Delivery, Feeding and Gender Differently Contribute to Infant Gut Microbiota Development

Juan Ding
the First Affiliated Hospital of Zhengzhou University
Xiao Ma
the First Affiliated Hospital of Zhengzhou University
Hongyan Ren
Shanghai Mobio Biomedical Technology Co., Ltd
Chenyang Zhi
People's Hospital of Zhengzhou
Qi Xin
Zhengzhou University
Zhen Li
the First Affiliated Hospital of Zhengzhou University
Liping Han
the First Affiliated Hospital of Zhengzhou University
Xianlan Zhao
the First Affiliated Hospital of Zhengzhou University
Zhihong Zhuo
the First Affiliated Hospital of Zhengzhou University
Ang Li
the First Affiliated Hospital of Zhengzhou University
Chao Liu
Shanghai Mobio Biomedical Technology Co., Ltd
Zhigang Ren (fccrenzg@zzu.edu.cn)
the First Affiliated Hospital of Zhengzhou University

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Abstract

To characterize gut microbiome of the infant during the first year of life and assess the different contributions of delivery mode, feeding mode and infant gender to gut microbial development. We collected 314 faecal samples from 80 infants at 5 time points of 0, 1st, 3rd, 6th and 12th months prospectively, and finally 213 samples completed Miseq sequencing and analysis. We characterized gut microbiome of the infant at the different phases and evaluated the different contributions of delivery mode, feeding mode and gender to gut microbial development. Delivery mode, gender and feeding mode were the strongest factors determining gut microbiome colonization at 0 months, from 1 month to 6 months and 12 months, respectively. Four genera including *Bifidobacterium*, *Bacteroides*, *Parabacteroides* and *Phascolarctobacterium* were increased, whereas 10 genera e.g. *Salmonella* and *Enterobacter* were reduced, in vaginal delivery versus cesarean section. Two genera including *Peptostreptococcaceae incertae sedis* and *Anaerococcus* were increased, whereas 3 genera e.g. *Coriobacteriaceae uncultured* were reduced, in exclusive breastfeeding versus combined feeding. This study indicated the contribution degrees of delivery mode, feeding mode and gender to gut microbial initiation and evolvement, and reported microbial differences induced by the different delivery mode, feeding mode and gender.

Introduction

The infant gut microbiota is critical to human health, playing significant roles in metabolism, immunity and development[1,2]. Colonization of the infant gut microbiota is a complex process dependent on multiple overlapping factors, such as delivery mode [3,4], feeding mode[5,6], gestational age[7] and environmental exposures[8].

There is growing evidence linking host health with the establishment of the infant gut microbiome. Cesarean delivery is associated with a number of human diseases, including obesity[9,10], asthma[11], celiac disease[12], and type 1 diabetes[13,14]. Infant gut microbiota colonization patterns differ between infants experiencing vaginal delivery and those delivered by cesarean Sect. [15]. Cesarean-delivered infants have particularly low richness and diversity compared with vaginally delivered infants. Breastfeeding is related to fewer illnesses, such as obesity[9,10], asthma[11] and metabolic syndrome[16], than formula feeding. Feeding mode also influences the establishment of the infant gut microbiota. Breastfed infants are reportedly colonized with bacteria in breast milk and with greater numbers of *Bifidobacterium* than formula-fed infants[17]. Gender-associated differences are relevant to the prevalence, manifestations, and outcomes of malignancies and autoimmune and infectious diseases[18]. Gender-associated differences in the gut microbiota can influence susceptibility to disease[19]. There is an undeniable need to explore gut microbiota characteristics caused by gender differences. However, the contribution degrees of these factors to the development of the infant gut microbiota remain unknown.

In this study, a total of 82 infants were prospectively enrolled, and fecal samples were separately collected at 0 months (meconium) and 1, 3, 6 and 12 months postpartum. Finally, 55 healthy infants were enrolled, and their samples were subjected to 16S rRNA gene sequencing. We characterized the gut microbiome at 5 time points during the first year of life, analyzed the bacterial community, and reported differences at crucial time points of infant development. Furthermore, we assessed the different contributions of delivery mode, feeding mode and infant gender to gut microbial development and compared microbial differences induced by the different delivery, feeding and gender.

Material And Methods

Study Participants

The primary objective of this study was to use longitudinal sampling to monitor changes in the infant gut microbiome over time. Specific variables were examined, including delivery mode, feeding mode and infant gender. All study procedures were consistent with the ethical guidelines of the Helsinki Declaration. The study was approved by the Institutional Review Board of the First Affiliated Hospital of Zhengzhou University (2017-KY-12). Recruitment took place in China between July 2017 and August 2017. Infants were excluded if they had a health condition or received antibiotics. Written informed consent was obtained from all parents of the infants before collecting data and fecal samples.

Basic demographics, delivery mode, feeding mode, and information concerning current or recent medications (including antibiotics) were obtained from hospital electronic medical records and questionnaires. Infants who had been breastfed and never given formula prior to fecal collection were assigned exclusively breastfed status. Infants who were fed both breast milk and formula prior to the time of stool collection were identified as having received combined feeding (both breast milk and formula milk). If an infant received formula milk at any collection time point, the infant was considered to have received combined feeding for the remainder of the study, as even short-term formula feeding causes profound and long-lasting shifts in the gut microbiome composition[44].

Sample Collection

The study population was recruited before mothers gave birth. Meconium samples were collected in the hospital, and other fresh fecal samples were collected at their homes. Fecal samples were collected by the infant's parents and stored in a household freezer (−20°C); samples were immediately shipped on dry ice to the laboratory, where they were placed at −80°C until DNA was extracted.

DNA extraction

DNA was extracted from the fecal samples using the E.Z.N.A.® Stool DNA Kit (Omega Bio-tek, Inc., GA) according to the instructions provided by the manufacturer. DNA was quantified by a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, United States), and fragment sizes were determined using 1.5% agarose gel electrophoresis.

PCR amplification

The extracted DNA samples were amplified with a set of primers targeting the hypervariable V3-V4 region (341F/805R) of the 16S rRNA gene. The forward primer (341F) was 5'-CCTACGGGNGGCWGCAG-3', and the reverse primer (805R) was 5'-GACTACHVGGGTATCTAATCC-3'. PCR was performed with an EasyCycler 96 PCR system (Analytik Jena Corp., AG) using the following program: 3 min of denaturation at 95 ℃; 21 cycles of 0.5 min at 94 ℃ (denaturation), 0.5 min for annealing at 58℃, and 0.5 min at 72 ℃ (elongation); and a final extension at 72 ℃ for 5 min. Paired-end sequencing was performed with the constructed sequencing libraries on the Illumina MiSeq platform by Shanghai Mobio Biomedical Technology Co., Ltd., China. The raw Illumina read data from all samples were stored in the European Bioinformatics Institute European Nucleotide Archive database (PRJNA665920).

Operational taxonomic unit (OTU) clustering and taxonomy annotation

Random reads were chosen from all fecal samples with equal numbers, OTUs were binned by the UPARSE pipeline, and OTUs were binned by the UPARSE pipeline with the following steps: (i) abundant sequences and singletons were first removed, (ii) unique sequences were binned into OTUs with the command "usearch-cluster_ otus", and (iii) randomly chosen sequences were aligned against OTU sequences with the command "usearch-usearch_global-id 0.97". The identity threshold was set as 0.97, and then an OTU composition table was created. We annotated sequences by using RDP classifier version 2.6[45].

Bacterial diversity and taxonomic analysis

Bacterial diversity was determined by sampling-based analysis of OTUs and represented by the Shannon index, Simpson index and Chao1 index, which were calculated using the R package "vegan"[46]. Principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS) based on OTUs were performed by the R package (http://www.r-project.org/) to analyze microbiome distances between samples. Unweighted UniFrac distances were calculated with the phyloseq package[47]. Taxonomic group comparisons, including comparisons of bacterial phyla, order, family and genera, were conducted using the Wilcoxon rank sum test. A heatmap that identified discriminatory variables was generated by a heatmap builder.

