sIgA carried glycan residues which can interact with symbiotic bacteria, including *Lactobacillus*, *Bifidobacteria*, *Escherichia coli*, and *Bacteroides* [37]. *Bifidobacteria* have been shown to induce high levels of IgA in the gut, which promote the production of sIgA targeting themselves [38]. This was confirmed by a study that revealed that the number of *Bifidobacteria* and Enterobacteriaceae were high in the IgA-coated fraction of infants [39].

Lactobacillus is another co-occurrence genus in sIgA-coated microbiota in the present study, which is one of the specific bacterial taxa in human faeces that are highly coated with IgA [25]. Dominant species were *L. reuteri* and *L. gasseri*, which are commonly isolated from breast milk [40][41]. We recently found that *L. reuteri* is a typical species in the sIgA-coated bacterial fraction of healthy pregnant women [33]. The occurrence of *L. reuteri* in breast milk was higher in breast milk samples from rural and pastoral areas of China than in those from the industrialised region [33]. Strains isolated from the sIgA–targeted fraction of the former improved intestinal epithelial barrier function in mice [42].

In KEGG functional categories shared in metagenomes of sample dyads of sIgA-coated microbiota, the identified pathway was related to sucrose metabolism, maltosaccharide utilisation, and that related to the ABC transport system. A recent study revealed that the ABC transporter is a key genetic factor for fucosyllactose utilisation in *Bifidobacteria* [43]. ABC-type transporters are also involved in the transport of B vitamins by gut commensal bacteria [44]. This suggests that co-occurrence bacteria can acquire essential compounds from the extracellular environment to survive in the gut.

Limitations of this study include a limited sample capacity and a lack of attention to strain-level identification. Additionally, gut bacteria-specific media were used to activate sIgA-coated bacteria, which may have led to a preference for specific genera. Whether GMM and De Man Rogosa Sharpe medium could restore the microbial structure of the intestine should be further confirmed.

Conclusions

Breastmilk and infant faeces samples showed unique microbial composition and diversity in the three lactation stages. Fractions of sIgA-coated microbiota of maternal faeces, breast milk, and infant gut showed a similar bacterial pattern, shared with genera including *Bifidobacterium*, *Enterococcus*, *Streptococcus*, *Lactobacillus*, *Klebsiella*, *Escherichia-Shigella* and an unclassified genus of Enterobacteriaceae. According to shotgun sequencing, the co-occurrence species of *Bifidobacterium* and *Lactobacillus* were dominated by *B. longum*, *L. reuteri,* and *L. gasseri*, sharing functional genes to survive in an ever-changing hostile environment.

Methods

Milk sample collection

Thirty-six mothers were recruited after delivery at The Affiliated Wuxi Maternity and Child Health Care Hospital of Nanjing Medical University from March 2018 to June 2020 (Fig. 8). The recruitment criteria for mother-infant dyads were as follows: (i) vaginal delivery at term (≥37 weeks gestation), (ii) exclusive breastfeeding during the sampling period, (iii) no antibiotic/probiotic exposure of either the mother or infant during pregnancy, intrapartum, or postnatal. Clinical data including maternal age and weight, delivery mode, neonate weight, lactation stage, gestational time, blood biochemistry, and routine blood tests were collected (Table 1). Ethical approval for this study was provided by The Affiliated Wuxi Maternity and Child Health Care Hospital of Nanjing Medical University, and all participants provided written informed consent.

Pregnancy duration was >38 weeks for all mothers, and samples were collected within 1 month after delivery. Breast milk was divided into three stages according to the time after delivery: colostrum (days 1–5), transitional (days 6–15), and mature (day 16 onwards) [45]. We collected breastmilk at three stages, and the corresponding infant and maternal faecal samples. Faecal and breastmilk sample collection was standardised for all subjects. Prior to milk sample collection, the maternal gland was sterilised with 75% alcohol and the first drops were discarded to minimise contamination [46]. Spot infant faecal samples were collected from paper diapers on a super clean bench. Maternal faecal samples were stored in a plastic box. Maternal and infant faecal and breastmilk specimens were stored in plastic containers at 4 °C in home refrigerators until they were brought to the study clinic no more than 24 h after collection. At the study clinic, samples were frozen at -80 °C until analysis.

Enrichment of sIgA-coated bacteria through MACS

Milk samples (10 mL) were divided into two parts, one (5 mL) for direct DNA extraction, and the other for identifying sIgA-coated bacteria. Nanoscale aminated magnetic beads and streptomycin affinity nanomagnetic beads were prepared using methods previously developed in our lab [18]. Thereafter, 5 mL of maternal breastmilk was centrifuged at 10,000 ×*g* for 10 min [47], and the precipitate was resuspended in phosphate-buffered saline (PBS). Either 5% goat serum (40 μL), biotin-labelled rabbit anti-human IgA (20 μL), or streptavidin-labelled nanomagnetic beads (250 μL) were added to the bacterial suspension with PBS, which was subsequently placed in an ice bath for 20 min. Nanomagnetic beads were adsorbed with a magnet, and the supernatant was discarded. Finally, the magnetic bead and bacterial cell combination were washed several times with PBS until the supernatant was clean and the enriched bacteria were resuspended in PBS. Aliquots (100 μL) were added to De Man Rogosa Sharpe medium and GMM [26], and anaerobically cultured for 48 h at 37 °C.

Infant and maternal faecal samples corresponding to maternal breastmilk samples were weighed and dissolved in peptone buffer to prepare a 20% faecal bacterial suspension. Half of the sample was stored with 30% glycerine for DNA extraction, and half was supplemented with 0.5% Tween 20, vortexed, and centrifuged at 1,000 ×g for 10 min. Thereafter, the supernatant was removed by washing the precipitate with peptone buffer three or four times. Breastmilk samples were prepared in the same way; however, peptone was used instead of PBS.

All steps were carried out in an anaerobic glove cabinet (DROID Instruments and Equipment Co., Ltd., Shanghai, China).

