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The origin and establishment process of gut microbiota in early life

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The origin and establishment process of gut microbiota in early life

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Editorial: The origin and establishment process of gut microbiota in early life

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Editorial on the Research Topic
The origin and establishment process of gut microbiota in early life

Early life represents a critical window of human growth and development, accompanied by the initial colonization and maturation of microbes (microbiota) and their genes (microbiome), which plays a crucial role in physiology, metabolism, nutrition, and immunity response. Maternal microbiome exerts key influence on early microbial establishment and maturation in infants. Through exposure to the birth cannel, postpartum breastfeeding and intimate skin contact, infant early microbiome is shaped by maternal microbiota from vaginal, fecal, skin, and breastmilk. In this specialized Research Topics collection of Frontiers in Microbiology, we investigate the establishment process and developmental trajectory of gut microbiota in early life, and the impact of maternal microbiota on the healthy conditions of offspring, such as metabolic diseases, food allergy, and growth development in both human and animal studies.

Several contributions in this Research Topics highlight the maternal microbiota and microbial metabolites on infant health. The review published Jiang et al. presented studies showing how researchers came to the path of investigating maternal-associated microbial metabolites and then to present studies linking them to the health conditions of offspring. Generally, most researchers investigated the possible relationship between gut microbiota alteration and risks of a wide range of diseases in the host, and following studies further found the associations may be mediated by the microbial metabolites, especially during pregnancy and lactation maternal-associated microbial metabolites may have crucial roles in participating the microbial regulation of offspring diseases development. However, few studies investigate the functions of maternal-associated metabolites. For the early prediction, early diagnosis, early prevention, or early treatment of child diseases, high-quality animal and clinical trials are needed. Also, studies comprehensively evaluating the effects of altered maternal-associated metabolites on overall maternal and infant health maintenance are required.

The study conducted by Wang S. et al. identify characteristics of the maternal gut microbiota in the third trimester and the infant gut microbiota in early life and the association of these microbiotas with infant food allergy. The results showed that maternal carriage of *Holdemania* during the third trimester strongly predicted the absence of food allergies in infants; However, this effect was not retained post-reconstruction of the infant gut microbiota after birth, suggesting that the effect of maternal gut microbiota on food allergy in the offspring may not be primarily mediated through the regulation of changes in the infant gut microbiota.

Preterm birth has an adverse effects of infant gut microbiota. Toubon et al. investigated the relationship between gut microbiota at 1 month after birth (hospitalization period) and 3.5 years of age in 159 preterm children. The results showed that the gut microbiota of preterm and full-term children at 3.5 years of age is characterized by two enterotypes dominated by either *Bacteroides* or *Prevotella*. Interestingly, we found that prematurity still imprints the gut microbiota of children, as the microbiota of preterm children showed lower diversity and different community composition than that seen in full-term children's microbiota. Additionally, the gut microbiota at 3.5 years of age was not related to that at 1 month in preterm children. At the same time, Chen et al. evaluate the characteristics of the gut microbiota of term Small for gestational age (SGA) infants and the associations between the gut microbiota in SGA infants and neurodevelopmental outcomes at 6 months of age. They found the gut microbial diversity of term SGA infants was significantly lower in the first week of life than that of term AGA infants. On the other hand, investigated microbial community assembly and dynamics in extremely low birth weight infants (ELBWI) over the first 2 weeks of life.

Maturation of the gut microbiota is shaped by numerous perinatal factors, and the delivery mode is a major determinant of the gut microbiota in the first weeks of life; cesarean section delivery disrupted the natural transmission of the gut microbiota from mothers to offspring. Matharu et al. highlight the strong impact of delivery mode on the gut microbiota developmental trajectories in healthy infants from 3 weeks to 1 year of age. The study shows a depletion of genus *Bacteroides* in 40% of the vaginally delivered infants result in the cluster to the cesarean section delivered infants, expanding the understanding of the impact of various early life factors on the colonization and dynamics of *Bacteroides* spp. in infants. In addition, the review submitted by Zhang et al. summarized the great significance of delivery mode on microbiota and health, as well as provided clinically feasible methods for the prevention and treatment of cesarean section related gut diseases.

This collection of articles provides an enhanced understanding of the developmental trajectory and effect factors of microbiota establishment in the early life, which also raises more questions. Studies comprehensively investigating the triangle relationship among the establishment of infant microbiota, various perinatal factors and long-term health maintenance are required.

Author contributions

YL drafted the editorial text. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Effects of Delivery Mode on the Gut Microbiota and Health: State of Art

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The delivery mode is an important factor driving alteration in the gut microbiota during the neonatal period. Several studies prove that the alteration of gut microbiota induced by cesarean section could influence the activation of intestinal epithelial cells and the development of immune system. Further, some autoimmune and metabolic disorders may be related to the microbiota dysbiosis in infants caused by cesarean section. It is noteworthy that probiotics could promote the intestinal microecology, which may further prevent and treat cesarean section related diseases. This review summarized the great significance of delivery mode on microbiota and health, as well as provided clinically feasible methods for the prevention and treatment of cesarean section related gut diseases.

Keywords: cesarean section, vaginal delivery, gut microbiota, intestinal epithelial cells, immune system, probiotics

INTRODUCTION

A major determinant of the composition of neonatal microbiota is the delivery mode (Penders et al., 2006). Cesarean section (c-section) is an important operation in the field of obstetrics, which is a lifesaving intervention to avoid the risk of mother and child (Sandall et al., 2018). However, in recent years, c-section has been overused. The proportion of c-section delivered infants has increased year by year, especially in China, which is close to 50%. The differences of delivery mode have been linked with the differences of gut microflora of infants (Dominguez-Bello et al., 2010). The birth canal contains probiotics such as *Lactobacillus reuteri*, *L. rhamnosus* and so on. Compared with the c-section infants, the oral cavity, nasal cavity, skin and other parts of vaginally delivered infants will be exposed to more beneficial bacteria. The bacterial communities in vaginally delivered infants are similar to those of the maternal vagine. Studies have confirmed that the gut microbiota of c-section infants is significantly different from that of vaginally delivered infants. The bacterial communities in c-section infants are similar to those found on their own mother's skin surface (Goedert, 2016). The mode of delivery may give rise to variations in the microbial development, which may then result in alterations in normal physiology or disease susceptibility (Dominguez-Bello et al., 2010). It was also found that the vaginal microbial transmission is conducive to the establishment of the initial gut microbial structure of neonates (Dominguez-Bello et al., 2010, 2016;

Shao et al., 2019). In this review, we summarize recent studies concerned that c-section is closely related to an increased risk of food allergy, asthma, diabetes, obesity and other autoimmune and metabolic diseases in children. We also try to explain the possible mechanism that the changes of gut microbiota induced by c-section influence the activation of intestinal epithelial cells and the development of immune system. Probiotics can improve the development of gut microbiota, intestinal barrier and immune system in infants, and may also be used to prevent and treat c-section related gut diseases. These findings contribute to understand the influences of the delivery mode on diseases *via* gut microbiota.

INFLUENCES OF DELIVERY MODE ON THE GUT MICROBIOTA

Human gut is the habitat of diverse and dynamic microbial ecosystems. Gut microbiota is involved in many basic metabolic pathways and the maturation of the immune system in host. The composition of gut microbiota is unique to each individual after bacterial colonization in infants, though more than 95% of gut microbiota can be divided into four major phyla (Landman and Quévrain, 2016). The establishment of gut microbiota during infancy may be affected by many factors, including the delivery mode (Rutayisire et al., 2016). A number of studies have correlated delivery mode with significant variations of gut microbiota in infants (**Table 1**).

Fecal samples were collected from 596 healthy, full-term infants, including 282 c-section delivered infants and 314 vaginally delivered infants (Shao et al., 2019). During neonatal period (≤1 month), fecal samples of these babies are collected at least once. Fecal samples are resampled later from 302 babies during infancy (8.75 ± 1.98 months). Besides, they also collect the fecal samples from 175 mothers paired with 178 babies. Metagenomic analysis of the total 1,679 fecal samples reveals that the delivery mode is the most important factor leading to the variation of gut microbiota during neonatal period. On the fourth day, the largest influence of the delivery mode is observed (R2 = 7.64%, $P < 0.001$). Some studies suggest that early-life antibiotic exposures and breastfeeding play an important role in the establishment of gut microbiota (Bokulich et al., 2016; Kim et al., 2019), however, in Shao's work, some meaningful clinical covariates which are related to hospital delivery (such as the duration of hospitalization and the perinatal antibiotic usage), as well as breastfeeding, exhibit smaller effects. The influence of delivery mode decreases with age but remains prominent when sampling during infancy (R2 = 1.00%, $P = 0.002$). Maternal-infant transmission of gut microbiota is an important mode of bacterial transmission. They profile the gut microbiota across 178 mother–baby dyads in order to evaluate whether the changes of neonatal microbiota are related to the differential transmission of maternal microbiota. The results indicated that 74.39% of the early microbiota in vaginally delivered infants is obtained from maternal microbial strains. In contrast, only 12.56% of the early microbiota in c-section delivered infants is obtained from maternal microbial strains. *Parabacteroides* spp., *Bacteroides* spp., *Bifidobacterium* spp., and *E. coli* are most commonly transmitted to infants from mothers *via* vaginal delivery. Most of c-section delivered infants still cannot acquire *Bacteroides* species, such as *B. vulgatus*, after neonatal period. These results emphasize that neonatal period is a crucial early window for transmission of gut microbiota from mother to infant. Dominguez-Bello et al. (2010) have found that the composition of gut bacterial communities in vaginally delivered infants is similar to that of the maternal vaginal microbiota, dominated by *Prevotella*, *Sneathia*, and *Lactobacillus* spp., while that of c-section delivered infants is similar to that of mothers' skin surface, dominated by *Propionibacterium*, *Corynebacterium*, and *Staphylococcus* spp. The maternal vaginal microbiota provides the physical habitats where the first natural microbial exposure to neonates. Differences in microbial succession modes of body habitats, such as the gut, may be caused by differences in initial communities, which will continue over time. The *Lactobacillus* are enriched in the gut at the early stage of vaginally delivered neonates, and then *Bacteroides* increased in the second week. However, c-section delivered infants do not have these above microbial features (Dominguez-Bello et al., 2016). In 2019, Akagawa et al. (2019) demonstrated that at 4 days of age, the bacterial diversity of vaginally delivered neonates was significantly higher than those delivered by c-section. *Enterobacteriales* and *Bacteroidales* in vaginally delivered neonates are overrepresented ($P = 0.011$ and $P = 0.0031$), while *Lactobacillales* and *Bacillales* in c-section delivered neonates are overrepresented ($P = 0.0016$ and $P = 0.012$). However, there is little difference in relative abundance and bacterial diversity among infants at 1 month of age. It was considered that c-section delivery appeared to decrease the diversity of gut microbiota in neonates, leading to dysbiosis. However, since breast milk may help correct the dysbiosis induced by c-section, this situation improves to the equivalent level seen in 1-month-old vaginally delivered infants. It was found that transplant the maternal vaginal microbiota to their children delivered by c-section could partially restore the above-mentioned microbiota characteristics, although the long-term effect of the transplantation had yet to be further confirmed by long-term observation (Dominguez-Bello et al., 2016). These studies indicate that delivery mode is a significant factor affecting the gut microbiota of infants.

Rutayisire et al. (2016) found that the colonization pattern and diversity of gut microbiota were obviously correlated with the delivery mode in the first three months of life, but the significant differences observed disappeared after 6 months of life. Meanwhile, Bokulich et al. (2016) showed that, compared to vaginally delivered infants, c-section delivered infants exhibited significantly decreased evenness, richness, and phylogenetic diversity at baseline during the first month of life. Subsequently, children delivered by c-section showed lower richness and diversity up to the age of 2, especially after 8 months of age. However, other studies suggested that microbiota dysbiosis may persist much longer, until the age of 2 or even 7 (Salminen et al., 2004; Jakobsson et al., 2013). Different confounding and concurrent factors can partly explain the differences in

TABLE 1 | Studies on the association between the delivery mode and gut microbiota.

	Author	Year	Countries	Study design	Method	Predominant gut microbiota in vaginally delivery infants	Predominant gut microbiota in cesarean section infants	References
1	Shao et al.	2019	United Kingdom	$n1^1$ = 314, $n2^2$ = 282. During their neonatal period (≤1 month), fecal samples are collected from all babies at least once. During infancy (8.75 ± 1.98 months), 302 babies are resampled later. Maternal fecal samples are obtained from 175 mothers paired with 178 babies.	Longitudinal sampling and whole-genome shotgun metagenomic analysis.	*Bifidobacterium* (such as *Bifidobacterium breve* and *Bifidobacterium longum*), *Bacteroides* (*Bacteroides vulgatus*), *Escherichia* (*Escherichia coli*) and *Parabacteroides* (*Parabacteroides distasonis*).	*Enterococcus faecium*, *Enterococcus faecalis*, *Streptococcus parasanguinis*, *Staphylococcus epidermis*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Clostridium perfringens*, and *Enterobacter cloacae*. Opportunistic pathogens associated with the hospital environment (including *Klebsiella* species, *Enterobacter* and *Enterococcus*).	Shao et al., 2019
2	Dominguez-Bello et al.	2010	Venezuela	$n1^1$ = 4, $n2^2$ = 6. Before delivery, maternal skin, oral mucosa, and vagina are sampled 1 h. After delivery, neonates' skin, nasopharyngeal aspirate, and oral mucosa are sampled <5 min, and meconium <24 h.	Multiplexed 16S rRNA gene pyrosequencing.	*Lactobacillus*, *Prevotella*, and *Sneathia* spp.	*Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp.	Dominguez-Bello et al., 2010
3	Dominguez-Bello et al.	2016	United States	$n1^1$ = 7, $n2^2$ = 11. During the first two minutes of birth, these neonates delivered by c-section are exposed to their maternal vaginal contents by wiping with gauze, beginning with the mouth, next the face, and lastly the rest of the body. A total number of 1,519 samples are obtained from the oral, skin and anal of infants and mothers. These samples are obtained at six time points (1, 3, 7, 14, 21, and 30 days) during the first month of life after birth.	Sequencing the V4 region of 16S rRNA gene.	*Lactobacillus* are enriched in the early stage followed by a bloom of *Bacteroides* from the second week.	There is no such phenomenon.	Dominguez-Bello et al., 2016
4	Akagawa et al.	2019	Japan	Fecal samples of 36 healthy Japanese neonates are obtained at 4 days and 1 month of age.	16S rRNA sequencing	*Bacteroidales* and *Enterobacteriales.*	*Bacillales* and *Lactobacillales.*	Akagawa et al., 2019

[1]n1 = the number of vaginally delivered infants; [2]n2 = the number of cesarean section infants.

the results obtained by different authors. These factors are not always correctly identified as neonatal exposure (such as feeding type), or fully reported (such as geographical or ethnic differences), or even due to factors associated with the experimental technology used (DNA extraction method, culture technology, etc.) (Arboleya et al., 2018).

LONG-TERM HEALTH CONSEQUENCES OF GUT MICROBIAL DYSBIOSIS INDUCED BY C-SECTION

The gut of human contains approximately 10^{14} bacteria and many other microorganisms (Landman and Quévrain, 2016). The symbiotic relationship between host and gut microbiota ensures proper development of the metabolic system in humans (Patterson et al., 2016). A variety of perinatal determinants, such as c-section delivery, could influence the pattern of bacterial colonization and lead to dysbiosis (Butel et al., 2018). Dysbiosis is usually driven by a set of host-related and environmental factors, which contribute to alterations in the composition and function of the gut microbiota (Levy et al., 2017). Dysbiosis is characterized by the expansion of potentially harmful microbiota, the loss of beneficial microbiota, and/or the loss of overall microbial diversity (Petersen and Round, 2014). Long-term dysbiosis of gut microbiota may have long-lasting functional effects and result in a variety of diseases. The alterations of the microbiota are important causes of increased risk of food allergy, asthma, diabetes and obesity.

Microbiota and Food Allergy

In recent decades, the prevalence of food allergy has been rising with economic growth and urbanization, which affects up to 10% of populations all over the world (Tang and Mullins, 2017; Sicherer and Sampson, 2018). The increased incidence of allergic diseases is related to changes in environmental factors and human lifestyle, including changes in external and internal microbial communities. C-section delivery may be susceptible to allergic disorders, which presumably due to the changes in the establishment of normal gut microbiota during early infancy (Papathoma et al., 2016; Mitselou et al., 2018). Recent evidence suggests that the increase in prevalence of food allergy is related to alterations in the gut microbial composition and function (Azad et al., 2015; Chen et al., 2016).

In 2016, Chen et al. (2016) conducted a case-control study on 22 healthy children and 23 children with food allergy. It was found that food allergy was related to the alterations of gut microbiota. There is lower diversity of the total microbiota ($P = 0.01$) in children with food allergy. Compared with healthy children, the number of Proteobacteria, Actinobacteria, and Firmicutes are obviously increased at the phylum level and that of *Bacteroidetes* bacteria is obviously reduced in children with food allergy. Besides, significant differences are observed in the children with food allergy at the genus level, including the elevated abundances of *Subdoligranulum* and *Clostridium* IV, and the depressed abundances of *Veillonella* and *Bacteroides*. In 2015, Azad et al. (2015) performed a general population cohort study of 166 infants and found that gut colonization in infancy might increase the risk of developing food allergy and atopic disease. The low abundance of gut microbiota and the elevated ratio of Enterobacteriaceae to Bacteroidaceae in early infancy are related to subsequent food allergy. There is a 55% decrease in the risk of food allergy at 1 year when the abundance of microbiota in infants increases by every quartile at 3 months (aOR 0.45, 95% CI: 0.23–0.87). Separately, a two-fold increase in risk is related to each quartile increase in the proportion of Enterobacteriaceae to Bacteroidaceae (2.02, 1.07–3.80). At 1 year, the proportion of Enterobacteriaceae to Bacteroidaceae remains elevated in infants with food allergy, while the abundance of Ruminococcaceae tends to decrease. These results suggest that the dysbiosis of gut microbiota is linked to an elevated risk of allergic diseases.

Microbiota and Asthma

Asthma is a common chronic and non-communicable diseases in children (Papi et al., 2018), which can be characterized by intermittent respiratory symptoms, reversible airflow obstruction and airway inflammation (Levy and Fleming, 2020). Asthma is thought to be a result of the complex interaction between environment and gene. Recent studies have proved c-section is associated with asthma (Moya-Pérez et al., 2017; Stokholm et al., 2020). As described before, c-section has a great impact on the earliest microbiota and its development (Dominguez-Bello et al., 2010, 2016; Shao et al., 2019). Studies have also found that early-life dysbiosis of microbiota is an important factor affecting the development of asthma (**Table 2**; Holgate, 2012; Arrieta et al., 2015; Fujimura et al., 2016; Stokholm et al., 2018).

In 2015, Arrieta et al. (2015) compares the gut microbiota of 319 infants and shows that infants at risk of asthma exhibit transient gut microbial dysbiosis during the first 100 days of life. The relative abundance of the bacterial genera *Rothia*, *Faecalibacterium*, *Veillonella*, and *Lachnospira* is significantly reduced in infants at risk of asthma. The decrease of bacterial taxa is accompanied by the dysregulation of enterohepatic metabolites and the decrease of fecal acetate levels. Inoculating these four bacterial taxa into germ-free mice can improve airway inflammation of their adult offspring, which proves the causal role of these four bacterial taxa in avoiding the development of asthma. Another important finding of this study is that infants with atopy (that is, allergy skin prick testing) and clinical wheeze data at 3 months of age have a significant decrease in fecal acetate. The conducted asthmatic animal models show that the butyrate, acetate, propionate, and SCFAs, which can stimulate dendritic cells and Tregs and prevent Th2-type immune responses (Holgate, 2012), have a protective effect on airway inflammation. In 2016, another study (Fujimura et al., 2016) indicated that neonatal gut microbiota dysbiosis led to CD4 + T cell dysfunction associated with childhood atopy, which affected the susceptibility of allergic asthma in children. These results proved that the first 100 days of human life represented a critical window of early life, in which dysbiosis of gut microbiotal was related to the risk of asthma and allergic disorders. In 2018, a study from Stokholm et al. (2018) demonstrated that the immature microbial composition of 1-year-old children could increase the risk of developing asthma at the age of 5. It is only evident in children born to mothers with asthma, which indicates that children born to mothers with asthma are vulnerable to gut microbiota. Meanwhile, maternal asthma status doesn't influence the composition and maturation of gut microbiota in early life, which points to mechanisms other than strict genetic effects. The immaturation of gut microbiota in the first year of life is a crucial determinant of the elevated risk of asthma.

Microbiota and Diabetes

Diabetes is a group of chronic metabolic diseases resulted from insulin secretion and (or) action deficiency. It is characterized by elevated blood glucose levels, which will seriously damage the eyes, heart, kidneys, nerves, and blood vessels over time (Al-Awar et al., 2016). According to etiological classification, type 1 diabetes (T1D) and type 2 diabetes (T2D) are the two most common types of diabetes. T1D accounts for about 5–10% of all types of diabetes, once known as insulin-dependent or juvenile diabetes. The pancreas of patients with T1D produces little or no insulin. The most common form is T2D, affecting 90 to 95% of patients with a family history of diabetes, which usually occurs in adults. T2D happens when the body is unable to produce enough insulin or resistant to insulin (Pascale et al., 2018).

T1D is an autoimmune disease. A meta-analysis based on 20 studies demonstrates that c-section delivery contributes a 20% increased risk of childhood-onset T1D, which may reflect differences in microbial exposure in early life (Cardwell et al., 2008). It has been shown that delivery mode has an important influence on the immunological function and the development of gut microbiota in infants (Huurre et al., 2008), both of

TABLE 2 | Studies on the association between the gut microbiota and asthma.

	Author	Year	Countries	Study design	Method	Analysis of the relationship between gut microbiota and the risk of asthma	References
1	Arrieta et al.	2015	Canada	Cohort of 319 infants. According to allergy skin prick testing (that is, atopy) and clinical wheeze data at the age of 1, these infants were divided into 4 different clinical phenotypes: controls (*n* = 74), atopy only (*n* = 87), wheeze only (*n* = 136), and atopy + wheeze (AW, *n* = 22).	16S rRNA gene sequencing, quantitative PCR and PICRUSt analysis, fecal SCFAs and urinary metabolomic analysis.	During the first 100 days of life, infants at risk of asthma show gut microbial dysbiosis transiently, which is characterized by the decrease of four bacterial genera-*Rothia*, *Veillonella*, *Faecalibacterium* and *Lachnospira*, and the increased risk of asthma. Children in the AW phenotype present this gut microbial characteristic at 3 months, and compared with the control group, the AW group at the age of 3 are 21.5 times more likely to develop asthma. Atopic dermatitis and antibiotic exposure in the first year of life were factors that increase infants' risk of being classified as AW compared controls.	Arrieta et al., 2015
2	Fujimura et al.	2016	Southeastern Michigan	Cohort of 298 infants. Assuming that there exist human neonatal intestinal microbiota (NGM) with different composition, which are associated with relative risk (RR) of childhood asthma. Newborn (median age, 35 days) were divided into three microbiota composition states (NGF1-3) according to fecal samples (age 1–11 months) and 16S rRNA sequencing.	16S rRNA sequencing, Bacterial- and fungal-community profiling, PICRUSt and metabolomic analyses.	Neonatal gut microbiota affect the susceptibility of allergic asthma in children, possibly affecting CD4 + T cell populations and function through changes in the gut microenvironment. Labeled NGM3, the highest risk group, exhibit higher relative abundance of particular fungi (for example, *Rhodotorula* and *Candida*), lower relative abundance of certain bacteria (*Faecalibacterium*, *Akkermansia* and *Bifidobacterium*), and a unique fecal metabolome rich in pro-inflammatory metabolites.	Fujimura et al., 2016
3	Stokholm et al.	2018	Copenhagen	Cohort of 700 children recruited in pregnancy. During the first 5 years of life, children were prospectively followed up for deep clinical phenotyping at 11 scheduled visits, including 1 week, 1, 3, 6, 12, 18, 24, 30, and 36 months, and then once a year.	16S rRNA gene amplicon sequencing of the V4 region.	The immature microbial composition of 1-year-old children could increase the risk of developing asthma at the age of 5. It is only evident in children born to mothers with asthma, which indicates that the immaturation of gut microbiota in the first year of life is a crucial determinant of the elevated risk of asthma. Maternal asthma status doesn't influence the composition and maturation of gut microbiota in early life. In the healthy adult human gut, the most abundant Firmicutes families are *Ruminococcaceae* (including *Faecalibacterium* and *Ruminococcus*) and *Lachnospiraceae* (including *Roseburia* and *Lachnospiraceae incertae sedis*). In children with later asthma, the relative abundance of the above genera is lower, which indicates an overall delay in microbial maturation.	Stokholm et al., 2018

which are related to the development of diabetes (Bonifacio et al., 2011). Leiva-Gea et al. (2018) have found that T1D is related to the differences in gut microbial composition. Compared with healthy children, the diversity of gut microbiota is significantly lower in patients with T1D. A case-control study (Murri et al., 2013), which conducted in 16 healthy children and 16 children with T1D, demonstrated that T1D was related to compositional changes in gut microbiota. It was found that compared with the healthy children, the bacterial number of Firmicutes and Actinobacteria decreased significantly while that of Bacteroidetes increased significantly in the children with T1D. And this change was associated with the glycemic level of the diabetic children. Moreover, the numbers of mucin-degrading bacteria, butyrate-producing bacteria and lactic acid-producing bacteria were significantly lower in the diabetic children than the healthy children, which were important to maintain gut integrity. The findings are helpful to develop strategies to control the development of patients with T1D by regulating their gut microbiota.

In 2020, Chavarro et al. (2020) suggested that c-section delivery was related to an increased risk of T2D of the offspring in adulthood. They conduct a prospective cohort study and find that, compared with vaginally delivered offspring, those delivered by c-section have a 46% higher risk of T2D in adulthood. It is

known that delivery mode has an important influence on the gut microbiota of offspring (Dominguez-Bello et al., 2010, 2016; Shao et al., 2019). A study have showed that the gut microbiota is an environmental factor controlling energy metabolism, which is closely related to metabolic disorders such as T2D (Cani and Delzenne, 2007). It was reported that T2D was related to the dysbiosis of gut microbiota (Zhang et al., 2020) rather than the effect of a simple increase in diversity or a single microbe (Wu et al., 2010). The fecal samples of 16 patients with T2D and 12 healthy individuals were collected in this study and found that the fecal microbial composition in diabetic group was different from the healthy group. There are significant differences in the number of *Bifidobacterium* and the similitude of bacterial community between diabetes group and healthy group. It is reported that the genera of *Bifidobacterium*, *Bacteroides*, *Roseburia*, *Akkermansia*, and *Faecalibacterium* are negatively correlated with T2D, while the genera of *Blautia*, *Fusobacterium*, and *Ruminococcus* are positively correlated with T2D (Gurung et al., 2020).

Microbiota and Obesity

In 2016, the World Health Organization (WHO) evaluated that 39% (39% of men and 40% of women) of adults older than 18 were overweight. Between 1975 and 2016, the global prevalence of obesity almost tripled. Obesity is a major risk factor of developing chronic diseases, which can lead to cardiovascular diseases (CVDs), type II diabetes (T2D), musculoskeletal disorders and cancers (breast, liver, gallbladder, kidney, colon, endometrial, ovarian, and prostate) (Li et al., 2017). An ongoing prospective cohort study, enrolls 116,671 women aged 24 to 44 years, conducted in 1989. Throughout the follow-up period, they find that being born by c-section is related to an 11% higher risk of obesity. There is growing evidence that obesity in adulthood among individuals born by c-section is associated with changes in gut microbiota (Cani and Delzenne, 2007). It was shown that mice in c-section group gain more body mass and the phenotype of females is even stronger after weaning (Martinez et al., 2017). Compared with the control mice, C-section-born mice lack the dynamic developmental changes of gut microbiota. Here, they found that the bacterial taxa which are related to vaginal delivery, such as *Clostridiales*, *Ruminococcaceae*, and *Bacteroides*, have previously been related to lean phenotypes in mice. The results prove that there is a causal relationship between c-section and weight gain, and could support that maternal vaginal bacteria participate in the normal metabolic development of offspring. The results also suggest that maternal vaginal microbiota is necessary for normal metabolic development during delivery and provide important new information in the context of the global obesity epidemics. Generally, the change of microbial composition may result in weight gain through three ways: (1) microbiota may modulate the expression of gut gene, resulting in the increases of adipose and free fatty acids levels; (2) microbiota can convert indigestible food into biochemical absorbable nutrients, which can increase energy harvest; and (3) gut microbiota induce an inflammatory state of obesity by activating lipopolysaccharides (Pei et al., 2014). Therefore, dysbiosis of gut microbiota induced by c-section would be a target for prevent obesity (Isolauri, 2017).

DYSBIOSIS OF THE GUT MICROBIOTA INDUCED BY CESAREAN SECTION AFFECTS ACTIVATION OF INTESTINAL EPITHELIAL CELLS AND IMMUNE SYSTEM

C-Section and Activation of Intestinal Epithelial Cells

Intestinal epithelial cells (IECs) are non-hematopoietic cells. They resist external antigens by forming a physical barrier (Goto, 2019) and are the first defensive line of mucosal surface barrier. The activity of IECs greatly influence the gut microenvironment and immunity (Adachi et al., 2016). Lotz et al. (2006) have found that vaginally born mice show spontaneous activation of IECs and tolerance to Lipopolysaccharide (LPS) within two hours after birth. LPS, a product of microorganisms and their cell wall, could stimulate the innate immune response of immature fetal enterocyte and increase the production of IL-8 by activating the transcription factor NF-κB (Fusunyan et al., 2001). These findings can be confirmed by the detection of IκB-α phosphorylation, nuclear translocation of the NF-κB subunit p65 and transcriptional activation of chemokine MIP-2. However, this phenomenon is not found in c-section delivered neonates or in TLR4-deficient mice. The loss of LPS responsiveness after birth is related to the down-regulation of IL-1 receptor-associated kinase 1 after transcription, which plays a significant role in epithelial TLR4 signaling *in vitro*. The decreasing levels of TLR4 combined with the increasing levels of IκB-α expression by IECs effectively increases the threshold of immune activation in intestinal epithelium (Francino, 2018). IECs acquire TLR tolerance directly by exposure to exogenous endotoxin immediately after birth, which can promote subsequent birth colonization and maintain a stable intestinal host-microbiota homeostasis. However, intestinal epithelium cells of c-section infants could not activate of immediately after birth by LPS and then result in dysbiosis of microbiota.

C-Section and Development of Immune System

Delivery mode strongly affect the early neonatal microbial exposure and immune environment. Labor can induce immune responses in the uterine cavity. These intrauterine immune responses will not occur in elective c-sections, which can affect the immune environment of neonate (Thornton et al., 2003). The first few months of life are a critical time window in the establishment of tolerance and the development of the immune system, delivered by c-section will affect the lifelong risk of developing immune diseases (Francino, 2018).

Delivery mode could make a difference in the frequencies of lymphocyte subset in full-term newborns (Pittard et al., 1989). The characteristics of full-term c-section neonates are that T cells and helper T cells increase significantly, while natural killer cells decrease significantly. In contrast, the characteristics of vaginally delivered neonates are that the frequency of T cells and helper T cells decrease significantly, while natural killer cells increase

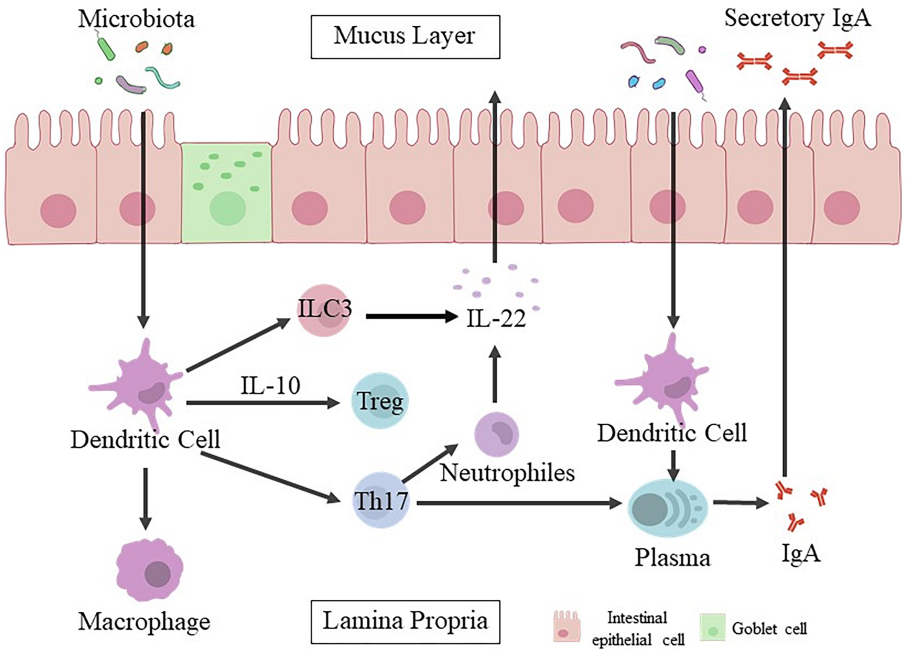

FIGURE 1 | Intestinal epithelial cells mediate the crosstalk between gut microbiota and host immune responses. The mucosal barrier formed by intestinal epithelial cells can prevent the conflict between gut microbiota and host immune cells, and keep bacteria away from the epithelial surface. Microbial signals are captured by epithelial cells through their specific receptors directly or transmitted by dendritic cells (DCs) to activate other innate immune cells, including innate lymphocytes (ILC3) and macrophages (Mφ). Dendritic cells activated by bacteria are also involved in the recruitment and activation of adaptive immune cells. Plasma cells release IgA. Once IgA is attached to the polymeric immunoglobulin receptor (pIgR), it will enter the gut lumen in the form of secretory IgA (sIgA). Besides, various populations of CD4 + T cells are induced simultaneously, and their function is balanced to maintain physiological inflammation, especially under the control of Treg and IL-10. Th17 production (T helper 17).

significantly (Samelson et al., 1992; Cuppari et al., 2015). To a large extent, immune tolerance to environmental antigens is mediated by peripheral Foxp3 + regulatory T cells (Knoop et al., 2017). It was found that the adult mice delivered by c-section had lower proportions of tolerogenic CD103 + dendritic cells and Foxp3 + regulatory T cells (Hanse et al., 2014; Zachariassen et al., 2019). In contrast, the Foxp3 + regulatory T cells of vaginally delivered neonates are elevated (Yildiran et al., 2011). T and B lymphocyte subpopulations of c-section delivered infants are increased. The positive rate of cell surface markers of B lymphocyte subpopulations is obviously higher in infants delivered by elective c-section (Gasparoni et al., 1992).

Changing bacterial colonization at the intestinal level by c-section might play a important role in affecting the development of immune system (Cho and Norman, 2013). Gut microbiota is closely related to immunity system (**Figure 1**), which could promote the development of mesenteric lymph nodes, isolated lymphoid follicles, gut-associated lymphoid tissue Peyer's patches and/or recruitment of mature immune cells (Renz et al., 2011; Olin et al., 2018). Abnormal intestinal colonization in c-section delivered infants may prolong postnatal immunological immaturity, and hinder proper immune initiation, leading to increase the risk of later immune diseases (Ly et al., 2006). Colonization of gut microbiota in neonates initiates the immune system and lead to the imbalance of Th1/Th2.56 The levels of Th1 related chemokines CXCL10 and CXCL11 are significantly lower in the blood of c-section delivered infants. C-section is associated with delayed colonization of the Bacteroidetes phylum, reduced diversity of total microbiota and decreased Th1 responses in the first two years after birth (Jakobsson et al., 2013).

Cytokines are of great importance in labor, which can affect the neonatal immunity (Yektaei-Kari et al., 2007). Vaginal delivery can promote the production of cytokines. However, cord blood from c-section delivered infants with decreased levels of IFN-γ and TNF-α can damage their production (Keelan et al., 2003). A prospective study find that the levels of IL-6, IL-1β, IFN-γ, and TNF-α in c-section delivered neonates are significantly lower than those in vaginally delivered neonates (Malamitsi-Puchner et al., 2005). Puff et al. (2015) have found that compared with vaginally delivered infants, the concentrations of cytokines IL-8 and IFN-γ is lower in c-section delivered infants. This study also shows that c-section delivered infants have lower cytokine release in early childhood than vaginally delivered infants. Besides, the level of immunoglobulin (Ig) in c-section delivered infants is lower than that in normal vaginal delivery. Bonifacio et al. (2011) devise a substantial cohort of young infants and find that compared with vaginally delivered infants, 1-year-old infants delivered by c-section contain more immune cells secreting IgA and IgG. The cord blood level of IgG in c-section delivered infants is significantly lower than that in normal vaginally delivered infants (Agrawal et al., 1996). However, the cord blood level of

IgE in c-section delivered infants is higher than that in vaginally delivered infants (Petrovičová et al., 2016).

PROBIOTICS MAY PREVENT AND TREAT CESAREAN SECTION RELATED GUT DISEASES

Probiotics are live micro-organisms which play a healthy role beyond the inherent general nutrition in the host after adequate intake. Probiotics could improve the dysbiosis of gut microbiota and restore gut function (Li et al., 2020). *Lactobacillus* and *Bifidobacterium* are dominant in maternal birth canal and also belong to probiotics (Yang et al., 2019). The undesired alterations in the composition and function of microbiota caused by caesarean section can be corrected by supplementing a probiotics mixture to infants (Korpela et al., 2018), which may be associated with the prevention and treatment of c-section related intestinal diseases.

Food allergy results from the deficiency of immune tolerance mechanism, which is regulated by the composition and function of gut microbiota. Selected probiotic strains could play the role of immune tolerance mechanisms (Paparo et al., 2019), and as a method of prevention and treatment of food allergy. The reintroduction of certain commensal microbes, such as Clostridia, could prevent or alleviate allergic symptoms in clinical trials and animal models (Shu et al., 2019). Besides, oral probiotics represent a possible treatment for asthma and allergic diseases (Kang et al., 2017). Studies show that probiotic supplementation can treat acute gastroenteritis in children and decrease the risk of developing necrotizing enterocolitis in premature infants (Bertelsen et al., 2016). Stewart et al. (2018) find that for people with potential T1D, early probiotic supplementation (within 27 days after birth) can reduce the risk of islet autoimmunity when compared with no probiotic supplementation or probiotic supplementation after 27 days. Minami et al. (2018) establish a randomized, double-blind, placebo-controlled trial and demonstrate that probiotic strain *B. breve* B-3 has the potential to reduce body fat in healthy pre-obese individuals. This probiotic strain can be used to prevent the accumulation of body fat and related metabolic disorders in pre-obese individuals. Moreover, although the composition of *in situ* microbiota remains unchanged, supplementation of *B. lactis* NCC2818 can further improve intestinal barrier function, immunity, host metabolism and host-microbiota co-metabolism (Lewis et al., 2017).

CONCLUSION

Delivery mode has impacts on the establishment and development of gut microbiota in infants. C-section could lead to dysbiosis of gut microbiota. Cesarean section affects the activation of IECs and the development of immune system through a variety of mechanisms. Long-term dysbiosis of gut microbiota will lead to some c-section related autoimmune and metabolic disorders. Probiotics can improve the dysbiosis of gut microbiota and restore gut function, which may be related to the prevention and treatment of c-section related intestinal diseases.

There are some questions that can be used in clinical research in the future:

1. There are few studies on the mechanisms of c-section induced alterations of immune system in offspring. What are the specific differences and mechanisms between the specific immune system of c-section and vaginal delivered infants? Further study is needed.
2. It is known that *L. acidophilus* and *Bifidobacterium longum* can upregulate the levels of SERT mRNA and protein in intestinal epithelial cells. Whether the supernatants of these two species can also improve gastrointestinal sensation and intestinal motility by regulating the expression of SERT?
3. Could probiotics activate the IECs to enable neonate delivered by c-section to acquire the same immune tolerance as vaginal delivered neonate?

AUTHOR CONTRIBUTIONS

CZ, LL, and ZL contributed to conception and design of the work. BJ and XX contributed to the acquisition, analysis, and interpretation of data for the manuscript. CZ and LL drafted the manuscript. XZ and YL critically revised and reviewed the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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Ecological Processes Shaping Microbiomes of Extremely Low Birthweight Infants

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The human microbiome has been implicated in affecting health outcomes in premature infants, but the ecological processes governing early life microbiome assembly remain poorly understood. Here, we investigated microbial community assembly and dynamics in extremely low birth weight infants (ELBWI) over the first 2 weeks of life. We profiled the gut, oral cavity and skin microbiomes over time using 16S rRNA gene amplicon sequencing and evaluated the ecological forces shaping these microbiomes. Though microbiomes at all three body sites were characterized by compositional instability over time and had low body-site specificity (PERMANOVA, r^2 = 0.09, p = 0.001), they could nonetheless be clustered into four discrete community states. Despite the volatility of these communities, deterministic assembly processes were detectable in this period of initial microbial colonization. To further explore these deterministic dynamics, we developed a probabilistic approach in which we modeled microbiome state transitions in each ELBWI as a Markov process, or a "memoryless" shift, from one community state to another. This analysis revealed that microbiomes from different body sites had distinctive dynamics as well as characteristic equilibrium frequencies. Time-resolved microbiome sampling of premature infants may help to refine and inform clinical practices. Additionally, this work provides an analysis framework for microbial community dynamics based on Markov modeling that can facilitate new insights, not only into neonatal microbiomes but also other human-associated or environmental microbiomes.

Keywords: neonatal microbiome, ecological processes, community states, Markov model, microbial community assembly

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BACKGROUND

Preterm birth remains one of the major risk factors for acute as well as long-term adverse health outcomes. Even in high- and middle-income countries, premature birth has been estimated to account for over 50% of neonatal deaths (Blencowe et al., 2013). Extremely low birthweight infants (ELBWI) are at high risk for suffering prematurity-related complications. Among the most critical complications are necrotizing enterocolitis (NEC) (Samuels et al., 2017), early- and late-onset sepsis (EOS and LOS, respectively) (Escobar et al., 2014;

Afonso and Blot, 2017), bronchopulmonary dysplasia (BPD) (Thekkeveedu et al., 2017), retinopathy of prematurity (ROP) (Quimson, 2015) and intraventricular hemorrhage (IVH) (Poryo et al., 2018). These complications are not only an acute risk, but can lead to lasting neurodevelopmental impairment (Stoll et al., 2004). Microbial colonization plays a key role in the maturation and function of the immune system (Geva-Zatorsky et al., 2017). This is particularly important for extremely preterm infants, as they are frequently exposed to invasive procedures such as catheterization, intubation and assisted ventilation, which are potent sources of nosocomial infections during intensive care (Ramasethu, 2017).

Extremely low birth weight infants, which harbor lower-diversity microbial communities compared to neonates with higher birth weight (Chernikova et al., 2018), are particularly prone to infections due to their naive immune system, less effective mucosal and epithelial barriers (Weström et al., 2020), diminished complement components, and impaired function of antigen-presenting cells (Sadeghi et al., 2007; Schüller et al., 2013; Wisgrill et al., 2016; Jong et al., 2017). Early gut colonization by pathogenic or immunomodulatory bacteria can distort the fragile immune homeostasis in the premature gut and predispose neonates to disease (Mazmanian et al., 2008; Wlodarska et al., 2015). For example, research suggests that NEC, one of the most severe complications for ELBWI, is preceded by alterations in the gut microbiome marked by decreased bacterial diversity and blooms of Gammaproteobacteria and bacilli (Morrow et al., 2013; Warner et al., 2016). Studies on the initial assembly of the human microbiota have been mostly focused on the gut community in term (Palmer et al., 2007; Koenig et al., 2011; Sharon et al., 2013; Hill et al., 2017) and preterm infants (Rosa et al., 2014; Ho et al., 2018). Recent efforts to describe spatiotemporal community dynamics within and across multiple body sites in premature infants have provided first insights into assembly patterns (Costello et al., 2013; Olm et al., 2017; Grier et al., 2018; Younge et al., 2018; Tirone et al., 2019), but the ecological processes underlying initial community assembly remain poorly understood. Generally, community dynamics can be described by four fundamental ecological processes: (1) selection, the fitness difference between species, (2) drift, stochastic changes in species abundance, (3) dispersal, the ability of species for movement across space to new sites, and (4) speciation (Vellend, 2010). Essentially, community assembly can be influenced by both deterministic and stochastic processes to a degree that is dependent upon changes of environmental factors over time and space (Stegen et al., 2012). In this study, we monitored amplicon sequence variant (ASV) relative abundances on the skin, gut and oral cavity over the first 2 weeks of life in 15 ELBWI in order to better understand the process of *de novo* assembly of bacterial communities and to determine the relationship between communities at different body sites. We characterized bacterial diversity and inferred the ecological processes governing community assembly. Additionally, we evaluated associations between microbiome data and clinically relevant parameters such as gestational age, delivery mode, inflammatory response, and disease diagnosis. We then developed an analysis framework that identifies discrete community structures and interprets the observed temporal microbiome trajectories as a Markov process of transitions between community states.

MATERIALS AND METHODS

Study Cohort and Sampling

We recruited a cohort of 15 ELBWI (defined as having a birth weight < 1000 g) hospitalized in the neonatal intensive care unit (NICU) at the General Hospital of Vienna/Medical University of Vienna. Exclusion criteria were chromosomal aberrations, congenital malformations, inherent metabolic disorders, and maternal chronic infections. Due to the high risk of necrotizing enterocolitis in this high-risk patient cohort, all infants received pasteurized human donor milk or their own mother's milk in the first few weeks of life. Infant nutrition was supplemented with parenteral nutrition during the study period (**Supplementary Figure 1**) and combinations of broad-spectrum antibiotics (**Supplementary Figure 2**) were administered prophylactically during the study period. Patients were sampled at four time points over the first 2 weeks of life (postnatal days 1, 3–4, 7–8, and 14–16). Stool was collected and chest skin and oral cavity were sampled using ESwab^{TM} (COPAN diagnostics) swabs. All samples were immediately stored at −80°C. The study was approved by the ethics committee of the Medical University of Vienna (EK No. 1175/2016).

Clinical Definitions

Bronchopulmonary dysplasia (BPD) was defined as supplemental oxygen treatment or oxygen plus respiratory support at 36 weeks postmenstrual age (Higgins et al., 2018). Retinopathy of prematurity (ROP) was diagnosed and staged according to the international consensus guidelines (International Committee for the Classification of Retinopathy of Prematurity, 2005). The severity of intraventricular hemorrhage (IVH) was defined as grades 1 to 4 according to the modified Papile classification (Papile et al., 1978). Statistical analyses relating ROP and IVH to microbiome data were performed without considering disease severity due to limited sample size of some disease grades.

Elevated IL-6 was defined as IL-6 $>$ 150 pg/ml. Early-onset clinically suspected inflammation (CSI) was defined as IL-6 $>$ 150pg/ml on day 1 or 2 of life, but with negative blood culture results (i.e., elevated inflammatory markers, but no clinical diagnosis of sepsis). Late-onset sepsis (LOS) was defined according to the NEO-KISS protocol (Gastmeier et al., 2004) for nosocomial infection surveillance for preterm infants. Clinical LOS as well as LOS with coagulase negative staphylococcus (CoNS) were defined as an episode with the following characteristics: $>$ 72 h of life, empiric antibiotic therapy $\geq$ 5 days, no apparent infection at another body site, and additionally fulfilling any two of the following criteria: temperature $>$ 38°C or $<$ 36.5°C, temperature instability, tachycardia, bradycardia, apnea, hypotension, hyperglycemia, metabolic acidosis, prolonged recapillarization time or positive blood infection parameter (C-reactive protein $>$ 2 mg/dl or IL-6 $>$ 50 pg/ml). Culture-positive LOS was defined as a clinical infection as described above with the additional growth

of a pathogen in the corresponding blood culture. Oxygen supplementation (DOS) was defined as the cumulative number of days with at least 12 h/day of $FiO_2 > 21\%$ up to the day of sampling. Days of mechanical ventilation (DMV) was defined as the number of days with mechanical ventilation support up to the day of sampling. Antibiotic administration was measured as a cumulative index of days of antibiotic given accounting for parallel antibiotics prescription (antibiotic prescriptions per day multiplied by the number of days of administration).

16S rRNA Gene Amplicon Sequencing

For nucleic acids extraction, we used 100 mg of stool or 500 ul swab solution for skin and oral samples, after vigorous vortexing to release cells from the swab. Nucleic acids were extracted using a phenol-chloroform bead beating protocol (Griffiths et al., 2000). Barcoded amplicon libraries were prepared according to a two-step PCR protocol as described previously (Herbold et al., 2015). Briefly, the extracted DNA was PCR amplified for 30 cycles in total, using a universal primer pair S-D-Bact-0341-b-S-17 [5′-CCTACGGGNGGCWGCAG-3′] and S-D-Bact-0785-a-A-21 [5′-GACTACHVGGGTATCTAATCC-3′] that targets the V3-V4 hypervariable regions of the 16S rRNA gene. Amplicon libraries were purified and normalized in equal molar quantities with the *SequalPrep*^{TM} Normalization Plate Kit (Invitrogen, United States) before pooling. The preparation was performed on an automated liquid handling workstation (Beckman Coulter, United States). Sequencing on the Illumina MiSeq platform (2 × 300bp) was performed at Microsynth AG (Balgach, Switzerland).

Sequence Data Pre-processing

Reads were demultiplexed using an in-house python script. A custom pipeline built on *Qiime2* (Bolyen et al., 2019) was developed for processing the sequence data. Specifically, reads were processed into amplicon sequence variants (ASVs) using the Divisive Amplicon Denoising Algorithm (*DADA2*) (Callahan et al., 2016) without the pooling option. We extracted sequences from the *SILVA* database (SILVA 132, 99% OTUs) and trained a classifier (bayesian module from *Qiime2*) specific to the amplified region for taxonomic assignment of ASVs (Bokulich et al., 2018). Due to the very low read yield of the negative control libraries, no reads passed the DADA2 pipeline. Thus, we examined libraries for potential contaminants by taxonomically classifying the raw reads from negative control samples by best BLAST hit to the NCBI 16S rRNA database. Genera with > 10 reads were considered contaminants (and accounted for on average ∼0.8% of total sequences in the libraries from the patient sample). We then removed any genus that was highly correlated across the dataset with the contaminant genera (Pearson r > 0.9), as well as previously described PCR reagent contaminants (∼11% of total sequences) (Salter et al., 2014). Additionally, we removed ASVs that were detected in higher abundance in samples from other datasets in the same sequencing run as potential cross-contamination (∼0.13% of total sequences). Libraries were rarefied to 600 reads per sample for subsequent analysis after evaluating rarefaction curves to ensure inclusion of the maximum number of samples given no richness underestimation (Good's coverage min = 0.995, max = 1, CI_{95} = [0.9986984, 0.99918778]; **Supplementary Figure 3**).

Ecological Statistical Analyses

We measured alpha diversity (Shannon index, evenness, and richness) with the skbio.diversity.alpha_diversity function from *scikit-bio* python module and Beta diversity (*Bray-Curtis* dissimilarities) with the scipy.spatial.distance.pdist function from *scipy* python module (Virtanen et al., 2020). Differential abundance analysis was carried out with the *MetagenomeSeq* R package (Paulson et al., 2013). For multivariate analysis, we used the *adonis* (PERMANOVA, using 1,000 permutations) and *envfit* functions from the *vegan* R package, with permutations constrained to the patient samples (Dixon, 2003). The significance between groups was assessed with ANOVA or the Wilcoxon rank-sum test with the *aov* and *compare_means* functions in R. We applied k-means clustering to partition samples based on their *Bray-Curtis* dissimilarities. We then evaluated clustering efficiency by comparing silhouette scores for individual clusters to the average Silhouette score (>0.67) as well as maximization of the Calinski harabasz criterion (**Supplementary Figure 4**). We constructed co-occurrence networks from pairwise spearman correlations that were calculated from ASV abundances across samples. Only ASVs present in at least 2 of the respective samples were used for the analysis. The significance of the calculated correlation coefficients was estimated by comparison to a null distribution. We obtained this null distribution by shuffling the ASV abundances across samples and re-calculating spearman correlations 1000 times. The resulting *p*-values were then corrected for multiple comparisons using the method of Benjamini and Hochberg (1995) and only observed correlation coefficients with a *p*-value < 0.01 were used for further analysis. Correlation coefficients were calculated using the function *cor* from the *stats* package in R. Network visualization was done with *Cytoscape* 3.8.0 (Shannon et al., 2003).

Analysis of Ecological Processes

A maximum likelihood phylogenetic tree was reconstructed with *IQ-TREE* (Nguyen et al., 2015) based on the TIM3e + R5 DNA model, inferred with *ModelFinder* (Kalyaanamoorthy et al., 2017). Distance based RDA and phylogenetic diversity was calculated in R using the *vegan* package and *ordistep* function, as well as the *picante* package (Kembel et al., 2010). Null-model analysis was used to analyze the β-mean-nearest taxon distance (βMNTD) for all pairwise comparisons within each body site. The difference between observed βMNTD and the null model is given in units of standard deviation (of the null distribution) and termed βNTI (β-nearest taxon index). We interpret βNTI values according to previously established criteria (Stegen et al., 2013) as follows: scores greater than + 2 indicate variable selection pressures; scores near zero indicate dominance of stochastic processes; and scores less than −2 indicate homogeneous selective pressures. In a second step, the RCBray index was used to characterize stochastic processes. | RCBray| < 0.95 indicates that in a pairwise comparison, communities share as many species as expected by chance,

indicating that drift acts alone. RCBray < -0.95 indicates homogenizing dispersal, as communities share more species than expected. RCBray > 0.95 indicates dispersal limitation, as fewer species than expected are shared.

Estimation of Conditional Probabilities

For each infant, we estimated the conditional probability of detecting an ASV at a certain body site conditioned upon its detection at another body site, as follows:

$$P(s_1|s_2) = \frac{P(s_2|s_1)\,P(s_1)}{P(s_2)}$$

where, s_1 is one body site and s_2 is another. Similarly, we obtained the transition probability between community states for each body site by estimating the probability of a current community state, conditioning upon the community state at the previous time point.

Markov Modeling

Markov models are stochastic models that assume that each future state depends solely on the current state. This assumption is reasonable for systems with frequent disturbances. We constructed a Markov chain from the previously estimated transition probabilities between community states and estimated their stationary frequencies. These are estimates of a future converging point in the process where the probability distributions will no longer change. Using these stationary or steady state frequencies, we can predict the equilibrium point of our transition model across community types. States in a Markov chain are categorized into transient and recurrent states. Recurrent states are those which are estimated to have a probability of one for returning to this state, whereas transient states do not. We used the functions *steadyStates* and *summary* from the *markovchain* package in R (Spedicato, 2017) in order to construct the Markov chain, estimate steady states, and explore their characteristics.

RESULTS

Description of the Cohort

In our ELBWI cohort, the mean gestational age (GA) was 24.67 (± 1.12) weeks and the mean birth weight was 731 (± 116) g. Patient characteristics and the frequency of short-term morbidities are summarized in **Table 1**. The same probiotic supplementation (Antibiophilus® - *Lactobacillus casei*) as well as parenteral and enteral feeding regimens were applied to all patients over the entire study period. Sampling was not synchronized across infants, although there were overlaps in hospitalization.

TABLE 1 | Summary of cohort characteristics.

	Mean ± SD
Gestational age (weeks)	24.67 ± 1.12
Birth weight (g)	731 ± 116.19
	N (%)
Gender	
Female	8 (53)
Male	7 (47)
Mode of delivery	
Cesarean section	11 (73)
Vaginal birth	4 (27)
IVH	
No	10 (67)
Stage 1	4 (27)
Stage 2	1 (6)
BPD	
No	10 (67)
Yes	5 (33)
ROP	
No	4 (27)
Grade 2	8 (53)
Grade 3	3 (20)
NEC	
No	15 (100)
LOS	
No	8 (53)
Yes	7 (48)
CSI	
No	2 (13)
Yes	13 (87)

IVH, intraventricular hemorrhage; BPD, bronchopulmonary dysplasia; ROP, retinopathy of prematurity; NEC, necrotizing enterocolitis; LOS, late onset sepsis; CSI, clinically suspected inflammation.

Diversity of the Extremely Low Birth Weight Infants Microbiome

Skin, oral cavity, and gut microbiome composition was obtained by amplicon sequencing of the V3-V4 regions of the 16S rRNA gene at four time points over the first 2 weeks after birth. In this period, ELBWI were colonized by low diversity microbial communities on all tested body sites. *Firmicutes* and *Proteobacteria* were the most abundant phyla across all body sites, predominantly due to the abundance of the genera *Staphylococcus*, *Escherichia/Shigella* and *Lactobacillus* (**Figures 1A,B**). *Staphylococcus* was on average four times more abundant (Cohen's $d_{\text{skin-gut}} = 1.5$) in skin samples compared to gut samples and twice as abundant (Cohen's $d_{\text{skin-oral cavity}} = 0.64$) compared to oral cavity samples, whereas *Lactobacillus* and *Escherichia/Shigella* were three times more abundant in gut communities compared to the skin (Cohen's $d_{\text{gut-skin}} = 0.67$, Cohen's $d_{\text{gut-skin}} = 0.55$, respectively). While *Escherichia/Shigella* was detected in similar abundance in gut and oral samples (Cohen's $d_{\text{gut-oral cavity}} = 0.08$), *Lactobacillus* abundance remained half as low in oral samples (Cohen's $d_{\text{gut-oral cavity}} = 0.5$) (*ANOVA* - *Staphylococcus*; $p < 0.001$, *Escherichia/Shigella*; $p = 0.003$, *Lactobacillus*; $p = 0.027$, respectively). However, similar communities were found at all body sites, and body site was only able to explain a minor, though statistically significant, fraction of the microbiome variation across the entire dataset (PERMANOVA: $r^2 = 0.09$, $p = 0.001$).

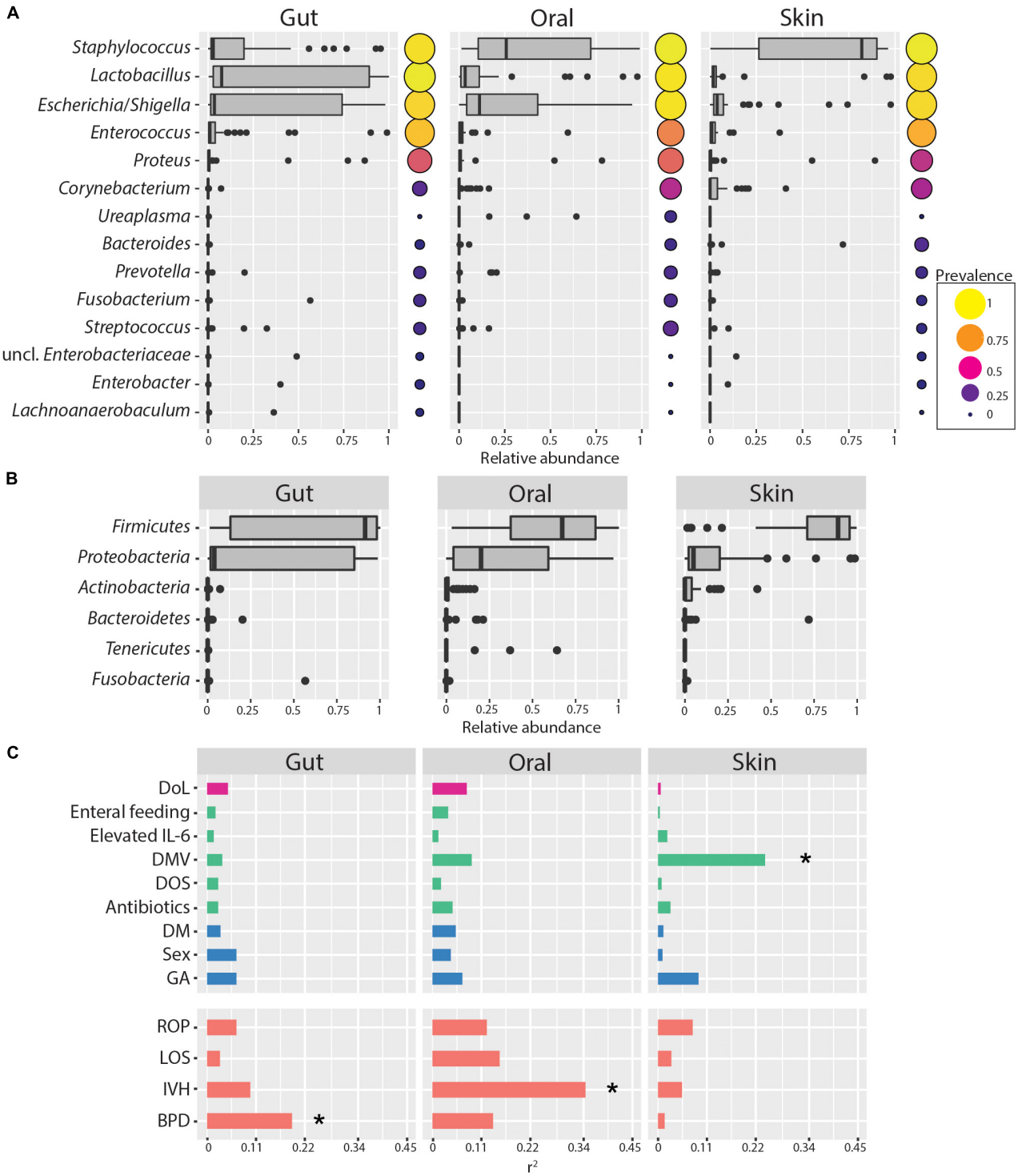

FIGURE 1 | Skin, oral cavity, and gut microbiome composition of ELBWI. **(A)** Relative abundances of bacterial genera across body sites. Genera with >0.2 relative abundance in at least one sample are shown. Circle size and color indicates genus prevalence in the dataset. **(B)** Relative abundances of phyla across body sites. **(C)** Association of microbiome composition with clinical parameters and disease outcome (using the *envfit* function. Pearson correlation coefficient [r^2] on *X*-axis). DoL, days of life; elevated IL-6 = IL-6 > 150 pg/ml in the time window of 2 days before to 2 days after sampling; DOS, number of days with at least 12 h per day of FiO_2 > 21% up to the day of sampling; DMV, days with mechanical ventilation support till the day of sampling. Antibiotics = cumulative index for days of antibiotic administration accounting for different compounds per day; GA, gestational age; DM, delivery mode; ROP, retinopathy of prematurity; IVH, intraventricular hemorrhage; BPD, bronchopulmonary dysplasia. *p-value < 0.01.

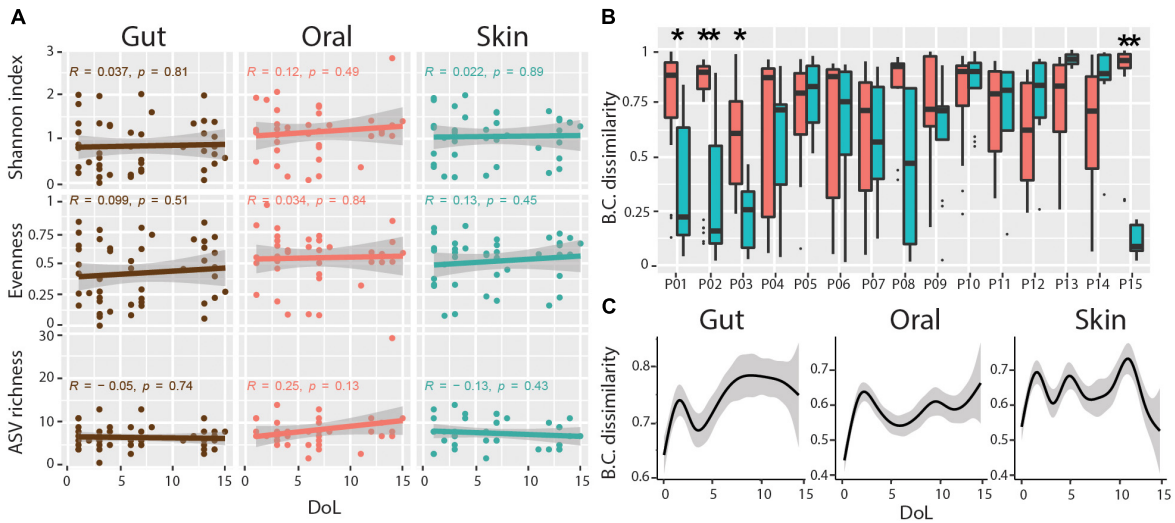

FIGURE 2 | Diversity and body-site-specificity of microbiomes. **(A)** Alpha diversity metrics over patient days of life. Linear regression Pearson *r* and *p*-values are shown within each subplot. **(B)** Bray-Curtis (BC) dissimilarities of samples from the same body site compared to samples across body sites for each infant. Wilcoxon Rank-sum test, adjusted *p*-values * < 0.01, ** < 0.001. **(C)** Progression of Bray-Curtis (BC) dissimilarities (*Y*-axis) in ELBWI with increasing distance over time (*X*-axis). Smoothed lines result from locally estimated scatterplot smoothing (LOESS) and indicate trends of development.

Clinical parameters such as delivery mode and gestational age at birth also explained little of the observed variability in the composition of the microbiome (**Figure 1C**), though the low number of patients delivered by spontaneous birth and the small range of gestational ages of study patients (24.67 ± 1.12) must be considered when interpreting these results. Strikingly, duration of mechanical ventilation was associated with changes in skin microbiome ($r^2 = 0.24$, $p = 0.007$). Considering clinical diagnosis, BPD and IVH were significantly associated with gut and oral cavity microbiome composition, respectively (**Figure 1C**). However, we did not identify any ASVs consistently associated with disease diagnosis, suggesting that there were no robust ASV-level markers for disease in this cohort.

Microbial community diversity remained low at all body sites over the first 2 weeks of life, with no significant trend for increasing ASV richness, evenness, or Shannon diversity during this period (**Figure 2A**). As previously mentioned, we observed a subtle association between microbial communities and body sites. However, only four of the 15 infants had significantly more similar communities within each body site compared to between body sites over the sampling period (**Figure 2B**). Additionally, we find no indication of community succession within this timespan, with no increase in Bray-Curtis distance of communities over time compared to the founder community within this period (**Figure 2C**). These data indicate that habitat filtering is largely overwhelmed by stochastic disturbance events.

Ecological Processes Affecting Microbiome Assembly

To characterize the ecological drivers of microbiome assembly in different body sites, we compared the observed phylogenetic diversity to ecological null models and determined the contributions of selection, dispersal, drift, and dispersal limitation to community turnover in ELBWI using an approach established by Stegen et al. (2013) and (**Table 2**). We observed that stochastic processes (βNTI $< |2|$) dominated community assembly across all body sites, but that deterministic processes were higher in the oral cavity than the other body sites (**Figure 3A**; ANOVA, $p < 0.0001$, Tukey *post hoc*: oral-gut and oral-skin $p < 0.001$). Analysis of the specific processes revealed that ecological drift dominated all body sites, but that there was elevated homogenizing dispersal in the gut (Chi-square test, $p < 0.001$), variable selection in the oral cavity (Chi-square test, $p < 0.001$), and homogenizing selection on the skin (**Figure 3B**; Chi-square test, $p < 0.001$). Canonical correlation analysis (CCA) revealed that mechanical ventilation, oxygen supplementation and antibiotic administration were significantly associated with βNTI values, though they only explained a small percentage of the observed variation (**Figure 3C**).

The detection of distinct community types that were not body-site-specific suggested limited dispersal limitation and/or environmental filtering of bacteria across body sites. In order to more deeply characterize the extent of habitat specificity of microbes and potential dispersal of microbes between body sites, we calculated the probability of detecting an ASV at a body site given that it is detected on another body site of the same patient (i.e., conditional probability). We found that very abundant ASVs are likely to be present in all sampled sites from the same patient, which is consistent with the neutral theory of community assembly (Volkov et al., 2003). However, the majority of ASVs were detected sporadically, and in some cases were exclusive to certain neonates (**Supplementary Figure 5**). Interestingly, ASVs were more likely to be detected in the gut if they were detected in the oral cavity (Posterior probability = 0.55), and vice-versa (Posterior probability = 0.56) compared to the other body site

TABLE 2 | Glossary of ecological terms.

Speciation	The creation of new species (Vellend, 2010)
Ecological drift	A stochastic element of the changes in the composition and diversity of species, due to their ecological equivalence (Vellend, 2010)
Environmental/habitat filtering/selection	Environmental factors which increase fitness of particular species (Vellend, 2010)
Dispersal limitation	The limitation of a species ability to spread/move across space, resulting in local communities that altogether define a metacommunity (Vellend, 2010)
Homogenizing dispersal	High levels of species dispersal which are able to homogenize community composition (Stegen et al., 2013)
Homogenizing selection	Strong selection that reduces compositional turnover for communities exposed consistently to the same environmental pressures (Stegen et al., 2015)

combinations. The oral cavity and skin had somewhat fewer ASVs with a high conditional probability of detection (Posterior probability = 0.42 and 0.37, respectively), and between skin and gut that was even lower (Posterior probability = 0.29 and P = 0.29, respectively) (**Figure 3D**). This suggests that either physical proximity between body sites or the relative similarity of environmental conditions (e.g., O_2 levels, water content, or immune factors) could influence establishment of certain ASVs.

Extremely Low Birth Weight Infants Microbiomes Have Distinct Community Structures

Principal coordinates analysis (PCoA) revealed clear clustering of samples (**Figure 4A**), though this clustering was not driven primarily by body site, age, or patient (**Supplementary Figure 6**). To further define and characterize these discrete community structures, we performed a cluster analysis optimization based on silhouette score maximization (Arumugam et al., 2011). This revealed four community structures (all clusters were well-supported, having an average silhouette score = 0.67), which were driven largely by differences in the relative abundance of the three most dominant ASVs in the dataset. Specifically, we identified three mono-dominated clusters (SC, EC, and LC), in which ASV_3: *Staphylococcus* [SC, mean = 0.61, $CI_{95\%}$ = (0.55,0.68)], ASV_1: *Escherichia/Shigella* [EC, mean = 0.6, $CI_{95\%}$ = (0.51,0.69)] and ASV_2: *Lactobacillus* [LC, mean = 0.91, $CI_{95\%}$ = (0.86,0.95)] were predominant, respectively (**Figure 4B**). We also observed a cluster of samples (IC) that had comparatively intermediate relative abundance for the above-mentioned ASVs, as well as a higher overall bacterial diversity (**Figure 4C**). The identified microbiome structures were unequally present at the studied body sites: we detected the LC cluster mainly in the gut, EC more frequently in the gut and less in the oral cavity, whereas SC was enriched in skin samples. Interestingly, the IC cluster appeared more evenly across all body sites (**Figure 4D**). The structural differences between those clusters were also reflected in their co-occurrence networks, which show distinct topologies and cluster-specific correlation patterns of ASVs (**Supplementary Figure 7**).

A Probabilistic Framework to Characterize Microbiome Dynamics

It has been suggested that initial microbial community assembly in low-birth-weight neonates is a stochastic process (Costello et al., 2013). As our data indicated that deterministic processes also play a role in assembly and that there are distinct microbiota compositional states, we next applied a probabilistic analysis framework to gain a deeper understanding of the observed dynamics. We modeled how the microbiome at each sample site changes over time as a Markov process, using conditional probabilities estimated from the data. Specifically, we estimated the probability of observing a certain state (community structure), conditioning on the state at the preceding time point, for each body site and each patient separately. This approach revealed new aspects of early-life microbiome dynamics (**Figure 5A**). In the gut, community structures were the most stable (mean prob. = 0.62) compared to the other body sites. Specifically, EC, IC, and LC were stable clusters (prob. $>$ 0.5), while SC had equal probability to transition to other community structures. In contrast, SC was more stable in the oral cavity, and there was a higher net transition probability from IC to SC communities. LC appeared sporadically in the oral cavity and transitioned only to EC or IC. For the skin, we observed an overall lower community stability compared to other body sites (mean prob. = 0.21). However, there was a higher transition probability to the SC cluster from all other clusters. These results suggest that microbiomes at different body sites have different levels of community stability and characteristic transitions between community states.

In order to better understand the implications of these community state transitions, we next evaluated the long-term consequences of these dynamics by calculating their steady-state probabilities using Markov chain theory. Briefly, Markov chains are "memoryless" processes in which the transition from one discrete state to another depends solely upon the present state, but not the past. This assumption is reasonable for systems with frequent disturbances, such as would be expected considering the intensive clinical care practices administered to ELBWI. States can be classified into recurrent or transient, based on their presence or absence at steady-state (i.e., equilibrium). At all body sites, all clusters were found to be recurrent states. However, their steady-state frequencies differed across body sites (**Figure 5B**). EC and IC shared similar frequencies in the gut and oral microbiomes at steady state, while LC was higher in the gut and SC higher in the oral cavity. Notably, SC comprised ∼70% of the communities at steady state in the skin.

We then evaluated the contribution of each community type to the observed deterministic processes in each body site (**Figures 3A,B**). This revealed that in the gut EC clusters

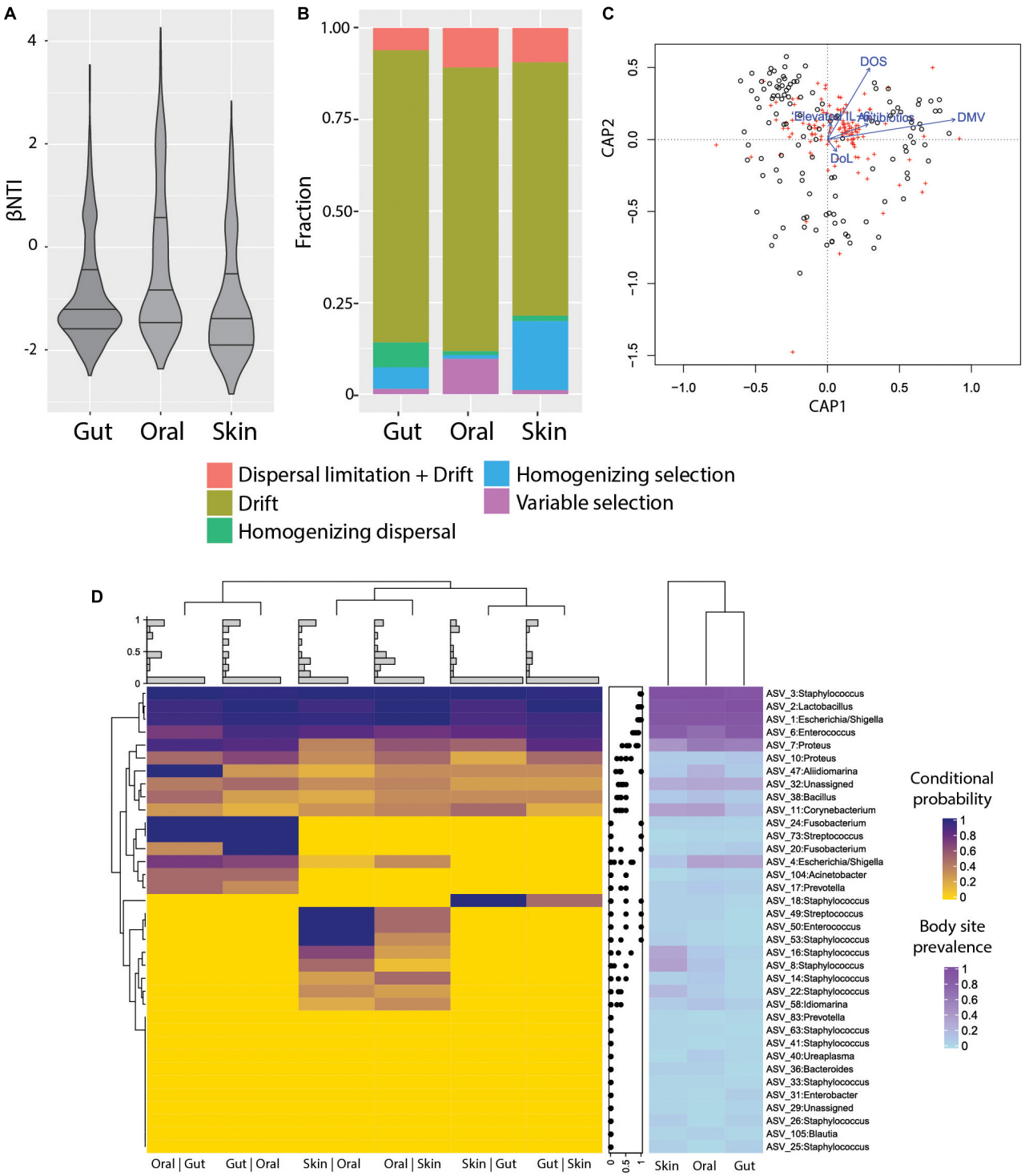

FIGURE 3 | Ecological processes and microbiome assembly. **(A)** βNTI (beta Nearest Taxon Index) per body site. βNTI greater than + 2 standard deviations from the null-model indicate variable selection pressures; scores near zero indicate dominance of stochastic processes; and scores less than -2 standard deviations indicate homogeneous selective pressures. **(B)** Fraction of ecological processes predicted for each body site. **(C)** Canonical correlation analysis (CCA) plot, showing the relative proportion of βNTI variance explained by DMV, DOS, Elevated IL-6, Antibiotics, and DoL (proportion of constrained variance = 0.04). DoL, days of life; Elevated IL-6, IL-6 > 150 pg/ml in the time window of 2 days before to 2 days after sampling; DOS, number of days with at least 12 h per day of FiO_2 > 21% up to the day of sampling; DMV, days with mechanical ventilation support till the day of sampling. Antibiotics = cumulative index for days of antibiotic administration accounting for different compounds per day. **(D)** Heatmap of conditional probability to detect an ASV at a body site when present at another (left). Distribution of values for each row (center). Heatmap for ASV prevalence in each body site (right).

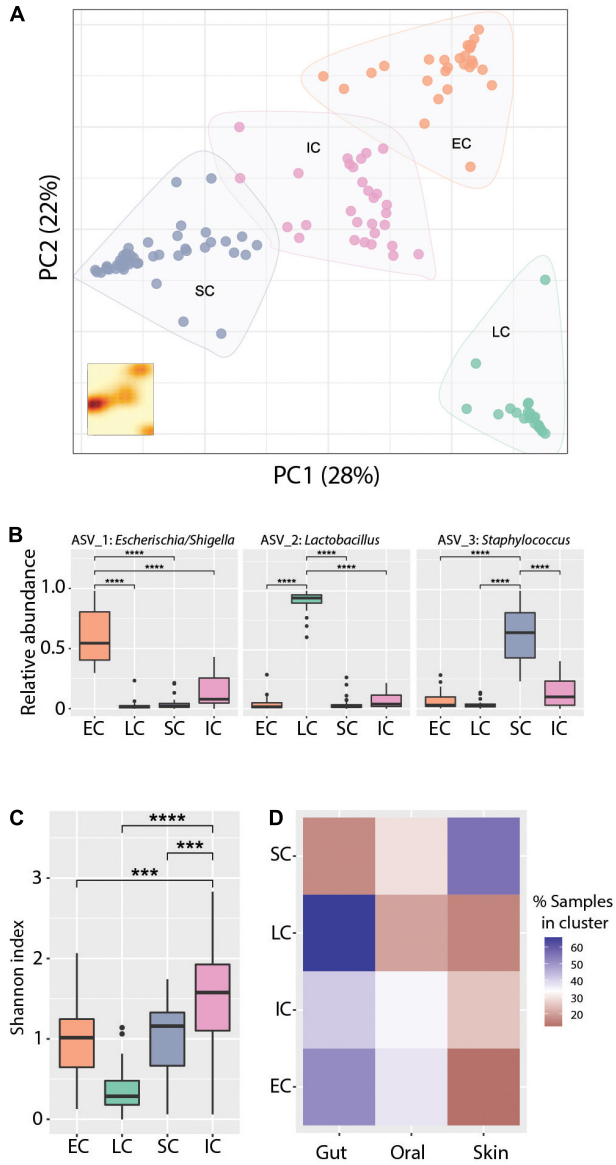

FIGURE 4 | Cluster analysis reveals distinct community states. **(A)** Principal coordinates analysis ordination of Bray-Curtis dissimilarities. K-means clustering analysis indicates four community types based on Silhouette score - Calinski harabasz maximization. KDE plot (bottom right) displays sample density on the first two principal components. **(B)** Relative abundance of the dominant ASVs in the respective clusters, Wilcoxon Rank-sum test ****p-value < 0.0001. **(C)** Alpha diversity measured with the Shannon index across community types. Wilcoxon Rank-sum test ***p-value < 0.001, ****p-value < 0.0001. **(D)** Representation of community types (clusters) in the gut, skin, and oral cavity respectively, as percentage of the total number of samples assigned to each cluster. Blue and red color gradients indicate positive or negative deviation, respectively, from a uniform cluster distribution across body sites (i.e., 33%).

contributed significantly to deterministic assembly [Odds ratio: 3.3, 95% CI = (1.4,7.9)], whereas in the oral cavity IC and SC contributed to deterministic assembly [Odds ratio: 2.5, 95% CI = (1.1,6.0) and Odds ratio: 2.8, 95% CI = (1.3,6.4), respectively], and in the skin SC contributed to deterministic assembly [Odds ratio: 9.3, 95% CI = (1.3,69.2); **Figure 5C**]. These results are largely in line with the Markov modeling results, suggesting that community state stability is governed in part by deterministic ecological processes.

DISCUSSION

Previous studies of gut microbiome assembly in preterm and term infants have suggested that over the course of months to years there is a succession in microbial communities (Koenig et al., 2011; Rosa et al., 2014; Hill et al., 2017; Stewart et al., 2018). However, studies that have focused on early-life microbiome development at multiple body sites in preterm infants have concluded that there are stochastic dynamics and lack of body-site-specificity in the initial community assembly in the first 2 weeks of life (Costello et al., 2013; Olm et al., 2017). As extremely preterm infants are highly vulnerable to inflammation, infections and associated adverse outcomes for which the microbiome is known to play a role (Dobbler et al., 2017; Younge et al., 2019), we sought to develop an improved framework to better understand early microbiome assembly and to identify ecological factors driving this process.

In this study, we show that the initial colonization of the gut, oral cavity, and skin in ELBWI is dominated by *Firmicutes* and *Proteobacteria* in the first 2 weeks of life. This is reflected in the abundances of three predominant ASVs classified as *Escherichia/Shigella*, *Staphylococcus,* and *Lactobacillus* genera, respectively (**Figure 1A**). These taxa are commonly observed in human microbiome studies in neonates as well as adults (Schloissnig et al., 2013; Sharon et al., 2013). Over the first 2 weeks following birth, alpha diversity of microbiomes across all body sites was variable and did not have an increasing trend, suggesting limited enduring microbial succession in this time period (**Figure 2A**). Only four of the 15 infants had body-site specific microbiomes across the time course, indicating extensive colonization of similar microbes at different body sites for most patients. Interestingly, after evaluating Silhouette score maximization to define clusters, we found that microbiomes could be classified into four distinct community types that could be observed at all body sites, three of which were dominated by either *Escherichia/Shigella, Staphylococcus* or *Lactobacillus*, and a fourth which was more diverse and included all three of these prevalent genera at similar levels (**Figure 4**).