The linear discriminant analysis (LDA) effect size (LEfSe) method was used to distinguish taxonomic types in microbial communities related to specific characteristics (http://huttenhower.sph.harvard.edu/lefse/)[48]. With a normalized relative abundance matrix, LEfSe used the Kruskal-Wallis rank sum test to detect the features related to markedly different abundances between assigned bacterial taxa and used LDA to assess the effect size of each feature[49].

Functional annotation of the 16S rRNA gene

phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) version 1.0.0 pipeline[50] and human version 0.99[51] were used to establish KEGG orthology (KO) and KEGG pathway/module profiles and predict the functional profiles of the gut microbiota based on 16S rRNA gene sequences.

Bioinformatics and statistical analysis

Clean data were extracted from raw data using USEARCH 7.1 with the following criteria: (i) sequences shorter than 400 bp after the merge or containing a homopolymer longer than four bases were discarded; (ii) sequences from each sample were extracted using each index with fewer than two mismatches, and (iii) sequences with an overlap shorter than 16 bp were discarded.

We evaluated the associations of delivery mode, feeding mode, and infant gender with microbiome community composition using generalized UniFrac analysis[52]. The phylogenetic tree required for UniFrac analysis was computed based on FastTree[53] and was midpoint rooted. Statistical comparisons of groups were conducted using ADONIS between groups with 10,000 permutations. We computed mean generalized UniFrac distances for both within and between delivery mode groups (vaginal delivery and cesarean section), feeding mode groups (exclusive breastfeeding and combined feeding) and infant gender. Statistical comparisons of mean UniFrac distances were performed using the Kruskal-Wallis rank sum test. The statistical significance threshold was set at $P < 0.05$.

Results

Study Population

In this study, a total of 82 infants (314 fecal samples) were recruited, and infant fecal samples were collected at 0 months (meconium), 1 month (27 to 33 days), 3 months (87 to 93 days), 6 months (177 to 183 days) and 12 months (357 to 363 days) after birth. After confirmation, 2 infants without fecal samples, 3 premature infants, 5 infants with incomplete information, and 17 infants with drug therapy were excluded. After DNA extraction, 16S rRNA gene sequencing and data quality control, 38 fecal samples were further discarded. Finally, 55 healthy infants with 213 fecal samples at 0 months (n = 19), 1 month (n = 49), 3 months (n = 50), 6 months (n = 47) and 12 months (n = 48) were included in the final analysis.

In total, 8,542,788 raw reads were obtained after sequencing. The mean read depth per sample was 40,107 ± 10,818 sequences per sample. After initial quality filtering, 6,904,876 sequences passed with a mean of 32,417 ± 7,111 sequences per sample. A flowchart describing the sample collection is shown in **Fig. 1**. All infants included in the study were fed complementary food after fecal samples were collected at 6 months. The complementary food mainly included vegetables, porridge and pasta.

Clinical information of the participants

Comprehensive clinical information for each enrolled individual was recorded (**Table 1).** We evaluated delivery and feeding characteristics in 55 infants (**Table 1)**. Samples showed that 36 (65.5%) of enrolled infants had vaginal deliveries, and 19 (34.5%) infants had cesarean section deliveries. Thirty-six (65.5%) infants were exclusively breastfed, and 19 (34.5%) infants received combined feeding.

Infant gut microbial diversity during the first year of life

Bacterial diversity was determined by sampling-based analysis of OTUs as estimated by the Shannon index (**Fig. 2a**) and the Simpson index **(Fig. 2b**). The fecal microbial diversity was significantly decreased at 0 months compared with the other 4 times ($p < 0.001$ and $p < 0.001$, respectively, **Supplementary Table S1**). Microbial diversity was markedly increased at 12 months compared with the other 4 times ($p < 0.001$ and $p < 0.001$, respectively, **Supplementary Table S1**). The microbial diversity presented no significant difference between 1, 3 and 6 months ($p = 0.4$ and $p = 0.87$, respectively, **Supplementary Table S2**). These data suggested that gut microbial diversity was consistently increased from 0 months to 12 months with the development of the infant.

To assess the overall diversity in bacterial composition, we performed PCoA and NMDS analysis based on unweighted UniFrac distances **(Fig. 2c-d andSupplementary Fig. S1**, **Supplementary Table S3)**. The PCoA based on the unweighted UniFrac distances indicated that there was a notable separation of samples at 0, 1, 3, 6 and 12 months (Adonis $p < 0.001$, $R^2 = 0.08$, **Supplementary Table S4**). There was an obvious separation in gut microbiome composition between 0 months and 1 month (Adonis $p = 0.001$, $R^2 = 0.04$). Meanwhile, the microbial community at 12 months was clustered together and significantly separated from that at 6 months in the PCoA (Adonis $p < 0.001$, $R^2 = 0.06$). No obvious separation was observed in the microbiota between 1, 3 and 6 months (Adonis $p = 0.22$, $R^2 = 0.02$). These results suggested that infants' gut microbiota was consistently altered from 0 months to 12 months with the development of the infant.

In addition, we calculated the average UniFrac distances within each group to assess the microbial differences between the individual infants. We found the greatest average distance within each group between the infants at 0 months. The distance within the group was significantly decreased at 1, 3, and 6 months versus 0 months. Notably, the distance within the group remarkably increased from 6 months to 12 months **(Fig. 2e, Supplementary Table S5)**. These data demonstrated that individual microbial variation of the infants was decreased from 0 months to 1 month but then increased from 6 months to 12 months.

Gut microbiota community structure during the first year of life

A heatmap from hierarchical clustering analysis revealed a discriminatory gut microbiome during the first year of life. Comparison of fecal microbiomes demonstrated that 60 key OTUs were significantly different at 0, 1, 3, 6, and 12 months (**Supplementary Fig. S2**). We further analyzed the infant gut microbiota composition and alterations during the first year of life. The fecal bacterial composition in each sample at the phylum and genus levels is shown in**Supplementary Fig. S3-**

4, respectively (**Supplementary Table S6-S7**). The average compositions of the microbial community at the phylum and genus levels during the first year of life are shown in **Fig. 3a-b** (**Supplementary Table S8-S9**). Moreover, bacterial compositions at the class and order levels during the first year of life are shown in **Supplementary Fig. S5a-b** (**Supplementary Table S10-S11**).

The bacterial phyla *Firmicutes, Proteobacteria*, *Bacteroidetes* and *Actinobacteria*, together accounting for up to 99% of sequences on average, were the four dominant phyla at 0 months. *Firmicutes, Proteobacteria* and *Actinobacteria*, together accounting for greater than 85%, were the three dominant phyla at 1, 3, 6 and 12 months, respectively. The average abundance of the phylum *Firmicutes* increased from 16.34% at 0 months to 48.15% at 12 months ($p < 0.001$, **Fig. 3c, Supplementary Table S12**), while the abundance of the phylum *Proteobacteria* decreased gradually from 67.43% at 0 months to 15.82% at 12 months ($p < 0.001$, **Fig. 3d**). Moreover, the average relative abundance of the phylum *Actinobacteria* consistently increased from 4.59% at 0 months to 33.34% at 3 months ($p < 0.001$) and then presented no significant alterations from 3 months to 12 months (26.25%) (**Fig. 3e**).