DNA extraction

Bacterial genomic DNA was extracted using a reaped bead-beating method using an Ezup Column Bacteria Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China) with a minor modification as previously reported [48]. Briefly, cell lysis was achieved by bead beating with zirconium beads (0.1 g, 0.7 mm: 0.3 g, 0.15 mm) on an oscillator at 6,000 rpm with two circulations (30 s per circulation; Precellys 24, Bertin Technologies, Montigny-le-Bretonneux, France) in the presence of 4% (w/v) sodium dodecyl sulphate, 500 mM NaCl, and 50 mM ethylenediaminetetraacetic acid. Ammonium acetate was used to precipitate and remove impurities, and sodium dodecyl sulphate in addition to isopropanol precipitation was used for nucleic acid recovery. RNA and proteins were removed or degraded using RNase and Proteinase K, respectively, followed using an Ezup Column Bacteria Genomic DNA Purification Kit. Genomic DNA concentration was determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and DNA integrity was determined by electrophoresis on 1% agarose gels. DNA concentration for all samples was standardised to 10 ng/μL.

16S rRNA gene sequencing

Microbial profiles were analysed by 16S rDNA sequencing at GENEWIZ, Inc. (Suzhou, China). To maximise the effective length of the MiSeq 250PE and 300PE sequencing reads, a region of approximately 469 bp encompassing the V3 and V4 hypervariable regions of the 16S rRNA gene was targeted for sequencing. The PCR primers used to amplify V3 and V4 hypervariable regions were as follows: forward 5'- CCT ACG GRR BGC ASC AGK VRV GAA T -3' and reverse 5'- GGA CTA CNV GGG TWT CTA ATC C -3'. In addition, an indexed linker was added to the end of the 16S rDNA PCR product for next-generation sequencing (Illumina, San Diego, CA, USA). First-round PCR products were used as templates for a second round of PCR amplicon enrichment (94 °C for 3 min, followed by 24 cycles at 94 °C for 5 s, 57 °C for 90 s, and 72 °C for 10 s, and a final extension at 72 °C for 5 min). PCR reactions were performed in triplicate using a 25 μL mixture containing 2.5 μL of TransStart Buffer, 2 μL of dNTPs, 1 μL of each primer, and 20 ng of template DNA. DNA library concentration was validated using a Qubit 3.0 Fluorometer. The libraries were quantified to 10 nM, and subsequently multiplexed and loaded on an Illumina MiSeq instrument according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was performed using PE250/300 paired-end; image analysis and base calling were performed using the MiSeq Control Software embedded in the MiSeq instrument.

Metagenomic sequencing

DNA was sequenced using an Illumina HiSeq 3000 at GENEWIZ Co. (Suzhou, China). Cluster generation, template hybridisation, isothermal amplification, linearisation, and blocking denaturing and hybridisation of sequencing primers were performed according to the workflow specified by the service provider.

Libraries were constructed with an insert size of approximately 500 bp, followed by high-throughput sequencing to obtain paired-end reads with 150 bp in the forward and reverse directions.

For data quality control, Prinseq [49] was employed to: 1) trim reads from the 3' end until reaching the first nucleotide with a quality threshold of 20; 2) remove read pairs when either read was <60 bp or contained “N” bases; and 3) de-duplicate reads. Reads that could be aligned to the human genome (*Homo sapien*, UCSC hg19) were removed (aligned with Bowtie2 [50] using –reorder –no-hd –no-contain –dovetail).

High-quality paired-end reads from each sample were used for de novo assembly with IDBA_UD [51] into contigs of at least 500 bp. Genes were predicted using MetaGeneMark [52]. A non-redundant gene catalogue of 4,893,833 microbial genes was constructed with CD-HIT using the parameters “-c 0.95 –aS 0.9”. High-quality reads were mapped onto the gene catalogue using SOAPaligner [53]. Aligned results were sampled and downsized to 21,399 per sample. The soap.coverage.script was used to calculate the gene-length normalised base counts in each downsizing step. The sampling procedure was repeated 30 times, and the mean abundance value was used in further analyses. Based on the orthologous genes from the KEGG database, gene function was annotated and quantified.

Sequence data analysis and statistical analysis

The QIIME data analysis package was used for 16S rRNA data analysis. The forward and reverse reads were joined and assigned to samples based on the barcode and truncated by cutting off the barcode and primer sequence. Quality filtering on joined sequences was performed, and sequences that did not fulfil the following criteria were discarded: sequence length <200 bp, no ambiguous bases, mean quality score ≥20. Thereafter, sequences were compared with those in the reference database (RDP Gold database) using the UCHIME algorithm to detect chimeric sequences; all chimeric sequences were removed. The remaining sequences were used in the final analysis. Sequences were grouped into OTUs using the VSEARCH clustering program (1.9.6) against the Silva 132 database, pre-clustered at 97% sequence identity. The Ribosomal Database Program classifier used the Silva 132 database, which contains taxonomic categories predicted to the species level, for assignment to all OTUs at a confidence threshold of 0.8.

Sequences were rarefied to the minimum library size and total sum scaling was applied. Alpha diversity indices were calculated in QIIME from rarefied samples using the Shannon index for diversity and Chao1 indices for richness. Beta diversity was calculated using PCoA based on the distance between the matrix Brary–Curtis and visualised through PCoA plots, and similarity was measured using the Anosim test (9,999 permutations) [54]. An UPGMA clustering tree was constructed using the weighted clustering hierarchy and the group average method.

The relative contribution of the mother’s gut and breast milk to the assembly of bacteria in the infant gut was analysed with feast (V.1.0) [55]. OTUs present in <1% of samples were first filtered, and the resultant OTU table was imputed with default parameters, with infant faeces as the “sink” and the samples from

the mother (faeces, milk) identified as the “source”. The results were aggregated into three categories: mother gut, milk, and other (unknown).