We observed that community assembly was driven largely by ecological drift, but that the oral cavity had elevated variable selection, which may be due to differences in oral immune factors among patients that impose different selection pressures. Homogenizing selection was highest for the skin, which may be due to the presence of *Staphylococcus* as a well-adapted skin bacterium. The gut had the lowest dispersal limitation, which may be because the gut has a higher number of bacterial cells compared to other body sites, which increases their dispersal probability. Our results suggest that deterministic assembly processes are detectable in the first 2 weeks of life, but that they are largely overwhelmed by stochastic processes. Indeed, chaotic

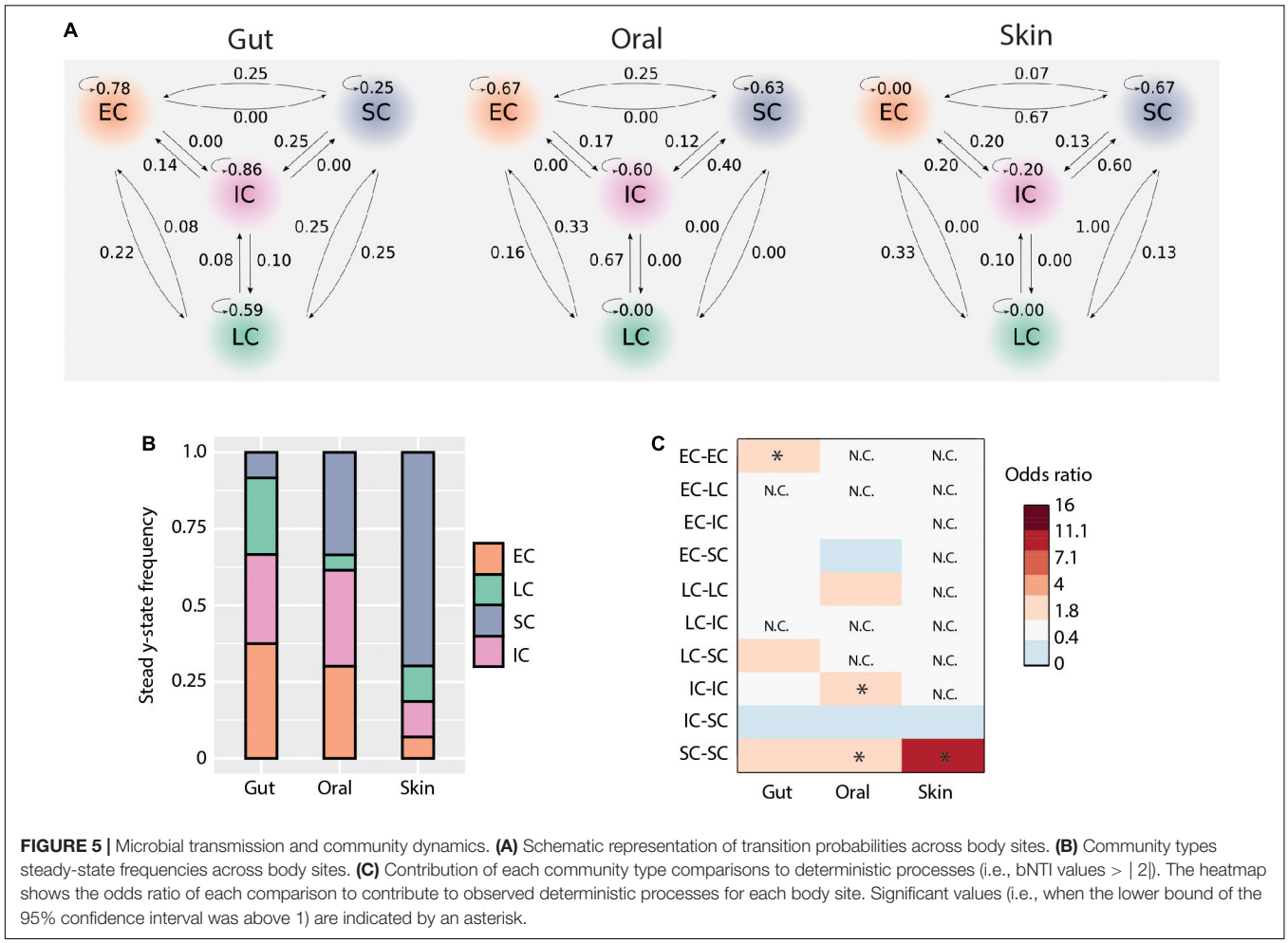

FIGURE 5 | Microbial transmission and community dynamics. **(A)** Schematic representation of transition probabilities across body sites. **(B)** Community types steady-state frequencies across body sites. **(C)** Contribution of each community type comparisons to deterministic processes (i.e., bNTI values > | 2|). The heatmap shows the odds ratio of each comparison to contribute to observed deterministic processes for each body site. Significant values (i.e., when the lower bound of the 95% confidence interval was above 1) are indicated by an asterisk.

microbiome dynamics may occur due to the many possible environmental disturbances encountered immediately after birth such as antibiotic administration, mechanical ventilation or nutrition (Aujoulat et al., 2014; Stewart et al., 2016, 2018). Despite the known beneficial role of breastfeeding in term infants (Bäckhed et al., 2015; Stewart et al., 2018), in part due to the enrichment of bifidobacteria (Makino et al., 2013; Lawson et al., 2020), we find that the amount of enteral feeding (mother or donor pasteurized milk) had no detectable effect on microbiome composition. This is in line with previous findings in preterm infants where nutritional exposures did not shape the microbiome within the first weeks (Gregory et al., 2016). The absence of bifidobacteria from our study could be attributed to the effect of broad-spectrum antibiotics administered throughout the study period, and also to the microaerobic conditions in the preterm intestine, which favors facultative anaerobes compared to strict anaerobes such as bifidobacteria (Arboleya et al., 2012). Similarly, delivery mode, which is a determinant of microbial colonization in term infants (Dominguez-Bello et al., 2010), had a minor effect in preterm infants, and it has been suggested that the NICU environment plays more of a role for shaping preterm gut microbiome (Brooks et al., 2017). Strains transmitted from the mother might also be unable to establish due to administration of antibiotics (Brooks et al., 2014). Inconsistencies between the results of different studies on preterm infant microbiota may result in part from differences in cohort inclusion criteria, such as range of patient gestational age or birth weight (Chernikova et al., 2018), as well as local differences in environmental microbial pools driven by factors such as seasonality or NICU hygiene practices (Taft et al., 2014).

In the initial microbial colonization of ELBWI, disturbances may also dramatically influence microbiome composition and make interpreting microbiome dynamics more challenging. In fact, the intermediate disturbance hypothesis posits that the magnitude and frequency of ecosystem disturbances can impact biological diversity (Hall et al., 2012), species dispersal, and colonization efficiency (Castorani and Baskett, 2020). In order to better understand initial assembly and succession processes in the face of these chaotic dynamics, we applied a probabilistic approach and analyzed state transitions through Markov chain modeling on our time-course data. Markov processes are widely used in many fields of science, from thermodynamics to phylogenetic inference and genome evolution (Erez et al., 2008; Kaehler et al., 2015; Sampid et al., 2018; Dhar et al., 2020; He et al., 2020), but have rarely been applied in microbial ecology (DiGiulio et al., 2015) and have not yet been used to

evaluate longitudinal transitions in microbiome composition in an individual. Despite the large influence of stochastic processes, this analysis revealed distinctive microbiome dynamics, as well as community stability, for each body site (**Figure 4**).

CONCLUSION

Moving forward, larger clinical studies are needed to establish the extent to which mono-dominated communities are associated with adverse outcomes and additional research is necessary to determine the mechanisms underlying this association. As sequencing technology now enables profiling of microbiomes within a few hours (Leggett et al., 2020), routine monitoring of neonate microbiomes coupled with time-integrated analysis of community diversity, structure and resilience, may prove to be a valuable complement to current diagnostic measurements. In summary, we have identified ecological factors determining the initial microbiome composition of oral cavity, gut, and skin samples of ELBWI and proposed a methodological framework for the analysis of microbiome dynamics based on Markov chain modeling. This framework has the potential to complement and refine existing clinical practices aimed at minimizing adverse outcomes in premature neonates.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA688751.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Medical University of Vienna (No. 1175/2016). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LW and DB designed the study. CZ and LW acquired and processed samples and patient data. CZ, DS, FB, CH, AB, LW, and DB analyzed and interpreted data. CZ and DB drafted the manuscript, with input from all other authors. All authors have approved the submitted version and have agreed both to be personally accountable for their contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.812136/full#supplementary-material

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The Protective Effects of Inulin-Type Fructans Against High-Fat/Sucrose Diet-Induced Gestational Diabetes Mice in Association With Gut Microbiota Regulation

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Background: Inulin-type fructans (ITF) have been used as prebiotics to alleviate glucose and lipid metabolism disorders. However, few studies evaluated the microbial mechanism of ITF in improving maternal metabolic status during pregnancy.

Methods: C57BL/6J mice were fed a high-fat/sucrose diet (HFD) for 4 weeks before and throughout pregnancy to induce a model of gestational diabetes mellitus (GDM). Body weight, glycolipid metabolic parameters, and fecal short-chain fatty acids (SCFAs) were assessed in the experimental process. The effects of ITF on the fecal microbiota were analyzed by 16S rRNA gene amplicon sequencing.

Results: Pregnant HFD-fed mice displayed significant insulin resistance and dyslipidemia. ITF (3.33 g/kg/day) treatment improved glucose and lipid metabolism disorder parameters in HFD-induced GDM mice and alleviated fat accumulation and glucose intolerance. The alpha diversity of the gut microbial community was increased in ITF mice, while the beta diversity returned to the level of normal chow diet (NCD) mice. Interestingly, Verrucomicrobia, *Bifidobacterium*, and *Akkermansia* were obviously enriched, while *Dubosiella* was obviously lessened after inulin treatment. Further analysis indicated that *Dubosiella* was positively correlated with markers of glycolipid metabolism disorders, whereas the ITF-supplemented diet partially reversed the changes.

Conclusion: Our results suggest that the ITF treatment may alleviate glucose and lipid metabolism disorders with the mediation of gut microbiota.

Keywords: inulin-type fructans, high-fat/sucrose diet, gut microbiota, maternal metabolism, gestational diabetes mellitus (GDM)

BACKGROUND

Gestational diabetes mellitus (GDM), carbohydrate intolerance, and insulin resistance during pregnancy are serious problems with increasing prevalence (American Diabetes, 2019), resulting in significant short-term and long-term adverse health outcomes in both mother and offspring (Miao et al., 2017; Song et al., 2018; Lowe, 2019). The physiological changes in insulin resistance

and lipid profiles are exacerbated in women with GDM and may indicate an underlying metabolic dysfunction that transiently manifests during pregnancy (Schneider et al., 2011; Zhu and Zhang, 2016).

Gut dysbiosis plays a vital role in abnormal host metabolism, as recently demonstrated in studies of type 2 diabetes (T2D) and obesity (Karlsson et al., 2013). *Prevotella* and *Bacteroides* have been identified as the main species contributing to insulin resistance and glucose intolerance (Pedersen et al., 2016). While the impact of gut microbiota on host metabolism and metabolic diseases is well-documented (Moller, 2001), only recently have studies focused on microbiota changes that influence metabolic mechanisms during pregnancy (Koren et al., 2012). *Parabacteroides* are significantly more abundant in GDM women than in healthy pregnant women (Kuang et al., 2017). A novel relationship between gut microbiome composition and the metabolic hormonal environment in overweight and obese pregnant women at the first trimester has also been described (Gomez-Arango et al., 2016). These studies suggest that major shifts in the gut microbiome during pregnancy may play a crucial part in the development of GDM.

Dietary intervention has become a potentially effective strategy to modulate the gut microbiota and improve the host health (Marchesi et al., 2016). Inulin-type fructans (ITF) are a type of dietary fiber present in vegetables, such as chicory roots, and can also be extracted to be used as food ingredients (Kalala et al., 2018). Isolated ITF have been considered to be typical prebiotics (Gibson et al., 2017). Prebiotics are defined as non-digestible compounds that are generated through fermentation by the gut. Prebiotics are able to modulate the composition and/or activity of the gut microbiota, thereby conferring a beneficial physiological effect on the host (Bindels et al., 2015; Salminen et al., 2021). *In vitro* studies and randomized controlled trials have shown that ITF can stimulate the growth of *Bifidobacterium* populations (Roberfroid et al., 1998; Sawicki et al., 2017) and certain butyrate-producing species (Ramirez-Farias et al., 2009; Scott et al., 2014) as well as reduce the abundance of Firmicutes (Everard et al., 2011, 2013; Dewulf et al., 2013). In addition, numerous randomized controlled trials have demonstrated direct health benefits of ITF, including inhibiting pathogens, protecting against cardiovascular diseases, and improving mineral bioavailability (Abrams et al., 2005; Kellow et al., 2014; Lohner et al., 2014). However, the relationships among dietary ITF, GDM and gut microbiota are still not clear.

Given that there are few studies aiming to evaluate the microbial mechanism of soluble dietary fiber in improving maternal metabolic status during pregnancy, our current research was undertaken to investigate the effects of adding ITF to a high-fat/sucrose diet (HFD) on the composition and metabolites of fecal microbiota from 4 weeks before conception and throughout gestation as well as maternal and neonatal health parameters in a GDM mouse model. In human intervention studies, doses ranging from 12 to 16 g/day are often given when testing for metabolic effects of ITF (Cani et al., 2009; Parnell and Reimer, 2009; Tarini and Wolever, 2010; Rahat-Rozenbloom et al., 2017), which equals to 2.46 and 3.33 g/kg body weight in mice, respectively (Nair and Jacob, 2016). In this study, the dose was based on our previously published study of ITF administration in mice (Miao et al., 2021). A high dose was chosen due to the short window of treatment allowed by pregnancy. Our study aimed to provide some microbial mechanistic insights into the application of ITF to a typical gestational diet characterized by high-fat/sucrose for improving maternal and neonatal health.

MATERIALS AND METHODS

Materials

Six-week-old C57BL/6J mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). ITF were procured from Fengning Ping'an hi tech Industry Co., Ltd. (Hebei, China), Thermo Scientific (Massachusetts, United States), Thermo Fisher (Massachusetts, United States), (Vilof^{TM} soluble dietary fiber powder) which contains 91% ITF and 9% mixture of sucrose, fructose, and glucose.

Animal Treatment and Experiment Design

Mice were housed in a temperature- and humidity-controlled laboratory. This animal experiment was approved by the Animal Protection Ethics Committee of Women's Hospital of Nanjing Medical University (No. 2018-49). All animal experiments were performed in accordance with Chinese national regulations on the administration of animal experimentation as well as international guidelines on animal experimentation. After 1 week of acclimatization, mice were randomly divided into three groups (n = 5): control [normal chow diet (NCD) + vehicle, n = 5], HFD (HFD + vehicle, n = 5), and ITF treatment (HFD + ITF, n = 5). In order to compare the changes of fecal microbiota before and after pregnancy, the three groups were renamed to normal chow diet in gestation (NCDG) group, HFDG group, and ITFG group after mating. The NCD mice were fed a low-fat diet (Research Diet AIN-93G, consisting of 20.3% protein, 63.9% carbohydrate, and 15.8% fat) for 4 weeks prior to mating and throughout pregnancy (18 days), while both HFD and ITF treatment groups were fed an HFD (Research Diet D12451, consisting of 35.2% protein, 63.9% carbohydrate, and 45% fat). The ITF treatment group received a dose of 3.33 g/kg of ITF each day *via* oral gavage, while the NCD and HFD groups received the same dose of a vehicle (DD H_2O). All mice were given free access to 100 g of fresh diet and 250 ml of fresh water daily per cage (five mice per cage).

Fasting Blood Glucose and Oral Glucose Tolerance Test

Blood samples were collected from the tail vein, and blood glucose levels were measured with a glucose meter (Roche Accu-Chek Active, Mannheim, Germany). FBG was monitored at different time points, including before dietary intervention, after 4 weeks of HFD, and on gestational age of 0 day (GD0), gestational age of 10 days (GD10), gestational age of 14 days (GD14), and gestational age of 18 days (GD18). OGTT was performed on GD14. The animals fasted for 6 h and then were gavaged with 2 g/kg glucose. The blood glucose levels at 0, 30, 60, 90, and 120 min were determined.

Detection of Biochemical Indexes

Mice were euthanized by CO_2 inhalation on GD18 (or equivalent) after fasting for 6 h from 8 a.m., and blood sample was collected. Blood was centrifuged at 3,000 *g* for 15 min at 4°C, and serum was isolated. The levels of fasting serum insulin (FINS), triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were measured using a commercial detection kit (NJJCBIO Co., Ltd., Nanjing, China) according to the kit instructions.

Based on the measured content of FBG and FINS, the homeostasis model of assessment (HOMA) for insulin resistance (IR) index (HOMA-IRI) was calculated and compared. HOMA-IRI was calculated as [fasting glucose (mmol/L) × fasting insulin (mU/L)]/22.5. Meanwhile, the area under the curve (AUC) of blood glucose was calculated (Lachine et al., 2016).

Hematoxylin–Eosin Staining

Liver and inguinal fat tissues were fixed in 4% paraformaldehyde, decalcified, paraffin embedded, and stored at 4°C. After tissues were sliced into 4 μm sections, hematoxylin–eosin staining was performed. First, sections were stained with hematoxylin for 5–10 min, immersed in 70% ethanol for 30 min to remove cytoplasm coloring, alkalized with alkaline solution, and washed with distilled water for 1 min. Second, sections were stained with eosin for 30–60 s, dehydrated with gradient ethanol, cleared two times with xylene, dried, and mounted. Finally, the morphological structures of the liver and inguinal fat tissues were observed under an optical microscope.

Fecal DNA Extraction

One day prior to mating and GD18, fecal samples were collected in individual sterilized cages and immediately frozen in liquid nitrogen. About 100 mg of stool samples was used to extract total genome DNA according to the DNA extraction kit (DP328, Tiangen Company, Beijing, China). The concentration and purity of the extracted bacterial DNA were detected using a Qubit 2.0 fluorometer (Thermo Scientific, United States). The 16S rRNA gene V4 region-specific primers are 515F (GTGCCAGCMGCCGCGGTAA) and 806R GGACTACHVGGGTWTCTAAT. The PCR products of sterile water were considered as the negative control for 16S rRNA sequencing. The PCR products were purified using the Gene JET Gel Extraction Kit (Thermo Scientific). The library was constructed using Ion Plus Fragment Library Kit 48 reactions (Thermo Fisher, United States). After Qubit quantification and testing, the library was sequenced by Thermo Fisher's Ion S5^{TM} XL.

Gut Microbiota Analysis

Raw data were obtained after data processed using Cutadapt (V1.9.1[1]). Then, chimera sequences were removed to obtain clean reads. Operational taxonomic units (OTUs) were assigned for sequences with ≥97% similarity. OTUs were annotated using the SILVA132 database.[2] The taxonomic information was obtained, and the community composition was counted at seven taxonomic levels: kingdom, phylum, class, order, family, genus, and species. Alpha diversity was analyzed by Chao 1[3] with QIIME software (version 1.9.1). Beta-diversity metrics were calculated by the non-metric multidimensional scaling (NMDS) model based on the Bray–Curtis distance. One-way analysis of similarities (ANOSIM) with multiple pairwise post-tests on all groups at the same time was performed to test whether the difference between the extra groups was greater than that between the intra-groups and to assess the significance of the difference in separation. Chao 1, Bray–Curtiss indexes, NMDS, and ANOSIM were calculated at the OTU level. Differentially abundant genera were analyzed by meta stats[4] with a non-parametric test, followed by the Benjamini and Hochberg false discovery rate approach to filter relevant *p*-values.

Fecal Short-Chain Fatty Acid Analysis

The feces from each mouse were collected and frozen at −80°C. Acetate, propionate, and butyrate in fecal samples were analyzed using gas chromatography–mass spectrometry (GC-MS) (Sun et al., 2015). Briefly, the feces were homogenized with a saturated sodium chloride solution and acidified with 10% sulfuric acid. Next, diethyl ether was used to extract SCFAs. After centrifugation, the supernatants were harvested for GC-MS.

Statistical Analysis

Data represent mean ± standard error of the mean. For parametric variables, the unpaired two-tailed Student *t*-test was used to assess the differences in mean values between two groups. For three groups, statistical analysis was performed with ANOVA with Tukey *post hoc* test. For non-parametric variables, the statistical significance of the differences was evaluated by the Mann–Whitney test or Kruskal–Wallis test. For the OGTT, two-way ANOVA was performed for the evolution of blood glucose levels with a *post hoc* test using the Bonferroni method. A *p*-value < 0.05 was considered statistically significant. GraphPad Prism 7 (GraphPad Software, San Diego, CA, United States) was used to do the statistical analyses.

RESULTS

Changes of Body Weight and Glycolipid Metabolic Parameters in Mice

To investigate the effect of inulin treatment on glycolipid metabolism disorders in HFD-induced gestational diabetes mice, we examined the body weight, daily food intake, and glycolipid metabolism-related parameters. The body weight, FBG, FINS, HOMA-IR, TG, TC, LDL-C, and the AUC of OGTT of the HFDG group mice were significantly elevated compared with those of the NCDG group mice (**Figures 1A–H**, **2A–D**), indicating severe glucose intolerance, insulin resistance and dyslipidemia.

[1]http://cutadapt.readthedocs.io/en/stable/

[2]http://www.arb-silva.de/

[3]http://scikit-bio.org/docs/latest/generated/skbio.diversity.alpha.chao1.html#skbio.diversity.alpha.chao1

[4]http://metastats.cbcb.umd.edu/detection.html

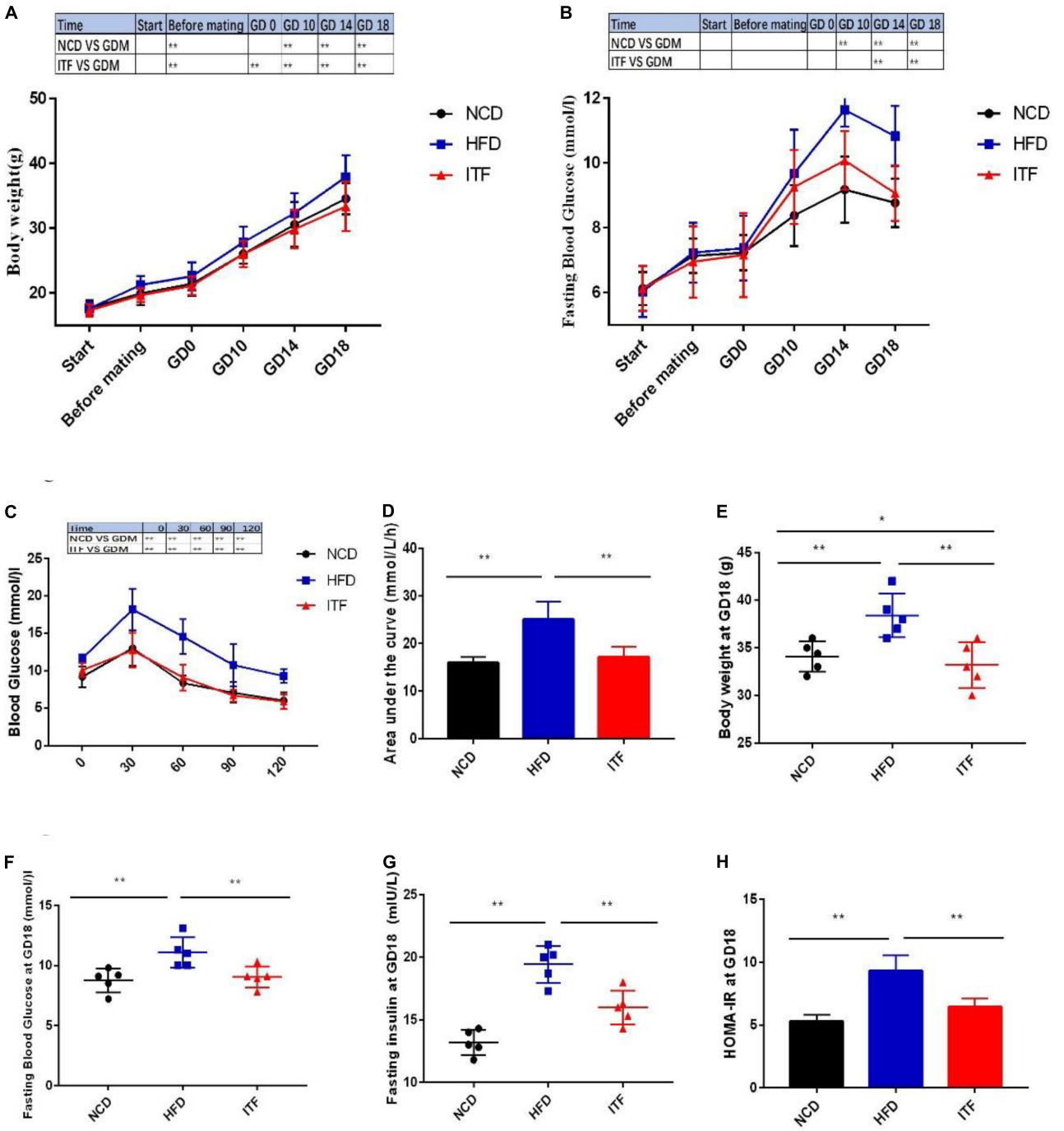

FIGURE 1 | Improvement in metabolic parameters in HFD-induced gestational diabetes mice by ITF. **(A)** Body weight. **(B)** FBG. **(C)** Plasma glucose profile. **(D)** Mean AUC measured during the OGTT. **(E)** Body weight at GD18. **(F)** FBG at GD18. **(G)** Fasting insulin at GD18. **(H)** HOMA-IR at GD18. AUC, area under the curve; OGTT, oral glucose tolerance test. Data are presented as mean ± SEM. Data were analyzed using two-way ANOVA followed by the Bonferroni *post hoc* test for panels **(A,B,F)** and using one-way ANOVA followed by the Tukey *post hoc* test for panels **(C–E,G)**. $^{*}p < 0.05$, $^{**}p < 0.01$.

In contrast, ITFG group mice fed the ITF-supplemented diet showed improved metabolic parameters (**Figures 1A–G**, **2A–D**). After ITF intervention, body weight, serum TG, TC, and LDL-C on GD18 reduced significantly by 4.54 g, 0.48 mmol/l, 1.04 mmol/l, and 0.494 mmol/l ($p < 0.05$, vs. HFDG group) (**Figures 1C**, **2A,B,D**), respectively. Additionally, the AUC of OGTT on GD14 and the FBG and serum insulin on GD18 were lowered by 7.95 mmol/L/h, 2.04 mmol/l, and 3.46 mIU/L, respectively ($p < 0.05$, vs. HFDG group), indicating a significant improvement in glucose tolerance (**Figures 1D,E,G**).

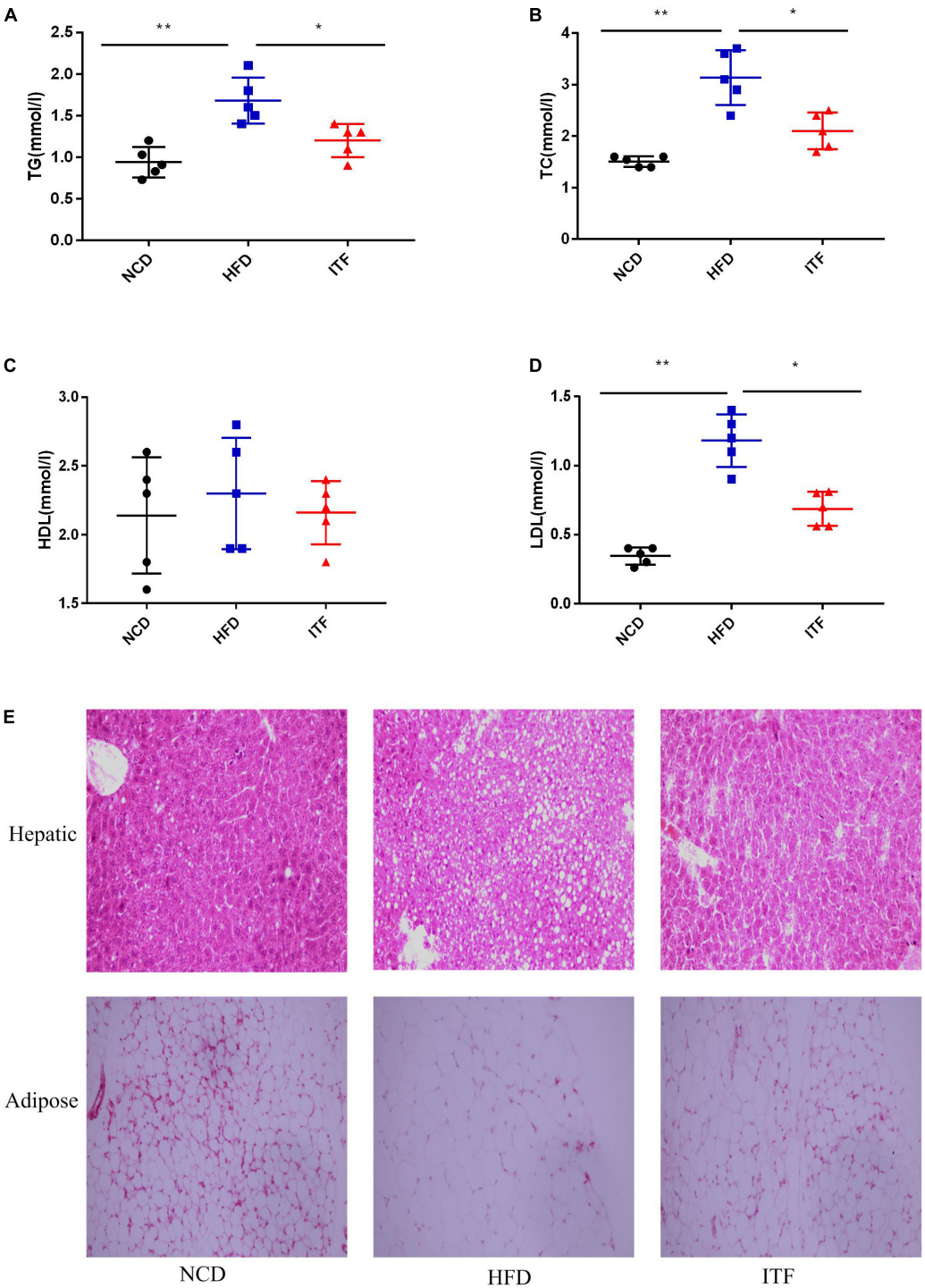

FIGURE 2 | Improvement in metabolic parameters in HFD-induced gestational diabetes mice by ITF. **(A)** Serum TG. **(B)** Serum TC. **(C)** Serum HDL-C. **(D)** Serum TC LDL-C. **(E)** Representative H&E-stained images of the hepatic and adipose tissues (×200). TG, triacylglycerol; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. Data were analyzed using one-way ANOVA followed by the Tukey *post hoc* test for panels **(A–D)**. $^{*}p < 0.05$, $^{**}p < 0.01$.

According to hepatic and adipose tissue staining (**Figure 2E**), the HFDG group mice exhibited severe hepatic lipid droplets and adipocyte hypertrophy, which were alleviated after ITF treatment. Overall, the above results indicate that ITF have a beneficial effect that ameliorates glycolipid metabolism disorders in HFD mice.

Reproductive Outcomes of Pregnant Mice

The number, body weight, and length of fetal mice in each group were compared. There was no significant difference in the number of fetal mice among groups (**Supplementary Figure 1A**). The average body weight and length of fetal mice born by HFD mothers (1.26 ± 0.16 g and 2.33 ± 0.09 cm, respectively) were significantly higher than those by ITF mothers (1.03 ± 0.05 g and 2.16 ± 0.07 cm, respectively) (**Supplementary Figures 1B,C**).

Changes of Fecal Microbial Diversity

We used the 16S rRNA gene amplicon sequencing method (V4 region) and generated 2,131,728 reads for a total of 25 samples, with an average of 85,269 ± 22,171 reads per sample. At each stage, NCD-HFD-ITF and NCDG-HFDG-ITFG pairs shared less common OTUs with each other. The Venn graph exhibited common OTUs for NCD-HFD-ITF and NCDG-HFDG-ITFG pairs, decreasing from 579 before mating to 438 on GD18 (**Figure 3A**).

To assess the fecal microbial community structure, richness (Chao 1 index) and diversity (Simpson index) were calculated (**Figures 3B,C**). For Chao 1 index, the data of the ITF group were significantly higher than those of NCD and HFDG groups ($p < 0.05$, $p < 0.01$). A remarkable increment in Simpson index with ITF supplementation was found compared with HFD and HFDG groups in the present study ($p < 0.05$). All the results above provided the view that ITF treatment could effectively improve the decline of Chao 1 index and Simpson index induced by HFD addition.

We then used principal co-ordinate analysis (PCoA) to categorize the OTU data into two main factors that explained 64.42% of the variance (**Figure 3E**), which showed that the microbiomes in NCD (NCD and NCDG), HFD (HFD and HFDG), and ITF (ITF and ITFG) treatment groups significantly differed from one another while the two groups of the same treatment shared some overlapping regions before and after conception, which indicated that the overall gut microbial community had been significantly modified. The four groups exhibited significant, tight clustering according to NCD or ITF diet. Independent biological replicates were generally consistent, but more variable among mice fed by HFD (**Figure 3D**).

Changes of the Relative Abundance at the Phylum Level

The phylum Bacteroidetes was dominant among the nine phyla (>1% in at least one sample) present in the gut microbiota from the six groups of mice, and the ratio of Firmicutes/Bacteroidetes (F/B) was increased in HFD and HFDG mice over the NCD and NCDG groups, but lower in the ITF and ITFG groups compared with HFD and HFDG mice (**Figure 4** and **Supplementary Table 1**). The gut microbiota in obese individuals has usually shown an increased F/B ratio (Ramirez-Farias et al., 2009). Therefore, the decreased F/B ratios of ITF and ITFG mean that the feature in HFD mice could be reversed by the ITF-supplemented diet. HFD treatment decreased the relative abundance of Proteobacteria before mating ($p < 0.01$). ITF supplementation increased the relative abundance of Verrucomicrobia compared with HFD before mating and on GD18 ($p < 0.01$). Relative abundances of the Deferribacteres group of HFD and the Cyanobacteria group of NCD were not detected in fecal samples on GD18. Moreover, relative abundances of Actinobacteria decreased in HFD before mating but increased substantially when reaching the perinatal period. The majority of genera were affected by the gestation stage, indicating that their relative abundances changed greatly over the pregnancy progress.

Changes of the Relative Abundance at the Genus Level

The relative abundances at the genus level (>1% in at least one sample) were present in **Figure 5** and **Supplementary Table 2**. Fat addition (HFD and HFDG) increased the relative abundances of *Dubosiella* and *Lactobacillus* and decreased those of *Romboutsia* and *Alloprevotella* compared to the NCD (NCD and NCDG). The abundance of *Bifidobacterium* increased, whereas that of *Dubosiella* decreased with the intervention of ITF before and after conception. Our results also indicated that the abundance of *Akkermansia* was significantly higher in the ITF-treated (ITF and ITFG) groups than in any other group. The heat map analysis of microbial community composition at the family level confirmed that the abundance of *Dubosiella* that causes obesity and metabolic syndrome-related inflammation was reduced after ITF treatment (**Figure 6**).

Next, to identify the changes in specific bacterial taxa after ITF-supplemented diet intervention before and after conception, we utilized the linear discriminant analysis (LDA) effect size (LEfSe) to compare the fecal microbiota composition between the NCD, HFD, and ITF groups; the LDA score was selected to discriminate specific taxa in different groups. Compared with the HFD group, the ITF mice had a higher abundance of f-Ruminococcaceae, f-Prevotellaceae, o-Verrucomicrobiales, *g-Akkermansia*, c-Verrucomicrobiae, p-Verrucomicrobia, and f-Akkermansiaceae but lower abundance of *g-Unidentified clostridiales*, *f-Unidentified clostridiales*, *g-Dubosiella*, c-Erysipelotrichia, o-Erysipelotrichales, and f-Erysipelotrichaceae (**Figures 7A–E**). Correspondingly, *g-Bacteroides*, f-Ruminococcaceae, and f-Bacteroidaceae were enriched in the ITFG group on GD18 (**Figures 8A–C**).

Changes in Fecal Short Chain Fat Acids Levels Upon Inulin-Type Fructans Intervention

Acetate, propionate, and butyrate levels in fecal samples were quantified by GC-MS. Over time, fecal acetic acid levels were significantly increased in ITF group mice when compared to HFD group mice before mating ($p < 0.05$) and on GD18 ($p < 0.01$) (**Figures 9A,B** and **Supplementary Table 3**). Butyric acid levels were significantly increased in ITF group mice compared to HFD group mice on GD18 ($p < 0.05$) (**Figure 9B** and **Supplementary Table 3**). However, we observed no differences in the propionate levels among the three groups at any of the time points (**Figures 9A,B** and **Supplementary Table 3**).

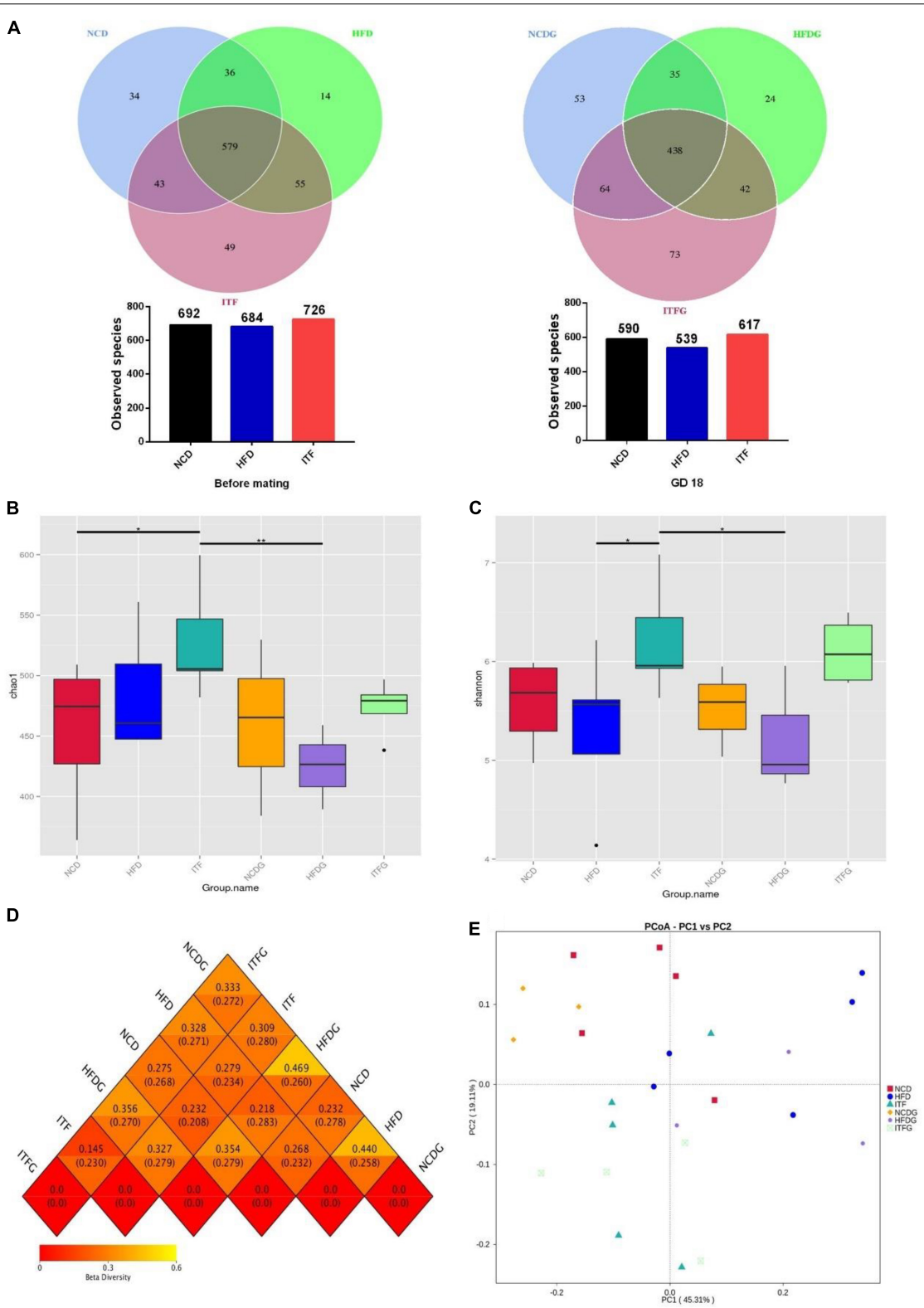

FIGURE 3 | ITF modify the composition of the cecal microbiota in ob/ob mice. **(A)** OTU number before mating and on GD18. **(B)** Chao 1 index of microbiota. **(C)** Shannon index of microbiota. **(D)** Heat map of beta diversity index. **(E)** The beta diversity of gut microbiota analyzed by PCoA. Data were analyzed using one-way ANOVA followed by the Tukey *post hoc* test for panels **(B,C)**. $^{*}p < 0.05$, $^{**}p < 0.01$.

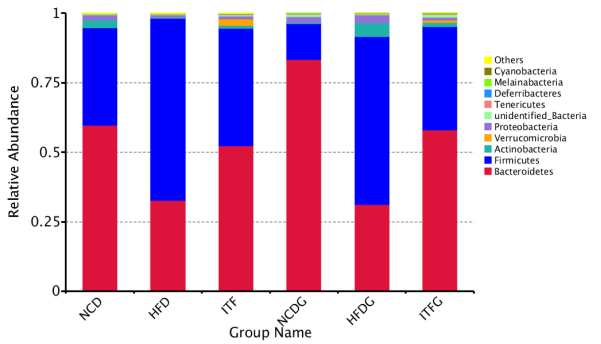

FIGURE 4 | Relative abundance of microbial species of the top 10 phyla in the feces of mice.

Correlations Between Glycolipid Metabolism Indicator and Bacterial Abundance

At the phylum level, we analyzed the correlations between significant glycolipid metabolism indicator and gut microbiota on GD18. Bacteroidetes abundance was negatively correlated with FBG, FINS, TG, and TC, whereas Firmicutes abundance was positively correlated with FBG, FINS, and TG (**Figure 10A**). Moreover, Actinobacteria abundance was positively correlated with FINS and TC (**Figure 10A**).

At the genus level, the relative abundance of *Dubosiella* was positively correlated with FBG, FINS, and TC (**Figure 10B**). *Romboutsia* abundance was positively correlated with FBG (**Figure 10B**).

DISCUSSION

Gut microbiota disorder has been considered as one of the contributing factors for metabolic disorders. The composition of the microbiome also changes during pregnancy. It has been recently proposed that fecal microflora and their metabolic activities may play a critical role in body weight control, energy homeostasis, fermentation, and absorption of non-digestible carbohydrate, as well as in the development of IR. Therefore, gut microbiota may also participate in the pathogenesis of several metabolic disorders, such as obesity, diabetes mellitus, and GDM (Cani et al., 2014; Zhang et al., 2015; Rowland et al., 2018; Cortez et al., 2019). Prebiotics can exert positive effects on the maintenance of host metabolic homeostasis, which are mainly mediated by the gut microbiota (Khanum et al., 2000; Wang et al., 2021). ITF, one of the crucial prebiotics, have been demonstrated to be effective in the treatment of T2DM (Dehghan et al., 2014a; Zhang et al., 2018), while data on the effects of symbiotic supplementation on markers of insulin metabolism and lipid concentrations in GDM are scarce. The aim of this study was to determine whether ITF taken before and during pregnancy could impact the development of HFD-induced glucose intolerance during pregnancy.

To induce features of GDM, C57BL/6J mice were fed an HFD for 4 weeks before and during pregnancy. This model has previously been used to induce features of GDM in mice, such as insulin resistance and dyslipidemia (Holemans et al., 2004; Jones et al., 2009; Liang et al., 2010). A period of only 4 weeks of HFD exposure before pregnancy is not sufficient to cause a diabetic phenotype; however, continued feeding throughout pregnancy leads to progressive glucose intolerance and insulin resistance, mimicking human disease. This mouse model allowed a factorial design to determine the interaction of treatments, as well as more thorough examination of potential mechanisms and whole-tissue analysis, which would not be possible in human trials.

In the present study, we chose the dose of 3.33 g/kg/day of ITF, which was equal to the highest dose reported for human consumption (16 g/day) to evaluate the potential antidiabetic effects of ITF in GDM mice. Consistent with a previous study showing that ITF administration significantly lowered the levels of FBG, IL-6, TNF-α, and plasma LPS in T2DM

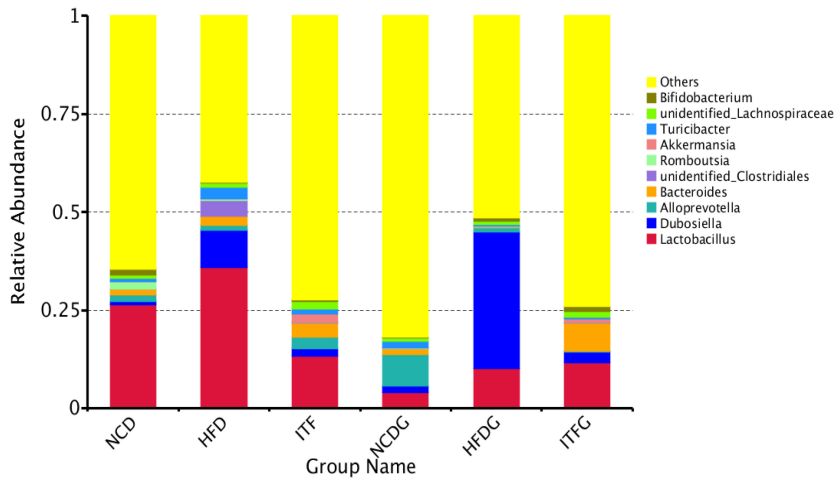

FIGURE 5 | Relative abundance of microbial species of the top 10 genera in the feces of mice.

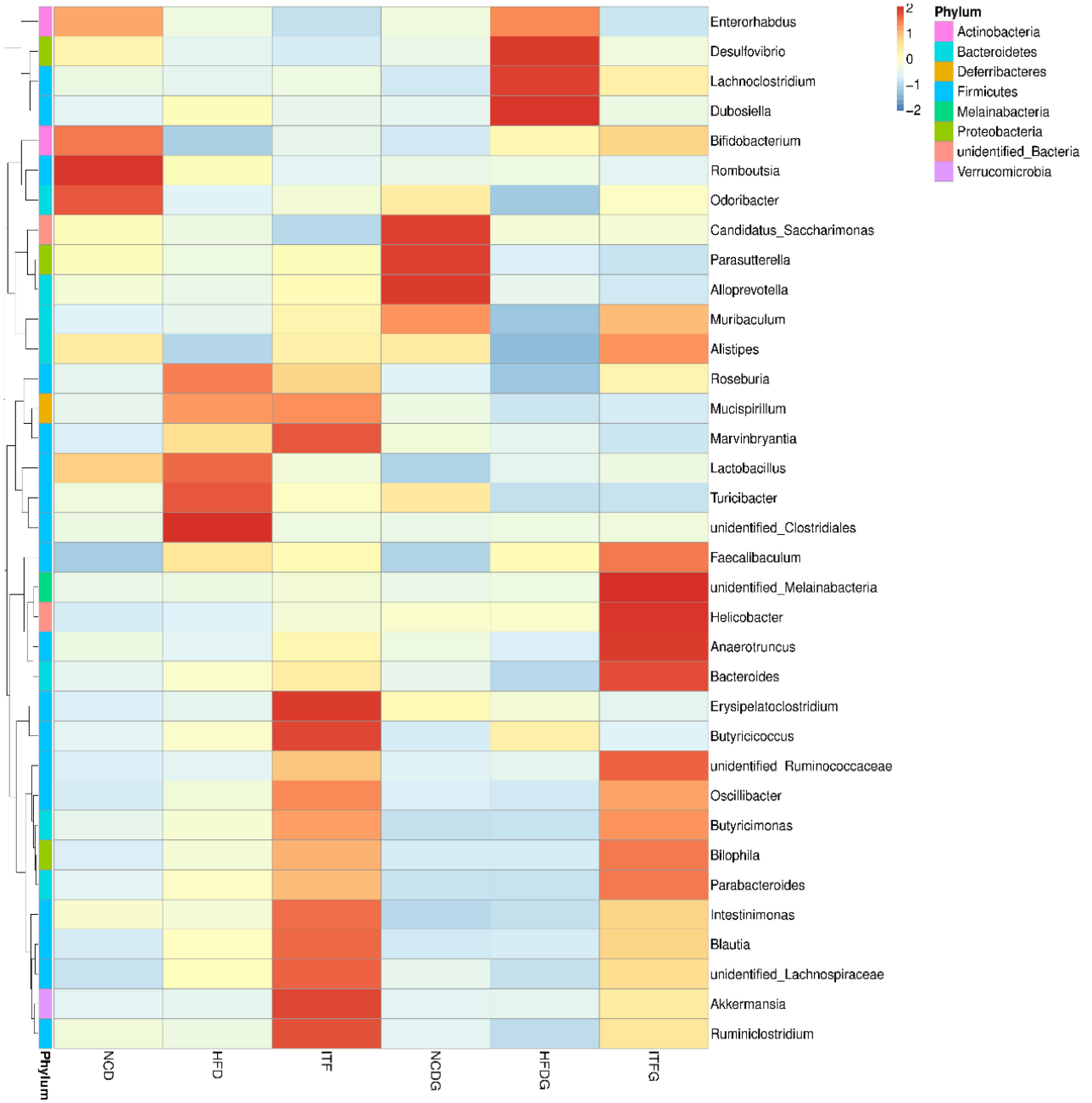

FIGURE 6 | Heat map of microbial species of at the genus level in the feces of mice.

patients (Dehghan et al., 2014b), we found that ITF relieved the gestational diabetic symptoms as evidenced by reduced body weight, blood glucose level, and insulin level. However, Farhangi et al. (2016) found that chicory inulin significantly reduced the fasting serum glucose level and HbA1C ratio but had little effect on the insulin level in patients with T2DM. We speculate that the different effects of chicory inulin on insulin may be due to different dosages (10 g/day for T2DM patients in Farhangi et al.'s study). Moreover, a strong hypolipidemic effect of ITF in GDM mice was observed. These results agree with a previous study showing that inulin promoted lipid metabolism by altering the expression of acetyl-CoA carboxylase and the activities of fatty acid synthase and xanthine oxidase (Lin et al., 2014).

Accumulating studies have been performed to reveal the underlying mechanisms of efficient treatment of ITF in GDM. The majority of mechanisms are attributed to gut microbiota alteration, immune inflammation, abnormal lipid metabolism, and oxidative stress. Growing evidences have demonstrated that the gut microbiota play a critical role in the development of GDM (Fugmann et al., 2015; Mokkala et al., 2017; Crusell et al., 2018;

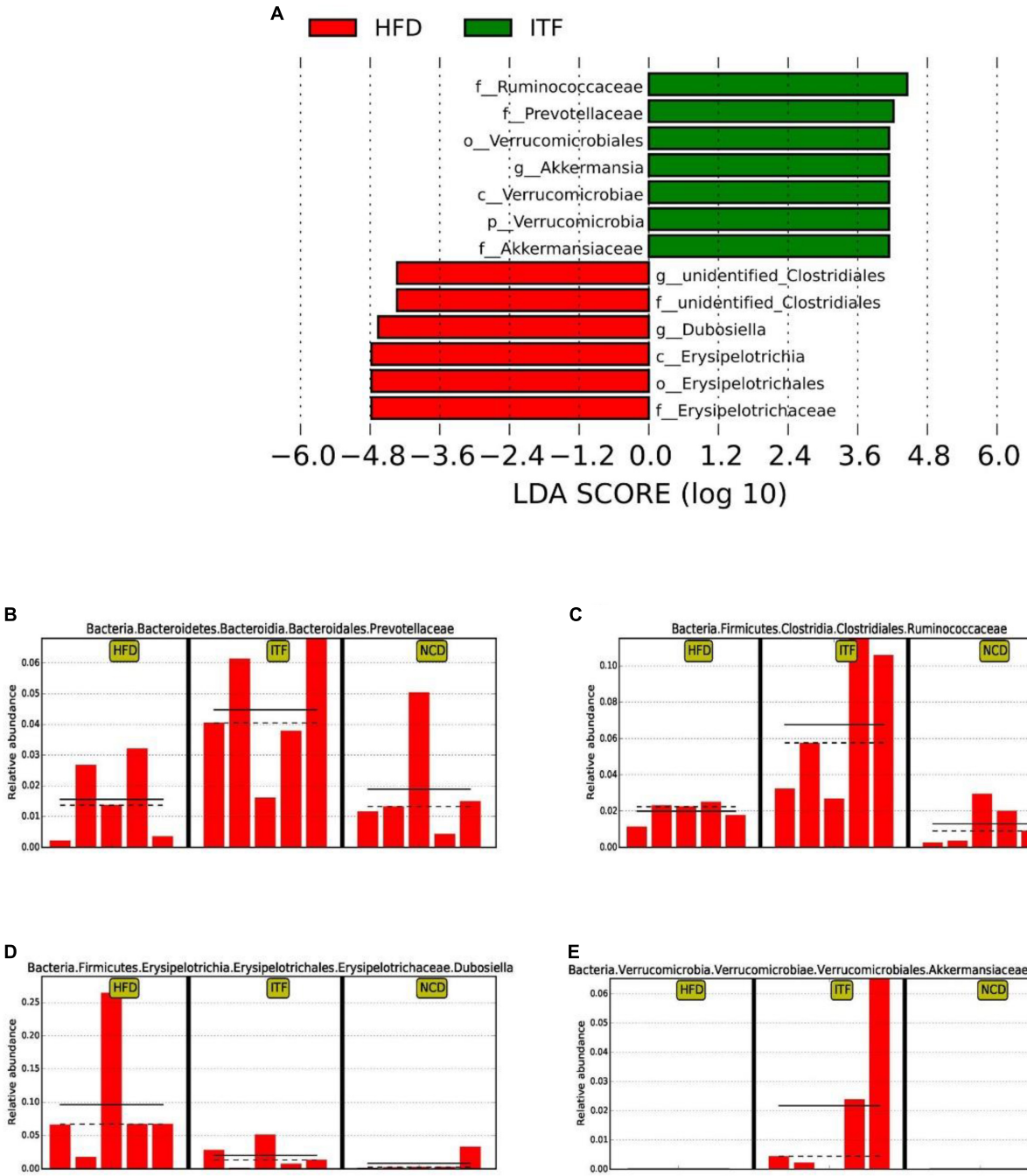

FIGURE 7 | Identification of the most differentially abundant among HFD, ITF, and NCD analyzed by the LEfSe method. **(A)** LDA scores of differentially abundant taxa. **(B)** Relative abundance of Prevotellaceae. **(C)** Relative abundance of Ruminococcaceae. **(D)** Relative abundance of *Dubosiella*. **(E)** Relative abundance of *Akkermansia*.

Hasan and Aho, 2018). In the present study, the alpha diversity index that was reduced by HFD could be effectively improved by inulin treatment. Stanislawski et al. (2017) reported that gestational weight gain was associated with lower alpha diversity. Beta-diversity analysis of unweighted UniFrac illustrated the distinct clustering of the relative abundances of OTUs after ITF treatment. Similar results were obtained from PCoA.

At the phylum level, a higher ratio of F/B was observed in the HFD group, which was supported by a study showing that the F/B ratio in overweight human adults was lower than that in lean controls (Lordan and Thapa, 2020). An imbalance in the F/B ratio is related to dysbiosis conditions (Ley et al., 2005; Nelson et al., 2010). The decreased F/B ratio of ITF and ITFG means that this feature in obesity could be

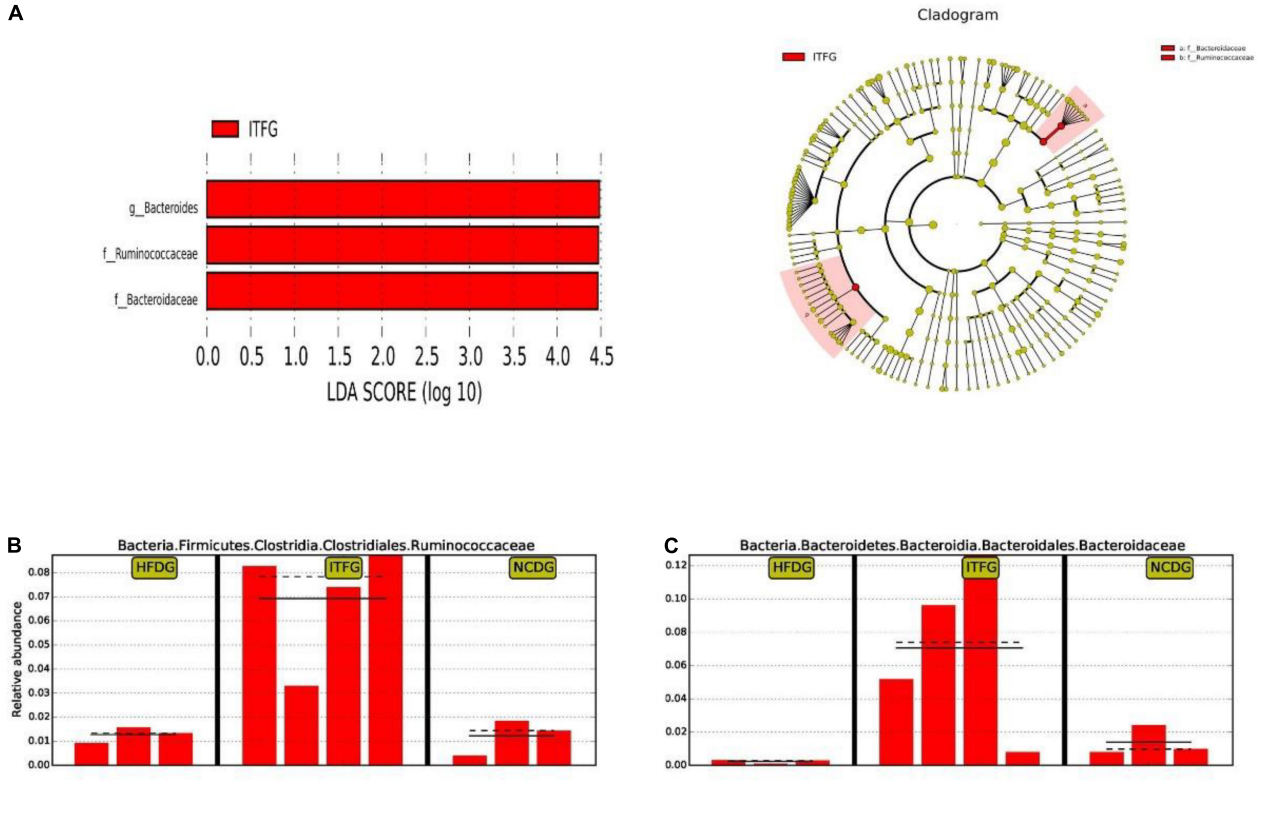

FIGURE 8 | Identification of the most differentially abundant among HFDG, ITFG, and NCDG analyzed by the LEfSe method. **(A)** LDA scores of differentially abundant taxa. **(B)** Relative abundance of Ruminococcaceae. **(C)** Relative abundance of Bacteroidaceae.

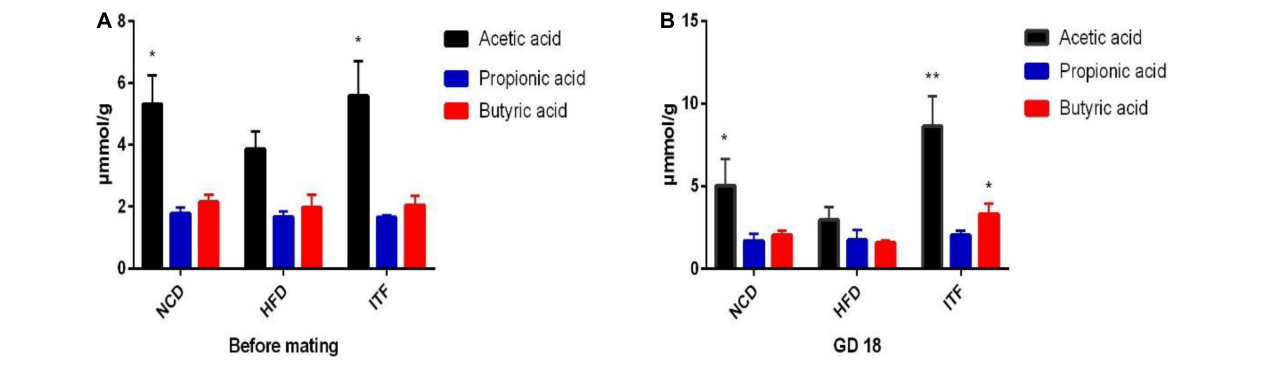

FIGURE 9 | (A) SCFA data before mating. **(B)** SCFA data on GD18. Data were analyzed using one-way ANOVA followed by the Tukey *post hoc* test for panels **(A,B)**. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to HFD.

reversed by the ITF-supplemented diet. Our analyses showed, after ITF treatment, an enhancement of the relative abundance of Verrucomicrobia in the HFD group before mating and on GD18, as well as an obviously lessened Actinobacteria on GD18. Verrucomicrobia is a member of the PVC (Planctomycetes–Verrucomicrobia–Chlamydiae) superphylum, which includes phylogenetically related bacteria with unusual characteristics such as the existence of a complex and dynamic endomembrane system that, in some aspects, makes them closer to eukaryotic cells. A recent study showed that the healthy Chilean subjects reveals a high abundance of the phylum Verrucomicrobia (Fujio-Vejar et al., 2017). Positive correlations of Actinobacteria with FINS caused aggravation of insulin resistance in the disease, which was reversed by inulin intervention.

At the genus level, ITF supplementation showed a significant effect on increasing the abundance of *Bacteroides*, which have been demonstrated to ameliorate inflammation in recent studies (Ejtahed et al., 2016; Li et al., 2017; Biruete et al., 2021). SCFAs, including acetate, propionate, and butyrate, derived from the gut microbiome are pivotal for rectifying host metabolism

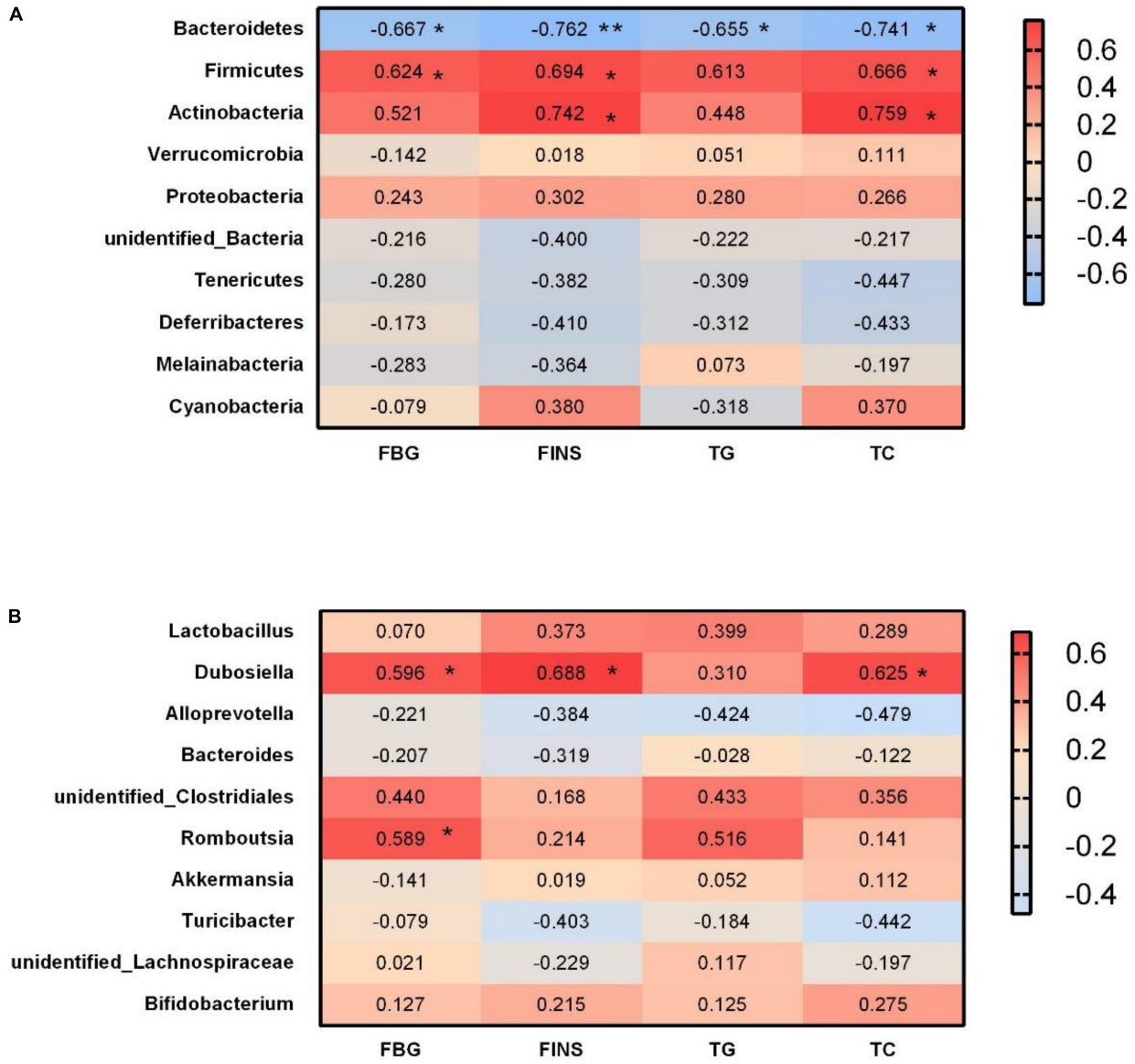

FIGURE 10 | Correlations between glycolipid metabolism indicator and bacterial abundance. **(A)** Heat map of Spearman correlations between the levels of metabolites/components and the abundances of gut microbial phyla. **(B)** Heat map of Spearman correlations between the levels of metabolites/components and the abundances of gut microbial genera. FBG, fasting blood glucose; FINS, fasting insulin; TG, triglyceride; TC, total cholesterol. *$p < 0.05$, **$p < 0.01$.

and immunity (Meijer et al., 2010). In the present study, we observed that acetic acid levels of the ITF group increased significantly before mating and on GD18, whereas butyric acid levels only increased on GD18, suggesting that changes of bacterial metabolites might be dependent on the intervention time. Consistent with our findings, ITF-fed mice increased the production of SCFAs, benefiting the balance of gut microbiota in the alleviation of diabetic mice (Chen et al., 2017). Significant elevation of SCFA-generating *Bacteroides* revealed that our ITF treatment may restore gut dysbiosis by promotion of *Bacteroides*. Another genus that we found increased abundance in ITF-fed mice was *Akkermansia*. Recent studies described this as an important probiotic genus, with systemic beneficial effects to the host (Cani, 2014; Cani and de Vos, 2017), including the control of metabolic syndromes (Christiansen, 2013; Dao et al., 2016). In rodents, probiotics supplementation with *Akkermansia* improved glucose tolerance and insulin sensitivity (Zhao et al., 2017). Our results suggest that *Akkermansia* might have another impact on host physiology during pregnancy than otherwise described or that we found another subspecies of *Akkermansia*. The applied 16S rRNA gene amplicon sequencing methods does, however, not make it possible to investigate this finding at a deeper taxonomic resolution. We observed that HFD mice have increased *Dubosiella*, which has been previously described in dysbiosis conditions such as GDM and obesity (Bai et al., 2019; Li et al., 2020; Sheng et al., 2020; Qiu and Macchietto, 2021;

Yi et al., 2021). Positive correlations of *Dubosiella* with FBG, FINS, and TC demonstrated that these bacteria may promote the glycolipid metabolism disorders, which could be reversed by ITF treatment.

Modulation of the human gut microbiome with dietary interventions has been extensively studied, mainly focusing on the supplementation of non-digestible carbohydrates (NDCs) (Ladirat et al., 2014; Elison et al., 2016; Zhao and Zhang, 2018). However, the impact of dietary components on the stability and resilience of the gut ecosystem has been barely addressed. We found ITF intervention evidently primed the mice with significant change in microbiota profile, and the gestational impact (IFTG-IFT) was largely ameliorated compared to the other two treatments. This may be partially due to the ability of ITF to improve gut microbiome resilience. A high microbial diversity, as well as the increase of the levels of fecal SCFA, seemed to be critical aspects for the resilience of ITF group mice. Thus, further studies are required to reveal the precise mechanism(s) behind these effects.

In summary, we show that ITF treatment (3.33 g/kg/day) alleviates glucose and lipid metabolism disorders in HFD-induced gestational diabetes mice. These actions are likely to be mediated *via* increasing the abundance of Verrucomicrobia, *Bifidobacterium*, and *Akkermansia* and reducing the abundance of *Dubosiella*. We further demonstrate that the abilities of inulin intervention to enhance the relative abundance of SCFA-producing bacteria and increase the levels of SCFAs play a key role in antidiabetic effects. Our findings suggest a potential value of ITF as an inexpensive supplement for the prevention and treatment of GDM patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: The sequences are available in the NCBI database (Accession number: Bioproject PRJNA789154; SRA submission: SUB10794397).

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Protection Ethics Committee of Women's Hospital of Nanjing Medical University (No. 2018-49). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MM and XZ conceived and designed the experiments. XW, CF, and TL performed the animal experiments and completed the analysis of metabolic parameters. QW, YZ, and LY collected the data and performed the bioinformatics and statistical analysis. MM, XW, and QW wrote the initial manuscript. XZ, YD, and PL reviewed and edited the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.832151/full#supplementary-material

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Analysis and Comparison of Gut Microbiome in Young Detection Dogs

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The detection dogs are well-known for their excellent capabilities to sense different kinds of smells, which can play an important role in completing various searching and rescuing missions. The recent studies have demonstrated that the excellent olfactory function of detection dogs might be related with the gut microbes *via* the bidirectional communications between the gastrointestinal tract and the brain. In this study, the gut microbial communities of three types of breeds of detection dogs (Springer Spaniel, Labrador Retriever, and German Shepherd) were studied and compared. The results revealed that the richness and the diversity of gut microbiome German Shepherd dogs were significantly higher than the Labrador Retriever dogs and the Springer Spaniel dogs. At the phylum level, the most predominant gut microbial communities of the detection dogs were comprised of Fusobacteriota, Bacteroidetes, Firmicutes, Proteobacteria, Campilobacterota, and Actinobacteriota. At the genus level the most predominant gut microbial communities were comprised of *Fusobacterium*, *Megamonas*, *Prevotella*, *Alloprevotella*, *Bacteroides*, *Haemophilus*, *Anaerobiospirillum*, *Helicobacter*, *Megasphaera*, *Peptoclostridium*, *Phascolarctobacterium*, and *Streptococcus*. However, the gut microbial communities of the three dogs group were also obviously different. The mean relative abundance of *Fusobacterium*, *Prevotella*, *Alloprevotella*, *Megamonas*, *Bacteroides*, and *Phascolarctobacterium* presented significant differences in the three groups. According to the portraits and characteristics of the gut microbiome in young detection dogs, multiple kinds of nutritional interventions could be applied to manipulate the gut microbiota, with the aim of improving the health states and the olfactory performances.

Keywords: detection dog, gut microbiota, olfactory function, Springer Spaniel, Labrador Retriever, German Shepherd

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INTRODUCTION

The detection canines have been widely used for executing various kinds of searches and rescue missions, and their excellent performances in identifying discriminations are also have intimate relations with the olfactory capabilities to detect different odors and chemical compounds (Jenkins et al., 2018). Current studies have revealed that the physical condition and olfaction performance of the working canines were closely related with the symbiotic microbiome, including the nasal, oral and gut microbiota (Suchodolski et al., 2009; Isaiah et al., 2017a; Emilie et al., 2021). Alterations of the gut microbiota induced by dietary structure, drug administration, and living environments

can all influence the health conditions and olfactory capabilities of the detection canines (Jenkins et al., 2016; Herstad et al., 2017; Essler et al., 2019). Therefore, manipulations of the gut microbiota through multiple strategies can be applied as novel interventions to improve the odor detecting properties of working dogs.

During the 30,000 years of domesticating process, the companion dogs gradually adapted to the human living environments and appeared to be interested in the human social cues (Wu et al., 2017). At the same time, the richness and diversity of the companion dogs' gut microbiome also changed significantly to adapt the domesticated living environment, while the hierarchical clustering of gastrointestinal metagenomes has proved the phylogenetic and metabolic similarities between the dogs and humans (Swanson et al., 2011). The high-throughput sequencing technique research data also proved that the gut microbiome of the companion dogs shared certain similarities with their owners (Deng and Swanson, 2015; Wang et al., 2022). Therefore, further study is required to identify the regulating effects of genetics, environments, and domestications on the composition of the canine microbiome, and to evaluate its role in canine immune function and gastrointestinal health.

In fact, the search and rescue performances of the detection dogs can be improved by extensive training and good dog-handler relationships (Diverio et al., 2016). Because the development of animal behavioral profiles can be powerfully influenced by the social experiences in the early life phases, the capabilities training of the detection dog to sense different smells can be started in the growing process (Sachser et al., 2011). The potential odor recognizing abilities of detection dogs can be improved by well-established training practice using the explosive samples or other organic chemicals (Lucia and David, 2014). By comparing the brain sizes between the ancestral species and the domesticated relatives, artificial domestication and social adaption could influence the brain function and behavioral development (Kruska, 2005). The bidirectional communications between the gut and the brain can be realized through the vagus nerve, the neuroendocrine pathways, and the bacteria-derived metabolites, and the brain function and the behavioral profiles can be influenced by the microbiota–gut–brain axis (Sandhu et al., 2017). Therefore, the olfactory performances of detection dogs might have certain relations with their unique gut microbial communities, and their physiological and behavioral conditions could be changed by gut microbiome alteration (Hooda et al., 2012).

Diet structure is commonly regarded as a critical influencing factor on the dog gut microbiota, which can produce important effects for gut health and overall well-being (Herstad et al., 2017). When the canine diet was changed from commercial dry food to mildly cooked diet (such as boiled minced beef), the microbial communities of the gut microbiota and the fecal metabolism profile were also changed (Tanprasertsuk et al., 2021). Compared with the commercial extruded diet, the administration of the raw-based diet supplemented with vegetable foods could promote the balance of dog–gut–microbial ecosystem and improve the gut function of healthy dogs (Sandri et al., 2017). Bones and raw food diets contained a high amount of meat, offal, and raw meaty bones, which combined with small amounts of vegetables and fruits and different sorts of oil and supplements. The previous study proved that the gut microbial communities and metabolome were significant different between the bones and raw food diets fed dogs and the commercially fed dogs (Schmidt et al., 2018). However, other studies revealed that a high protein diet could increase the abundance of butyrate-producing bacteria in Beagles, and had a greater impact on the microbial communities of the obese dogs (Xu et al., 2017). Therefore, the regulating role of diet consumption on the gut microbiome should also not be neglected besides the genetic portraits.

In the early life period, the acquisition of gut microbiome in young detection canines can be influenced by many factors, and it is a critical phase to establish the well-balanced microbial community and the maturated and developed immune system. The probiotics interventions targeting gut microbiota can confer health benefits for the host and forbid the invasion of foreign pathogens into the gastrointestinal tract. Fecal microbiota transplantation (FMT) is also recognized as an effective treatment for recurrent *Clostridioides difficile* infection; therefore, FMT can also provide valuable benefits for dogs with acute and chronic digestive diseases (Chaitman and Gaschen, 2021). Moreover, supplementations of probiotics can also prevent and treat the allergy and acute gastroenteritis of the companion dogs (Grześkowiak et al., 2015). In this study, the extraordinary compositions of gut microbiota in different breed types of young detection dogs were investigated and compared, and their possible functions, the gastrointestinal tract health, and the olfactory properties were predicted and analyzed.

MATERIALS AND METHODS

Animals and Diet

A total of 14 healthy dogs (five females and nine males) belonging to three different kinds of breed were recruited at the Shanghai Jialiang Working Dog Center (**Figure 1**). The participant dogs were categorized into three groups, including German Shepherd dogs (group D, $n = 5$), Labrador Retriever dogs (group L, $n = 5$), and Springer Spaniel dogs (group S, $n = 4$; **Table 1**).

All the dogs were fed with a commercial diet (Fubei PetCare, Shanghai, CN; **Table 2**). All the dogs were housed under the same environment and had free access to drink water, and all the dogs were maintained routinely by a professional breeder without any additional treatment. None of the enrolled dogs presented a history of medication, neutralization, or diarrhea before and after the experiment. All experimental procedures in this study were approved by the Committee for Accreditation of Laboratory Animal Care and the Guideline for the Care and Use of Laboratory Animals of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Science (approval number: 20210615).

The dogs were maintained open during the sampling process, and sterile swabs (Copan®, FLOQSwabs ™, 553C, Brescia, Italy) were introduced through the anus up to one third of the distal rectum. Three gentle complete circular movements were used to brush the mucosa before the withdrawal of the swab

FIGURE 1 | Three breed types of detection dogs recruited in this study. **(A)** Springer Spaniel dog **(B)** Labrador retriever dog **(C)** German Shepherd dog.

TABLE 1 | Basal characteristics of the enrolled detection dogs.

Breed groups	Sex (female/male)	Age (months)	Body weight (kg)
S	2/2	2.3	3.1
L	2/3	2.5	5.5
D	1/4	2.8	7.3

(Bell et al., 2020). Then the tops of the fecal swabs were cut and stored in sterile cryotubes at −80°C.

DNA Isolation

The collected fecal swabs were aliquoted into a sterile 2 ml tube containing 250 μl of 0.1 mm zirconia-silica beads, and then the samples were homogenized for a duration of 1 min at a speed of 5 m/s (Guard et al., 2015). Then the DNA was extracted with the QIAamp Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. The extracted genomic DNA quality was verified by agarose gel electrophoresis, and the total DNA concentration was measured by optical density ratio at 260 nm/280 nm using a spectrophotometry reader (NanoDrop, Thermo Scientific). The extracted DNA was stored at −20°C for further analyses.

16S rDNA Amplicons Sequencing

The V3/V4 hypervariable regions of the bacterial 16S rRNA gene were amplificated using the following primers: 341F(5′-CTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC−3′). All the 16S rRNA gene amplicons were used for constructing DNA libraries and were sequenced using the Illumina NovaSeq PE250 platform (You and Kim, 2021).

TABLE 2 | Primary ingredients and nutritional composition of the utilized diets.

Ingredients	Nutrient composition
Crude protein	26%
Crude fat	12%
Crude fiber	5%
Crude Ash	10%
Calcium	1.1%
Phosphorus	0.9%
Tysine	1.0%
Chloride	0.5%
Moisture	10%
Energy	442 kcal/100 g

Microbial Community Analysis

The bioinformatic analysis was performed using the quantitative insights into microbial ecology (QIIME) package (version 2). The sequencing results were firstly converted to FASTQ files based on the Illumina index sequences, and then the adapter sequences were trimmed using FASTP and the overlapping regions were demultiplexed. After removing the low-quality sequences, the remaining reads were clustered into operational taxonomic units (OTUs) with 97% sequence similarity. The 16S rRNA gene sequences were aligned by PyNAST and clustered under 100% sequence identity by UCLUST, and the microbial community analysis of the observed OTUs was performed based on the SILVA database (Emilie et al., 2021; You and Kim, 2021). The sequence biodiversity and richness were evaluated by the rank–abundance curves, and the Venn diagram was calculated to assess the microbiota structure in different samples. The alpha diversity analysis was calculated by the Chao1, ACE, Shannon and Simpson index, and the richness and diversity of the

microbial community within different groups were measured using QIIME 2. The beta diversity was evaluated using the visualized principal coordinate analysis (PCoA) based on the unweighted UniFrac distances, and the microbial compositions between different groups were estimated and compared (Xu et al., 2019). The significant differences in OTUs abundance among the three groups were analyzed by non-parametric Kruskal–Wallis test. The PICRUSt package were used to predict the contribution of bacterial community genes for potential function through the EggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) database (http://eggnog.embl.de/). All other analyses and visualizations were performed with R software version 3.0.1 and the boxplot package (Suchodolski et al., 2012; Minamoto et al., 2015; Zhang C. et al., 2015; Isaiah et al., 2017b; Li et al., 2021).

Statistical Analysis

In the comparison of microbial diversity index and relative abundance, the Kruskal–Wallis test or Wilcoxon rank sum test were used, and $p < 0.05$ was determined statistically significant for other statistical analyses.

TABLE 3 | Summary of sequencing data in the dog gut microbiota.

Amplified region	Input	Filtered	Denoised	Merges	Non-chimera
341F_805R	5104464	4829067	4740078	4147147	2790859

Sequence Data Accession Numbers

The 16S rRNA gene sequencing data reported in this study have been submitted to the GenBank Sequence Read Archive database (accession numbers of PRJNA803605).

RESULTS

Quality Control of the Sequenced Data and OTUs Analysis

The raw data obtained from the sequencing instrument was demultiplexed and quality filtered using QIIME2. Firstly, a total of 4,829,067 filtered reads were obtained from the 5,104,464 raw reads, and then the denoised analysis was performed by discarding the ambiguous reads and the primer matched barcoded reads were merged. Finally, a total of 2,790,859 clean reads were assembled into qualified sequences and used for further analysis (**Table 3**).