Eighty-nine genera in the infant gut microbiota were detected during the first year of life. The infant gut microbiota was dominated by *Bifidobacterium, Streptococcus, Escherichia−Shigella, Klebsiella,Bacteroides,Clostridium sensu stricto 1,Veillonella,Lactobacillus*and *Lachnospiraceae incertae sedis*, and their total abundance exceeded 70%. The average relative abundance of *Bifidobacterium* significantly elevated during the period from 0 months (2.66%) to 3 months (28.15) ($p < 0.001$) and had no obvious increase from 3 months to 12 months (23.81%) (**Fig. 3f, Supplementary Table S13)**. The average amount of *Streptococcus* remarkably increased from 0 months (6.19%) to 1 month (14.11%) ($p < 0.001$) and presented no significant changes from 1 month to 12 months (11.91%) **(Fig. 3g)**. Additionally, *Veillonella* consistently increased from 0 months (2%) and 1 month (3.22%) to 12 months (5.48%) **(**$p < 0.001$, **Fig. 3h)**.

To identify specific bacterial taxa associated with the time points, we compared the fecal microbiota using LEfSe. A cladogram representative of fecal microbial structures and their predominant bacteria displayed the greatest differences in taxa among different times (all $p < 0.05$, **Supplementary Fig. S6a-b, Supplementary Table S14**). The gut microbial community metabolic function profiles and the predominant microbial functions at 0, 1, 3, 6 and 12 months were analyzed by a cladogram and LDA (**Supplementary Fig. S7a-b, Supplementary Table S15**).

Gut microbial differences of the infants at the crucial development time points

Compared with that at 1 month, as estimated by the Shannon index, Simpson index and Chao1 index, fecal microbial diversity at 0 months was significantly decreased ($p < 0.001$, $p < 0.001$ and $p < 0.05$, respectively, **Supplementary Fig. S8a-c, Supplementary Table S16**). A heatmap revealed a discriminatory gut microbiome and demonstrated that 15 key OTUs were significantly different between 0 months and 1 month (**Supplementary Fig. S9**)**.** We analyzed differences in bacterial taxonomic compositions at the phylum and genus levels between 0 months and 1 month.(all $p < 0.05$, **Supplementary Fig. S10a-b,**

Supplementary Table S17-S18). The predominant fecal microbiome at 0 months and 1 month was determined by LEfSe and LDA **(**all $p < 0.05$, LDA > 3, **Supplementary Fig. S11a-b,Supplementary Table S19).** The predominant fecal microbial functions at 0 and 1 month were visualized by a cladogram and LDA (all $p < 0.05$, LDA > 2, **Supplementary Fig. S12a-b, Supplementary Table S20**).

Compared with that at 12 months, fecal microbial diversity at 6 months was significantly decreased (all $p < 0.001$, **Supplementary Fig. S13a-c, Supplementary Table S21).** A heatmap revealed a discriminatory gut microbiome and demonstrated that 35 key OTUs were significantly different between 6 months and 12 months (**Supplementary Fig. S14**). We analyzed differences in bacterial taxonomic compositions at the phylum and genus levels (all $p < 0.05$,**Supplementary Fig. S15a-b**, **Supplementary Table S22-S23**). The predominant fecal microbiome at 6 months and 12 months was determined by LEfSe and LDA (all $p < 0.05$, LDA > 2, **Supplementary Fig. S16a-b, Supplementary Table S24)**.
The fecal predominant microbial functions at 6 months and 12 months were visualized by a cladogram and LDA (all $p < 0.05$**,** LDA > 2, **Supplementary Fig. S17a-b, Supplementary Table S25**). These data revealed significant differences in the gut microbiota between these two groups.

Delivery, feeding and gender contribute differently to microbiota development

Each infant has unique gut microbial characteristics. We analyzed gut microbial similarity within each group, compared the within-group average UniFrac distances between infants within specific delivery mode, feeding mode and gender groups, and revealed individual microbial differences of the infant. Greater average UniFrac distances were observed between infants who were born by vaginal delivery versus cesarean delivery **(Fig. 4a, Supplementary Table S26)**. A greater average distance was observed between infants who received combined feeding versus those exclusively breastfed **(Fig. 4b, Supplementary Table S26).** Within-group distances revealed no significant difference between boys and girls **(Fig. 4c, Supplementary Table S26)**. These data suggested that vaginal delivery and combined feeding played an important role in the development of the infant gut microbiota.

We further calculated between-group average UniFrac distances to assess the microbial compositional similarities. After birth, the UniFrac distance between vaginal delivery and cesarean section was the largest, followed by the distances between feeding modes and gender differences **(Fig. 5a, Supplementary Table S27)**. At 1, 3 and 6 months, the average distance was greater between boys and girls than between vaginal delivery and cesarean section, as well as exclusively breastfed and combined feeding **(Fig. 5b-d, Supplementary Table S27).** By 12 months, the contributions to the development of gut microbiota had changed. The greatest distance was observed between exclusively breastfed and combined feeding; then between vaginal delivery and cesarean section, as well asbetweenboys and girls **(Fig. 5e, Supplementary Table S27).** These data demonstrated that for the development of the infant gut microbiota, the delivery mode was the main contribution at birth, gender accounted for the dominant contribution at 1, 3 and 6 months, and feeding mode played the main role after 12 months.

The effect of delivery, feeding and gender on the gut microbiota

We analyzed fecal bacterial composition and differences between vaginal delivery and cesarean section. A heatmap revealed a discriminatory gut microbiome between vaginal delivery and cesarean section, and demonstrated that 17 key OTUs were significantly different between the two groups (**Supplementary Fig. S18**). The average composition of the microbial community at the phylum and genus levels between the two groups is shown in **Fig. 6a** and **Supplementary Fig. S19a** (**Supplementary Table S28-S29**). At phylum level, 2 phyla including Actinobacteria and Bacteroidetes were increased, whereas 2 phyla including Firmicutes and Proteobacteria reduced, in vaginal delivery versus cesarean section (all $p < 0.05$, **Fig. 6b, Supplementary Table S30**). At genus level, 4 genera including *Bifidobacterium*, *Bacteroides*, *Parabacteroides* and *Phascolarctobacterium* were increased, whereas 10 genera mainly including *Clostridium sensu stricto 1, Salmonella* and *Enterobacter* reduced, in vaginal delivery versus cesarean section (all $p < 0.05$, **Fig. 6c**, **Supplementary Table S31**). The predominant fecal microbiome for vaginal delivery and cesarean section were determined by LEfSe (**Supplementary Fig. S20a**). Based on LDA, 14 bacteria mainly including *Bacteroidetes*, *Bacteroidales*, *Bacteroidia* and *Actinobacteria* were enriched, while 14 bacteria mainly including *Firmicutes*, *Proteobacteria* and *Clostridiales*, were reduced for vaginal delivery compared with cesarean section (all $p < 0.05$, LDA > 2.5, **Supplementary Fig. S20b, Supplementary Table S32**). The predominant fecal microbial functions for vaginal delivery and cesarean section were analyzed by a cladogram (**Supplementary Fig. S21a**). Based on LDA, 81 functions mainly including Genetic Information Processing, Replication and Repair, Metabolism and Translation were enriched, while 40 functions mainly including Environmental Information Processing, Membrane Transport and Transporters, were reduced for vaginal delivery compared with cesarean section (all $p < 0.05$, LDA > 2, **Supplementary Fig. S21b, Supplementary Table S33**). We further analyzed bacterial taxonomic compositions and alterations at the phylum level between vaginal delivery and cesarean section infants under fixed exclusive breastfeeding ($p < 0.05$, **Supplementary Fig. S22a-b,Supplementary Table S34-S35)**.The predominant fecal microbiome associated with delivery mode under fixed exclusive breastfeeding was determined by LEfSe and LDA (all $p < 0.05$, LDA > 2, **Supplementary Fig. S23a-b, Supplementary Table S36**).