The core community was defined as the selection of information on genus taxonomy with a relative abundance of at least 0.01% and present in more than 90% of tested samples. Differentially represented bacterial families for all comparisons were identified using edgeR in R Bioconductor [56].

Abbreviations

BM, mature breast milk; BMC, sIgA-coated microbiota in breast milk of mature stage; IF, infant faeces; IFC, sIgA-coated microbiota in infant faeces of mature stage; MF, maternal faeces; MFC, sIgA-coated microbiota in maternal faeces.

Declarations

Ethics approval and consent to participate

This study was approved by the Internal Ethics Committee of the Institute of Chinese Medical Sciences, Jiangnan University according to a protocol (JN20130918) developed by The Affiliated Wuxi Maternity and Child Health Care Hospital of Nanjing Medical University according. All subjects provided informed consent to be included in the study.

Consent for publication

Not applicable.

Availability of data and material

The data sets supporting the results of this article are available in the NCBI Sequence Read Archive, BioProject is PRJNA542027 (accession # SUB5578272)

(https://submit.ncbi.nlm.nih.gov/subs/sra/SUB5578272/overview).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MD and JS participated in data collection performed the analyses and wrote the paper. CQ and RY designed the study, collected the data and participated in data analysis. DL, RY, HX and QZ participated in data analysis and writing of the paper. DC performed data pre-processing.

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Data availability.

The data sets supporting the results of this article are available in the NCBI Sequence Read Archive, BioProject is PRJNA542027 (accession # SUB5578272) for 16s sequencing data and PRJNA687137 (accession # SUB8771143) for shotgun sequencing data.

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Tables

Table 1. Selected anthropometric and descriptive variables of 19 lactating women participating in this study[1]

Age, y	30 ± 6
Height, cm	162 ± 6
Pre-pregnancy weight, kg	54 ± 8
Prenatal weight, kg	70 ± 7
Prenatal BMI, kg/m^2	26.5 ± 2.6
Pre-pregnancy BMI	20.5 ± 3.7
Maternal weight gain	17 ± 8
Birth weight	3.3 ± 0.3
Delivery mode	
Vaginal, n	19
Delivery location	
Hospital, n	19
Parity, n	1.3 ± 0.4
Female infants, %	39
Exclusively breastfed before sampling, %	100

[1]Values are the mean ± SD or unit of measure as indicated; a total of 19 women and their infants were studied.

BMI, body mass index

Figures

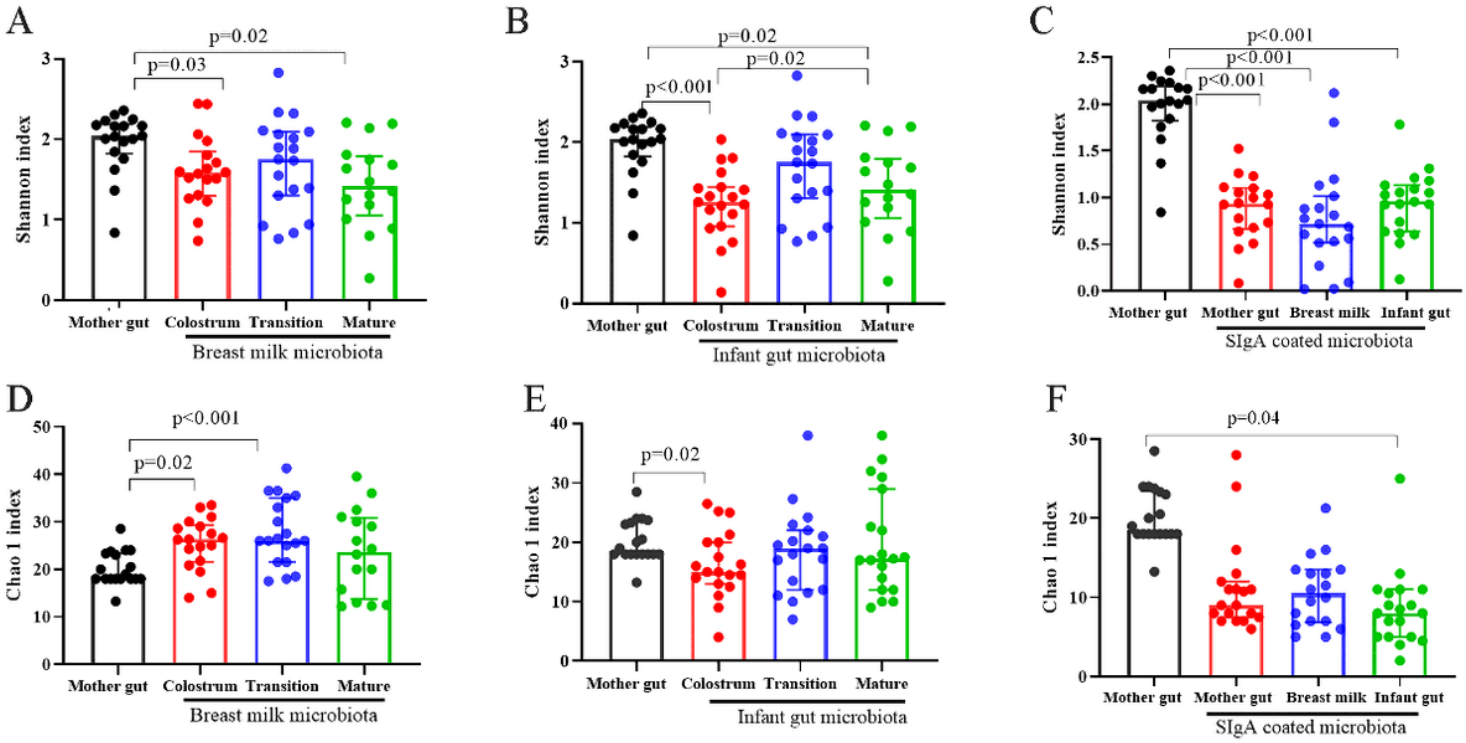

Figure 1

Alpha diversity of total microbiota in breast milk (A, D) and faeces (B, E) from different lactation periods, and sIgA-coated microbiota of mature period (C, F), indicated by Shannon index (A, B, C) and Chao 1 index (D, E, F). Data were shown as median with interquartile range and compared using the Wilcoxon paired test.