The high-quality non-chimera sequences were clustered into OTUs with at least 97% sequence identity. As showed in **Figure 2A**, there were 244 shared OTUs among the three groups; however, each group also had its corresponding unique OTUs. The Venn diagrams demonstrated that the OTUs numbers of the German Shepherd group (2,135) was the highest, which was

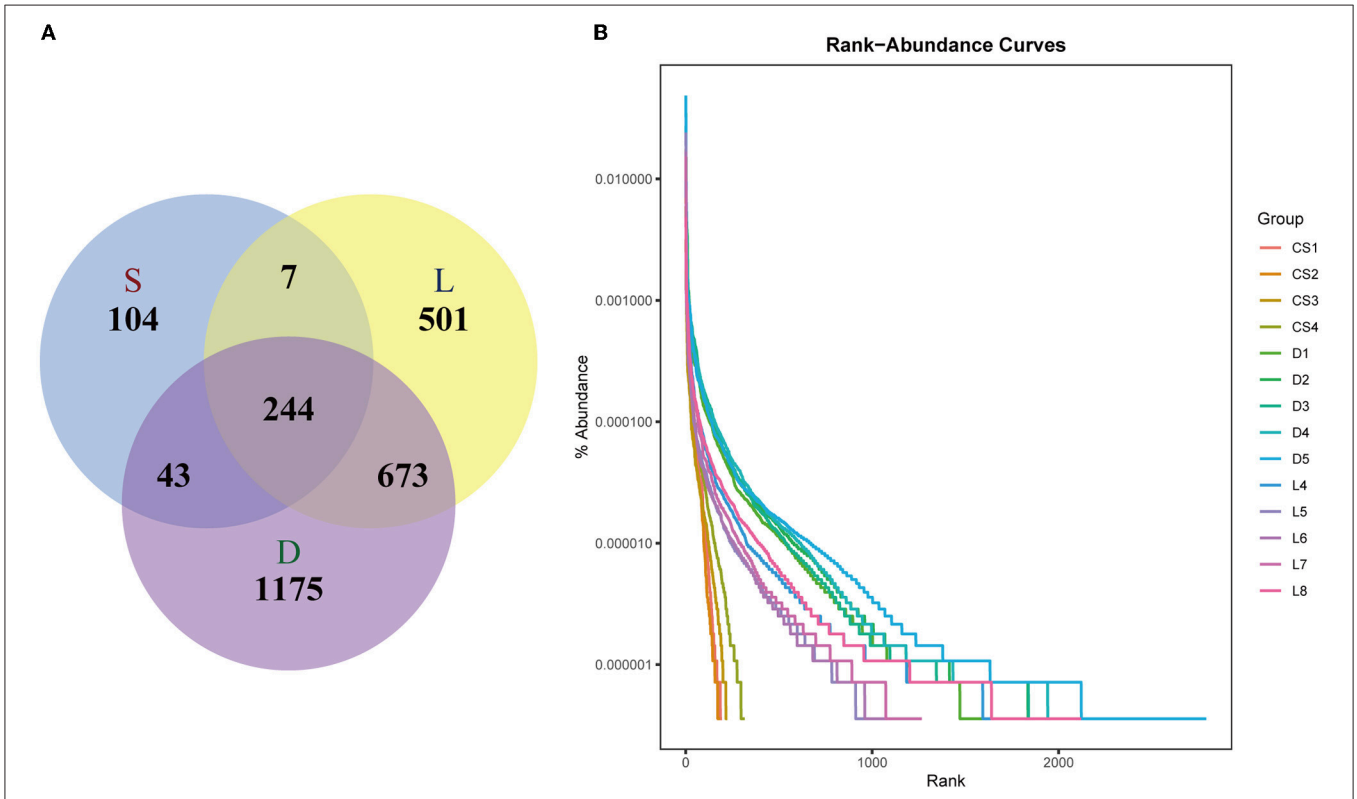

FIGURE 2 | Venn diagram **(A)** and the rank–abundance curves **(B)**. There were 244 shared OTUs among the three groups, wherein the OTUs number of German Shepherd group (2,135) was the highest, and then the OTUs number of Labrador retriever group (1,425) was much higher than that of the Springer Spaniel group (398). The vertical axis of the rank–abundance curves showed the percent of OTUs after sampling, while the horizontal axis showed the number of sequences, and the tail length of rank–abundance curves revealed the bacterial community richness.

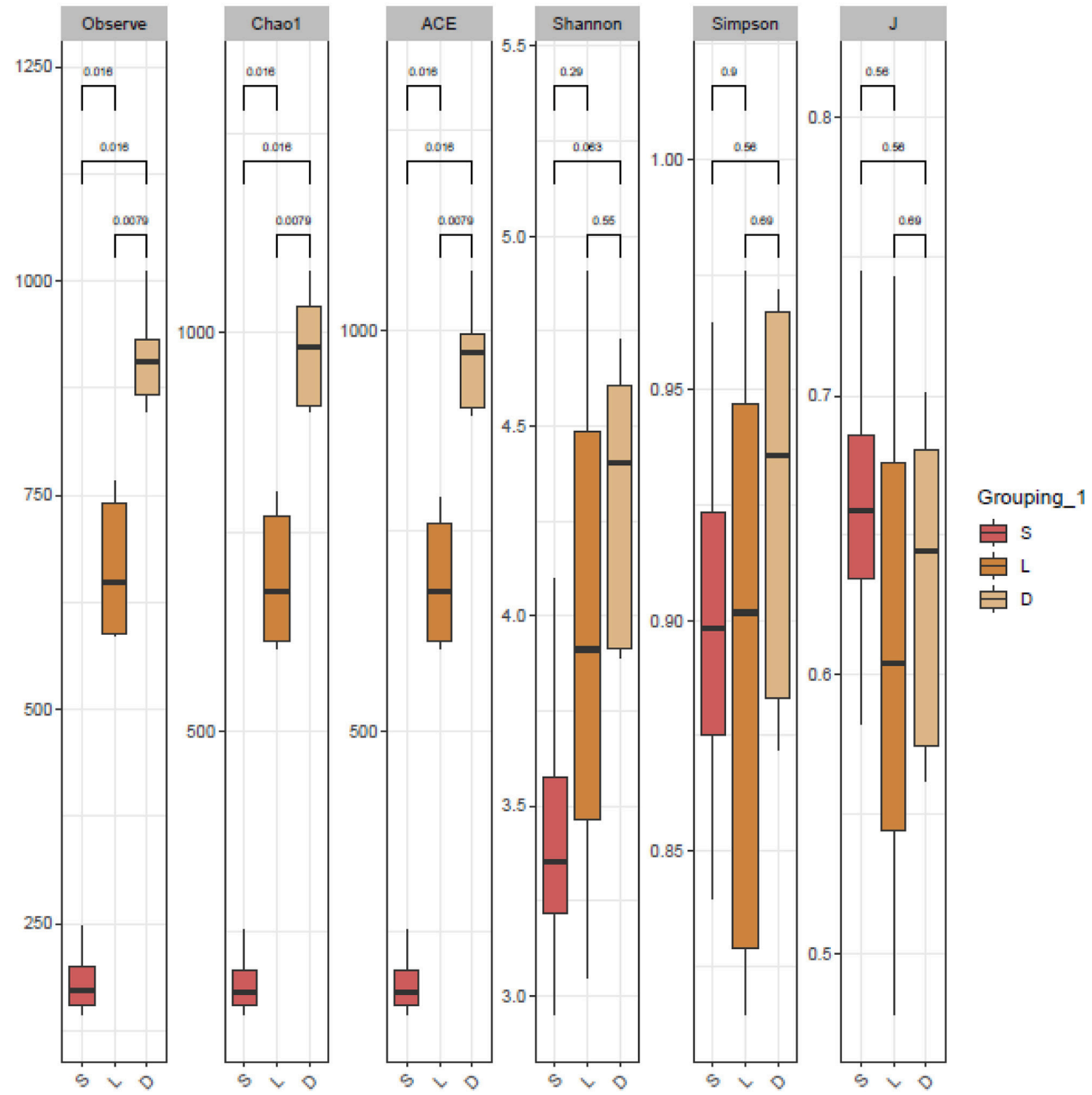

FIGURE 3 | Alpha diversity analysis of the dog gut microbial community. The richness and diversity estimators of observe, Chao1, ACE, Shannon, Simpson, and J indices were calculated, respectively. The observe, ACE, and Chao1 indices indicated that the bacterial richness of German Shepherd group was much higher than the other two groups, and Shannon and Simpson indices revealed that bacterial community diversity of German Shepherd was the highest.

much higher than those of the Labrador Retriever group (1,425) and the Springer Spaniel group (398). The comparison results of OTUs number indicated that the gut microbial diversity of German Shepherd dogs was much higher than the Labrador Retriever dogs and the Springer Spaniel dogs. The tail lengths of rank–abundance curves at the horizontal axis showed the bacterial community richness of the German Shepherd group was much higher than those of the Labrador Retriever group and the Springer Spaniel group (**Figure 2B**).

Diversity Analysis of Gut Microbial Communities

The clustered OTUs were analyzed using the RDP Classifier against the SILVA database with a confidence threshold of 70%. The alpha diversity analysis of the gut microbial community was evaluated by the observe, Chao1, ACE, Shannon, Simpson, and J indices (**Figure 3**). The calculated observe, ACE, and Chao1 indices indicated that the bacterial richness of the German Shepherd group was significantly higher than the Labrador

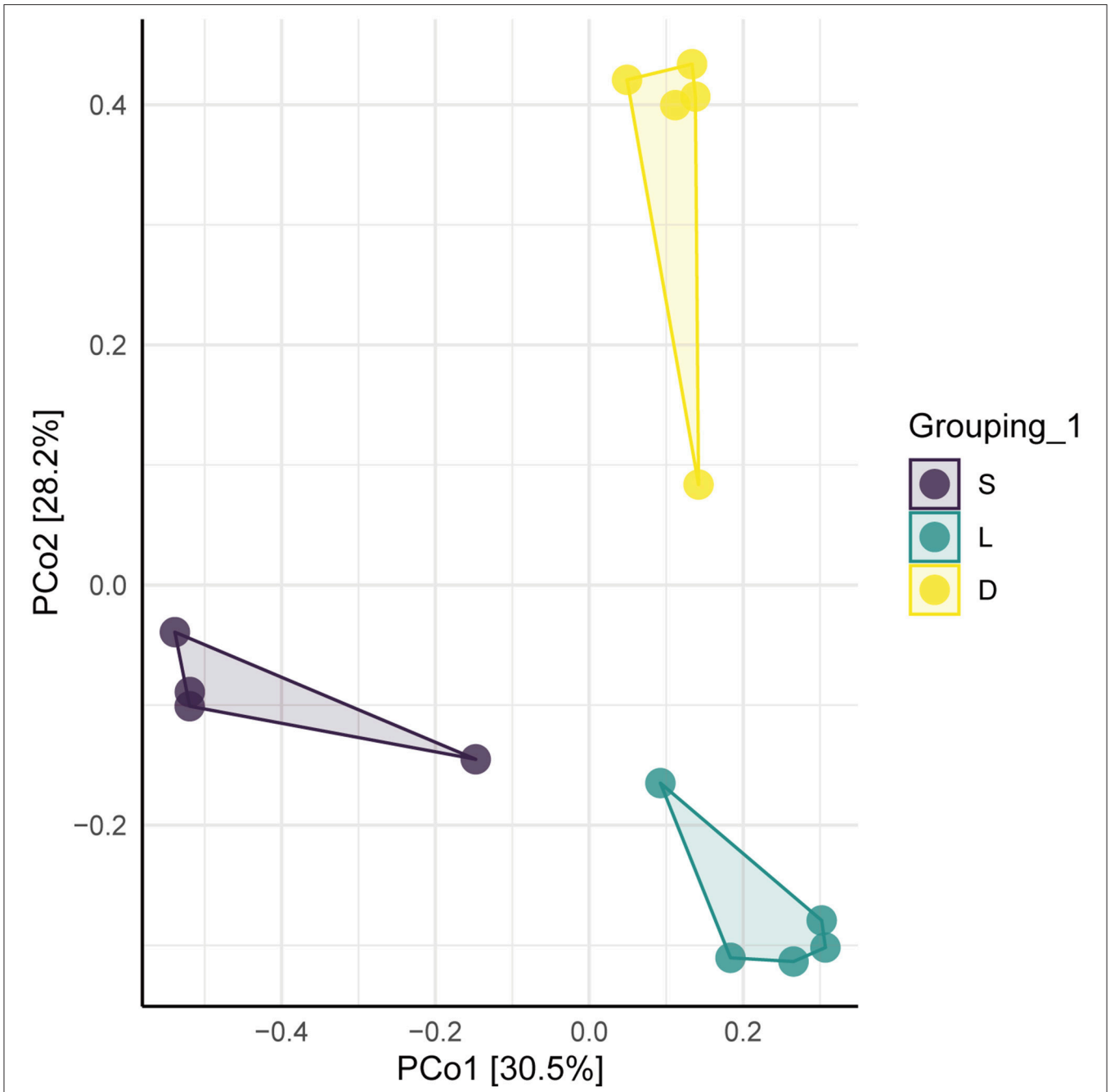

FIGURE 4 | Plot of PcoA of the dog gut microbial community. The three different groups were segregated into different community clusters.

Retriever group and the Springer Spaniel group. At the same time, the Shannon and Simpson indices revealed that the gut bacterial diversity of the German Shepherd group was much higher than the other two groups. The beta diversity analysis was estimated by the PCoA based on the unweighted UniFrac distance, and the clustering results demonstrated that the three different groups were segregated into different clusters (**Figure 4**).

Microbial Compositions of the Gut Microbiota

The taxonomic composition of the detection dogs' gut microbial communities was analyzed at the phylum level and the genus level, respectively (**Figures 5A,B**). Across all the sequenced samples, the most predominant microbial communities were showed in **Table 4**. However, the microbial communities of the three groups at the phylum level differed apparently.

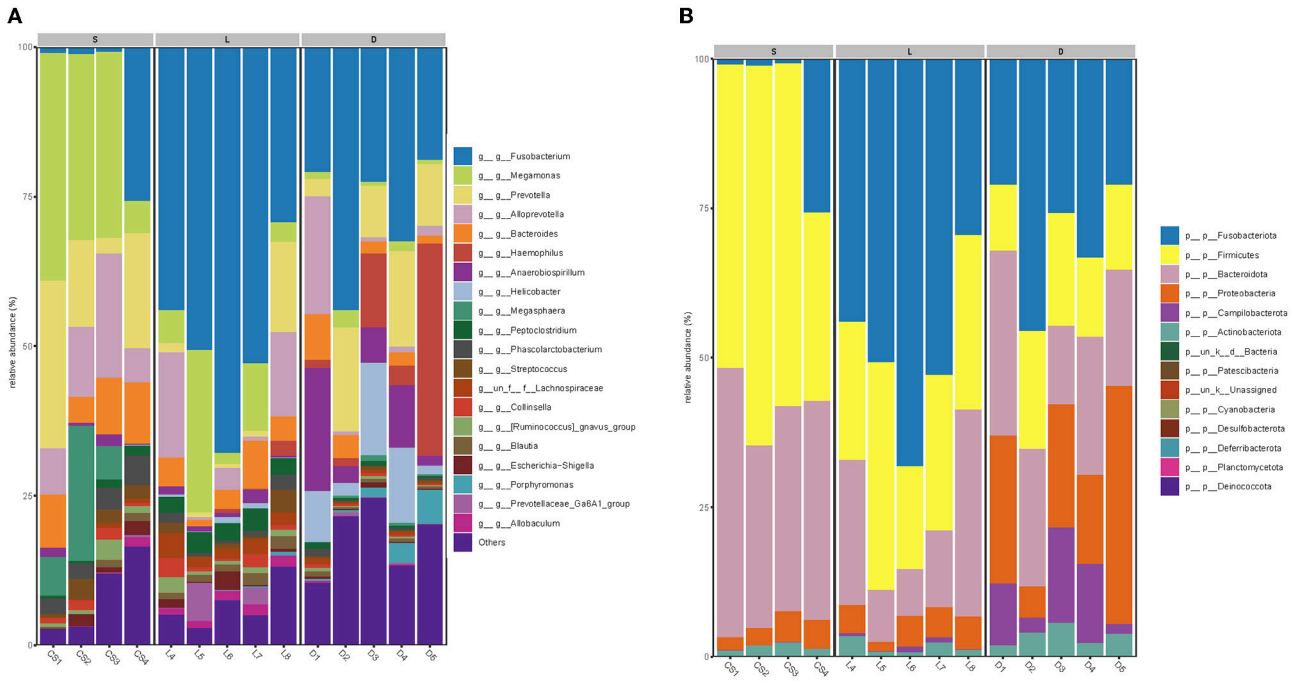

FIGURE 5 | The compositions of dog gut bacterial community at the phylum **(A)** and genus **(B)** levels. Less than 1% abundance of bacterial taxa at the phyla or genus levels was merged into others, and the microbial communities of the three groups differed obviously.

TABLE 4 | The predominant taxonomic profiles of the dog gut microbiota.

Sample group	Taxonomic level	Phyla	Relative abundance (%)
	Phylum	Fusobacteriota	29.81
		Firmicutes	29.78
		Bacteroidetes	24.54
		Proteobacteria	10.17
		Campilobacterota	3.29
		Actinobacteriota	2.27
	Genus	*Fusobacterium*	29.19
		Megamonas	11.53
		Prevotella	9.85
		Alloprevotella	7.54
		Bacteroides	5.09
		Haemophilus	4.04
		Anaerobiospirillum	3.63
		Helicobacter	3.01
		Megasphaera	2.65
		Peptoclostridium	1.63
		Phascolarctobacterium	1.57
		Streptococcus	1.22

The most predominant gut microbial communities at the phylum level were comprised of Fusobacteriota (29.81%), Bacteroidetes (29.78%), Firmicutes (24.54%), Proteobacteria (10.17%), Campilobacterota (3.29%), and Actinobacteriota (2.27%; showed in **Figure 5A**). As showed in **Figure 5B**, the most predominant gut microbial communities at the genus level were comprised of *Fusobacterium* (29.19%), *Megamonas* (11.53%), *Prevotella* (9.85%), *Alloprevotella* (7.54%), *Bacteroides* (5.09%), *Haemophilus* (4.04%), *Anaerobiospirillum* (3.63%), *Helicobacter* (3.01%), *Megasphaera* (2.65%), *Peptoclostridium* (1.63%), *Phascolarctobacterium* (1.57%), and *Streptococcus* (1.22%). The gut microbial communities of the three group of detection dogs at the genus level were also obviously different.

Comparisons of the Gut Microbial Communities

To compare the mean percentage of the predominant genera among the three groups, the significant differences in the relative abundance of detection dogs' gut microbiota were analyzed using the Kruskal–Wallis test. The percentages of *Fusobacterium*, *Prevotella*, *Alloprevotella*, *Megamonas*, *Bacteroides*, and *Phascolarctobacterium* presented significant differences in the three groups (**Figures 6**, **7**). In detail, the percentage of *Fusobacterium* in the Labrador Retriever group (48.39%) was the highest, which was much higher than those of the German Shepherd group (27.60%) and the Springer Spaniel group (7.20%). The percentage of *Prevotella* in the Springer Spaniel group (16.06%) was the highest, which was higher than those of the German Shepherd group (10.99%) and the Labrador Retriever group (3.76%). Similarly, the percentage of *Alloprevotella* in the Springer Spaniel group (11.50%) was also the highest, which was higher than those of the Labrador Retriever group (7.23%) and the German Shepherd group (4.68%). Moreover, the percentage of *Megamonas* in the Springer

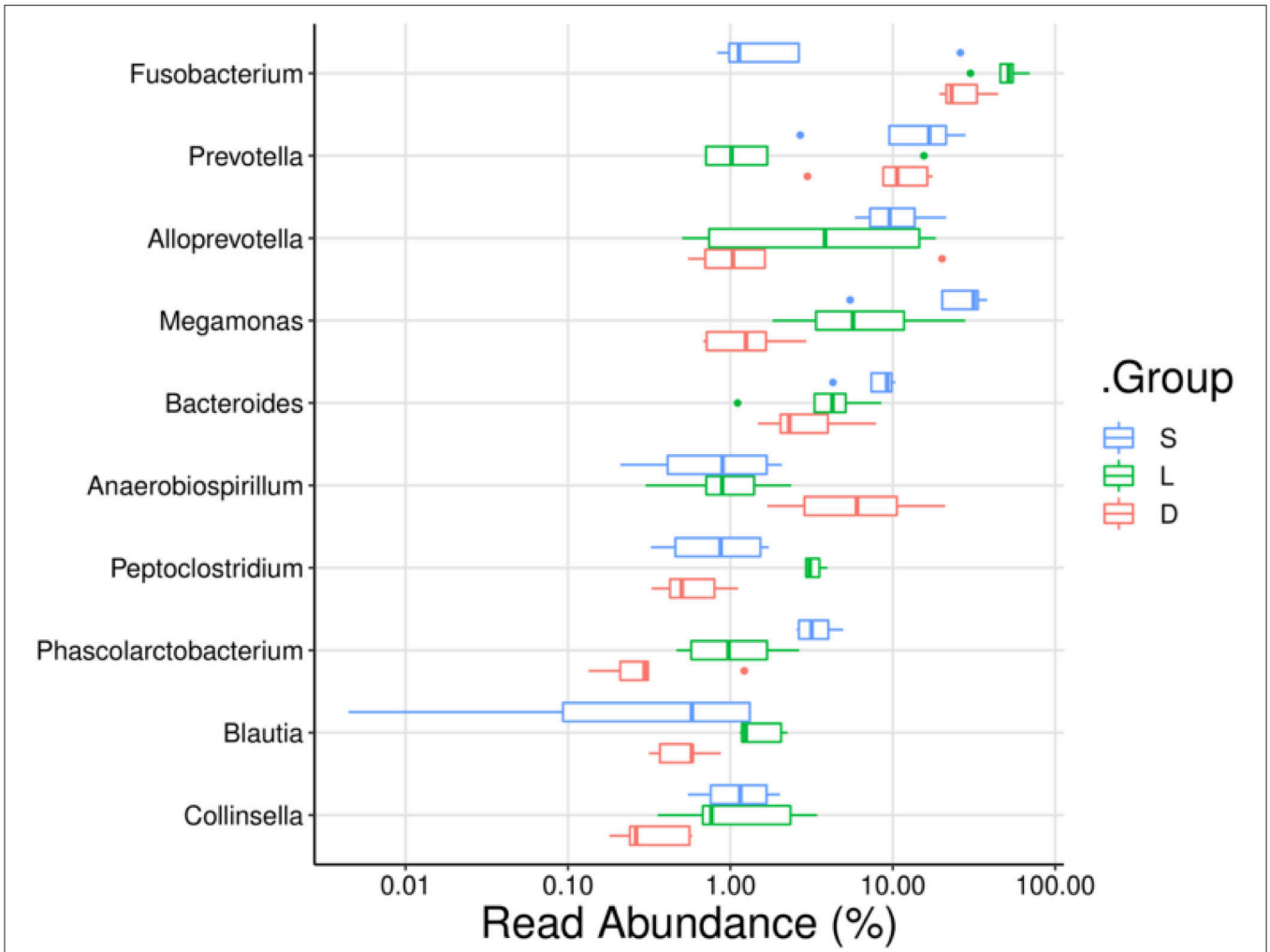

FIGURE 6 | Comparisons of the relative abundance of dog gut microbiota at the genus level. The ordinate indicated the bacterial name at genus levels, and the abscissa indicates the abundance percentage values of the samples.

Spaniel group (26.41%) was also the highest, which was higher than those of the Labrador Retriever group (9.74%) and the German Shepherd group (1.41%). The relative abundances of *Bacteroides* and *Phascolarctobacterium* in the Springer Spaniel group were also higher than the other two groups ($p < 0.01$). However, the relative abundance of *Lactobacillus* in the German Shepherd group (0.87%) was much higher than those in the Springer Spaniel group (0.61%) and the Labrador Retriever group (0.12%). Due to the reason that all these three groups of dogs lived in a similar environment and consumed the same diet, the marked differences in gut microbial communities might be associated with their unique breed types.

PICRUSt Functional Prediction

The predicted functions were calculated based on PICRUSt in EggNOG database, and a total of 22 pathways related to the dog gastrointestinal tract diseases were identified (**Figure 8**). The predicted microbial genes related to the defense mechanisms in the German Shepherd group (3,125,562) were much higher than those in the Labrador Retriever group (2,135,376) and the Springer Spaniel group (859,726), indicated that the German Shepherd dog might had a stronger immunity to fight against the gastrointestinal tract infectious diseases. Moreover, the predicted microbial genes related to the carbohydrate transport and metabolism in the German Shepherd group (8,747,138) were also much higher than those in the Labrador Retriever group (6,763,335) and the Springer Spaniel group (2,904,780), which meant that the German Shepherd dog had a stronger carbohydrate metabolic ability. Correspondingly, the relative abundances of lipid transport and metabolism, amino acid transport and metabolism, and energy production and conversion microbial genes in the German Shepherd group were also much higher than the other two groups. In all, the predicted functions of microbial genes demonstrated that the gut microbiome of the German Shepherd dog might provide more effective energy supply and stronger immune protection.

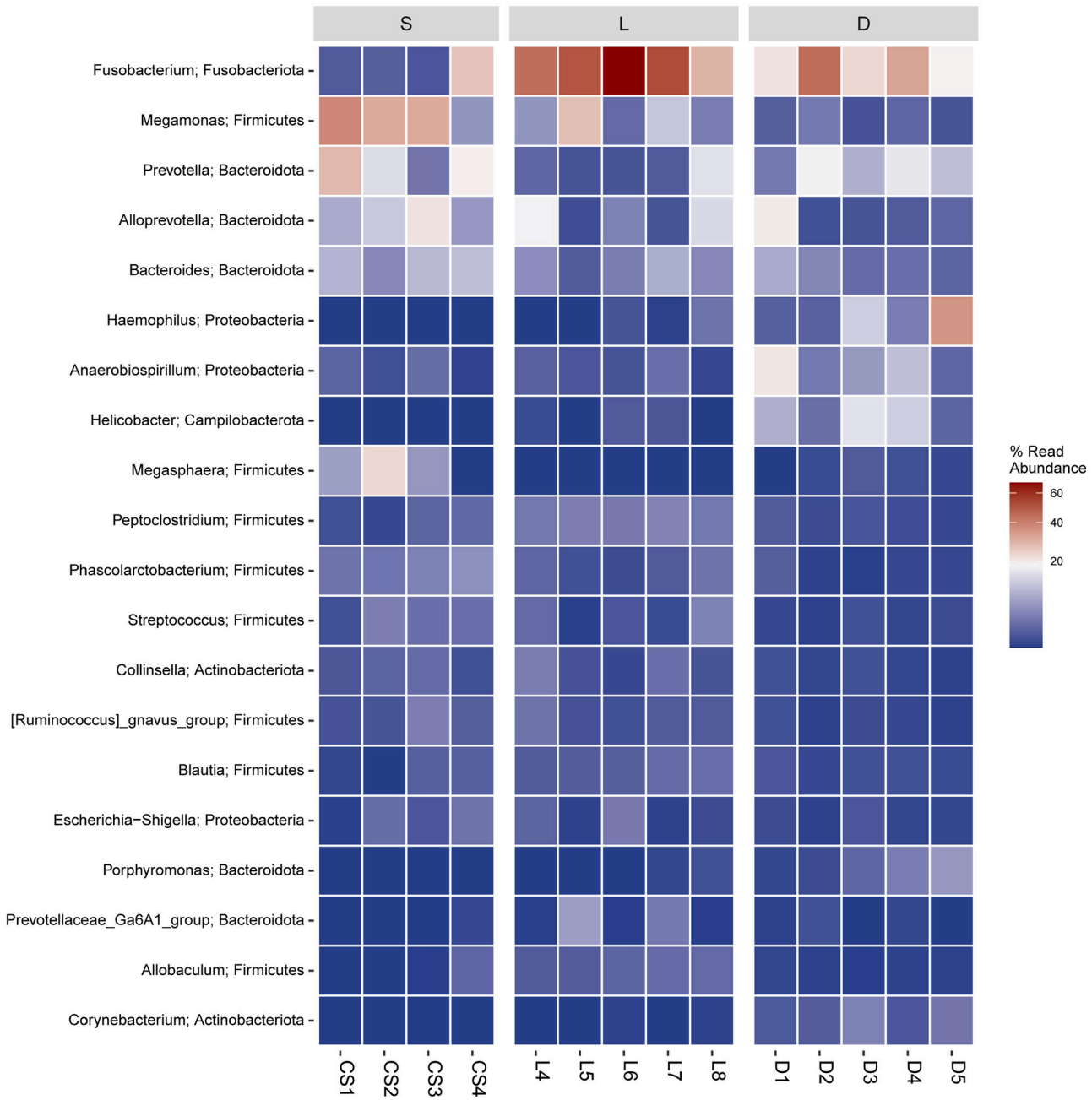

FIGURE 7 | Heatmap of hierarchy cluster results for the abundance of genus. The genus names of the OTUs are shown on the left, and the different colors of the spots indicated the normalized and log-transformed relative abundance.

DISCUSSION

During the long term of mammal animal evolutionary process, the human artificial domestication and hybridization on companion dogs had generated obvious morphology differences and behavioral responses when compared with their wild ancestors. The typical changes in the appearance characteristics (such as curly tails, floppy ears, white patches, and shorter muzzles) and behavioral profiles (such as social behaviors, cognitive abilities, and emotional responses) of domestic canines might have close relations with the physiological shifts and genetic alterations (Kaiser et al., 2015; Lesch et al., 2022). In this study, the extraordinary compositions of gut microbiota in different breed types of young detection dogs were investigated and compared, and the possible relations between their gut microbiota and working performances were also analyzed and discussed.

The gut microbial composition of detection dogs might be influenced by diet structure, living environment, exercise management, and other impacting factors. However, the

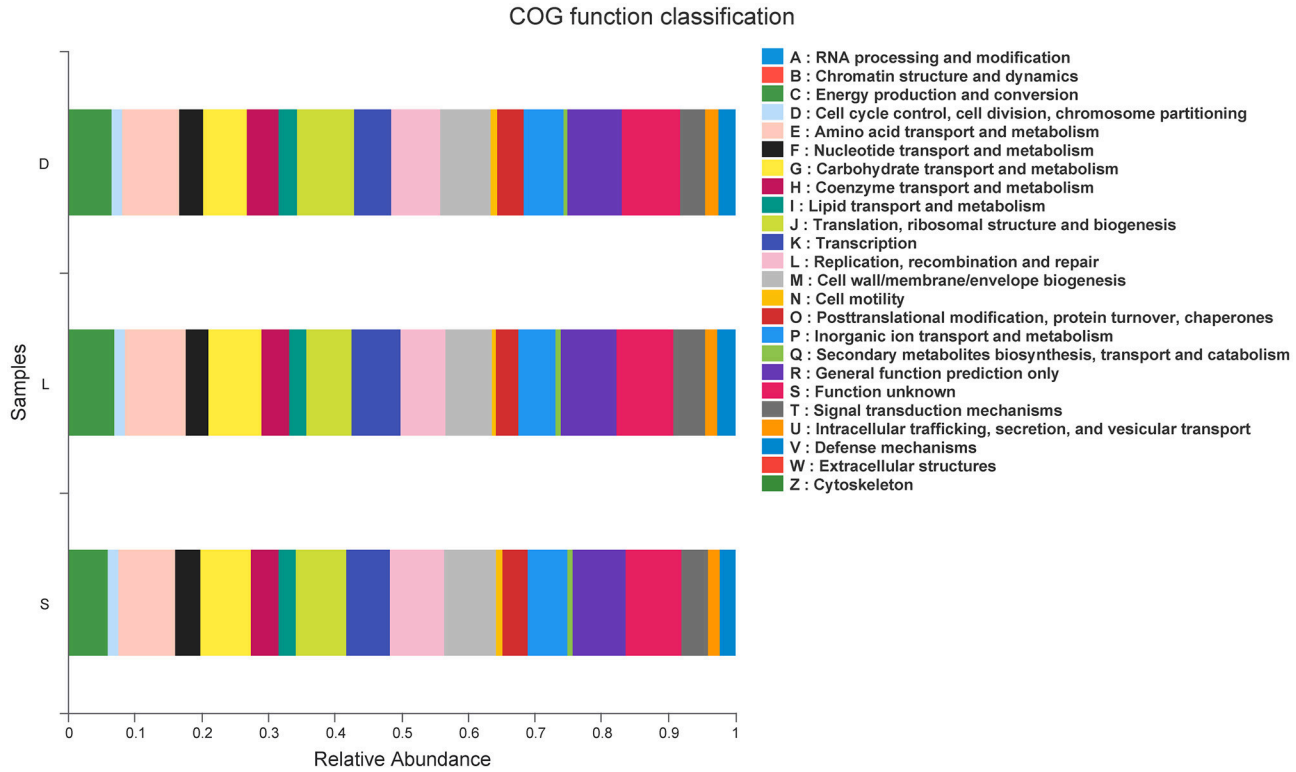

FIGURE 8 | PICRUSt functional prediction was performed using EggNOG database, and pathways related to dog gastrointestinal diseases were identified.

microbial species differed apparently upon the different canine breeds according to the results shown in this study. Venn diagrams showed that the OTUs number of German Shepherd was the highest, which meant that the gut microbial diversity of German Shepherd dogs was much higher than the Labrador Retriever dogs and the Springer Spaniel dogs (showed in **Figure 2A**). The longer rank–abundance curves tails of the German Shepherd dogs at the horizontal axis indicated the bacterial community richness of German Shepherd dogs was also much higher than the other two groups (**Figure 2B**). For the alpha diversity analysis, the calculated observe, ACE, and Chao1 indices indicated that the bacterial richness of German Shepherd dogs was the highest among the three groups, while the Shannon and Simpson indices revealed that the diversity of German Shepherd dogs' gut microbiota was also higher than the Labrador Retriever group and the Springer Spaniel group (**Figure 3**). For beta diversity analysis, the PCoA based on the unweighted UniFrac distance demonstrated that the gut microbes of the three groups were clustered into different communities (**Figure 4**). Therefore, the current research data revealed that the richness and diversity of gut microbiota in different breeds of detection dogs differed obviously, which suggested that the genetic portrait might be a major determining factor on the gut microbiota.

According to the previous studies, the gut microbiome played an important role in maintaining the host health state, for the reason that the gut microbiome could educate the immune system, regulate the energy metabolism, and fight against the invading pathogens (Mondo et al., 2019; Pilla and Suchodolski, 2020). Therefore, the multiple physiological functions of certain members in the gut microbiome are worthy to be further studied. In the present study, the taxonomic compositions of the gut microbial communities were separately analyzed at the phylum level and the genus level (**Figures 5A,B**). At the genus level, the members of *Phascolarctobacterium*, *Blautia*, *Ruminococcus*, and *Coprococcus* were identified in the gastrointestinal tract of detection dogs. These four genera are well-known for the abilities of fermenting carbohydrate to produce short-chain fatty acids (SCFAs), which can help the host to maintain the immune homeostasis and regulate the energy metabolism (Zhang J. et al., 2015). Results also demonstrated that the gut microbial communities of the three group of detection dogs were obviously different, and then the most predominant gut microbial taxa were investigated and compared. The mean relative abundance of *Fusobacterium*, *Prevotella*, *Alloprevotella*, *Megamonas*, *Bacteroides*, and *Phascolarctobacterium* presented significant differences in the three groups (**Figures 6**, **7**). In detail, the percentage of *Fusobacterium* in the Labrador Retriever group was much higher than those of the German Shepherd group and the Springer Spaniel group. However, the percentage of *Prevotella*, *Alloprevotella*, *Megamonas*, *Bacteroides*, and *Phascolarctobacterium* in the Springer Spaniel group was higher than those of the German Shepherd group and the Labrador. Interestingly, the relative abundance of *Lactobacillus* in the German Shepherd group (0.87%) was found to be

much higher than the Springer Spaniel group (0.61%) and the Labrador Retriever group (0.12%). The *Lactobacillus* might generate multiple kinds of beneficial metabolites to protect the gastrointestinal tract function (Hang et al., 2013). Therefore, the remarkable differences of the gut microbiome in the three groups might be associated with their corresponding genetic backgrounds, and the characteristics of the gut microbiome might reveal the breed portraits of the detection dogs.

The canine gastrointestinal microbes had important roles for the nutritional, immunological, and physiologic functions, while the microbiome dysbiosis caused by various reasons could induce canine chronic diarrhea and inflammatory bowel diseases (Hooda et al., 2012; Omori et al., 2017). The predicted PICRUSt functions *via* EggNOG database revealed the immune regulating roles of the gut microbiome. The predicted microbial genes related to the defense mechanisms, carbohydrate transport and metabolism, lipid transport and metabolism, amino acid transport and metabolism, and energy production and conversion in the German Shepherd group were found to be higher than the Labrador Retriever group and the Springer Spaniel group, which meant that the gut microbiome of the German Shepherd dog could provide more effective energy supply and stronger protection against the gastrointestinal tract diseases. The most important characteristics of the detection dogs are their excellent capabilities to sense different kinds of smells, and their olfaction could be impacted by gut microbes through the bidirectional communications between the gastrointestinal tract and brain. However, dietary fiber, prebiotics, probiotics, and other dietary interventions could be applied to regulate the canine gut microbiome and improve the health indices (Bell et al., 2020). Therefore, novel techniques to manipulate the gastrointestinal microbiota could be explored to improve the olfactory performance of working canines.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA803605.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee for Accreditation of Laboratory Animal Care and the Guideline for the Care and Use of Laboratory Animals of Shanghai Veterinary Research Institute.

AUTHOR CONTRIBUTIONS

All authors researched data for the article, made substantial contribution to discussion of content, and wrote, reviewed and edited the manuscript before submission. All authors contributed to the article and approved the submitted version.

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Developmental Profiling of Dietary Carbohydrate Digestion in Piglets

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Carbohydrates are the main source of energy in the diet, accounting for the largest proportion in the diets of humans and monogastric animals. Although recent progress has been made in the study of intestinal carbohydrate digestion in piglets, there is a lack of comprehensive study on the dynamic changes in intestinal carbohydrate digestion with age in the early growth stage of piglets. To fill in this gap of knowledge, we collected samples of the small intestine, pancreatic tissues, and colonic digesta from 42 piglets during newborn [day (d) 0], lactation (d 7, 14), weaning (d 21), and nursery (d 28, 35, and 42) stages. Intestinal and pancreatic tissues and colonic digesta were collected at necropsy and analyzed for morphology, digestive enzyme activities, short-chain fatty acids (SCFA), and microbial abundance. Villus height reached a maximum at 1 week (d 7) in the duodenum and jejunum ($P < 0.01$), and a higher ratio of villus height to crypt depth and lactase activity were observed on d 0 and 7 ($P < 0.001$) compared to other ages. However, the sucrase and maltase activities were increased with piglets' age. Similar activities of sucrase and maltase were found in the small intestine. In addition, amylase, lipase, and protease activities were assayed in the pancreas. The activity of amylase increased with age, while lipase and protease decreased gradually from birth to weaning (d 21, 28) and then increased after weaning (d 35, 42). Compared with d 0, d 42 increased the abundance of *Firmicutes* and *Bacteroidetes* with a higher concentration of total SCFA ($P < 0.001$) and decreased the abundance of *Proteobacteria*, but weaning (d 21, 28) increased the abundance of *Proteobacteria* in the colon. These results indicate that with the increase in piglet age, the carbohydrate digestive function gradually increased, but weaning hindered the development of intestinal function. These results provide us with new insights into the healthy development of piglets' intestines, which may help us to better regulate the physiological health of piglets in the future.

Keywords: carbohydrate, microbiome, birth, weaning, piglet

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INTRODUCTION

In recent years, the impact of dietary carbohydrates on health has become one of the main focuses in the field of public health. As one of the three essential nutrients, carbohydrates provide about 60% of energy in the Asian diet (Cui and Dibley, 2012). Therefore, it plays an important role in the nutrition supply of human or monogastric animals during lactation and nursery stages. During lactation, lactase is the main source of energy; after weaning, the starch in food is the main

source of energy (Corring et al., 1978). However, due to an immature gastrointestinal tract, young children may experience temporary malabsorption when consuming starchy foods, which may lead to disease in serious cases (Lin, 2018; Shulman, 2018). In contrast to the slow weaning process of human infants, piglets in commercial animal production experienced a huge shift from high-fat, low-carbohydrate breast milk to high-carbohydrate, and low-fat solid feed in 21–28 days of life. This change will lead to diarrhea and intestinal damage in piglets and then affect growth performance (Xiong et al., 2019). Therefore, this may bring an economic burden on the swine industry (Dou et al., 2017). Over the last decade, animal nutritionists have improved the overall health of weaned piglets by continuously optimizing feed formulations to meet the needs of weaned piglets and exploring different nutritional factors or management (Xiong et al., 2019). Studies have found that the gut of mammals is home to trillions of microbes that play a crucial role in nutrient absorption and metabolism (Hu et al., 2016). Intestinal microbial composition and ecological succession are determined by some complex internal and external factors, such as age, weaning, and diet (Kim et al., 2012; Yatsunenko et al., 2012). Because microorganisms reproduce in the host and change with age, it is very important to study the community structure of intestinal microorganisms (Green et al., 2006; Kim et al., 2012; Krajmalnik-Brown et al., 2012). In addition, the role of microbiota in health has attracted more and more attention (Ramayo-Caldas et al., 2016). Miniature piglets are similar to humans in physiological, anatomical, and endocrine systems, especially in infancy. Therefore, it is of great significance to study the changes in intestinal digestive enzymes and microbiota in miniature weaned piglets (Hu et al., 2016). Few studies have examined changes in intestinal digestive enzymes and microbes in piglets at different time periods, and there are currently no longitudinal studies that follow the correlation between digestive enzymes and microbes in piglets from birth to nursery.

In this study, we followed the changes in the digestive function, microbial communities, and the correlation between intestinal microorganisms and digestive enzymes and microbial metabolites in piglets from birth through weaning, up to 6 weeks of age. More importantly, this experiment covered the effects of diet, weaning, and age on the health of piglets. It is hoped that the results of this experiment will contribute to a deeper understanding of the changes in the digestive physiology of piglets and pave the way for the formulation of better nutritional strategies to promote the overall health of infancy and piglets.

MATERIALS AND METHODS

Ethics Statement

The experimental protocols used in the present study were approved by the Sichuan Agricultural University Institutional Animal Care and Use Committee No. 69130079.

Animals and Experimental Treatments

Six multiparous sows were chosen for this study, with similar parity and health status. Upon delivery, the neonatal piglets were cohoused with sows by litter. Before weaning at 21 days of age, neonatal piglets are allowed to feed freely and, as far as we know, they do not eat sow feed. After weaning, piglets were removed from the sow and transferred to separate housing and fed *ad libitum* with the same basal diet (maize–soybean meal diet) formulated by the National Research Council (NRC, 2012) (**Table 1**). Consequently, a total of 42 piglets (Duroc × Landrace × Yorkshire) with similar body weights were studied. The piglets had no access to antibiotics.

Tissue Collection and Processing

At Days 0, 7, 14, 21, 28, 35, and 42 after birth, one piglet per group from each of the six litters was euthanized with intravenous injection of sodium pentobarbital (200 mg per kg, BW). After the slaughter, the abdominal cavity had been opened. The intestinal segments of the distal duodenum, mid-jejunum, and ileum were flushed gently with ice-cold phosphate-buffered saline (PBS) and then fixed in 4% formaldehyde-phosphate buffer for intestinal histology. Tissue samples of the small intestine, pancreatic, and colonic contents were immediately frozen in liquid nitrogen and then stored at −80°C for further analysis.

Intestinal Morphology

Intestinal morphology was measured using standard procedures (Pluske et al., 1996). In brief, fixed samples (duodenum, jejunum,

TABLE 1 | Ingredients and chemical composition of experimental diets (as-fed basis).

Ingredients	Content, %	Nutrient composition[c]	Content, %
Corn (7.8% crude protein)	24.8	Digestible energy, Mcal/kg	3.54
Extruded corn (7.8% crude protein)	28.00	Crude protein	19.69
Extruded soya bean	7.00	Calcium	0.80
Soybean meal, dehulled	14.00	Available phosphorus	0.36
Fish meal (62.5% crude protein)	5.00	Lysine	1.35
Whey powder	9.00	Methionine	0.39
Soy protein concentrate	5.00	Methionine + Cysteine	0.70
Soybean oil	3.00	Threonine	0.81
Glucose	2.00	Tryptophan	0.23
NaCl	0.35		
Limestone	0.93		
Dicalcium phosphate	0.26		
L-Lysine–HCl (78%)	0.21		
DL-Methionine	0.05		
Chloride choline	0.15		
Vitamin premix[a]	0.05		
Mineral premix[b]	0.20		
Total	100		

[a] *The vitamin premix provided the following per kg of diets: 6,000 IU vitamin (V) A, 3,000 IU VD3, 24 mg VE, 3 mg VK3, 1.5 mg VB1, 6 mg VB2, 3 mg VB6, 0.02 mg VB12, 14 mg niacin, 15 mg pantothenic acid, 1.2 mg folic acid, and0.15 mg biotin.*

[b] *The mineral premix provided the following per kg of diets: 100 mg Fe, 10 mg Cu, 80 mg Zn, 4 mg Mn, 0.30 mg I, and 0.35 mg Se.*

[c] *Values are calculated composition.*

and ileum) were dehydrated and embedded in paraffin, sectioned (5 μm thickness), and stained with hematoxylin and eosin (H&E). The specimens were examined using an Eclipse Ci-L microscope at 40× magnification. A minimum of 10 well-oriented villi and crypts from each section were measured, and the ratio of villus height to crypt depth was calculated. The villus height and crypt depth were measured and analyzed using an Image-pro plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Digestive Enzyme Activities

The activity of brush border enzymes was determined in intestinal (duodenum, jejunum, and ileum) tissue, and trypsin, chymotrypsin, lipase, and amylase activities were determined in the pancreatic tissue. After thawing, 0.3–0.9 g of tissue was homogenized with ice-cold physiological saline and centrifuged for 10 min at 2,500 g at 4°C. The supernatant was collected for the determination of digestive enzyme activities and total protein. Total protein content was measured using the Bradford brilliant blue method (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The activities of lactase, maltase, and sucrase were measured by kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Except for lipase and chymotrypsin, enzymatic activity was expressed as nanomoles of substrate hydrolysis per minute per gram of protein (U/g protein). For other enzymes, the enzymatic activity was expressed as nanomoles of substrate hydrolyzed per minute per mg protein (U/mg protein).

Short-Chain Fatty Acids

The SCFA concentrations in the colonic digesta were analyzed according to the method described by Porter and Murray (2001). In brief, 0.7 g of colonic digesta was put into a 2 ml centrifuge tube with 1.5-ml distilled water, then incubated on ice for 30 min, and mixed and centrifuged (15,000 rpm) at 4°C for 15 min. The supernatant (1 ml) was transferred into centrifuge tubes (2 ml) and mixed with 0.2 ml of metaphosphoric acid and 23.3 μl of crotonic acid. After 30 min at 4°C, the tubes were centrifuged (15,000 rpm) again at 4°C for 10 min. Then, 300 μl supernatant was transferred to another sterile tube, mixed with 900 μl methanol, and homogenized. After this, the mixture was centrifuged (1,000 rpm) at 4°C for 5 min. The supernatant was obtained and then filtered using a 0.22-μm nylon membrane filter (Millipore, Bedford, OH, USA). Finally, aliquots of the supernatant (1 μl) were injected into a gas chromatographic system (VARIAN CP-3800, Varian, Palo Alto, CA, America) to separate and quantify the SCFA.

DNA Extraction and Illumina MiSeq

Total genomic DNA from the individual samples of colonic digesta was extracted using an E.Z.N.A Stool DNA Kit (Omega Bio-Tek, Doraville, GA) according to the manufacturer's instructions. The V3–V4 hypervariable regions of 16S rRNA were PCR-amplified from microbial genome DNA which was harvested from colonic digesta samples with the forward primer 341F (CCTACGGGNGGCWGCAG) and reverse primer 806R (GACTACHVGGGTATCTAATCC). The amplification mix contained 2 × Hieff® Robust PCR Master Mix (dNTP and Mg^{2+}) (Yeasen Co. Ltd., Shanghai, China), 1 μl of each primer, 10–20 ng of PCR products, and 9–12 μl H_2O in a reaction volume of 30 μl. The PCR program initially started with 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 54°C for 20 s, and 65°C for 30 s, and then followed by a single final extension step at 72°C for 10 min. The PCR reaction system which was used to add a specific tag sequence was 30 μl, containing 2 × Hieff® Robust PCR Master Mix (dNTP and Mg^{2+}) (Yeasen Co. Ltd., Shanghai, China), 1 μl of each primer, 20–30 ng of PCR products, and 9–12 μl of H_2O. The PCR conditions were 95°C for 3 min, followed by five cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 30 s, and then followed by a single final extension step at 72°C for 5 min. PCR product was excised from a 2% agarose gel, purified by Hieff NGS™ DNA Selection Beads (Yeasen Co. Ltd., Shanghai, China), and quantified by a Qubit 3.0 fluorometer (Invitrogen, USA). Library construction and Illumina MiSeq sequencing were carried out in Sangon Biotech (Shanghai) Co., Ltd. The information on DNA sequences was analyzed by QIIME software (Caporaso et al., 2010).

Statistical Analysis

The data in the present study were analyzed by IBM SPSS 23.0 (Chicago, IL, USA) and expressed as means ± SEM. All parameters were assessed using each slaughtered piglet as an experimental unit. The data were evaluated by one-way ANOVA with Duncan's *post-hoc* test. A value of $P < 0.05$ was used to indicate statistical significance, whereas a *P*-value between 0.05 and 0.10 was considered to indicate a trend toward significance.

RESULTS

Small Intestinal Morphology

The small intestinal morphology of the piglets is shown in **Table 2** and **Figure 1**. The results showed that in the duodenum, villus height, and villus/crypt ratio increased from birth to lactation, decreased during weaning, and then began to recover at 35–42 d ($P < 0.001$). In the jejunum, crypt depth increased at each step from birth to nursery with increasing piglet age ($P < 0.001$), while in the ileum villus height gradually decreased from birth to weaning and began to recover at 35–42 d ($P = 0.008$).

Digestive Enzyme Activity in the Intestine and Pancreas

The effect of age on digestive enzyme activity in the small intestine and pancreas is shown in **Figures 2**, **3** (**Supplementary Tables S1, S2**). Compared with lactation and weaning, piglets in the nursery period (35–42 d) showed higher sucrase and maltase activities, and there was no significant difference between lactation and weaning. However, the activity of lactase was the highest at birth and gradually decreased with the increase in age, especially during weaning ($P < 0.001$).

Compared with lactation and weaning, piglets at birth had higher activities of trypsin ($P = 0.018$) and chymotrypsin ($P = 0.010$) in the pancreas. The activity of pancreatic lipase in piglets was low either during lactation or weaning and increased with

TABLE 2 | Effect of piglet age on intestinal morphology.

Item	0d	7d	14d	21d	28d	35d	42d	Pooled SEM	*P*-value
Duodenum									
Villus height, μm	319.93^{bc}	429.47^{a}	465.88^{a}	339.88^{b}	200.13^{d}	255.38^{cd}	275.16^{bc}	15.96	<0.001
Crypt depth, μm	103.99	124.17	120.48	143.77	123.55	117.51	129.91	3.61	0.109
Villus height/crypt depth	3.12^{bc}	3.52^{ab}	3.89^{a}	2.48^{cd}	1.61^{e}	2.22^{de}	2.01^{de}	0.15	<0.001
Jejunum									
Villus height, μm	472.73^{ab}	533.51^{a}	475.65^{ab}	353.74^{bc}	269.97^{c}	317.40^{bc}	347.03^{bc}	22.88	0.007
Crypt depth, μm	80.20^{d}	100.85^{cd}	105.37^{bc}	125.04^{ab}	136.10^{ab}	149.79^{ab}	153.37^{a}	6.47	0.010
Villus height/crypt depth	6.03^{a}	5.38^{ab}	4.84^{ab}	3.45^{bc}	2.05^{c}	2.23^{c}	2.44^{c}	0.34	<0.001
Ileum									
Villus height, μm	447.32^{a}	349.62^{ab}	302.02^{b}	273.03^{b}	251.63^{b}	340.35^{ab}	360.54^{ab}	15.37	0.008
Crypt depth, μm	81.25^{b}	105.72^{ab}	109.00^{ab}	113.86^{ab}	138.46^{a}	116.55^{a}	118.51^{a}	4.48	0.037
Villus height/crypt depth	5.74^{a}	3.34^{b}	3.10^{b}	2.72^{b}	1.83 ^{b}	3.01^{b}	3.14^{b}	0.26	0.001

Mean values with their standard errors.
^{a,b,c,d,e}Mean values within a row with different superscript letters were significantly different ($P < 0.05$).

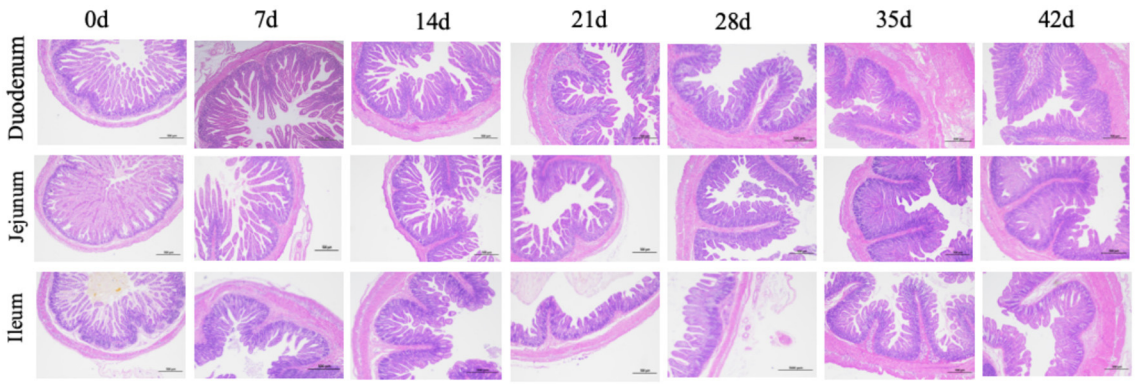

FIGURE 1 | Histological evaluation of small intestine tissue in piglets with increasing age.

the age after weaning ($P < 0.001$). As for amylase, the activity increased with the piglet age, especially in lactation ($P < 0.001$).

The Concentration of Short-Chain Fatty Acids in Colonic Digesta

Table 3 shows the concentration of SCFA determined in the colonic digesta. At 42 d, we found significantly higher concentrations of acetate, propionate, butyrate, and total SCFA than those during lactation and weaning ($P < 0.001$). However, the concentration of SCFA was low at birth and weaning. In general, the concentration of SCFA increased with the age of piglets from birth to nursery ($P < 0.001$), but decreased during weaning and began to recover after weaning.

Bacterial Composition and Diversity

In this study, an average of 59,433 clean tags was obtained for each group, and the length of the sequences ranged between 415 and 426 bp. α-Diversity analysis showed a sharp contrast among newborn, lactating, and nursery piglets ($P < 0.001$, **Figure 4**), and when examined over time, a gradual increase in the α-diversity (phylogenetic distance, PD) from 0 to 21 d is observed. This trend stabilized after 21 d until other time points in the study, but the differences between litters were not significant ($P > 0.05$). Furthermore, 3 weeks after weaning (28–42 d), the colonic digesta microbiota appeared to be more diverse and had greater evenness than that of lactation and weaning, according to the Shannon index ($P = 0.009$, **Figure 5A**) and observed OTUs ($P < 0.001$, **Figure 5B**). This trend stabilized after 28 d to other time points studied, but differences between litters were not significant ($P > 0.05$).

On the contrary, bacterial community composition was significantly different between animals when measured by analysis of similarities (ANOSIM) of unweighted UniFrac distance ($P < 0.001$). The unweighted UniFrac principal coordinate analysis (PCoA) plot (**Figure 5C**) visually confirmed the distinct separation of microbial communities among different ages of piglets. The first group of samples in the upper left quadrant were from 0-d piglets, which were more dispersed than the other age groups. The second cluster in the top-right quadrant of the PCoA chart consists of a sample of 42-d piglets. The third cluster in the middle of the four quadrants comprises samples from 7- to 35-d piglets clustered together but with

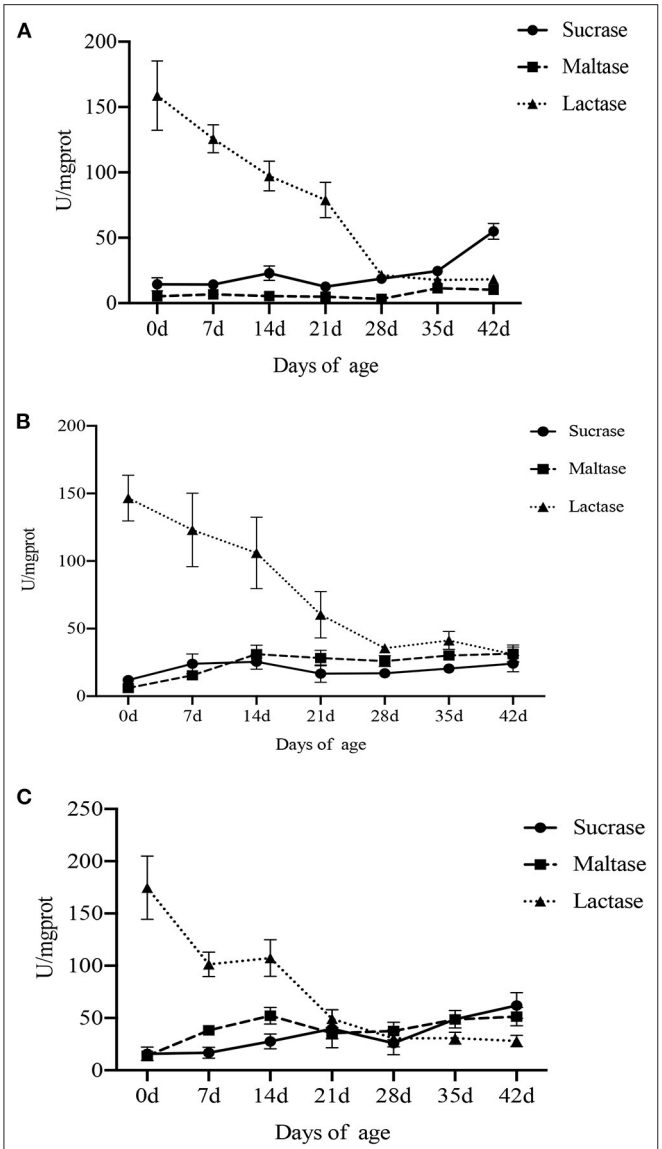

FIGURE 2 | Effect of piglet age on intestinal disaccharidase activity. **(A)** Duodenum. **(B)** Jejunum. **(C)** Ileum. Values are expressed as means ± SEM.

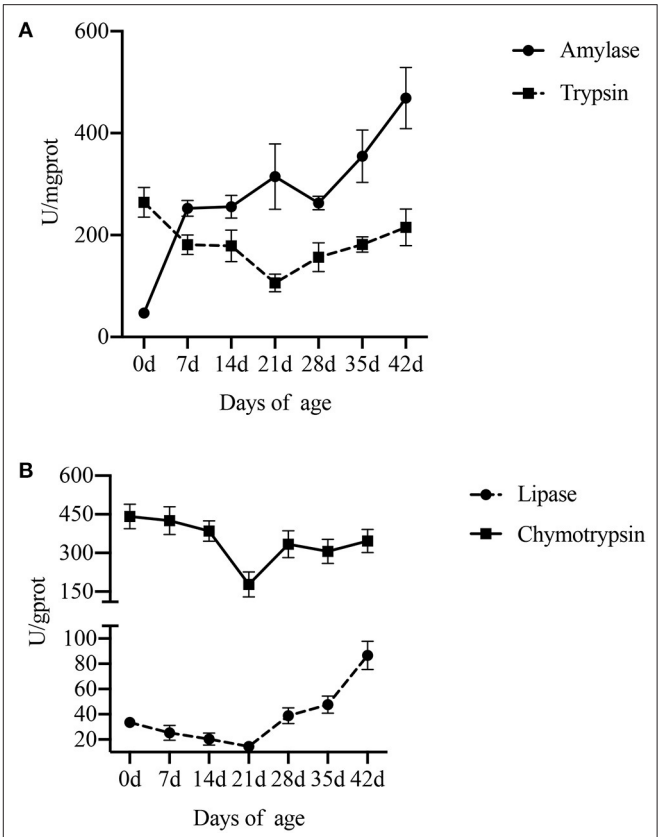

FIGURE 3 | Effect of piglet age on pancreatic digestive enzyme activity. **(A)** Amylase and trypsin. **(B)** Lipase and chymotrypsin. Values are expressed as means ± SEM.

a relatively low degree of aggregation. Overall, these results suggested that the β-diversity of the colonic digesta microbiota of piglets increased with age.

The relative abundance of intestinal microflora at phylum, family, and genus levels of piglets at different ages is shown in **Figure 6**. At the phylum level, the seven growth stage groups are mainly composed of seven phyla as follows: *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Fusobacteria*, *Actinobacteria*, *Verrucomicrobia,* and *Spirochaetes*. *Firmicutes* ($P = 0.001$), *Proteobacteria* ($P < 0.001$), and *Bacteroidetes* ($P = 0.003$) were the most dominant among the seven phyla in the samples, regardless of age, and comprised more than 85% of the total sequences. The bacterial abundances of distinct phyla differed in the seven groups. *Firmicutes* ($P = 0.001$) was the most predominant phylum after birth (7–42 d), accounting for more than 46% of the sequences, while *Proteobacteria* ($P < 0.001$) was the main phylum of newborn piglets (0 d), accounting for more than 78% of the sequence. At 42 d, a higher percentage (83%) of the sequences was assigned to *Firmicutes*, but there was no significant difference from 7 to 35 d. *Bacteroidetes* and *Proteobacteria* were the second largest phylum at 7 d (33%), 14 d (22%), 35 d (20%), 42 d (10%), 0 d (78%), 21 d (26%), and 28 d (17%), respectively (**Figure 6A**).

Surprisingly, at the family level, *Enterobacteriaceae* of newborn piglets was the most abundant bacterial family, accounting for ∼70% of the sequences. The abundance of *Enterobacteriaceae* decreased gradually during lactation, increased after weaning, and then decreased gradually from 28 to 42 d, but the change in *Lactobacillaceae* was the opposite. In addition, weaning increased the abundance of *Ruminococcaceae* compared with lactation and post-weaning. When comparing the microbiota during lactation and after weaning, there were significant differences. First, *Prevotellaceae* decreased nearly 4-fold from an average of 4.7% during lactation to 1.3% during weaning. This coincided with a decrease in the population of *Bacteroidaceae* and *Fusobacteriaceae* from 6.4 and 5.0% during lactation to 2.1 and 1.5% during weaning, respectively (on

TABLE 3 | Effect of piglet age on the yield of short-chain fatty acids (μmol/g of wet digesta) in colonic digesta.

Item	0d	7d	14d	21d	28d	35d	42d	Pooled SEM	*P*-value
Acetate	2.15^{d}	14.42^{c}	17.52^{c}	13.10^{c}	18.78^{bc}	24.42^{b}	36.64^{a}	1.76	<0.001
Propionate	1.20^{d}	4.07^{cd}	7.00^{bc}	3.52^{cd}	6.67^{bc}	9.42^{b}	17.25^{a}	0.89	<0.001
Butyrate	0.48^{c}	2.08^{bc}	3.28^{b}	1.66^{bc}	1.37^{bc}	3.10^{b}	7.69^{a}	0.45	<0.001
Isobutyrate	0.26^{c}	0.55^{c}	0.72^{bc}	0.48^{c}	0.57^{bc}	1.07^{ab}	1.46^{a}	0.08	<0.001
Isovalerate	0.38^{c}	0.73^{bc}	1.05^{bc}	0.71^{bc}	0.66^{bc}	1.23^{bc}	1.99^{a}	0.11	<0.001
Valerate	0.24^{b}	1.11^{ab}	0.73^{ab}	0.46^{b}	0.34^{b}	0.51^{b}	1.79^{a}	0.15	0.048
Total short-chainfatty acids	3.80^{d}	22.96^{c}	30.30^{bc}	19.94^{c}	28.39^{bc}	39.74^{b}	66.82^{a}	3.24	<0.001

Mean values with their standard errors.
[a,b,c,d]Mean values within a row with different superscript letters were significantly different ($P < 0.05$).

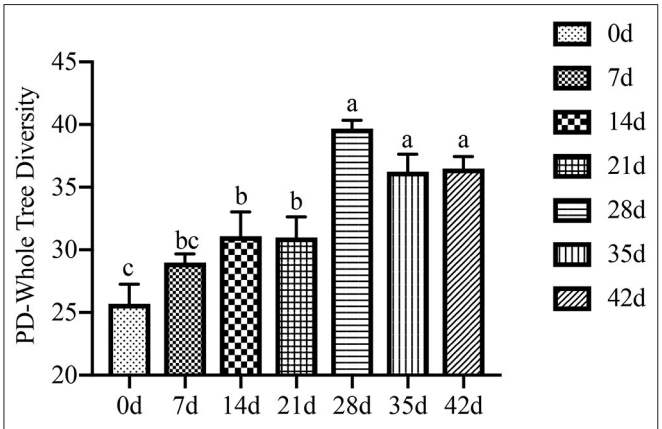

FIGURE 4 | Effect of piglet age on colonic chyme α-diversity (phylogenetic distance, PD). Values are means with standard errors represented by vertical bars. a, b, c Mean values with unlike letters were significantly different within a cluster of bars, not across the clusters of bars ($P < 0.05$).

average). There is also an increase in *Streptococcaceae* from 0.2 to 2.8% after weaning. *Lactobacillaceae* increased over time from 13.5 to 40.5% after weaning. In contrast, *Lachnospiraceae*, *Porphyromonadaceae,* and *Campylobacteraceae* did not change dramatically throughout the study (**Figure 6B**).

To further investigate the taxonomic compositions of piglets at different days of age, a total of 281 genera were identified from the bacterial community of colonic digesta of piglets. Among these genera identified, 13 abundant genera were detected, which contained more than 5% of the total sequence in at least one sample. The 13 abundant genera were as follows: *Lactobacillus*, *Escherichia_Shigella*, *Bacteroides*, *Fusobacterium*, *Prevotella*, *Gemmiger*, *Streptococcus*, *Roseburia*, *Akkermansia*, *unclassified_Ruminococcaceae*, *unclassified_Porphyromonadaceae*, *unclassified_Lachnospiraceae,* and *unclassified_Bacteria* (**Figure 6C**). All the 13 abundant genera plus the unclassified genera accounted for over 62% of the total sequences in the samples, regardless of the age of piglets. Genus *Escherichia_Shigella* belonged to phylum *Proteobacteria* and had the highest abundance at 0 and 21 days of age, while *Lactobacillus* belonged to phylum *Firmicutes* and had the highest abundance at 7–42 d. With the increase in piglets' age, the abundance of *Escherichia_Shigella* and *unclassified_Ruminococcaceae* decreased, but increased at weaning, while the change in *Lactobacillus* was the opposite. Similarly, the abundance of *Prevotella*, *Roseburia*, *Akkermansia*, *Pasteurella*, *Bifidobacterium,* and *Megasphaera* was higher during lactation than after weaning, while the change in *Gemmiger*, *Blautia*, *Treponema*, *Faecalibacterium*, *Clostridium_IV,* and *unclassified_Bacteria* was opposite.

Microbiota–Metabolite Correlation

The triplot of redundancy analysis (RDA) was conducted based on genus-level microorganisms and their environmental factors (amylase, SCFAs, and disaccharidase, **Figure 7A**), indicating that piglets of different ages were separated on the first constraint axis. RDA indicated that there were positive correlations among SCFAs, amylase, and sucrase and a negative correlation between lactase and SCFAs. In addition, a correlation between the top 50 microbial genera and the environmental factors was determined by calculating Spearman's correlation coefficients and was directly reflected by a heatmap (**Figure 7B**). The threshold $|R| > 0.4$ is considered relevant. The results indicated that *Lactobacillus*, *unclassified_Ruminococcaceae*, and *Faecalibacterium* were positively correlated with amylase and SCFAs, while *unclassified_Lachnospiraceae*, *unclassified_Bacteroidetes,* and *Roseburia* were positively correlated with sucrase and maltase. *Escherichia_Shigella*, *Fusobacterium,* and *Phascolarctobacterium* were positively correlated with lactase, while *unclassified_Bacteria*, *unclassified_Clostridiales*, *Gemmiger,* and *unclassified_Firmicutes* were negatively correlated with lactase. *Unclassified_Clostridiales* was positively correlated with acetate and negatively correlated with valerate. *Lactobacillus*, *unclassified_Bacteroidetes,* and *Roseburia* were positively correlated with amylase, sucrase, and maltase, while *Escherichia_Shigella* and *Bifidobacterium* were negatively correlated with them.

DISCUSSION

The first goal of this study was to better understand the changes in the digestive function of piglets from birth to post-weaning. So far, under the background that early weaning models are widely used, there are few studies on the changes in the intestinal

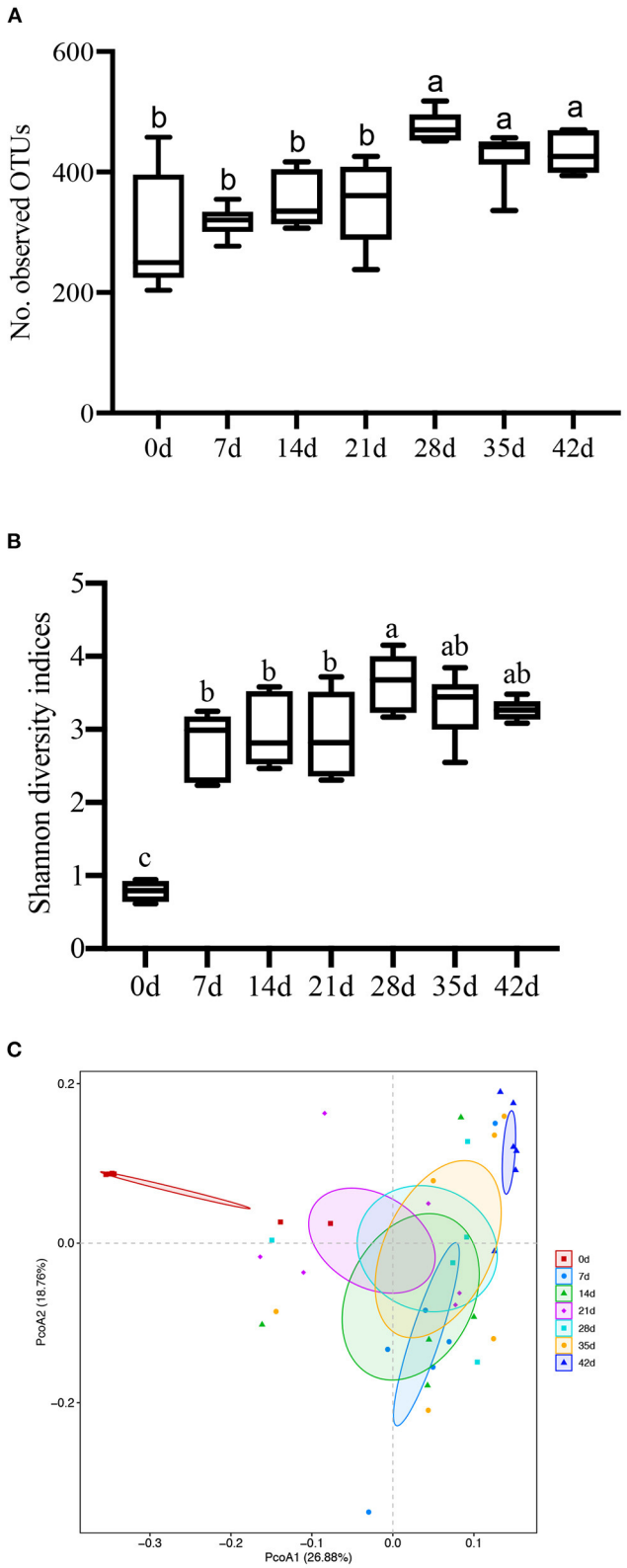

FIGURE 5 | Effect of piglet age on α- and β-diversity of microbial communities in colonic digesta. **(A)** Bacterial α-diversity determined by no. of observed OTUs. **(B)** Bacterial α-diversity determined by the Shannon index. **(C)** Scatter

(Continued)

FIGURE 5 | plot from PCoA, based on weighted UniFrac distance in bacterial communities (0, 7, 14, 21, 28, 35, and 42 days after birth). Different letters above the bars denote a significantly different α-diversity index among groups.

digestive function with age in piglets (Corring et al., 1978; Owsley et al., 1986; Hedemann et al., 2003). Therefore, there are differences between the early research and the current situation of piglets, and new research is needed to provide a theoretical basis to guide production.

The intestine is the main place for digestion and absorption of nutrients, and the early postnatal period is a key time for intestinal development. The tissue mass and absorption surface area of the small intestine of newborn piglets increased significantly, for example, the number of mucosal cells increased by 50% on the first day after birth and doubled on the third day after birth (Widdowson and Crabb, 1976; Xu et al., 1992). These studies show that the intestine develops most rapidly in the early stages of piglets. In this study, our measurement of intestinal tissue characteristics is basically consistent with the data in the literature, which confirms that the animals used in this study are healthy and normal (Skrzypek et al., 2018; Verdile et al., 2019). Research showed that the morphological structure of the duodenum will change with the growth of piglets during lactation, such as the increase in villus height, crypt depth, and intestinal wall thickness (Wiyaporn et al., 2013). For instance, the villus height of the small intestine of piglets increases by 33% in the first week after birth and reaches its maximum in the second week after birth (Skrzypek et al., 2005). Consistent with previous studies, duodenal villus height increased continuously from 0 to 14 d, peaked at 14 d, decreased at weaning (21–28 d), and then increased at 35–42 d. However, the changing trend of jejunum and ileum is inconsistent with that of the duodenum, and the maximum value appeared at 7 and 0 d, respectively. One plausible explanation for the inconsistent peak time of villus height may be that the development of the small intestine structure is basically completed and the villus height is high at birth. Therefore, through this experiment, we once again demonstrated that the structure and digestive function of small intestine of piglets began from prenatal.

At birth, the small intestine is almost complete and digestion can begin after the first intake of colostrum. The integrity of intestinal development will affect the utilization efficiency of carbohydrates because it will affect the disaccharidase activity of piglets (Tsukahara et al., 2016). Since the pancreas is an important organ involved in nutrient digestion, in order to further understand the changes in the digestive ability of piglets with the increase in age, we measured the digestive enzymes secreted by the pancreas. Our results showed that the activities of sucrase and maltase in the intestine and amylase in the pancreas increased with age during lactation, decreased during weaning, and then increased during nursery, while the activities of lactase continued to decrease with age. The activities of lipase, trypsin, and chymotrypsin decreased with age during lactation and weaning but increased during the nursery. These results are consistent with previous studies (Bellinge et al., 2005; Ito

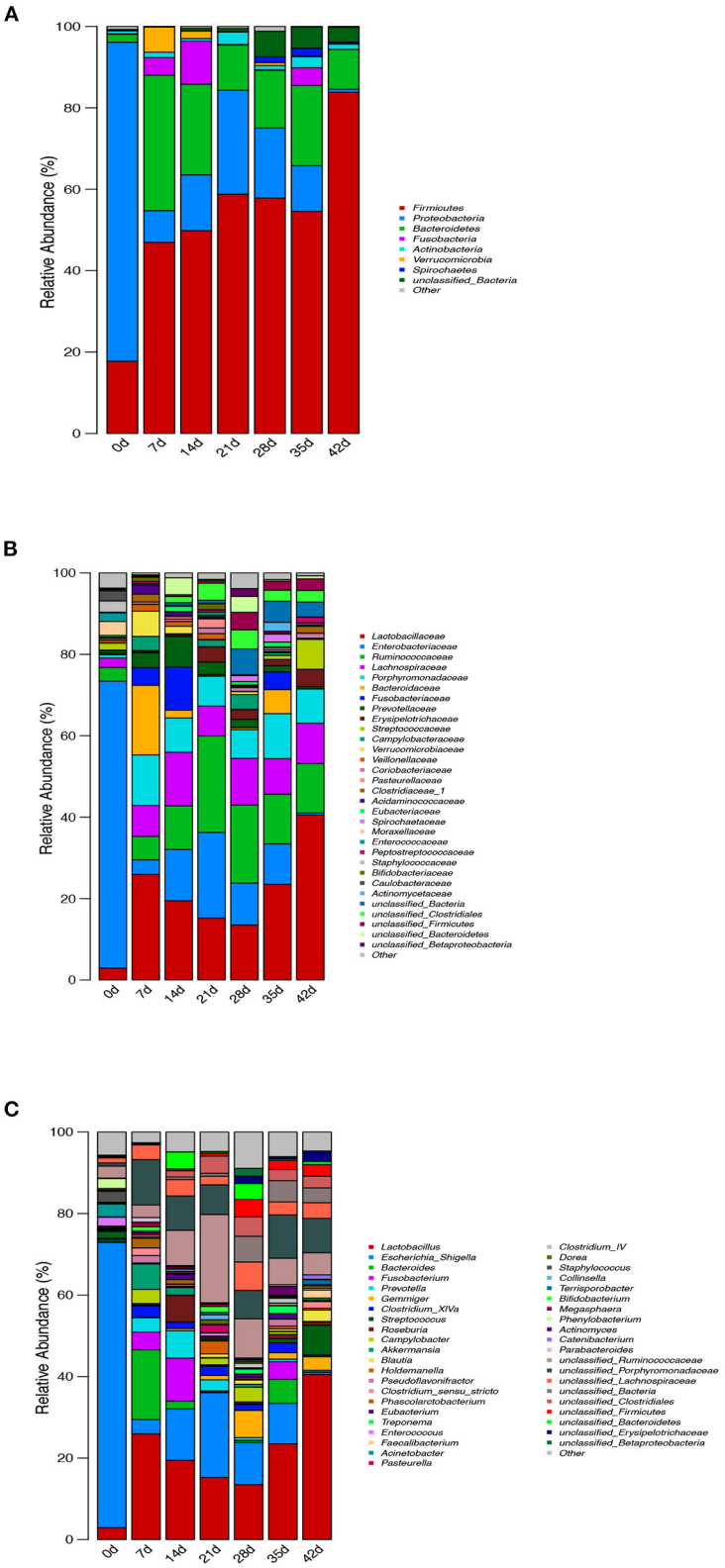

FIGURE 6 | Effect of piglets' age on taxonomic classification of the 16S rRNA gene sequences at **(A)** phylum, **(B)** family, and **(C)** genus levels.

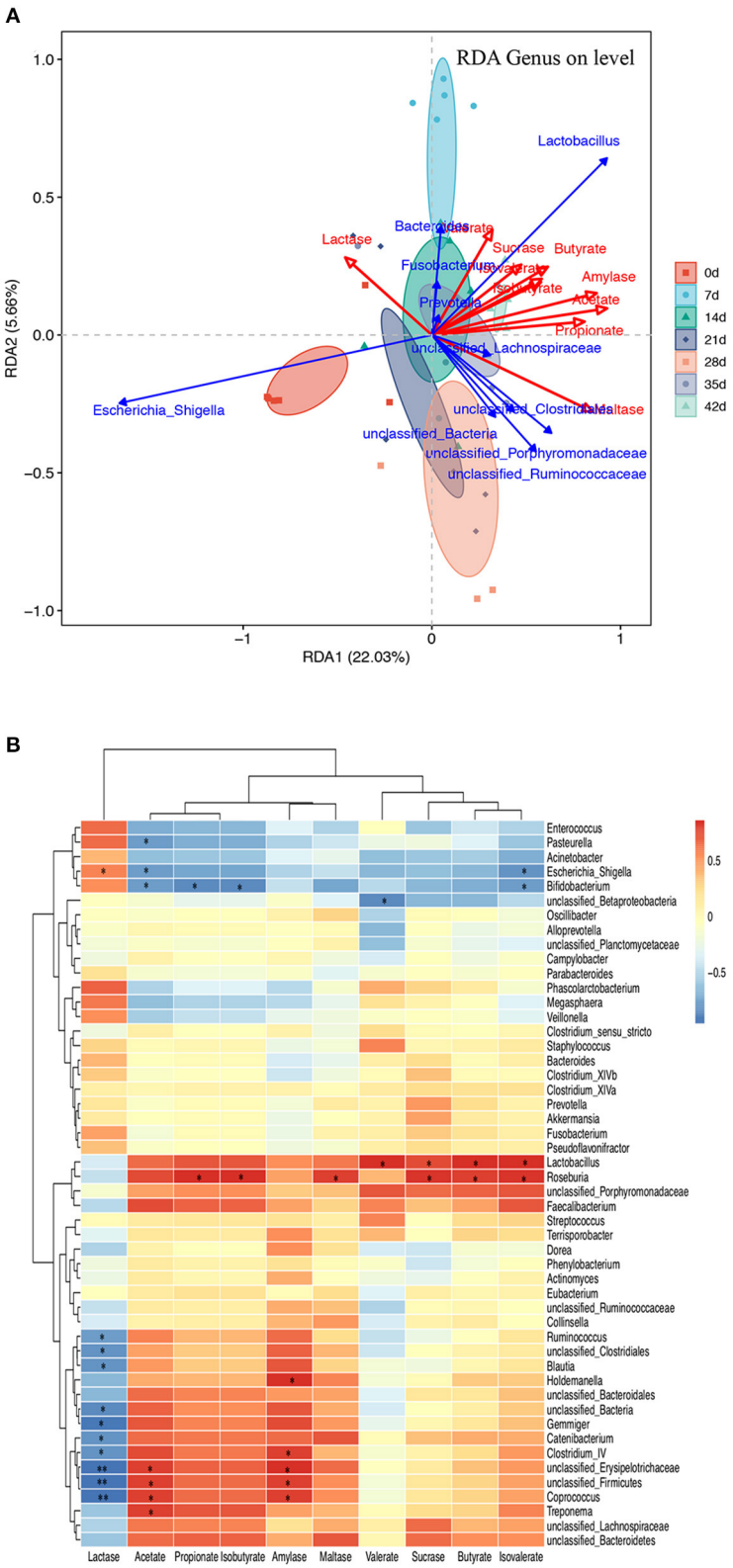

FIGURE 7 | Microbiota–metabolite correlation. **(A)** Triplot of RDA of the colonic microbial composition at genus level relative to pancreatic amylase, jejunal disaccharidase, and colonic SCFAs. The microbiota of different age groups are represented by different colors. Constrained explanatory variables (amylase, SCFA, and disaccharidase) are indicated by red arrows. Responding taxa are indicated by blue arrows, and only those with a higher fit in the ordination plot are labeled. The first (22.03% interpretation) and second coordinates (5.66% interpretation) are plotted. **(B)** A heatmap of the correlation analysis was conducted between the top 50 bacterial genera and the environmental factors. *$0.01 < P \leq 0.05$ and **$0.001 < P \leq 0.01$.

et al., 2019). In addition, sucrase and maltase activities are important markers for evaluating intestinal development, and the activity intensity of pancreatic enzymes is an indicator to measure digestive ability (Huygelen et al., 2015; Pieper et al., 2016; Yuan et al., 2017). Thus, increased sucrase, maltase, and pancreatic enzyme activities imply rapid maturation of the small intestine and digestive capacity, respectively. Furthermore, higher lactase activity during lactation and higher sucrase and maltase activities during nursery were helpful to degrade polysaccharides in sow milk and feed into monosaccharides, respectively. This facilitates the absorption and utilization of carbohydrates by the body, thereby promoting intestinal maturation and host growth. Overall, these results suggest that the digestive capacity of the intestine and pancreas increases with age, and the increased carbohydrate degradation rate in the diet promotes the digestion and absorption of nutrients by increasing the activity of amylase and disaccharidase.

This study also measured short-chain fatty acids in the hindgut, which are key metabolites interacting with the intestinal microbiota and can affect intestinal health and systemic metabolism (Yao et al., 2020; Zhou et al., 2020). Our results showed that the concentration of SCFAs increased with age but decreased at weaning. Studies have confirmed that higher concentrations of SCFAs in the intestine contribute to better growth performance (Le Gall et al., 2009). In addition, butyrate has been shown to have positive effects on pathogen control and intestinal barrier function, particularly energy utilization in the colon, gastrointestinal cell proliferation, and pH stabilization in the intestinal lumen (Guilloteau et al., 2010; Kelly et al., 2015).