We also analyzed gut microbial composition and alterations between exclusive breastfeeding and combined feeding. A heatmap revealed a discriminatory gut microbiome between exclusive breastfeeding and combined feeding, and demonstrated that 17 key OTUs were significantly different between the two groups (**Supplementary Fig. S24**). The average composition of the microbial community at the phylum and genus levels between the two groups is shown in **Supplementary Fig. S19b** and **Fig. 6d** (**Supplementary Table S37-S38**). We found no significant between two groups at phylum level (all $p < 0.05$, **Supplementary Table S39**). At genus level, 2 genera including *Peptostreptococcaceae incertae sedis* and *Anaerococcus* were increased, whereas 3 genera including *Lachnospiraceae incertae sedis*, *Coriobacteriaceae uncultured* and *Erysipelotrichaceae norank* reduced, in exclusive breastfeeding versus combined feeding (all $p < 0.05$, **Fig. 6e**, **Supplementary Table S40**). The predominant fecal microbiome for vaginal delivery and cesarean section were determined by LEfSe (**Supplementary Fig. S25a**). Based on LDA, 3 bacteria including *Anaerococcus*, *Serratia* and *Peptostreptococcaceae* were enriched, while 2 bacteria including *Lachnospiraceae* and *Incertae Sedis*,

were reduced for exclusive breastfeeding compared with combined feeding (all $p <$ 0.05, LDA > 2, **Supplementary Fig. S25b, Supplementary Table S41**). The predominant fecal microbial functions for exclusive breastfeeding compared with combined feeding were analyzed by a cladogram (**Supplementary Fig. S26a**). Based on LDA, 2 functions including Fatty acid metabolism and Glycine serine and threonine metabolism were enriched, while 2 functions including Sporulation and Methane metabolism, were reduced for exclusive breastfeeding compared with combined feeding (all $p <$ 0.05, LDA > 2, **Supplementary Fig. S26b, Supplementary Table S42**). We further analyzed bacterial taxonomic compositions and alterations at the phylum level between exclusive breastfeeding and combined feeding infants under fixed vaginal delivery ($p < 0.05$, **Supplementary Fig. S27,Supplementary Table S43-S44)**.The predominant fecal microbiome associated with feeding mode under fixed vaginal delivery was determined by LEfSe and LDA (all $p < 0.05$, LDA > 2, **Supplementary Fig. S28, Supplementary Table S45**).

Moreover, we analyzed gut microbial composition and alterations between boys and girls. A heatmap revealed a discriminatory gut microbiome boys and girls, and demonstrated that 17 key OTUs were significantly different between the two groups (**Supplementary Fig. S29**). The average composition of the microbial community at the phylum and genus levels between the two groups is shown in **Supplementary Fig. S19c-d** (**Supplementary Table S46-S47**). We found no significant between two groups at phylum level (all $p < 0.05$, **Supplementary Table S48**). At genus level, 2 genera including *Alistipes* and *Anaeroglobus* were increased, whereas 4 genera including *Parasutterella*, *Eubacterium, Peptoniphilus* and *Anaerosporobacter* reduced, in boys versus girls (all $p < 0.05$, **Fig. 6f**, **Supplementary Table S49**). The predominant fecal microbiome for vaginal delivery and cesarean section were determined by LEfSe (**Supplementary Fig. S30a**). Based on LDA, 3 bacteria including *Veillonellaceae*, *Selenomonadales* and *Negativicutes* were enriched, while 2 bacteria including *Burkholderiales* and *Family XI*, were reduced for boys compared with girls (all $p < 0.05$, LDA > 2, **Supplementary Fig. S30b, Supplementary Table S50**). We further identified different bacterial taxa associated with gender under fixed vaginal delivery and feeding with exclusively milk conditions (**Supplementary Fig. S31-S32, Supplementary Table S51-S53**).

Functional prediction of microbial genes associated with delivery mode and feeding mode at 12 months

The gut microbial community metabolic function profiles and the predominant microbial functions at 12 months related to vaginal delivery and cesarean sectionare shown in a cladogram (**Supplementary Fig. S33a**). Based on LDA, *nitrogen metabolism* was enriched, while 3 functions, including*replication recombination and repair proteins, valine leucine and isoleucine biosynthesis,* and*basal transcription factors*, were reduced for vaginal delivery compared with cesarean section (all $p < 0.05$, LDA >2, **Supplementary Fig. S33b**, **Supplementary Table S54**).

Moreover, the gut microbial community metabolic function profiles and the predominant microbial functions at 12 months for exclusively breastfed and combined feeding are also shown in a cladogram (**Supplementary Fig. S34a**). Based on LDA, compared to the combined feeding group, 20

functions, mainly *ABC transporters, ascorbate and aldarate metabolism,* and*nitrogen metabolism,* were increased, whereas 9 functions, mainly *amino sugar and nucleotide sugar metabolism*,*replication recombination* and *repair proteins,* were decreased in the breastfed group **(**all $p < 0.05$, LDA >2, **Supplementary Fig. S34b**,**Supplementary Table S55)**.

Discussion

We characterized the gut microbiome of 55 infants at 0, 1, 3, 6, and 12 months after birth; analyzed the influence of delivery mode, feeding mode and gender on the gut microbiota; and evaluated the contributions of these factors to gut microbial development. Although delivery mode[20] and feeding mode[21] together influenced infant gut microbial composition and evolution, our study demonstrated the contribution degrees of these factors to gut microbial development. We found that delivery mode was the main contribution at birth and that feeding mode played the main role in infant gut microbiota development after 6 months. Interestingly, we found that gender accounted for the dominant contribution to infant gut microbial development at 1, 3 and 6 months after birth, which overturned our common theory.

In our study, individual microbial variation dynamically changed with infant growth. Individual microbial variation in the infants decreased from 0 months to 1 month but then increased from 6 months to 12 months. Individual microbial variation at birth was initiated by differences in the maternal microbiota[22] and vaginal[23], maternal skin[24] and hospital environments[25]. Vaginally delivered infants are exposed to the vaginal and fecal microbiota, leading to gut colonization by vagina-associated microbes such as Lactobacillus[23]. In contrast, cesarean section-delivered infants do not experience direct contact with maternal microbes and are thus more likely to become colonized by maternal skin, the hospital environment, or hospital staff[20]. With the development of infants, complementary foods, different living environments and different contact populations together contributed to the evolution of the infant gut microbiota, subsequently resulting in increased individual variation after 6 months. The shift in dietary patterns from exclusive breastfeeding to the consumption of solid foods induces the development of the mature gut microbiota[26]. Our study indicated that due to the complementary introduction of a variety of novel food substances, alpha diversity and individual variation increased.

We observed individual differences between vaginally delivered infants and cesarean delivered infants. We found that the variation in the microbial communities of vaginally delivered infants was greater than that of cesarean delivered infants. In healthy infants, delivery is the initial encounter with bacteria capable of colonizing the gut. In a previous study of 24 infants aged 3 to 4 months in Canada, *Bacteroides* was depleted in cesarean delivered infants relative to those who were vaginally delivered[6]. This result was also underscored in a study of 24 infants in Sweden that reported that the depletion of *Bacteroides* in infants delivered by cesarean section persisted until 12 months[27]. Our findings indicated that *Bacteroides* levels were differentially abundant between vaginally and cesarean delivered infants, in line with the results of earlier studies. These high levels of *Bacteroides* in infancy are associated

with increased risks of asthma and obesity later in life[28,29]. Consistent with a previous study on premature infants, we observed that delivery mode was the main contribution at birth[13,15]. Independent of whether prenatal colonization takes place in the fetus, delivery marks the moment of extensive exposure to bacterial communities of vaginal, fecal, skin and environmental origins[30]. During vaginal birth, specific microbial strains associated with the mother's vaginal microbiota are vertically transmitted from mothers to infants[24,31]. Therefore, delivery mode has a profound impact on the earliest microbial colonization of the neonatal gut.