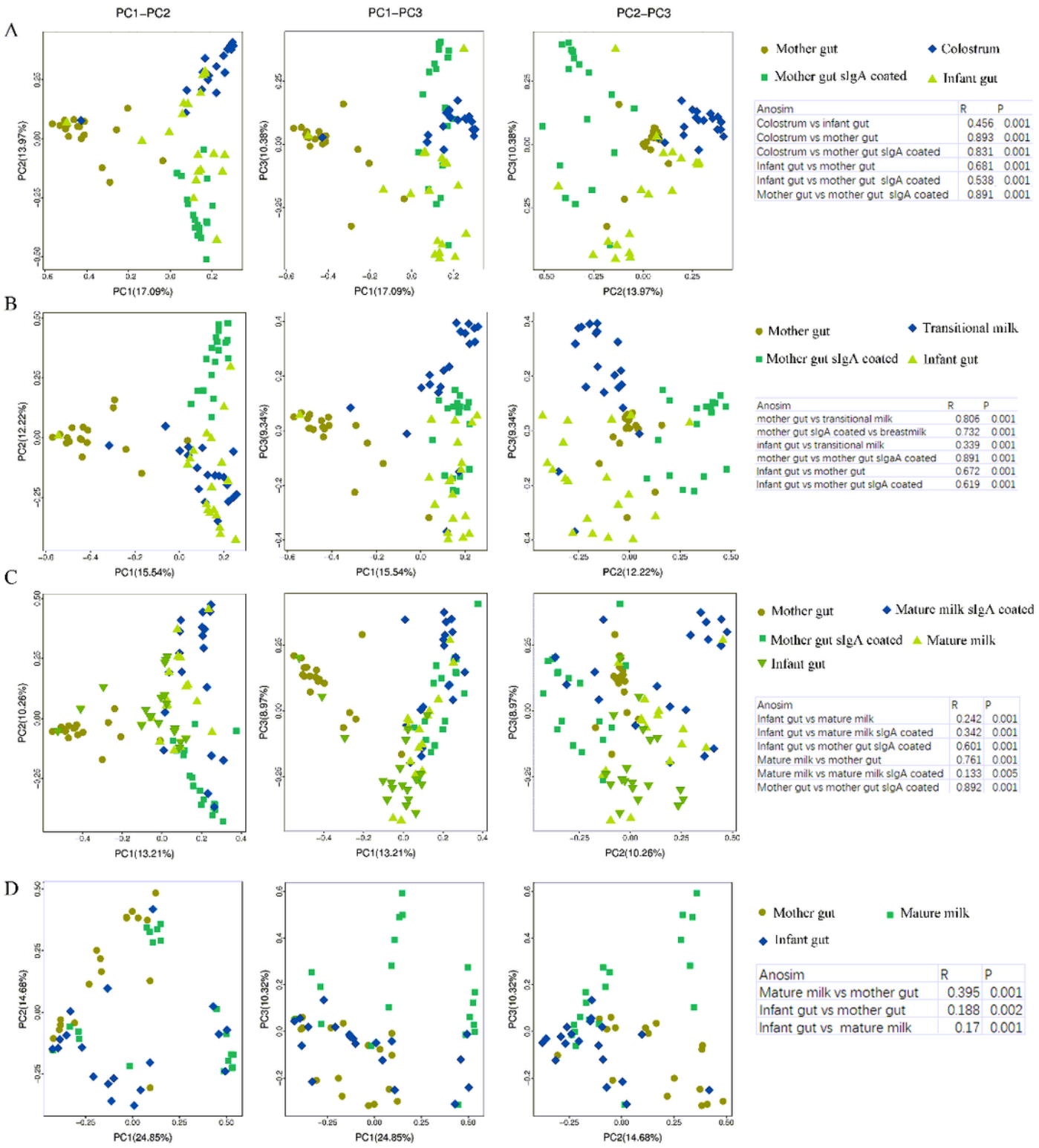

Figure 2

Beta diversity (Bray-Curtis distance of the operational taxonomic units) of the total microbiota of breast milk (A) and infant gut (B) from different lactation periods and sIgA-coated microbiota from mother gut, breast milk, and infant gut (C). To examine the effect of lactation periods or source of sIgA-coated microbiota on β-diversity, the Anosim test was conducted for pairwise comparison.