The mammalian gastrointestinal tract contains a wide variety and active microbial community that serves as an important barrier against pathogens and plays an integral role in promoting the development of the intestinal immune system and maintaining normal intestinal function (Buffie and Pamer, 2013; Kamada et al., 2013). The present study shows a significantly increased α-diversity in the intestinal bacterial community with the age of piglets but decreased at the weaning stage. Moreover, recent studies showed that the α-diversity of intestinal bacteria increased significantly with the time interval of ∼1 month after weaning (Niu et al., 2015; Zhao et al., 2015). One of the most striking observations in this study was that with the exception of *Firmicutes* and *Proteobacteria*, which were the two most dominant phyla in the piglet intestinal microbiota at 0 and 21 d, the predominant phylum in the other groups was *Firmicutes* and *Bacteroidetes*. Our results are consistent with the finding of Zhao et al. which indicated the maximum abundance of *Proteobacteria* at birth and then it begins to decline (Zhao et al., 2015). In this study, *Escherichia_Shigella* was the most abundant bacteria in the intestinal of newborn piglets and subsequently, its abundance decreased during lactation and increased again during weaning. Piglet diarrhea can be attributed to the presence of certain bacteria in the microbiota, such as *Escherichia_Shigella* (Schokker et al., 2015; Hu et al., 2016). This result suggests that pathogenic species are often present in the gastrointestinal tract of infants or early piglets, waiting for a potential opportunity to become pathogens. The results of this study also showed that *Lactobacillus* belonging to the phylum *Firmicutes* was the most abundant genera in intestinal bacterial communities during lactation and nursery periods. It is well known that *Lactobacillus* will produce acetate, and the increase in *Lactobacillus* abundance will increase the concentration of acetate in the hindgut, which is consistent with our previous results on SCFAs. Furthermore, *Lactobacillus* and *Escherichia_Shigella* are the core microbiota in pre-weaning piglets, suggesting that they play a key role in the establishment and maintenance of the postnatal intestinal microbiota in piglets. In addition, the abundance of *Prevotella* increased with age during lactation, which is in line with the results of the study based on the intestinal microbiota of infants (from newborn to 12 months) (Bäckhed et al., 2015). *Prevotella* is a key microbe member of the animal gastrointestinal tract, and it is not only important for the degradation of starch and plant polysaccharides but also has strong catabolism of mucin (Kovatcheva-Datchary et al., 2015; Fang et al., 2017).

Using RDA, amylase, SCFAs, and disaccharidase were significantly associated with colonic microbiota. Furthermore, Spearman's correlation analysis of the top 50 microbiota genera and hindgut environmental factors showed that the microbiota bacteria with positive effects on SCFAs were *Lactobacillus*, *unclassified_Porphyromonadaceae,* and *Faecalibacterium*, while the microbiota negatively correlated with SCFAs were *Escherichia_Shigella*, *Acinetobacter,* and *Bifidobacterium*. Previous studies have found that *Lactobacillus* bacteria promote early piglet development and improve the intestinal health of newborn piglets by modulating intestinal microbiota (Dowarah et al., 2017; Yang et al., 2018; Zhang et al., 2019). The abundance of *Bifidobacteria* decreased with increasing age. As *Bifidobacteria* have been proved to inhibit the inflammatory response of intestinal epithelial cells, the decrease in its relative abundance with age may be related to the increase in chronic inflammation in the elderly. In addition, *Faecalibacterium* is a butyrate-producing bacterium, which increases with age. *Unclassified_Bacteroidetes* were positively correlated with amylase, sucrase, and maltase. *Bacteroidetes* are involved in nutrient metabolism, including carbohydrate fermentation, polysaccharides, and steroid metabolism, and are essential for maintaining the normal physiological function of the intestine (Yang et al., 2019).

Overall, our results suggested that the most significant difference between weaning, lactation, and the nursery was the decrease in the abundance of bacteria producing SCFAs and bacteria involved in carbohydrate metabolism. Many of the bacterial taxa that showed reduced numbers in piglets with diarrhea were related to the carbohydrate production of SCFAs, which was also observed in humans with Crohn's disease, suggesting that abnormal carbohydrate metabolism may be an important feature of diarrheal disease in piglets (Erickson et al., 2012). Therefore, we should further study the carbohydrate metabolism of piglets in future research.

CONCLUSION

In this experiment, we explored the digestive function and microbial changes in piglets from birth to nursery and the

correlation between intestinal microorganisms and digestive enzymes and metabolites. Based on the results, we infer that age and growth environment, including diet composition and weaning, are the key factors for the formation of piglet intestinal microbiota. Diarrhea caused by carbohydrate digestion deserves the attention of nutritionists. Supplementing bacteria conducive to carbohydrate digestion at an appropriate time will be beneficial to the health of piglets and infants. Our results broaden the understanding of the digestive function and microbial changes in piglets at different stages. These results will provide help for the experimental design of host–microbial interaction.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject—PRJNA816983.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care Advisory Committee for Sichuan Agricultural University.

AUTHOR CONTRIBUTIONS

JH and DC designed the whole experiment. XG performed the experiment, including chemical analysis, statistical analysis, and manuscript writing. BY, ZH, and JY verified the validity of the experiment and checked the results. PZ, JL, and YL participated in the experimental design and provided valuable advice. All authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.896660/full#supplementary-material

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Evaluating Starter Feeding on Ruminal Function in Yak Calves: Combined 16S rRNA Sequencing and Metabolomics

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For young ruminants, starter feeding can effectively facilitate the growth and development of rumen in ruminants, but the development of rumen is an important physiological challenge as it remains unclear for the mechanism of starter feeding stimulating. In this study, we performed an analysis of ruminal microbiota and their metabolites in yak calves to explore how the ruminal microbiota and their metabolites stimulate the ruminal function. This study associated 16S rRNA sequencing with liquid chromatography-mass spectrometry (LC-MS)-based metabolomics to evaluate the effects of starter feeding on ruminal microbiota diversity and metabolites in yak calves. We designed the experiment using 20 yak calves that were assigned equally into 2 groups, based on feeding milk replacer; the control (RA) group was fed with alfalfa hay while the treatment (RAS) group was fed with alfalfa hay and starter. After the experiment, we investigated the ruminal microbiota and metabolites through 16S rRNA sequencing and LC-MS-based metabolomics. During the preweaning period, the RAS group significantly promoted the growth performance and ruminal development in yak calves, including increases in body weight, chest girth, and development of rumen ($P < 0.05$). The RAS group increased the relative abundance of *Bacteroidota*, *Proteobacteria*, *Chloroflexi, Synergistota,* and *Spirochaetota* and decreased the abundance of *Firmicutes*, *Desulfobacterota*, *Actinobacteriota,* and *Actinobacteriota* at the phylum level ($P < 0.05$). At the genus level, the ruminal content of the RAS group was significantly enriched for *Rikenellaceae_RC9_gut_group* and *Ruminococcus,* while depleted for *Prevotella*, *Christensenellaceae_R-7_group*, and *NK4A214_group* ($P < 0.05$). A total of 37 metabolites were identified between the RA group and the RAS group, of which 15 metabolites were upregulated and 22 metabolites were downregulated compared with the RA group. Metabolic pathway analyses indicated that upregulated the metabolites of the RAS group yak calves were related to carbohydrate metabolism, ubiquinone, and other terpenoid-quinone biosynthesis, while the downregulated metabolic pathway was relevant to xenobiotic biodegradation, metabolism, and nucleotide metabolism. In summary, starter feeding before weaning significantly increased the dry matter intake and body weight of yak

calves, changed the diversity and abundance of ruminal microbiota, and positively regulated the good development of ruminal morphology and function, providing an important basis for high-quality cultivation and the nutritional level of nutrition of yak calves in the Qinghai Tibet plateau. This study is based on the availability of 16S rRNA sequencing and LC-MS-based metabolomics in clarifying the function of starter feeding in the yak calves.

Keywords: feeding strategies, ruminal development, ruminal microbiota, ruminal metabolomics, yak calves

INTRODUCTION

Yak calves are the foundation of the yak industry in the Qinghai-Tibetan Plateau, and the quality of yak calf rearing directly contributes to the performance of adults (Long et al., 2008). In the early stage of raising, the suckling period has a long-term impact on various biological functions (Baldwin et al., 2004; Soberon and Van Amburgh, 2013). Traditionally, yak calves are weaned naturally or artificially at 1.5–2 years of age under a wide range of conditions (Ding et al., 2013). Premature weaning of yaks is being attempted to improve ruminal growth and function (Mohr et al., 2002). However, under natural grazing conditions, there is a high mortality rate of calves due to the lack of nutrition and poor environmental conditions in the Qinghai-Tibetan Plateau (Liu et al., 2018). Taking this into account, Khan et al. (2016) proposed that a mixture of roughage and grain could be used for early weaning under the condition of house feeding. Diet, as one of the most important factors, affects the digestive systems, especially the structure and function of the ruminal microbiota, which can promote ruminal development (Berends et al., 2015; Latham et al., 2018; Ogunade et al., 2019). Coincidentally, a supplement of alfalfa stimulated the ruminal development consistent with the changes in ruminal microbiota and animal performance before and after weaning (Yang et al., 2018). Meanwhile, starter feeding decreased mRNA expression of cytokines, namely, TNF-α and IFN-γ in the colonic tissue and also in the digestive tract (Liu et al., 2017).

The digestive tract mainly develops in the early growth stage of calves, which effectively influences their long-term performance (Davis Rincker et al., 2011). As the fermentation pot of the ruminant animal, rumen fermentation produces volatile fatty acids (VFAs) that can directly stimulate the proliferation and development of the ruminal epithelium (Górka et al., 2018). Besides, the ruminal microbial composition is an effective way to resist external stimulation and maintain ruminal environmental stability (Shen et al., 2017). Therefore, the ruminal microbiota plays a central role in the efficiency of digestion in ruminants (Morgavi et al., 2013).

Although it had already been proved in dairy calves that alfalfa hay could promote ruminal epithelial and muscular development (Yang et al., 2015), but the inferior quality of alfalfa hay to be fed to calves could not provide efficient ruminal VFA required for ruminal papilla development (Mirzaei et al., 2015; Hosseini et al., 2016). Fortunately, the previous study found that the supplement of milk replacer and starter feeding can relieve the stress of weaning and raise the level of ruminal development (Sweeney et al., 2010). Therefore, in this study, 16S rRNA sequencing and metabolomics technology were used to investigate starter feeding based on alfalfa hay for weaning yak calves, providing an important reference for the research of milk replacer breeding technology after weaning in yak calves.

In recent years, next-generation high-throughput sequencing (*via* 16S rRNA sequencing) has been used to assess the influences of dietary on the ruminal microbial community (Pinloche et al., 2013; Kim et al., 2014). The application of metabolomics analysis provides an opportunity to measure large numbers of small molecule metabolites in cells, tissues, and biofluids (Goldansaz et al., 2017). Recent studies have applied metabolomics to predict feed efficiency and residual feed intake (Karisa et al., 2014; Artegoitia et al., 2017), examine disease conditions (Hailemariam et al., 2014), evaluate dietary responses to different feeds (Saleem et al., 2012), and assess milk quality of ruminants (Abarghuei et al., 2014). Nevertheless, variations in the types of metabolites produced as a result of starter feeding in yak calves have not been completely described. Therefore, a better comprehension of the relationship between starter feeding and ruminal factors (i.e., fermentation, morphology, microbiota community, and their metabolites) in yak calves will facilitate more accurate estimations of the starter supplement and demand under current feeding patterns.

MATERIALS AND METHODS

Ethical Approval Statement

All yak calves and experimental protocols in this study were conducted following the recommendations of the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in 2004).

Animals and Experimental Design

All the yak calves were maternally nursed and grazed in the Datong Yak Breeding Farm of Qinghai Province before the trail. The experiment was conducted from July to November 2020, with 20 male yak calves at the age of 30 days [body weight (BW) of 33.67 ± 3.52 kg, mean ± standard deviation (SD)] with similar body conditions randomly recruited and assigned into two groups, with ten calves per group, nursed at the Haibei Tibetan Autonomous Prefecture Plateau Ecological Animal Husbandry Science Park Management Committee. All the calves were supplied with the same milk replacer (Beijing Precision Animal Nutrition Research

Center, Q/HDJZA0007-2019); while the control (RA) group received only alfalfa hay, the treatment (RAS) group was fed with alfalfa hay and starter (Beijing Precision Animal Nutrition Research Center). The ten calves in each group were individually fed in ten different pens. We fed all the yak calves twice a day, at 08:00 and 16:30 with 100–700 g of milk replacer powder dissolved in 42°C water five times. The supplementation of milk replacer increased along with the increase of body weight, approximately 80 g per week until 3 months of age, and then gradually decreased by the same rate until the end of the study. Freshwater was supplied freely to the yak calves.

In brief, during the experimental period, the alfalfa hay and starter offered were adjusted daily to ensure at least 10% orts, while daily feed supplied was recorded at 3-day intervals, and the orts were gathered as well and then pooled and weighed at 3-day intervals for the calculation of the averaged dry matter intake (DMI) over 3 days until the average daily DMI achieved 1 kg each of the yak calves. At the end of the experiment, this resulted in the numbers of the feed intakes for each calf, and the means of those intakes were used as individual replicates for the statistical analysis of the difference in feed intake between the two treatments.

Samples of the starter feed, alfalfa hay, and milk replacer were measured (AOAC International, 2000) for dry matter (oven method 930.15), sugar (colorimetric method), starch (α-amylase method), crude protein (Kjeldahl method 988.05), ether extract (alkaline treatment with Röse-Gottlieb method 932.06 for milk replacer; diethyl ether extraction method 2003.05 for starter and alfalfa hay), NDF with ash without sodium sulfite or α-amylase, ADF with ash, calcium (Ca), and phosphorus (P) (dry ashing, acid digestion, and analysis by inductively coupled plasma, method 985.01), and the nutrient compositions of the milk replacer, alfalfa hay, and starter are given in **Table 1**.

TABLE 1 | Nutrient composition of the milk replacer, alfalfa hay, starter used in the present study.

Items (% of dry matter)	Milk replacer[1]	Alfalfa hay	Starter feed
Dry matter (% as fed)	95.00	93.70	87.80
Sugar	–	–	6.50
Starch	–	–	40.50
Crude protein	26.24	12.51	20.01
Ether extract	27.79	0.89	4.70
Neutral detergent fiber	–	56.45	10.90
Acid detergent fiber	–	40.40	4.10
Calcium	2.50	0.99	0.79
Phosphorus	1.40	0.16	0.46
Lysine	2.20	0.84	1.05
Methionine	1.00	0.16	0.34

[1] *The milk replacer was stored as a powder and contained whole milk powder, whey powder, protein concentrate, vitamin A, vitamin D3, vitamin E, nicotinic acid, pantothenic acid, lysine, methionine, threonine, sodium chloride, copper, zinc manganese, and iron.*

Sample Collection

When the average daily DMI achieved 1 kg each of the yak calves, yak calves were fasted for 24 h, and then, the body weight, height, length, and the chest girth of all the yak calves were recorded. Five yak calves were selected randomly from each group, killed by exsanguination, and then dissected at once.

The rumen was separated, and the content within the rumen was collected for sampling. We collected 5 ml of homogenized ruminal content samples in triplicates from the ventral sac of the rumen and stored them at -80°C for microbial DNA extraction and untargeted metabolomics. Ruminal fluid samples were collected from individual yak calves and strained through 4 layers of sterile cheesecloth, and the pH was measured immediately using a portable pH meter (HI 9024C; HANNA Instruments, Woonsocket, RI, United States). Meanwhile, another 5 ml of the ruminal fluid was collected and stored at −20°C for VFA and NH_3-N analyses. Specifically, a solute with metaphosphoric acid and crotonic acid was added to 2 ml of these 5 ml ruminal fluid samples before further analyses of the VFA concentrations in gas chromatography (GC-14B, Shimadzu, Japan) (Wang et al., 2017).

Subsequently, three segments of the tissue sample (2 × 2 cm) from the ventral sac of the rumen were collected, immediately washed with saline solution, then fixed in 10% buffered formalin, and stored at 4°C until papilla length and width, and the thickness of ruminal base was measured (Ishii et al., 2005). All the tissue samples were taken from the same location in each animal. Additionally, all the other collected samples were first stored in liquid nitrogen for 24 h, unless noted otherwise, and then stored at −80°C before analyses.

Determination of the Ruminal Morphology

By using the routine method of the wax section, the development of rumen was studied. After fixing in 10% buffered formalin for 24 h, the ventral sac of the ruminal tissue samples was gradually dehydrated at different concentrations (60, 70, 80, 90, and 100%) of ethanol and cleaned. Then, the ruminal samples were trimmed into small pieces and inserted into cassettes, which were embedded in liquid paraffin. Notably, 5-μm paraffin sections were sliced using the microtome and stained with hematoxylin-eosin. Using the phase-contrast microscope (Nikon NiE200, Tokyo, Japan) the papillae length and width of the ruminal tissue and the thickness of the ruminal base were measured (Wu et al., 2018).

Determination of Volatile Fatty Acid and NH_3-N Concentrations in Ruminal Fluid

The ruminal fluid samples were centrifuged at 13,000 × *g* for 10 min at 4°C before the VFA and NH_3-N concentration measurement. Using the Agilent 6850 gas chromatograph (Agilent Technologies Inc., Santa Clara, CA, United States) equipped with a polar capillary column (HP-FFAP, 30 m × 0.25 mm × 0.25 μm) and a flame ionization detector to analyze the supernatant ruminal fluid samples is previously described (Xue et al., 2017). The NH_3-N concentration in each

TABLE 2 | Effects of the starter feeding supplementation in the preweaning period on the growth performance and development of rumen in yak calves.

Items		Treatment[1]		SEM	*P*-value
		RA	RAS		
Growth performance	Body weight (kg)	72.07 ± 2.47	80.58 ± 1.26	1.38	<0.001
	Body length (cm)	90.80 ± 3.63	93.80 ± 1.92	1.34	0.141
	Body height (cm)	76.33 ± 2.08	81.25 ± 0.96	1.20	0.008
	Chest girth (cm)	104.80 ± 2.68	116.40 ± 2.61	2.22	<0.001
	DMI (g)	608.36 ± 60.68	678.71 ± 6.53	17.04	0.033
Rumen weight	Rumen (g)	1.07 ± 0.07	1.17 ± 0.04	0.23	0.036
Rumen index, expressed as kg of organ/kg of BW	Rumen ($\times 10^{-2}$)	1.47 ± 0.08	1.56 ± 0.13	0.17	0.221

[1] *The alfalfa (RA) was fed with the milk replacer and alfalfa hay, the alfalfa hay and starter (RAS) group was fed with milk replacer, alfalfa hay and the starter.*

supernatant sample was measured using a continuous-flow analyzer (SKALAR San, Skalar Co., Breda, Netherlands).

Microbial DNA Extraction, 16S rRNA Gene Amplification of the V3 + V4, and Bioinformatics Analysis

Total genome DNA from the 10 ruminal content samples of yak calves from two different treatments was extracted using the cetyltrimethylammonium bromide (CTAB) method in accordance with Henderson et al. (2013). Meanwhile, DNA extraction was assessed through the QIAamp DNA Stool Mini Kit (Qiagen, Dusseldorf, Germany). DNA concentration was monitored on 1% agarose gels. The purity was assessed from the 260: 280 nm ratio (>1.8) using a NanoDrop ND2000 spectrophotometer (Thermo Scientific,Waltham, MA, United States), and the DNA was stored at −80°C until it was used in sequencing analysis.

16S rRNA genes of 16S V3-V4 regions were amplified using specific primer set 515F (5′-GTGCCAGCMGCCGCGG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) with barcodes (Yu et al., 2005; Sundberg et al., 2013). All PCR reactions were carried out with 15 μl of Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Beijing, China), 2 μM of forward and reverse primers, and approximately 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s, and finally, 5 min at 72°C. PCR product quantification and qualification: the same volume of 1 × loading buffer (contained SYB green) was mixed with PCR products and electrophoresis on 2% agarose gel was performed for detection. PCR products were mixed in equidensity ratios. Then, the mixture of PCR products was purified using the Qiagen Gel Extraction Kit (Qiagen, Dusseldorf, Germany). Sequencing libraries were generated using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, United States) following the manufacturer's recommendations and adding index codes. The library quality was assessed using the Qubit@ 2.0 Fluorometer (Thermo Scientific, Waltham, MA, United States) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina NovaSeq platform, and 250 bp paired-end reads were generated.

Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags (Bokulich et al., 2013), according to the QIIME 2 (Bolyen et al., 2019). The tags were compared with the reference database (Silva database) using the UCHIME algorithm (UCHIME) (Edgar et al., 2011) to detect chimera sequences, then the chimera sequences were removed (Haas et al., 2011), and effective tags were finally obtained.

Sequence analyses were performed using Uparse software (Uparse version 7.0.1001) (Edgar, 2013). Sequences with ≥97% similarity were assigned to the same OTUs. A representative sequence for each OTU was screened for further annotation. For each representative sequence, the Silva database (Edgar et al., 2011) was used based on the Mothur algorithm to annotate taxonomic information. To study the phylogenetic relationship of different OTUs and the difference of the dominant species in different groups, multiple sequence alignments were conducted using the MUSCLE software (Version 3.8.31) (Edgar, 2004). OTU abundance information was normalized using a standard sequence number corresponding to the sample with the least sequences. Subsequent analyses of alpha diversity and beta diversity were all performed based on this output normalized data.

The taxon abundance for each sample was determined according to phylum, class, order, family, and genus. The microbiota were compared for beta diversity using the distance matrices generated from weighted UniFrac analysis, principal coordinated analysis (PCoA), and analysis of similarities (ANOMIS). The *P*-value was set as <0.05, and the threshold of the linear discriminant analysis (LDA) score was set at a default value of 2.0.

Untargeted Metabolomics

The ruminal content samples (1 ml) were freeze-dried and resuspended with prechilled 80% methanol and 0.1% formic acid using a good vortex. Then, the samples were incubated on ice for 5 min and centrifuged at 15,000 *g*, 4°C for 15 min. Some supernatant was diluted to the final concentration containing 53% methanol by LC-MS grade water. The samples were subsequently transferred to a fresh Eppendorf tube and then centrifuged at 15,000 *g*, 4°C for 15 min. Finally, the supernatant was injected into the LC-MS system analysis.

TABLE 3 | Effects of starter feeding on the ruminal fermentation parameters and ruminal epithelium development in yak calves.

Items		Treatment		SEM	*P*-value
		RA	RAS		
Ruminal epithelium	Papilla width (μm)	952.69 ± 105.45	824.07 ± 191.11	25.15	0.283
	Papilla length (μm)	569.82 ± 100.37	748.72 ± 128.53	33.29	0.049
	Muscle thickness (μm)	1915.07 ± 439.49	2031.12 ± 643.69	105.68	0.757
Ruminal fermentation characteristics	pH	7.35 ± 0.12	7.35 ± 0.116	0.03	0.978
	Ammonia nitrogen, NH_3-N (mg/dL)	2.46 ± 0.28	3.29 ± 0.23	0.16	0.001
	Total VFA (mmol/L)	57.40 ± 5.27	50.23 ± 5.25	2.07	0.081
	Acetate (mmol/L)	35.78 ± 3.51	29.60 ± 1.05	0.23	0.034
	Propionate (mmol/L)	9.39 ± 1.29	8.28 ± 1.07	0.44	0.232
	Butyrate (mmol/L)	5.41 ± 1.55	4.62 ± 1.18	0.43	0.389
	Isobutyrate (mmol/L)	1.53 ± 0.084	1.54 ± 0.25	0.06	0.936
	Valerate (mmol/L)	3.58 ± 0.43	4.23 ± 0.05	0.15	0.021
	Isovalerate (mmol/L)	1.20 ± 0.14	1.27 ± 0.17	0.05	0.592

Discoverer 3.1 (CD3.1, ThermoFisher, Waltham, MA, United States) was used to perform peak alignment, peak picking, and quantitation for each metabolite. Later, peak intensities were normalized to the total spectral intensity. The normalized data were used to predict the molecular formula based on additive ions, molecular ion peaks, and fragment ions. Then, peaks were matched using the mzClou, mzVault, and MassList database to obtain accurate qualitative and relative quantitative results.

Statistical analyses were performed using the statistical software R (R version R-3.4.3), Python (Python 2.7.6 version), and CentOS (CentOS release 6.6). When data were not normally distributed, normal transformations were attempted using area normalization.

These metabolites were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Principal component analysis (PCA) was performed at metaX (Wen et al., 2017) (a flexible and comprehensive software for processing metabolomics data). We applied univariate analysis (*t*-test) to calculate the statistical significance (*P*-value). The metabolites with VIP $>$ 1, *P*-value $<$ 0.05, and fold change (FC) $\geq$ 2 or FC $\leq$ 0.5 were considered to be differential metabolites. Volcano plots were used to filter metabolites of interest-based on $\log_2$(FC) and $-\log_{10}$(*P*-value) of metabolites using ggplot2 in R language.

The KEGG pathway enrichment of differential metabolites was performed, the ratio was satisfied by x/n $>$ y/N, and the metabolic pathway was considered an enrichment, while when the *P*-value of the metabolic pathway $<$ 0.05, the metabolic pathway was considered a statistically significant enrichment.

Statistical Analysis

In this study, all data were tested and presented as a normal distribution. At the beginning of the experiment, the initial body weight, height, length, and chest girth were tested using the *t*-test, no significant differences were identified, and the initial indices were almost constant. Therefore, for other indices except for the DMI data, analysis was performed using the one-way ANOVA procedure using SPSS 22.0 software (SPSS, Inc., Chicago, IL, United States) with replicates as experiment units, and a value of $P < 0.05$ was regarded as statistically significant. The differences in DMI between two groups of yak calves were further analyzed using the following model: $Yij = \mu + Di + Tj + \varepsilon ij + DT$, using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, United States, 2007), which considered the significant differences of these indices induced by the time effect in the same treatment. Yij is the response variable, in particular, μ is the overall mean, Di is the fixed effect of treatment (*i* = maternal grazing or barn feeding), Tj is the fixed effect of time (10 days as a unit) of the experiment, DT is the interaction of dietary and time, and εij is the residual error. If a significant diet and time effect were observed, the significance between the treatment and time differences was separately identified using the Tukey's test multiple comparison test. All data are expressed as the means with the standard error.

The statistical evaluation of 16S rRNA sequencing and the untargeted metabolomics results were analyzed using the bioinformatics methods described above. Spearman's correlation test was used to examine relationships between the RA group and the RAS group. The correlation coefficient rho between the relative abundance of differential metabolites and the total abundance of differential bacteria was calculated using the Spearman statistical method (rho ≥ 0, $P \leq 0.05$).

RESULTS

The Effect of Starter Feeding on Growth Performance, Dry Matter Intake, and Development of the Rumen

Significant differences in daily DMI were found between the two groups of the yak calves over the whole experimental period ($P = 0.033$), where the higher intake was found for yak calves in the RAS group. Compared with the RA group, the RAS group had a significantly greater body weight ($P < 0.001$), height ($P = 0.008$),

TABLE 4 | Effects of the starter feeding on richness and diversity index of ruminal microbiota in yak calves.

Items	Treatment		SEM	*P*-value
	RA	RAS		
OTUs	1457.25 ± 94.49	1265.00 ± 120.69	50.78	0.046
Good's coverage	0.99 ± 0.001	0.99 ± 0.001	0.01	0.557
ACE value	1619.81 ± 162.03	1404.46 ± 132.99	63.33	0.086
Chao 1 value	1596.47 ± 143.09	1385.30 ± 136.44	60.72	0.077
Shannon indices	7.50 ± 0.29	7.18 ± 0.89	0.21	0.468
Simpson indices	0.97 ± 0.01	0.96 ± 0.03	0.01	0.468

and chest girth ($P < 0.001$) (**Table 2**). Meanwhile, a significant increase in organ development was found in the RAS group, which included the weight of the rumen ($P = 0.036$) (**Table 2**).

Development of Ruminal Morphology and Ruminal Fermentation Profiles

Over the whole experimental period, when compared with the RA group, the RAS group had significantly greater papilla length ($P = 0.049$). The RAS group could significantly increase the ruminal fluid NH_3-N concentration compared with the RA group ($P = 0.001$). Besides, the RA group had a significantly higher acetate concentration than the yak calves in the RAS group ($P = 0.034$), while the valerate concentration was significantly greater in the RAS group ($P = 0.021$) (**Table 3**).

Ruminal Microbiota

According to further analysis of ruminal microbiota, we found significantly decreased the OTUs in the RAS group (**Table 4**, $P = 0.046$), whereas the two groups showed no significant difference in Chao 1 value, Shannon indices, and Simpson index (**Table 4**, $P = 0.468$). Moreover, beta diversity analyses showed that the compositions of the gastrointestinal prokaryotic community of the yak calves in different feeding groups were different in the rumen (**Figure 1A**).

Rumen whose phylum-level relative abundance was in the top 15 of all the microbiota communities is shown in **Figure 1B**. The genus of the top 15 is given in **Figure 1C**. Differential ruminal microbiota are identified between two different feeding groups. Among these, *Firmicutes* ($P < 0.001$), *Desulfobacterota* ($P = 0.002$), and *Actinobacteriota* ($P = 0.002$) were significantly greater in the RA group, while *Bacteroidota* ($P = 0.001$), *Proteobacteria* ($P = 0.011$), *Chloroflexi* ($P = 0.004$), *Synergistota* ($P = 0.005$), and *Spirochaetota* ($P = 0.017$) were significantly higher in the RAS group (**Table 5**). At the genus level, *Prevotella* ($P = 0.018$), *Christensenellaceae_R-7_group* ($P = 0.013$), and *NK4A214_group* ($P = 0.018$) were significantly greater in the RA group. *Rikenellaceae_RC9_gut_group* ($P = 0.004$) and *Ruminococcus* ($P = 0.041$) were identified significantly greater genera in the RAS group. Furthermore, similar results that contained similar but less differential bacteria were also identified using the Mann–Whitney *U* test (**Table 5**).

Microbiological Metabolism of the Rumen

Based on the relative quantitative value of the metabolites, the Spearman correlation coefficient between the QC samples is calculated (**Figure 2A**). The correlation between the QC samples was closer to 1 ($R^2 < 1$), indicating that the test process was stable and the quality of data high (Rao et al., 2016).

In contrast, metabolism mainly participated in amino acid metabolism and nucleotide metabolism in ruminal content (**Figure 2B**). We detected the metabolites in the rumen by metabolomics. In our research, the relative concentrations of 37 of the total 1,057 identified metabolites were altered by starter feeding (**Figure 2C**), of which 15 metabolites were upregulated and 22 metabolites were downregulated in yak calves in the RAS group compared with those in the RA group (**Table 6**).

As shown in **Figure 2D**, UMP, hydroquinone, xylitol, and delta-tocopherol were enriched in the KEGG pathway "metabolic pathways." Hydroquinone was enriched in the KEGG pathway "Microbial metabolism in diverse environments," "Tyrosine metabolism," "Aminobenzoate degradation," "Benzoate degradation," and "Chlorocyclohexane and chlorobenzene degradation." Xylitol was enriched in the KEGG pathway "ABC transporters" and "Pentose and glucuronate interconversions." UMP and delta-tocopherol may be related to "Pyrimidine metabolism" and "Ubiquinone and other terpenoid-quinone biosynthesis," respectively.

Correlation Between Ruminal Microbiota and Fermentation Profiles

There is an interaction between ruminal microbiota and growth performance of yak calves and ruminal fermentation profiles, which affects the feed efficiency and tissue development of ruminants. The correlation between them is illustrated in **Figure 3**.

At the phylum level (**Figure 3A**), finally, body weight of yak calves was significantly positively correlated with *Actinobacteriota*, *unidentified-Archaea,* and *Cyanobacteria*. The DMI of yak calves was significantly negatively correlated with *Bacteroidota*, *unidentified-Archaea,* and *Cyanobacteria*. The ruminal weight of yak calves was significantly negatively correlated with Verrucomicrobiota, *unidentified-Archaea*, *MBNT15*, *Sva0485*, *Cyanobacteria,* and *Nitrospinota*. The width of ruminal papilla was significantly negatively correlated with *Spirochaetota*. The length of ruminal papilla was significantly positively correlated with *Proteobacteria*, *unidentified-Bacteria,* and *Kapabacteria* and significantly negatively correlated with *Bacteroidota* and *unidentified-Archaea*. The NH_3-N concentration in the ruminal fluid was significantly negatively correlated with the *Bacteroidota*, *unidentified-Archaea,* and *Verrucomicrobiota* and was significantly positively correlated with *Desulfobacterota* and *Kapabacteria*. The MCP concentration in the ruminal fluid was significantly negatively correlated with the relative abundance of *unidentified-Archaea* and *Cyanobacteria*. Acetate concentration was significantly positively correlated with *Verrucomicrobiota* and *unidentified-Archaea* and significantly negatively correlated with Kapabacteria.

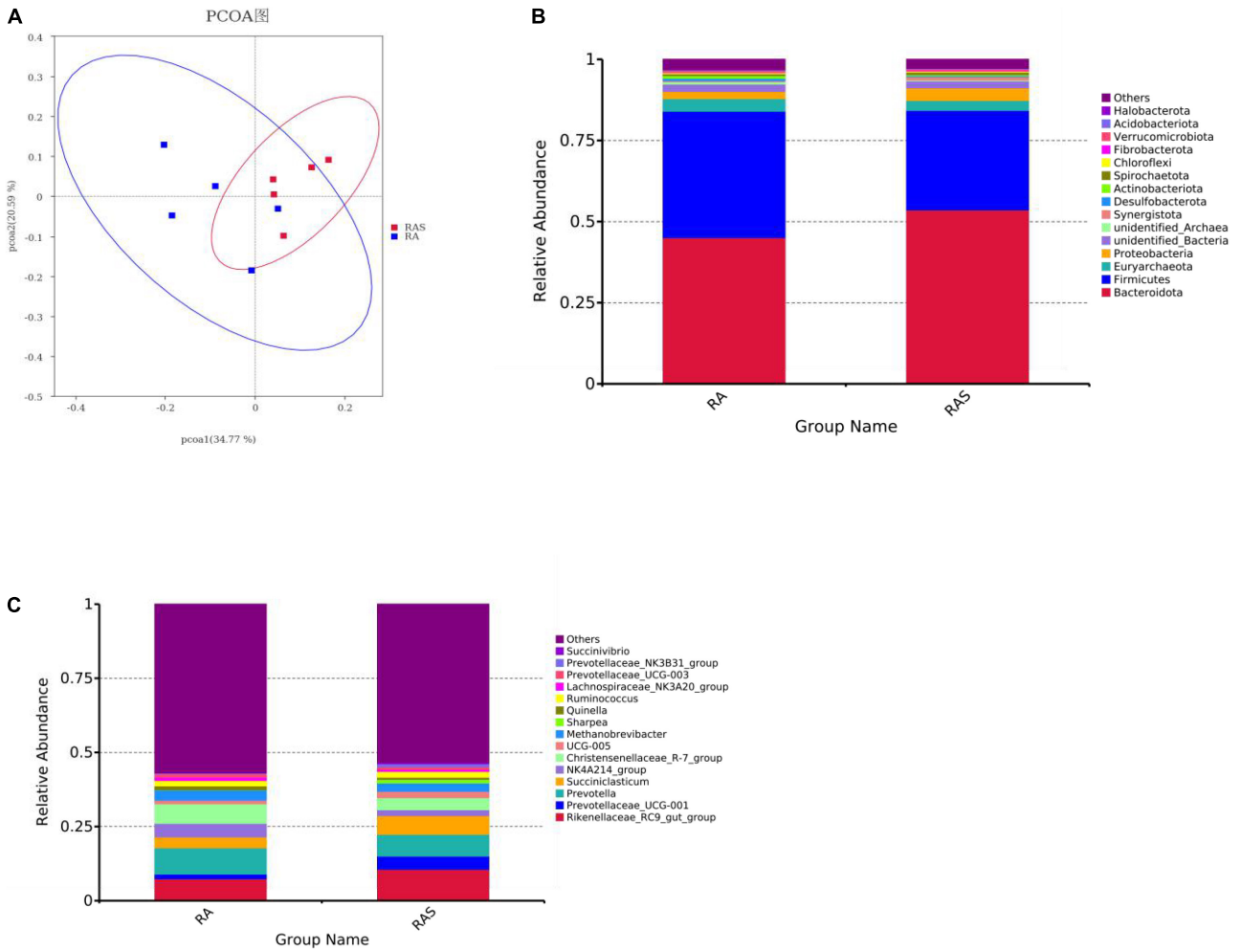

FIGURE 1 | (A) Diversity analyses based on the ruminal microbiota; phylum **(B)** and genus level **(C)** composition of the ruminal bacteria (top 15).

Propionate concentration was significantly positively correlated with *Chloroflexi*. The concentration of valerate was significantly negatively correlated with *Bacteroidota*, *unidentified-Archaea*, MBNT15, Sva0485, and *Cyanobacteria*. TVFA concentration was significantly positively correlated with *Cyanobacteria*.

At the genus level (**Figure 3B**), there were significant positive correlations between the final body weight, DMI, ruminal weight, NH_3-N, MCP concentration, and valerate concentration and the relative abundance of *Ruminococcus*, significant negative correlations between acetate and TVFA concentration and the relative abundance of *Ruminococcus*. MCP and valerate concentrations were significantly positively correlated with *Sharpea*. Butyrate concentration was significantly positively correlated with the *Prevotaceae-UCG-001*. The concentration of acetate was significantly negatively correlated with *Ruminobacter*. The final body weight, DMI, ruminal weight, ruminal papilla length, NH_3-N, and MCP concentration and valerate concentration of yak calves were significantly negatively correlated with *Lachnospiraceae-XPB1014-group*.

Correlation Between Ruminal Microbiota and Metabolites

In this study, the correlation analysis results of metabolites and 16S rRNA are shown. As shown in **Figure 4A**, a total of 44 correlations significantly differ at the phylum level, with 36 significantly positive correlations and 8 significantly negative correlations. Hydroquinone was significantly positive with *Desulfobacterota* and *Actinobacteriota*. UPM was significantly positive with *Firmicutes* and *Sva0485*, while significantly negative with *Bacteroidota*. Xylitol was significantly positive with *Spirochaetota*.

There were 104 significantly positive and 19 significantly negative correlations between ruminal microbiota and their metabolites at the genus level (**Figure 4B**), with *Rikenellaceae_RC9_gut_group* significantly positive with UPM and significantly positive with xylitol. *Prevotella*, *NK4A214_group*, *Lachnospiraceae_NK3A20_group*, *Desulfovibrio*, *Butyrivibrio*, and *Methanosphaera* were significantly positive with Hydroquinone, while *Succiniclasticum*

TABLE 5 | Ruminal microbiota community difference between the two groups by using the Mann–Whitney *U* test.

Items	Groups		SEM	*P*-value
	RA	RAS		
Phylum				
Bacteroidota	38.38 ± 4.96	53.61 ± 1.41	2.963	0.001
Firmicutes	43.98 ± 2.77	30.70 ± 2.41	2.567	<0.001
Euryarchaeota	3.16 ± 1.16	2.20 ± 0.92	0.387	0.240
Proteobacteria	2.68 ± 0.38	3.87 ± 0.59	0.263	0.011
Verrucomicrobiota	0.38 ± 0.13	0.43 ± 0.15	0.046	0.577
Chloroflexi	0.13 ± 0.04	0.32 ± 0.07	0.040	0.004
Synergistota	0.27 ± 0.05	0.90 ± 0.27	0.140	0.005
Desulfobacterota	0.68 ± 0.11	0.34 ± 0.08	0.069	0.002
Actinobacteriota	1.06 ± 0.28	0.28 ± 0.02	0.169	0.002
Spirochaetota	0.46 ± 0.19	1.00 ± 0.22	0.130	0.017
Genus				
Prevotella	11.04 ± 0.10	8.10 ± 1.43	0.707	0.018
Methanobrevibacter	2.67 ± 0.74	1.20 ± 0.85	0.290	0.274
Quinella	0.56 ± 0.08	0.56 ± 0.35	0.080	0.993
Rikenellaceae_RC9_gut_group	6.73 ± 1.74	12.33 ± 0.79	1.240	0.004
Prevotellaceae_UCG-001	1.56 ± 0.31	1.39 ± 0.54	0.164	0.659
Succiniclasticum	3.66 ± 0.92	4.57 ± 0.50	0.337	0.203
NK4A214_group	2.72 ± 0.71	1.43 ± 0.25	0.311	0.018
Christensenellaceae_R-7_group	6.60 ± 1.11	4.07 ± 1.40	0.565	0.013
UCG-005	1.37 ± 0.46	1.08 ± 0.11	0.139	0.351
Ruminococcus	1.59 ± 0.41	3.93 ± 0.23	0.660	0.041

was significantly negative with Hydroquinone. Delta-tocopherol was significantly positive with *Succiniclasticum*, *Sharpea*, *Ruminococcus,* and *UCG-002*, while it was significantly negative with *Christensenellaceae_R-7_group*. UMP was significantly positive with *Acetitomaculum* and *UCG-002*.

DISCUSSION

Due to the slow development of rumen, the growth performance of newborn calves was related to the digestion of wrinkles and the intestine. The ruminal epithelium is responsible for nutrient absorption and transportation in the rumen. This study shows that starter feeding with alfalfa hay significantly increased DMI, body weight, and chest girth in yak calves, which presumably stimulated both the development of ruminal epithelia and performance (Baldwin et al., 2004; Norouzian et al., 2011; Yang et al., 2015; Lin et al., 2019), in line with the results of the previous studies in calves' and lambs' early life (Saro et al., 2018; Cui et al., 2020). Starter feeding also significantly promoted ruminal development in yak calves. These improvements probably attributed to the improved dry matter intake and ruminal fermentation. With the growth of the age and the solid feed intake, the ruminal epithelium can influence the net use of digestion and utilization of diet, serving as a barrier to the ruminal epithelium's contents (Sander et al., 1959; Anderson et al., 1987). Similar to the previous study, the starter feeding contributed to the ruminal papilla length, which is also consistent with Xie et al. (2013) research on Holstein calves.

Due to various in-nutrient supplementation, significant changes occurred in the ruminal fermentation, which represented the increased carbohydrate fermentation in the rumen (Raghuvansi et al., 2007; Xue et al., 2017; Yang et al., 2018). The starter feeding contains grains, thus providing easily fermentable carbohydrates for microbial fermentation. When supplied with a starter, the RAS group produced a bigger NH_3-N concentration compared with the RA group, which fed with only alfalfa hay. NH_3-N concentration in the rumen is in a dynamic balance, which can provide raw materials for generating the microbial protein (Firkins et al., 2007). Increased NH_3-N concentration in RAS group yak calves, this is in line with this study (Agle et al., 2010). As suggested in previous studies (Stams et al., 1984; Poudel et al., 2018; Gao et al., 2019), the alfalfa hay supplementation could increase the ruminal acetate-producing bacteria, further influencing the ruminal acetate concentration. Likewise, an increased acetate concentration level in the RA group calves that fed with the alfalfa hay reflected increased fiber digestibility because the acetate is the primary product of cellulolytic bacteria (Lu et al., 2005). The significantly increased valerate concentration happened in the starter feeding group, which demonstrated that more nutrients could be provided to increase growth performance and organ development (Orskov, 2012).

Both dietary and additives could influence the abundance of ruminal microbiota (Abecia et al., 2014), while the abundance of the microbiota also affects the use of the host's utilization of the diet (Carberry et al., 2014). *Bacteroidetes* and *Firmicutes* are the most dominant phylum in yak calves' rumen, which

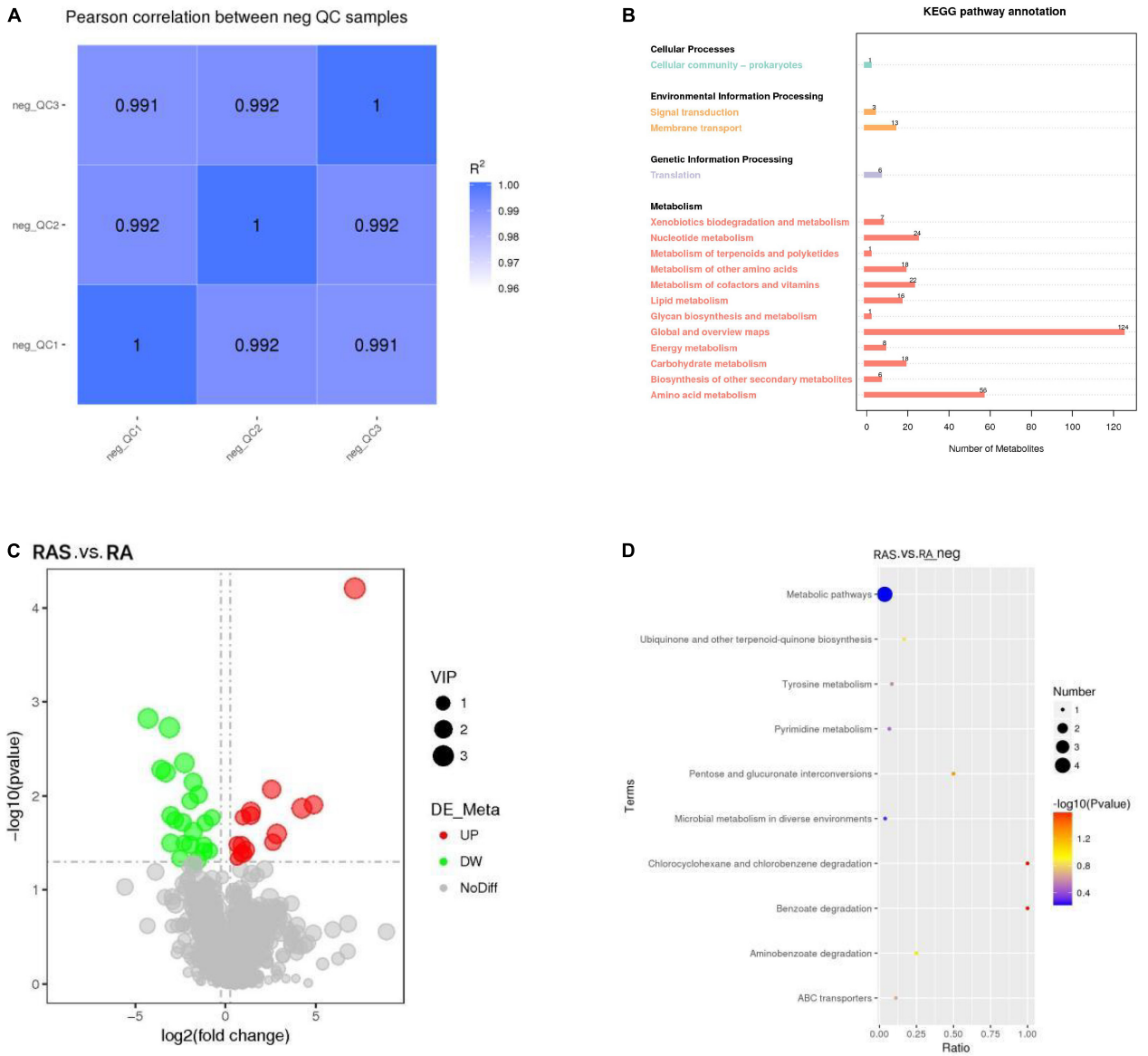

FIGURE 2 | (A) QC samples correlation analysis; **(B)** The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation; **(C)** The volcano plot of difference metabolites; **(D)** enrichment of differential metabolites in KEGG pathways. Rich factor, ratio of the proportion of differential metabolites to the proportion of all metabolites in the pathway; the size of the dots in the graph represents the number of distinct metabolites enriched in the corresponding pathways.

are consistent with the previous studies on ruminants (Shen et al., 2017; Wu et al., 2021). The depressed ratio of *Firmicutes* vs. *Bacteroidetes* in the rumen resulted in increasing lignocellulose digestion (Mu et al., 2019). Similar to other studies (Fernando et al., 2010; Iqbal et al., 2017), the dominant phylum was identified in this study, including *Euryarchaeota* and *Proteobacteria*. Through the correlation analysis, *Proteobacteria* and *Synergistota* were significantly positive with the NH_3-N concentration, and these three indicators were also significantly higher in the starter feeding group, which indicated that starter feeding can promote ruminal microbiota to produce available NH_3-N and strengthen the absorption and utilization of nutrients. *Proteobacteria* have the function of degrading soluble carbohydrates; the crude protein and ether extract were much higher in the feeding of the RAS group, along with the significantly increased abundance of *Proteobacteria* in the RAS group. Therefore, we predicted that the abundance of *Proteobacteria* was positive with the crude protein and ether extract content in the dairy feed.

Prevotella was the dominant genus of abundance, which had the ability to degrade fiber sources (Emerson and Weimer, 2017). Members of the genus *Prevotella* in the ruminal microbial community have been reported to play an important role in the utilization of dietary nutrition

TABLE 6 | Differential metabolites in the rumen of yak calves in the RA and RAS groups.

Metabolites	mzmed	rtmed	log2FC	*P*-value	VIP	Regulated
Saccharin	181.992	6.042	7.160	0.000	3.387	Up
6-Keto-prostaglandin f1alpha	369.228	12.182	2.556	0.009	2.381	Up
delta-Tocopherol	401.343	16.697	4.883	0.012	2.348	Up
Pyridoxine *O*-Glucoside	330.120	2.068	4.228	0.014	2.896	Up
2′-*O*-Methyluridine	257.079	5.528	1.411	0.015	2.638	Up
15(S)-HpETE	353.234	12.929	1.405	0.016	2.137	Up
FAHFA (16:1/18:3)	529.424	14.506	0.964	0.017	1.374	Up
Isopentenyladenine	202.110	10.799	2.845	0.025	2.731	Up
*N*1-isopropyl-2-(1H-2-pyrrolylcarbonyl)-1-hydrazinecarboxamide	419.212	9.289	2.629	0.031	1.717	Up
*N*7-Methylguanosine	298.115	1.361	0.638	0.033	1.566	Up
Pepstatin	684.455	15.539	0.884	0.033	2.031	Up
(+/-)8(9)-DiHET	337.239	13.317	1.122	0.038	2.039	Up
Xylitol	151.061	1.426	0.862	0.041	1.692	Up
(+/-)9,10-dihydroxy-12Z-octadecenoic acid	313.239	12.924	0.962	0.041	2.172	Up
Cinnamoylglycine	204.067	8.755	0.639	0.046	1.025	Up
3,5-Dihydroxybenzoic acid	153.020	7.550	−4.292	0.001	2.951	Down
(3R)-8-hydroxy-3-(4-methoxyphenyl)-3,4-dihydro-1H-2-benzopyran-1-one	269.083	9.621	−3.107	0.002	3.140	Down
2-Hydroxyhippuric acid	194.046	7.134	−2.277	0.004	2.801	Down
FAHFA (2:0/23:0)	411.349	16.143	−3.557	0.005	2.496	Down
Hydroquinone	109.030	7.551	−3.315	0.006	2.674	Down
D-Glucosyl-beta-1,1-*N*-palmitoyl-D-erythro-sphingosine	698.560	16.209	−1.790	0.007	2.303	Down
3-(methylsulfanyl)-5H-[1,2,4]triazino[5,6-b]indole	215.039	10.572	−1.497	0.010	2.102	Down
PC (15:1/18:2)	800.546	16.225	−1.955	0.011	1.749	Down
Isorhapontigenin	257.082	10.283	−3.028	0.016	2.153	Down
FAHFA (2:0/18:1)	357.265	13.102	−0.751	0.017	1.655	Down
1-(2,4-dihydroxyphenyl)-2-(3,5-dimethoxyphenyl)propan-1-one	301.108	7.583	−2.793	0.018	1.856	Down
2-Furoylglycine	168.031	5.992	−2.379	0.019	2.147	Down
PE (16:0/16:0)	690.509	14.288	−1.134	0.019	1.724	Down
D-Glucuronic acid	193.036	12.638	−1.792	0.024	2.331	Down
PG (20:0/20:4)	825.567	15.518	−3.036	0.032	2.263	Down
PG (20:0/20:3)	827.584	16.042	−2.299	0.032	1.490	Down
13,14-dihydro Prostaglandin F1α	393.243	14.550	−1.955	0.033	2.025	Down
PG (18:1/22:4)	823.552	15.392	−1.183	0.033	1.623	Down
3-methyl-4-[2-(2-methylphenyl)hydrazono]-4,5-dihydro-1H-pyrazol-5-one	215.093	1.915	−0.860	0.038	1.362	Down
UMP	323.029	1.460	−1.210	0.039	2.018	Down
*N*1-[4-(cyanomethyl)phenyl]-4-chlorobenzamide	269.046	10.375	−2.514	0.046	1.759	Down
Catechin	289.072	7.111	−1.567	0.049	2.133	Down

FC, fold change; mzmed, mass-to-charge ratio of metabolites; rtmed, retention time of metabolites; VIP, variable importance in the projection. Regulated "up" represents a higher abundance in calves in the RAS group, "down" represent a higher abundance in calves in the RA group.

(Latham et al., 2018). In this study, the abundance of *Prevotella* was significantly negative with isobutyrate, and the abundance of *Prevotella* was significantly increased in the RA group, due to the higher neutral detergent fiber and acid detergent fiber level in the alfalfa hay. Part strains of *Butyrivibrio* degraded cellulose in the rumen, which also shows that the abundance of *Butyrivibrio* increased in the RA group. The abundance of *Rikenellaceae_RC9_gut_group* was significantly enriched in the RAS group and positive with the NH_3-N and valerate concentration, which was consistent with the result of rumen fermentation. The relative abundance of *UCG-002* was negative with the propionate, butyrate, valerate, and isovalerate concentration, which could be resistant to the growth performance when propionate was absorbed and converted to glucose, amino acids, and lipids (Orskov, 2012). Very few studies referred to the significant valerate concentration decrease in the RAS group, with completely unknown and unexplored functions in ruminal physiology.

In brief, the colonization of ruminal microbiota during preweaning could further influence the subsequent ruminal microbiota of adult ruminants (Ben Salem et al., 2005; Kim et al., 2016). Specifically, the effect of differentially supplementing carbohydrates in the early life of ruminants has been demonstrated (Khan et al., 2016; Meale et al., 2016). Therefore, starter feeding yak calves in early life was beneficial to the digestion and absorption function of yaks both during

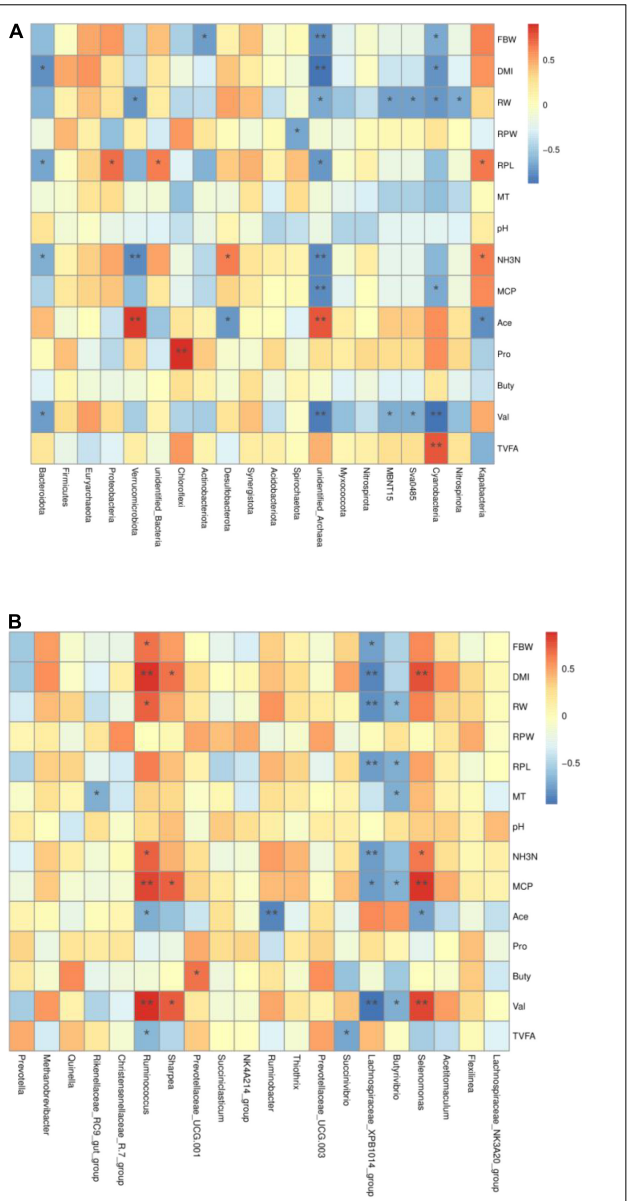

FIGURE 3 | Heatmap of the correlation between microbiota [**(A)** phylum and **(B)** genus] and fermentation parameters in the rumen of yak calves. RBW, finally body weight; DMI, dry matter intake; RW, ruminal weight; RPW, ruminal papilla width; RPL, ruminal papilla length; MT, muscle thickness; NH3N, NH_3-N; MCP, microbiota crude protein; Ace, acetate; Pro, propionate; But, butyrate; Ibu, isobutyrate; Val, valerate; Iva, isovalerate.

preweaning and in the subsequent adult period, which could be helpful for the growth of yaks.

The alteration in the ruminal metabolites we discovered as a result of feeding starter was not surprising since alteration of the ruminal microbial community generally affects the types of compounds produced by the rumen microbiota. We identified 37 different metabolites using the currently available metabolite databases.

Ruminal metabolites in pathway analysis expressed the difference in yak calves, and based on the *t*-test and FC analysis, nine pathways were revealed: chlorocyclohexane and chlorobenzene degradation, benzoate degradation, pentose and glucuronate interconversions, aminobenzoate degradation, ubiquinone, and other terpenoid-quinone biosynthesis, ABC transporters, tyrosine metabolism, pyrimidine metabolism, and microbial metabolism in diverse environment pathways. All relevant pathways were significantly upregulated in the starter feeding group due to increased concentrations of the associated metabolites xylitol and delta-tocopherol and decreased UMP and hydroquinone. This study has found that ruminants can meet up to 70% of their energy requirements and 50–70% of their protein requirements through rumen microbial metabolic activities (Wu et al., 2021). Xylitol, as an intermediate of sugar metabolism, is mainly involved in carbohydrate metabolism. In the case of the decreased insulin level in the body, xylitol is transported across the membrane to participate in the generation of liver glycogen and provide energy for the body cells (Di Rienzi and Britton Robert, 2020; Meyer-Gerspach et al., 2021). In the metabolic process of the body, xylitol is utilized by microorganisms and converted into xylulose, which enters the pentose phosphate pathway and generates short-chain fatty acids (propionate) to ensure the smooth metabolic pathway of pentose and glucuronic acid conversion (Uebanso et al., 2017). In this study, xylitol, a differential metabolite of yak calves, was significantly upregulated after supplying with starter feeding, indicating that starter feeding is helpful with propionate metabolism and thus improves the growth and development of yak calves. Hydroquinone was significantly downregulated, resulting in significant degradation of cyclochloroethane and chlorobenzene as well as benzoic acid degradation pathway, indicating that the metabolic pathway of xenobiotic degradation was significantly changed after starter feeding supplementation. UMP is a short uridine acid, consisting of uracil base, phosphate group, and ribose, it can promote the biosynthesis of glucuronic acid, uridine acid, and its derivatives play a key role in host immune response and metabolic regulation (Zhang et al., 2014). In feed production, the use of the internucleotide combination, including UMP, can promote the growth and development of animals and improve the immune function of the body (Grimble and Westwood, 2001). In this study, the metabolites of yak calves were significantly changed after the early intake of concentrate, which also caused changes in related metabolic pathways, further suggesting that early supplementation of concentrate can improve the growth performance of calves by changing rumen metabolites.

The relative abundance of *Bacteroidota* significantly decreased with the increase of DMI and showed a positive correlation with *Proteobacteria*. *Bacteroidota*, as fiber-degrading bacteria (Zapata and Quagliarello, 2015), were significantly higher in the rumen of yak calves fed only on alfalfa hay, while the nutritional monoculture and poor palatability of herbage resulted in a decrease in DMI of yak calves. *Proteobacteria* can use polysaccharides as a nutrient source for ruminants (Zimmermann, 1990), ruminal papilla length was significantly positively correlated with *Proteobacteria*, which may be due to the increased *Proteobacteria* abundance after starter feeding supplementation, which improves nutrient utilization efficiency of yak calves, promotes ruminal papilla development of yak

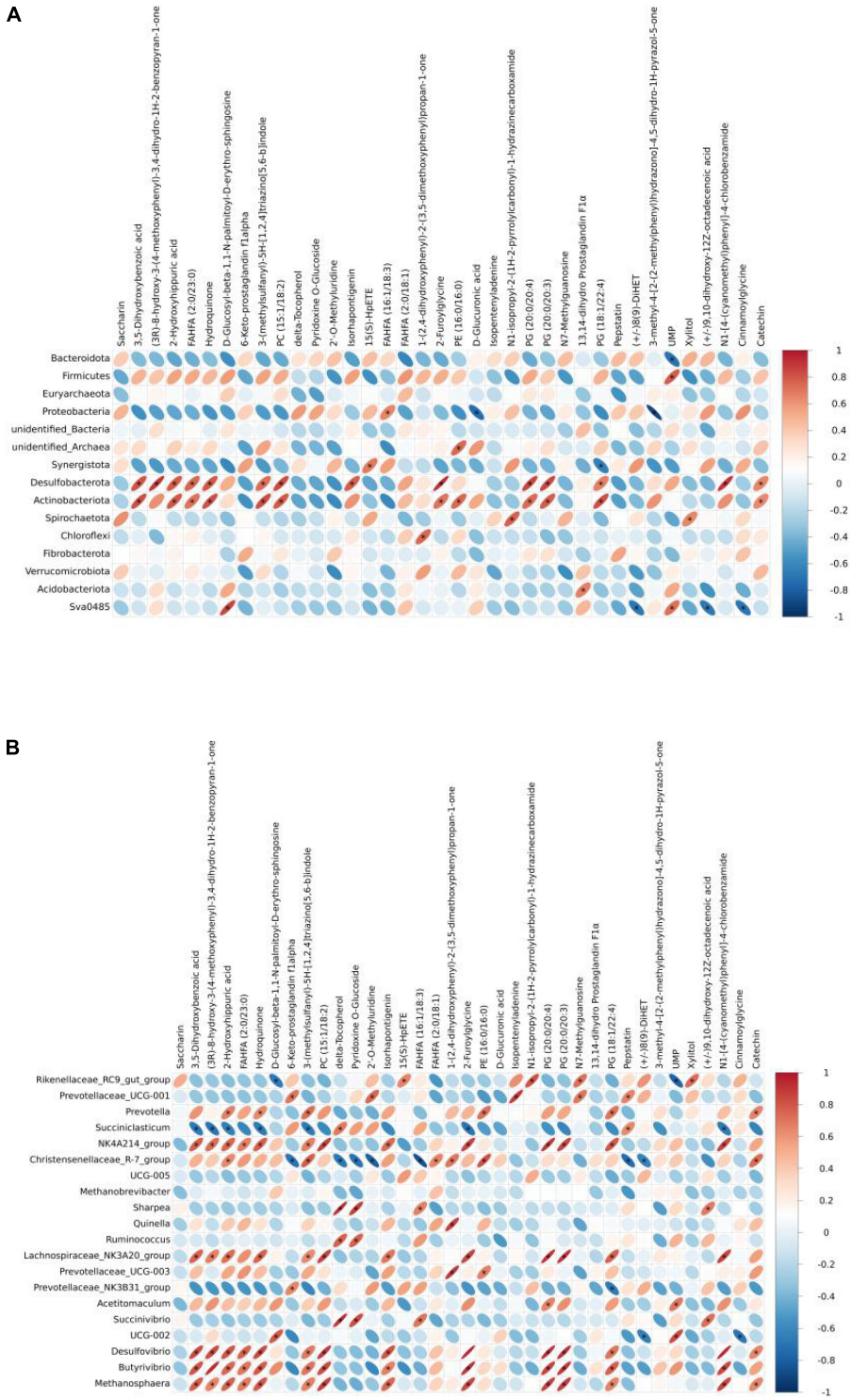

FIGURE 4 | Heatmap of the correlation between ruminal microbiota [**(A)** phylum and **(B)** genus] and metabolites of yak calves.

calves, and thus improves production performance. Studies have shown that *Ruminococcus*, as a producer of carbohydrate-active enzymes (Rosewarne Carly et al., 2012), can decompose dietary fiber and improve the decomposition of hemicellulose and dietary fiber (Wang L. et al., 2019). The final body weight, DMI, and concentrations of NH_3-N, MCP, and valerate in the rumen were proportional to the relative abundance of *Ruminococcus*, suggesting that starter feeding may improve the

relative abundance of *Ruminococcus* and lead to the improvement of body growth performance.

Ruminal microbial interaction can decompose carbohydrate carbohydrates such as starch and fructose in the diet that cannot be directly absorbed by the digestive tract into monosaccharides or disaccharides. After microbial fermentation, glycogen is resynthesized and then transported to the intestine for decomposition and utilization in the small intestine, which is one of the important pathways for body function (Zhao et al., 2018). In ruminants, there are a large number of ciliates and other fiber-degrading bacteria in the rumen, and a large amount of cellulose in herbages is decomposed into short-chain fatty acids under the action of ruminal microbial fermentation, providing carbon skeleton for the synthesis of microbial proteins in the rumen and participating in the body circulation pathway (Gill and King, 2002; Zhang et al., 2017). Ruminal microbial community response changes with the change of dietary nutrient composition, which affects the types of small molecule metabolites in the digestive tract, and even threatens the immunity and health of the body (Yang and Duan, 2018). Therefore, exploring the relationship between ruminal microbiota and metabolomics is helpful to reasonably improve the diet structure and early prevention of diseases. In the correlation analysis, the metabolite UMP was positively correlated with microbial *Bacteroidota* in the rumen, and negatively correlated with *Firmicutes*. In the 16s rRNA sequencing, the relative abundance of *Bacteroidota* in yak calves fed with starter feeding significantly decreased, and the difference metabolite UMP significantly decreased, and the trend was consistent. In the functional analysis of differential metabolites, UMP was involved in the metabolism of pyrimidine in the nucleotide metabolic pathway. At the genus level, delta-tocopherol was significantly positively correlated with *Ruminococcus* and *Sharpea*, while the *Ruminococcus* was significantly increased after the yak calves were fed with starter feeding, and the metabolite delta-tocopherol was also significantly upregulated, which promoted the metabolism of coenzyme factors and vitamins. Therefore, UMP and delta-tocopherol can be used as potential markers for exploring the dominant ruminal microbiota. Studies have shown that the correlation between ruminal microbiota and metabolomics plays an important role in the treatment of ruminant ruminal acidosis and other diseases, and there is a direct or indirect correlation between microbiota candidate metabolomics (Mao et al., 2016; Xue et al., 2020). Wang changed the diet structure of dairy calves and found that the changes in the ruminal environment were caused by microbial community structure and metabolites (Wang B. et al., 2019). Pickard used high-throughput sequencing to detect the activity of intestinal microbiota and suggested that when metabolomics was associated with the abundance of microbiota, in-depth exploration of the correlation mechanism between the two had important advantages for monitoring the physiological state of the body and preventing diseases (Pickard Joseph and Chervonsky Alexander, 2015). How ruminal microbiota and metabolomics affect and associate with each other remains to be further explored.

The untargeted metabolomics approach depends on comparing peak intensity to evaluate differences in the relative abundance of metabolites with the disadvantage of a lack of accuracy and precision (Veenstra Timothy, 2012). Furthermore, identifying metabolites accurately is a tough challenge due to the complexity and chemical diversity of the metabolome (Aretz and Meierhofer, 2016). Another limitation is the small number of yak calves in similar conditions. Even with these limitations, our study enhances the understanding of the effects of starter feeding and confirms the usefulness of 16S rRNA sequencing and untargeted metabolomics analyses in ruminant nutrition studies, especially yak calves.

CONCLUSION

In summary, a supplement to the starter promoted organ development and increased the abundance of some amylolytic bacteria. Ruminal microbiota have positive associations with metabolites involved in carbohydrate metabolism, nucleotide metabolism, xenobiotic biodegradation, and metabolism, demonstrating that the starter feeding can improve the nutritional status of the yak calves.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. These data can be found here: https://dataview.ncbi.nlm.nih.gov/object/PRJNA808822, BioProject: PRJNA808822.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of College of Agriculture and Animal Husbandry in Qinghai University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

YW, SL, and ZC conceived and designed the experiments. YW, ZC, SL, HX, QY, and DY mainly performed the experiments. YW and ZC analyzed the data. ZC and SL contributed the reagents, materials, and analysis tools, had primary responsibility for final content. YW and ZC wrote the manuscript. All authors read and approved the final manuscript.

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Temporal Changes in Fecal Unabsorbed Carbohydrates Relative to Perturbations in Gut Microbiome of Neonatal Calves: Emerging of Diarrhea Induced by Extended-Spectrum β-lactamase-Producing Enteroaggregative *Escherichia coli*

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Early gut microbiota development and colonization are crucial for the long-term health and performance of ruminants. However, cognition among these microbiota is still vague, particularly among the neonatal dairy calves. Here, extended-spectrum β-lactamase-producing enteroaggregative *E. coli* (ESBL-EAEC)-induced temporal changes in diversity, stability, and composition of gut microbiota were investigated among the neonatal female calves, with the view of discerning potential biomarkers of this arising diarrhea cases in local pastures. Nearly, 116 newborn calves were enrolled in this time period study during their first 2 weeks of life, and a total of 40 selected fecal samples from corresponding calves were used in this study. The results revealed that differentiated gut microbiome and metabolome discerned from neonatal calves were accompanied by bacterial infections over time. Commensal organisms like *Butyricicoccus*, *Faecalibacterium*, *Ruminococcus*, *Collinsella*, and *Coriobacterium*, as key microbial markers, mainly distinguish "healthy" and "diarrheic" gut microbiome. Random forest machine learning algorithm indicated that enriched fecal carbohydrates, including rhamnose and N-acetyl-D-glucosamine, and abundant short-chain fatty acids (SCFAs) existed in healthy ones. In addition, Spearman correlation results suggested that the presence of *Butyricicoccus*, *Faecalibacterium*, *Collinsella*, and *Coriobacterium,* key commensal bacteria of healthy calves, is positively related to high production of unabsorbed carbohydrates, SCFAs, and other prebiotics, and negatively correlated to increased concentrations of lactic acid, hippuric acid, and α-linolenic acid. Our data suggested that ESBL-EAEC-induced diarrhea in female

calves could be forecasted by alterations in the gut microbiome and markedly changed unabsorbed carbohydrates in feces during early lives, which might be conducive to conduct early interventions to ameliorate clinical symptoms of diarrhea induced by the rising prevalence of ESBL-EAEC.

Keywords: enteroaggregative *E. coli*, extended-spectrum β-lactamase producing *E. coli*, neonatal dairy calves, unabsorbed carbohydrates, gut microbiome, metabolome

INTRODUCTION

Young ruminants are susceptible to diarrhea during their early lives, which is mainly induced by pathogenic *E. coli*, *Cryptosporidium*, Rotavirus, Coronavirus, and certain remarkable changes in the environmental factors (Cho and Yoon, 2014). Recent developments in the pathogenesis of ESBL-EAEC highlight the disorder of the host immune system, associated with the production of extrinsic toxins, adhesins, and siderophores, and gut microbiota dysbiosis (Chevalier et al., 2021). Indeed, increasing evidence has emphasized the long-time impact of early-life gut microbiome structures on the gut health status of adults (Kerr et al., 2015). Besides, efficient blockage of bacterial adhesion to the surface of intestinal epithelial cells is an effective strategy to control the rising ESBL-EAEC infection cases (Boll et al., 2020). Unfortunately, antimicrobials are still widely used for both prevention and treatment of common infectious pathogens, such as EAEC, in food-producing animals during their early lives (Mathew et al., 2007; Yang et al., 2017), which consequently induce perturbations in the diversity of gut microbiota and produce long-term adverse impacts, including rising susceptibility to pathogens, immunological defects, and incidence of multi-drug resistance (MDR) (Zeissig and Blumberg, 2014; Bakkeren et al., 2019). Indeed, the consumption of rational antibiotics is a powerful tool for disease control; however, the overuse of antibiotics is more cost-effective (Cho and Yoon, 2014). Thus, tracking the diarrheic onset following the changes in the gut microbiome supports early interventions in dairy calves, thus leading to a cut down in antibiotic usage. Previous findings based on neonatal dairy veal calves have predicted temporal changes in the gut microbiota of diarrheal calves (Ma et al., 2020). Therefore, we deduced that the composition of the early-life gut microbiome in these female neonatal calves could reflect the intestinal health status and thus indicate the onset of diarrhea induced by ESBL-EAEC infection.

Actually, temporal changes in the gut metabolome are correlated to perturbations in the gut microbiome of neonatal calves, such as the production of SCFAs and unabsorbed carbohydrates. The SCFAs are mainly produced by colonic bacterial fermentation of unabsorbed carbohydrates and dietary fiber (Cummings et al., 1987). The total SCFA concentrations in the hindgut are significantly different depending on the calf age, considering that total SCFA production increases from day 7 onward, with the highest concentration observed on day 21 (Song et al., 2018). Different fermentation substrates produce different types of SCFAs, and the unabsorbed sugar L-rhamnose mostly yields propionate (Darzi et al., 2016). Actually, L-rhamnose is a natural monosaccharide that is widely found in foods, such as carrots, cabbage, and oranges (Cummings and Englyst, 1987). It cannot be absorbed in the small intestine until it reaches the colon in humans and animals (Byrne et al., 2018). Interestingly, fermentation of L-rhamnose by the rumen bacteria significantly reduces the rate of methanogenesis and upregulates the production of SCFAs (Reinhardt et al., 2019). Among these bacteria, previous studies have shown that various *Clostridial* species are involved in the fermentation of L-rhamnose (Petit et al., 2013), and specific species are able to produce acetic acid, propionic acid, butyric acid, 1,2-propanediol, and *n*-propanol when L-rhamnose is used as the sole carbon and energy source (Diallo et al., 2019). Besides, it is an important ingredient of surface-associated exopolysaccharide (EPS) in many probiotics, thus mediating the displacement of pathogenic organisms through the competitive occupancy of adhesion sites and stimulation of the immune system (Guglielmetti et al., 2010; Hidalgo-Cantabrana et al., 2012).

Herein, we used a female calf as an animal model to represent young ruminants, and aimed to detect core intestinal flora and fecal metabolites post-ESBL-EAEC infection. We hypothesized that ESBL-EAEC infection could induce perturbations in the gut microbiome and temporal changes in the fecal metabolome, particularly with regard to the relative abundance of unabsorbed carbohydrates. Based on the previous publications and existing data, we inferred that alterations in ceratin specific commensal microbiota and fecal metabolite content could be exploited as potential biomarkers for diarrhea induced by ESBL-EAEC infection. In addition, we tried to shed light on the potential hazards and risks associated with emerging ESBL-EAEC infections in young calves, and the alterations in the gut microbiota and fecal metabolome among healthy and diarrheic calves could be employed as predictive biomarkers for diarrhea induced by ESBL-EAEC infection.

MATERIALS AND METHODS

Bacterial Strains and Bacterial Culture

The identified ESBL-EAEC strain 1587 was isolated from our previous clinical trial and was found to be prevalent in the pasture of this study. Genomic information and antibiotic susceptibility of the strain are presented in **Supplementary Tables 2**, **3** (He et al., 2021). Except for specific instructions, all isolates from fecal samples were incubated on MacConkey agar plates (Luqiao company, Beijing, China) or Luria-Bertani broth (LB, Qingdao Hope Bio-technology) at 37°C. Antibiotics were obtained from the China Institute of Veterinary Drug Control. All *E. coli* isolates were screened for the phenotypic identification of ESBL

producers on MacConkey agar containing cefotaxime (2 mg/L), and further confirmation was made using double-disc synergy testing in accordance with CLSI recommendations. Isolates were considered positive when the clear zone of inhibition produced by ceftazidime plus clavulanic acid or cefotaxime plus clavulanic acid was at least 5 mm larger than their respective single disks (CLSI, 2016).

Calf Health Status Assessment and Fecal Sample Collection

Neonatal female Holstein dairy calves sourced from conventional cow pasture in north China and free from antibiotic treatments were selected for this time period study. All animals were transferred to the separate calf hutches after birth to avoid direct contact, and health conditions were appraised daily to ensure that they were free from disease, injury, and dehydration. The animal experimental protocols were approved by Beijing Association for Science and Technology (ID no. SYXK, 2016-0008). The calves were fed 4 L of colostrum during post-natal care service. The general appearance, fecal score, and respiratory score were recorded according to the previously published methods (Villot et al., 2019). Briefly, a total of 116 neonatal calves were included in our trial, and they were drived to separate fences in 2 days old after birth. They were bucket-fed with 4.5 L of colostrum daily during the first 2–7 days (phase I) and 5.5 L during 8–14 days (phase II). The milk replacer contained 67% of skim milk powder with 260 g/kg crude protein, 160 g/kg crude fat, 10 g/kg crude ash, and 19.2 MJ/kg metabolizable energy on a dry matter basis (Nutrifeed, IN, Netherlands). All these calves were the offsprings of the same litter where feeding multiparous cows of 2 to 3 parity. Besides, the average birth weight of all these calves was 37 kg with a similar body measurement index (rump height, thurl width, and body trunk index). Calves with diarrhea were identified over the time period and were defined as the "diarrhea" group (fecal score was $\geq$3 for at least 2 days and positive for only ESBL-EAEC) (Lesmeister and Heinrichs, 2004). The remaining calves of the same time period were classified as the "healthy" group (fecal score was $\leq$2 for at least 2 days and free from main pathogens) (**Supplementary Table 1**). No calves were treated with antibiotics during this trial. Appropriate equipment and sterile gloves were used for the collection of rectal fecal samples ($\sim$10 g) to prevent cross-contamination. Initially, all fecal samples were subjected to the detection of ESBL-EAEC isolates according to a previous method (He et al., 2021) and were further screened using the culture methods mentioned earlier. The common genes encoding ESBL and EAEC were tested by PCR as described previously with minor modifications and further confirmed by sequencing analysis (Dallenne et al., 2010). Then, the positive and negative samples were further tested for the presence of Rotavirus, Coronavirus, and *Cryptosporidium* antigens using commercial ELISA kits, and all the three negative samples were chosen for further analysis (Feldmann et al., 2019). Finally, 40 fecal samples (positive for ESBL-EAEC and negative for Rotavirus, Coronavirus, and *Cryptosporidium* antigens) were enrolled in phase I (9 healthy: H_1 and 11 diarrheal: D_1) and phase II (11 healthy-H_2 and 9 diarrheal-D_2) trials over the entire study period. All the collected fecal samples were immediately stored at -80°C for gut microbiome and metabolome analyses.

DNA Extraction, PCR Amplification, and 16S rRNA Gene Sequencing

The respective bacterial genomic DNA was extracted from 40 fecal samples, phase I (9 healthy-H_1 and 11 diarrheal-D_1) and phase II (11 healthy-H_2 and 9 diarrheal-D_2), using QIAamp DNA Isolation Kit (Qiagen, Hilden, Germany). Amplicons of V3-V4 hypervariable regions of the 16S rRNA gene were amplified using optimized primers (338F: 5′-ACTCCTACGGGAGGCAGCAG-3′, 806R: 5′-GGACTACHVGGGTWTCTAAT−3′). The amplicon DNA with an optimal size ($\sim$450 bp) was purified on 1.2% agarose gel using a QIAquick PCR purification kit (Qiagen Science, MD). The quality and quantity of purified PCR products were checked using Quant-iT PicoGreen dsDNA Assay Kit (Microplate reader, BioTek, FLx800) to ensure that all DNA concentrations were above 25 ng/μl. For the Illumina Miseq sequencing, the PCR product library was prepared using the TruSeq Nano DNA LT Library Prep Kit (Illumina), following sequencing on the Illumina Miseq platform (2 $\times$ 300, pair-end).

Gut Microbiota Profiling

The paired sequences were denoised, quality-filtered, and merged using the DADA2 plugin (version 3.11) to obtain the amplicon sequence variants (ASVs) feature table (Callahan et al., 2016). Taxonomic classification was performed using q2-feature-classifier (QIIME2 microbiome analysis platform, version 2020.02) (Bolyen et al., 2019). Taxonomy was assigned to filtered ASVs using a pretrained QIIME2-compatible SILVA version 132 database, with 99% identity for the bacteria and representative sequences (Quast et al., 2013). Species diversity was determined using q2-diversity of QIIME2 version 2020.02 (http://www.r-project.org/). Bray–Curtis, Jaccard, unweighted UniFrac, and weight UniFrac outputs were assessed and visualized using unsupervised PCoA analysis to contrast bacterial communities between groups using the "ggplot2" package of the R software (version 3.3.1) (http://www.r-project.org/). Differences between the groups were determined using PERMANOVA, ANOSIM, and PERMDISP, with 999 Monte Carlo permutations in the "vegan" package in R software. Differentially abundant genera were identified by performing linear discriminant analysis (LDA) effect size (LEfSe) after analyzing all features using Kruskal–Wallis test and checking whether all the pairwise comparisons between subclasses within different classes significantly agree with the class level trend using the pairwise Wilcoxon test (http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=lefse_upload) (Segata et al., 2011). Alpha values for the Kruskal–Wallis and pairwise Wilcoxon tests were 0.05. A size-effect threshold of 3 on the logarithmic LDA score and average relative abundances >0.01% were used to differentiate the discrepant taxa. Gene family abundance of gut microbial communities was predicted using PICRUSt analysis according to the 16S rRNA gene composition (Langille et al., 2013). The constructed ASV feature table was converted into the PICRUSt format and normalized to 16S rRNA gene

copy number to correct for over- and under-estimation of microbial abundance. For potential functional profiles, the normalized dataset was analyzed using the MetaCyc dataset (https://metacyc.org/) and the KEGG database (https://www.kegg.jp/). The abundance of KEGG Orthology (KO), KEGG enzymes, and pathways was normalized to counts per million reads (CPM) for downstream analysis. To establish the model for predicting diarrhea, random forest algorithms ("Random Forest Classifier" package in QIIME2) were used to identify "healthy" and "diarrheal" microbiota based on the relative abundance of specific bacterial genera. The accuracy and feature importance of specific genera were further analyzed. To minimize the potential over-fitting in the model, the three-fold cross-validation was done till model accuracy was determined for each permutation, and then an overall accuracy was estimated (Yatsunenko et al., 2012).

Untargeted Metabolomic Analyses

Lyophilized fecal samples (5 mg) of healthy or diarrheal calves were homogenated using zirconium oxide beads for 3 min, and 145 μL of extraction solution (containing 25 μL of water and 120 μL of methanol) was further added to extract the metabolites. The samples were homogenated for another 3 min using a high-throughput tissue disruptor and then centrifuged at 1,800 $\times g$ for 20 min. The acquired supernatant was transferred to a 96-well plate and mixed with 20 μL of derivative reagents at 30°C for 60 min, following procedures of Eppendorf epMotion Workstation (Eppendorf Inc., Humburg, Germany). The sample was further diluted with 330 μL of ice-cold 50% methanol, stored at −20°C for 20 min, and followed by centrifugation (4,000 $\times g$ for 30 min at 4°C). The supernatants were subjected to LC-MS analysis.