Moreover, we observed gut microbial differences between combined feeding infants and exclusively breastfed infants. The microbial variation between the combined feeding infants was greater than that between exclusively breastfed infants. In a previous study of 102 infants aged 6 weeks, infants who were fed breast milk supplemented with formula had greater individual differences than exclusively breastfed infants[3]. The large individual difference in the gut microbiota in combined feeding infants may be caused by the use of different brands of formula. Feeding mode has the potential for lasting effects on the gut microbiota, and these effects may be associated with childhood and lifelong health[32]. Studies show that breastfeeding confers protection against gastrointestinal tract and respiratory infections and allergic diseases in addition to reducing the risk of chronic diseases, such as inflammatory bowel disease, obesity and diabetes[5,33,34]. Our study indicated that feeding mode played the main role after 6 months in the development of the infant gut microbiota. A previous study showed that bacteria from breast milk are most prominent in infants' guts in the first month, accounting for nearly 40% of the gut bacteria in primarily breastfed infants[5]. Ding et al. recently reported that breastfeeding during infancy was a major life-history characteristic that affected microbial community composition in adults[35]. The breast milk microbiota that seeds the infant's gut first influences and selects for the microbiota that follow, leaving a footprint that can be detected even in adulthood. Although the differences in breast milk bacteria increase between mothers during the first 6 months of infancy[5], the other factors that affect the gut microbiota, including delivery mode and gender, still have greater impacts than the feeding mode on the gut microbiota at 0 months and at 1 month to 6 months, respectively.

Interestingly, we found that gender accounts for the dominant contribution at 1, 3 and 6 months after birth. Gender differences in the gut microbiota are driven by gender hormones, which in turn affect gender differences in susceptibility to a large number of chronic diseases and infections[36-38]. Three populations were found to significantly increase over time in the boy group: *Selenomonadales,Negativicutes* and *Veillonellaceae*. A recent study suggests that lower abundances of *Selenomonadales* and *Veillonellaceae* are associated with autism spectrum disorder [39,40]. Dysregulation of *Selenomonadales* affects gut secretion and in turn affects brain function by modulating the efficacy of the vagal response[41]. A relative paucity of *Negativicutes* might predispose patients to necrotizing enterocolitis[42]. From another perspective, the difference between boys and girls begins with the gut microbiota in infancy. Children manifest secondary genderual characteristics after gender hormone stimulation at puberty[43]. We hypothesize that the gender-specific early gut microbiota development precedes the

development of secondary genderual characteristics. These studies and our results hint that the gender of the newborn affect the gut microbiome and can be valuable for optimizing prevention and treatment strategies. There is currently a paucity of data addressing how the gender of the infant affects the gut microbiota profiles; thus, this study provides important insights into the impact of the gender of the infant on gut microbiota development.

In conclusion, based on a gut microbial characterization during infant development, this study found an increase in gut microbiota diversity that increased over time and indicated that individual microbial variation in infants decreased from 0 to 1 month but then increased from 6 to 12 months. Importantly, we demonstrated the contribution degrees of delivery mode, gender and feeding mode to gut microbial establishment and evolution, which overturned our common theory and provided a novel concept. Understanding the patterns of infant gut microbial colonization and development is critical for both short- and long-term health and may provide a solid foundation for future health outcomes through microbiota intervention.

Abbreviations

PCoA, principal coordinates analysis; NMDS, nonmetric multidimensional scaling; OTUs, operational taxonomic units; LEfSe, linear discriminant analysis effect size;

PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states.

Declarations

Acknowledgement

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Availability of data and materials

The raw Illumina read data for all samples were deposited in the European Bioinformatics Institute European Nucleotide Archive database under the accession number PRJNA665920.

Author contributions: RZG and DJ designed the study. DJ, MX, XQ, LZ, HLP, ZXL, ZCY and ZZH collected clinical samples. RHY, LA and LC performed Miseq sequencing and data analysis; DJ, RZG, and MX wrote the manuscript. All authors reviewed and approved the manuscript.

Compliance with ethics guidelines

All author declare that they have no conflict of interest. This study was approved by the Institutional Review Board of the First Affiliated Hospital of Zhengzhou University (2017-XY-012). The study was performed in accordance with the Helsinki Declaration and Rules of Good Clinical Practice. All participants signed written informed consents after the study protocol was fully explained.

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Tables

Table 1. Characteristics of 55 participants

Variables	No. (%)
Newborn sex	
Male	28 (50.9)
Female	27 (49.1)
Delivery mode	
Vaginal delivery	36 (65.5)
Cesarean section delivery	19 (34.5)
Feeding patterns	
Exclusive breastfeeding	36 (65.5)
Combined feeding	19 (34.5)
Birth weight at birth (kg), mean±SD	3.34±0.39
Birth weight at 12 month (kg), mean±SD	10.01±1
Gestational age (days), mean±SD	278±5

Figures

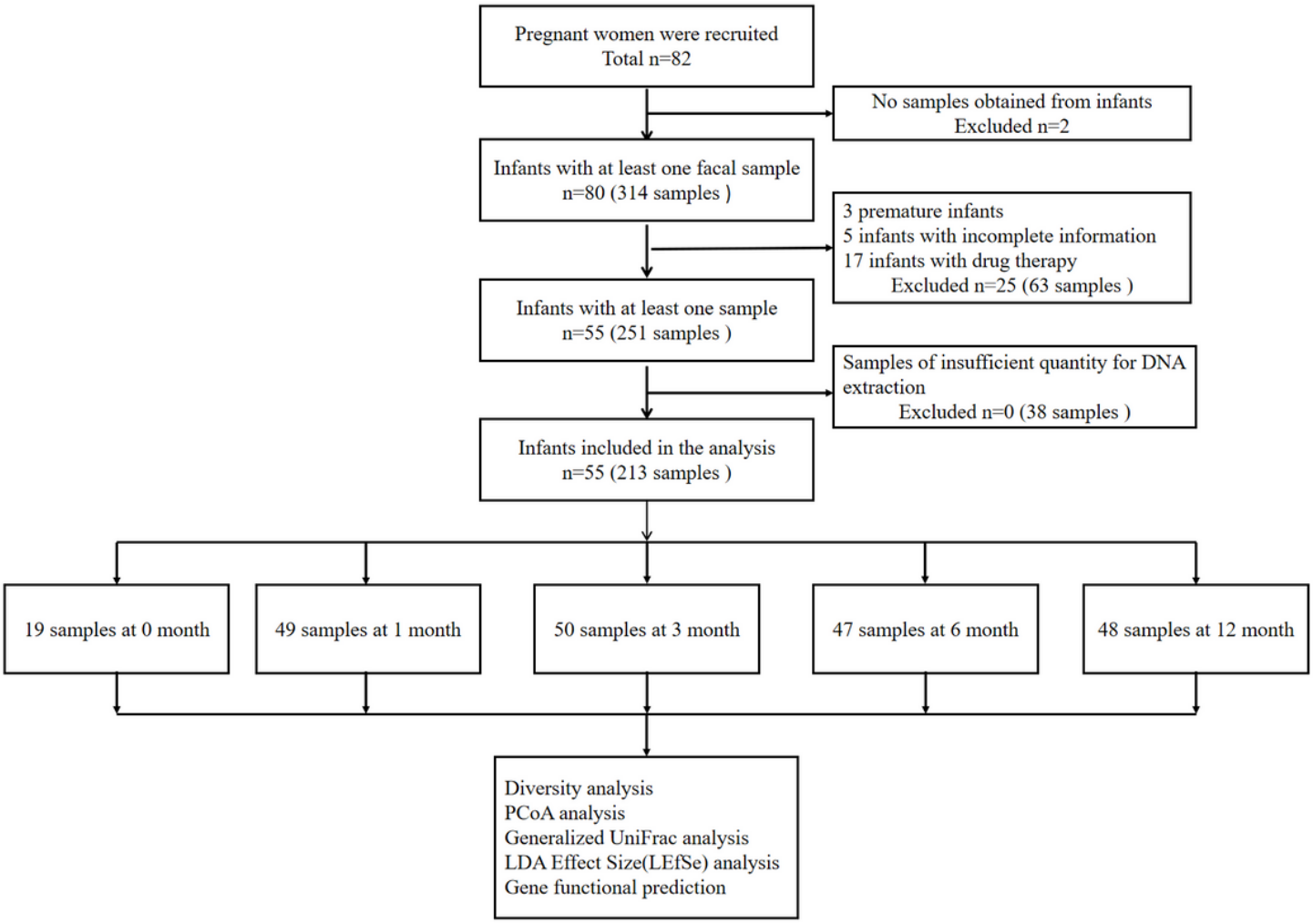

Figure 1

Study design and flow diagram. A total of 82 infants (314 fecal samples) were recruited, and samples were collected at 0, 1, 3, 6 and 12 months postpartum. After an exclusion process, the remaining samples were used for DNA extraction, 16S rRNA gene sequencing and data quality control. Finally, 213 fecal samples from 55 infants at 0 months (n = 19), 1 month (n = 49), 3 months (n = 50), 6 months (n = 47) and 12 months (n = 48) were included for the final analysis.