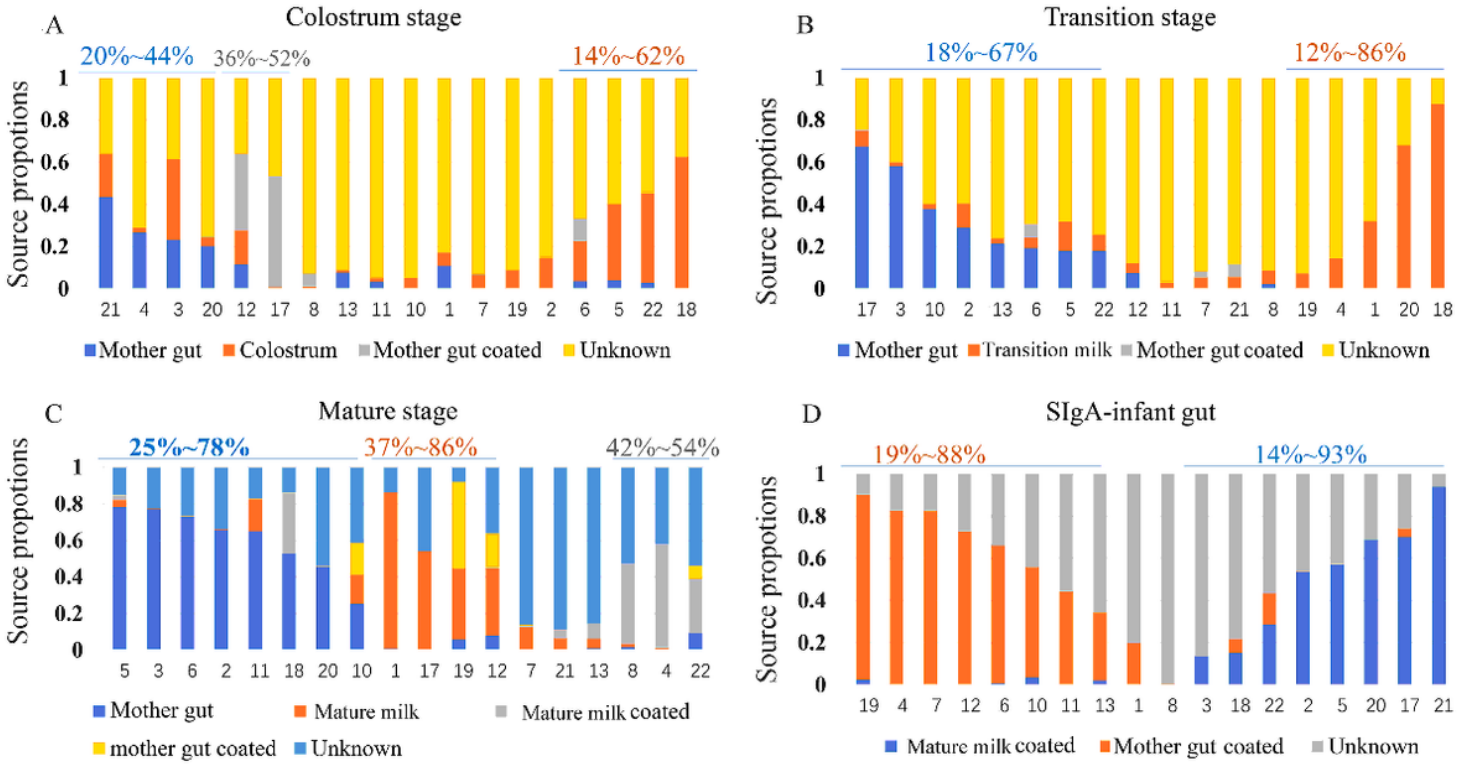

Figure 3

Source of infant faecal microbiota at the lactation stage of colostrum (A), transitional (B), and mature stage (C), and contribution of sIgA-coated microbiota in mother gut and breast to that in infant gut (D).