The extracted metabolites were analyzed using a UPLC-MS/MS system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA, USA). Chromatographic separation was performed using a BEH C18 column (2.1 mm×100 mm, 1.7 μm, Waters). The desolvation and source temperatures were set at 500 and 150°C, respectively. Mobile phases containing acetonitrile/isopropanol (1:1, 0.1% formic acid) and 0.1% formic acid were used as carried liquids at a constant flow rate of 0.4 mL/min.

The acquired raw data were processed using the MassLynx software (version 4.1, Waters, Milford, MA, USA). Each sample was analyzed by UPLC-MS/MS in both positive and negative ionization modes to acquire metabolite profiles. The order of analysis of all test samples was randomized. The quality control (QC) samples were pooled samples in which both the metabolite composition of the samples and sample matrix were mixed, and then analyzed using the same methods to evaluate the quality and variance of the acquired data. Self-developed platform iMAP (version 1.0, Metabo-Profile, Shanghai, China) was used for further statistical analyses of metabolite profiles based on the differences in concentrations between individuals and groups. Bray–Curtis dissimilarity was assessed and visualized using PCoA. The relative concentrations of the metabolites were presented as a heatmap. To establish the model for predicting diarrhea, random forest algorithms ("Random Forest" package in R) were used to identify "healthy" and "diarrheal" microbiota based on the relative abundance of specific metabolites. The accuracy of the selected bacterial genera was then assessed by calculating the area under the receiver-operating characteristic (AUC) ("roc.curve"package) in R. To further minimize the potential over-fitting in the model, a three-fold cross-validation approach ("trainControl" package in R) was applied (Cawley and Talbot, 2010).

Statistical Analysis

The distance in the coefficient of variation (CV) with regard to the relative abundance of genera in calf feces and predicted genes on each day of growth between the two groups of calves were assessed using the nonparametric Kruskal–Wallis test, and multiple comparisons were conducted using Mann–Whitney–Wilcoxon U-test. A significant difference was observed following the interpretation and visualization of the results. For metabolome studies, two types of statistical analyses were extensively performed: (1) multivariate statistical analyses, such as principal component analysis (PCA), partial least square discriminant analysis (PLS-DA), orthogonal partial least square discriminant analysis (OPLS-DA), random forest, and so on; and (2) univariate statistical analyses, including the nonparametric Kruskal–Wallis test, student's *t*-test, Mann–Whitney–Wilcoxon U-test, ANOVA, correlation analysis, and so on. Statistical algorithms were adapted from the widely used software packages for statistical analysis in R studio (http://cran.r-project.org/). STAMP software was applied to detect the differentially abundant KEGG pathways with false discovery rate correction. Spearman correlation coefficient analysis was used to analyze the correlation between gut microbiota and metabolites using the "ggplot2" and "pheatmap" packages of R software (version 3.3.1). All data were presented as mean ± SEM values unless otherwise indicated. Statistical significance was determined for $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

RESULTS

Perturbations in Microbial Diversity and Stability of Gut Microbiota Post-ESBL-EAEC Infection

To obtain more information regarding the impact of ESBL-EAEC infection on neonatal calves, the selected fecal samples were subjected to 16S rRNA gene sequencing to evaluate the diversity, community, and stability of fecal microbiota in different phases, including H_1, H_2, D_1, and D_2 (**Supplementary Table 1**). A total of 4,756,074 high-quality sequences were acquired from 40 fecal samples of 40 corresponding calves after quality control and filtration, and they were assigned to 7,766 ASVs based on a 99% nucleotide sequence similarity. A total of 10 bacterial phyla were shared among the four groups. Among these, Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, and Fusobacteria accounted for a major proportion, while more members of Bacteroidetes and Fusobacteria were found in the healthy groups (**Supplementary Figure 1A**). Simultaneously, the relative abundance of *Coriobacteriaceae*, *Ruminococcaceae*,

Veillonellaceae, *Bacteroidaceae*, and *Lachnospiraceae* was higher in the healthy groups, while the populations of *Bifidobacteriaceae*, *Lactobacillaceae*, and *Streptococcaceae* were enriched in the diarrheal group (**Supplementary Figure 1B**). Among them, remarkably abundant populations of *Collinsella*, *Prevotella*, *Ruminococcus*, *Faecalibacterium*, *Comamonas*, *Butyricicoccus*, *Blautia*, and *Oscillospira* were found in both H_1 and H_2 groups at the genus level (**Figures 1A,B**). In the diarrheal group, at 2–7 days, the relative abundance of *Escherichia-Shigella* tended to be lower, while it increased during 8–14 days of the D_2 phase. The relative abundance of almost all top 50 bacterial genera changed a lot over the whole period, depending on the affecting pathogen and the age of the animal. Besides, a decline in the microbial composition was observed in the D_1 and D_2 groups relative to H_1 and H_2 groups, as shown by Chao1 and Simpson indices (**Figure 2A**). The perturbations in microbial diversity and stability were further investigated via detecting beta diversity according to principal co-ordinate analysis (PCoA) based on weighted UniFrac distance. Indeed, the overall difference in the microbial structure of the diarrheal groups was distinct from that of the healthy ones, particularly the significantly changed distance between H_1 and D_1 groups (**Figure 2B**, $P = 0.002$). Furthermore, the linear discriminant analysis (LDA) effect size (LEfSe) algorithm ranked *Collinsella*, *Faecalibacterium*, *Enterococcus*, *Prevotella*, *Butyricicoccus*, and *Ruminococcus* as the main distinguished bacterial taxa in H_1 calves, with conspicuous *Streptococcus* and *Gallibacterium* in D_1 calves (**Figure 2C**). Similarly, *Collinsella*, *Faecalibacterium*, *Butyricicoccus*, *Coriobacterium*, *Blautia*, and *Ruminococcus* were identified as the differentiated bacteria in the H_2 group and *Bifidobacterium* and *Flavobacterium* in the D_2 group (**Figure 2D**). Besides, the differences between two healthy groups and two diarrheal groups were also detected. The results revealed Actinomycetaceae as the main distinguished taxa in H_2 calves and Micrococcaceae in H_1 calves (**Figure 2E**). Coprococcus, Ruminococcus, Lachnospira, and Aquabacterium were the differentiated bacteria in the D_2 group (**Figure 2F**). The random forest supervised machine learning algorithm was utilized to construct the model linking to the diarrheic prediction of neonatal female calves. A total of 29 genera were included in the differentiation of groups to establish the machine learning model, and this model showed an overall accuracy of 80% for predicting healthy and diarrheal gut microbiota. In the light of three-fold cross-validation, our results demonstrated that the same five bacteria, belonging to the 29 identified genera, did not change among the sampling days, including *Ruminococcus*, *Butyricicoccus*, *Faecalibacterium*, *Collinsella*, and *Coriobacterium*, which mostly resulted in the discrimination power of health status with improved performance (**Figure 3**). The Importance Index was used to plot the relative rank of relative abundances of five microbial markers, and the relative abundances of the five bacteria were found to be higher in the healthy groups. Briefly, these data demonstrated that ESBL-EAEC infection was closely related to the alteration in the structure of the gut microbial community, and the difference in ranks between these taxa suggested that *Gallibacterium*, *Flavobacterium*, *Bifidobacterium*, and *Streptococcus* were more ubiquitous in diarrheal gut microbiota, while *Ruminococcus*, *Butyricicoccus*, *Faecalibacterium*, *Collinsella*, and *Coriobacterium* were more prevalent microbial markers in healthy neonatal female calves relative to other taxa.

Comparison of Temporal Changes in Fecal Metabolome Composition With the Growing Age and Specific Fecal Metabolites Mediating Diarrhea Resistance

The fecal metabolome was further analyzed using untargeted metabonomics to obtain a systematic understanding of the interactions between intestinal epithelium, gut microorganisms, and their associated metabolites. Herein, fecal metabolites of healthy ($n = 20$) or diarrheal calves ($n = 20$) were analyzed with ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) system. Classification of different metabolites indicated that 35.02% of the compounds were amino acids, 23.4% were SCFAs, 21.65% were fatty acids, and 11.35% were carbohydrates (**Figure 4A**). Similarly, the healthy fecal metabolome separated widely from the diarrheal groups based on PLS-DA analysis, considering their allocated groups (Component 1, $P = 2.10e{-}06$; Component 2, $P = 1.63e{-}05$; **Figure 4B**). Unsurprisingly, dispersed data points on the plots of the metabolome were clearly displayed between H_1 and D_1, H_2 and D_2, H_1 and H_2, and D_1 and D_2 groups (**Supplementary Figure 2**). According to the markedly altered metabolites, enriched KEGG pathway analyses were involved with pyruvate metabolism, valine, leucine, and isoleucine biosynthesis, TCA cycle, glycolysis or gluconeogenesis, propanoate metabolism, and α-linoleic acid metabolism (**Figure 4C**). Importantly, the random forest supervised machine learning algorithm was also utilized to construct the model linking to diarrheic metabolome prediction of neonatal female calves. Herein, 10 most prominent fecal metabolites contributed to the discrimination power of health status in the dairy calf, including rhamnose, N-acetyl-D-glucosamine, and butyric acid. The relative rank of the relative abundance of these 10 metabolite biomarkers was plotted against the healthy status represented by the score of MeanDecreaseGini (**Figure 4D**). Among them, various unabsorbed carbohydrates were listed as potential biomarkers on comparison of the healthy calves with the diarrheal calves, which included rhamnose ($P = 1.5e{-}06$), N-acetyl-D-glucosamine ($P = 1.9e{-}06$), and xylose ($P = 1.3e{-}02$) (**Figures 5A–C**). The production of SCFAs correlated with colonic bacterial fermentation of unabsorbed carbohydrates and dietary fiber (Cummings et al., 1987). The relative production levels of fecal SCFAs, such as acetic acid ($P = 3.2e{-}04$), butyric acid ($P = 2.5e{-}06$), and isovaleric acid ($P = 9.3e{-}04$), were also compared in this study (**Figures 5D–F**). In addition, enriched concentrations of lactic acid ($P = 1.9e{-}02$), hippuric acid ($P = 1.3e{-}03$), and α-linolenic acid ($P = 1.3e{-}02$) were found in diarrheal groups (**Figures 5G–I**). Thus, the difference in the ranks of these metabolites and the corresponding production levels

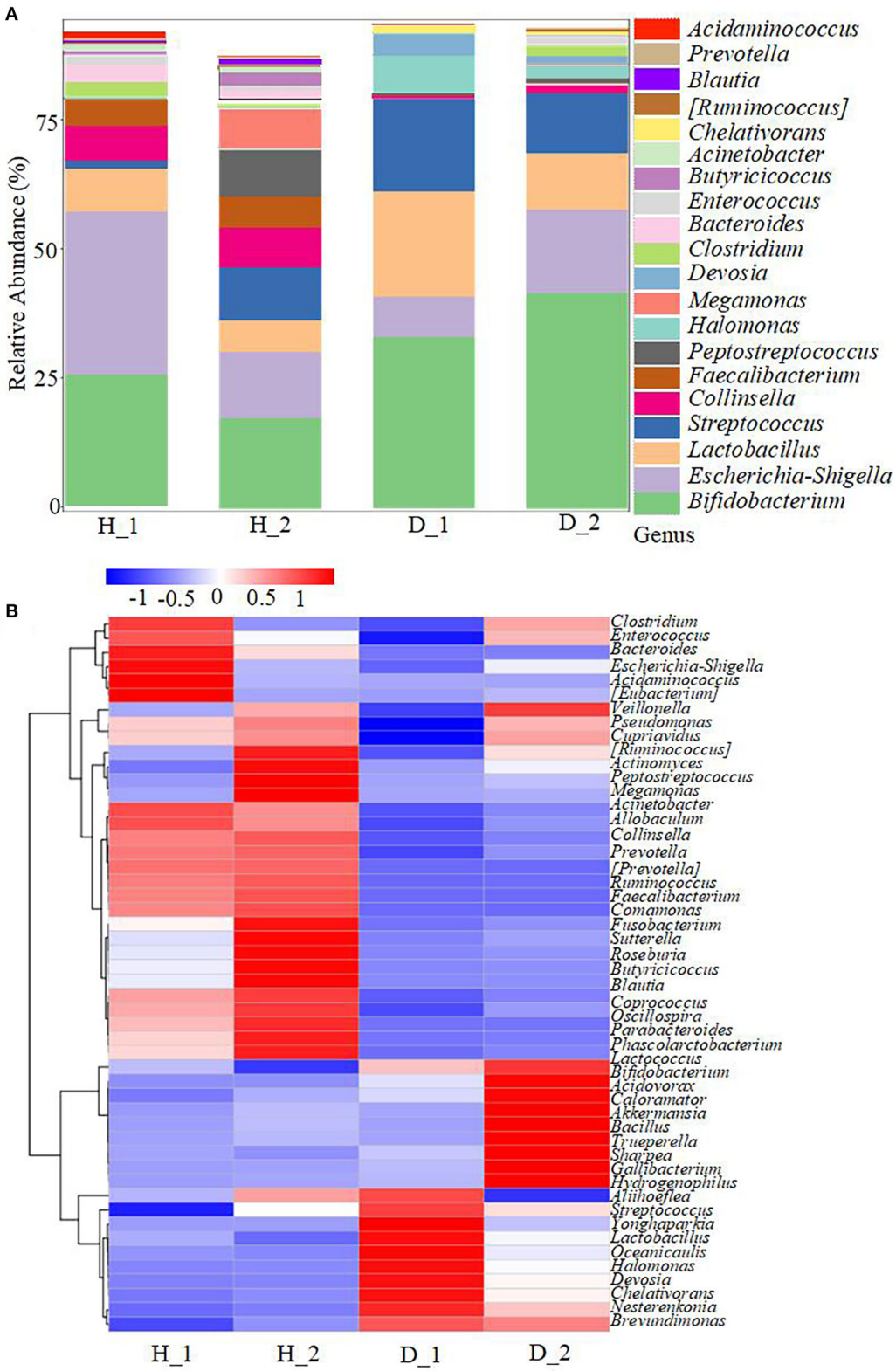

FIGURE 1 | Gut microbiota assembly of neonatal calves post-ESBL-EAEC infection. **(A)** The relative abundance of top 20 bacterial genera in calf feces. **(B)** Top 50 bacteria of fecal samples presented using cluster heatmap analysis in diarrheal (D) or healthy (H) calves. In the graph of species clustering, the default species are UPGMA clustered according to the Pearson correlation coefficient matrix of their constituent data and arranged according to the clustering results. The red color block indicates that the abundance of the genus in this group is higher than in the other groups, while the blue color block indicates that the abundance of the genus in this group is lower than in the other groups. The corresponding relationship between the color gradient and the value is shown in the gradient color block.

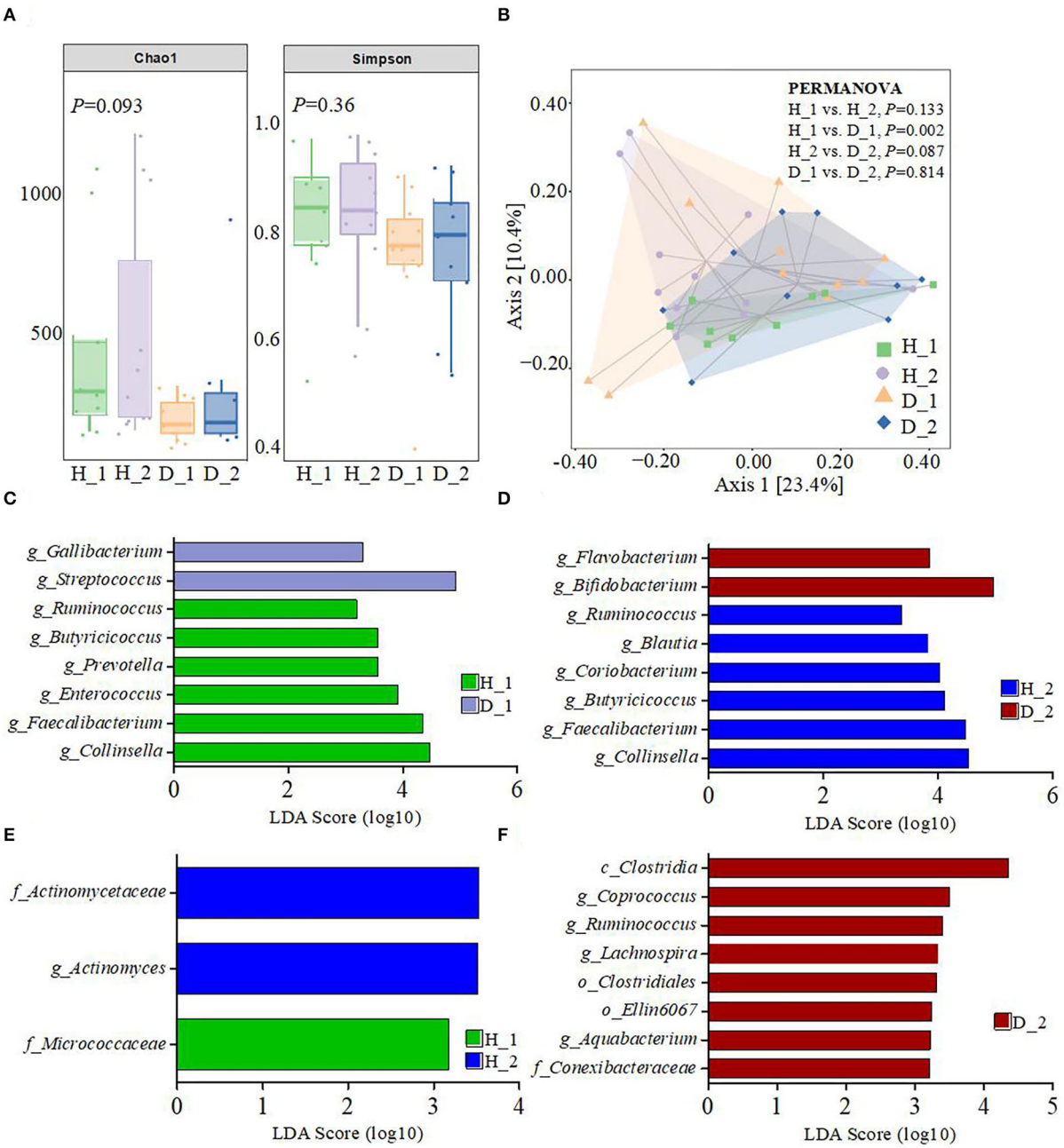

FIGURE 2 | Gut microbiota diversity of neonatal calves post-ESBL-EAEC infection. **(A)** The α-diversity of different groups by Chao1 and Simpson indices. Data were presented as mean ± SEM values. *P*-values were determined using the nonparametric Kruskal–Wallis test. **(B)** Principal coordinate analysis (PCoA) of fecal bacteria was performed based on the weighted UniFrac distance matrix. The statistical tests were accomplished using PERMANOVA, with 999 permutations. The enriched gut microbiota taxa were shown by LEfSe [linear discriminant analysis (LDA) coupled with effect size measurements] of H_1 vs D_1 **(C),** H_2 vs D_2 **(D)**, H_1 vs H_2 **(E)**, and D_1 vs D_2 **(F)**.

revealed that rhamnose, N-acetyl-D-glucosamine, xylose, acetic acid, butyric acid, and isovaleric acid were more prevalent in the healthy fecal metabolome of neonatal female calves, while lactic acid, hippuric acid, and α-linolenic acid were more prevalent in the diarrheal metabolome relative to other detected metabolites.

Correlation Between Bacterial and Metabolite Markers in Disease Onset Prediction in Neonatal Intestine

The correlation of significantly altered metabolites with specific differentiated microbial taxa was revealed directly. Spearman rank correlation analysis indicated a strong positive

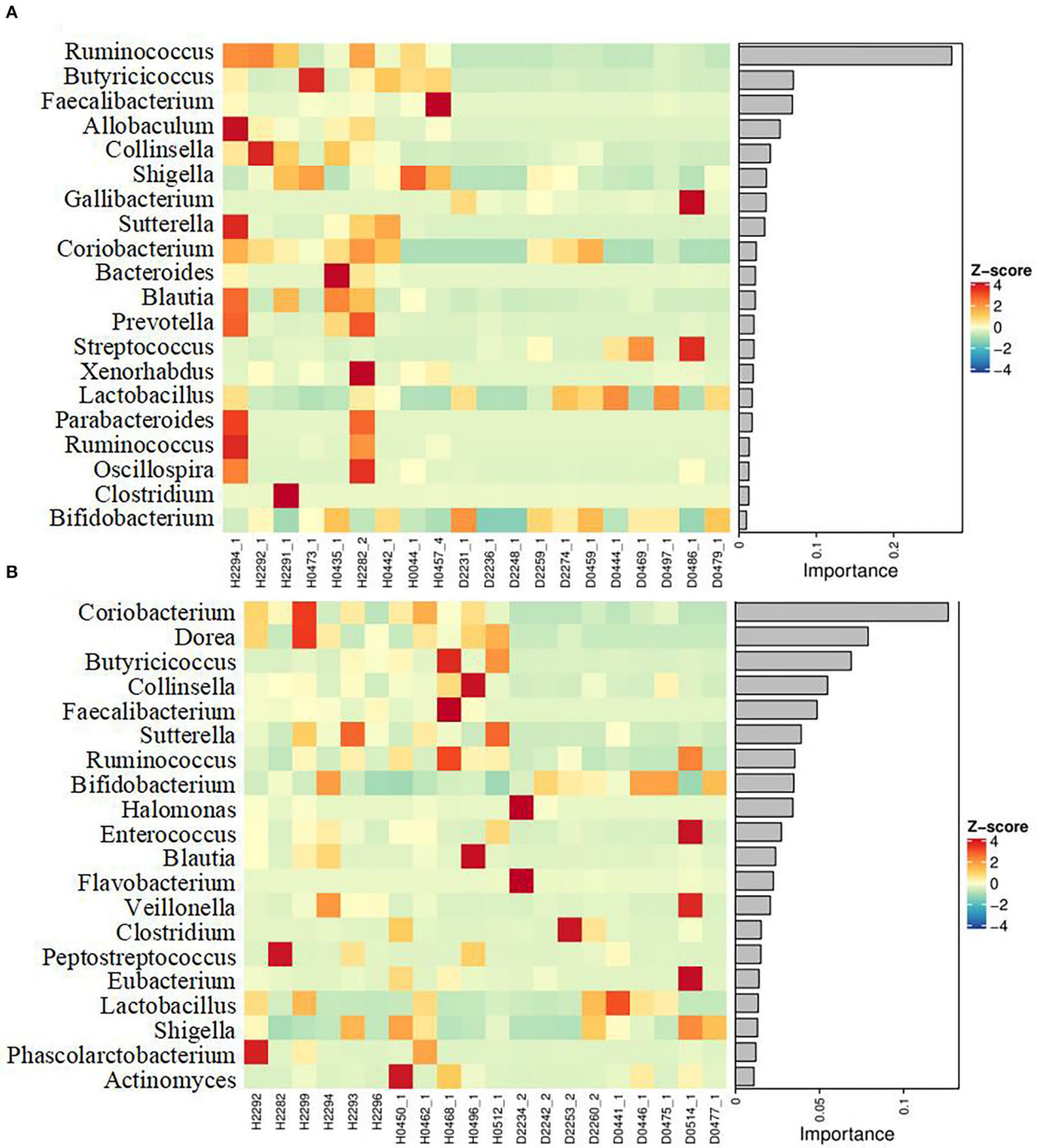

FIGURE 3 | Differentiated genera were displayed using a random forest supervised machine learning algorithm between H_1 vs D_1 **(A)** and H_2 vs D_2 **(B)**. The respective name of the bacterial genus is shown on the left. The relative abundance of genera was clustered using a UPGMA dendrogram and showed in a heatmap. The color indicates the relative abundance of the genus in the group of samples, and the corresponding relationship between the color gradient and the value is shown in the gradient color block. The genus variation is shown using Z-Score. The top 29 genera in the fecal samples were included and the rank values are shown using the Importance Index.

correlation between abundant rhamnose and *Sutterella* ($R > 0.74$, $P = 5.4e{-}08$), *Butyricicoccus* ($R > 0.71$, $P = 3.2e{-}07$), *Faecalibacterium* ($R > 0.68$, $P = 1.4e{-}06$), *Dorea* ($R > 0.60$, $P = 4.0e{-}05$), *Collinsella* ($R > 0.51$, $P = 0.00081$), and *Coriobacterium* ($R > 0.48$, $P = 0.0014$) in H_1 and H_2 groups (**Figure 6**). Similarly, a significantly positive correlation between

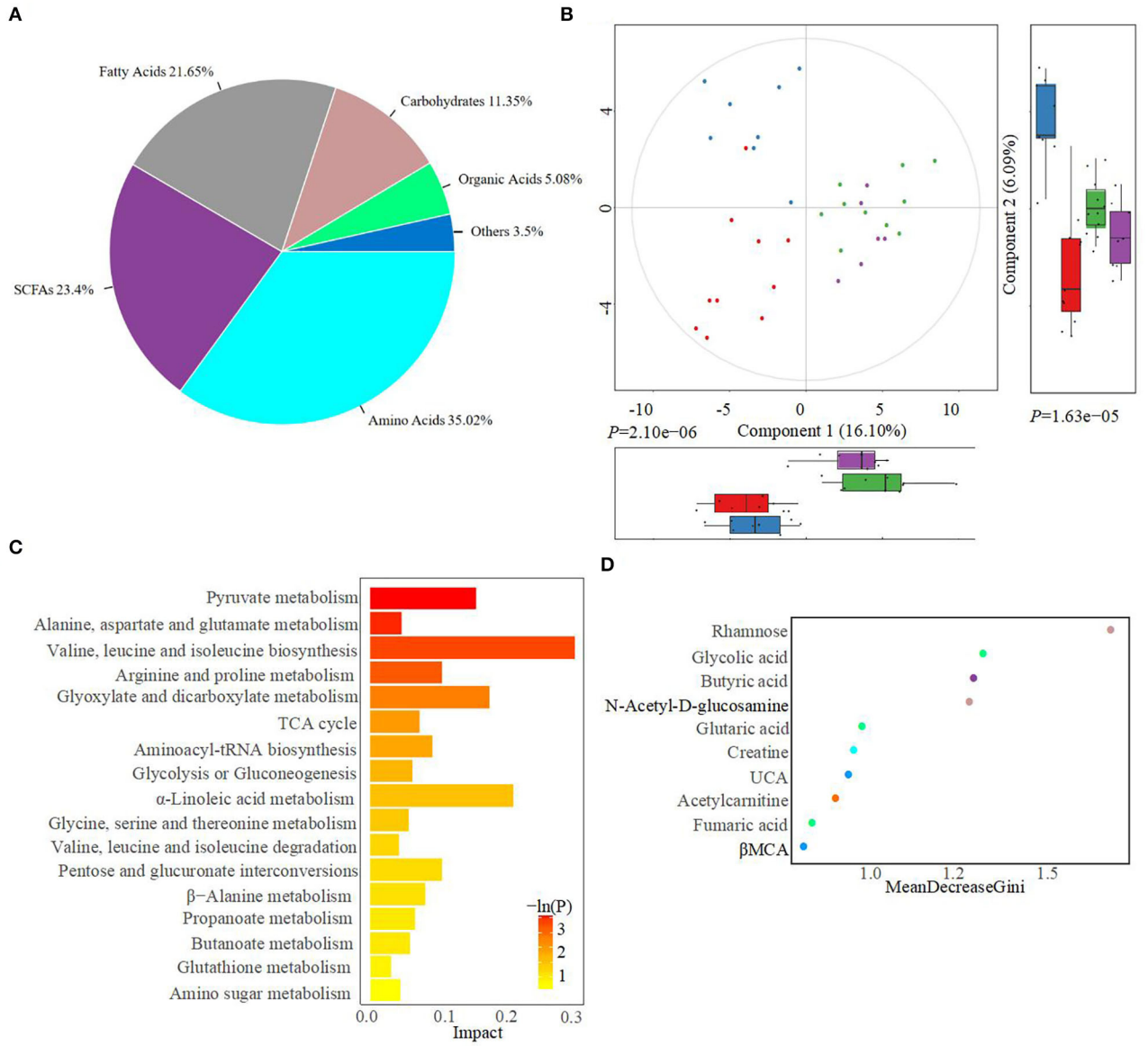

FIGURE 4 | Alterations in the fecal metabolome profiles of neonatal calves post-ESBL-EAEC infection. **(A)** The classifications of total metabolome compounds in H_1, H_2, D_1, and D_2 groups. The total number of significantly changed metabolites in this class is indicated and the corresponding proportions are shown in parentheses. **(B)** Partial least squares discriminant analysis (PLS-DA) was used here to cluster the fecal metabolome profiles of calves. Metabolome profile for the H_1, H_2, D_1, or D_2 groups is shown in the same color, respectively. Data were presented as mean ± SEM values. *P*-values were acquired using the nonparametric Kruskal–Wallis test. **(C)** KEGG pathway enrichment analysis was associated with dramatically changed metabolites. The respective name of the KEGG pathway is shown on the left, and the corresponding *P*-value is shown on the right with a gradient color. *P*-values were acquired following two-side Fisher's exact tests with Benjamini-Hochberg correction for multiple testing. **(D)** Differentiated metabolites were displayed using a random forest supervised machine learning algorithms among H_1, H_2, D_1, and D_2 groups. The respective name of the metabolite is shown on the left. The top 10 metabolites in fecal samples are shown in different colors, and the rank values are shown as MeanDecreaseGini.

abundant N-acetyl-D-glucosamine and *Butyricicoccus* ($R > 0.77$, $P = 6.3e{-}09$), *Sutterella* ($R > 0.68$, $P = 1.6e{-}06$), *Collinsella* ($R > 0.61$, $P = 4.0e{-}05$), *Faecalibacterium* ($R > 0.59$, $P = 5.8e{-}05$), *Coriobacterium* ($R > 0.56$, $P = 0.00018$), and *Dorea* ($R > 0.48$, $P = 0.0018$) (**Figure 6**) was noticed. Notably, *Butyricicoccus* also showed the strongest correlation with enriched butyric acid ($R > 0.75$, $P = 3.2e{-}08$), isobutyric acid ($R > 0.55$, $P = 0.00022$), isovaleric acid ($R > 0.46$, $P = 0.0031$), glycolic acid ($R > 0.75$, $P = 1.8e{-}08$), and UDCA ($R > 0.69$, $P = 1.0e{-}06$), while *Collinsella* exhibited the strongest correlation with enriched acetic acid ($R > 0.56$, $P = 0.00022$). The microbes mentioned above were also strongly linked to other unabsorbed carbohydrates, SCFAs,

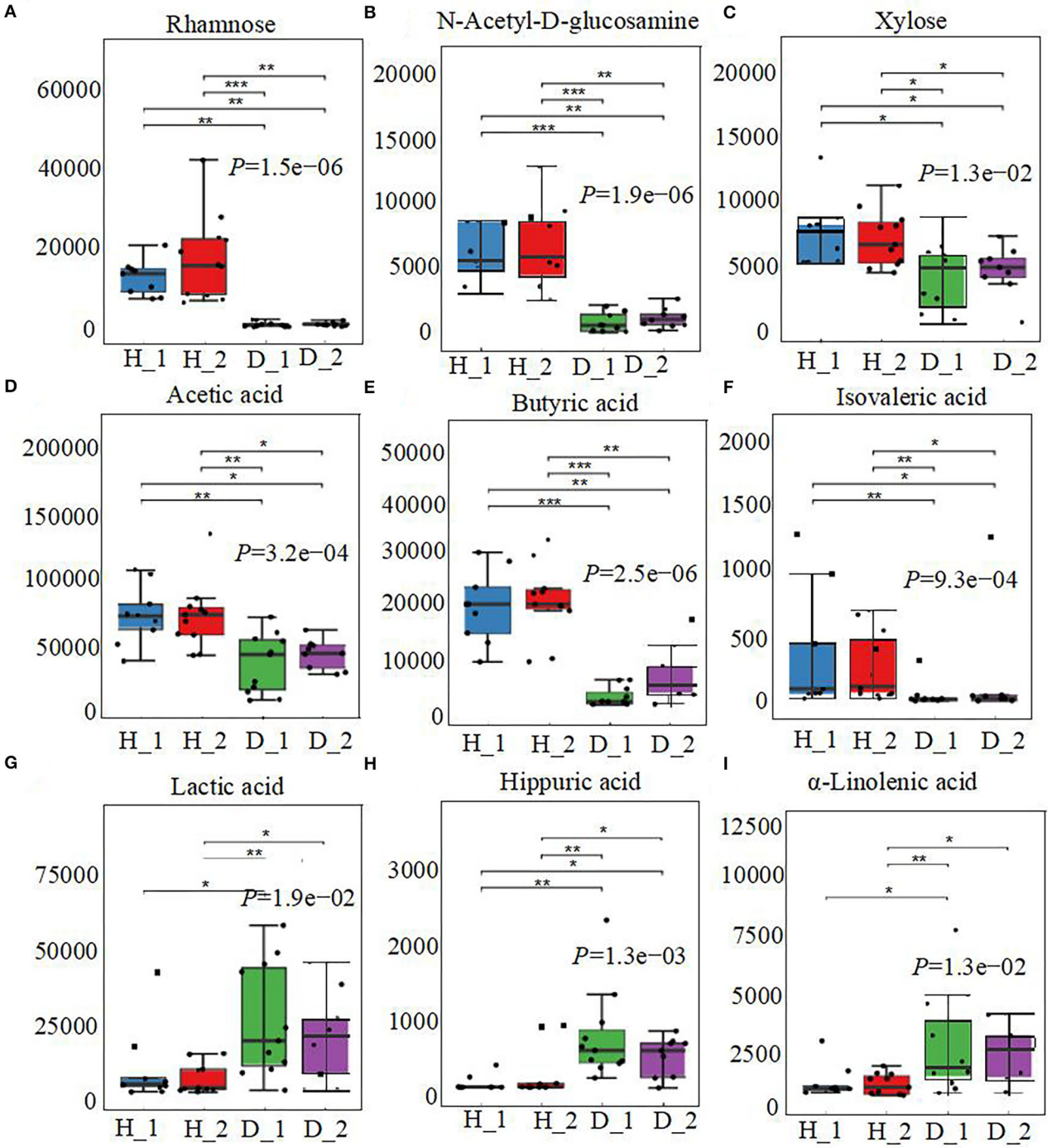

FIGURE 5 | The alterations in fecal metabolome profiles of neonatal calves post-ESBL-EAEC infection. The concentrations of fecal rhamnose **(A)**, N-acetyl-D-glucosamine **(B)**, xylose **(C)**, acetic acid **(D)**, butyric acid **(E)**, isovaleric acid **(F)**, lactic acid **(G)**, hippuric acid **(H)**, and α-linolenic acid **(I)** are displayed as box and dot plots. Data were presented as mean ± SEM values. *P*-values were determined using the nonparametric Kruskal–Wallis test. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

bile acids, and indole upregulations, and negatively related to α-linolenic acid, hippuric acid, lactic acid, and amino acid consumption. Also, it should be noted that there existed a strong correlation between *Gallibacterium* and hippuric acid ($R > 0.50$, $P = 0.00099$) and lactic acid ($R > 0.40$, $P = 0.01$). Thus, massive reduction in the unabsorbed carbohydrates, SCFAs, and

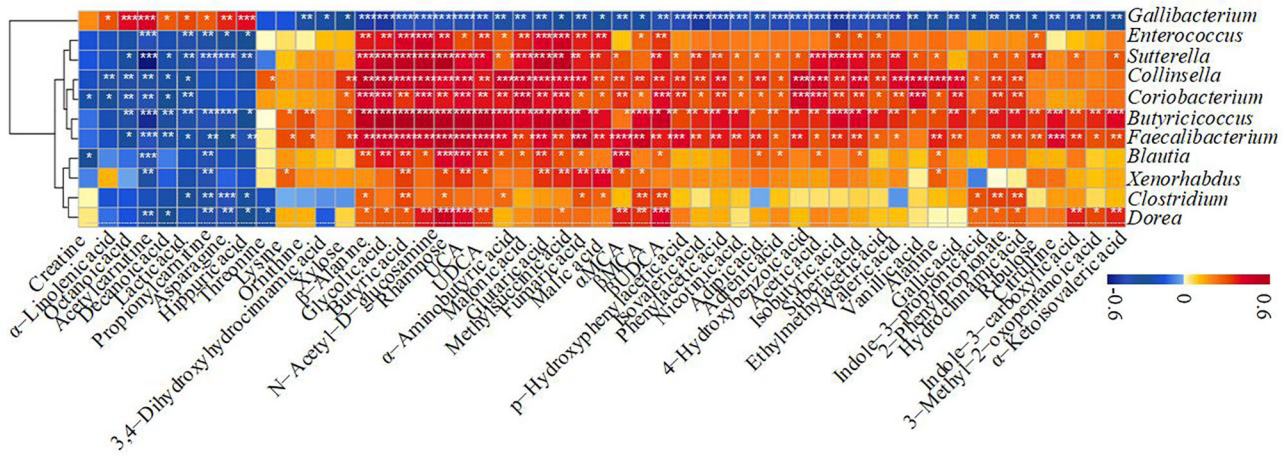

FIGURE 6 | The Spearman correlation between differentiated gut microbial taxa and fecal metabolites in H_1 vs H_2 vs D_1 vs D_2 groups. Red squares indicate a positive correlation, and blue squares indicate a negative correlation. The intensity of the color was proportional to the strength of the Spearman correlation. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

some other prebiotics was probably due to the decrease in the abundance of *Sutterella*, *Butyricicoccus*, *Faecalibacterium*, *Dorea*, *Collinsella*, and *Coriobacterium* observed in healthy calves, while increased hippuric acid and lactic acid content related to affluent *Gallibacterium*. These observations were similar to the obtained microbial and metabolite biomarkers. Thus, a decline in some of the commensal bacteria caused by ESBL-EAEC infection was apparently linked to a drop in fecal unabsorbed carbohydrates and derived SCFA production (including acetic acid, butyric acid, isobutyric acid, and isovaleric acid), thus inducing temporal destruction of intestinal homeostasis. Of note, our findings suggested that unabsorbed carbohydrates or early dietary fiber administration could ameliorate the intestinal health status of neonatal female calves.

DISCUSSION

Calves are a group of animals that are highly susceptible to various enteric infections considering the immature immune system and gastrointestinal tract. According to statistics on the morbidity of 450,000 heifers of China, our research group concluded that the morbidity of suckling calves accounted for 51.4% of the cases, of which the incidence of calf diarrhea accounted for 72.8% of the cases (White Paper on China Dairy Replacement 2020). Hence, prevention of calf diarrhea and timely intervention still pose a great challenge. The prevalent diarrheagenic *Escherichia coli* (DEC) causes an aggravation of the sporadic clinical diarrhea cases, resulting in the outbreak of gastroenteritis around the world (Schultsz et al., 2000). They can be categorized into six pathotypes: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic (Shiga-toxin producing) *E. coli* (EHEC/STEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (Kaper et al., 2004). Even worse, the pathogenesis of ESBL-EAEC remains unclear, and antibiotic abuse accelerates the rapid spread of MDR, which poses a severe threat to public health (Boucher et al., 2009). Multi-drug resistant ESBL-EAEC infection correlates with the extensive clinical diarrheal cases among animals and humans, particularly the rising occurrence of extended-spectrum beta-lactamase (ESBL)-producing isolates (Valat et al., 2014). Here, we aimed to attenuate the suffering associated with the post-ESBL-EAEC infection among neonatal calves by investigating predictive biomarkers with the aim of blocking further dissemination of resistance. We directly isolated clinical ESBL-EAEC isolates from neonatal calves in the conventional pasture, which had been proved to harbor MDR genes and enterotoxin EAST1. ESBL-EAEC strains with highly expressed EAST1 have been proved to cause diarrhea principally in humans, and later facilitate rapid adaption and propagation of bacteria in calves, piglets, and the other animals (Menard and Dubreuil, 2002). In most cases, ESBL-EAEC infections represent asymptomatic carriage and are self-limiting in the host, but some external factors, such as colostrum, diet, and environmental microbial community, correlate closely leading to the development and progression of calf diarrhea (Cho and Yoon, 2014). Currently, we only separated EAEC strains due to geographical restrictions and limited large ranch opening permissions. So, the data here could only reveal the antibacterial effects of the host on ESBL-EAEC. Nevertheless, we will surely proceed with further investigations on the regulatory role of the host in the pathogenesis of other DEC strains thoroughly in our subsequent clinical studies, along with the isolation of these strains from the calves of other conventional pastures in the other parts of China, thus systematically clarifying the systematic mechanism of DEC intervention action on gut health and development during the early days of life.

Alterations in the gut microbial colonization in the early life often contribute to longstanding effects on rumen microbes and host phenotype (Furman et al., 2020). In the current study, the abundance of nearly all top 50 bacterial genera changed

a lot from 2–7 days to 8–14 days of age. Such findings were similar to the shift in the hindgut microbiota in healthy dairy calves during the first 6 weeks of age (Song et al., 2018). Diet composition correlates with the alterations in the gut microbial diversity in these young calves (Dill-McFarland et al., 2017), and the detected changes in the hindgut microbiota of healthy calves correlate with colostrum feeding, milk replacer components, and calf starter feeding (Song et al., 2019). To avoid these limitations, the calves in this trial were fed with the same batch of heated colostrum (60°C, 60 min) and milk replacer (same amount under the same feeding phase), to facilitate the detection of the main host factors involved in mediating diarrhea resistance against ESBL-EAEC in neonatal female calves. Although the temporal changes in gut microbiota between H_1 and H_2 groups or D_1 and D_2 groups were similar, and the relative abundance of total bacterial genera showed no obvious difference among the groups, a significant tendency toward higher Chao1 and Simpson indices and dispersed bacterial community in healthy groups than diarrheal groups revealed that perturbations owing to ESBL-EAEC infection resulted in the fluctuation of the colonization of commensal bacteria and microbial dysbiosis. In our study, no calves received therapeutic antimicrobials or medical treatments, thus avoiding severe negative impact on the early development of neonatal microbial diversity and stability over the first 2 weeks of life and reflecting the real self-regulation ability of the host.

To gain insight into the alteration of fecal microbiome post-ESBL-EAEC infection, taxonomic and bacterial compositions of all fecal samples were compared immediately. Similar to the reports of a previous publication (Ma et al., 2020), most of the diarrheal cases occurred in female calves from 4 to 10 days. Various species of *Escherichia-Shigella* are widely known as the major enteric pathogens and cause calf diarrhea (Bartels et al., 2010). In this study, a lower relative abundance of *Escherichia-Shigella* was observed at 2–7 days in the D_1 than in H_1 groups, while a higher abundance was noticed at 8–14 days in the D_2 than in H_2 groups, indicating the gradual colonization and a favorable position of ESBL-EAEC in the gut lumen post-infection. The detected higher abundance of *Escherichia-Shigella* during the first week suggested that calves were more susceptible to infections considering their exposure to more number of opportunistic pathogens during this period (Song et al., 2018). Importantly, gut commensal bacteria are closely linked to immune recognition, host nutrient acquisition, and pathogen exclusion, thus linking endogenous and exogenous factors (Littman and Pamer, 2011; Clemente et al., 2012). Our data demonstrated that ESBL-EAEC infection promoted the diarrheal process by altering the composition of gut microbiota, which included a severe reduction in the abundance of *Sutterella*, *Collinsella*, *Prevotella*, *Faecalibacterium*, *Butyricicoccus*, *Ruminococcus*, *Blautia*, and *Oscillospira*. A higher prevalence of mucin- or SCFA-producing bacteria has been reported in the colon or fecal microbiota in healthy calves (Sokol et al., 2009; Lee et al., 2013; Graziani et al., 2016; Zhou et al., 2018; Vacca et al., 2020). However, the interference of those commensal bacteria colonizations correlated with diarrhea in animals (AlShawaqfeh et al., 2017). Besides, the enriched antibiotic resistance genes detected in diarrheal groups reflect the potential hazard of increased gene propagation and the risk of failure of future antimicrobial treatment. In view of the negative effect of the perturbation on the temporal development of microbial community due to ESBL-EAEC infection, a timely prediction of diarrhea based on microbial and metabolite biomarkers could be a promising approach to avoid the occurrence of such perturbations. LEfSe analysis of H_1 vs D_1 and H_2 vs D_2 groups highlighted that *Ruminococcus*, *Butyricicoccus*, *Faecalibacterium*, *Collinsella*, and *Coriobacterium* could be deemed as the indicator phylotypes of active intestinal tract. A similar change in the gut microbiota has been validated in patients with inflammatory bowel disease (IBD) (Pittayanon et al., 2020). However, diarrheal calves were associated with prevalent *Gallibacterium*, *Flavobacterium*, *Bifidobacterium*, and *Streptococcus*. Notably, the microbial community of the hindgut was dominated by lactic acid bacteria, including *Lactobacillus*, *Streptococcus*, and *Bifidobacterium*, as indicated by abundant lactic acid in the diarrheal groups, which can be attributed to the prominent contribution of hindgut fermentation in metabolizable energy supply during the first weeks after birth (Castro et al., 2016). Genus *Bifidobacterium* is known to prevail in the neonatal gut, especially when the colostrum is fed during the first 12 h of life (Malmuthuge et al., 2015). In our study, the relative abundance of digesta-associated *Bifidobacterium* was higher during 2–7 days than during 8–14 days, indicating that the consumption of milk oligosaccharides could result in a higher abundance of this genus (Lozupone et al., 2013). ESBL-EAEC infection could markedly increase the *Bifidobacterium* population, and also enrich *Lactobacillus* and *Streptococcus* in the diarrheal groups. Based on these findings, the results demonstrated that the hindgut microbiota of the neonatal female calves was similar to the microbial composition of monogastric animals, harboring the capabilities of adaption to an anaerobic environment and utilization of available substrates to construct their colonization niches. *Gallibacterium* is associated with a wide range of pathological changes in poultry (Persson and Bojesen, 2015), thus indicating the potential risks of intestinal infections in neonatal calves. *Flavobacterium* is detected to be a common species among the bacterial communities in diarrhea-affected cattle (Ateba et al., 2021). Further studies are needed to probe into the mutual influence of these microbial markers on improved gut health in young ruminants.

As for metabolome data and modeling algorithms of metabolites, various unabsorbed carbohydrates were listed as potential biomarkers, including rhamnose, N-acetyl-D-glucosamine, and xylose, accompanied by enriched acetic acid, butyric acid, and isovaleric acid. Increased amounts of lactic acid, hippuric acid, and α-linolenic acid were observed in the diarrheal groups, which highlighted that the differences in the fecal production of these nine metabolite markers closely reflect their absolute difference between "healthy" and "diarrheal" metabolomes. Plunged commensal bacteria, such as *Sutterella*, *Butyricicoccus*, *Faecalibacterium*, *Dorea*, *Collinsella*, and *Coriobacterium,* strongly correlated with the reduced unabsorbed carbohydrates, SCFAs, and bile acids, and indole upregulation, and negatively related to α-linolenic acid, hippuric acid, lactic acid, and amino acid consumption over the whole

time period, underlining the importance of both improved microbiota and medicinal benefits of their derived metabolome on the ESBL-EAEC infection state. There was also a strong correlation between *Gallibacterium* and hippuric acid and lactic acid. Thus, a simple prediction model using gut microbial markers was still challenging and limited to predict diseases of humans and animals. Early-life hindgut microbiota and fecal metabolome analyses should be combined for the accurate prediction of the diarrheal processes (such as ESBL-EAEC infection) in young calves.

Other kinds of metabolites were also differentiated in our study, including UDCA and indoles. UDCA, a natural secondary bile acid derived from gut microbiota, is discovered to possess an excellent effect on colonic epithelial cell protection against oxidative damage and cell apoptosis (Barrasa et al., 2013). Indoles belong to gut microbiota-derived tryptophan metabolites, which could influence inflammatory responses (Krishnan et al., 2018). Importantly, the beneficial effects of SCFA on the gut mucosal immune response also provided adjunctive effects in the fight against ESBL-EAEC infection (Hiltz and Laarman, 2019; Liu et al., 2021). These aforementioned metabolites could also display prebiotic properties against ESBL-EAEC infection. In our future research, we would explore the direct role of these above-mentioned commensals (*Butyricicoccus*, *Faecalibacterium*, *Ruminococcus*, *Collinsella*, and *Coriobacterium*) in mediating the metabolism of unabsorbed carbohydrates, SCFA, and other prebiotics and antibacterial effects using culture technique, proteomics technique, and targeted metabolomics. It can be achieved by directly comparing ESBL-EAEC-infected diarrheal neonatal calves with those purely isolated from the hindgut bacteria of healthy calves. Linoleic acid, a dietary polyunsaturated fatty acid (PUFA), can serve as a key biomarker for the progression of ulcerative colitis and gut microbiota dysbiosis, and destroy the cell membrane of some probiotics (Lv et al., 2020; Tang et al., 2020), which is consistent with enriched linoleic acid metabolism pathway in our KEGG analysis. In addition, hippuric acid, a protein-bound uremic toxin, correlated with the upregulation of pro-inflammatory cytokines and oxidative stress, which could accelerate the deterioration of disease and indicated its utility in calf feces as a plausible hallmark of frailty post-ESBL-EAEC infection (Watanabe et al., 2011). Future research exploring the direct relationship between *Gallibacterium* and hippuric acid and lactic acid metabolisms was also needed. Our limitations were that limited sampling time points (2–14 days) were available, which could not correctly reflect the dynamic changes of the microbiota and metabolites during different infection stages, and the inherent influential mechanisms remained elusive. The sampling number is limited to assess the accuracy of the current model and its broad application to different herds. Therefore, further research is needed to determine the exact role of these biomarkers in the progression of calf diarrhea with much more sampling time points and adequate populations to detect their prediction specificity of diarrhea induced by ESBL-EAEC infection.

The successful colonization of hindgut microbiota and increased microbial diversity and stability are vital features for optimizing the better performance of neonatal calves. ESBL-EAEC infection in the early days led to drastic changes in the hindgut microbial community and altered fecal metabolites especially during the first 2 weeks of age, suggesting that early infections by these bacteria could probably have a negative impact on the long-term health of female calves. Previous studies clearly indicated the early life stage as a critical window for gut microbiota manipulation to mediate the metabolome and immunity of neonatal calves. Thus, future studies concerning the impact of early control of pathogens and supplementation with unabsorbed carbohydrates or dietary fiber on gut health and productivity of calves are urgently needed, utilizing multi-omics analyses to elucidate the effect of the interaction between these biomarkers on the gut health.

CONCLUSION

Collectively, multi-omics analyses of fecal samples of neonatal calves indicated the differences in the hindgut microbiota and fecal metabolites. Using the random forest model and Spearman correlation analysis, the data provided innovative insights into the exact predictions of diarrhea induced by ESBL-EAEC using commensals and associated unabsorbed carbohydrates among neonatal calves. In addition, the results highlighted the possibilities of employing hindgut microbiota and associated metabolites for predicting many other intestinal infections or diseases in neonatal food-producing animals, thus facilitating the reduction of antimicrobial usage.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Ethics Committees of China Agricultural University.

AUTHOR CONTRIBUTIONS

ZC, ZH, YM, YW, WW, HY, and SLi designed the experiments. YM and ZH conducted the experiments and analyzed the data. SY, YM, SZ, SLiu, XC, and JX collected the samples and performed the analysis of the samples. ZC and ZH wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.883090/full#supplementary-material

Supplementary Table 1 | Sampling information of calves used in the current study.

Supplementary Table 2 | Genomic analysis of 1587 strain.

Supplementary Table 3 | Antibiotic susceptibility analysis of 1587 strain.

Supplementary Figure 1 | Gut microbiota assembly of neonatal calves post-ESBL-EAEC infection. The relative abundance of fecal bacterial phylum **(A)** and family **(B)** represented 99.5% of the community.

Supplementary Figure 2 | Cognate metabolomics analyses of fecal samples in healthy and diarrheic calves. Partial least squares discriminant analyses (PLS-DA) for neonatal calves in H_1 vs D_1 **(A)**, H_2 vs D_2 **(B)**, H_1 vs H_2 **(C)**, and D_1 vs D_2 **(D)**. H, healthy calves; D, diarrheic calves.

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Very Preterm Children Gut Microbiota Comparison at the Neonatal Period of 1 Month and 3.5 Years of Life

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Prematurity is a risk factor for dysbiosis of the gut microbiota due to particular birth conditions and frequent prolonged hospitalization of neonates. Although gut microbiota colonization after birth and its establishment during the hospitalization period have been studied in preterm infants, data on gut microbiota following discharge, particularly during early childhood, are scarce. The present study investigated the relationship between gut microbiota at 1 month after birth (hospitalization period) and 3.5 years of age in 159 preterm children belonging to the French EPIFLORE prospective observational cohort study. Analysis using bacterial 16S rRNA gene sequencing showed that the gut microbiota of preterm neonates at 1 month was highly variable and characterized by six distinct enterotypes. In contrast, the gut microbiota of the same children at 3.5 years of age showed less variability, with only two discrete enterotypes. An absence of association between enterotypes at 1 month and 3.5 years of age was observed. While the alpha diversity of gut microbiota significantly increased between 1 month and 3.5 years of age, for both alpha and beta diversities, there was no correlation between the 1-month and 3.5-years time points. Comparison at 3.5 years between children born either preterm (n = 159) or full-term (n = 200) showed no differences in terms of enterotypes, but preterm children harbored a lower Shannon diversity index and a different overall composition of microbiota than full-term children. This study suggests that the characteristics of the early gut microbiota of preterm children are not predictive of the microbial community composition at 3.5 years of age. However, the impact of gestational age is still noticeable on the gut microbiota up to 3.5 years of age.

Keywords: prematurity, gut microbiota, DOHaD, children, enterotypes

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INTRODUCTION

The human microbiota has become a research topic of utmost interest in recent years, with several studies highlighting its potential impact on physiology and, as a result, its possible effects on health and disease (Fan and Pedersen, 2021; Sarkar et al., 2021). Recently, substantial efforts have focused on studying infant gut microbiota with the emergence of the concept of Developmental

Origins of Health and Disease (DOHaD) (Butel et al., 2018) and the critical first 1.000 days of life window hypothesis (Wopereis et al., 2014; Robertson et al., 2019). Prematurity, accounting for approximately 10% of births worldwide (Blencowe et al., 2012; Chawanpaiboon et al., 2019), is a risk factor for early gut dysbiosis, which is suggested to be associated with necrotizing enterocolitis (Rozé et al., 2017), growth delay (Tirone et al., 2019), cognitive impairment (Rozé et al., 2020), and brain damage (Seki et al., 2021). In very preterm neonates, gut microbiota is characterized by high inter-individual variability (Yee et al., 2019; Rozé et al., 2020) that can be attributed to varying birth conditions and extended exposure to the neonatal intensive care unit (NICU) environment and practices. Indeed, preterm infants are more often born *via* cesarean section and are frequently administered broad-spectrum antibiotics. Furthermore, gestational age influences the dynamics of the intestinal bacterial establishment (La Rosa et al., 2014). The early establishment of preterm gut microbiota is characterized by a dominance of opportunistic and potentially pathogenic bacteria, such as *Enterobacter*, *Enterococcus*, and *Staphylococcus*, and a delay in colonization by *Bifidobacterium* and *Bacteroides* (Arboleya et al., 2012; Barrett et al., 2013; La Rosa et al., 2014; Hill et al., 2017; Korpela et al., 2018). Several studies have highlighted the influence of the NICU environment on the establishment of preterm neonate gut microbiota (Hartz et al., 2015; Moles et al., 2015; D'Agata et al., 2019; Tauchi et al., 2019; Yap et al., 2021), notably during hospitalization (Rozé et al., 2020), and the existence of overlap of microbial taxa between NICU surfaces and the gut of preterm neonates (Brooks et al., 2014). Thus, while the infant gut microbiota establishment during the first weeks and months of life is documented, only a few studies have reported on the late development of gut microbiota in preterm children. Additionally, the relationship between gut microbiota and early-life neonatal factors, such as birth mode, breastfeeding, and antibiotic treatments after NICU discharge and during early childhood of preterm children, has been rarely studied, although it has been emphasized as a research priority (Groer et al., 2014). Indeed, to date, only a few studies with small cohorts have explored the development of gut microbiota in preterm children after 1 year of age (Gómez et al., 2017; Fouhy et al., 2019; Yee et al., 2019). These studies report an increase in alpha diversity over time and lower inter-individual variability in the gut microbiota between NICU discharge and early childhood. Furthermore, it has been suggested that gestational age at birth results in distinct microbial profiles extending up to 4 years of age (Fouhy et al., 2019). To the best of our knowledge, these data constitute the only available longitudinal evidence describing the gut microbiota of preterm children during early childhood. Therefore, given the paucity of data regarding this subject, there is a need to add substantial evidence related to prematurity and gut microbiota during early childhood, particularly in large cohorts.

This study aims to compare and characterize the gut microbiota of 159 preterm children from the EPIPAGE2 French nationwide cohort at two time points: at 1 month of life (NICU hospitalization) and at 3.5 years of age, when the child's gut microbiota supposedly stabilizes toward a mature adult-like gut microbiota (Stewart et al., 2018). Additionally, we investigated factors previously reported to influence early gut microbiota development, such as gestational age (Groer et al., 2014; Gritz and Bhandari, 2015; Chernikova et al., 2018), birth mode (Dominguez-Bello et al., 2010; Portela et al., 2015; Stewart et al., 2018), birth weight (Groer et al., 2014; Unger et al., 2015), sex (Martin et al., 2016), antibiotics (Gibson et al., 2015; Tamburini et al., 2016), breastfeeding (Portela et al., 2015; Lemas et al., 2016; Pannaraj et al., 2017; Stewart et al., 2018), skin-to-skin contact (Hartz et al., 2015), and NICU strategies (Rozé et al., 2020). This study provides informative data concerning prematurity and the gut microbiota in early childhood based on a large and well-documented French multicentric cohort of preterm children.

MATERIALS AND METHODS

EPIFLORE Study and ELFE Cohort

The preterm children included in this study were part of the EPIFLORE study (Ancel and Goffinet, 2014), an ancillary study of the EPIPAGE2 French national birth cohort that was launched in 2011 and included preterm neonates born between 24 and 31 weeks of gestation (Lorthe et al., 2021). The aim of EPIFLORE was to investigate the development of the intestinal microbiota and its relationship with early-life factors that influence its establishment and subsequent health outcomes in early childhood among very preterm children (<32 weeks of gestational age). The EPIFLORE study included clinical data and stool samples collected from 24 French NICUs (Rozé et al., 2020). In the present study, full-term children who were born in 2011 from the ELFE French national birth cohort (Charles et al., 2020) were included as controls to investigate the differences between the gut microbiota of preterm and full-term children at 3.5 years of age. Full-term singletons born after 37 weeks of gestation with fecal samples available at 3.5 years of age and at least 2 years of subsequent follow-up were randomly selected from the ELFE cohort.

Participants and Sample Collection

The EPIFLORE study included 729 neonates, and stool samples were collected from 574 preterm neonates during hospitalization at a median age of 23 days (interquartile range [IQR], 22–26 days), which is referred to as 1-month sampling. An additional stool sample was obtained for 208 preterm children at a median age of 42.6 months (IQR, 42.2–43.4 months), referred to as 3.5 years of age sampling. In the current study, 159 preterm children with fecal samples collected at both time points were included. Additionally, stool samples of 200 full-term children from the ELFE cohort were collected at 3.5 years of age (median age, 42.1 months; IQR, 41.4–43.6 months) during the same period and using the same methodology as for preterm children (**Supplementary Figure 1**).

DNA Extraction, Sequencing, and Data Processing

The total fecal DNA was extracted according to the International Human Microbiome Standards operating procedure (Dore

et al., 2015), as previously reported (Rozé et al., 2020). We included negative controls that went through the same process as samples, from DNA extraction to sequencing. Sequencing was performed on the GeT-PlaGe platform of Génopole (Toulouse Midi-Pyrénées, France) using Illumina Miseq technology (V3, 2 × 250 bp) targeting the V3-V4 primers (V3fwd: ACGGRAGGCAGCAG, V4rev: AGGATTAGATACCCTGGTA) regions of the 16S bacterial rRNA gene. Raw sequences were analyzed using the pipeline "Find Rapidly OTU with Galaxy Solution" (FROGS) version 3.2 (September 2021) from the Galaxy software framework (Escudié et al., 2018). Sequences were checked for quality using FastQC (threshold 20–38). After trimming barcodes, the sequences were filtered (length of the reads ranged from 435 to 459 bp) and clustered into operational taxonomic units (OTUs) using the swarm clustering method implemented in the FROGS version 3.2 pipeline. OTUs representing less than 0.005% (788,210 reads) of all the sequences, the majority corresponding to singleton OTUs, were discarded (Bokulich et al., 2013). Taxonomic affiliation was assigned to the OTUs using the Silva 138.1 pintail100 database (September 2021) yielding 99% of the sequences affiliated with ≥99% identity and 100% coverage. A total of 11,526,400 reads (median, 22,619 reads per sample) were obtained from 16S rRNA gene sequencing. To adjust for the influence of uneven sampling depth, each sample was rarefied to the minimum sampling depth of the dataset (5,201 reads, **Supplementary Figure 2**), and rarefied data were used for all downstream analyses unless stated otherwise.

Microbiota Analysis: Diversity and Composition

The Chao1 richness estimate (richness) and Shannon index (diversity) were calculated to investigate the patterns of microbial community diversity in the gut microbiota of preterm and full-term children. For beta diversity analysis, we computed dissimilarity matrices using Bray–Curtis and Unifrac distances. The Bray–Curtis dissimilarity distance reflects community composition considering the abundance of taxa, while the Unifrac distance considers the phylogenetic relationships among members of the bacterial communities. Gut microbiota inter-individual variability was visualized using principal coordinate analysis (PCoA/MDS) based on dissimilarity matrices. Stratification of the cohort based on gut microbial composition was performed according to the previously reported enterotyping guidelines (Arumugam et al., 2011). Briefly, the relative abundances of the classified genera were used to produce a Jensen–Shannon divergence (JSD) distance matrix between samples. Based on the obtained distance matrix, samples were clustered using Partitioning Around Medoids (PAM) algorithm. The optimal number of clusters was chosen by maximizing the Calinski–Harabasz index and was cross-validated with the silhouette index and prediction strength. The results of PAM clustering were visualized on PCoA biplots, and driver genera vectors were assessed using the "envfit" function. The vectors show the direction in the ordination space toward which genera change most rapidly and to which they have maximal correlations within the ordination configuration. The genus with the highest relative abundance in each group was considered the main contributor to each enterotype.

Neonatal Characteristics and Neonatal Intensive Care Unit Strategies

In each NICU, data on children and mothers during the perinatal and neonatal periods were collected prospectively during hospitalization until discharge. Available clinical data included gestational age, birth mode, birth weight, sex, antibiotics, breastfeeding, skin-to-skin contact, and NICU strategies. The NICU strategies were previously characterized in the EPIPAGE2 study regarding early extubation or no intubation, use of sedation, direct breastfeeding, skin-to-skin practice, speed of progression of enteral feeding, and duration of primary and secondary antibiotic therapy (Rozé et al., 2020). All these practices concern the early period of hospitalization before the 1-month stool sampling period. The methods to characterize these strategies were applied as previously described (Rozé et al., 2020).

Statistical Analysis

For the comparative analysis of alpha diversity and pairwise beta diversity between enterotypes and time points, Wilcoxon rank-sum tests and Kruskal–Wallis tests were performed. Hypothesis testing was carried out using permutational multivariate analysis of variance (PERMANOVA) based on 999 permutations to analyze the influence of neonatal factors on the gut microbiota and to compare the gut microbiota between preterm and full-term children. For all PERMANOVA analyses, Benjamini–Hochberg FDR (false discovery rate) correction was used to adjust for multiple testing. We implemented a differential abundance testing analysis in order to assess significant changes in abundance at the genus level between samples at 1 month and 3.5 years in the preterm population. As these methods can produce heterogeneous results (Nearing et al., 2021; Wallen, 2021), we used two different methods: Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) (Lin and Peddada, 2020) and ANOVA-Like Differential Expression tool for compositional data (ALDEx2) (Fernandes et al., 2014). Differences in abundance were based on non-rarefied counts for both ANCOM-BC and ALDex2, as both methods used their own normalization methods, and only genera with an abundance of 0.1 in at least 1% of the samples were used. We considered only concordant results between the two methods. *P*-values were corrected using the Benjamini–Hochberg method. Correlations of alpha- and beta-diversity community structures between 1 month and 3.5 years of age samples were assessed as follows: pairwise Spearman rank correlation test for alpha-diversity indexes; Mantel test for beta-diversity matrices using the Spearman rank correlation method based on 999 permutations. The association between enterotypes at both ages was tested using Fisher's exact test. To analyze the association between microbiota enterotypes found at 3.5 years of age and both neonatal characteristics and NICU strategies (neonates hospitalized at day 7), we used the chi-squared tests for categorical variables and student tests for continuous variables in univariate analyses.

Mixed-effects logistic regression with a random hospital intercept to consider the correlation between newborns in the same NICU was performed in multivariate analyses. All analyses were performed using the R software version 4.0.4 (R Foundation) and the packages phyloseq (v1.34.0), vegan (v2.6-0), cluster (v2.1.2), clusterSim (v0.49-2), fpc (v 2.2-9), ade4 (v1.7-16), ggplot2 (v 3.3.5), and lme4 (v1.1-27.1).

RESULTS

Enrolled Children Population

Our analyzed sample of preterm children ($n = 159$) was compared with the total population enrolled in the EPIFLORE study ($n = 570$), without stool samples available at the two time points, that is, 1 month and 3.5 years of age (**Supplementary Table 1**). We found that the characteristics of our study population did not significantly differ from the total population enrolled in the EPIFLORE study except in terms of the mother's age, the rate of mothers born outside France, and maternal level of education (**Supplementary Table 1**).

Preterm Gut Microbiota at 1 Month of Age

Among the 159 stool samples analyzed at 1 month, 18 were non-amplifiable. The 141 neonates for whom sequencing data were obtained were partitioned into five bacterial enterotypes similar to those previously described by Rozé et al. (2020). In the present study, enterotypes 1–5 were characterized by a dominance of *Enterobacter* ($n = 69$), *Clostridium sensu stricto 1* ($n = 18$), *Escherichia* ($n = 25$), *Enterococcus* ($n = 18$), and *Staphylococcus* ($n = 11$), respectively (**Figures 1A,B**). At the OTU level, enterotypes 1–5 were characterized by the dominance of OTU_1_*E. hormaechei*, OTU_13_*C. neonatale*, OTU_2_*E. coli*, OTU_4_*E. faecalis*, and OTU_19_*S. caprae*, respectively (**Supplementary Figure 3**). Enterotype 2 (dominance of *Clostridium sensu stricto 1*) and enterotype 3 (dominance of *E. coli*) were associated with greater gestational age (**Supplementary Figure 4**). Enterotype 3 was considered to be the more mature microbiota and defined as the reference enterotype at 1 month, as previously reported (Rozé et al., 2020). Concerning the 18 non-amplifiable samples, they represented the enterotype 6 gathering infants with a low bacterial load (Rozé et al., 2020).

Community complexities of the five different enterotypes were compared at the genus level according to alpha (Chao 1 richness and Shannon index) and beta (Bray–Curtis and Unifrac distances) diversities (**Figure 1C**). Significant differences were observed in alpha diversity across all enterotypes (Shannon diversity index, $p = 0.003$). Compared to reference enterotype 3, enterotypes 4 and 5 showed a significantly lower alpha diversity (Shannon index, $p < 0.05$ and $p < 0.001$, respectively). However, pairwise beta-diversity comparisons between enterotypes showed significant overall differences across groups (Bray–Curtis and Unifrac distance dissimilarities, $p < 10^{-4}$ for both). Higher distance dissimilarities among infants belonging to enterotypes 1 and 2 were observed when compared to the reference enterotype 3 (Bray–Curtis, $p < 0.0001$ for both enterotypes), suggesting higher inter-individual variation. Enterotypes 4 and 5 were characterized by lower pairwise distance dissimilarities within groups (Bray–Curtis, [$p < 0.0001$], Unifrac [$p = 0.007$] for Ent4, and Bray–Curtis [$p < 0.0001$] for Ent5). At the OTU level, significant differences were observed in alpha diversity across all enterotypes (Chao1 and Shannon diversity index, $p = 0.005$ and $p = 0.008$, respectively). Differences at the OTU level are further presented in the **Supplementary Figure 3**.

Preterm Gut Microbiota at 3.5 Years of Age

At 3.5 years of age, the gut microbiota of the 159 preterm children was characterized by the presence of six different phyla as follows: *Actinobacteriota*, *Bacteroidota*, *Desulfobacterota*, *Firmicuteota*, *Fusobacteriota*, and *Proteobacteriota*, with *Bacteroidota* and *Firmicuteota* being the most abundant phyla (**Supplementary Figure 5**). Concerning the stratification of the gut microbiota, it is optimally separated into two distinct enterotypes (**Figure 2A**). Clustering based on the genus taxonomic level showed that *Prevotella* had the strongest positive correlation and *Bacteroides* had the strongest negative correlation with the PCoA 1 dimension (**Figure 2A**). The enterotypes were enriched in either *Prevotella* (P_type) ($n = 31$) or *Bacteroides* (B_type) ($n = 128$) (**Figure 2B**). Interestingly, *Prevotella* was depleted in most children with B_type stools, with a median abundance of 0.02% (IQR, 0.02–0.06%), whereas *Bacteroides* remained the second most abundant genus in children with P_type stools, with a median abundance of 14% (IQR, 6–19%). At the OTU level, the B_type enterotype was characterized by the dominance of OTU_3_*B. vulgatus*, and the P_type enterotype was characterized by the dominance of OTU_30_*P. copri* (**Supplementary Figure 6**).

When comparing the microbiota of both P_type and B_type enterotypes according to alpha and beta diversities, the P_type enterotype showed greater richness (Chao1 index, $p = 0.031$, genus level) (**Figure 2C**). At the OTU level, no difference in richness was observed between the two enterotypes (**Supplementary Figures 6A,C**).

The Bray–Curtis distance pairwise dissimilarities between P_type and B_type enterotypes based on the genus taxonomic profile showed that B_type and P_type children shared a similar bacterial community within each enterotype (**Figure 2D**). The dissimilarity, indicated by the heatmap color and boxplot of pairwise dissimilarities, was significantly higher in the P_type enterotype than in the B_type enterotype, suggesting that the P_type community was more heterogeneous among children (**Figure 2D**). In contrast, at the OTU level, the P_type community was less heterogeneous and was characterized by lower pairwise dissimilarity distances (**Supplementary Figure 6**).

Preterm and Full-Term Children Gut Microbiota Comparison at 3.5 Years of Age

To investigate the differences between the gut microbiota of preterm and full-term children at 3.5 years of age, we included

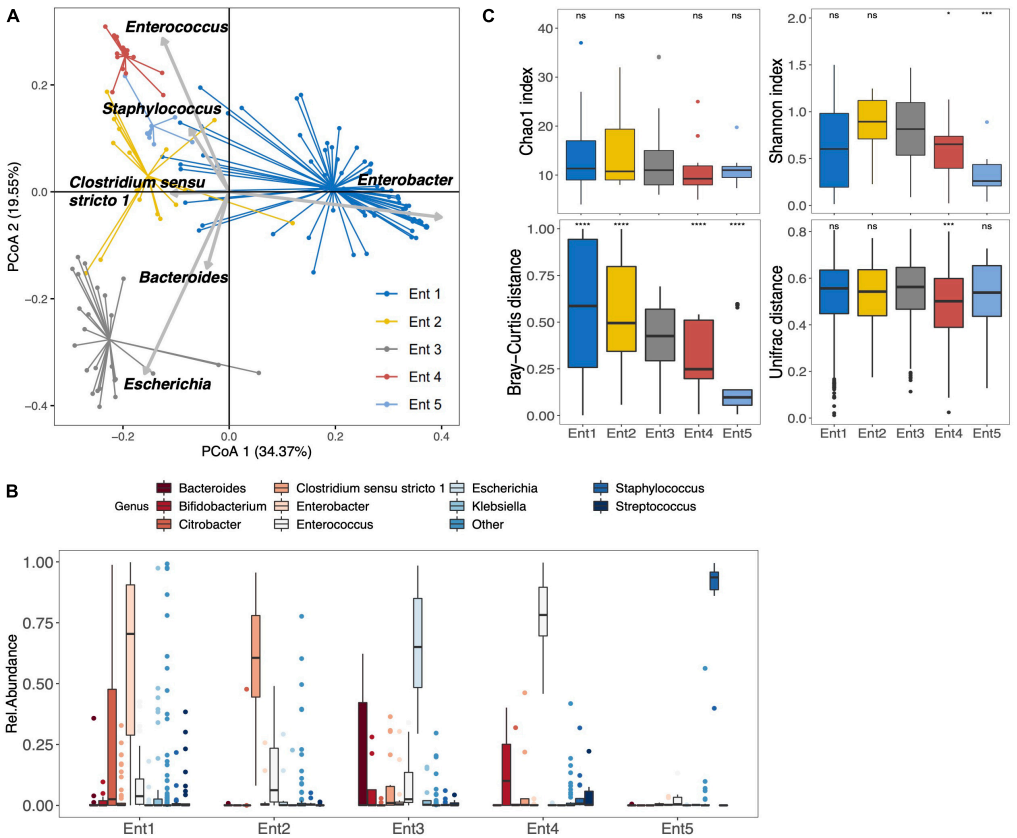

FIGURE 1 | Gut microbiota composition and diversity of the 141 preterm infants at 1 month after birth. **(A)** Clustering based on the genus taxonomic profiles. Biplot arrows indicate the top six genera that drive the sample to different locations on the plot. **(B)** The boxplots represent the relative abundance of the top 10 genera distributed among the five enterotypes. The sixth enterotype is not represented because it is constituted by infants without sample amplification (low bacterial load). The boxplots show the smallest and largest values, 25 and 75% quartiles, the median, and outliers. **(C)** Boxplots of alpha diversity (top) assessed by Chao1 and Shannon index, and pairwise beta diversity dissimilarities (bottom) assessed by Bray–Curtis and Unifrac distances within enterotypes based on genus taxonomic profiles (*$p < 0.05$, ****$p < 0.001$, ns = $p > 0.05$, Reference group = Ent3); Ent, Enterotype.

200 full-term children (ELFE cohort). Full-term children were significantly different in terms of gestational age, delivery mode, birth weight, the rate of mothers born outside of France, and the level of mother's education (**Supplementary Table 2**). *Firmicuteota* and *Bacteroidota* were the main phyla identified in the gut microbiota of full-term children. Moreover, the top 10 genera found in preterm children (except for the genus *Blautia*) also characterized the gut microbiota of full-term children (**Figure 3A**). Enterotyping at the genus taxonomic level showed that the gut microbiota in full-term and preterm children was optimally clustered into two discrete B_type (n = 293) and P_type (n = 66) enterotypes, with the two largest predictors being *Bacteroides* and *Prevotella*, respectively (**Figure 3B**). Interestingly, full-term children showed the same patterns as those seen in preterm children, where *Prevotella* was depleted from the majority of B_type children [median abundance of 0.04% (IQR, 0.02–0.08%)], whereas *Bacteroides* remained the second most abundant genus in P_type children (median abundance of 14% [IQR, 9–19%]) (**Figure 3A**). No differences in enterotype proportion distribution were observed between preterm and full-term children (p = 1.000; **Figure 3C**).

Analysis of alpha diversity showed that the gut microbiota of preterm children was characterized by a significantly lower Shannon diversity index than that of full-term children at the genus (p = 0.012) (**Figure 3D**) and OTU levels (p = 0.037). Overall, there were no differences in richness assessed using the Chao1 index at both the genus and OTU levels.

Using both Bray–Curtis and Unifrac distances at the genus level, the PERMANOVA suggested differences in the microbial community between preterm and full-term children (p = 0.012 and p = 0.006, FDR-corrected, respectively, **Supplementary Figure 7**).

Preterm Gut Microbiota Comparison at 1 Month and 3.5 Years of Age

Figures 1B, **2B** present the top 10 abundant genera at 1 month and 3.5 years of age, respectively, showing that the major genera present in the gut microbiota of preterm children changed between the two ages. In addition to *Bifidobacterium* and *Bacteroides*, the major genera initially found at 1 month (*Enterobacter*, *Clostridium sensu stricto*,

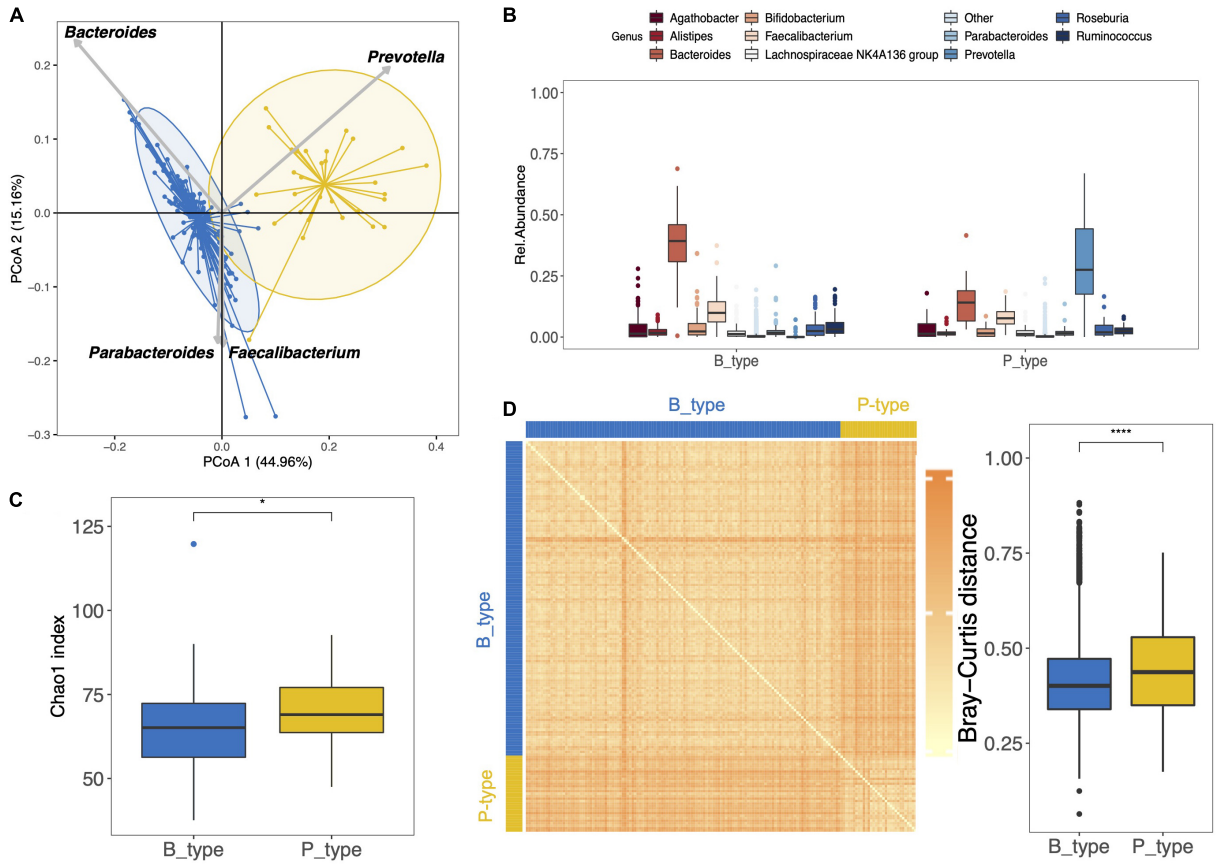

FIGURE 2 | Gut microbiota composition and diversity of the 159 preterm children at 3.5 years of age. **(A)** Clustering based on the genus taxonomic profile. Biplot arrows indicate the top four genera that drive samples to different locations on the plot. **(B)** The boxplots represent the relative abundance of the top 10 genera distributed among the P_ and B_type enterotypes. The boxplots show the smallest and largest values, 25 and 75% quartiles, the median, and outliers. **(C)** Boxplot of alpha diversity assessed by Chao1 estimate based on the genus taxonomic profile. **(D)** Inter-individual dissimilarity of the genus community based on the Bray–Curtis distance and represented by the heat map and boxplot. Each cell in the heat map, ordered according to B_ and P_type enterotypes, represents the dissimilarity level as per the color scale beside the box plot (*$p < 0.05$, ****$p < 0.0001$).

Escherichia, *Enterococcus*, and *Staphylococcus*) were replaced by other genera (*Agathobacter*, *Faecalibacterium*, *Roseburia*, *Prevotella*, and *Ruminococcus*). Except for *Bifidobacterium* and *Prevotella*, all genera cited above were significantly differentially abundant between the two time points (**Supplementary Table 3**). Based on the genus composition, the alpha diversity (Chao1 richness and Shannon index) of the gut microbiota significantly increased between both ages (**Figure 4A**). Pairwise dissimilarities based on genus composition within each age group showed that the gut microbiota of children at 1 month of age harbored higher beta diversity (Bray–Curtis and Unifrac) than at 3.5 years of age ($p < 0.0001$ for both; **Figure 4A**). Investigation of the relationship between gut microbiota at 1 month and 3.5 years of age in preterm children showed the absence of correlations of diversities between the two age groups either according to alpha diversity (*rho* = −0.054, $p = 0.521$ [Chao1 index]; *rho* = 0.063, $p = 0.458$ [Shannon index]) or beta diversity ($r = 0.008$, $p = 0.410$ [Bray–Curtis]; $r = 0.030$, $p = 0.176$ [Unifrac]). Comparison of the proportions between the two 3.5-year and the six 1-month enterotypes showed that enterotypes 3, 4, and 5 tended to harbor the smallest proportion of P_type, but overall, the association was not significant (Fisher's exact test, $p = 0.490$) (**Figure 4B**). Compared to the reference enterotype 3, the gut microbiota of children originally clustered into enterotype 2 presented a higher alpha diversity at 3.5 years of age, but no overall differences were observed (Shannon index, $p = 0.355$; **Figure 4C**). No overall differences were noted at the OTU level ($p = 0.173$).