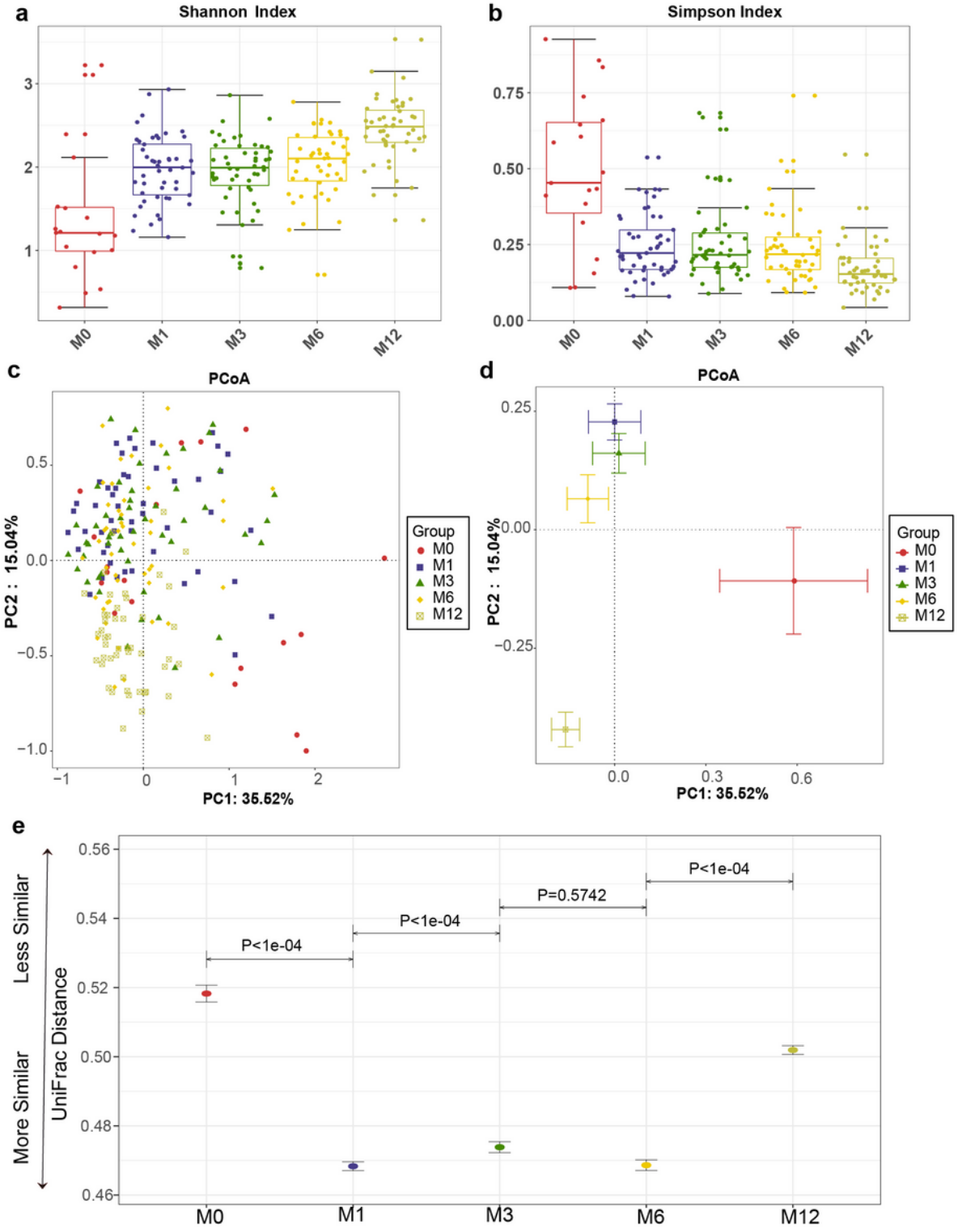

Figure 2

Infant gut microbial diversity during the first year of life. Fecal microbial diversity, as estimated by the Shannon index (A) and the Simpson index (B), significantly increased over developmental time ($p < 0.001$ and $p < 0.001$, respectively). (C, D) Overall diversity in bacterial composition was calculated using unweighted UniFrac distances by PCoA, and it indicated a notable separation of samples at 0, 1, 3, 6 and 12 months (Adonis $p < 0.005$, $R2 = 0.08$). (E) Individual microbial differences of the infants were

assessed using average UniFrac distances. The greatest distance was observed among those infants at 0 months, and the distance significantly decreased at 1, 3, and 6 months versus 0 months. Notably, the distance within each group increased from 6 months to 12 months. PCoA: principal coordinates analysis; M0: 0 months; M1: 1 month; M3: 3 months; M6: 6 months; M12: 12 months.