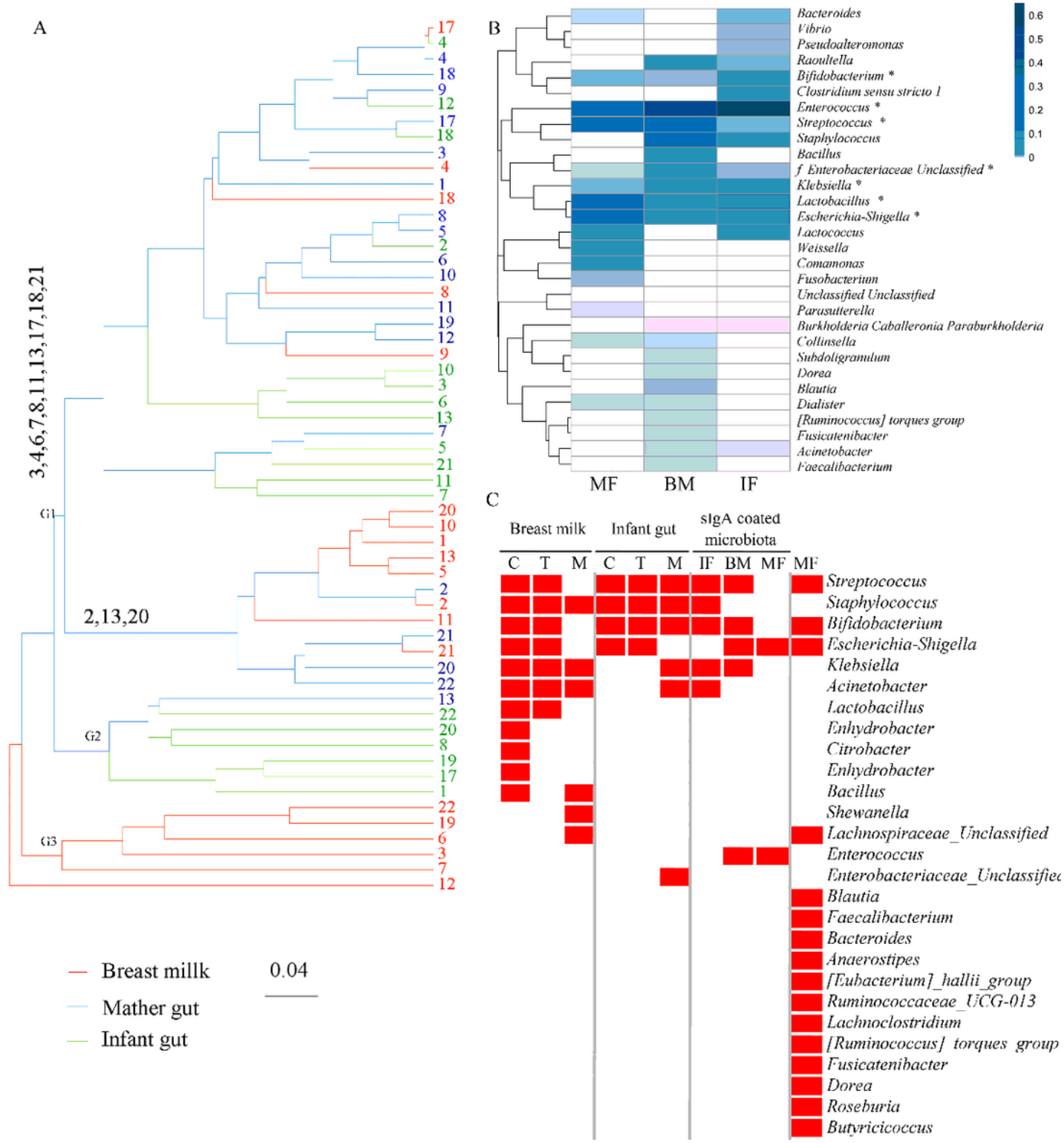

Figure 4

Co-occurrence of specific genera among different types of samples. A, Unweighted pair group method with arithmetic mean clustering tree constructed via weighted clustering hierarchy and the group average method, for samples of sIgA-coated microbiota; B, Species abundance heatmap showing normalised values of 30 differentially abundant genera of three type of sIgA-coated microbiota samples. The asterisk indicates the common genus of bacteria among samples. C, Core genus present in at least 90% of

individuals with a minimum of 0.01% mean relative abundance in specific types of samples. C, T, and M stands for sample from colostrum, transitional and mature milk stage, respectively. IF, MF, and BM stand for infant faeces, maternal faeces, and breast milk, respectively.

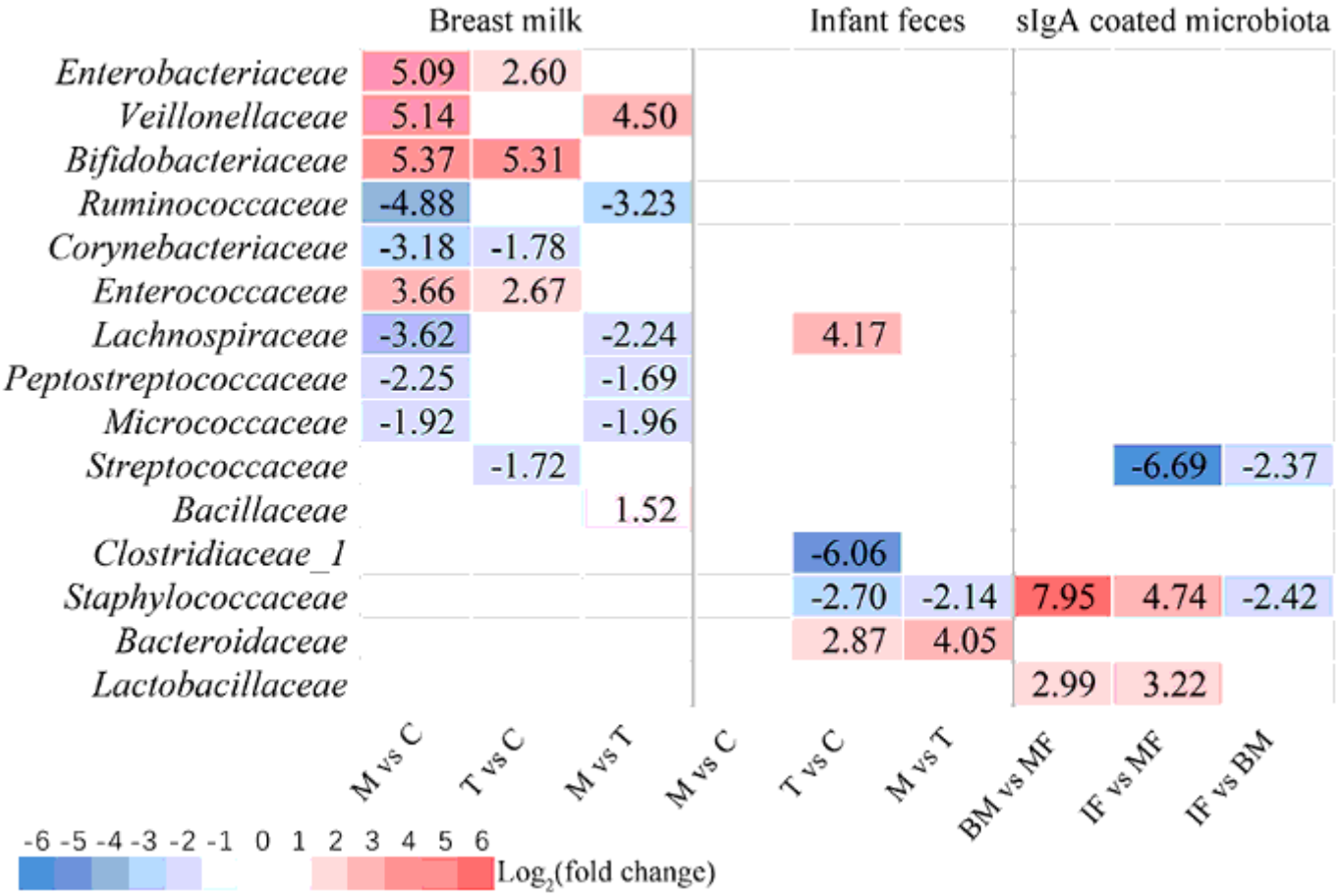

Figure 5

Significantly different family between different sample types. Different families between samples type were identified in edgeR and expressed as the log2 fold-change; the threshold was set to FDR < 0.5, |log2 fold-change | > 1.5, and log CPM > 10.0. C, T, and M stands for sample from colostrum, transitional, and mature milk stage, respectively. IF, MF and BM stands for infant faeces, maternal faeces, and breast milk, respectively.