Preterm Gut Microbiota at 3.5 Years of Age and Neonatal Factor Relations

Since we found that enterotypes at 3.5 years of age explained most of the variance in the gut microbiota (PERMANOVA analysis; **Supplementary Table 4**), we further explored the association between neonatal characteristics and enterotypes. Higher birth weight was significantly associated with the P_type enterotype in the univariate analysis (**Table 1**). In the multivariate model adjusted for maternal age, education level, and country of birth, we included covariates known to influence early gut microbiota development, such as gestational age, birth

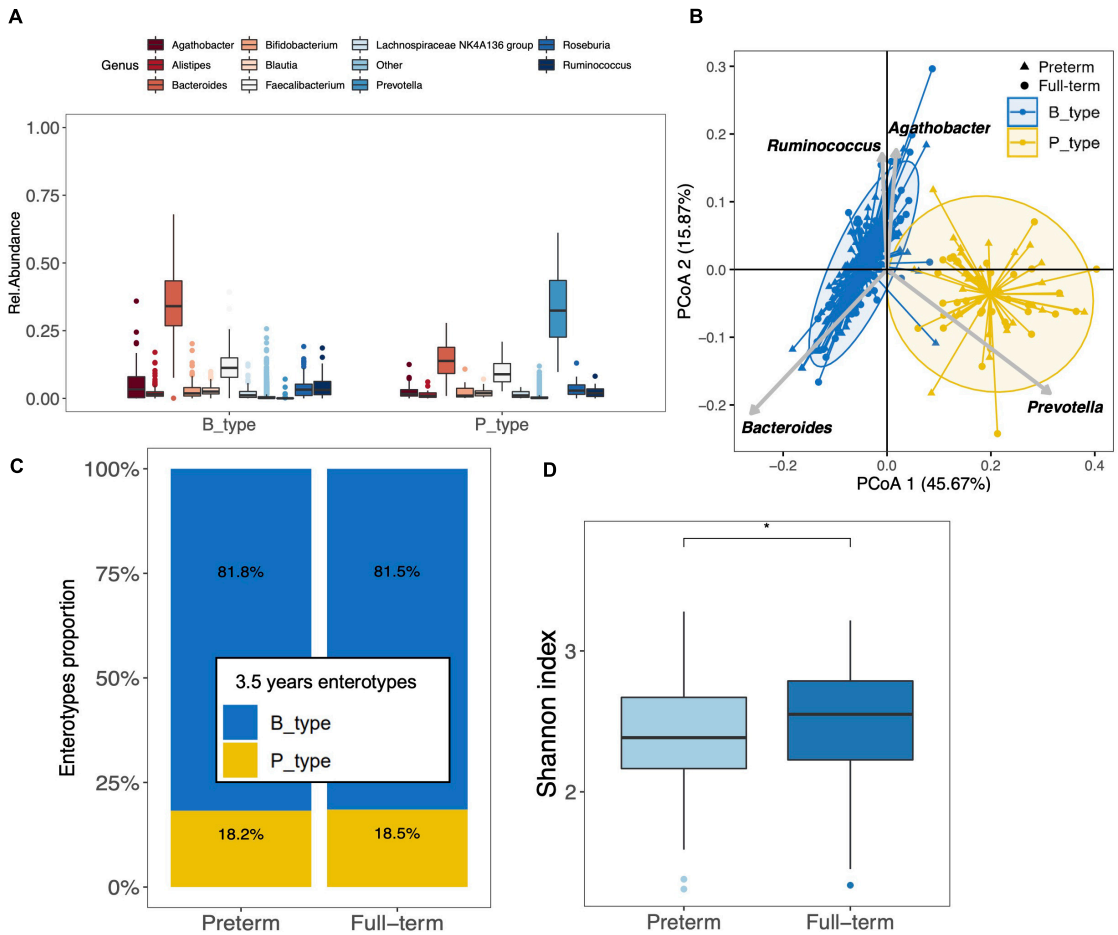

FIGURE 3 | Clustering of the gut microbiota of preterm (n = 159) and full-term (n = 200) children at 3.5 years of age. **(A)** The boxplots represent the relative abundance of the top 10 genera distributed between the B_ and P_types among the full-term children. The boxplots show the smallest and largest values, 25 and 75% quartiles, the median, and outliers. **(B)** Gut microbiota clustering of the 159 preterm and 200 full-term children at 3.5 years of age based on the genus taxonomic profile. Biplot arrows indicate the top four genera that drive samples to different locations on the plot. **(C)** Distribution of enterotypes among full-term and preterm children. The distribution is expressed as the proportion of each enterotype among each group of children. **(D)** Boxplot of alpha diversity assessed by Shannon index based on the genus taxonomic profile between preterm and full-term children (*$p < 0.05$). The boxplots show the smallest and largest values, 25 and 75% quartiles, the median, and outliers.

mode, birth weight, sex, antibiotics, breastfeeding, skin-to-skin contact, and NICU strategies. The results showed that birth weight was no longer associated with enterotypes, and no other neonatal factors were associated with enterotypes (**Supplementary Table 5**). Concerning the NICU strategies, we observed a tendency between the practice of a low volume of enteral nutrition and an increased probability of belonging to the P_type enterotype (OR: 5.3, 95% confidence interval [0.9–30.8]) (**Supplementary Table 5**).

DISCUSSION

This study showed that the gut microbiota of preterm and full-term children at 3.5 years of age is characterized by two enterotypes dominated by either *Bacteroides* or *Prevotella*. Interestingly, we found that prematurity still imprints the gut microbiota of children, as the microbiota of preterm children showed lower diversity and different community composition than that seen in full-term children's microbiota. Additionally, the gut microbiota at 3.5 years of age was not related to that at 1 month in preterm children.

We confirmed that the gut microbiota of preterm neonates at 1 month of age is highly variable and clustered into six distinct enterotypes. Preterm neonate enterotypes were driven by a specific genus (i.e., *Enterobacter*, *Clostridium sensu stricto 1*, *Escherichia*, *Enterococcus*, and *Staphylococcus*), except for the sixth enterotype, which was characterized by a low bacterial load, leading to the absence of DNA amplification. These findings are in agreement with those of previous studies indicating that the early intestinal microbiota composition in preterm neonates is characterized by a strong dominance of only a few taxa (Stewart et al., 2016; Korpela et al., 2018). In a previous study, one of four genera, *Bifidobacterium*, *Enterobacter*, *Staphylococcus*, or

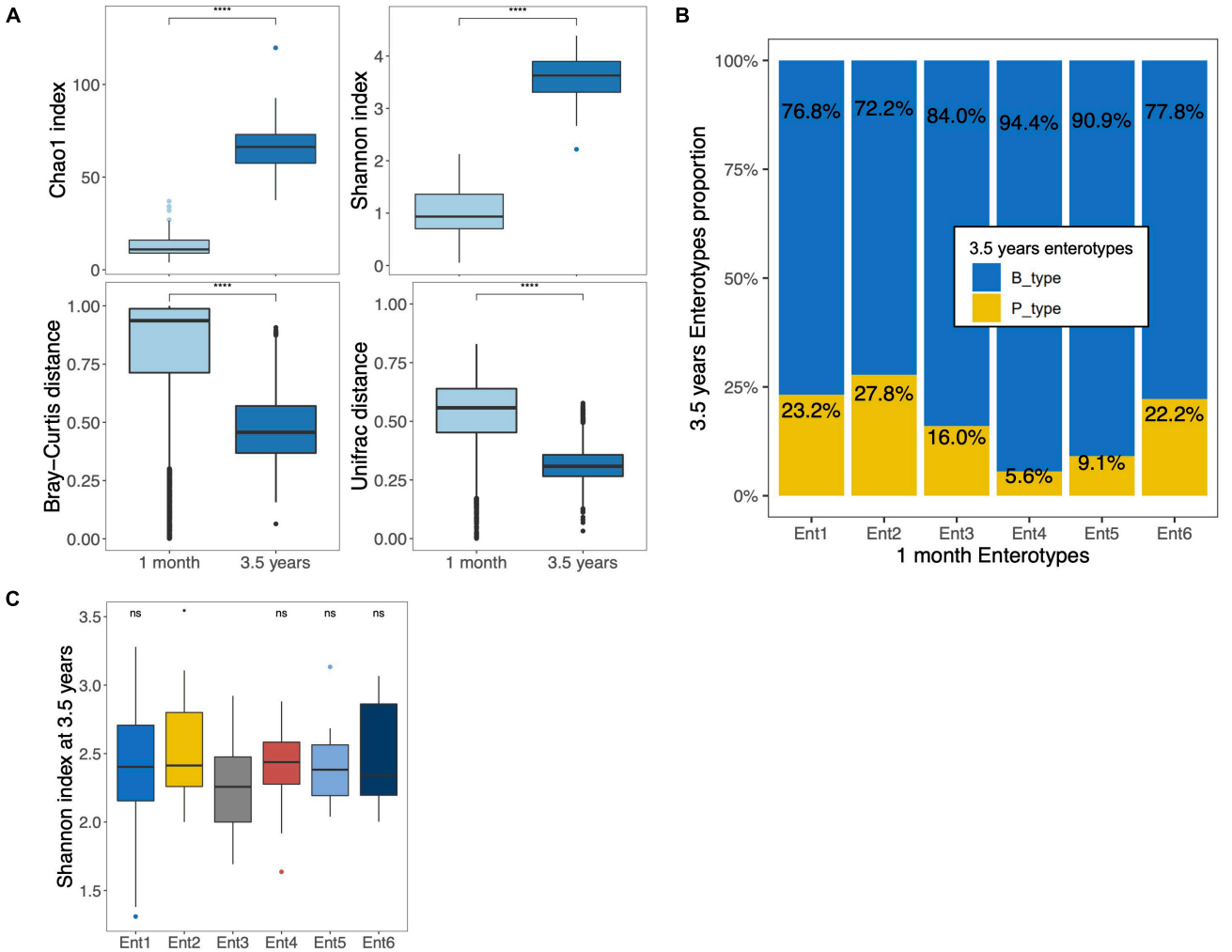

FIGURE 4 | Comparison of the gut microbiota of the same preterm children at 1 month and 3.5 years of age. **(A)** Boxplots of alpha (top) and beta diversities (bottom) based on both Chao1 and Shannon index and both Bray–Curtis and Unifrac dissimilarity distances, respectively, at genus taxonomic level in the 141 preterm children with sequencing data at two time points. **(B)** Distribution of B_ and P_type enterotypes among the six enterotypes at 1 month in 159 preterm children with a fecal sample at two time points. The values are expressed as proportions of each enterotype at 3.5 years of age among each enterotype at 1 month. **(C)** Boxplots of Shannon diversity based on genus level at 3.5 years of age according to enterotype at 1 month (*$p < 0.05$, ns = $p > 0.05$; Reference group = Ent3, Ent, Enterotype). The boxplots show the smallest and largest values, 25 and 75% quartiles, the median, and outliers. ****$p < 0.0001$.

Enterococcus, represented > 50% of the reads in a given sample (Korpela et al., 2018). The authors associated *Staphylococcus* and *Enterococcus* dominance with younger postmenstrual age. In our cohort, *Staphylococcus*- and *Enterococcus*-driven enterotypes were also characterized by a lower gestational age (**Supplementary Figure 4**). Additionally, we found a dominance of *Clostridium sensu stricto 1* and *Escherichia*, and an absence of a *Bifidobacterium*-driven enterotype, even though *Bifidobacterium* was among the top 10 dominant genera at 1 month. Korpela et al. (2018) reported an association between postnatal age and *Bifidobacterium* colonization. As their study was conducted with stool samples collected up to 60 days after birth, the lack of a *Bifidobacterium*-driven enterotype in our study could be explained by the fact that in our cohort, stool samples were collected at a median age of 23 days (IQR, 22–26 days), which is too early for this genus to reach a state of dominance. In a study by Stewart et al. (2016), the gut microbiota of preterm neonates, examined using stool samples collected from birth to 100 days of life, was grouped into six clusters characterized by the dominance of *Klebsiella*, *Staphylococcus*, *Enterococcus*, *Escherichia*, and *Bifidobacterium*, or dominance of both *Klebsiella* and *Enterococcus*.

The present study showed that alpha diversity of the gut microbiota in preterm children increases with age and that the gut microbiota community is significantly different between 1 month and 3.5 years of age. The latter is characterized by two discrete enterotypes driven by two dominant genera, *Bacteroides* and *Prevotella*. Previous studies monitoring the gut microbiota of preterm children between the NICU stay and 2 years of age also showed a significant increase in alpha diversity with age

TABLE 1 | Univariate association between preterm children enterotypes at 3.5 years of age, neonatal characteristics, and NICU strategies.

Variables	Enterotype		
	B_type (n = 128)	P_type (n = 31)	*P*-value
Gestational age (weeks)			
Mean (SD)	28.7 (1.96)	29.3 (1.89)	0.117
Maternal age (years)			
<25	6 (4.7%)	3 (9.7%)	0.354
25–35	85 (66.4%)	23 (74.2%)	
≥35	32 (25.0%)	5 (16.1%)	
Missing	5 (3.9%)	0 (0%)	
Country of birth of the mother			
France	112 (87.5%)	26 (83.9%)	0.810
Other	16 (12.5%)	5 (16.1%)	
Maternal level of education			
<High school	24 (18.8%)	11 (35.5%)	**0.011**
High school	19 (14.8%)	8 (25.8%)	
High school diploma +1 +2	30 (23.4%)	7 (22.6%)	
>High school diploma +3	55 (43.0%)	4 (12.9%)	
Missing	0 (0%)	1 (3.2%)	
Neonatal factors			
Male sex	66 (51.6%)	20 (64.5%)	0.272
Birth weight (g), Mean (SD)	1130 (335)	1280 (317)	**0.021**
Birth weight (Z-score), Mean (SD)*	−0.913 (1.38)	−0.483 (1.36)	0.123
C-section delivery mode	82 (64.1%)	16 (51.6%)	0.283
Practice of skin-to-skin contact during the first week of life			
Started between 0 and 3 days	29 (22.7%)	8 (25.8%)	0.359
Started between 4 and 7 days	39 (30.5%)	13 (41.9%)	
Not practiced	53 (41.4%)	9 (29.0%)	
Missing	7 (5.5%)	1 (3.2%)	
Antibiotic therapy during neonatal period**	104 (81.3%)	28 (90.3%)	1.000
Missing	11 (8.6%)	0 (0%)	
Did the child receive human milk during the neonatal period**	109 (85.2%)	25 (80.6%)	0.796
Missing	3 (2.3%)	1 (3.2%)	
NICU strategy* **			
Direct breastfeeding during the first week	11 (8.6%)	2 (6.5%)	0.980
Skin-to-skin contact during the first week	74 (57.8%)	21 (67.7%)	0.419
Longer duration of primary antibiotic therapy	38 (29.7%)	13 (41.9%)	0.273
Longer duration of secondary antibiotic therapy	60 (46.9%)	17 (54.8%)	0.551
Sedation during the first week	81 (63.3%)	20 (64.5%)	1.000
No intubation or extubation at day 1	46 (35.9%)	14 (45.2%)	0.457
Low volume of enteral nutrition at day 7	35 (27.3%)	14 (45.2%)	0.087

Complete case analysis. Data are presented as number of events (percentages). Missing data are noted if any (B_type, enterotype enriched in Bacteroides; P_type, enterotype enriched in Prevotella).
NICU, neonatal intensive care unit.
**Score based on Olsen curves.*
***Neonatal period is defined as the first 28 days of life.*
****Favorable strategy: the observed percentage was greater than the expected percentage of newborns receiving the treatment or practice.*
P-values marked with bold indicate statistically significant p-values ($p < 0.05$).

(Gómez et al., 2017; Yee et al., 2019) and reported differential beta-diversity structures between NICU stay and 4 years of age (Yee et al., 2019). We observed greater variability in the diversity of preterm children's gut microbiota at 1 month than at 3.5 years; this finding is in accordance with previous reports (Barrett et al., 2013; Stewart et al., 2016; Korpela et al., 2018). This indicates that preterm neonates' early gut microbiota has greater inter-individual variability and lower stability but evolves and stabilizes over time. Furthermore, between 1 month and 3.5 years, 68 genera were significantly differentially abundant (25 negatively and 43 positively associated with the gut microbiota at 3.5 years). Indeed, in addition to *Bifidobacterium* and *Bacteroides*, the major genera shifted from organisms previously found to be abundant in preterm neonates during the NICU stay to common

organisms found in adults, such as *Faecalibacterium*, *Roseburia*, and *Ruminococcus* (Roswall et al., 2021). Altogether, these data support the fact that the intestinal microbiota of children born preterm evolves toward a richer and more diverse adult-like microbiota over time.

Analyses of correlations between preterm children's gut microbiota diversity during the NICU stay and early childhood showed no significant relationship. Children with higher alpha diversities at 1 month of age did not harbor higher alpha diversities at 3.5 years of age. Based on beta diversity, we found no correlation when examining community composition among close-together (i.e., lower dissimilarity distance) preterm children at 1 month and at 3.5 years of age, demonstrating that close-together newborns do not conserve their proximity over time. Similarly, there was no association between preterm neonate enterotypes at 1 month and 3.5 years of age, suggesting that belonging to one of the six enterotypes at 1 month did not predispose to harbor a certain type of enterotype at 3.5 years. If clustering methods are sensitive to sample size (Koren et al., 2013; Henry et al., 2015; Preud'homme et al., 2021), in the present study, we demonstrated good reproducibility of the clustering at 1 month based on our population of 141 EPIFLORE preterm neonates (i.e., 159 neonates excluding the 18 preterm neonates belonging to enterotype 6) compared to the previous clustering performed on 484 EPIFLORE preterm neonates (Rozé et al., 2020). Because we identified the same clustering patterns based on smaller sample size, we can assume that the shift from six to two enterotypes that we observed is not an artifact of the smaller sample size of the present study.

In the present study, the analysis between preterm and full-term children at 3.5 years of age showed that both populations shared similar microbiota profiles characterized by *Bacteroides*- and *Prevotella*-driven enterotypes. This clustering is consistent with the gut microbiota studies among full-term children (Bergström et al., 2014; Nakayama et al., 2015; Méndez-Salazar et al., 2018; Xiao et al., 2021) and adults (Roager et al., 2014; Levy et al., 2020). A comparison of the microbiota community complexities at 3.5 years of age indicated that preterm and full-term children shared a similar richness composition, but preterm children had a lower alpha diversity and a different beta diversity (**Supplementary Figure 7**). It is known that the early gut microbiota differs between full-term and preterm children because of differences in exposure to prenatal and postnatal determinants (Groer et al., 2014; Tauchi et al., 2019; Aguilar-Lopez et al., 2021). Nonetheless, little is known about how early differences in gut colonization and establishment evolve as preterm neonates grow older. Differences in gut microbiota diversity between preterm and full-term children are observed within the first 6 months of life and are either still present (Vandenplas et al., 2020) or disappear (Dahl et al., 2018; Yap et al., 2021) at 12 months of age. Recently, the transition of enterotypes shaping the gut microbiota of children at an early age revealed that gut microbiota maturity of the preterm children was delayed up to 12 months compared to that of full-term children (Xiao et al., 2021). The present study indicates that at 3.5 years of age, the gut microbiota of the preterm children differs in terms of composition and is less diverse than that of full-term children's gut microbiota, suggesting that the transition recovery of the initially delayed gut microbiota colonization in preterm children still continues at 3.5 years of age. Our results are in accordance with those of Fouhy et al. (2019), who reported that gestational age imprints gut microbiota up to 4 years of age. Over the course of 3.5 years, we observed the evolution of preterm children's gut microbiota toward an enterotype-like gut microbiota similar to those found in full-term children. Because there is currently a lack of data on the development of preterm children's gut microbiota during early childhood and its impact on later health outcomes, the present study provides another insight into our understanding of the impact of gestational age on gut microbiota development up to 3.5 years of age.

The neonatal factors, such as gestational age, delivery mode, breastfeeding, early antibiotic therapy medication, skin-to-skin practice, and NICU practices, are known to influence early gut microbiota colonization (Rodríguez et al., 2015; Martin et al., 2016; Tamburini et al., 2016; Kumbhare et al., 2019; Rozé et al., 2020; Vandenplas et al., 2020). However, little is known about the long-term impact of these factors on the gut microbiota. Therefore, we investigated the potential effects of these factors on the gut microbiota of preterm children aged 3.5 years. We found no effects on the gut microbiota composition or any significant associations with the enterotype prevalence in preterm children at 3.5 years of age, indicating that these factors may not persist up to this age. Nonetheless, we observed a tendency between a low enteral volume at 7 days and an increased probability of belonging to the *Prevotella*-driven enterotype; the odds ratio indicates a strong association, but the wide 95% confidence interval indicates poor precision, which may be due to the limited sample size. Previously, the EPIFLORE study reported that gestational age and birth mode were associated with enterotypes at 1 month of age. Furthermore, no assisted ventilation on day 1 was associated with a decreased risk of belonging to enterotypes 5 or 6, while sedation and low volume of enteral nutrition were associated with an increased risk (Rozé et al., 2020), [enterotypes 5 and 6 were the less mature enterotypes characterized by a smaller gestational age (**Supplementary Figure 4**)]. If NICU practices are associated with preterm neonates' gut microbiota during the NICU stay, some of these practices may remain weakly associated with the microbiota 3.5 years later.

Study Limitations and Strengths

The use of the 16S rRNA gene sequencing approach limits this study to the description of bacterial composition. Analysis using shotgun metagenomics would allow greater sequencing depth, thus providing additional information regarding microbiome functional profiles and meaning at the species level of the microbiome that can differ between preterm and full-term populations, despite similar bacterial composition. Microbiota analysis was only performed for two times, that is, at 1 month and 3.5 years of age, which does not take into account possible dynamic changes in microbiota between these two time points. Moreover, we studied the influence of prenatal factors on the gut microbiota at 3.5 years in a preterm population, but we did not take into account more contemporary factors occurring during childhood, such as the use of antibiotics or dietary

habits that might influence the gut microbiota at 3.5 years. Finally, the number of children included may represent a limitation to definitely conclude about the association between the gut microbiota at 3.5 years of age and neonatal factors, but a tendency was observed, indicating a potential lack of statistical power.

In terms of major strengths, the present study is rare in the number of preterm children included within a multi-year follow-up and was performed on a large nationwide multicentric cohort, providing an accurate description of the neonatal characteristics of preterm children and characterization of the NICU strategies during hospitalization. Therefore, it allows a confident description of the preterm gut microbiota at both 1 month and 3.5 years of age, when the children's gut microbiota is supposed to mature toward an adult-like microbiota.

CONCLUSION

This study demonstrates that during the NICU stay, the gut microbiota of preterm neonates is characterized by high inter-individual variation and low stability shaped by the NICU environment. However, NICU-shaped gut microbiota was not predictive of microbial community composition in the same child at 3.5 years of age. At 3.5 years of age, preterm children share gut microbiota similar to that of children born full-term and correspond to a mature adult-like microbiota in terms of enterotypes. However, the gut microbiota of preterm children was characterized by a lower Shannon diversity and different overall microbial composition compared to that seen in the gut microbiota of full-term children, indicating that prematurity still imprints the gut microbiota up to 3.5 years of age. These results must be replicated in other large-scale longitudinal cohorts to confirm the relationship between gestational age and other perinatal factors and the gut microbiota during early childhood.

DATA AVAILABILITY STATEMENT

Personal data of children from the ELFE and EPIPAGE2 cohorts cannot be made publicly available for ethical reasons. They are available upon reasonable request from the authors under data-security conditions. The 16S rRNA gene reads are publicly available from the National Center for Biotechnology Information (nih.gov) Sequence Read Archive (SRA) under the Bioproject accession number: PRJNA798897, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA798897.

ETHICS STATEMENT

This study was approved by the National Data Protection Authority (Commission Nationale de l'Informatique et des Libertes [CNIL]), by the national advisory committee on information processing in health research (Comité Consultatif sur le Traitement de l'Information en matière de Recherche dans le domaine de la Santé [CCTIRS]) and by the Committee for the Protection of People Participating in Biomedical Research (Comité de Protection des Personnes [CPP]). Recruitment and data collection occurred only after the families had received information and agreed to participate in these cohorts with informed consent.

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AUTHOR CONTRIBUTIONS

J-CR, M-JB, M-AC, P-YA, and JA conceived and designed the study. M-JB, PL, GT, JD, and J-CR generated the data. JD and GT analyzed the data. GT drafted the manuscript. M-JB, J-CR,

PL, JD, P-YA, M-AC, and JA critically revised the manuscript. JA, M-JB, and M-AC supervised the study. All authors approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.919317/full#supplementary-material

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Characteristics of gut microbiota of term small gestational age infants within 1 week and their relationship with neurodevelopment at 6 months

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Introduction: Small for gestational age (SGA) infants are at a higher risk of neurodevelopmental delay than infants appropriate for gestational age (AGA). Previous studies have confirmed that gut microbiota in early life influences subsequent neurodevelopment. However, few studies have reported corresponding data in SGA populations.

Objective: We aimed to evaluate the characteristics of the gut microbiota of term SGA infants and the associations between the gut microbiota in SGA infants and neurodevelopmental outcomes at 6 months of age.

Methods: Fecal samples were collected on days 1, 3, 5, and 7 from term SGA and AGA infants born between June 2020 and June 2021 at the Peking University First Hospital. 16S ribosomal deoxyribonucleic acid amplicon sequencing was used to analyze the fecal microbiota. We followed up for 6 months and used the Ages and Stages Questionnaires-3 (ASQ-3) to evaluate the neurodevelopmental outcomes among SGA infants.

Results: A total of 162 neonates were enrolled, with 41 SGA infants (25.3%) in the study group and 121 AGA infants (74.7%) in the control group. The gut microbial diversity in the SGA group was lower than that in the AGA group on days 1, 3, 5, and 7. Non-metric multidimensional scaling and analysis of similarities showed significant differences between the two groups. The SGA group had increased relative abundances of *Ralstonia* (3, 5, and 7 days) and *Clostridium* (3 and 7 days). The dominant microorganisms of the SGA group were *Ralstonia* on day 1, *Escherichia_Shigella* on days 3 and 7, and *Clostridia* on day 5. We found that the gut microbial diversity of SGA infants with poor communication scores was higher than that of SGA infants with good communication scores on day 3. Fine motor scores were negatively correlated with the relative abundance of *Bacteroides_fragilis* on day 1. A negative correlation was observed between gross motor scores and relative abundance of *Clostridium_saccharobutylicum* on day

7. *Bacteroidota*, *Bacteroidia*, *Bacteroides*, and *Bacteroides_fragilis* were the dominant microorganisms in the good communication score group on day 7. Communication scores were positively correlated with the relative abundance of *Bacteroidota*, *Bacteroides,* and *Bacteroides_fragilis* on day 7.

Conclusion: The gut microbial diversity of term SGA infants was significantly lower in the first week of life than that of term AGA infants. Certain pathogenic and conditional pathogenic bacteria, such as *Escherichia_Shigella*, *Ralstonia* and *Clostridium* increased or formed the dominant microbiota in SGA infants. Alpha diversity, *Bacteroidota*, *Bacteroides*, *Bacteroides_fragilis*, and *Clostridium_saccharobutylicum* found in SGA infants may be associated with neurodevelopmental outcomes at 6 months of age, indicating possible therapeutic targets for clinical intervention.

KEYWORDS

gut microbiota, *Bacteroides*, SGA, neonates, neurodevelopment

Introduction

Small for gestational age (SGA) infants, defined as having a birth weight less than the 10th percentile of the birth weight of the same sex at the same gestational age, comprise a heterogeneous group (Chen et al., 2017; McCowan et al., 2018; Chawla, 2019). The incidence of SGA in China is ∼ 6.5%, ranking fifth worldwide (Lee et al., 2013). The development of each SGA system is imperfect, and the incidence of neurodevelopmental delay is significantly higher than that in appropriate for gestational age (AGA) infants (Sharma et al., 2016; McCowan et al., 2018; Kesavan and Devaskar, 2019). At present, the mechanisms leading to neurodevelopmental delays are unclear.

In recent years, gut microbiota has become a research hotspot in the fields of biology and medicine. Researchers have realized that the gut microbiota plays an important role in digestion, immune response, nutrient absorption, growth, and metabolism. The gut microbiota is involved in the regulation of many diseases such as inflammatory bowel disease, metabolic syndrome, and diabetes (Adak and Khan, 2019; Dabke et al., 2019; Ma et al., 2019; Mentella et al., 2020). Studies have found that microbiota plays a significant role in early neurological development (Carlson et al., 2018; Cohen Kadosh et al., 2021; Seki et al., 2021).

The so-called "gut–brain axis" represents a two-way communication network between the gut microbiota and the brain. The gut–brain axis theory proposes that the gut microbiota participates in the regulation of brain development and maturation, thus impacting brain functions, including anxiety-like behavior, locomotor behavior, social cognition, learning, and working memory (Al-Asmakh et al., 2012; Cryan et al., 2019; Long-Smith et al., 2020; Saurman et al., 2020). Compared with mice with normal gut microbiota, germ-free mice showed more obvious short-term cognitive and working memory impairments, whereas probiotic treatment prevented memory impairment after an inflammatory response in mice (Gareau et al., 2011). Gut microbiota affects various normal psychological processes and phenomena, participating in the pathophysiology of several psychological and neurological diseases (Liang et al., 2018). It has been reported that the gut microbial composition is altered in children with autism (Finegold et al., 2010; Plaza-Díaz et al., 2019; Dan et al., 2020; Saurman et al., 2020; Wong et al., 2022) and adults with Parkinson's disease (Vascellari et al., 2020; Hirayama and Ohno, 2021) and Alzheimer's disease (Zhuang et al., 2018; Bostancikliоğlu, 2019). Many studies have shown that probiotics are effective against anxiety, depression, autism spectrum disorder (ASD), and obsessive-compulsive disorder, and can also improve cognitive function, learning, and memory ability (Wang et al., 2016; Eastwood et al., 2021; Kim et al., 2021; Alemohammad et al., 2022). The intake of probiotics may ameliorate neurodegenerative disorders, including Alzheimer's disease, multiple sclerosis, Parkinson's disease, and amyotrophic lateral sclerosis (Cheng et al., 2019; Roy Sarkar and Banerjee, 2019).

Research focusing on children has indicated associations between gut microbiota in the first year of life and subsequent early neurodevelopment (Carlson et al., 2018; Sordillo et al., 2019; Tamana et al., 2021). Researchers have found that the alpha diversity of the gut microbiota in 1-year-old children could predict cognitive function at 2 years of age (Carlson et al., 2018). A cohort study found strong evidence of positive associations between *Bacteroidetes* in late infancy and subsequent cognitive and language performance

(Tamana et al., 2021). Another cohort study observed an association between the gut microbiome composition of infants aged 3–6 months and communication—personal and social—and fine motor skills at 3 years of age (Sordillo et al., 2019).

At present, there are many studies on the development and establishment of gut microbiota in healthy neonates. However, studies on the characteristics and evolution of the gut microbiota in SGA infants and their relationship with long-term neurodevelopmental outcomes remain scarce. Therefore, the objective of this study was to explore the characteristics of the gut microbiota of SGA infants during the first week of life using high-throughput sequencing technology. Additionally, this study aimed to further explore the potential relationship between gut microbiota and neurodevelopmental prognosis of SGA infants at 6 months of age. The discovery of the effects of specific microbiota on neural development would provide important insights into potential therapeutic targets for the clinical improvement of neurological development in SGA infants.

Subjects and methods

Subjects

Term SGA and AGA neonates hospitalized in the pediatric neonatal ward of Peking University First Hospital between June 2020 and June 2021 were recruited for this study.

Inclusion criteria

The following inclusion criteria were used: (a) neonates in the study group needed to meet the diagnostic criteria of SGA infants: newborns whose birthweight was less than the 10th percentile of the birth weight of the same sex at the same gestational age (1); (b) gestational age was defined as $\geq$ 37 and $<$ 42 weeks; (c) neonates without asphyxia, neonatal hypoxic-ischemic encephalopathy, severe intracranial hemorrhage, cerebral infarction, cytomegalovirus infection, recurrent hypoglycemia, bilirubin encephalopathy, and genetic metabolic diseases were enrolled; (d) informed consent was provided by the legal guardian(s); and (e) neonates were only enrolled with the agreement of cooperation with the follow-up by the legal guardian(s).

Exclusion criteria

The following exclusion criteria were used: (1) critical clinical conditions, such as sepsis and multiple organ failure; (2) gastrointestinal malformation, abdominal distension, vomiting, diarrhea, bloody stool, necrotizing enterocolitis, and other gastrointestinal diseases within 1 week; and (3) the presence of diseases that might affect neurological development during the follow-up period, such as severe brain trauma, epilepsy, meningitis, and genetic metabolic diseases.

Methods

Data collection

Clinical data, including sex, gestational age, birth weight, mode of delivery, and antibiotic application within 1 week after birth, were collected. Feces produced on postnatal days 1, 3, 5, and 7 were collected. Fecal samples were stored in sterile freezing tubes (Haimen Morder Experimental Equipment Factory) at −20°C and subjected to microbiota analysis within 1 week.

Gut microbiota test and analysis

Microbiota sequencing

A biological information database was built using an Illumina TruSeq§ DNA PCR-Free Sample Preparation Kit. Quality was evaluated with the assistance of the Qubit@ 2.0 and Agilent Bioanalyzer 2100 system. High-throughput sequencing was performed using an Illumina NovaSeq 6000 platform.

Bioinformatics analysis

The effective data were obtained by filtering the original data. The sequences were then clustered into operational taxonomic units (OTUs) with 97% identity, and the OTUs sequences were compared with the silva138 database for species annotation to obtain the basic analysis results of the OTUs and taxonomic pedigree for each sample. Finally, the analysis of OTUs, including alpha and beta diversity, was completed according to species annotation.

- Alpha diversity analysis: The richness and diversity of microbiota can be indicated by alpha diversity, wherein Observed species, Chao1, abundance-based coverage estimator (ACE), Shannon, Simpson, and goods coverage are major evaluation indices of alpha diversity. Observed species represents the actual number of OTUs in the sample. The Chao1 and ACE indices use different calculation methods to estimate the number of OTUs in a sample; the higher the number of OTUs, the higher the diversity of the sample. The abundance and uniformity of the gut microbiota can be expressed using the Shannon and Simpson indices. If all the OTUs contained in the sample were the same, the diversity was the lowest; if they were different, the diversity was the highest. The larger the values of the Shannon and Simpson indices, the higher the diversity of the samples. Good coverage index indicates the sequencing depth; the higher the value, the better the sequencing. The closer the value is to 1, the closer the sequencing depth is to cover all bacteria in the test sample. Rarefaction and rank variance curves are

common curves that describe the diversity of the samples in the group. The rarefaction curve directly reflects the rationality of the sequencing data and indirectly reflects the richness of species in the sample, whereas the rank variance curve intuitively reflects the richness and uniformity of species in the sample.

- Beta diversity analysis: Beta diversity analysis focuses on the differences in the microbial community composition of different samples, which is used for the analysis of differences between groups. Principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) directly reflects the differences in community composition between groups based on the distance between samples. Each point in the figure represents a sample; points of the same color belong to the same group, and the distance between points represents the degree of difference. The distance is directly proportional to the difference between points. A stress score < 0.2 indicates that NMDS can accurately reflect the degree of difference between groups. Analysis of similarities (Anoism) is a non-parametric test used to test the significance of differences. An R-value > 0 indicates significant differences between groups, an R-value < 0 indicates that the differences within groups were greater than those between groups, and a P-value < 0.05 indicates statistical significance.
- Differential analysis of gut microbiota: (1) Differential relative abundance: We compared the differences in microbial distribution between groups according to the relative abundance of communities at different levels of phylum, class, order, family, genus, and species. In this study, we used the Metastat method to analyze microbial differences between the two groups at the phylum, family, and genus levels. (2) Differentially dominant microorganisms: We found microbial differences between groups using linear discriminant analysis effect size (LEfSe) analysis. The LEfSe calculation method not only has statistical significance, but also focuses on biological correlation by using linear discriminant analysis (LDA) to reduce the dimension and evaluate the impact of species with significant differences (i.e., LDA score). The default LDA score was 2, which can be increased according to the characteristics of the community distribution to obtain more accurate data.

Follow-up of study group

We followed up with the SGA infants until 6 months after birth and assessed their neurodevelopmental outcomes using the Age and Staging Questionnaire-3 (ASQ-3). The same neurodevelopmental professional evaluation doctor, proficient in ASQ-3 scoring criteria, conducted the evaluation. ASQ-3 mainly includes five parts, namely communication, gross motor, fine motor, problem-solving, and personal–social, with each part containing six specific assessment questions.

Statistical analysis

Statistical software (SPSS 25.0) was used to analyze the data. Measurement data consistent with a normal distribution are expressed as the mean ± standard deviation ($x \pm s$). Student's t-test was used to compare two groups, while analysis of variance (ANOVA) was used to compare three or more groups. The measurement data that were not in line with the normal distribution were expressed as median (IQR) or median (P25, P75). The Wilcoxon Mann–Whitney U test was used for the comparison between two groups, while the Kruskal–Wallis H test was used for the comparison of three groups and above. The enumeration data were expressed as the number of cases and percentages, and comparisons between groups were performed using the χ^2 test. If the total number n was < 40 or at least one actual frequency, $t < 1$, Fisher's exact test method was applied. For the correlation analysis of two quantitative datasets, Pearson correlation analysis was adopted if it conformed to the bivariate normal distribution; otherwise, Spearman correlation analysis was adopted. Statistical significance was set at $P < 0.05$.

Ethical approval

The study was approved by the Ethics Committee of the Peking University First Hospital. The legal guardians of each participant provided written informed consent.

Results

Clinical characteristics of neonates in the small for gestational age and appropriate for gestational age groups

A total of 41 SGA neonates were enrolled in the SGA group, including 19 males (46.3%) and 24 neonates (58.5%) delivered *via* cesarean section. A total of 121 AGA neonates were enrolled in the AGA group, with 75 males (62.0%) and 33 neonates (27.5%) delivered *via* cesarean section. All neonates were born at a gestational age of 37–42 weeks and fed a mixed feed (breast milk + formula). The clinical characteristics of the enrolled neonates are presented in **Table 1**. A total of 31 neonates (75.6%) in the SGA group and 112 neonates (92.6%) in the AGA group had aspiration pneumonia or increased non-specific inflammatory indices. The proportion of ampicillin users in the AGA group was significantly higher than that in the SGA group. The gestational age and birth weight of the neonates in the SGA group were significantly lower than those in the AGA group, and the proportion of cesarean sections was significantly higher in the SGA group. The following clinical characteristics differed significantly between the SGA and AGA groups: infants from twin pregnancies, premature rupture of membranes, hospital

stay of neonates, chorioamnionitis, and maternal antibiotics. There were no significant differences in sex, Apgar scores at 1 and 5 min, region, siblings, mother's pregnancy weight gain, pregnancy complications (diabetes or gestational hypertension), or pet ownership between the two groups. None of the enrolled neonates were infected with the novel coronavirus, and neither their mothers nor their family members showed the emergence of pandemic-related mental or personality disturbances.

Gut microbiota analysis of the small for gestational age and appropriate for gestational age groups

In this study, an average of 76,816 tags were measured per sample, and an average of 75,108 valid data points was obtained after quality control. The sequence was clustered into OTUs with 97% identity and 13,747 OTUs were obtained.

Sequencing depth and rationality

After obtaining all the OTUs, a rarefaction curve was drawn to evaluate whether the current sequencing depth of each sample could fully reflect the microbial diversity in the community samples. When the dilution curve tended to be flat (**Supplementary Figure 1**), the sequencing data gradually became reasonable. The coverage index of these samples fluctuated between 0.974 and 1, indicating that the sequencing depth was close to covering all bacterial communities in the tested samples.

Alpha and beta diversities

A comparison of the alpha diversity of fecal microbiota in the SGA group on different days in the first week of life revealed no statistical difference in the Chao1, ACE, Observed species, Simpson, and Shannon indices, indicating that there was no significant difference in the richness and diversity of gut microbiota within the SGA group at postnatal days 1, 3, 5, and 7 (**Supplementary Table 1**).

A comparison of alpha diversity of fecal microbiota between the SGA and AGA groups revealed that the SGA group's gut microbial diversity was significantly lower than that of the AGA group in the Chao1, ACE, Observed species, Simpson, and Shannon indices on the first day (< 0.05). On days 3, 5, and 7, the Chao1, ACE, and Observed species indices of fecal microbiota of the SGA group remained significantly lower than the AGA group ($P < 0.05$), whereas there was no significant difference in the Simpson and Shannon indices (**Supplementary Figure 2** and **Supplementary Table 2**).

PCoA showed significant differences in the gut microbiota on days 1, 3, 5, and 7 between the two groups. The R-value > 0, and statistical analysis between groups showed significant differences ($P < 0.05$) (**Supplementary Figures 3A–H** and **Supplementary Table 3**). The NMDS analysis (**Supplementary Table 4**) indicated that the gut microbiota of the two groups differed significantly on days 1, 3, 5, and 7 (stress score < 0.2) (**Figure 1**).

Analysis of differential gut microbiota

Differential relative abundance

The main microbiota in the AGA group at the phylum level were *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* (**Supplementary Figures 4**, **5**); at the family level, *Enterococcaceae*, *Streptococcaceae*, *Enterococcaceae*, *Staphylococcaceae*, *Vibrionaceae* (**Supplementary Figures 6**, **7**); and at the genus level, *Enterococcus*, *Streptococcus*, *Escherichia-Shigella*, *Staphylococcus*, and *Vibrio* (**Supplementary Figures 8A**, **9**).

On day 1, the SGA group showed a decreased relative abundance of *Cyanobacteria*, *Vibrionaceae*, *Parabacteroides*, *Lactiplantibacillus*, *Serratia*, *Citrobacter*, and *Cutibacterium*. However, the relative abundance of *Ileibacterium* was higher in the SGA group than in the AGA group (**Table 2**, **Figure 2**, and **Supplementary Figures 10**, **11**).

On day 3, the SGA group showed decreased relative abundances of *Actinobacteria*, *Streptococcaceae*, *Vibrionaceae*, *Streptococcus*, *Vibrio*, *Pseudoalteromonas*, *Uruburuella*, *Parabacteroides,* and *Lactiplantibacillus*, whereas *Burkholderiaceae,* and *Ralstonia were* higher in the SGA group than in the AGA group (**Table 2**, **Figure 2**, and **Supplementary Figures 10**, **11**).

On day 5, the SGA group showed decreased relative abundances of *Streptococcaceae*, *Lysinibacillus*, *Streptococcus*, *Lactiplantibacillus*, *Cutibacterium*, *Serratia* and *Citrobacter*, while those of *Campylobacteria*, *Verrucomicrobiota*, *Burkholderiaceae*, *Erysipelotrichaceae*, *Micrococcaceae*, *Helicobacteraceae*, *Ileibacterium,* and *Akkermansia* were higher in the SGA group than in the AGA group (**Table 2**, **Figure 2**, and **Supplementary Figures 10**, **11**).

On day 7, the SGA group showed decreased relative abundances of *Actinobacteria*, *Cyanobacteria*, *Streptococcaceae*, *Lysinibacillus*, *Lactiplantibacillus,* and *Serratia*, whereas those of *Burkholderiaceae*, *Erysipelotrichaceae*, *Ralstonia*, *Ileibacterium*, *Akkermansia*, *Halomonas,* and *Rhodococcus* were higher in the SGA group than in the AGA group (**Table 2**, **Figure 2**, and **Supplementary Figures 10**, **11**).

Differential dominant microorganisms

LEfSe was used to analyze differentially dominant microorganisms, and the LDA value was set to 4. On day 1, the dominant microorganisms in the SGA group were *g-Ralstonia* and *s-Ralstonia_pickettii*, while *s-Streptococcus_sp_FDAARGOS_192*, *f-Vibrionaceae*, and *g-Vibrio* were dominant in the AGA group. On day 3, the dominant microorganisms in the SGA group were *s-Ralstonia_pickettii* and *g-Escherichia_Shigella*, while *f-Streptococcaceae*, *g-Streptococcus*, and *s-Streptococcus_sp_FDAARGOS_192*

were dominant in the AGA group. On day 5, the dominant microorganisms in the SGA group were *c-Clostridia*, *g-Rothia*, *s-Bacteroides_fragilis*, and *o-Clostridiales*, while *f-Streptococcaceae*, *g-Streptococcus*, and *s-Streptococcus_sp_FDAARGOS_192* were dominant in the AGA group. On day 7, the dominant microorganisms in the SGA group were *s-Ileibacterium_valens*, *g-Ileibacterium*, *f-Enterobacteriaceae*, *g-Helicobacter*, and *g-Escherichia_Shigella*, while *s-Streptococcus_sp_FDAARGOS_192* were dominant in the AGA group (**Figure 3** and **Supplementary Figures 12A–D**).

Correlation analysis between the top six microbiota at the genus and species levels in the small for gestational age group and ASQ-3 scores at 6 months of age

Neonates in the SGA group were followed up to 6 months of age, of which, 38 (92.7%) infants completed the follow-up and three infants were lost to follow-up (7.3%). The fine motor scores of ASQ-3 were negatively correlated with the relative abundance of *s-Bacteroides_fragilis* on day 1 ($r = -0.412$, $P = 0.041$). On day 7, the communication scores were positively correlated with the relative abundances of *g-Bacteroides* ($r = 0.875$, $P = 0.004$) and *s-Bacteroides_fragilis* ($r = 0.886$, $P = 0.003$), whereas a negative correlation was observed between gross motor scores and the relative abundance of *s-Clostridium_saccharobutylicum* ($r = -0.736$, $P = 0.037$; **Table 3**).

Analysis of gut microbiota of neonates in small for gestational age group with different neurological prognosis (communication)

We followed SGA infants until 6 months of age, and 38 of them completed the follow-up. A communication score ≥ 40 was considered normal. There were 31 (81.6%) infants with normal communication scores and 7 (18.4%) infants with poor communication scores in the SGA group. The two subgroups showed no significant differences in sex, gestational

TABLE 1 Clinical characteristics of the SGA and AGA groups.

Descriptive variable	SGA group $n = 41$	AGA group $n = 121$	Statistic value	*P*
Male	19 (46.3%)	75 (62.0%)	3.076	0.079
Gestational age (weeks)	37.6 (1.3)	39.3 (1.6)	−4.946	< 0.001
Birthweight (grams)	2352.7 ± 300.6	3282.3 ± 331.7	15.66	< 0.001
Infants from twin pregnancy	10 (24.4%)	2 (1.7%)	19.887	< 0.001
Cesarean	24 (58.5%)	33 (27.5%)	12.872	< 0.001
Premature rupture of membranes	2 (4.9%)	36 (29.8%)	10.553	0.001
Apgar score at 1 min	10 (0)	10 (0)	0.428	0.669
Apgar score at 5 min	10 (0)	10 (0)	0.852	0.394
Mixed fed (formula + breast-feeding)	41 (100%)	121 (100%)	-	> 0.999
Ampicillin to neonates (first week)	31 (75.6%)	112 (92.6%)	6.942	0.008
Hospital stay of neonates (days)	7 (2)	6 (1)	−3.712	< 0.001
Sibling	14 (34.1%)	30 (24.8)	1.354	0.245
Mother's age (years)	33.0 ± 3.6	32.2 ± 3.8	−0.343	0.732
Mother's pregnancy weight gain (kg)	12.0 (4.8)	13.6 (5.0)	−1.273	0.203
Maternal smoking	0 (0%)	0 (0%)	-	> 0.999
Gestational hypertension	8 (19.5%)	10 (8.3%)	2.866	0.090
GDM or DM	10 (24.4%)	36 (29.8%)	0.433	0.511
Antenatal TG (mmol/L)	2.22 (1.82)	2.56 (1.67)	−0.286	0.775
Antenatal TCHO (mmol/L)	5.87 (2.38)	5.93 (2.40)	−0.485	0.628
Antenatal HDL (mmol/L)	1.63 (1.00)	1.69 (0.00)	−0.080	0.936
Antenatal LDL (mmol/L)	3.14 (1.40)	2.82 (1.54)	−1.115	0.265
Chorioamnionitis	11 (26.8%)	16 (13.2%)	4.082	0.043
Antibiotics to mother	8 (19.5%)	48 (39.7%)	5.501	0.019
Antenatal corticosteroids	0 (0%)	1 (0.8%)	-	> 0.999
Inclusion site-countryside	2 (4.9%)	6 (5.0%)	0.00	> 0.999

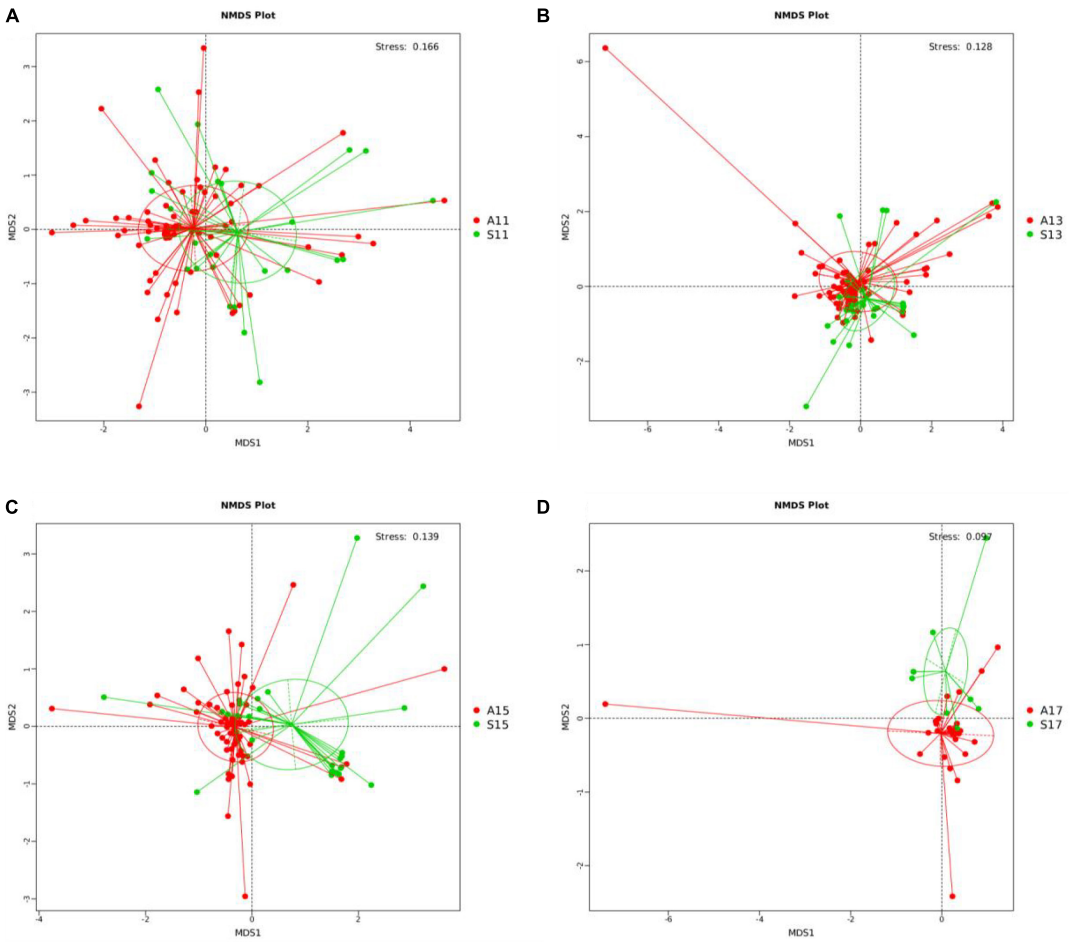

FIGURE 1
NMDS analysis between the SGA and AGA groups on days 1, 3, 5, and 7. **(A)** NMDS analysis on day 1. **(B)** NMDS analysis on day 3. **(C)** NMDS analysis on day 5. **(D)** NMDS analysis on day 7. NMDS, non-metric multi-dimensional scaling. The red points represent belonging to the AGA group, and the green points represent belonging to the SGA group. S11, gut microbiota of the SGA group on day 1; A11, gut microbiota of the AGA group on day 1; S13, gut microbiota of the SGA group on day 3; A13, gut microbiota of the AGA group on day 3; S15, gut microbiota of the SGA group on day 5; A15, gut microbiota of the AGA group on day 5; S17, gut microbiota of the SGA group on day 7; A17, gut microbiota of the AGA group on day 7.

age, birth weight, mode of delivery, feeding pattern, or antibiotic application within 1 week after birth (Table 4).

Alpha and beta diversities

A comparison of the alpha diversity of fecal microbiota between the good and poor communication score groups revealed no statistical difference in the Chao1, ACE, Observed species, Simpson, and Shannon indices on days 1, 5, and 7. On day 3, the gut microbial diversity of the poor communication score group was significantly higher than that of the good communication score group in the Chao1, ACE, and Observed species indices ($P < 0.05$), whereas there was no significant difference in the Simpson and Shannon indices (Figure 4 and Supplementary Table 5).

PCoA and Anosim showed no significant differences in the gut microbiota on days 1 and 7 between the good and poor communication score groups; although the R-value was > 0, but the statistical analysis between the groups showed no significant difference ($P > 0.05$). There were no significant differences in gut microbiota on days 3 and 5 in the good and poor communication score groups; the R-value was < 0, but the statistical analysis within groups showed no significant difference ($P > 0.05$) (Supplementary Figures 13A–D and Supplementary Table 6). However, NMDS analysis indicated that the gut microbiota of the two groups differed significantly on days 1, 3, 5, and 7 (stress score < 0.2) (Supplementary Figures 14A–D).

Analysis of differential gut microbiota

Differential relative abundance

The main microbiota in the good communication score group included *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and

TABLE 2 Gut microbiota analysis of the SGA and AGA groups on days 1, 3, 5, and 7 at levels of phylum, family, and genus.

Taxonomy	Days	Microbiota	SGA group	AGA group	*P*
Phylum ($P < 0.05$)	D1	*Cyanobacteria*	3.22×10^{-4}	1.18×10^{-2}	0.005
	D3	*Actinobacteria*	3.32×10^{-2}	6.69×10^{-2}	0.030
	D5	*Campylobacteria*	3.31×10^{-4}	7.20×10^{-6}	0.010
		Verrucomicrobiota	8.33×10^{-4}	1.62×10^{-5}	0.017
	D7	*Actinobacteria*	1.45×10^{-2}	5.12×10^{-2}	0.011
		Cyanobacteria	4.09×10^{-5}	4.72×10^{-3}	0.005
Family ($P < 0.05$)	D1	*Vibrionaceae*	4.95×10^{-3}	4.37×10^{-2}	0.021
	D3	*Streptococcaceae*	7.23×10^{-2}	1.74×10^{-1}	0.003
		Burkholderiaceae	8.85×10^{-2}	7.22×10^{-4}	0.002
		Vibrionaceae	7.40×10^{-4}	6.88×10^{-3}	0.003
	D5	*Streptococcaceae*	1.18×10^{-1}	2.58×10^{-1}	0.002
		Burkholderiaceae	9.37×10^{-3}	4.69×10^{-4}	0.022
		Erysipelotrichaceae	1.40×10^{-3}	9.85×10^{-5}	0.048
		Micrococcaceae	2.85×10^{-2}	1.94×10^{-3}	0.016
		Helicobacteraceae	3.22×10^{-4}	5.85×10^{-6}	0.016
	D7	*Streptococcaceae*	9.06×10^{-2}	2.85×10^{-1}	0.027
		Burkholderiaceae	1.29×10^{-2}	7.39×10^{-5}	0.003
		Erysipelotrichaceae	6.98×10^{-2}	1.69×10^{-4}	0.017
Genus ($P < 0.01$)	D1	*Parabacteroides*	5.46×10^{-5}	1.02×10^{-2}	0.001
		Lactiplantibacillus	3.15×10^{-6}	4.84×10^{-4}	0.001
		Serratia	2.31×10^{-5}	1.17×10^{-3}	0.005
		Citrobacter	2.10×10^{-6}	1.29×10^{-3}	0.008
		Cutibacterium	2.04×10^{-4}	2.93×10^{-3}	0.009
		Ileibacterium	1.04×10^{-3}	5.75×10^{-6}	0.008
	D3	*Streptococcus*	7.19×10^{-2}	1.73×10^{-1}	0.003
		Vibrio	7.40×10^{-4}	6.88×10^{-3}	0.003
		Pseudoalteromonas	1.74×10^{-4}	1.50×10^{-3}	0.004
		Uruburuella	3.28×10^{-5}	2.60×10^{-4}	0.004
		Parabacteroides	1.75×10^{-4}	2.41×10^{-3}	0.006
		Lactiplantibacillus	9.74×10^{-6}	1.86×10^{-4}	0.006
		Ralstonia	8.85×10^{-2}	6.61×10^{-4}	0.001
	D5	*Lysinibacillus*	0.00	2.52×10^{-3}	0.001
		Streptococcus	1.18×10^{-1}	2.58×10^{-1}	0.002
		Lactiplantibacillus	4.36×10^{-6}	1.68×10^{-3}	0.002
		Cutibacterium	2.29×10^{-5}	1.88×10^{-4}	0.008
		Serratia	2.18×10^{-6}	2.11×10^{-5}	< 0.001
		Citrobacter	0.00	1.75×10^{-5}	< 0.001
		Ileibacterium	9.00×10^{-4}	1.35×10^{-6}	0.001
	D7	*Lysinibacillus*	0.00	1.01×10^{-3}	0.009
		Lactiplantibacillus	6.30×10^{-6}	4.74×10^{-3}	0.005
		Serratia	0.00	1.44×10^{-3}	0.001
		Ralstonia	1.28×10^{-2}	5.57×10^{-5}	0.003
		Ileibacterium	6.45×10^{-2}	1.01×10^{-6}	0.002
		Akkermansia	2.09×10^{-3}	8.10×10^{-6}	0.001
		Halomonas	2.71×10^{-4}	0.00	0.001
		Rhodococcus	5.29×10^{-4}	1.01×10^{-6}	0.002

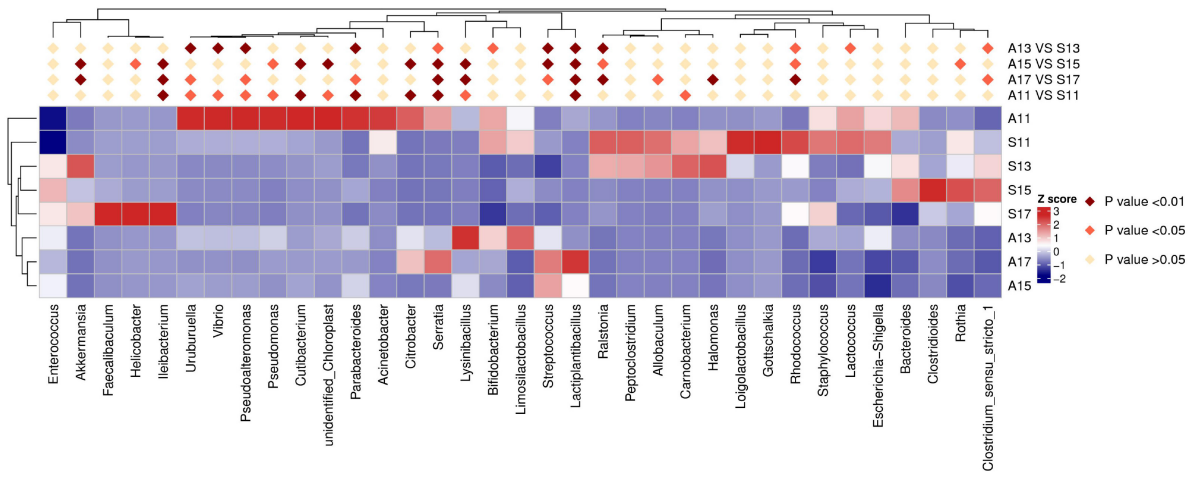

FIGURE 2
Results of heatmap analysis of species with significant differences between the SGA and AGA groups at the genus level. S11, gut microbiota of the SGA group on day 1; A11, gut microbiota of the AGA group on day 1; S13, gut microbiota of the SGA group on day 3; A13, gut microbiota of the AGA group on day 3; S15, gut microbiota of the SGA group on day 5; A15, gut microbiota of the AGA group on day 5; S17, gut microbiota of the SGA group on day 7; A17, gut microbiota of the AGA group on day 7.

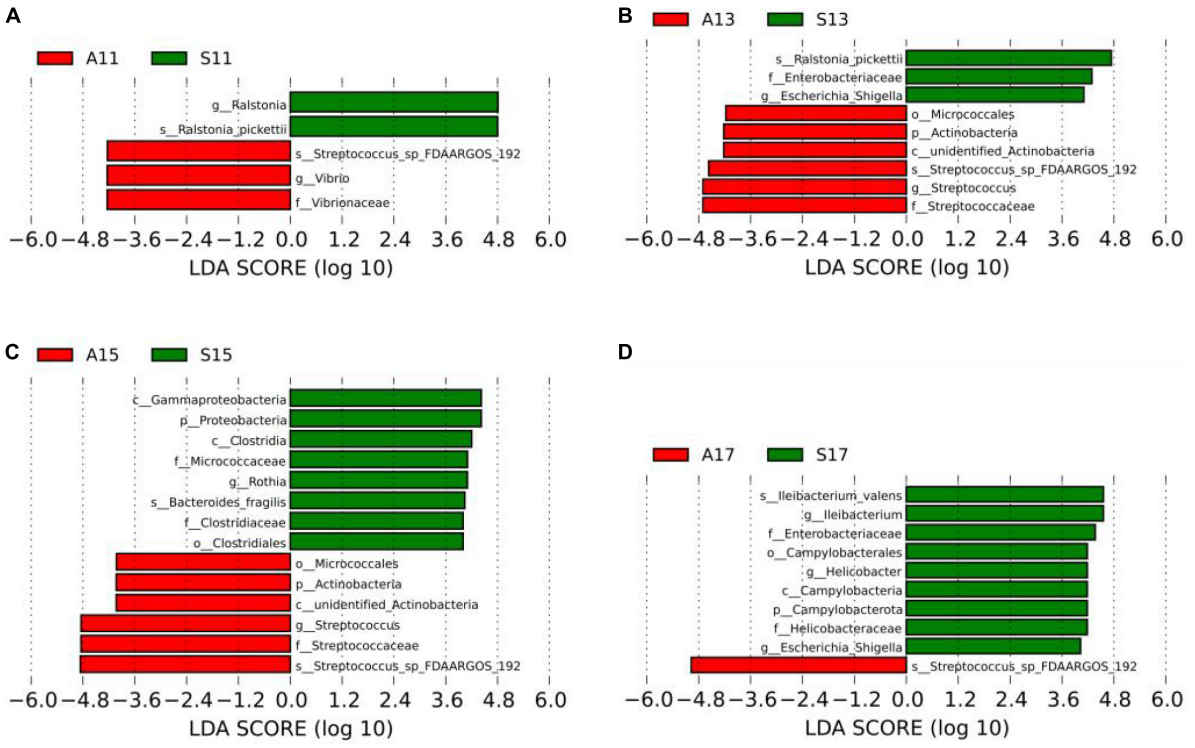

FIGURE 3
LEfSe comparison between the SGA and AGA groups. **(A)** LDA score histogram of differential microbiota of the two groups on day 1. **(B)** LDA score histogram of differential microbiota of the two groups on day 3. **(C)** LDA score histogram of differential microbiota of the two groups on day 5. **(D)** LDA score histogram of differential microbiota of the two groups on day 7. p_ represents phylum level, c _ represents class level, o_ represents order level, f_ represents family level, g_ represents genus level, and s_ represents species level. The length of the column represents the LDA score, and the greater the score, the greater the influence of the dominant microbiota. LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size. S11, gut microbiota of the SGA group on day 1; A11, gut microbiota of the AGA group on day 1; S13, gut microbiota of the SGA group on day 3; A13, gut microbiota of the AGA group on day 3; S15, gut microbiota of the SGA group on day 5; A15, gut microbiota of the AGA group on day 5; S17, gut microbiota of the SGA group on day 7; A17, gut microbiota of the AGA group on day 7.

TABLE 3 Correlation analysis between the top six microbiota at genus and species level in the SGA group and ASQ-3 scores at 6 months postnatal age.

Days and microbiota	ASQ-3 scores	Correlation index (*r*)	*P*
D7 *Bacteroides*	Communication	0.875	0.004
	Gross motor	0.012	0.977
	Fine motor	0.111	0.793
	Problem solving	0.101	0.811
	Personal–social	0.506	0.201
D7 *Bacteroides_fragilis*	Communication	0.886	0.003
	Gross motor	0.050	0.907
	Fine motor	0.050	0.906
	Problem solving	0.103	0.809
	Personal–social	0.050	0.207
D1 *Bacteroides_fragilis*	Communication	−0.055	0.794
	Gross motor	0.030	0.886
	Fine motor	−0.412	0.041
	Problem solving	−0.012	0.954
	Personal–social	−0.049	0.818
D7 *Clostridium_saccharobutylicum*	Communication	−0.230	0.583
	Gross motor	−0.736	0.037
	Fine motor	−0.221	0.599
	Problem solving	−0.053	0.900
	Personal–social	−0.299	0.472

Bacteroidetes at the phylum level (Supplementary Figure 15); *Enterococcaceae*, *Burkholderiaceae*, *Enterobacteriaceae*, *Streptococcaceae*, and *Staphylococcaceae* at the family level (Supplementary Figure 16); *Enterococcus*, *Streptococcus*, *Escherichia-Shigella*, *Staphylococcus*, *Bacteroides*, and *Ileibacterium* at the genus level (Supplementary Figure 17); and *Ralstonia_pickettii*, *Streptococcus_sp_FDAARGOS_192*, *Bacteroides_fragilis*, *Ileibacterium_valen*, *Rothia_mucilaginosa*, and *Clostridium_saccharobutylicum* at the species level (Supplementary Figure 18). On day 1, the poor communication score group showed decreased relative abundances of *Enterobacteriaceae*, *Streptococcaceae*, and *Streptococcus* (Supplementary Table 7 and Supplementary Figures 19A–D). On day 3, the main microbiota between the good and poor communication score groups showed no significant differences in the phylum, family, genus, and species levels (Supplementary Table 7 and Supplementary Figures 20A–D). On day 5, the poor communication score group showed decreased relative abundances of *Staphylococcaceae* and *Staphylococcus*, whereas the relative abundance of *Enterococcus* was higher in the poor communication score group (Supplementary Table 7 and Supplementary Figures 21A–D). On day 7, the poor communication score group showed decreased relative abundances of *Bacteroidota*, *Bacteroides*, and *Bacteroides_fragilis*, whereas the relative abundance of *Corynebacterium* was higher in the poor communication score group (Supplementary Table 7 and Supplementary Figures 22A–D).

Differential dominant microorganisms

On day 1, the dominant microorganisms in the poor communication score group were f-*Erysipelotrichaceae*, *f-Carnobacteriaceae*, and *g-Allobaculum*. On day 3, they were *p-Actinobacteria*, *c-unidentified Actinobacteria*, and *f-Peptostreptococcaceae*. There were no dominant microorganisms in the poor communication score group on day 5. On day 7, the dominant microorganism in the poor communication score group was *f-Peptostreptococcaceae*. *p-Bacteroidota*, *c-Bacteroidia*, *o-Bacteroidales*, *f-Bacteroidaceae*, *g-Bacteroides*, and *s_Bacteroides_fragilis* formed the dominant microorganisms in the good communication score group on day 7 (LDA score > 3). There were no dominant microorganisms in the good communication score group on days 1, 3, and 5 (Figure 5 and Supplementary Figure 23).

Analysis of differential microbiota and communication scores at 6 months of age

We analyzed the correlation between differentially abundant microbiota and communication scores at 6 months postnatal, and found that *p-Bacteroidota*, *g-Bacteroides*, and *s-Bacteroides_fragilis* were positively correlated with communication scores, on day 7. Moreover, there was no correlation between the rest of the

TABLE 4 Clinical characteristics of the good and poor communication score groups in the SGA population.

Descriptive variable	Good communication score group	Poor communication score group	Statistic value	*P*
Male	13 (41.9%)	4 (57.1%)	-	0.678
Gestational age (weeks)	37.9 ± 1.1	39.2 ± 1.7	1.850	0.106
Birthweight (grams)	2325.7 ± 288.5	2519.3 ± 335.3	1.559	0.128
Birthweight percentile < P3	9 (29.0%)	3 (42.9%)	-	0.656
Cesarean	18 (58.1%)	3 (42.9%)	-	0.678
Ampicillin to neonates (first week)	23 (74.2%)	5 (71.4%)	-	> 0.999

differentially abundant microbiota and communication scores (**Supplementary Table 8**).

Effect of delivery mode on gut microbiota of small for gestational age neonates and ASQ-3 scores

A total of 41 SGA neonates were enrolled, including 24 neonates (58.5%) delivered by cesarean section and 17 neonates (41.5%) through vaginal delivery. The two subgroups showed no significant differences in sex, gestational age, birth weight, feeding patterns, or antibiotic application within 1 week after birth (**Supplementary Table 9**).

Alpha and beta diversities

A comparison of alpha diversity of fecal microbiota between the cesarean birth and vaginal delivery groups revealed no statistical difference in the Chao1, ACE, Observed species, Simpson, and Shannon indices on days 1, 3, and 5 (**Supplementary Table 10** and **Supplementary Figure 24**).

PCoA and Anosim showed no significant differences in gut microbiota between the cesarean birth and vaginal delivery groups; the R-value was > 0, but the statistical analysis between the groups showed no significant difference ($P > 0.05$; **Supplementary Figures 25A–C**, **26A–C** and **Supplementary Table 11**). However, NMDS analysis indicated that the gut microbiota of the two groups differed significantly on days 1, 3, and 5 (stress score < 0.2; **Supplementary Figures 27A–C**).

Differential relative abundance and dominant microorganisms

Between the two groups, there were differences in the relative abundances of *Campylobacterota*, *Bacteroidota*, and *Actinobacteria* at the phylum level (**Supplementary Figures 28A–C** and **Supplementary Table 12**); *Bacteroidaceae* and *Tannerellaceae* at the family level (**Supplementary Figures 29A–C** and **Supplementary Table 12**); and *Bacteroides*, *Eubacterium_hallii_group*, and *Ileibacterium* at the genus level (**Supplementary Figures 30A–C** and **Supplementary Table 12**). On day 1, the dominant microorganisms in the vaginal delivery group were *g-Ileibacterium* and *s-Ileibacterium_valens* (**Supplementary Figure 31A**); *f-Bacteroidaceae*, *g-Bacteroides*, and *s-Bacteroides_fragilis* on day 3 (**Supplementary Figure 31B**); and *p-Bacteroidota*, *c-Bacteroidia*, *o-Bacteroidales*, *f-Bacteroidaceae*, *g-Bacteroides*, and *s-Bacteroides_fragilis* on day 5. The dominant microorganisms in the cesarean birth group were *o-Staphylococcales*, *f-Staphylococcaceae*, and *g-Staphylococcus* (**Supplementary Figure 31C**).

In summary, the gut microbiota with differences between the two delivery modes mainly included *g-Bacteroides*, *g-Staphylococcus*, and *g-Ileibacterium*, whereas the gut microbiota with differences between the SGA and AGA groups mainly included *g-Escherichia_Shigella*, *g-Ralstonia*, *g-Clostridium*, and *g-Streptococcus*.

Effect of delivery mode on ASQ-3 scores of small for gestational age infants at 6 months of age

The ASQ-3 scores of SGA neonates at 6 months of age were not associated with the delivery mode (**Supplementary Table 13**).

Effect of antibiotic application on gut microbiota of small for gestational age neonates and ASQ-3 scores

A total of 41 SGA neonates were enrolled, including 31 neonates (75.6%) treated with antibiotics and 10 neonates (24.4%) treated without antibiotics, according to their condition. The two subgroups showed no significant differences in sex, gestational age, birth weight, feeding patterns, or delivery mode (**Supplementary Table 14**).

Alpha and beta diversities

A comparison of the alpha diversity of fecal microbiota between the group with antibiotics and the group without antibiotics revealed no statistical difference in the Chao1, ACE, Observed species, Simpson, and Shannon indices on days 1, 3, and 5 (**Supplementary Table 15** and **Supplementary Figure 32**).

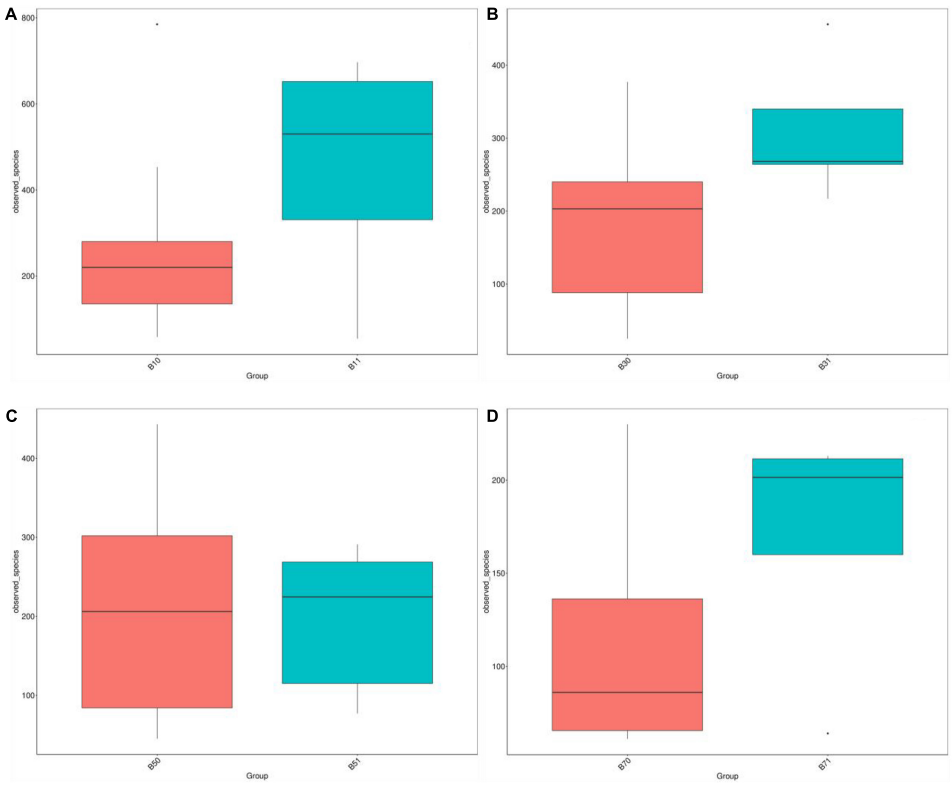

FIGURE 4
Distribution box plots of alpha diversity index between the good and poor communication score groups on days 1, 3, 5, and 7. **(A)** Distribution box plots of the alpha diversity index on day 1. **(B)** Distribution box plots of alpha diversity index on day 3. **(C)** Distribution box plots of alpha diversity index on day 5. **(D)** Distribution box plots of alpha diversity index on day 7. B11, gut microbiota of the poor communication score group on day 1; B10, gut microbiota of the good communication score group on day 1; B31, gut microbiota of the poor communication score group on day 3; B30, gut microbiota of the good communication score group on day 3; B51, gut microbiota of the poor communication score group on day 5; B50, gut microbiota of the good communication score group on day 5; B71, gut microbiota of the poor communication score group on day 7; B70, gut microbiota of the good communication score group on day 7.

The R- and *P*-values were > 0.05 on days 1 and 5, indicating that there was no statistical difference between the two groups on days 1 and 5. On day 3, R-value < 0 and $P > 0.05$ 3, indicating that there was no statistical difference between the two groups (**Supplementary Figures 33A–C**, **34A–C** and **Supplementary Table 16**). However, NMDS analysis indicated that the gut microbiota of the two groups differed significantly on days 1, 3, and 5 (stress score < 0.2; **Supplementary Figures 35A–C**).

Differential relative abundance and dominant microorganisms

Between the two groups, there was a difference in the relative abundance of *Gemmatimonadota* at the phylum level (**Supplementary Figures 36A–C** and **Supplementary Table 17**); *Carnobacteriaceae*, *Enterococcaceae*, *Enterobacteriaceae,* and *Burkholderiaceae* at the family level (**Supplementary Figures 37A–C** and **Supplementary Table 17**); *Aminobacter*, *Georgenia,* and *Loigolactobacillus* at the genus level (**Supplementary Figures 38A–C** and **Supplementary Table 17**). On day 1, the dominant microorganisms in the antibiotic-treated group were *f-Micrococcaceae*, *g-Rothia*, *o-Micrococcales,* and *s-Rothia_mucilaginosa* (**Supplementary Figure 39A**); *s-Clostridium_saccharobutylicum*, *g-Staphylococcus*, *o-Staphylococcales*, *f-Staphylococcaceae*, *s-Clostridium_perfringens*, *s-Enterococcus_faecalis*, *s-Clostridioides_difficile,* and *g-Clostridioides* on day 3 (**Supplementary Figure 39B**); and *o-Lactobacillales*, *f-Enterococcaceae*, *g-Enterococcus,* and *c-Bacilli* on day 5. The dominant microorganisms in the group without antibiotics were *o-Enterobacterales*, *f-Enterobacteriaceae*, *g-Escherichia_Shigella*, *g-Clostridium_sensu_stricto_1*, *f-Peptostreptococcaceae*, *g-Clostridioides,* and *s-Clostridioides_difficile* (**Supplementary Figure 39C**).

In summary, the gut microbiota with differences between the two groups with different applications of antibiotics mainly included *g-Rothia*, *g-Clostridioides*, *g-Staphylococcus, g-Enterococcus*, *g-Escherichia_Shigella,* and *g-Clostridium*; while the gut microbiota with differences between the SGA and AGA groups mainly included *g-Escherichia_Shigella*, *g-Ralstonia*, *g-Clostridium*, and *g-Streptococcus.*

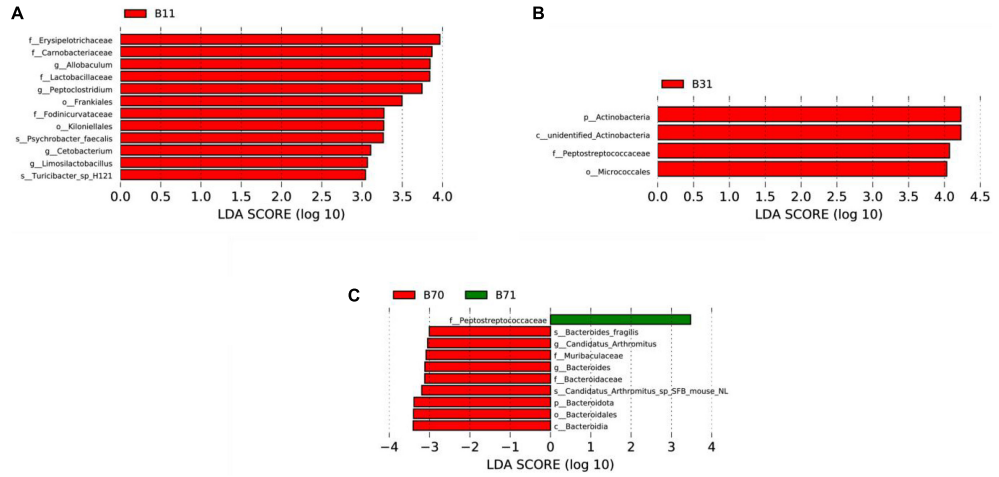

FIGURE 5
LEfSe comparison between the good and poor communication score groups. **(A)** LDA score histogram of differential microbiota of the two groups on day 1. **(B)** LDA score histogram of differential microbiota of the two groups on day 3. **(C)** LDA score histogram of differential microbiota of the two groups on day 7. p_ represents phylum level, c_ represents class level, o_ represents order level, f_ represents family level, g_ represents genus level, and s_ represents species level. B11, gut microbiota of the poor communication score group on day 1; B31, gut microbiota of the poor communication score group on day 3; B71, gut microbiota of the poor communication score group on day 7; B70, gut microbiota of the good communication score group on day 7.

Effect of application of antibiotics on ASQ-3 scores of small for gestational age infants at 6 months of age

The ASQ-3 scores of SGA neonates at 6 months of age were not associated with the use of antibiotics (**Supplementary Table 18**).

Discussion

SGA infants exhibit more significant long-term health issues, including a variety of major and subtle neurodevelopmental delays, than their appropriate gestational age counterparts (Sharma et al., 2016; McCowan et al., 2018; Kesavan and Devaskar, 2019). At present, the mechanisms underlying neurodevelopmental delay in SGA infants are unclear. A growing number of studies have indicated that the gut microbiota plays an important role in early neural development (Carlson et al., 2018; Cohen Kadosh et al., 2021; Seki et al., 2021). Colonization and maturation of the gut microbiota overlap with the critical period of early brain development, and an imbalance in gut microbiota during the early postnatal period may disrupt the developmental programming of the brain through the gut–brain axis, leading to brain injury and long-term neurodysplasia later in life (Al-Asmakh et al., 2012; Cryan et al., 2019; Seki et al., 2021). Therefore, it is of great significance to study the association between gut microbiota and neural development in SGA infants and to explore the impact of specific microbiota on neural development.

In our study, the alpha diversity of gut microbiota in the SGA group was significantly lower than that in the AGA group on days 1, 3, 5, and 7, consistent with the findings of a previous study (Zhang et al., 2019). At the phylum level, the main microbiota of the SGA group were *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidota*, similar to the results of previous studies (Martí et al., 2021; Zhang et al., 2021). With respect to the differential abundance of gut microbiota between the SGA and AGA groups, *Actinobacteria* (3, 7 days) and *Cyanobacteria* (1, 7 days) were significantly lower in the SGA group. Furthermore, another study found that pigs with intrauterine growth restriction had a lower relative abundance of *Actinobacteria* (Che et al., 2019), consistent with observations of our study. *Cyanobacteria* have been reported to exhibit good anti-inflammatory, antioxidant, cholesterol-lowering, and antimicrobial activities (Ferrazzano et al., 2020). *Campylobacterota*, considered to be associated with intestinal and extraintestinal infections (Fitzgerald, 2015; Same and Tamma, 2018), were more abundant on day 5 and were the dominant microbiota on day 7 in the SGA group.

At the genus level, the main microbiota of the SGA group included *Enterococcus*, *Ralstonia*, *Staphylococcus*, *Streptococcus*, *Escherichia-Shigella*, and *Bacteroides*, consistent with findings of previous research (Bäckhed et al., 2015; Gabriel et al., 2018; Zhang et al., 2021). Among the differential microbiota between the SGA and AGA groups, *Ralstonia* and *Ralstonia_pickettii* were the dominant microorganisms in the SGA group on day 1, and *Ralstonia* was higher in the SGA group on

days 3, 5, and 7. Reports have shown that *Ralstonia* is related to the pathogenesis of nervous system diseases such as autism spectrum disorder (ASD) and Parkinson's disease (PD), and its mechanism may involve neuroinflammation and immune activation (Keshavarzian et al., 2015; Ragusa et al., 2020). *F-Enterobacteriaceae* (7 days), *g-Escherichia_Shigella* (3, 7 days), and *g-Helicobacter* (7 days) were the dominant microorganisms in the SGA group. *Escherichia_Shigella* is well-known as a pathogenic enterobacterium; some strains of *Helicobacter* are recognized as important pathogens in gastrointestinal diseases, such as peptic ulcers and gastric cancer, and some strains are associated with bacteremia in immunocompromised and immunocompetent human hosts (On et al., 2002; Boltin et al., 2019). *c-Clostridia*, *o-Clostridiales*, and *g-Rothia* were the dominant microorganisms in the SGA group on day 5, and *Clostridium_sensu_stricto_1* was higher in the SGA group on days 3 and 7, consistent with a previous study on the gut microbiota of neonates with asphyxia (Zhang et al., 2021). Researchers have found that the predominance of *o-Clostridiales* in infants is associated with poorer communication performance at 3 years of age (Sordillo et al., 2019). *Clostridium_sensu_stricto_1* is associated with a variety of inflammatory genes and is considered an opportunistic pathogen associated with intestinal inflammation (Wang et al., 2018; Li et al., 2019; Wen et al., 2021). Studies have found that *Rothia* is an opportunistic pathogen associated with various infections in humans, and most reported *Rothia* infections have occurred in patients with pneumonia, endocarditis, peritonitis, and septicemia (Fatahi-Bafghi, 2021). *Burkholderiaceae* was higher in the SGA group on days 3, 5, and 7. An increased relative abundance of *Burkholderiaceae* was observed in the gut of rats with cognitive impairment (Rong et al., 2021), and *Burkholderiaceae* was elevated in the brain tissue of patients with Alzheimer's disease (AD) (Alonso et al., 2018). The SGA group showed decreased relative abundances of *Lactiplantibacillus* (1, 3, 5, and 7 days), *Streptococcus* (3, 5, 7 days), *Bifidobacterium,* and *Lactococcus* (3 days). Some strains of these four genera are considered probiotics (Guo et al., 2019; Prete et al., 2020; Wright et al., 2020; Griffin et al., 2022) because they improve memory impairment and cognitive function by reducing inflammation and oxidative stress (Den et al., 2020; Sivamaruthi et al., 2020; Griffin et al., 2022). *Ileibacterium* was higher in the SGA group on days 1, 5, and 7, and it was the dominant microbiota on day 7. *Ileibacterium* is a gram-positive anaerobic bacterium related to metabolic health that can decompose polysaccharides (Wang et al., 2021). An increased relative abundance of *Ileibacterium* has been observed in animal models of intestinal microecological disorders (Xiao et al., 2021; Wu et al., 2022). *Akkermansia* was higher in the SGA group on days 5 and 7. *Akkermansia* is the predominant member of *Verrucomicrobiota* (Wagner and Horn, 2006), with elevated levels in patients with multiple sclerosis (MS) linked to lower disability, and *Akkermansia* isolated from these patients ameliorates experimental autoimmune encephalomyelitis (EAE), suggesting that increased *Akkermansia* in MS may be a compensatory beneficial response (Cox et al., 2021).