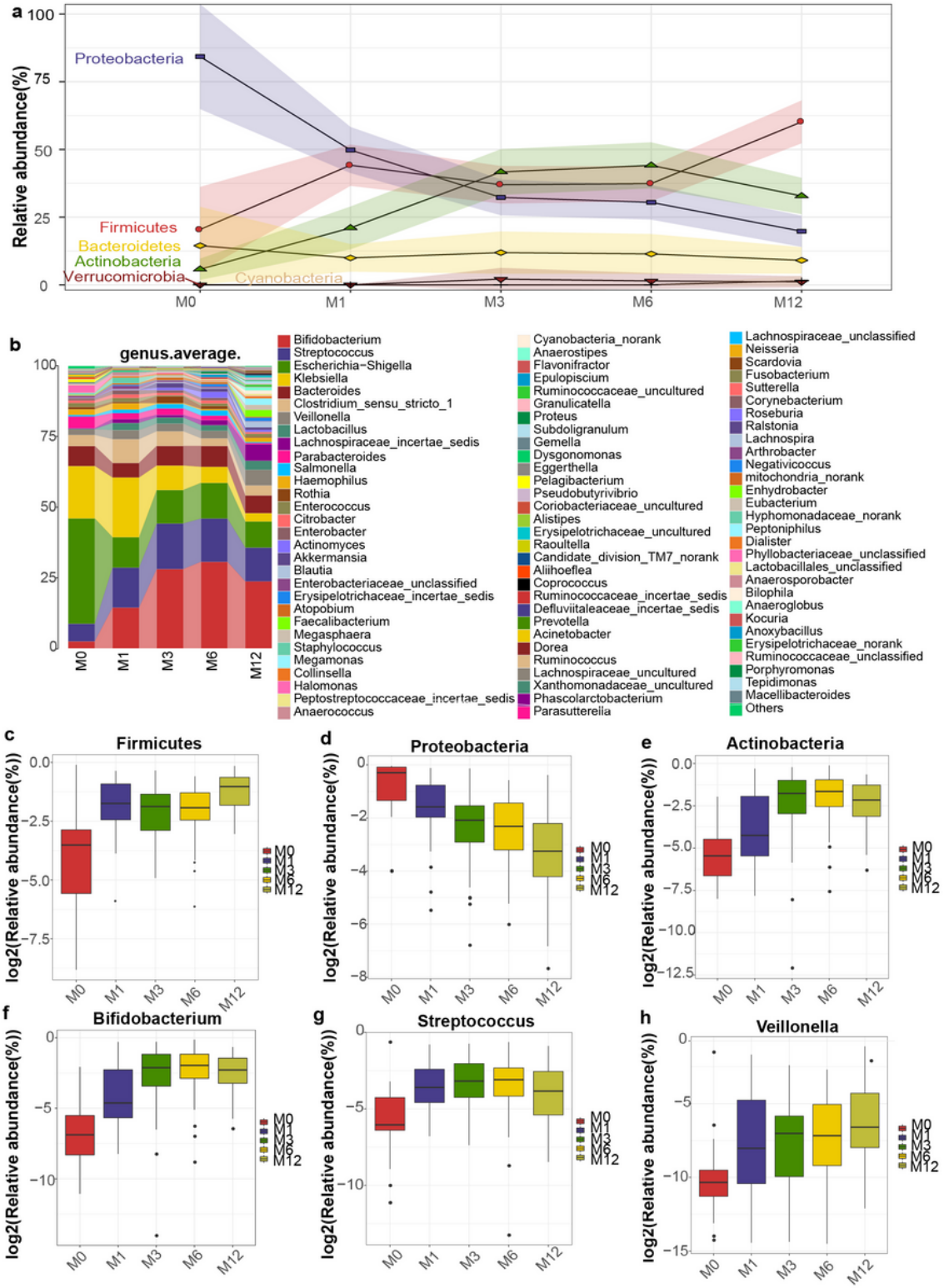

Figure 3

Gut microbiota community structure during the first year of life. Fecal microbiota composition and at the phylum (A) and genus levels (B) during the first year of life. Alterations in the bacterial phyla Firmicutes (C), Proteobacteria (D) and Actinobacteria (E) at 0, 1, 3, 6 and 12 months postpartum. Alterations in the bacterial genera Bifidobacterium (F), Streptococcus (G) and Veillonella (H) at 0, 1, 3, 6 and 12 months postpartum. The box presents the 95% confidence intervals, and the line inside denotes the median. M0: 0 months; M1: 1 month; M3: 3 months; M6: 6 months; M12: 12 months.

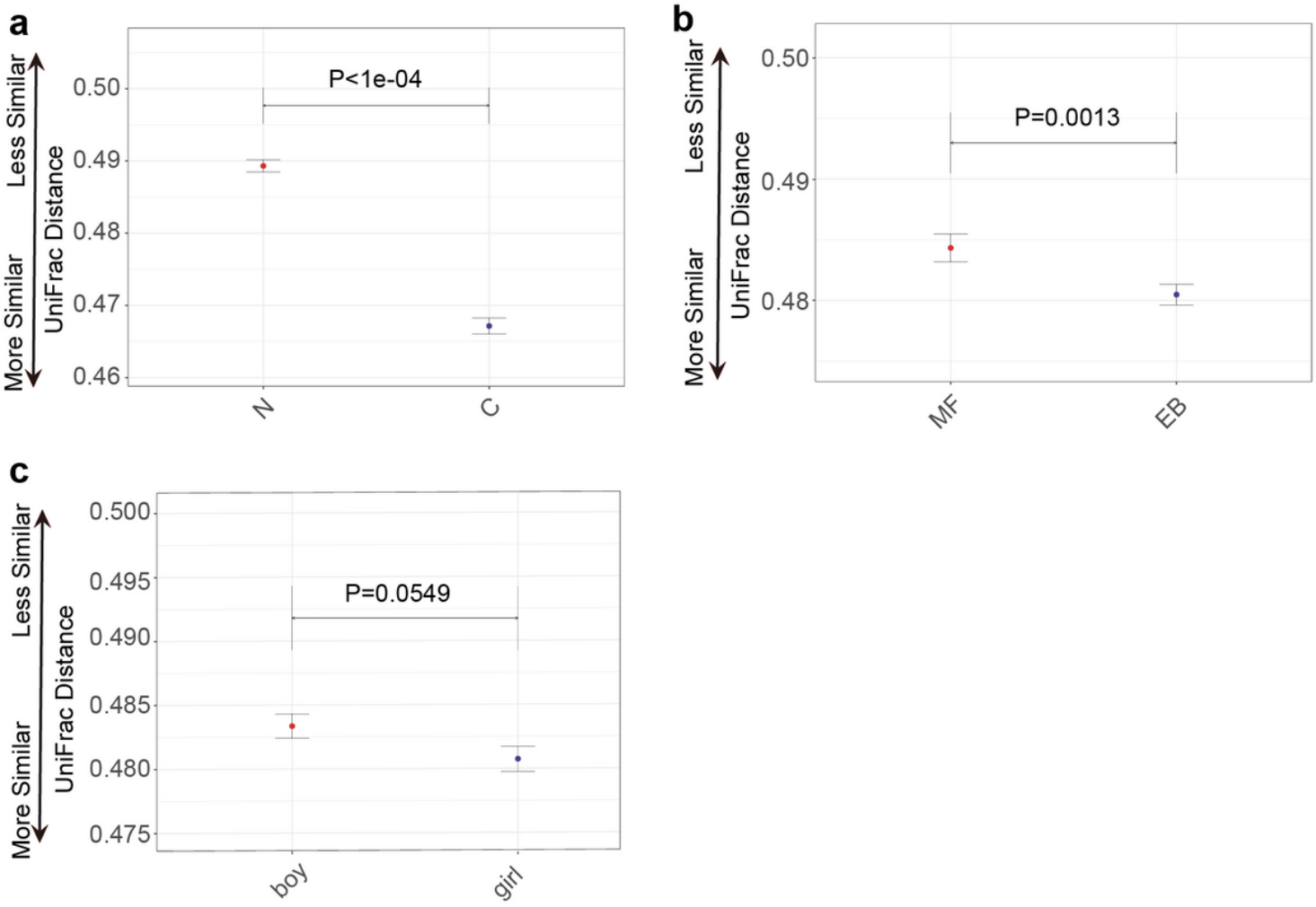

Figure 4

Individual microbial differences of the infants were analyzed within specific groups. (A) Microbial composition variation in vaginally delivered infants was greater than that in cesarean section infants; (B) differences in combined feeding infants were greater than exclusively breastfed infants; and (C) the distances revealed no significant difference between boys and girls. N: vaginally delivered; C: cesarean section. EB: exclusive breastfeeding; MF: combined feeding.