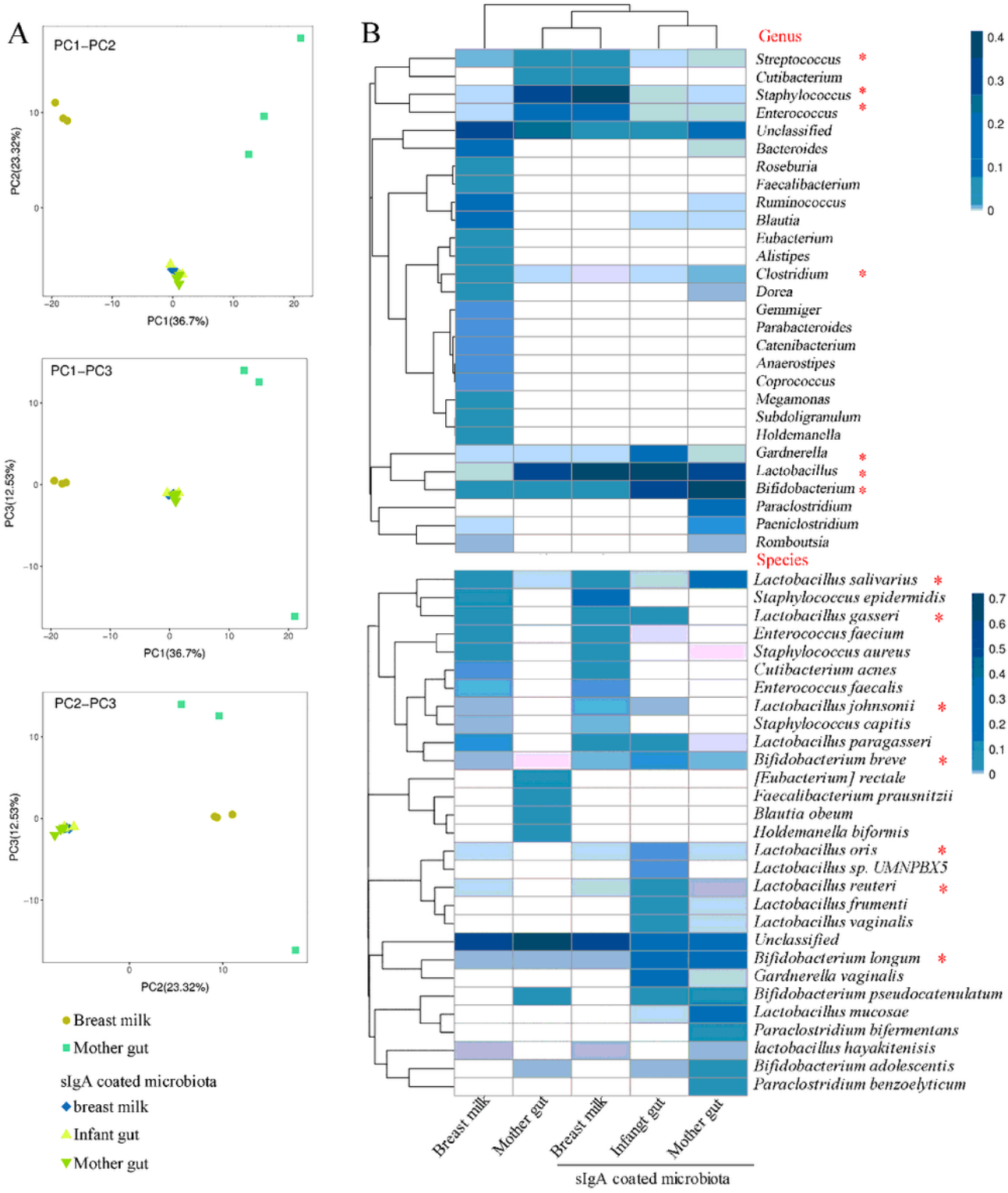

Figure 6

Principal component analysis (A) and genus/species heatmap of shotgun sequence data (B).