In summary, large differences were observed in the gut microbiota between the SGA and AGA groups in the first week of life, which can be summarized as follows: (1) compared with AGA infants, the alpha diversity of term SGA infants was significantly lower; (2) certain pathogenic and conditional pathogenic bacteria increased or formed the dominant microorganisms in SGA infants, such as *Escherichia_Shigella*, *Ralstonia,* and *Clostridium*.

After a 6 month follow-up, we found an association between higher alpha diversity on day 3 and communication problems in SGA infants. This finding was surprising because a more mature gut microbial community in infancy usually has a high level of biological diversity (Carlson et al., 2018; Coker et al., 2021), and decreased microbial diversity is related to adverse health outcomes in adults (Rinninella et al., 2019; Nikolova et al., 2021). However, the relationship between microbial diversity and health conditions in children is mixed (Abrahamsson et al., 2014; Kostic et al., 2015; Aatsinki et al., 2019). Our findings are consistent with recent evidence that increased microbial diversity in infancy is not necessarily beneficial for subsequent neurocognitive outcomes (Carlson et al., 2018; Gao et al., 2019; Loughman et al., 2020). One study showed that a higher alpha diversity of the gut microbiota in 1-year-old children was associated with lower overall composite, visual reception, and expressive language scores at the age of 2 years (Carlson et al., 2018). Their team found a correlation between higher levels of microbiome diversity in infancy and weaker thalamus-amygdala connectivity a year later (Gao et al., 2019), while the amygdala regulates emotion and controls learning and memory (McDonald and Mott, 2017). The same study also found positive associations between alpha diversity and functional connectivity between the supplementary motor area (SMA) and the inferior parietal lobule (IPL), while SMA-IPL connectivity at 1 year of age was negatively correlated with cognitive outcomes at 2 years of age (Gao et al., 2019).

With respect to the differential gut microbiota of SGA infants with different neurodevelopmental outcomes at 6 months, *g-Allobaculum* was the dominant microbiota in the SGA group with a poor communication score on day 1. *Allobaculum* may provide good anti-inflammatory functions by producing free long-chain fatty acids and short-chain fatty acids (SCFAs) which are related to glucose and lipid metabolism (Wu et al., 2020; Pujo et al., 2021). Therefore, we assumed that *Allobaculum*-dominant gut microbiome may be a compensatory beneficial response for SGA infants with poor communication scores. We found that *p-Bacteroidota*, *g-Bacteroides*, and *s_Bacteroides_fragilis* increased in abundance and were the dominant microorganisms

in the good communication score group on day 7. In addition, we observed correlations between increased abundances of *p-Bacteroidota*, *g-Bacteroides,* and *s-Bacteroides_fragilis* on day 7 and improved communication scores at 6 months, consistent with findings of previous studies on the association between gut microbiota in late infancy and subsequent neurodevelopment (Carlson et al., 2018; Tamana et al., 2021). We found that the relative abundance of *s-Bacteroides_fragilis* on day 1 was negatively correlated with the fine motor scores, similar to observation of a previous study on the association between *Bacteroides*-dominant gut microbiota at 3–6 months and subsequent delayed fine motor skills (Sordillo et al., 2019). *Bacteroides_fragilis* is a gram-negative obligate anaerobe with two subtypes. Enterotoxigenic *B. fragilis* (ETBF), identified as a common opportunistic pathogen in clinical infections, mainly causes colitis and systemic inflammation with the stimulation of toxins or lipopolysaccharides. The second subtype, non-toxigenic *B. fragilis* (NTBF), has been suggested as a potential probiotic in recent studies because of its ability to produce immunomodulatory substances such as polysaccharide A (PSA) and SCFAs (Sun et al., 2019; Qu et al., 2022). Non-toxigenic *B. fragilis* was found to be capable of protecting mice from central nervous system demyelination; this protective mechanism depended on the production of IL-10 (Ochoa-Repáraz et al., 2010). In addition, it was found to improve communication, stereotyped movement, anxiety-like behavior, and sensorimotor behavior in ASD model mice, as well as reduce the increased expression of IL-6 (Hsiao et al., 2013). Decreased levels of intestinal *Bacteroides* are also characteristic of children diagnosed with ASD (Dan et al., 2020; Iglesias-Vázquez et al., 2020). *Clostridium_saccharobutylicum* has a strong ability to produce butyrate (Huang et al., 2018; Miguel et al., 2019), and butyrate, as a microbial metabolite, can indirectly affect host metabolism through the gut–brain axis (Stilling et al., 2016). It has been reported that butyrate can reduce the inflammatory response of microglia and the hippocampus, inhibit inflammatory activities, promote the production of BDNF, and repair injured nerves (Kundu et al., 2019). What puzzled us was the negative correlation between the relative abundance of *s-Clostridium_saccharobutylicum* on day 7 and the gross motor scores at 6 months postnatal. This finding was unexpected and it is unclear why there was such a connection. Thus, we believe further investigation is required to confirm these results.

Briefly, gut microbial characteristics associated with neurological prognosis in SGA infants can be summarized as follows: (1) higher alpha diversity on day 3 was associated with poor communication performance in SGA infants at 6 months of age; (2) *Bacteroidota*, *Bacteroides*, *Bacteroides_fragilis*, and *Clostridium_saccharobutylicum* may be related to the neurodevelopmental outcomes of SGA infants at 6 months of age.

The development of neonatal gut microbiota is affected by several factors (Bäckhed et al., 2015; Rutayisire et al., 2016; Kapourchali and Cresci, 2020; Vandenplas et al., 2020). Most early colonists in the gut of neonates are maternal, and the mode of delivery strongly affects the formation of the early gut microbiota in term infants (Bäckhed et al., 2015; Rutayisire et al., 2016; Kapourchali and Cresci, 2020; Coelho et al., 2021). Studies have shown that vaginally-delivered neonates have more abundant *Bacteroides*, *Bifidobacterium*, and *Lactobacillus* (Bäckhed et al., 2015; Rutayisire et al., 2016; Coelho et al., 2021), whereas neonates delivered by cesarean section (CS) are enriched in *Staphylococcus*, *Streptococcus,* and *Clostridium* (Coelho et al., 2021). In our study, there was no difference in the alpha diversity of SGA neonates born by vaginal delivery and CS on days 1, 3, and 5, while the alpha diversity of SGA neonates delivered by CS showed a decreasing trend on day 7. Some researchers also found lower microbial diversity in the gut of infants delivered by CS than in vaginally-delivered infants in the first week of life (Bäckhed et al., 2015; MacIntyre et al., 2015; Rutayisire et al., 2016; Shi et al., 2018). LEfSe showed that the dominant microorganisms in vaginally-delivered SGA neonates were *g-Bacteroides* and *g-Ileibacterium*. *Bacteroides* seem to increase in abundance in vaginally-delivered infants compared with CS-delivered infants (Gronlund et al., 1999; Kabeerdoss et al., 2013; Hesla et al., 2014; Jakobsson et al., 2014; Rutayisire et al., 2016; Coelho et al., 2021). In addition, *Bifidobacterium* and *Lactobacillus* found in the vaginal delivery group did not increase significantly, in contrast to other findings (Bäckhed et al., 2015; Rutayisire et al., 2016; Coelho et al., 2021). The dominant microbiota of the cesarean birth group was *g-Staphylococcus* on day 5, similar to findings of previous studies (Li et al., 2018; Wampach et al., 2018; Coelho et al., 2021). However, *Streptococcus* and *Clostridium* found in the cesarean birth group did not increase significantly, in contrast to previous findings (Coelho et al., 2021). Therefore, SGA and delivery mode may have different effects on the development of gut microbiota in term neonates.

Antibiotic therapy can greatly alter the diversity and composition of neonatal gut microbiota (Nobel et al., 2015; Gasparrini et al., 2016). Associations between antibiotic therapy and decreased microbial diversity, increased abundance of *Firmicutes*, and decreased abundance of *Bacteroides* and *Bifidobacterium* have been reported (Gasparrini et al., 2016; Ficara et al., 2020). In our study, despite no statistical difference between the alpha diversity of the group with antibiotics and the group without antibiotics, the alpha diversity of SGA neonates treated with antibiotics was lower in the first week of life, similar to previous findings (Gasparrini et al., 2016; Ficara et al., 2020). Hence, we assumed that the therapeutic duration of antibiotics was insufficient to significantly disrupt gut microbial diversity. We also found that the composition of neonatal gut microbiota was altered by antibiotic therapy. However, there was no difference in

the relative abundance of *Firmicutes*, *Bifidobacterium,* and *Bacteroides* between the two groups, which differed from previous findings (Ficara et al., 2020).

This study is the first to analyze the characteristics and evolution of the gut microbiota of term SGA infants in the first week of life, and also the first to explore the potential association between specific microbiota and neural development in SGA infants. Alongside the novelty of this study, it is worth mentioning the following limitations. (1) This was a single-center study; there may be differences in the gut microbiota of SGA infants from other hospitals. The results need to be further confirmed by multicenter and large-exponent investigations. (2) We only studied the gut microbiota of term SGA infants within 1 week after birth and the neurological prognosis at 6 months of age; we will follow up with SGA infants and discuss the relationship between neonatal gut microbiota and neurological development by comprehensively considering various factors, such as education mode and society. (3) The ASQ-3 is a screening scale, and because of the prevalence of the novel coronavirus, it was difficult to use diagnostic scales such as the Bayley scale to evaluate the prognosis of neurodevelopment.

Conclusion

Compared to AGA infants, the gut microbial diversity of term SGA infants was significantly lower in the first week of life. Certain pathogenic and conditional pathogenic bacteria increased or formed the dominant microbiota in SGA infants, such as *Escherichia_Shigella*, *Ralstonia,* and *Clostridium*. This study suggests that there may be associations between alpha diversity, certain gut microbiota, and neurodevelopmental outcomes in SGA infants. The results showed that higher alpha diversity on day 3 was associated with poor communication performance in SGA infants at 6 months of age, and the gut microbiota factors affecting the prognosis of SGA infants included *Bacteroidota*, *Bacteroides*, *Bacteroides_fragilis*, and *Clostridium_saccharobutylicum.* SGA infants are at a higher risk for adverse neurodevelopmental outcomes; however, current methods for the clinical treatment of neurodevelopmental delay in SGA infants are limited. With further studies of the gut microbiota of SGA infants, we hope to provide further insights into the early treatment of SGA infants.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary material**.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Peking University First Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

All authors listed have made great contributions to this work and have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.912968/full#supplementary-material

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Association between body weight and distal gut microbes in Hainan black goats at weaning age

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Gut microbiota plays a critical role in the healthy growth and development of young animals. However, there are few studies on the gut microbiota of young Hainan black goats. In this study, 12 three-month-old weaned lambs with the same birth date were selected and divided into the high body weight group (HW) and low body weight group (LW). The microbial diversity, composition, and predicted function in the feces of HW and LW groups were analyzed by collecting fecal samples and sequencing the 16S rRNA V3-V4 region. The results indicated that the HW group exhibited higher community diversity compared with the LW group, based on the Shannon index. The core phyla of the HW and LW groups were both *Firmicutes* and *Bacteroidetes*. *Parabacteroides*, *UCG-005*, and *Bacteroides* are the core genera of the HW group, and *Bacteroides*, *Escherichia-Shigella*, and *Akkermansia* are the core genera of the LW group. In addition, genera such as *Ruminococcus* and *Anaerotruncus*, which were positively correlated with body weight, were enriched in the HW group; those genera, such as *Akkermansia* and *Christensenellaceae*, which were negatively correlated with body weight, were enriched in the LW group. Differential analysis of the KEGG pathway showed that Amino Acid Metabolism, Energy Metabolism, Carbohydrate Metabolism, and Nucleotide Metabolism were enriched in the HW group, while Cellular Processes and Signaling, Lipid Metabolism, and Glycan Biosynthesis and Metabolism were enriched in the LW group. The results of this study revealed the gut microbial characteristics of Hainan black goats with different body weights at weaning age and identified the dominant flora that contributed to their growth.

KEYWORDS

gut microbiota, 16S rRNA, Hainan black goats, body weight, association

Introduction

The gut microbiota (GM) is the largest and most intricate micro-ecosystem in animals, of which bacteria are the most numerous (Bhattarai and Janaswamy, 2022). GM is considered to be the "hidden metabolic organ" of the body, which plays a significant role in physiological activities such as energy metabolism, nutrient digestion and absorption, immune regulation, and health maintenance (Bohan et al., 2019). GM can affect animal growth performance by regulating these physiological activities, and animal growth performance is an important factor affecting the profitability of animal husbandry.

Average daily gain (ADG) is an important indicator of animal growth in animal husbandry. Recently, one study found that GM is closely associated with ADG in animals (Fang et al., 2020). It has also been reported that some specific GMs can improve feed conversion rates and promote animal growth (Stanley et al., 2012). A study on swine found that early-life establishment of the gut microbiota can influence the phenotype of animals (Mach et al., 2015). Lu et al. (2018) studied the change trends of fecal microorganisms in weaned piglets, 15-week-old, and 18-week-old pigs by 16S sequencing and proved that GM is involved in the regulation of host body weight. Previous research has demonstrated that microorganisms may affect animal growth performance by regulating energy production in animals. For example, microorganisms can hydrolyze carbohydrates under anaerobic conditions and ferment them to generate short-chain fatty acids (SCFAs) (acetic acid, propionic acid, butyric acid), to provide energy for the host (Wang M. et al., 2019). Taken together, the regulatory effects of these GMs are all associated with the increase in animal body weight.

At present, there are few studies on the relationship between GM and body weight in sheep and goats. A recent study on sheep found that there were significant differences in the distribution and composition of intestinal flora in sheep with different body mass indices (Cheng et al., 2022). However, little research has been done on lambs. As we all know, young ruminants are in the period of the fastest growth of the body, the rapid improvement of the tissue and organ functions, and the accelerated construction of the immune system. Research has shown that early microbial exposure is a potentially effective intervention strategy to modulate host health and metabolism (Li et al., 2018). Manipulation of the early microbiome has been reported in newborn calves to improve growth performance and health (Malmuthuge et al., 2015). Therefore, the development of GM in young ruminants largely determines the product quality in adult animals.

Due to the huge quantity and complex composition of GM, it was impossible to accurately describe the composition and function of microorganisms by traditional methods in the past. Over the past ten years, the rise of 16S sequencing technology has greatly enriched the research content of GM. Many studies by 16S sequencing technology have analyzed the correlation between body weight and GM of weaned piglets and investigated the relationship between obesity and the GM of diabetic patients (Han et al., 2017; Pai et al., 2022). Therefore, 16S sequencing has become a well-established technique to study GM.

Hainan black goat is the dominant goat breed in Hainan Province, which has excellent qualities such as rough feeding resistance, strong disease resistance, and good meat performance. It is a valuable breed resource for large-scale breeding in tropical regions (Quanwei et al., 2019). However, Hainan black goat has the disadvantage of a slow growth rate, which becomes an important factor in restricting its industrial development.

Hence, we use 16S sequencing techniques for the sake of studying the traits of GM of Hainan Black goats with high body weight and low body weight. This study provides a theoretical basis for further determining the dominant flora and improving the growth traits of Hainan black goats at weaning age.

Materials and methods

Ethics statement

The sampling method and all subsequent methods were approved by the Ethical Committee of the Hainan University (Haikou, China, Permit number: HNUAUCC-2022-000122). This experiment did not involve any endangered or protected species.

Experimental animals and sample collection

A total of 20 male Hainan black goats with the same birth date raised on a commercial farm in Hainan Province were used for this study. These lambs were self-bred by the farm with similar genetic backgrounds and fed with the ewes in each stage and suckled freely, after 21 days of age, they began to feed freely with the ewes on concentrate and high-quality forage and drank freely. Lambs were weighed when they reached 90 days of age. The fecal samples were collected using sterile tools and delivered to the lab within 2 h and stored at −80°C. All individuals were sorted according to body weight, and the six heaviest lambs were selected as the HW group (each sample was represented by H.1, H.2, H.3, H.4, H.5, H.6, respectively), and the six lightest ones as the LW group (each sample was represented by L.1, L.2, L.3, L.4, L.5, L.6, respectively). The average body weight of the HW group was 11.89 ± 1.67 Kg, and that in the LW group was 8.69 ± 1.22 Kg (**Table 1**). There was a significant difference in body weight (BW) and average daily gain (ADG) between HW and LW groups ($P < 0.01$; **Supplementary Figure 1**). No significant differences in the

weight of the newborn lambs were observed. In the process of the experiment, the lambs were not exposed to any specific pathogens, diseases, or antibiotics.

DNA extraction

Genomic DNA was extracted from each sample by using TIANGEN® Magnetic Soil and Stool DNA Kit (Qiagen, Valencia, California, USA). The quality of the extracted DNA was measured by 1% agarose gel electrophoresis.

16S rRNA gene polymerase chain reaction amplification, purification, and sequencing

In this study, the V3-V4 region was amplified by polymerase chain reaction (PCR), and the universal primer used was 341F:5′-CCTAYGGGRBGCASCAG-3′ and 806R:5′-GGACTACNNGGGTATCTAAT-3′. The total volume of the reaction was 30 μL, containing 15 μL Phusion Master Mix (2 ×), 3 μL Primer (2 μM), 10 μL DNA (1 ng/μL), and 2 μL H_2O. After denaturation at 98°C for 1 min, 30 amplification cycles were performed comprising a denaturation step at 98°C for 10 s, an annealing step at 50°C for 30 s, an extension at 72°C for 30 s, followed by the last extension at 72°C for 5 min. 2% agarose gel electrophoresis was used to examine the quality of PCR products, and qualified PCR products were purified and quantified by enzyme labeling. The purified PCR products were then used to construct the sequencing library by TruSeq DNA PCR-Free Library Preparation Kit. Illumina NovaSeq6000 was used for sequencing after the library was qualified.

Bioinformatics and data analysis

After splitting each sample data from the primer reads according to the Barcode sequence and PCR amplification primer sequence, truncating Barcode and primer sequence, using FLASH to splice, filter, and detect the reads of each sample, and finally, the chimera was eliminated to obtain the effective tags. Uparse algorithm was used to cluster all Effective Tags of all samples, and the sequence was clustered into OTUs with 97% consistency by default. Representative sequences for each OTU were taxonomically identified and phylogenetically analyzed. The alpha diversity indices [Chao1, Abundance-based coverage estimator (ACE), and Shannon index] of each sample were calculated using QIIME (Version 1.9.1), and the dilution curve and Rank abundance curve were drawn using R software (Version 2.15.3). Beta diversity was calculated using QIIME (Version 1.9.1) to compare the difference and similarities among different samples. Linear discriminant analysis effect size (LEfSe) was performed on the samples according to different grouping conditions. Based on the OTUs clustered from 16S rRNA sequencing data, this study employed a phylogenetic investigation of the community by reconstructing the unobserved state (PICRUSt) to infer the functional potential of the gut microbiota community. The inferred genes and their functions were precomputed in the database of the Kyoto Encyclopedia of Genes and Genomes (KEGG). $P < 0.05$ were considered to be statistically significant. GraphPad Prism (version 8.0c) and SPSS 17.0 were applied to statistical analysis. The data were expressed as means ± standard deviation (SD). The raw data of this study has been uploaded to the NCBI database under the number PRJNA827021.

Results

DNA sequences analysis

In this study, the 16S rRNA genes isolated from 12 fecal samples of Hainan black goats were sequenced and yielded 797799 sequences. After chimera checking and filtering out, 600862 sequences were produced from 12 samples and each sample had 50071 sequences on average. Following taxonomic assignment, the whole number of OTUs reached 2962 based on 97% nucleotide-sequence similarity and the HW and LW groups obtained 2399 and 2279 OTUs, respectively (**Figure 1A**). The OTUs of each sample in the HW and LW groups were shown in **Supplementary Figure 2**. The Good's Coverage index values of all samples in groups HW and LW exceeded 99% (**Figure 1B**). The rarefaction curve presented a steady trend when the number of effective sequences overtops 36000. It indicated that the sequencing depth and quantity meet the requirement of the analysis (**Figure 1C**). Moreover, the rank abundance curve of each group of samples spanned a wide range in the horizontal direction and was relatively flat in the vertical direction, which indicated that the samples had good species richness and evenness (**Figure 1D**). These results indicated that the amount of sequencing data from this study was sufficient.

Microbial diversity index analysis

For alpha diversity measurements of the GM population, the species abundance and community diversity were assessed by using the Chao1, ACE, and Shannon indices. We observed that the average of the Shannon index of group HW was 6.94 ± 0.80 and group LW was 5.48 ± 0.91 (**Table 2**). The Shannon index of HW was significantly higher than that of the LW group. Chao1 and ACE in group HW were 1065.74 ∼ 1479.21 and 1078.98 ∼ 1516.64, respectively, and those in group LW were 881.13 ∼

TABLE 1 Body weights of the Hainan black goats used in this study.

Groups	Number	Body weight	Maximum	Minimum	*P* value
HW	6	11.89 ± 1.67	14.15	10.45	<0.01
LW	6	8.69 ± 1.22	9.95	7.15	<0.01

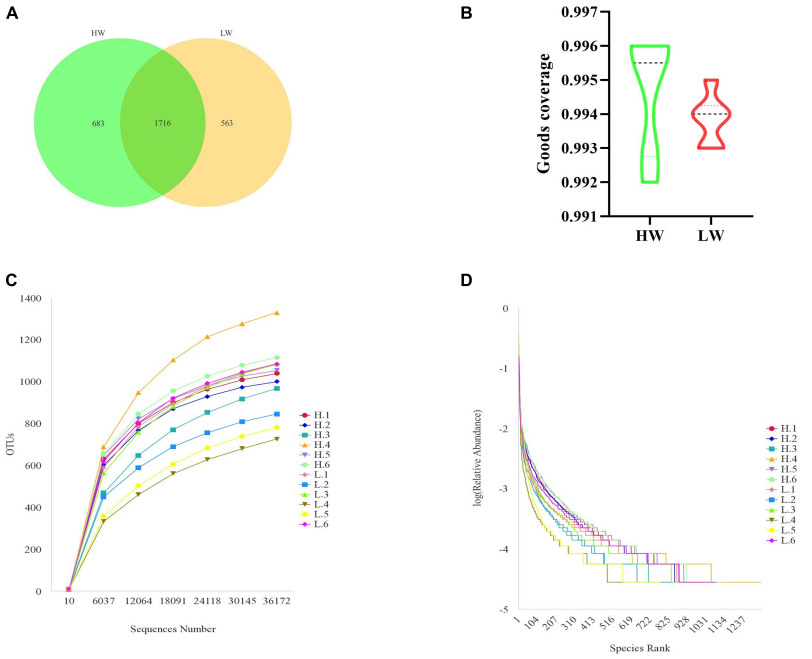

FIGURE 1
DNA sequence data analysis. **(A)** Venn diagram. The numbers in the figure show the unique or shared OTUs of each group. **(B)** It shows the sequencing depth of the HW and LW groups. Rarefaction **(C)** and rank-abundance curve **(D)** analysis of the different fecal samples at 97% sequences identity.

TABLE 2 The alpha diversity index of each sample.

Sample	Shannon	Chao 1	ACE	Good's coverage
H.1	7.25	1129.17	1131.68	0.996
H.2	7.16	1065.74	1078.96	0.996
H.3	5.72	1099.82	1186.89	0.993
H.4	6.21	1479.21	1516.64	0.992
H.5	7.66	1124.00	1137.05	0.996
H.6	7.65	1219.77	1240.85	0.995
L.1	5.96	1185.51	1229.84	0.994
L.2	5.36	947.86	1007.77	0.995
L.3	6.17	1222.16	1260.38	0.993
L.4	4.25	981.07	961.45	0.993
L.5	4.59	881.13	958.66	0.994
L.6	6.54	1206.28	1220.93	0.994

1222.16 and 958.66 ~ 1260.38, respectively. Although the Chao1 and ACE indices of HW were slightly higher than the LW group, the differences were not significant (Figure 2A).

Combining the OTU species of the samples and their relative abundance, the PCoA was plotted based on the unweighted Unifrac distances. Figure 2B clearly showed the differences between all samples. The result showed that the HW group was mainly clustered on the upper side of the coordinate axis, while the LW group was mainly clustered on the lower side of the coordinate axis. The samples of the HW group and the LW group showed a relatively obvious separation. It indicated that the structure of GM was different between HW and LW groups.

The analysis of community composition between high body weight group and low body weight group groups

The relative proportions of dominant taxa at phylum and genus levels were assessed by the distribution of microbial taxa in different groups. At the phylum level, we found 48 phyla in

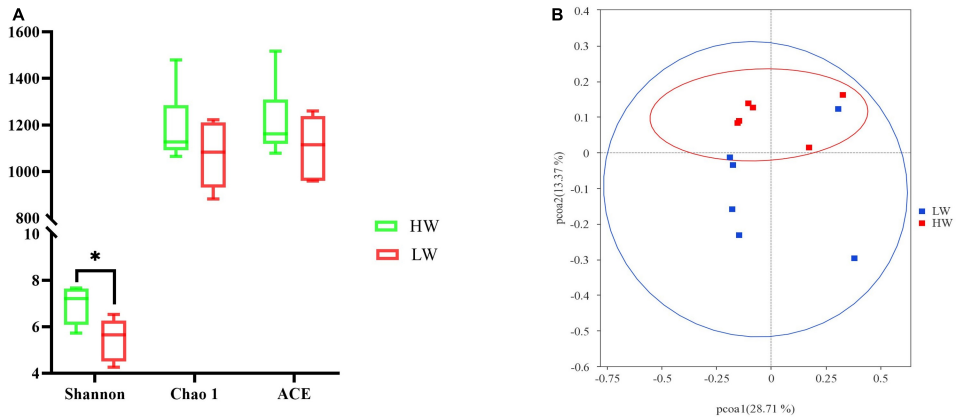

FIGURE 2
The different gut microbiota communities between the HW and LW groups. **(A)** Bacterial alpha diversity is based on Shannon diversity, Chao1, and ACE index. *Means the significant difference in statistics ($P < 0.05$). Shannon diversity represents microbial community diversity, and Chao1 and ACE represent species richness. **(B)** Differences in Principal Coordinate Analysis (PCoA) of GM structures HW and LW groups. The red dots represent the samples of the HW group and the blue dots represent the samples of the LW group. The distance between the two points represents the difference in GM.

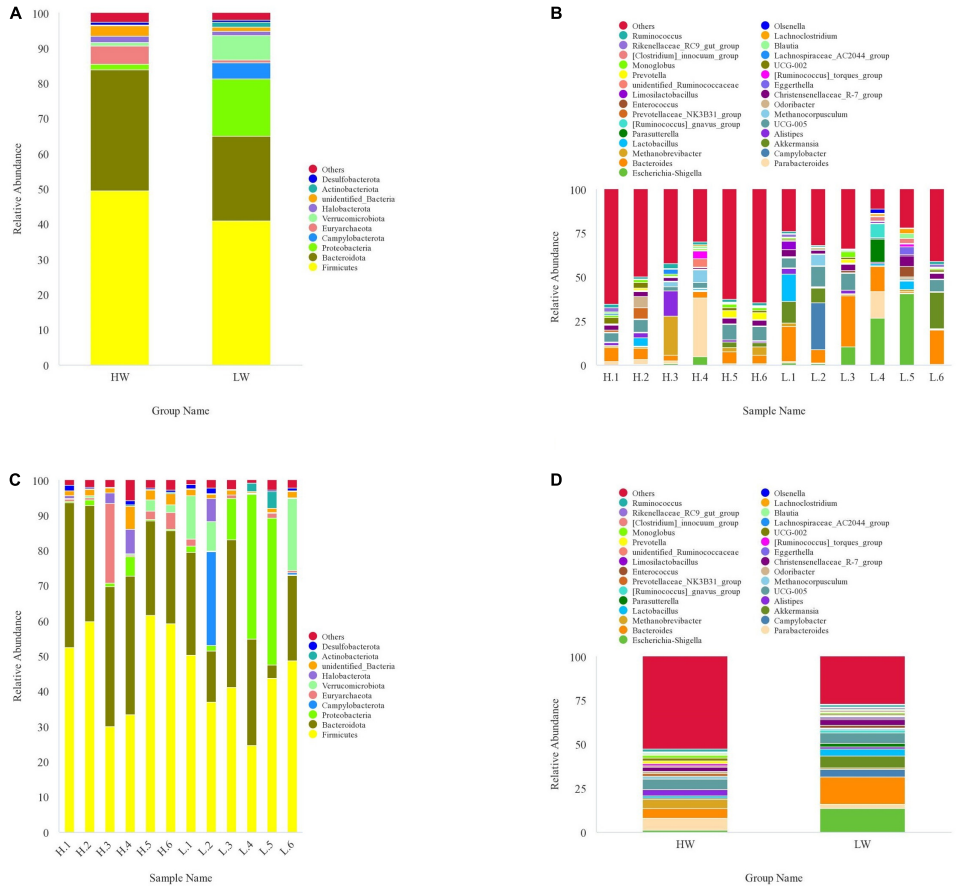

FIGURE 3
The taxonomic distribution between HW group and LW group samples (each color represents the relative abundance of a taxonomic bacterium). **(A)** at phylum level (top 10). **(B)** at genus level (top 30). **(C)** Between-group at phylum level (top 10). **(D)** Between-group at genus level (top 30).

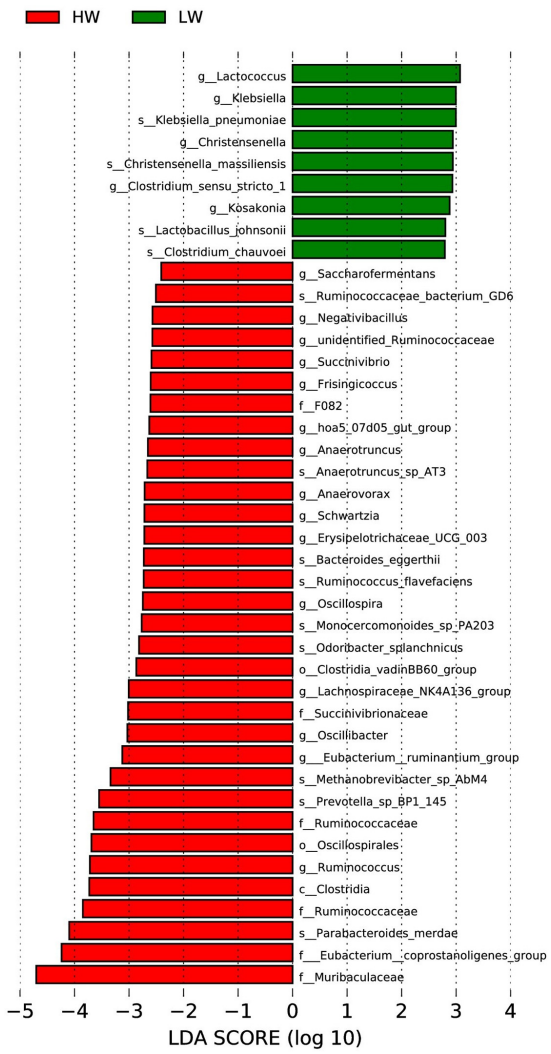

FIGURE 4
Significant differentiation of bacterial taxa between HW and LW groups was determined by linear discriminant analysis and effect size (LEfSe). LDA scores were calculated for bacterial taxa that were differentially enriched between different groups.

total in all samples from the HW and LW groups. According to the phylum assignment result (Figure 3), *Firmicutes* were the most abundant phylum of all samples, and *Bacteroidetes* were the secondary phylum. In the HW group, the relative abundance of *Firmicutes* (49.43%) and *Bacteroidetes* (34.42%) reached 83.85%, and the relative abundance of *Euryarchaeota* (5.23%) was also high. *Firmicutes* (40.93%) and *Bacteroidetes* (24.01%) remained in the highest relative abundance in the LW group, and interestingly, *Proteobacteria* (16.34%) reached equally high proportions in the LW group.

At the genus level, the core genera of the HW group were *Parabacteroides* (6.78%), *UCG-005* (5.84%), *Bacteroides* (5.51%), *Methanobrevibacter* (5.22%), *Alistipes* (3.49%), and *Christensenellaceae-R-7-group* (2.39%). The genera with high relative abundance in LW group mainly included *Bacteroides* (15.15%), *Escherichia-shigella* (13.36%), *Akkermansia* (6.87%), *UCG-005* (5.77%), *Campylobacter* (4.53%), *Lactobacillus* (3.69%), and *Christensenellaceae-R-7-group* (3.21%).

Differences between HW and LW groups were analyzed by Lefse analysis (LDA > 2) based on the classification of phylum and genus. Figure 4 showed that there was no significant difference between the HW and LW groups at the phylum and genus level; a total of 15 genera (e.g., *Ruminococcaceae*, *Oscillibacter*, *Oscillospia*, *unidentified-Ruminococcaceae*) were enriched in the HW group; 5 genera (e.g., *Lactococcus*, *Klebsiella*, *Christensenlla*) were enriched in the LW group.

Spearman analysis

To investigate the microflora that may be associated with growth performance, we performed Spearman's rank correlation coefficient analysis between the GM and BW or ADG. The results showed *Firmicutes*, *Bacteroidetes*, and *Euryarchaeota* were positively correlated with BW and ADG; *Proteobacteria* was negatively correlated with BW and ADG at the phylum level (Figure 5A). At the genus level, we selected the top 35 genera correlated with BW and ADG, of which 23 genera had a positive correlation and 12 genera showed a negative correlation (Figure 5B). *Parabacteroides* showed the strongest positive correlation with BW and ADG. In addition, *Ruminococcus*, *Alistipes*, *UCG-005*, and *unidentified-Ruminococcaceae* were all positively correlated with BW and ADG. *Bacteroides*, *Akkermansia*, and *Lactobacillus* were negatively correlated with BW and ADG.

Microbial function prediction

In this study, we used PICRUSt to predict and explore the molecular functions of microbial communities in two groups of samples. Figure 6A showed 41 functional genes predicted for Cellular Processes, Environmental Information Processing, Genetic Information Processing, Human Diseases, Metabolism, and Organismal Systems pathways. At the KEGG level 2, 41 functional genes in total were detected in the HW and LW groups. The majority of these functional genes belonged to Membrane Transport (11.78% of total genes inferred by PICRUSt), Carbohydrate Metabolism (10.54%), Amino Acid Metabolism (9.63%), Replication and Repair (8.71%), Energy Metabolism (6.81%), and Translation (5.73%).

The analysis showed that gut microbes in the HW group were mainly focused on Amino Acid Metabolism, Energy Metabolism, Carbohydrate Metabolism, Immune System, and Nucleotide Metabolism, while the gut microbes in the LW group were mainly focused on Infectious Diseases and Metabolism of Other Amino Acids (Figures 6B,C). We

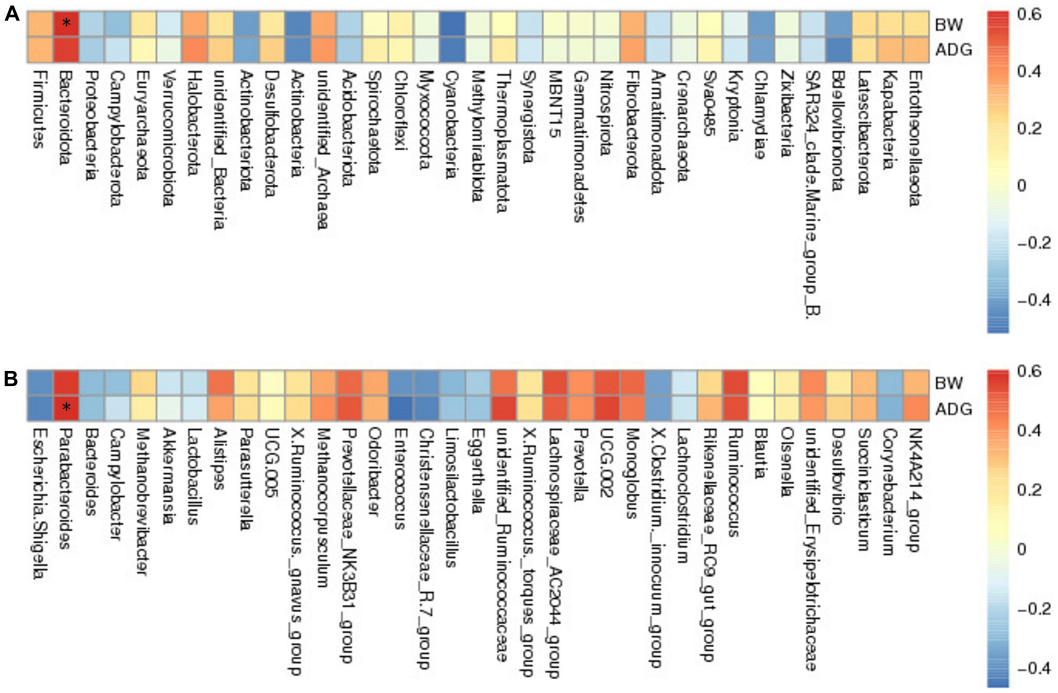

FIGURE 5
The results of Spearman's analysis show BW (body weight) and ADG (average daily gain) in the longitudinal direction, and each bacterial information in the transverse direction. **(A)** at phylum level. **(B)** at genus level. The value corresponding to the intermediate heatmap is the spearman correlation coefficient r, which is between −1 and 1, $r < 0$ is negative correlation, $r > 0$ is positive correlation, and marked * indicates significance test $p < 0.05$.

observed significant differences in many KEGG pathways in the fecal microbiota between the two groups by *T*-test ($P < 0.05$). The relative abundance of genes (**Figure 6D**) involved in Amino Acid Metabolism, Replication and Repair, Translation, and Nucleotide Metabolism in the HW group was significantly higher than in the LW group ($P < 0.05$), whereas the relative abundance of genes involved in Cellular Processes and Signaling, Lipid Metabolism and Glycan Biosynthesis and Metabolism were significantly lower than those in the LW group ($P < 0.05$).

Discussion

Gut microbiota (GM) is known as the "second genome," and its importance to the metabolic activities of animals is naturally self-evident (Zhu et al., 2010). Previous studies have shown that body weight changes in host animals can be affected by altering the gut microbes (Angelakis, 2017). The identification of GM that is beneficial for weight gain will provide an important aid to the healthy growth and production of animals. Therefore, in this study, 16S rRNA sequencing was used to analyze the composition of GM of Hainan black goats at weaning age and the differences in microbiota among goats of different body weights.

According to the results of the Shannon index, we found that the community diversity of the HW group was significantly higher than that of the LW group. Overall, the alpha diversity of the HW group was higher than that of the LW group, which was consistent with the research results on weaned piglets (Han et al., 2017). However, another study on rats found that the alpha diversity of obese rats was lower than that of lean rats (Resch et al., 2021). We speculate that this situation is due to possible differences in the composition and development of the GM between species. Furthermore, whether there is an inevitable correlation between GM alpha diversity and body weight needs further in-depth study.

At the phylum level, we found that *Firmicutes* and *Bacteroidetes* were dominant phyla in both the HW and LW groups, which was consistent with the results observed in Chinese small-tailed Han sheep, Mongolian sheep, and Boer goats (Zeng et al., 2015; Wang et al., 2018; Zhang et al., 2018). Although *Firmicutes* and *Bacteroidetes* were slightly more abundant in the HW group than in the LW group, the difference was not significant. Combined with the previous results, it was found that neither species nor body weight affected the dominant position of *Firmicutes* and *Bacteroidetes* in GM. *Firmicutes*, as the dominant bacteria in the intestine, were positively correlated with BW and ADG, which played a crucial role in the degradation and digestion of fiber and cellulose

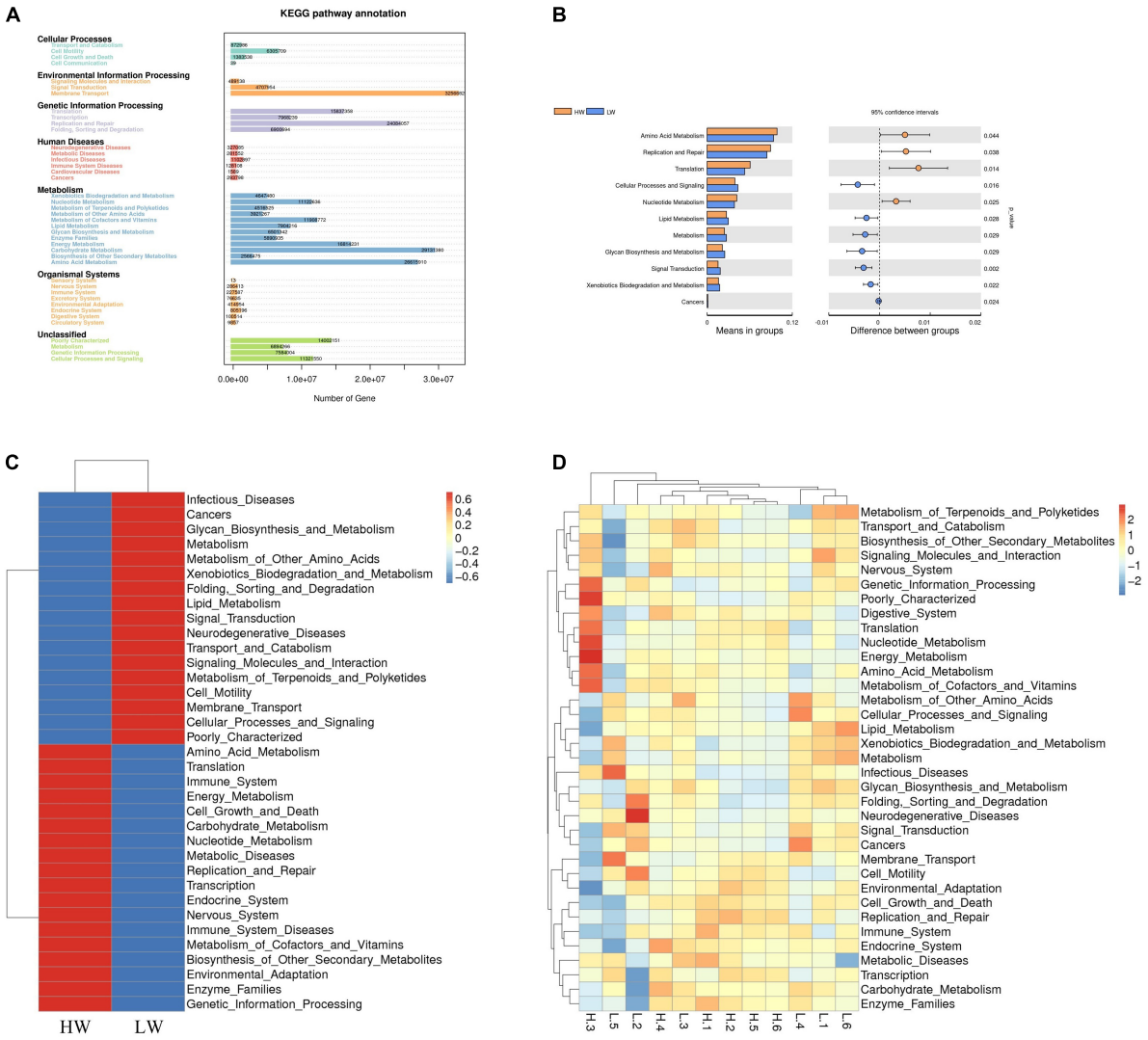

FIGURE 6
Genomic functional predictions. **(A)** KEGG pathway annotation. **(B)** Heatmap clustered based on functional prediction of each sample in level 2 KEGG pathway. **(C)** Heatmap clustered based on functional prediction of the different groups in level 2 pathway. **(D)** Comparison of HW and LW groups in differential metabolic pathways.

in animals (Hook et al., 2011). Another dominant phylum, *Bacteroidetes* had the main function of degrading non-fibrous carbohydrates and proteins and breaking down polysaccharides, thereby facilitating the digestion and absorption of nutrients (Huo et al., 2014). In addition, *Bacteroidetes* could also promote the formation of the intestinal mucosa and maintain the intestinal microecological balance of host animals (Béchon and Ghigo, 2022).

It is noteworthy that the relative abundance of *Euryarchaeota* was higher in the HW group, however, the relative abundance of *Proteobacteria* was higher in the LW group. By Spearman's analysis, *Euryarchaeota* was positively correlated with body weight, and *Proteobacteria* was negatively correlated with body weight. Many studies found that *Euryarchaeota* accounts for a higher percentage of the obese group and it may be one of the strongest predictors of obesity measures, which is in agreement with our findings (Bortolin et al., 2018). *Proteobacteria* included many pathogenic bacteria, such as *Escherichia coli*, *Salmonella*, *Vibrio*, and *Helicobacter*, which were prone to cause metabolic disorders and inflammatory bowel disease (Rizzatti et al., 2017). Besides, *Proteobacteria* have been reported as a marker of microbial dysbiosis in GM (Shin et al., 2015). Therefore, we speculate that the high abundance of *Proteobacteria* might be a reason for the slow growth of lambs in the LW group.

By comparing the GM at the genus level, we found that most of the core genera were different in the HW and LW groups. *Parabacteroides* was the most abundant genus in the

HW group. Spearman's analysis showed that *Parabacteroides* had the strongest positive correlation with BW and ADG, suggesting that it was crucial to the improvement of lamb growth performance. Studies have found that *Parabacteroides* is a comprehensive probiotic that can produce succinic acid, generate propionic acid through hydrogenation, and has a positive regulatory effect on the glycolipid metabolism and protein synthesis (Wang M. et al., 2019).

Ruminococcus was significantly higher in the HW group than in the LW group, and it was positively correlated with BW and ADG. One study found higher *Ruminococcus* concentrations in obese patients, by comparing the fecal microbiota of the obese group and the control group in humans (Kasai et al., 2015). This was consistent with the results of this study. *Stanley et al.* found that *Ruminococcus* was enriched in the group with a high feed conversion ratio, indicating that *Ruminococcus* could indeed improve the digestion and absorption of feed and promote the increase of animal body weight (Stanley et al., 2016). Additionally, *Ruminococcus* was able to degrade indigestible fibers and fermented complex sugars by producing cellulases and played an important role in degrading resistant starch (Terry et al., 2019; Hong et al., 2022).

In addition, some genera enriched in the HW group were considered potential probiotics. For example, *Schwartzia* could ferment succinic acid to produce propionic acid, which provided energy for protein synthesis (Ran et al., 2021). *Anaerotruncus*, a succinate-producing bacterium, had a positive correlation with obesity (Goodrich et al., 2014; Shang et al., 2016). Some genera that were enriched in the LW group were also correlated with body weight. Studies have manifested that *Christensenellaceae* was enriched in individuals with low body mass index and was inversely correlated with obesity. We observed that *Akkermansia* was negatively correlated with BW and ADG. Previous studies have confirmed that *Akkermansia* can reduce the risk of diabetes, obesity, and inflammation by reducing the fat formation and inhibiting insulin, which may be a reason for the lower weight of lambs in the LW group (Derrien et al., 2017). Besides, there are many other genera enriched in the HW group, however, the functions of these genera are still unclear, and further research is needed.

The involvement of GM in different metabolic pathways may be a reason for the difference in lamb body weight. Therefore, we also analyzed the functional capacity of the GM. The results showed that at KEGG level 2, the HW group was enriched in Amino Acid Metabolism, Energy Metabolism, Carbohydrate Metabolism, and Nucleotide Metabolism, which was consistent with the findings in obese children (Li et al., 2021). Amino Acid Metabolism played a vital role in the synthesis of acetate, malonate, and butyrate, and could promote the production of SCFA in the intestine, thereby providing energy for the host (Neis et al., 2015). Meanwhile, Energy Metabolism and Carbohydrate Metabolism were beneficial to glycogen synthesis and glycolysis *in vivo* and ensured adequate energy intake and storage of animals (Cipolla-Neto et al., 2014; Mul et al., 2015). The metabolic pathways that were significantly enriched in the HW group were all essential routine metabolic functions of the body. At the same time, these metabolic pathways also proved that GM in the HW group had a more powerful capacity for food digestion and nutrient absorption than in the LW group.

It is well established that the differences in lamb body weight involved multiple factors, and GM is one of the core factors. Different GM may cause differences in lamb growth through host-microbe interactions, and PICRUSt provides a significant basis for identifying the function of goat gut bacterial communities. Notably, a large number of the bacteria detected belong to unclassified genera, therefore, weight-related genera still need to be continuously explored and studied.

Conclusion

In this study, we compared the fecal microbiota of Hainan black goats in HW and LW groups at weaning age. There were significant differences in the microbiota structure between the two groups, and some microbes were detected to be significantly correlated with body weight. These results provide new information on the relationship between GM and growth traits of Hainan black goats at weaning age, and also provide directions for the use and development of gut microecological preparations.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA827021.

Ethics statement

This animal study was reviewed and approved by the Ethical Committee of the Hainan University (Haikou, China, Permit number: HNUAUCC-2022-000122).

Author contributions

LL and FW designed the experiments. LL, KL, ZB, ZC, and FW performed the experiments. KL and KC did the data analysis. LL, FW, and KL wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.951473/full#supplementary-material

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Comparative study on the microbiota of colostrum and nipple skin from lactating mothers separated from their newborn at birth in China

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Increasing studies have found breast milk (BM) contains its own microbiota. However, the route through which microbes enter the BM is still unclear. In order to verify the entero-mammary pathway of BM, we designed a rigorous study that prevented oral bacteria from contaminating the breast and nipple skin (NS) during baby nursing. Thirty-one healthy, postpartum mothers living in southern China who were immediately separated from their newborn after delivery were enrolled in this study. Using an aseptic protocol for sampling, sterile water was used to wash the NS and was then collected. Then the first drop of BM was discarded and colostrum was collected manually. Amplicon sequencing was performed targeting the V3–V4 region of the bacterial 16S rRNA gene, and the differences between the microbiota of the colostrum and NS were analyzed. Additionally, the effects of environmental factors, such as the delivery mode and intrapartum antibiotic exposure, on the diversity of the colostrum microbiota were also analyzed. We found significant differences in the α diversity and richness between the BM and NS as evidenced by richness, Chao1, and Simpson indices. There were 170 operational taxonomic units (OTUs) shared by colostrum and NS, while 111 and 87 OTUs were unique, respectively, as well as a clear distinction in OTUs was observed by unifrac binary analysis between them. Linear discriminant analysis effect size analysis found that anaerobes, such as *Bifidobacterium* and *Pantoea* at the genus level and enterobacteria including Enterobacteriaceae at the family level, were predominant in the colostrum, while the predominant bacteria on the NS were *Bacteroides*, *Staphylococcus,* and *Parabacteroides* at the genus level. BM is colonized by bacteria prior to baby suckling, and the diversity of the colostrum microbiota differs from that of the NS. The predominant microbiota taxa in BM indicated that they were likely to be transferred to the breast through the intestinal tract. Our study provides direct evidence for the revolutionary

active migration hypothesis. Additionally, factors like intrapartum antibiotic exposure did not significantly affect the diversity of the microbiota in the BM. Therefore, it is suggested that mothers continue to provide BM for their newborns during separation.

KEYWORDS

microbiota, diversity, colostrum, nipple skin, mother–infant separation

Introduction

Human breast milk (BM) is the best nutrition for infants, as it provides essential nutrients, immune cells, and bioactive components. Breastfeeding is associated with considerable health benefits for infants, including protection from diarrhea (Duijts et al., 2010), necrotizing enterocolitis (Herrmann and Carroll, 2014), respiratory infections (Lanari et al., 2013; Tromp et al., 2017), as well as obesity, type 2 diabetes, and cardiovascular disease (Arenz et al., 2004). These benefits are, especially, relevant to infants with increased susceptibility to infections, such as preterm or sick infants. Additionally, increasing evidence has shown that BM contains its own microbiota, which is important for maintaining both mammary and infant health (Zimmermann and Curtis, 2020). Breastfeeding was considered to be a smart investment in people and in economies (Hansen, 2016), thus World Health Organization (WHO) currently recommends an exclusively breastfed diet until 6 months of age, with solid food and breast milk continuing thereafter (WHO, 2001).

In recent years, the study of the BM microbiota has become increasingly extensive. BM is recognized as a source of commensal and potentially probiotic bacteria (Jost et al., 2014) and may also influence both neonatal gut colonization (Gueimonde et al., 2007) and the maturation of the immune system (Jost et al., 2014). In the past, bacteria isolated from BM were thought to be a contaminant from the infant's oral cavity, mother's skin, or due to incorrect handling or processing (Heikkila and Saris, 2003). One study estimated that average consumption of 800 ml of BM per day correlates to an infant ingesting approximately 8×10^7 to 10^{10} bacteria per day (Zimmermann and Curtis, 2020). Traditional culture and isolation techniques and culture-independent molecular methods, such as quantitative PCR, cloning and sequencing of bacterial 16S rRNA gene fragments (Cabrera-Rubio et al., 2012, 2016; Jost et al., 2013; Sakwinska et al., 2016; Ruiz et al., 2019), and metagenomics shotgun sequencing (Ward et al., 2013; Jimenez et al., 2015), have revealed the diversity and complexity of bacteria in BM.

The origin of the BM microbiota is the subject of much debate. There are currently two hypotheses regarding the source of the BM microbiota: the traditional pollution theory and the active migration theory. According to pollution theory, the microorganisms in breast milk come from the mother's nipple and areola and the baby's mouth. During the process of maternal feeding, the negative pressure of sucking generated by the infant allows the microorganisms colonizing the skin surrounding the nipple and areola to enter the mammary gland along the breast tube, or the newborn's mouth is contaminated by microorganisms from the mother's intestinal tract and vagina during delivery, which enter the mammary gland during feeding. Other studies have suggested that the microbiota in the mother's intestines and vagina can naturally "migrate" to the newborn's intestines during delivery (Martin et al., 2007a; Makino et al., 2011; Sanz, 2011).

The other is the hypothesis that maternal bacteria translocate through the intestinal epithelial barrier, migrate to the mammary glands *via* an endogenous cellular route, such as a bacterial entero-mammary pathway, and subsequently colonize the gut of the breastfed neonate (Martin et al., 2007b; Jost et al., 2014).

In order to test the hypothesis regarding an entero-mammary pathway of microbiota translocation in breast milk, we washed the skin surrounding the nipple and collected the cleaning fluid, in addition to the colostrum, from 31 mothers who were separated from their infants immediately after delivery. We used 16S rRNA gene sequencing to detect and analyze differences between the microbiota profile of the BM and nipple skin. We also investigated the impact of newborn gender, delivery mode, parity, gestational age, and intrapartum antibiotic exposure on the microbiota composition of colostrum.

Materials and methods

Participants

The study was conducted from January to February 2021 in the Luohu Maternal and Child Hospital in Shenzhen, China. Thirty-one healthy volunteers who met the inclusion and exclusion criteria were included in the study. The inclusion criteria were as follows: 20–45 years old,

TABLE 1 Average demographics of the participants.

Variable	Data
Mother age (years), Mean (SD)	29.3 (4.6)
Mother gain weight during pregnancy (kg), Mean (SD)	13.0 (4.5)
Pregnancy number, X (*N*)	1 (21)
	2 (7)
	3 (3)
Gestational age (weeks), w (*N*)	37–42 w (24)
	< 37 w (7)
Delivery mode (*N*)	Vaginal (17)
	C-section (14)
Intrapartum antibiotics during delivery (*N*)	Yes (22)
	No (9)
Infant gender (*N*)	Male (19)
	Female (12)
Newborn weight (kg), Mean (SD)	3.0 (0.6)
Sampling days after birth (days), Mean (SD)	3.0 (1.1)

healthy during pregnancy, without any common pregnancy complications, did not use probiotics during pregnancy, settled in Shenzhen, the Han nationality, and normal lactating woman. Exclusion criteria included pregnant women with gestational diabetes, pregnancy-induced hypertension syndrome, acute communicable diseases, and ethnic minorities. After birth, the newborns were immediately transferred to the neonatal intensive care unit (NICU) for monitoring or treatment due to premature rupture of membranes or other reasons, resulting in the separation of mother and baby. Thus, the newborn did not nurse from the mother's breast immediately after delivery. Further, BM samples were collected only from mothers who are yet to nurse their newborn babies. The postpartum mothers were usually hospitalized for 3−5 days and then discharged with their baby or discharged before the baby. In order to ensure the samples were all in the same ward environment, we collected BM from lactating mothers during their hospitalization. Since we collected BM within 5 days after delivery, the milk collected was colostrum. Additionally, mothers generally did not pump BM before delivery.

The participants completed a questionnaire that included basic maternal information (name, age, telephone number, and address), pregnancy and childbirth information (number of pregnancies, parity, the number of gestational weeks at delivery, education level, pre-pregnancy weight, pre-partum weight, breast changes during pregnancy, diet, delivery method, and prophylactic use of antibiotics before or after delivery), and information about the infant, including gender, height, and weight, as well as the reason for separation from the mother at birth (**Table 1** and **Supplementary Table S1**).

Sample collection

During hospitalization, colostrum was collected from all 31 lactating mothers within 5 days after delivery. The first BM and NS samples of the mother were collected on 5 January 2021, and the last samples were collected on 25 January 2021. Colostrum was collected by an International Board Certified Lactation Consultant (IBCLC) in our study group, and her assistant using an aseptic protocol. Briefly, 8–10 ml of sterile water was used to wash one nipple and the surrounding nipple skin (NS). The sterilized water for rinsing the nipple skin was collected in an enzyme-free, aseptic centrifugal tube. The first drop of breast milk was discarded with an aseptic yarn block, and the colostrum (3–5 ml) was manually collected into an enzyme-free, aseptic centrifugal tube by researchers with sterile gloves. After sealing the tube with sealing film, the BM and NS samples were quickly frozen in liquid nitrogen and then transferred to a −80°C freezer for storage. The total genomic DNA was extracted on 19th February 2021. These processes prevented the microbiota from replicating.

DNA extraction and PCR amplification

The bacterial DNA was extracted from the BM and lotion samples using the TGuide S96 Magnetic Soil/Stool DNA Kit [Tiangen Biotech (Beijing) Co., Ltd., China], and PCR amplification was conducted with barcoded-specific bacterial primers targeting the variable region 3–4 (V3–V4) of the 16S rRNA gene. The primers used were 335F: 5′-CADACTCCTACGGGAGGC-3′ and 769R: 5′-ATCCTGTTTGMTMCCCVCRC-3′.

The PCR was performed in a total reaction volume of 10 μl: DNA template (5−50 ng), *Vn F (10 μM, 0.3 μl), *Vn R (10 μM, 0.3 μl), KOD FX Neo Buffer (5 μl), dNTP (2 mM, 2 μl), KOD FX Neo (0.2 μl), and ddH2O (up to 10 μl). The amplification conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 50°C for 20 s, and 72°C for 40 s, and a final extension at 72°C for 7 min. The PCR amplified products were mixed and purified by an Omega DNA purification column (Norcross, GA, United States). The mixed PCR amplified products were then purified and recovered using 1.8% agarose gel electrophoresis.

Processing of the sequencing data

Construction of sequencing libraries and paired-end sequencing was performed on an Illumina NovaSeq6000 platform at Biomarker Technologies Co., Ltd. (Beijing, China) according to standard protocols. Paired-end reads were merged

using FLASH v1.2.7 (Yang et al., 2020), and tags with more than six mismatches were discarded. The merged tags with an average quality score < 20 in a 50-bp sliding window were determined using Trimmomatic (Sheng et al., 2019), and those shorter than 350 bps were removed. Possible chimeras were further removed, and the denoised sequences were clustered into operational taxonomic units (OTUs) with 97% similarity using USEARCH (version 10.0) (Edgar, 2010), and the OTUs with reabundance < 0.005% were filtered. Taxonomy was assigned to all OTUs by searching against the Silva databases (Release128) using USEARCH software (version 10.0). We used Decontam (Version 0.0.1) to minimize the sources of probable external contamination.

Statistical analysis

The statistical analyses were performed in R (Version 4.0.1). Microbiota profiles were included in the estimation of alpha diversity (referring to diversity within a particular region or ecosystem) and beta diversity (comparing the similarity of species diversity among different samples) analyses as described by Liu et al. (2021). We obtained the richness, chao1, and Shannon indices of alpha diversity and compared these differences between BM and NS by LSD *t*-test.

Dissimilarities between BM and NS were estimated with the Bray–Curtis dissimilarity index and Unifrac indices (Lozupone et al., 2007) and analyzed with principal coordinate analysis (PCoA). Moreover, permutational multivariate analysis of variance was used to describe the strength and significance that a categorical factor has in determining the variation of ecological distances. The differential OTU abundance and taxa were analyzed by Wilcoxon rank-sum tests in R version 4.0.1 (FDR < 0.05, the mean relative abundance > 0.1%).

Furthermore, we employed linear discriminant analysis (LDA) effect size (LEfSe; Segata et al., 2011) to identify the significant taxonomic difference among influencing groups, with edgeR (Robinson et al., 2010) for verification if necessary. A logarithmic LDA score of 2.0 was set as the threshold for discriminative features. Adobe Illustrator was used to plot the results.

Ethics statement

This study was conducted according to the guidelines set forth in the Declaration of Helsinki. The experiments in this study were approved by the Ethics Committee of the Luohu Maternal and Child Health Hospital (No. LL2022051337). Written consent was obtained from each volunteer.

Results

Participants' characteristics

The study was conducted on samples collected from 31 healthy mothers (aged 21–42 years) who were separated from their infants immediately after delivery without skin-to-skin contact with babies. The causes of mother-infant immediate separation after delivery included neonatal factors, such as pulmonary sequestration, premature birth, and amniotic fluid inhalation, and maternal factors, such as premature rupture of membranes, placental abruption, and extended vaginal delivery. Details are presented in **Table 1** and **Supplementary Table S1**. The average age of mothers was 29.3 ± 4.6 years. Nearly, 68% of mothers gave birth to their first child, 23% for the second baby, and less than 9% for the third. Among the 31 newborns, 19 were male and 12 were female, and 17 were delivered vaginally and 14 were born by cesarean section (C-section). The average birth weight of babies was 3.0 ± 0.6 Kg. Twenty-two mothers received intrapartum antibiotic prophylaxis (IAP) through intravenous infusion, including 14 who delivered *via* C-section and 7 who delivered vaginally. The main antibiotics were cefazolin sodium, which is a first-generation cephalosporin, and cefuroxime sodium, which is a second-generation cephalosporin. All 31 normal lactating mothers lived in Shenzhen, south of China, and experienced anxiety after being separated from their newborns.

Microbiota profiling by sequencing of 16S rRNA gene amplicons

The quantity of microbiota in the BM and NS samples collected *via* an aseptic protocol was sufficient for microbiota profiling based on 16S rRNA gene sequencing. All 62 samples, which included 31 BM and 31 NS samples, yielded quantifiable PCR products and were sequenced. The analysis resulted in the generation of a total of 4,646,066 clean reads, representing an average of 74,937 clean reads per sample. The average reads were 78,158 per sample in the BM samples and 71,714 reads per sample in the NS samples. Reads were clustered at the similarity level of 97.0%, and OTUs were obtained. Totally, 11,190 OTUs were retained for diversity analyses, with an average OUT abundance of 243 in BM and 212 in NS.

Alpha diversity analysis of the microbiota in the breast milk and nipple skin

A dilution curve (Wang et al., 2012) (rarefaction curve) randomly takes a certain number of sequences from a sample, counts the number of species represented by the sequences,

and constructs a curve based on the number of sequences and species. This curve is then used to verify whether the amount of sequencing data is sufficient to reflect the species diversity in the sample, and indirectly reflects the species richness in the sample. The rarefaction curves of the OTUs from the BM and NS samples approached the plateau phase with more than 40,000 sequences per sample, indicating that these values were saturated and there was no need to determine more sequences (**Figure 1A**). The α diversity of the microbiota from the BM and NS was estimated through sample richness (**Figure 1C**), Chao1 (**Figure 1B**), and Shannon index (**Figure 1D**). The BM samples displayed higher α diversity values than the NS samples, as determined *via* the three indices mentioned above (LSD *t*-test $p = 0.000$, 0.001, and 0.001, respectively) (**Figures 1B–D**). In short, the microbiota in BM and NS detected by 16S rRNA gene sequencing was diverse. Moreover, the microbial abundance in BM was higher than that in the NS.

Analysis of taxa shared or differentially represented in breast milk and nipple skin samples

Focusing on the differences and similarities of specific phylum between the BM and NS microbiotas (**Figure 2A**), we found that Firmicutes was the dominant phylum in both sample types, followed by Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria. These phyla were detected across all the samples analyzed. Analysis at the genus level (the microbial relative abundance was above 0.1% and the top 10 were displayed) (**Figure 2B**) found that the common genera in both the BM and NS microbiotas were *Bacteroides*, *Faecalibacterium*, *Pantoea*, *uncultured_bacterium_f_Lachnospiraceae*, *Prevotella_9*, *Roseburia*, *Lachnospira*, *Agathobacter*, *Staphylococcus*, *Parabacteroides*, and others. On the other hand, there were also differences in the abundance of some genera between the BM and NS samples. *Faecalibacterium* (9.39% vs. 9.11%) and *Pantoea* (9.4% vs. 0.51%) were dramatically higher in the BM microbiota when compared to the NS microbiota, while *Lachnospira* (2.04% vs. 3.48%), *Staphylococcus* (1.52% vs. 3.03%), and *Parabacteroides* (1.30% vs. 3.12%) were dramatically lower in the BM microbiota when compared to the NS microbiota.

According to the microbial abundance top10 statistics, the unique bacterial genera of the BM samples included *Pantoea*, *Pseudomonas*, *Bifidobacterium*, *Klebsiella*, and *Escherichia-Shigella* (**Figure 2C**), which were higher than 0.1%. The unique bacterial genera in the NS samples were *Lachnospira*, *Parabacteroides*, *Staphylococcus*, *Agathobacter*, and *Lachnoclostridium* (**Figure 2D**). Additionally, the shared genera between the BM and NS microbiotas were *Bacteroides*, *Faecalibacterium*, *uncultured_bacterium_f_Lachnospiraceae*, *Prevotella_9*, *Roseburia*, and others (**Figures 2C,D**). Additionally, high variability in the microbiota composition of both BM and NS was observed among the 31 mothers. Thus, the bacterial communities were generally complex and showed individual-specific profiles.

Beta diversity analysis of the microbiota in the breast milk and nipple skin

We examined differences in the microbiota of BM and NS at the OTU level. First, a Venn diagram that showed 170 overlapped OTUs between BM and NS was constructed. The unique OTUs were 111 for BM and 87 for NS (**Supplementary Figure S1A**). Through differential abundance analysis, 77 OTUs were depleted and 107 were enriched in the BM when compared with the NS (**Supplementary Figure S1B**). We found that the composition of the bacterial microbiota of BM was different from that of NS. Unifrac binary analysis (PCoA) of Bray–Curtis distance revealed that the microbiota of BM and NS formed two distinct clusters, which separated along the first coordinate axis (**Figure 3A**), indicating that the BM microbiota differed from the NS microbiota.

We then analyzed the enrichment of OTUs according to their taxonomy using Manhattan plots (**Supplementary Figure S1C**). Compared with the NS, OTUs enriched in the BM belonged to the genera *Prevotella and Erwinia*, while OTUs depleted in the BM were commonly in the genera *Bacteroides*, *Phocaeicola*, *Staphylococcus*, *Agathobacter,* and *Kineothrix* (FDR < 0.05, Wilcoxon rank-sum test; **Supplementary Figure S1C**). Additionally, the LEfSe analysis including taxonomic cladogram (**Figure 3B**) and linear discriminant analysis (LDA) scores (LDA values higher than 4.0, $p = 0.05$) (**Supplementary Figure S1D**) also determined microbiota unique to the BM, including *Bifidobacterium* at the genus level, *Enterobacteriaceae* at the family level, *Lactobacillales* and *Pseudomonadales* at the order level, *Actinobacteria* and *Gammaproteobacteria* at the class level, and *Proteobacteria* at the phylum level, which was totally different from the NS. The unique NS microbiota included *Bacteroides*, *Staphylococcus,* and *Parabacteroides* at the genus level, *Lachnospiraceae*, *Bacteroidaceae,* and *Porphyromonadaceae* at the family level, and *Bacillales* at the order level.

Analysis of various factors may influence the colostrum (breast milk) microbiota diversity

Possible factors, such as the parity, delivery method, IAP, gestational age, and gender of the newborn, that might affect the microbiota diversity were further explored. On the other

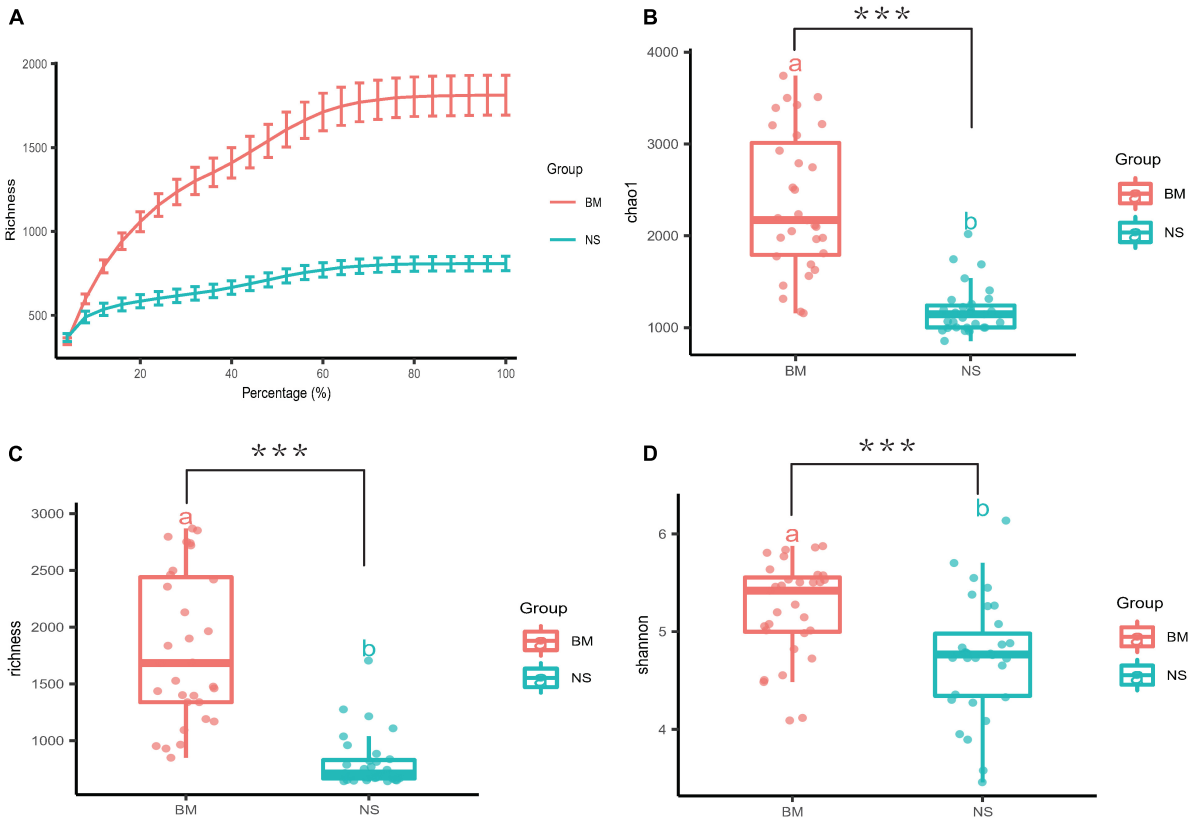

FIGURE 1
Measures of α diversity for BM and NS. **(A)** A rarefaction curve was used to evaluate the sequencing saturation of the samples, and it showed the mean value and standard error of the sequencing depth and the quantity of OTUs. The smoothing of the curves indicated that the sequencing was saturated. **(B–D)** Box plots of three different α diversity measures: Chao1, richness, and Shannon. The three indices were based on OTUs clustered at 97% similarity for the BM and NS samples (BM, n = 31 vs. NS, n = 31). There were significant differences in Chao1, richness, and Shannon between BM and NS groups (a and b above boxes represent statistical differences) with FDR-adjusted p = 0.000, 0.001, and 0.001 with LSD t-test, respectively. Each scatter represents a sample. The horizontal bars within boxes represent the median, and the tops and bottoms of the boxes represent the 75th and 25th quartiles, respectively. ***$p \leq 0.001$.

hand, we tried to apply LEfSe to analyze the characteristic microbiota taxa for different influencing factors and verify them using edgeR for subsequent studies on the characteristic microbiota. Only taxa with LDA values higher than 3.0 were presented (Supplementary Figure S2), and the LDA value for each lineage was listed. The gender of the newborn had no significant effect on the α and β diversity values of the BM microbiota (Figures 4A,B). The LEfSe analysis showed that the BM of female newborns was rich in *Streptococcaceae* at the family level, while the BM of male newborns was rich in *Roseburia* and *Alcaligenaceae* (Figure 4A). In addition, the edgeR verified that *Roseburia* was enriched at the genus level (data not shown). Parity had no significant effect on the α and β diversity values of the BM microbiota (Figures 4E,F). Also, the delivery mode had no significant effect on the α and β diversity of the BM microbiota (Figures 4C,D). However, LEfSe analysis showed that the BM microbiota from mothers who delivered *via* CS was rich in *Bifidobacterium* (Figure 4B), which was supported by edgeR (data not shown). IAP during delivery also had no significant effect on the α and β diversity of the BM microbiota. Moreover, the two antibiotics administered to mothers (cefazolin sodium and cefuroxime sodium) had no significant effect on the microbiota diversity of BM (Figures 4G,H). However, LEfSe analysis found that mothers who did receive IAP had BM that was rich in *Lachnospiraceae* at the genus level, which was verified by edgeR. Those who did not receive IAP were rich in *Lactobacillus* in BM (Supplementary Figure S2C). Gestational age also had no significant effect on the α and β diversity of the BM microbiota (Figures 4I,J). However, mothers who delivered a full-term infant(s) had BM that was rich in *Bifidobacteria*, as verified by LEfSe and edgeR (Supplementary Figure S2D).

Discussion

The ideal and most common state for mother and newborn is to be together, that is, the infant is immediately placed on

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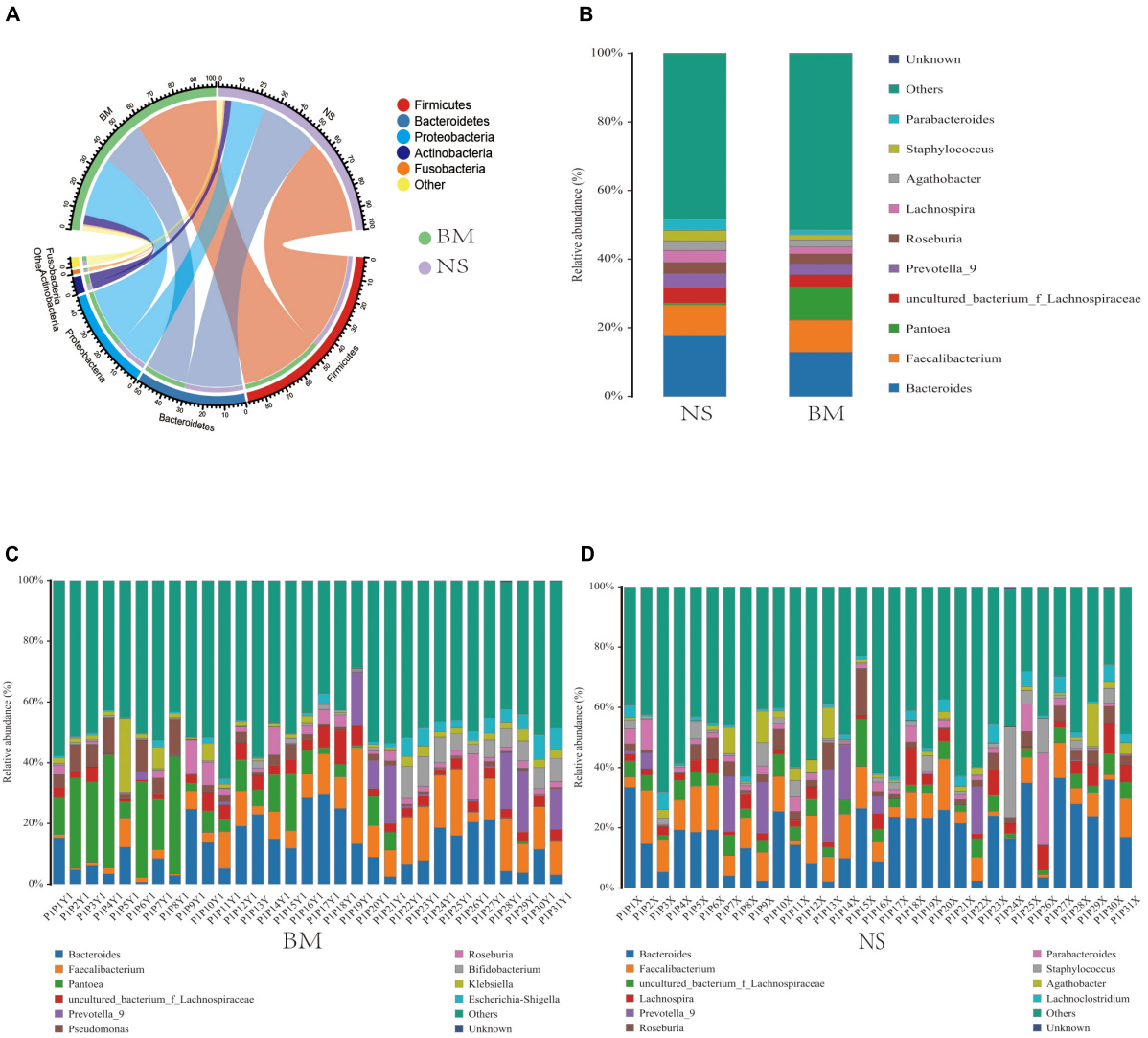

FIGURE 2
The common **(A,B)** and unique **(C,D)** relative abundances of predominant microbiota taxa in BM and NS. The common microbiota at the panels **(A)** phylum level and **(B)** genus level. The unique microbiota at the genus level of panels **(C)** BM and **(D)** NS. The proportion of microbial abundance was more than 0.1%, and the top 10 were displayed.

the mother's skin after birth and encouraged to nurse. This allows the newborn to consume BM, which provides a variety of nutrients that promote healthy programming (Cortes-Macias et al., 2021), as well as a multitude of immunomodulating components, such as soluble immunoglobulin A and growth factors that can reduce the risk of infectious diseases (Chi et al., 2021). However, not all mothers and newborns can be together after birth due to various factors, such as premature birth and amniotic fluid inhalation. Once the mother and baby are separated due to neonatal diseases or some other reasons in China, some mothers struggle to maintain and/or produce BM, resulting in a low supply of BM, which may affect the health of the newborn. The participants in this study were mothers who were separated from their newborns immediately after delivery. Their BM was sampled for studying the microbiota and exploring the origin of microbiota. The aim of this study was to provide a basis for continuous lactation in mothers separated from their newborns and encourage the mothers to provide BM to their newborns during separation.

The breast milk (colostrum) and nipple skin microbiota in lactating mothers separated from their newborns at birth is diverse

Through breastfeeding, newborns and infants are exposed to microorganisms in BM, which stimulate the intestinal immune

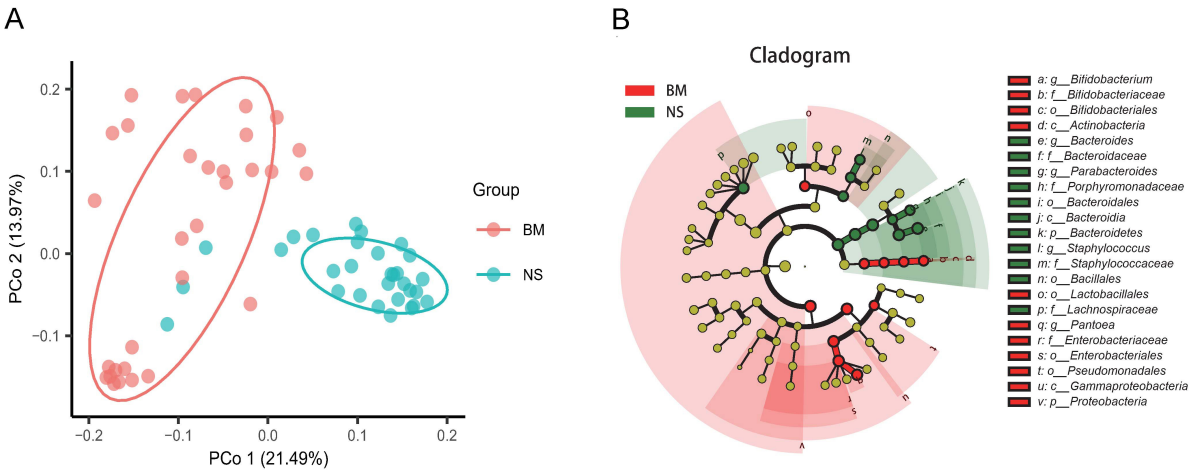

FIGURE 3
Differences in the microbiota between BM (colostrum) and NS. **(A)** Unconstrained principal coordinate analysis with Bray–Curtis distance showing that the BM microbiota separated from those of NS in the first axis (21.49% of variance explained, $P < 0.001$, permutational multivariate analysis of variance (PERMANOVA) by Adonis; $n = 31$ in each group). Ellipses cover 68% of the data for BM and NS samples. The percentage of variation indicated in each axis corresponds to the fraction of the total variance explained by the projection. **(B)** Taxonomic cladogram from linear discriminant analysis (LDA) effect size (LefSe) showing microbiota differences in BM and NS taxa. Dot size is proportional to the abundance of the taxon. The yellow circles represent the classification with no significant difference. P, phylum; c, class; f, family; o, order; g, genus.

function of newborns and infants. The BM microbiota aids the development and functional maturity of the intestinal immune system and reduces the susceptibility to disease (Gollwitzer and Marsland, 2015). Thus, BM is recognized as a major force that shapes the infant gut microbiome in early life. Currently, little is known about the composition of the BM microbiota, and even less is known about the factors that determine it (Zimmermann and Curtis, 2020). To our knowledge, this is the first study to explore the source and composition of the colostrum (first stage of BM) microbiota in a large cohort of Southern Chinese mothers immediately separated from their newborns after delivery. We carefully designed and adopted an aseptic sampling protocol and sample preservation, as well as the elimination of the first drip, which included a few microliters to milliliters of BM, to avoid external contamination and ensure the accuracy and reliability of the results.