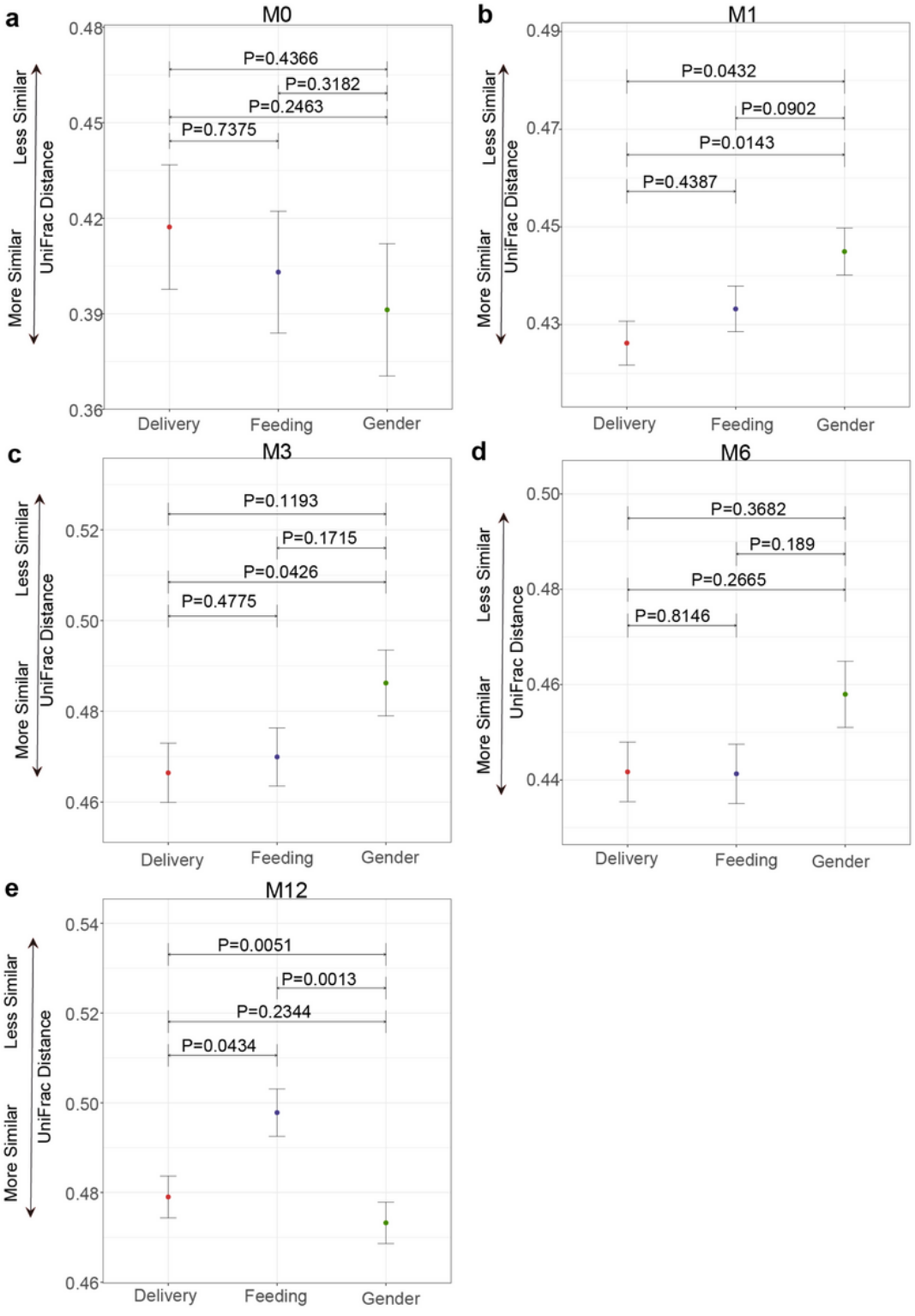

Figure 5

The microbial compositional similarities of the infants were calculated between groups. (A) At 0 months, the UniFrac distance between vaginal delivery and cesarean section was the largest, followed by the distances between feeding modes and gender differences. (B, C, D) At 1, 3 and 6 months, the average distances were greater between boys and girls than those between vaginal delivery and cesarean section and those between exclusively breastfed and combined feeding. (E) At 12 months, the greatest distance

was observed between exclusively breastfed and combined feeding, which was followed by that between vaginal delivery and cesarean section and that between boys and girls. N: vaginally delivered; C: cesarean section. EB: exclusive breastfeeding; MF: combined feeding. M0: 0 months; M1: 1 month; M3: 3 months; M6: 6 months; M12: 12 months.

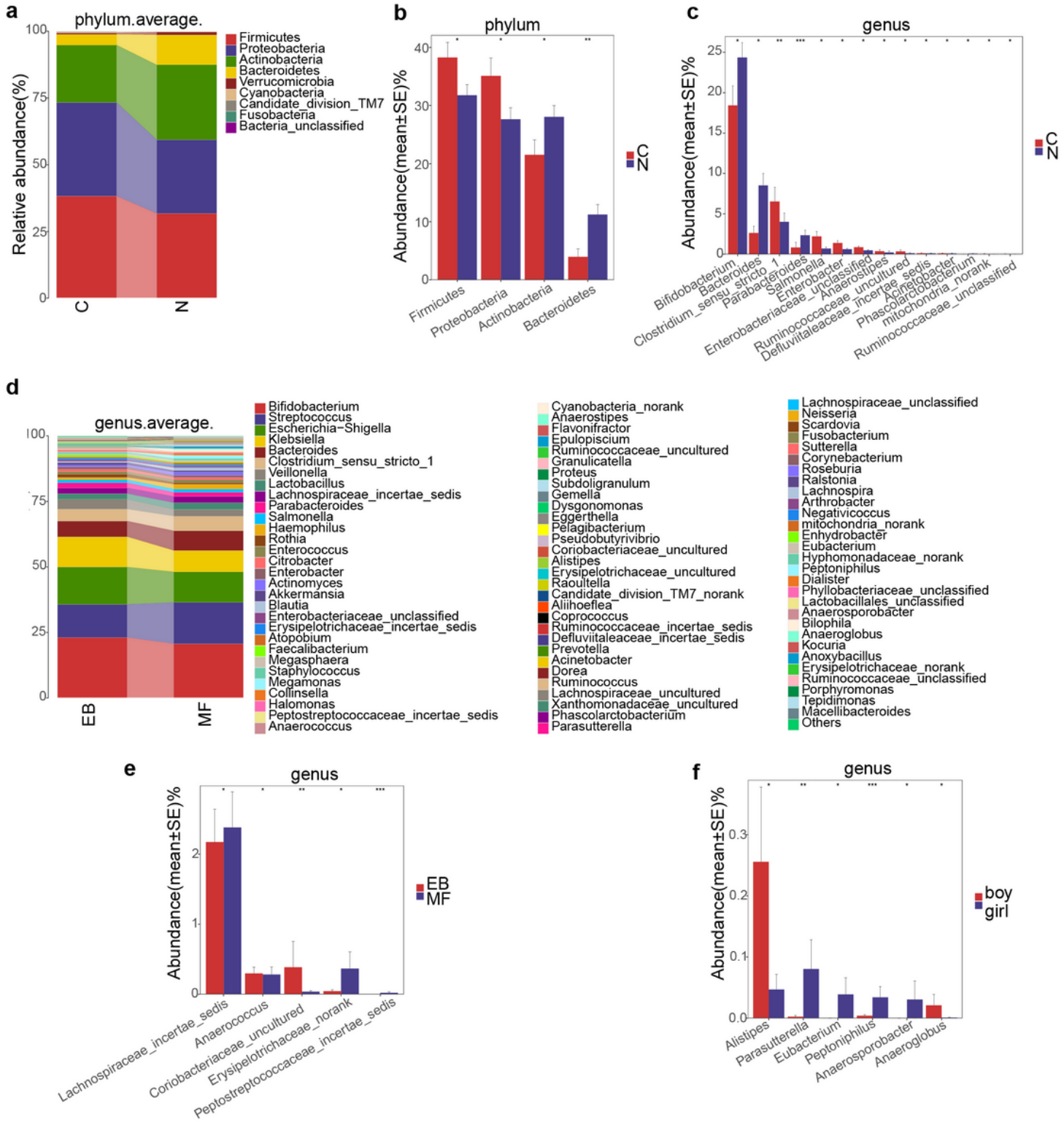

Figure 6

Differences in fecal microbial communities between groups. (A) Composition of the fecal microbiota at the phylum level between vaginal delivery and cesarean section groups. (B) Compared with their levels in cesarean section infants, 2 phyla were significantly increased, while 2 phyla were significantly decreased in vaginally delivered infants (all $p < 0.05$). (C) Four genera were increased, whereas 10 genera were decreased in vaginally delivered versus cesarean section infants (all $p < 0.05$). (D) Composition of the fecal microbiota at the genus level between exclusive breastfeeding and combined feeding infants. (E) Two genera were enriched, whereas 3 genera were decreased in exclusive breastfeeding versus combined feeding infants (all $p < 0.05$). (F) Two genera were increased, whereas 4 were reduced in boys versus girls (all $p < 0.05$). N: vaginally delivered; C: cesarean section. EB: exclusive breastfeeding; MF: combined feeding.

Supplementary Files

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