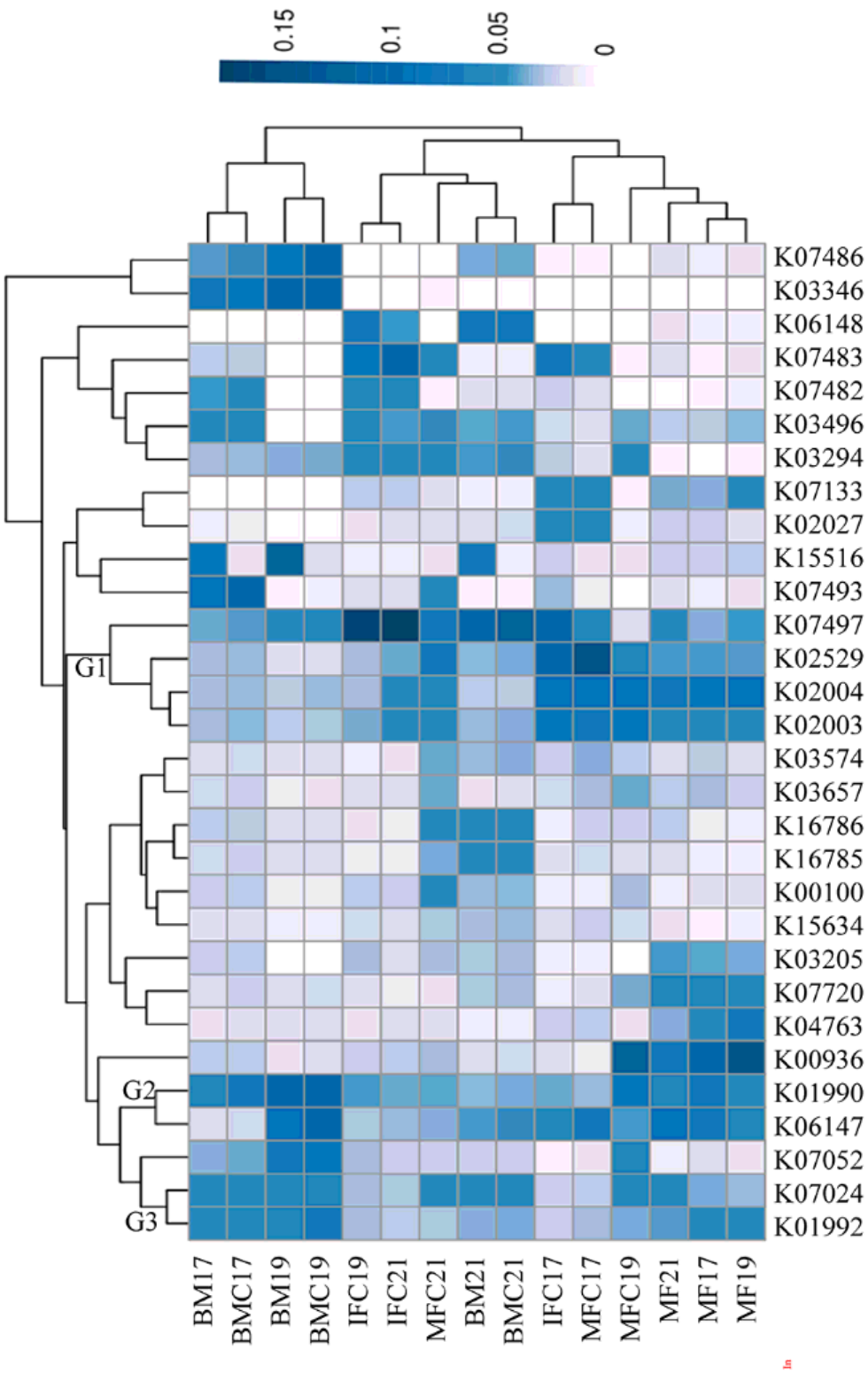

Figure 7

Heatmap of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway level 4 categories. K07497, putative transposase; K02529, LacI family transcriptional regulator; K02004, putative ABC transport system permease protein; K02003, putative ABC transport system ATP-binding protein; K01990, ABC-2 type transport system ATP-binding protein; K06147, ATP-binding cassette, subfamily B, bacterial; K07024, sucrose-6-phosphatase; K01992, ABC-2 type transport system permease protein.

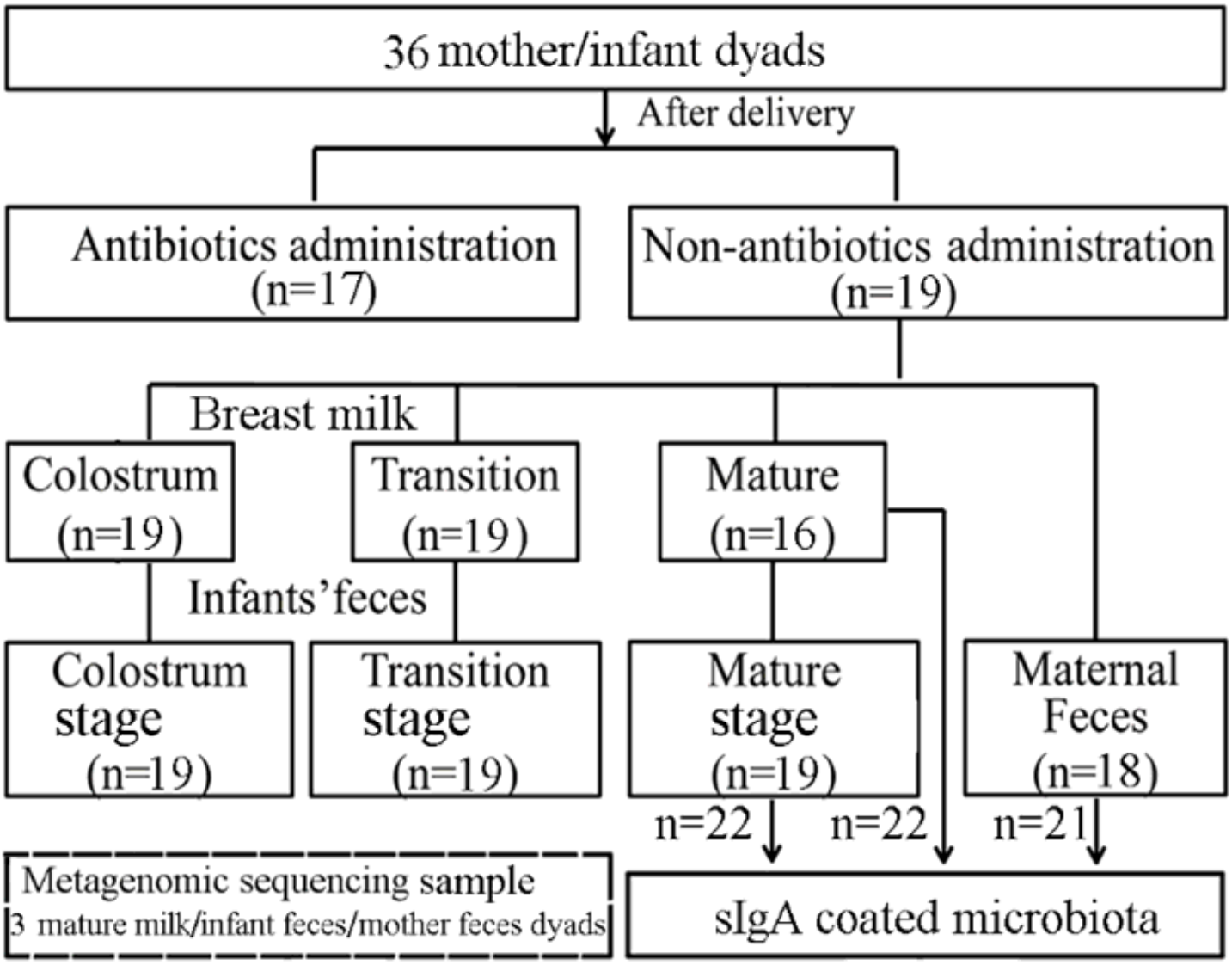

Figure 8

Enrolment, breast milk sample collection, infant faeces, maternal faeces, and samples selected for further analysis.

Supplementary Files

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- Supplementarymaterials.docx