Colostrum, generally produced within the fifth day after delivery, is the first stage of BM during lactation. It is rich in proteins and minerals, and contains many immune-active substances, such as antibodies and complement factors (Ballard and Morrow, 2013). It has been reported that colostrum is also rich in microorganisms. Our study analyzed the microbiota of colostrum and NS from mothers who were immediately separated from their newborns after delivery. The rarefaction curves demonstrated that all BM and NS samples reached standard quality requirements for sequencing, and all samples were sequenced sufficiently (**Figure 1A**). Moreover, α diversity of the BM microbiota was assessed *via* richness, Chao1, and Shannon indices (**Figures 1B–D**). The dominant phyla in colostrum (BM) were *Firmicutes* and *Proteobacteria*, which was consistent with previous studies (Urbaniak et al., 2016; Biagi et al., 2017; Murphy et al., 2017; Pannaraj et al., 2017; Simpson et al., 2018; Hermansson et al., 2019; Moossavi et al., 2019). However, the next most abundant phyla were *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria* (**Figure 2A**), which differed from previous reports.

At the genus level, the bacteria unique to the colostrum (BM) were *Pantoea*, *Pseudomonas*, *Bifidobacterium*, *Klebsiella*, and *Escherichia-Shigella* (**Figure 2C** vs. **Figure 2D**). The microbiota shared with the NS was *Bacteroide*, *Faecalibacterium*, *uncultured_bacterium_f_Lachnospiraceae*, *Prevotella_9*, and *Roseburia* (**Figure 2B**). These results differed from full-term newborns who routinely suck their mother's breast after birth, which had higher relative abundances of *Weisella*, *Leuconostoc*, *Streptococcus* (Urbaniak et al., 2016), and *Lactococcus* (Cabrera-Rubio et al., 2012). Notably, the genus *Staphylococcus* has been reported to have higher relative abundances during the first 10 days of life (Cabrera-Rubio et al., 2012; Sakwinska et al., 2016; Simpson et al., 2018), in addition to being universally observed in mature BM (Solis et al., 2010; Jost et al., 2013) by pyrosequencing. However, in our study, *Staphylococcus* was found to be predominant in the NS microbiota (3.03%). *Staphylococcus* was also found in the colostrum, although at a much lower abundance (1.52%), and was particularly depleted in BM compared with NS (**Supplementary Figure S1C**). Thus, the origin and types of *Staphylococcus* in BM and NS are worthy of further study.

The composition of the microbiota in reports varies widely, suggesting that the purpose of the studies, geographical location (Kumar et al., 2016; Li et al., 2017; Ding et al., 2019), ethnic

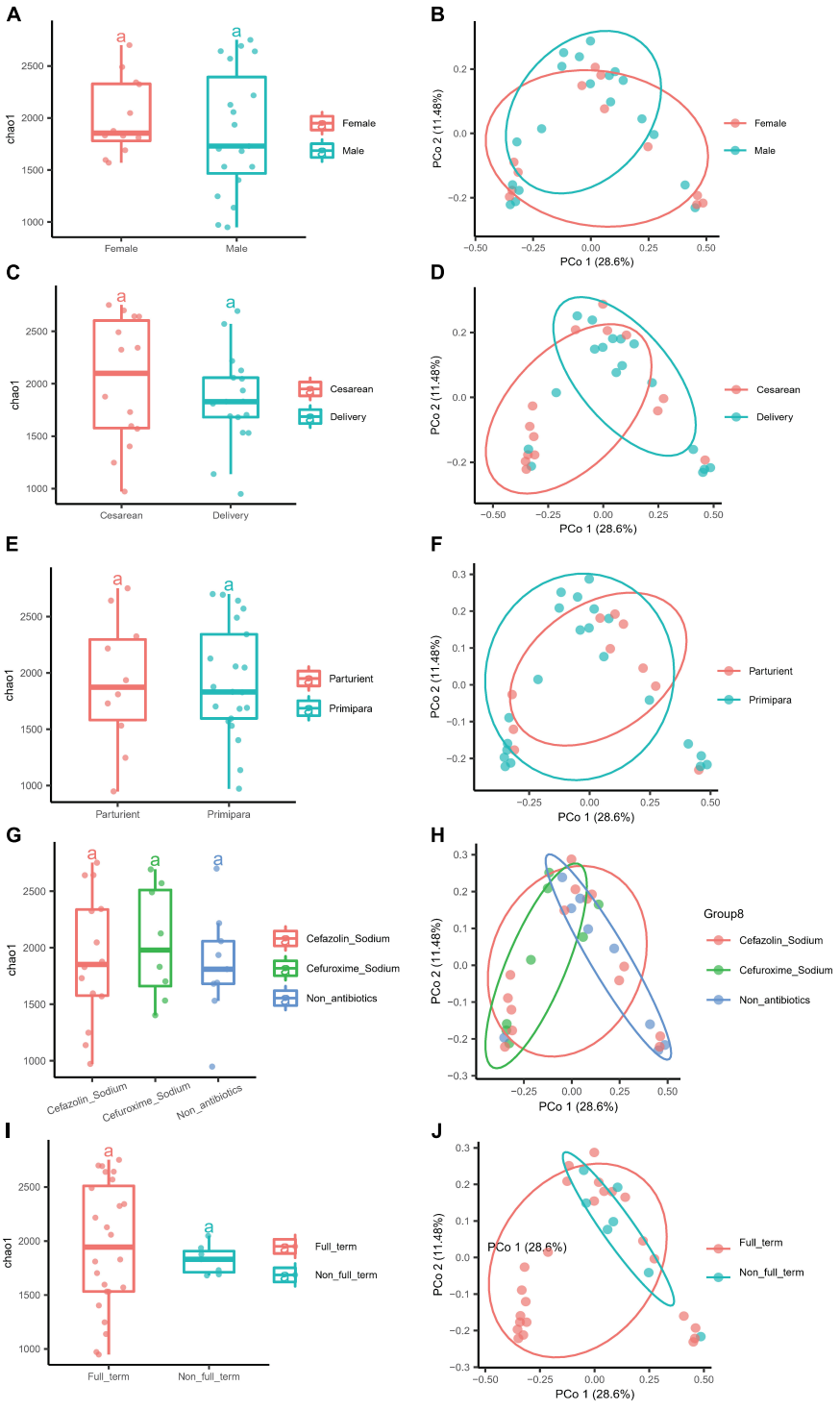

FIGURE 4
Different factors had no influence on the α and β diversity of the microbiota of BM. **(A)** The α diversity of newborn gender (Female and Male). **(B)** The β diversity of newborn gender (Female and Male). **(C)** The α diversity of the delivery mode (Cesarean and Delivery). **(D)** The β diversity of the delivery mode (Cesarean and Delivery). **(E)** The α diversity of parity (Parturient and Primipara), and **(F)** the β diversity of parity (Parturient and Primipara). **(G)** The α diversity of intrapartum antibiotic prophylaxis (Cefazolin sodium, Cefuroxime sodium, and Non-antibiotics), and **(H)** the β diversity of intrapartum antibiotics prophylaxis (Cefazolin sodium, Cefuroxime sodium, and Non-antibiotics). **(I)** The α diversity of gestational age (Full-term and Non-full-term). **(J)** The β diversity of gestational age (Full-term and Non-full-term).

differences, sampling volunteers (Ruiz et al., 2019), collection methods, and methodological differences (Sakwinska et al., 2016) may influence the results.

The microbiota of BM has been hypothesized to originate from commensal and potential probiotic bacteria colonized in the intestine of newborns. Through temperature gradient gel electrophoresis (TGGE) (Perez et al., 2007), pyrosequencing (Jost et al., 2012, 2014), and culture methods (Solis et al., 2010; Jost et al., 2012), *Bifidobacterium* strains, which are gut-associated obligate anaerobes, were found to be shared between maternal feces, BM, and neonatal feces, especially during the first week. Accordingly, our results also found that the relative abundance of *Bifidobacterium* at the genus level in colostrum was high (**Figures 2C**, **4B** and **Supplementary Figure S1D**). Thus, our results support that mothers separated from their newborns should maintain lactation and feed colostrum to their babies in the NICU in order to promote the establishment of normal intestinal microbiota.

Evidence of entero-mammary pathway of breast milk microbiota

Currently, it is still unclear how microbes reach the BM. Most previous studies have suggested the traditional contamination hypothesis (such as the mother's skin and infant's oral cavity). One suggested that mammary ducts become colonized by the infant oral microbiota during suckling, as the retrograde flow of BM into mammary ducts has been documented (Ramsay et al., 2004). However, other studies arrived at different conclusions. For example, buccal administration of colostrum to low-birth-weight newborns in the NICU changed their oral microbiota when compared to infants who were given standard care, suggesting that the BM might be responsible for colonizing the infant's mouth (Sohn et al., 2016). Additionally, precolostrum already contains bacteria before suckling has occurred (Ruiz et al., 2019). Other studies have also suggested that bacteria from the skin (such as *Corynebacterium*, *Cutibacterium,* and *Staphylococcus*) colonize the mammary ducts. However, studies in recent years have suggested the revolutionary active migration hypothesis, which supports the existence of an entero-mammary pathway.

One of the main purposes of this study was to provide a practical and reliable basis for determining the source of bacteria in the BM. In order to avoid contamination by the infant's oral cavity, we enrolled healthy, lactating women who were immediately separated from their newborn(s) after delivery in this study. The newborns did not suck the mother's breast. To avoid the influence of skin microbiota, we washed the surrounding NS with sterile water, collected cleaning water, and then collected the BM after squeezing out the first drop of colostrum. Both the NS and BM samples were analyzed by 16S rRNA gene sequencing targeting the V3–V4 region to compare the composition differences between the microbiota.

Our results showed that there were shared microorganisms between the BM and NS microbiota. As shown in the Venn diagram, there were 170 overlapping OTUs (**Supplementary Figure S1A**) and no significant OTUs in the Volcanic map (**Supplementary Figure S1B**), as well as no significant bacterial classification level in the Manhattan plot (**Supplementary Figure S1C**). However, there were also significant differences in the microbiota diversity between BM and NS. The Venn diagram displayed 111 OTUs unique to BM and 87 unique to NS, while 77 OUTs were depleted and 107 were enriched in BM compared with NS through a Volcanic map (**Supplementary Figure S1B**) for advanced differential abundance analysis. PCoA for the β diversity demonstrated two different populations, BM and NS, in the principal coordinate PCo1, explaining 21.49% of the variance (**Figure 3A**). The unique microbiota in BM was screened by LEfSe analysis. Anaerobic bacteria found in the colostrum (BM), which are not found on the skin (Aakko et al., 2017; Damaceno et al., 2017; Williams et al., 2017; Parnanen et al., 2018), include *Bifidobacterium* at the genus level, *Enterobacteriaceae* at the family level, *Lactobacillales* and *Pseudomonadales* at the order level, *Actinobacteria* and *Gammaproteobacteria* at the class level, and *Proteobacteria* at the phylum level. Similar to our results regarding *Lactobacillales* at the order level in the BM, a study displayed that Lactobacillus present in BM are genotypically different from those detected on the skin within individuals (Martin et al., 2007b). While in NS, the predominance of *Staphylococcus* at the genus level was universally observed in other studies (Solis et al., 2010; Jost et al., 2013).

Excluding neonatal oral contamination, breast skin contamination, and microbiota shared by the NS, the source of bacteria in the breast is quite amazing. As many of the bacteria found in BM can also be found in the intestine, it is plausible that an entero-mammary pathway exists, that is, intestinal organisms, or their DNA, can be transferred from the intestine to the mammary ducts (Zimmermann and Curtis, 2020). Gut-associated obligate anaerobic genera, like *Bifidobacterium* and *Lactobacillales*, have been identified by pyrosequencing to be shared among maternal feces, BM, and neonatal feces (Jost et al., 2014; Cortes-Macias et al., 2021).

Therefore, the entero-mammary pathway hypothesis of BM microbiota might be able to explain this. Our results of unique taxa enriched in the BM, including *Bifidobacterium*, *Enterobacteriaceae*, *Lactobacillales,* and *Pseudomonadales*, as well as *Gammaproteobacteria* and even *Proteobacteria*, were likely transferred from the intestine to the breast through the intestinal tract.

Previous studies have verified that when *Lactobacillus* is administered as a probiotic to women, the same strain can be identified in BM (Jimenez et al., 2008; Abrahamsson et al., 2009). Translocation of bacteria from the intestine to the mammary

glands is thought to mainly occur through gut-associated lymphoid tissues (Vazquez-Torres et al., 1999; Qutaishat et al., 2003) and involves dendritic cells and macrophages (Vazquez-Torres et al., 1999; Rescigno et al., 2001; Qutaishat et al., 2003). Moreover, the translocation of the microbiota has been reported to increase in pregnant or lactating women (Perez et al., 2007). A previous study reported the transmission of *Salmonella enterica* to infants through the mother's BM. It was proposed that there is a biologically feasible mechanism of transport of *Salmonella* from the gastrointestinal tract to BM. *Salmonella* is resistant to the acidic environment of the stomach and usually invades or is phagocytosed by cells lining the Peyer's patches in the small intestine. Specialized epithelial M cells overlying the lymphoid follicles of Peyer's patches provide a portal of entry for *Salmonella enterica* Typhimurium. The pathogenicity island 1 of *S. enterica* Typhimurium was required to penetrate these intestinal epithelial M cells. *S. enterica* Typhimurium is transported from the gastrointestinal tract to the bloodstream by CD18-expressing phagocytic leukocytes and may use macrophages and dendritic cells as a conduit to deeper tissue (Qutaishat et al., 2003).

For non-pathogenic microorganisms, a previous study reported that dendritic cells may express tight junction proteins, which open the tight junctions between epithelial cells, allowing them to penetrate the gut epithelial monolayers to sample bacteria (Rescigno et al., 2001). In future studies, we will conduct a series of animal experiments to follow non-pathogenic bacteria in maternal rats to explore their effects on the BM microbiota, intestinal-breast pathway, establishment of intestinal microbiota, and intestinal immunity.

Various factors had no effect on the microbiota diversity of colostrum (breast milk) but on differences in the unique bacteria in each group

In this study, various factors, including the newborn gender, delivery mode, parity, gestational age, and intrapartum antibiotics exposure (**Supplementary Table S1**), were considered to influence the microbiota diversity of colostrum (BM). Concerning the impact of the newborn gender, a study reported a lower diversity and richness in the microbiota of 3–4 months postpartum BM (mature milk) among mothers of male infants. We found no differences in the α and β indices of colostrum microbiota (**Figures 4A,B**). This may be due to the fact that BM has different lactation stages. The mothers of female newborns had a higher relative abundance of *Streptococcaceae* at the family level, which was the opposite result of another study which reported that mothers of male infants had a higher relative abundance of *Streptococcus* in mature milk (Williams et al., 2017). Mothers of male newborns in our study had a higher relative abundance of *Roseburia* at the genus level than those of female newborns (**Supplementary Figure S2A**). Gamez-Valdez et al. (2021) found that the BM microbiota was influenced by the infant gender in the obesity mother subgroup, with phylogenetic diversity of the female newborns more diverse than in males. Thus, the complex relationship between women's obesity and gestational diabetes mellitus and the influence of newborn gender on the microbiota composition may be worthy of deep study.

We concluded that the delivery mode did not affect the α and β indices of the BM microbiota diversity (**Figures 4C,D**), which was similar to another study (Li et al., 2017). Furthermore, we found that women who delivered by CS had a higher relative abundance of *Bifidobacterium* and *E. coli-Shigella* in colostrum (BM) (**Figure 4B**). Thus, it could be suggested that CS might lead to increased intestinal permeability, enhanced bacterial translocation in the maternal gut, and consequently, a higher transfer of bacteria to the BM.

The conclusion of the studies showed that the effect of parity on the microbiota of BM included two aspects. One aspect was that the diversity in the BM microbiota was lower in infants who were first born as compared with those who had one or more siblings (Moossavi et al., 2019). The second aspect was that parity had no effect on the diversity of the BM microbiota. Our results were in favor of the latter (**Figures 4E,F**), and all microbiota were shared by parturient and primipara mothers.

It has been reported that women who receive IAP had a higher α diversity and richness in their BM microbiota, *Bifidobacterium* was not found (Hermansson et al., 2019), and a lower relative abundance of *Lactobacilli* was found (Soto et al., 2014). We found that IAP (cefazolin sodium was given intravenously to 14 mothers and cefuroxime sodium to 8 mothers) had no effect on the microbiota diversity of colostrum (BM) (**Figures 4G,H**). Though there was no *Bifidobacterium* in non-antibiotics BM as assessed by LDA scores, the BM was rich in *Lactobacillus* at the genus level (**Supplementary Figure S2C**). According to the American College of Obstetricians and Gynecologists (ACOG), IAP is recommended for all women undergoing C-section, and the first generation of cephalosporin, such as cefazolin sodium, is preferred. Fourteen mothers undergoing C-section in this study were given cephalosporin, including cefazolin sodium and cefuroxime sodium. The other eight mothers received cephalosporin prophylactically because of maternal *Streptococcus* B infection or premature rupture of membranes. They accepted antibiotics according to the guidelines. The half-life of cephalosporins is short, so the effect of these antibiotics on the BM microbiota was minimal by the time we collected the mothers' BM on the third day after delivery (the average sampling days were 3 after delivery). Moreover, the type or level of antibiotics did not significantly affect the diversity of the colostrum microbiota. Additionally, *Streptococcus* was not found in the colostrum of mothers infected with *Streptococcus* B, indicating that this pathogenic bacterium did not enter the BM, or at least,

was not found in the colostrum after the administration of antibiotics.

A few studies have explored the influence of gestational age on the BM microbiota (Khodayar-Pardo et al., 2014; Urbaniak et al., 2016). One study investigated a higher relative and absolute abundance of *Bifidobacterium* in women who delivered at full-term (Khodayar-Pardo et al., 2014), which was the same as our results in colostrum (BM) (**Supplementary Figure S2D**). Otherwise, we found no effect of gestational age on the microbiota composition of colostrum (BM) (**Figures 4I,J**), which was consistent with another study (Urbaniak et al., 2016).

As the first attempt to explore the microbiota of BM, we only analyzed the colostrum and NS microbiota in this study, without the collection and analysis of stool from mothers. Therefore, we will expand the sample number and continuously explore the microbiota changes in colostrum, transitional milk, and mature BM compared to the maternal stool to provide further evidence regarding the origin of the BM microbiota. Overall, illuminating the potential factors affecting the differential bacterial taxa of the BM microbiota, such as delivery mode, gestational age, and IAP, was meaningful and allows for in-depth research in the future.

Conclusion

Our study further substantiates the presence and diversity of a microbiota specific to colostrum (BM) among mothers separated from their newborns. Furthermore, the comparison of the microbiota diversity between BM and NS provides reliable evidence for verifying the entero-mammary bacterial translocation hypothesis. According to our results of probiotics, such as *Bifidobacterium* in colostrum, and no effect of intrapartum antibiotics on microbiota abundance, we support mothers to maintain lactation and pump BM for newborns in NICU.

However, our results need to be further studied for confirmation. The impact of the BM microbiota on neonatal gut microbiota establishment, immunity function, and health consequences are worthy of further study.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI—PRJNA848210.

Ethics statement

The studies involving human participants were reviewed and approved by the Shenzhen Luohu Maternity and Child Healthcare Hospital (No. LL2022051337). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YD, ZHH, and RX performed the conception and design of the work and drafted the article. YD, QQ, and LW collected the samples and the data. ZOH and XW performed the data analysis and interpretation. ZOH and GJ performed the critical revision of the article. XW and GJ performed the final approval of the version to be published. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.932495/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Supplementary methods to describe the differences in the microbiota between BM (colostrum) and **(A)** A Venn diagram representing the overlapping and unique OTUs between BM and NS. **(B)** Taxonomic and functional characteristics of differential bacteria between the BM and NS microbiota. **(C)** Manhattan plot showing OTUs enriched in the BM as compared to NS. Each triangle represents a sing out OTU. OTUs enriched in BM or NS are represented by filled or empty triangles, respectively (FDR < 0.005, Wilcoxon rank-sum test). OTUs are arranged in taxonomic order and colored according to the genus. The size of the triangle represents the abundance of OTUs. **(D)** Linear discriminant analysis (LDA) scores computed for differentially abundant taxa in the microbiomes of NS (blue) and BM (red). Length indicates effect size associated with a taxon; $P = 0.05$ for the Kruskal–Wallis H-test; LDA score = 4.

SUPPLEMENTARY FIGURE S2
Differential bacterial taxa in the microbiome of NS (green) and BM (red) in different groups by linear discriminant analysis (LDA) scores. The length indicates the effect size associated with a taxon; $p = 0.05$ for the Kruskal–Wallis H-test; LDA score = 3.

SUPPLEMENTARY TABLE S1
The demographics of the participants.

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Bacteroides abundance drives birth mode dependent infant gut microbiota developmental trajectories

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Background and aims: Birth mode and other early life factors affect a newborn's microbial colonization with potential long-term health effects. Individual variations in early life gut microbiota development, especially their effects on the functional repertoire of microbiota, are still poorly characterized. This study aims to provide new insights into the gut microbiome developmental trajectories during the first year of life.

Methods: Our study comprised 78 term infants sampled at 3 weeks, 3 months, 6 months, and 12 months (n = 280 total samples), and their mothers were sampled in late pregnancy (n = 50). Fecal DNA was subjected to shotgun metagenomic sequencing. Infant samples were studied for taxonomic and functional maturation, and maternal microbiota was used as a reference. Hierarchical clustering on taxonomic profiles was used to identify the main microbiota developmental trajectories in the infants, and their associations with perinatal and postnatal factors were assessed.

Results: In line with previous studies, infant microbiota composition showed increased alpha diversity and decreased beta diversity by age, converging toward an adult-like profile. However, we did not observe an increase in functional alpha diversity, which was stable and comparable with the mother samples throughout all the sampling points. Using a *de novo* clustering approach, two main infant microbiota clusters driven by *Bacteroidaceae* and *Clostridiaceae* emerged at each time point. The clusters were associated with birth mode and their functions differed mainly in terms of biosynthetic and carbohydrate degradation pathways, some of which consistently differed between the clusters for all the time points. The longitudinal analysis indicated three main microbiota developmental trajectories, with the majority of the infants retaining their characteristic cluster until 1 year. As many as 40% of vaginally delivered infants were grouped with infants delivered by C-section due to their clear and persistent depletion in *Bacteroides*. Intrapartum antibiotics, any perinatal or postnatal factors, maternal microbiota composition, or other maternal factors did not explain the depletion in *Bacteroides* in the subset of vaginally born infants.

Conclusion: Our study provides an enhanced understanding of the compositional and functional early life gut microbiota trajectories, opening avenues for investigating elusive causes that influence non-typical microbiota development.

KEYWORDS

***Bacteroides*, shotgun metagenomics, infant gut microbiota, functional maturation, hierarchical clustering, vaginal delivery, cesarean section**

Introduction

The acquisition and development of early life gut microbiota have been linked to health outcomes during infancy and later life (Avershina et al., 2021; Sarkar et al., 2021). The establishment of gut microbiota is affected by several maternal factors, perinatal and postnatal exposures such as mode of birth, breastfeeding, infections, antibiotics, hosts genetics, and living environment (Penders et al., 2006; Tamburini et al., 2016; Korpela and de Vos, 2018). Delivery mode is a crucial factor especially in the first months of life (Arboleya et al., 2018; Kumbhare et al., 2019; Korpela, 2021). As infants develop, their microbiota matures through a phase characterized by high dynamics and low diversity in early infancy (from birth to 6 months) and reaches a more stable and diverse microbial community during early childhood (3–5 years old) (Milani et al., 2017; Korpela and de Vos, 2018; Korpela, 2021). Importantly, the maturation of infant gut microbiota is affected by the timing of weaning and breastfeeding cessation, as these events contribute to shifting of the microbial community composition toward an adult-like profile (Bäckhed et al., 2015).

Several authors have reported the critical impact of delivery mode on early-life microbiota acquisition. Typically, Cesarean-section delivered (CSD) infants show a strong depletion in *Bacteroides* that typically resolves between 6 and 12 months of age in relation to weaning (Shao et al., 2019; Yang et al., 2019; Korpela, 2021; Princisval et al., 2021). Some studies suggest that birth mode-related impact on early life microbiota may take 3–5 years to normalize (Roswall et al., 2021). On the other hand, vaginally delivered (VD) infants' gut microbiota is typically dominated by *Bifidobacterium* and *Bacteroides spp.*; both genera are known to be central in digestion of human milk oligosaccharides (Marcobal et al., 2010). The CS birth-related scarcity of *Bacteroides spp.* has been linked to increased risk of intestinal colonization by opportunistic pathogens (Reyman et al., 2019; Shao et al., 2019) and altered immunostimulatory potential in infants (Wampach et al., 2018). Overall, *Bacteroides spp.* have been suggested to play a pivotal role in infant health because of their role in modulating and training the immune system (Vatanen et al., 2016), and higher ratio of *Enterobacteriaceae* to *Bacteroidaceae* in CSD infants has been implicated in increased risk of development of atopy and *Clostridioides difficile* infection in late infancy (Vu et al., 2021). Some species of *Bacteroides* are involved in cross-feeding nutrients with other gut microbes, producing antimicrobial toxins and competing against pathogens (Wexler, 2007; Zafar and Saier, 2021). The lack of exposure to mother's gut microbiota at birth explains the delayed establishment of *Bacteroides* spp. in CSD babies (Korpela et al., 2020; Korpela, 2021). However, some VD infants are also essentially devoid of *Bacteroides* spp. (Yassour et al., 2016; Shao et al., 2019; Wilson et al., 2021). Characterization of individual variations influencing the abundance of *Bacteroides* spp. in the infant gut will contribute to the enhanced understanding of the physiological role of this key early life colonizer.

Most studies to date have described the dynamics of infant gut microbiota development through 16S rRNA gene amplicon sequencing approaches. When combined with unsupervised clustering approaches, these studies have provided important insights into temporal, ethnicity related, and age-specific development of microbiota trajectories as well as the stability, inferred functions, and maturation rates of community types (Stewart et al., 2018; Borewicz et al., 2019; Roswall et al., 2021; Tun et al., 2021; Xiao et al., 2021). Since amplicon-based studies restrict exploring the functional capacities of microbial communities, much less is known about the functional aspects of infant microbiota development. A recent Canadian study based on the well-characterized CHILD birth cohort used clustering approaches and conducted metagenomic imputation to provide novel insights into the potential role of infant's gut microbiota-mediated sphingolipid metabolism in food sensitization, linking *Bacteroides*-depleted microbiota clusters to higher odds of developing atopic sensitization in a host genotype-dependent manner (Tun et al., 2021). A recent meta-analysis of multiethnic infant microbiota enterotypes also characterized the functional aspects of microbiota maturation during the first years and

Abbreviations: VD, Vaginally delivered; CSD, Cesarean-section delivered; CS, Cesarean section; HMOs, Human milk oligosaccharides; IAP, Intra-partum antibiotic prophylaxis; HELMi, Health and Early Life Microbiota; RBB, Repeated bead beating; FDR, False discovery rate; PERMANOVA, Permutational multivariate analysis of variance; ANOVA, Analysis of variance; PCoA, Principal coordinate analysis; qPCR, Quantitative polymerase chain reaction.

showed that *Bacteroides*- and *Prevotella*-dominated infant enterotypes share enriched metabolic pathways, e.g., glycolysis and starch degradation (Xiao et al., 2021). Earlier metagenome studies have provided important insights into the temporal development of gut microbiota mainly in VD infants (Bäckhed et al., 2015; Stewart et al., 2018; Busi et al., 2021). However, the anticipated impact of delivery mode on the functional capacities of infant gut microbiota is still not fully understood. A recent metagenome study reported that the characteristic signature of low *Bacteroides* abundance in CSD infants led to the underrepresentation of several biosynthetic pathways at the age of 3 months (Wilson et al., 2021). Our study leverages the shotgun metagenomic sequencing approach to explore the dynamics of infant gut microbiota development. This study comprises samples spanning 3 weeks to 1 year of age in a group of 78 term infants from the well-characterized Finnish Health and Early Life Microbiota (HELMi) birth cohort (Korpela et al., 2019). Using a clustering approach, we defined three main microbiota development trajectories differing in both taxonomic and functional compositions. While the trajectories were strongly dependent on delivery mode, we identified a group of VD infants presenting depletion in *Bacteroides* spp. comparable to that in CSD infants. We further characterized this unusual microbiota composition for VD infants and investigated the underlying factors as well its impacts on the functional capabilities of the microbiota.

Materials and methods

Sample collection and sequencing

The HELMi birth cohort study ($N = 1{,}055$) is a prospective follow-up study on early life microbiota and health (Korpela et al., 2019). Healthy infants born on gestational weeks 37–42 without known congenital defects and exceeding the birth weight of 2.5 kg were included in the cohort. For this study, 90 infants representative of key early exposures (N = 25 born by CS, N = 24 VD with intrapartum antibiotics (IAP), and N = 41 VD without IAP) were selected from the HELMi cohort. The infants were selected from the broader HELMi cohort based on the number of samples available and their birth mode. Infants who had a long-term non-allergic disease reported by the age of 2 years were excluded. All CS deliveries involved IAP, and among the CSD infants, 14 were born by planned CS and 11 were by emergency CS. No restrictions in terms of maternal health status or maternal antibiotic treatment during the pregnancy period were applied in selecting the infants for this study.

Fecal samples were collected at the age of 3 weeks and 3, 6, and 12 months, and their mothers' samples were collected within 2 weeks prior to childbirth and were subjected to shotgun metagenome sequencing. The study was approved by the ethical committee of the Hospital District of Helsinki and Uusimaa and performed in accordance with the principles of the Helsinki Declaration. The parents signed informed consent for enrolment. They collected maternal and infant fecal samples and stored them at home in freezers at −20°C. The samples were transported frozen to the study center within 6 months of collection and were stored in freezers at −80°C upon arrival. Information on early life exposures was collected through online questionnaires and hospital records. The infants were generally healthy, although two were diagnosed with an allergic disease by the age of 1 year.

Fecal DNA was extracted from fecal material (0.026 to 2.46 g, median = 0.266 g) by suspension in 1 ml of sterile ice-cold PBS, and 250 μl of the fecal suspension was combined with 340 μl of RBB lysis buffer (500 mM NaCl, 50 mM Tris-HCl (pH 8), 50 mM EDTA, and 4% SDS) in a bead-beating tube from Ambion Magmax™ Total Nucleic Acid Isolation Kit (Life Technologies, Carlsbad, CA, United States). After repeat bead-beating, 200 μl of the supernatant was used for DNA extraction with a KingFisherTM Flex automated purification system (Thermo Fisher Scientific, Waltham, MA, United States) using MagMAXTM Pathogen High Vol. DNA was quantified using the Quanti-iT™ Pico Green dsDNA Assay (Invitrogen, San Diego, CA, United States). Sequencing libraries were prepared according to the Nextera DNA Flex Library Prep Reference Guide (v07) (Illumina, San Diego, CA, United States), with the exception that the reaction volumes were scaled down to ¼ of the protocol volumes. Sequencing was performed with an Illumina NovaSeq system using S4 flow cells with a lane divider (Illumina, San Diego, CA, United States) at the sequencing laboratory of the Institute for Molecular Medicine Finland (FIMM) Technology Center, University of Helsinki. Each pool was sequenced in a single lane. The read length for the paired-end run was 2 × 151 bp.

Quality control and human read filtering

Quality filtering and removal of human sequences were performed on Fastq files from NovaSeq using fastqc v0.11.9 and trimGalore v0.6.6 with default parameters. Quality-filtered sequences were screened to remove human sequences using bowtie2 v2.4.2 against a non-redundant version of the Genome Reference Consortium Human Build 38, patch release 7 (available at https://genome-idx.s3.amazonaws.com/bt/GRCh38_noalt_as.zip). After quality control and human read filtering, metagenomes at the early time points (3 and 12 weeks) containing <10 million paired-end reads were discarded based on the observed drop in species richness in shallow sequenced samples. For the later time points (6 and 12 months) and mother samples, metagenomes with <20 million paired-end reads were discarded. Infants with <3 samples remaining were excluded from the analysis. Overall, 12 infants ($N = 2$ born by CS, $N = 9$ VD delivery, and $N = 1$ VD with IAP) were excluded because

of low sequencing depth or insufficient number of samples. A total of 78 infant samples were kept for further analysis.

Taxonomic and functional profiling

Taxonomic profiling of the metagenomic samples was performed using Kraken2 (Wood et al., 2019) and Braken (Lu et al., 2017). Briefly, Kraken2 v2.1.1 was run on the paired read using the HumGut database (Hiseni et al., 2021), and Bracken v2.6.1 was run on Kraken2 outputs. Functional profiling was realized on the quality-controlled and human-filtered reads using HumaNn 3.0 (Beghini et al., 2021). The translated search was carried out using the UniRef90 database and hits were mapped to their corresponding Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthogroups (KOs). Gene family counts were normalized as count per million reads before the analysis. Gene counts were mapped to MetaCyc pathway definitions, and MinPath was used to calculate pathway abundances.

Alpha diversity measure (Shannon index), richness (Chao1 index), and beta diversity distances between samples (Bray-Curtis) for the taxonomic and functional profiles were estimated with the R packages *vegan* (Dixon, 2003) and *microbiome* (Lahti and Shetty, 2012-2019). The stability of bacterial composition during growth was explored by calculating the presence-absence Jaccard index for successive time points for each child. The Jaccard index was computed on the relative abundances at the genus level using the package *vegan*.

kmer-composition profiling

Simka v1.5.3 (Benoit et al., 2016) was run on metagenomic datasets with the following parameters: -abundance-min 2 -max-reads [MINCOUNT] -simple-dist, where [MINCOUNT] is the smallest sequence count across the analyzed samples. A Bray-Curtis distance computed on the content in the kmer of metagenomes was obtained and used for further analysis.

Hierarchical clustering of the samples based on taxonomic composition

The compositional analyses were performed using R (version 3.4.4). First, the taxonomy data were aggregated at the family level. Low-abundance families (<1% relative abundance and <0.5% prevalence) were filtered out, and zero values were replaced by an estimate of 1. Then, the dataset was normalized by taking the centered log ratio (CLR), and a distance matrix was calculated from the transformed data using the Euclidean distance (Aitchison distance) (Aitchison et al., 2000; Tsilimigras and Fodor, 2016). Hierarchical clustering was carried out separately for each time point with the function *hclust* and with the Wald.D2 method. To determine the appropriate number of clusters, the silhouette coefficient was calculated and the number of clusters allowing the highest silhouette coefficient was selected. The significance of the obtained clusters was confirmed by PERMANOVA at the family level using the Aitchinson distance. The association of the sample clusters to exposures such as intrapartum and postnatal antibiotics was assessed by Fisher's exact test and was not found to be significant at any time point.

Defining VD high- and low-*Bacteroides* groups

The VD infants were grouped as "*Bacteroides*-present VD" and "*Bacteroides*-depleted VD" according to the relative abundance of the *Bacteroides* genus in the samples collected at 3 weeks. A cut-off of 1% relative abundance at the genus-level was used to define the groups. The 1% cut-off was chosen as all the CS infants had a relative abundance of *Bacteroides* below this threshold. The association of the infant groups with IAP and postnatal antibiotic exposure was assessed by Fisher's exact test and was found to be non-significant.

MaAsLin2 differential abundance analysis

To assess the associations between infant microbiota clusters at each time point and MetaCyc pathway abundances, pathways with at least 0.0001% relative abundance in at least 10% of the samples were selected. The associations between pathways and clusters were determined with generalized linear and mixed models, using Multivariate Association with Linear Models (MaAsLin2) (Mallick et al., 2021). Any pathway association with an FDR- adjusted p-value < 0.1 was considered statistically significant.

Associations between maternal microbiota composition and the grouping of VD infants according to their relative abundance of *Bacteroides* were assessed using the Negative Binomial model from the MaAsLin2 package (-m NEGBIN parameter). Taxonomic profiles were aggregated at the species level, low abundance hits were filtered out (detection below 0.001% and prevalence below 1%), and counts were normalized by TMM (Trimmed Mean by M-Values). The results obtained through negative binomial models were confirmed using generalized linear models from the MaAsLin2 package. Any species association with an FDR-adjusted p-value < 0.1 was considered statistically significant.

Association between infant microbiota and environmental variables

A directed acyclic graph (DAG) was built detailing causal relationships affecting the association between perinatal and postnatal exposures and infant gut microbiota development (Supplementary Figure 1). Nodes represent the exposure of interest, and each node is interrelated by directional arrows that represent theoretical associations based on the researchers' assessment of previous literature and confirmed by association testing in the HELMi cohort. From this representation, we assessed that birth mode is a potential confounder of the effect of perinatal variables (intrapartum antibiotics, gestational age, time of first skin-to-skin contact, and number of previous deliveries) and breastfeeding variables (length of breastfeeding time, length of exclusive breastfeeding, and age of first solids). Tests of associations between these variables and the infant gut microbiota were therefore controlled for birth mode effects.

For testing the association between infant microbiota trajectories and environmental variables (Results Section Taxonomic and functional features of the birth mode-driven infant microbiota clusters) as well as between the VD *Bacteroides* infant group and environmental variables (Results Section CS-like *Bacteroides* depletion in early life microbiota can be observed in a subset of VD infants), a large number of tests were carried out in an attempt to identify any variables that may be significantly associated to a group of samples. An FDR correction was applied to account for the increased risk of Type I error in multiple testing settings, and FDR adjusted p-values <0.1 were considered statistically significant.

A *post-hoc* sensitivity analysis was conducted with the G*Power software version 3.1.9.2 to retrospectively examine the observed power of the study. The effect size required for a power level of 0.8 was calculated with the number of samples used at the significance level ($p = 0.05$) for the Wilcoxon–Mann–Whitney test.

Other statistical analysis

The statistical analysis was conducted using R (version 3.4.4) with the packages *vegan* and *microbiome* (Dixon, 2003; Lahti and Shetty, 2012-2019). Alluvial diagrams were constructed with the R package ggalluvial (Brunson, 2020).

For univariate data comparison, a statistically significant difference was evaluated by the unpaired Wilcoxon test when applied to numeric variables or the Fisher test when comparing categorical groups, and p-values <0.05 were considered statistically significant. Additionally, when a pair-wise Wilcoxon test was carried out on more than two groups of samples, an FDR correction was applied, and FDR adjusted p-values <0.05 were considered statistically significant.

Results

Cohort overview

The present study included 78 infants from the HELMi cohort [NCT03996304 (Korpela et al., 2019)], born in Finland at hospitals at term, and followed up for 1 year. A total of 55 infants were born *via* VD. All the 23 CS deliveries and 23 VDs involved IAP. Baseline characteristics and antibiotic exposures over the first year of life for all 78 infants, stratified by mode of delivery and IAP exposure, are summarized in Supplementary Table 1. Perinatal and background variables were evenly distributed over the three birth groups except for two environmental exposures (siblings and pets at home) and antibiotic exposures during the first year of life (Supplementary Table 1). All the children were breastfed during the first months of life. Most of the children were breastfed for more than 9 months, and only 8 were breastfed for 6 months or less. The introduction of solid food took place between 18 and 31 weeks of age. Seventeen (22%) children received at least one antibiotic treatment during their first year of life, and some received multiple courses (30 courses in total for all the children) (Supplementary Table 1). Infants' gut microbiota was assessed by shotgun metagenomic sequencing of fecal samples collected at 3 weeks ($n = 75$) and 3 months ($n = 77$), 6 months ($n = 71$), and 1 year (n=57). Each infant was sampled a minimum of 3 times, with 60% of the infants ($n = 46$) having all 4 samples. For 50 infants, metagenomic data were also available from their mother's stool samples taken during the last 2 weeks before delivery.

Microbiota maturation over the first year of life

After quality filtering and removing human sequences, the metagenomes had an average of 39 million paired-end reads per sample. These high-quality, human-filtered reads were subjected to taxonomic annotation with Kraken2 (Wood et al., 2019) using the HumGut database (Hiseni et al., 2021). The taxonomic assignment obtained with Kraken2 was recalculated using Braken (Lu et al., 2017). This method has been previously shown to achieve high taxonomic precision for gut taxonomic profiling (Tamames et al., 2019; Ye et al., 2019; Allnutt et al., 2021). On average, 91% of the total reads at the phylum level and 77% at the family level were successfully annotated. In the infant samples, the most abundant bacterial families were *Enterobacteriaceae* (27% global average relative abundance), *Bifidobacteriaceae* (22%), *Bacteroidaceae* (15%), *Lachnospiraceae* (7%), *Veillonellaceae* (4%), *Clostridiaceae* (4%), *Oscillospiraceae* (4%), and *Streptococcaceae* (1%). Other families represented in total 14% of the sequences (Supplementary Figure 2). The infants' microbiota maturation was characterized by a global decrease in interindividual beta diversity and convergence

toward an adult-like profile (Figure 1A) and had a progressive increase in *Lachnospiraceae* and *Oscillospiraceae* during the first 12 months (Supplementary Figure 2). However, at 1 year, the infants' gut microbiota composition was still significantly different from that of the mothers' microbiota (PERMANOVA on genus-level Aitchinson distance, $p = 0.001$; 999 permutations). The alpha diversity (Shannon diversity index) did not significantly increase between 3 weeks and 3 months but significantly increased between 3 and 6 months (Wilcoxon $p < 0.05$) (Figure 1B), coinciding with the age when solid foods were introduced to the children's diet (median = 22 weeks in the cohort). Significant differences in alpha diversity were also observed between 6 and 12 months, and between 12 months and adult microbiota. Similarly, species richness (Chao1 index) increased during infancy, especially between 6 and 12 months, but was significantly higher in the adult samples than in the 12-month samples (Wilcoxon $p < 0.05$) (Figure 1B). The results were similar after rarefying the samples to the same sequencing depth (data not shown) (Bäckhed et al., 2015).

We also analyzed gut microbiota maturation using an annotation-free approach that computes distances between microbial communities directly based on the number and abundance of shared words of length k (k-mers) (Benoit et al., 2016). Using this approach, we observed a global maturation of the infant samples toward an adult-like sequence composition (Supplementary Figure 3A) and a significant decrease in the inter-individual beta diversity during growth. However, no interindividual beta diversity decrease was observed between the 12-month samples and the mother samples (Supplemental Figure 3B). This could be explained by the increased heterogeneity in subspecies diversity in the adult samples.

Next, we explored how the maturation of the microbiota affected the microbial metabolic and functional pathways. KEGG Orthogroup (KO) counts were obtained for all the samples and mapped to the main KEGG cellular metabolic pathways. KOs involved in amino acid metabolism, carbohydrate metabolism, and energy metabolism were found to be the most abundant at all infant ages and in the mother samples (Supplementary Figure 4B). Similar to the taxonomic maturation, we observed functional maturation of the infant microbiota, with the infant samples slowly converging toward an adult-like composition during the first year of life (Figure 1C). The functional alpha diversity (Shannon diversity index on KO counts) was globally stable during the first year of life and comparable between the infant and mother samples. On the other hand, the gene richness (Chao1 index on KO counts) significantly increased in infants between 3 and 6 months but was surprisingly found to be lower in the mother samples (Figure 1D). To control for potential biases in the functional annotation as an explanation for the decreasing trend of gene richness, we looked at the proportion of sequences left out of functional annotation. Indeed, the proportion of sequences unmapped to a gene family increased significantly between 6 and 12 months in the infants and was highest in the mother samples (Supplementary Figure 4A), suggesting increased complexity of the microbiome in late infancy and further in adulthood.

Taxonomic and functional features of the birth mode-driven infant microbiota clusters

To identify the main microbiota establishment trajectories, we clustered the infant samples on their taxonomic composition at the family level for each sampling time point. We performed hierarchical clustering of the samples using the ward linkage on the Aitchinson distance. The bacterial communities clustered into two groups at each sampling time point (Figure 2). The clusters were confirmed by PERMANOVA and showed that the composition of the samples was significantly different between clusters at the family level at each time point (PERMANOVA on family-level Aitchinson distance, $p = 0.001$; 999 permutations). However, the alpha diversity was not significantly different between the clusters, and their taxonomic richness was significantly distinct between the clusters only in the 3-week and 12-month samples (Wilcoxon $p < 0.05$) (Supplementary Figure 5), suggesting that the identified clusters differed in terms of composition but not in terms of taxonomic complexity. The PCA bi-plot for the clusters revealed that the clustering was driven at all time points by the abundance of the *Bacteroidaceae*, *Tannerallaceae, Rikennellaceae*, and *Ordoribacteriaceae* families in the first cluster, and the presence of *Clostridiaceae* in the second (Supplementary Figure 6). The cluster membership was strongly associated with the birth mode at all time points (Fisher's exact test $p < 0.05$) but was not associated with IAP exposure (Fisher's exact test $p > 0.05$ between VD and VD + IAP infants) or postnatal antibiotic exposure (Fisher's exact test $p > 0.05$).

We next compared the identified clusters in terms of their functional composition. At all the time points, the clusters had a significantly different functional composition (PERMANOVA on KO Bray-Curtis distance, $p = 0.001$; 999 permutations). The KO alpha diversity (Shannon index) was not significantly distinct between the clusters at any sampling time point; however, the KO richness (Chao1 index) was found to be significantly lower in cluster 2 at 3 weeks (cluster 3w-2) (Supplementary Figure 5). To provide an overview of the variability patterns of microbiota functions between the clusters, we mapped the gene families to 401 prokaryotic MetaCyc pathways and identified differentially abundant pathways between the clusters for each time point. At 3 weeks, 49 pathways were identified as significantly differentially abundant in the two clusters; at 3 months, 55 pathways; at 6 months,

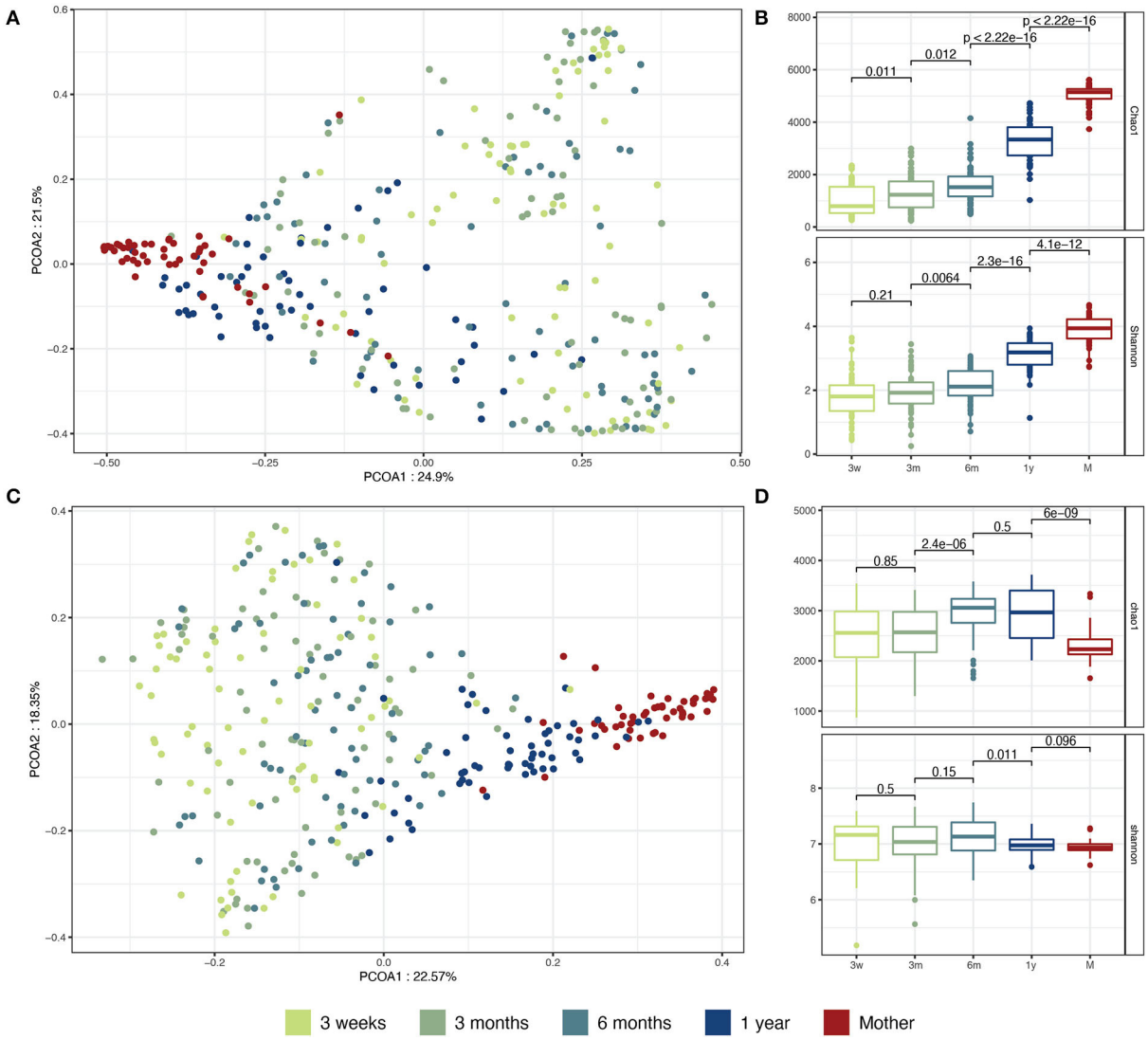

FIGURE 1
Taxonomic and functional maturation of infant microbiota during the first year of life. **(A)** PCoA on genus level taxonomic profiles. Taxonomic profiles were aggregated at the genus level. The beta diversity Bray-Curtis distance was computed between sample pairs and plotted as a PCoA. **(B)** Taxonomic alpha diversity and richness. Taxonomic profiles were aggregated at the species level. The Shannon alpha-diversity index and the Chao1 richness index were computed for each sample. **(C)** PCoA on KO functional profiles. The KO profiles were normalized as count per million reads. The beta diversity Bray-Curtis distance was computed between each sample pair and plotted as a PCoA. **(D)** Functional alpha diversity and richness. The KO profiles were normalized as previously described. The Shannon alpha-diversity index and the Chao1 richness index were computed for each sample. The comparisons between groups were conducted by unpaired Wilcoxon test.

73 pathways and at 12 months, 120 pathways were found significantly differentially abundant between the clusters (linear model, $q < 0.1$). Across all the time points, in total, 68 unique pathways were enriched in clusters 3w-2, 3m-2, 6m-2, and 1y-2, in particular pathways involved in nucleotide biosynthesis (18% of the differentially abundant pathways), carbohydrate biosynthesis (13%), and cofactor biosynthesis (10%). On the other hand, 124 pathways were enriched in clusters 3w-1, 3m-1, 6m-1, and 1y-1, in particular pathways involved in amino acid biosynthesis (15%), cofactor biosynthesis (14%), and carboxylate degradation (8%) (Supplementary File 1). Pathways involved in carbohydrate degradation were consistently differentially abundant between the two clusters at all the time points and were mostly associated with *Bacteroidaceae*-driven cluster 1, especially in the early time points (Figure 2). Interestingly, pathways involved in the degradation of beta-mannan, a plant-based dietary polysaccharide, were consistently enriched in this cluster throughout the first year and already before

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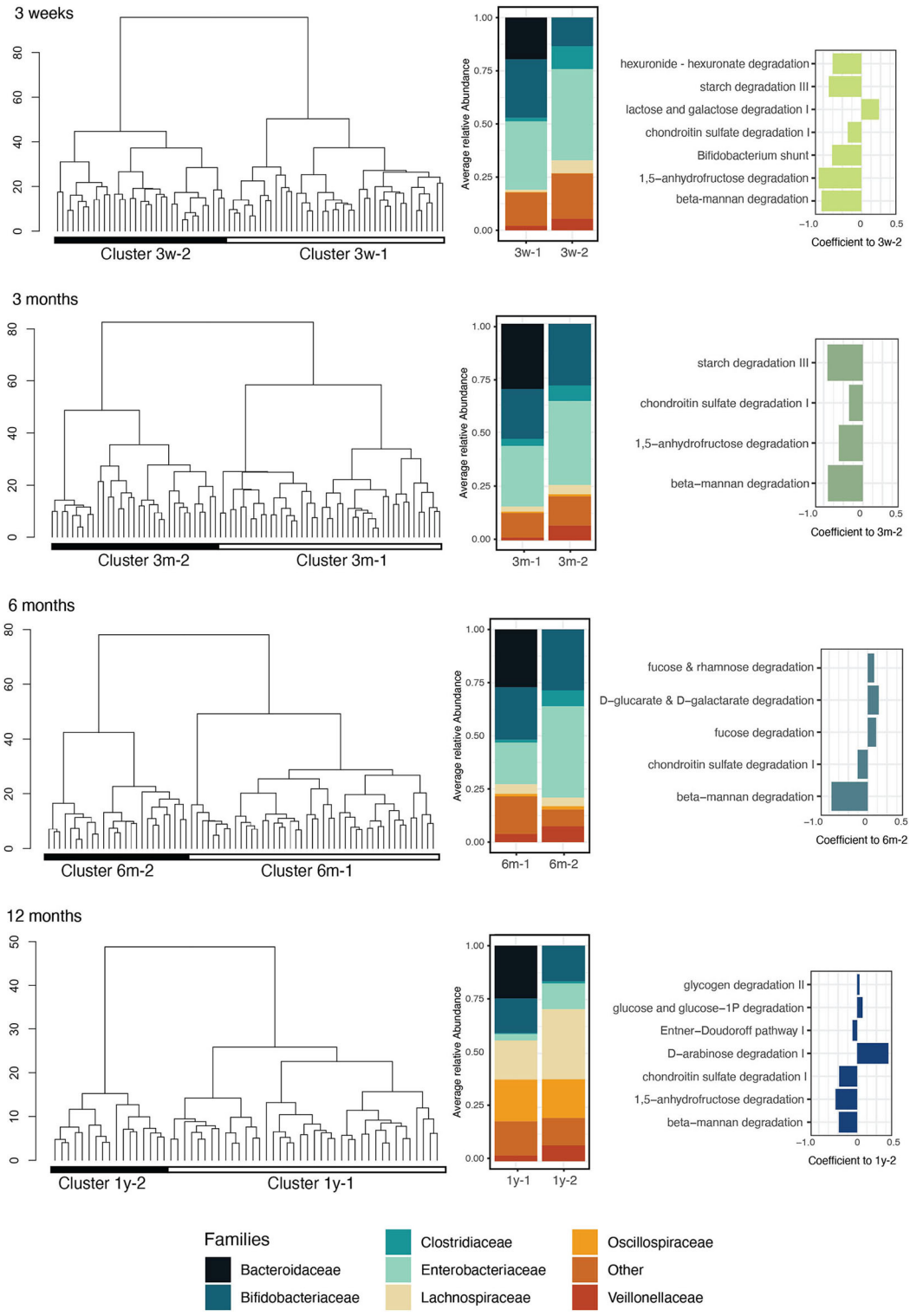

FIGURE 2
Infant gut microbiota clusters, representative bacterial families, and enriched functions related to carbohydrate metabolism. At each sampling time point (3 weeks, 3 months, 6 months, and 1 year), the infants' samples were clustered by hierarchical clustering on taxonomic profiles aggregated at the family level using the Aitchison distance (Column 1). The color blocks below the dendrograms represent the clusters identified using the silhouette maximization method (Column 2). The average relative abundance of the families in each cluster was represented in the associated bar plots. Families with detection and prevalence below 10% were grouped into the "Other" category (Column 3). Bar plot showing the coefficients of differentially abundant carbohydrate metabolism-related pathways (MaAsLin2, linear model, $q < 0.1$) in the two identified clusters. Negative coefficients indicate association to cluster 1.

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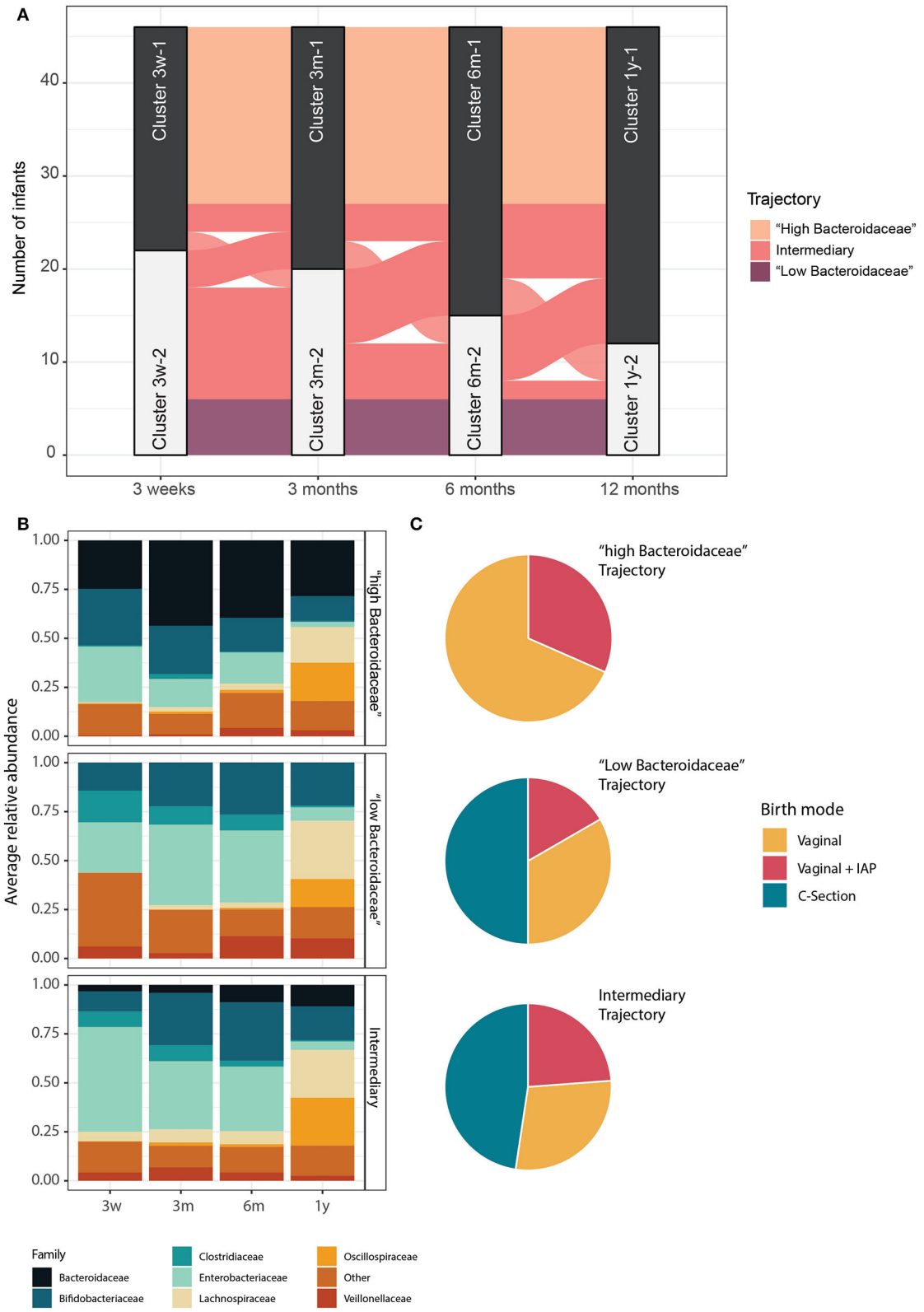

FIGURE 3
Three major microbiota development trajectories identified. **(A)** Alluvial plot showing the evolution of infants' microbiota trajectories through the first year of life, grouped according to the microbiota clusters identified at each time point. Groups of infants are colored according to the type of their trajectories. **(B)** Average relative abundance of bacterial families for infants within the same trajectory. Families with below 10% of relative abundance and prevalence are summed and grouped as "Other." **(C)** The proportion of infants born by CS and VD infants with or without IAP in each trajectory.

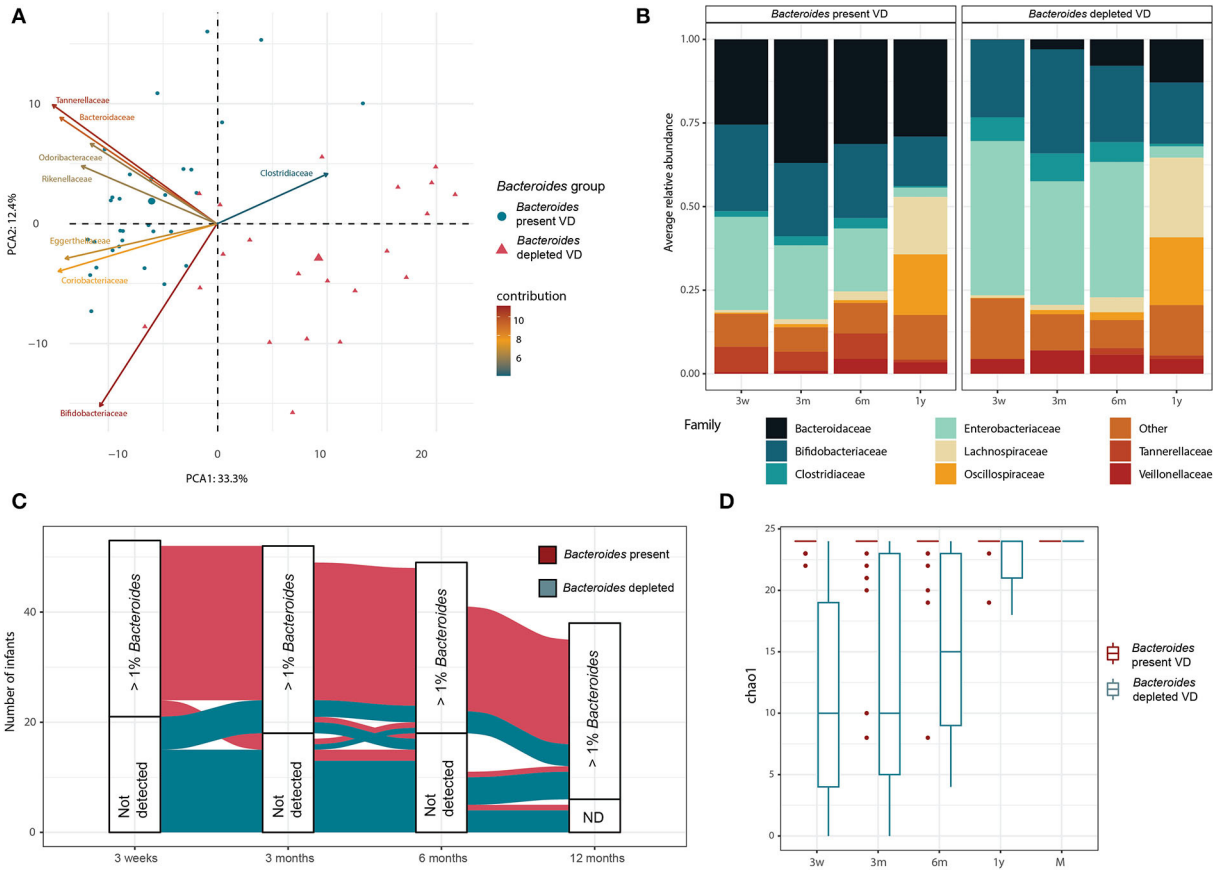

FIGURE 4
Depletion of genus *Bacteroides* in a subset of VD infants. **(A)** PCA biplot based on family-level taxonomic profiles for VD infants at 3 weeks of age. Taxonomic profiles obtained for the 3-week samples were aggregated at the family level, and low abundance taxa were excluded before computing an Aitchinson distance between samples. Samples in the PCA biplot are colored as per the groups based on relative abundance of *Bacteroides* genus. Bacterial families contributing most to the observed variation were plotted as arrows on the biplot. **(B)** Average microbiota composition for infants grouped by the abundance of *Bacteroides* at 3 weeks. Taxonomic profiles are aggregated at the family level, and families with below 10% of relative abundance and prevalence are summed and grouped as "Other." **(C)** Alluvial plot showing the evolution over the first year of life of infants according to the relative abundance of *Bacteroides*. A cutoff of 1% relative abundance of *Bacteroides* was chosen to group the samples, and the infants are colored by the relative abundance of *Bacteroides* at 3 weeks. **(D)** The taxonomic richness of the *Bacteroides* species (Chao1 index) in the infants and their respective mothers according to *Bacteroides* relative abundance at 3 weeks.

weaning age (Figure 2). The complete list of identified pathways and their differences between the clusters are listed in Supplementary File 1.

We next investigated the temporal stability of the identified clusters in individual infants. For this, we focused on a subset of 46 infants for which all four samples were available. Interestingly, 54% of infants belonging to one of the two clusters, either *Bacteroidaceae*- or *Clostridiaceae*-driven cluster at 3 weeks, stayed in the same cluster throughout all the time points, suggesting that the initial microbiota composition after birth has a critical effect on future microbiota developmental trajectory until 1 year (Figure 3A). Consequently, two stable microbiota development trajectories and an unstable trajectory emerged. One microbiota developmental trajectory was followed by 19 (41%) infants, all VD, and was characterized by a high relative abundance of the family *Bacteroidaceae* (mean $>$ 25% at all time points, IQR = 36%). Another microbiota trajectory was followed by 6 (13%) infants, with 50% CS infants and 50% VD infants, and was characterized by a virtual lack of *Bacteroidaceae* at all the time points (mean $<$ 0.05% at all time points, IQR = 2%) and higher relative abundance of *Clostridiaceae*. Finally, the temporally instable intermediary trajectory was followed by 21 (46%) infants, with 48% CS infants and 52% VD infants (Figures 3B,C). The relative abundance of *Bacteroidaceae* was significantly different between all the trajectories, and *Clostridiaceae* was significantly different in the second and third trajectories compared to the first one (Pair-wise Wilcoxon, FDR-adjusted, $p < 0.05$). Interestingly, the abundance of *Bifidobacteriaceae*, *Enterobacteriaceae*, *Lachnospiraceae*, *Oscillospiraceae*, and *Veillonellaceae* did not

differ consistently between the trajectories (Pair-wise Wilcoxon, FDR-adjusted, $p > 0.05$). Additionally, the alpha diversity (Shannon diversity index) was similar for all the trajectories at all the time points (Pair-wise Wilcoxon, FDR-adjusted, $p > 0.05$, on Shannon diversity index). We compared the trajectories in terms of stability of bacterial composition by calculating the presence-absence Jaccard index of the genus-level composition for successive time points for each child. As expected, the "intermediary" trajectory presented a less stable composition over time, with successive communities for each infant being more dissimilar than for the first and second trajectory (pair-wise Wilcoxon, FDR-adjusted, $p < 0.05$ on presence/absence Jaccard distance). The trajectories were strongly associated with delivery mode (Fisher test, FDR-adjusted, $p < 0.1$) but not with IAP exposure (Fisher test, FDR-adjusted, $p > 0.1$ between VD and VD + IAP infants). The trajectories were also not significantly associated with the number of antibiotic courses received by the infants during the first year of life (Fisher's exact test, FDR-adjusted, $p > 0.1$). Additionally, starting age of solid food and breastfeeding habits (exclusive breastfeeding and breastfeeding period length) were not significantly different between the three trajectories (Fisher's exact test, FDR-adjusted, $p > 0.1$). All the comparisons between trajectories are available in Supplementary File 5.

CS-like *Bacteroides* depletion in early life microbiota can be observed in a subset of VD infants

We further investigated the abundance and dynamics of the genus *Bacteroides* that was driving the clustering and trajectories. At 3 weeks, the microbiota of all the CSD infants contained a low relative abundance of the genus *Bacteroides* (mean = 0.2% relative abundance, IQR = 0.2%). At 3 weeks, we had 53 VD infants as opposed to 55, since samples from two infants at this time point were not available. Strikingly, roughly 40% ($n = 21$) of the VD infants ($n = 10$ in group VD with IAP, $n = 11$ in group VD without IAP) presented an equivalently low relative abundance (below 1%) of the genus *Bacteroides* at 3 weeks. To explore this unusual microbiota composition for the VD infants, we defined the two groups of VD infants according to the relative abundance of the genus *Bacteroides* at the first sampling point, i.e., at the age of 3 weeks. A group of 21 infants, called hereafter "*Bacteroides*-depleted VD" had a <1% relative abundance of *Bacteroides* in their microbiota. The rest of the VD infants ($n = 32$) were called "*Bacteroides*-present VD." The "*Bacteroides* VD" groups were consistent with the trajectories defined previously, with 90% of the "*Bacteroides*-present VD" infants following the first trajectory and 92% of the "*Bacteroides*-depleted VD" infants following the second or intermediary trajectory (Supplementary File 6). The global bacterial composition of the two groups was significantly distinct (PERMANOVA on family-level Aitchinson distance, $p = 0.001$; 999 permutations) at 3 weeks (Figure 4A). The two groups were also distinct in terms of their functional composition (PERMANOVA on KO Bray-Curtis distance, $p = 0.001$; 999 permutations). Altogether, 62 MetaCyc prokaryotic pathways were significantly differentially abundant in the two VD infant groups at 3 weeks (linear model, $q < 0.1$), in a large part, overlapping with the pathways identified as differentially abundant in clusters 3w-1 and 3w-2 (Supplementary File 2).

When comparing the microbiota developmental trajectories of the two groups of VD infants, the "*Bacteroides*-depleted VD" infants showed a gradual acquisition of *Bacteroides* during the first year of life (Figure 4B). Yet, while strongly depleted in *Bacteroides* at 3 weeks, 75% of the "*Bacteroides*-depleted VD" infants had a *Bacteroides* relative abundance above 1% at 12 months (average 13%). On the other hand, only three infants from the "*Bacteroides*-present group" showed a loss in *Bacteroides* relative abundance of <1% at any time point, while the remaining infants for that group had a *Bacteroides* relative abundance above 1% at all the sampling time points (Figure 4C). Globally, the richness of the *Bacteroides* species increased during growth, while the "*Bacteroides*-depleted VD" infant group had significantly lower *Bacteroides* richness at all the time points (Wilcoxon $p < 0.05$) (Figure 4D). A total of 24 species of *Bacteroides* were detected in the dataset, with *B. fragilis*, *B. dorei*, and *B. vulgatus* being the most abundant. However, no *Bacteroides* species were found to be present in one only infant group, indicating differences in the abundances of *Bacteroides* species but not in their prevalence (Supplementary Figure 7). Taken together, these results suggest that the *Bacteroides* depletion observed in the "*Bacteroides*-depleted VD" infant group at 3 weeks is general rather than a lack of specific species of *Bacteroides*, and has a long-term effect that impacts the microbiota development of the infants during the first year of life.

To explore the potential reasons for the depletion of *Bacteroides* in VD infants, we compared the prenatal and perinatal variables between the "*Bacteroides*-present VD" and "*Bacteroides*-depleted VD" infants. We first explored birth-related differences between the two groups, such as potential IAP exposure, intensive care, the time between water breakage and delivery, gestational age, and birth weight. None of these variables were significantly associated with the two infant groups. Next, we explored maternal variables such as the mother's age, maternal health during pregnancy, exposure to antibiotics or probiotics during pregnancy, number of prior pregnancies, and pre-pregnancy BMI. Once more, none of these variables were significantly associated with the two infant groups. We also tested against group membership the antibiotics received by the children in the first 3 weeks of life and breastfeeding habits; but once again, no significant association could be found. A complete list of the variables and their

definition, and the result of the statistical comparison are available in Supplementary File 3.

We next compared the composition of the maternal gut microbiota in the samples collected prior to delivery for the two VD groups (Supplementary Figure 8), and in particular in the *Bacteroides* species (Supplementary Figure 7). Overall, no differences were observed between the microbiota composition of the mothers of "*Bacteroides*-present VD" and "*Bacteroides*-depleted VD" infants at the family level (PERMANOVA on family-level Aitchinson distance, $p = 0.26$; 999 permutations), and we could not identify any significantly differentially abundant taxa between the two groups of mothers using MaAsLin2 at the family and species levels (negative binomial, $q > 0.1$). In order to rule out any effect of technical variations, we explored the association between the two groups of infants with the length of sample storage, the person performing DNA extraction, DNA extraction yield, and sequencing depth. Interestingly, DNA yield (ng of DNA per gram of feces) was significantly higher in the "*Bacteroides*-present VD" than in the "*Bacteroides*-depleted VD" (FDR-adjusted $p < 0.1$, Wilcoxon test). Similarly, the DNA yield obtained from 3 weeks samples of CSD infants was significantly lower (median 1,762 ng/g of feces) compared to the "*Bacteroides*-present VD" (median 5,495 ng/g of feces) but was similar to the "*Bacteroides*-depleted VD" samples (median 1,575 ng/g of feces, FDR-adjusted $p < 0.1$ Wilcoxon test). Importantly, the lack of significant associations between birth and maternal variables and the "*Bacteroides*-depleted VD" group could be due to our limited cohort sampling size. Indeed, our study was powered to only reliably detect large effect sizes (d = 0.82 for the power of 0.8 by the Wilcoxon test).

Discussion

Birth mode is a major factor influencing the development of infant gut microbiota and has anticipated effects on infants' immune systems and long-term health (Mueller et al., 2015; Korpela, 2021). More specifically, CSD infants have a characteristically reduced colonization and abundance of *Bacteroides* spp. (Dominguez-Bello et al., 2010; Bokulich et al., 2016; Wampach et al., 2018; Guittar et al., 2019; Reyman et al., 2019; Busi et al., 2021). In this study, we leveraged the shotgun metagenomics approach on 78 infants from a well-characterized longitudinal birth cohort followed up from 3 weeks to 1 year of age. Our results recapitulate the previous findings that show a strong influence of birth mode on infant microbiota acquisition. Apart from comparing the infant microbiota composition and function based on birth groups, we zoomed into data-driven infant microbiota clusters and trajectories. Our results show a strong depletion of genus *Bacteroides* in 40% of the VD infants. This study expands our understanding of the impact of various early life factors on the colonization and dynamics of *Bacteroides* spp. in infants.

As previously reported in several longitudinal infant cohorts, we observed the maturation of the infant gut microbiota over the first year of life in terms of taxonomic composition and diversity, with an increase in taxonomic alpha diversity and richness especially observed around the age of weaning (Bäckhed et al., 2015; Beller et al., 2021). Consistent with previous studies (Yatsunenko et al., 2012; Bäckhed et al., 2015), the compositional microbiota maturation during the first year of life resulted in reduced beta diversity and global convergence in the gene content of the infant gut microbiota toward an adult-like composition. At the age of 1 year, the taxonomic composition of the infant gut microbiota was still distinct from that of the adult gut microbiota. Interestingly and contrary to the taxonomic maturation, the functional microbiota maturation was not characterized by an increase in gene family richness. This result also observed in previous studies (Yatsunenko et al., 2012; Wang et al., 2021), is likely due to a smaller fraction of sequences with assignable KEGG annotations, in particular in the adult samples, as a result of increased complexity. We did not observe any significant differences in the abundance of broad functional categories between the infant and mother samples or in the infant samples across the different time points. As reported previously (Wang et al., 2021), unlike taxonomic signatures, there is a high similarity between the microbial metabolic functions between infants and adults, underlining the importance of conserved core microbial functions.

We used a clustering approach to identify the main microbiota community type characteristics for each sampling time point. We applied hierarchical clustering on the Aitchinson distance, which allows us to take into account the compositionality of microbiome data and reduces effects due to sequencing depth differences between samples (Galloway-Pena et al., 2017). This method allowed us to identify two main infant microbiota clusters at each time point, driven in part by the relative abundance of the *Bacteroidaceae* family. Using cut-offs different from those in our study, Eck et al. reported comparable patterns, in particular describing two distinct infant gut microbiota settler types based on a cut-off of 30% of relative abundance of *Bacteroidetes* (Eck et al., 2020). A large meta-analysis covering > 10,000 microbiota samples from 17 countries and derived from children sampled between birth and 3 years identified four robust infant enterotypes typified by *Firmicutes*, *Bifidobacterium*, *Bacteroides*, and *Prevotella* (Xiao et al., 2021). While age was the strongest predictor of enterotype and all enterotypes included children from both birth modes, interestingly, the *Bacteroides*-dominated enterotype was detected in Northern European countries such as Finland, Norway, and Estonia in most sampling months. Hence, our results on Finnish infants may not be generalized across populations. In any case, many previous studies using the clustering approach have also reported the importance of *Bacteroidaceae* in infant or child gut microbiota datasets and its associations to geography, breastfeeding duration, butyrate

synthesis, birth mode, human milk oligosaccharide (HMO) metabolizing capacity, orthogonal or collateral relationship with clusters driven by other taxa, and developmental stages (Yatsunenko et al., 2012; Stewart et al., 2018; Zhong et al., 2019; Berger et al., 2020; Casaburi et al., 2021; Roswall et al., 2021). Importantly, we observed the infant microbiota clusters to be associated with differences in several functional pathways, including pathways implicated in carbohydrate metabolism. In particular, degradation pathways of two glycans, β-mannans, and chondroitin sulfate were significantly associated with the *Bacteroidaceae*-rich cluster at all the time points. Chondroitin sulfate is a glycosaminoglycan prevalent in human milk and is associated with anti-inflammatory properties and protective effects in neonates (Knowles et al., 2021); it can be degraded by *Bacteroides* species (Shang et al., 2016). β-Mannans are complex plant-based sugars widespread in the human diet and can be degraded and used by *Bacteroides* species apart from some *Firmicutes* species (la Rosa et al., 2019). Overall, the results suggest that depletion in *Bacteroidaceae* contributes to a significant alteration in the ability of an infant's microbiota to metabolize complex sugars (Marcobal et al., 2011; Bäckhed et al., 2015; Casaburi et al., 2021). Our results expand on previous reports that have addressed the functional differences of infant gut microbiota in relation to CS birth and reduced abundance of *Bacteroides*. A previous study has reported that during the first 3 months of life, CSD infants have underrepresented biosynthetic pathways and that the vast majority of which (14/20) could be assigned to *Bacteroides* spp. (Wilson et al., 2021). A recent meta-analysis on infant metagenomes reported functions such as starch degradation, glycolysis, and queuosine biosynthesis to be enriched in a *Bacteroides*-dominated community type, which comprised 90% of VD infants (Xiao et al., 2021). In a recent randomized controlled trial, functional differences between CSD and VD infants were investigated on days 7 and 27, and the results indicated that 133 and 663 functional genes differed between the two birth groups, respectively (T. Dierikx et al., 2022). Wang et al. have also reported differences between metabolic functions in relation to birth mode, with functions such as vitamin, sugar, and cell wall biosynthesis higher in VD infants (Wang et al., 2021).

Strikingly, considering the infants that had samples available at all the time points, 54% of the infants clustering together at 3 weeks clustered together throughout all the sampling time points. This indicates the lasting influence of the pioneering community assemblance and aligns with previous reports about deterministic and largely predictable transition patterns between infant gut microbiota community types (Stewart et al., 2018; Xiao et al., 2021). The fact that more than half of the infants retained their characteristic high or low abundance of *Bacteroidaceae* allowed us to define three infant microbiota trajectories strongly associated with the birth mode. As expected, the "high *Bacteroidaceae* trajectory" was only observed in the VD infants, while the "low *Bacteroidaceae* trajectory" and "intermediary trajectory," characterized by complete or milder depletion in *Bacteroidaceae* until 1 year of age, respectively, were observed not only in the CS born infants but also in the VD infants. While this phenomenon has not been well-discussed in the scientific literature, we are not the first to report it. Our results confirm previous observations that *Bacteroides* depletion is not only present in CS-born infants but can also be observed in 20–49% of VD infants (Yassour et al., 2016; Shao et al., 2019; Wilson et al., 2021). We further explored the plausible causes of *Bacteroides* depletion in VD infants. The depletion was prolonged and was only partially resolved after 1 year. While many studies (e.g., Shao et al., 2019) and a systematic review (T. H. Dierikx et al., 2020) have documented that intrapartum antibiotic exposures lead to depletion of *Bacteroides* in VD infants, we did not confirm such an association in our cohort. Similarly, Stearns et al. (2017) did not find IAP to decrease the relative abundance of *Bacteroides* among VD infants at 12 weeks, and a clinical trial where no VD infants were exposed to IAP found that 20% of their VD samples had a low *Bacteroides* signature (Wilson et al., 2021). Also, Yassour et al. observed a similar *Bacteroides* depletion profile in seven Finnish VD infants; however, with such a small subset of infants, they were unable to search for significant correlations with any clinical variables such as IAP (Yassour et al., 2016). Our results from the same cohort using absolute abundance estimates from 16S rRNA gene amplicon data (Jokela et al., In Press), as well as earlier targeted qPCR studies, identified no statistically significant differences in the absolute abundance of the *Bacteroides fragilis* group in VD infants in relation to IAP exposure (Aloisio et al., 2014; Corvaglia et al., 2016). In this study, the incidental courses of postnatal antibiotics did not explain the microbiota clustering or trajectories at any time point.

Despite extensive exposure and other metadata available for the study infants, we were not able to associate the differences in the relative abundance of *Bacteroides* in VD infants to any prenatal variable such as the mother's pre-pregnancy BMI or parity, or to perinatal and postnatal factors such as gestational age, stay in intensive care, time between water break and delivery, and infant weight at birth. Importantly, all the infants in our study were partially or exclusively breastfed until 3 months, with the majority of infants breastfed until 6 months, and no association was found between breastfeeding habits and the *Bacteroides* depletion observed. Similarly, an earlier study found *Bacteroides* depletion within the first 3months of life not to be related to feeding mode or antibiotic exposure after birth (Wilson et al., 2021). We also comprehensively explored the possible effect of technical variations such as time of storage of samples before processing, extraction date, and sequencing run. Interestingly, we found DNA extraction yield per gram of feces to be significantly reduced in the *Bacteroides*-depleted VD infants. While the relative abundance of this genus is known to be sensitive to sample storage and DNA extraction methods (Salonen et al., 2010; Bahl et al., 2012; Rinninella et al., 2019;

Zhang et al., 2021), in our study, all the samples were frozen without delays with stabilization buffers and were extracted using the same DNA extraction protocol. Also, the sequencing libraries were normalized for the input DNA. Hence, unlike across different studies, we believe that within this study, the link between DNA yield and community composition is a biological effect rather than a technical effect. As in the fecal samples, the majority of the extracted DNA is from bacteria (Qin et al., 2010; Li et al., 2014; Sender et al., 2016; Matijašić et al., 2020), and our findings suggest that infants with *Bacteroides*-depleted community type may have a lower absolute abundance of bacteria in their stools. While outside of the scope of this study, this hypothesis could be explored by qPCR, potentially in conjunction with sequencing, to convert the relative abundance of microbiota profiles into estimates of absolute abundances (Jian et al., 2020).

Several studies have documented the prominent transmission of *Bacteroides* spp. from mothers to infants (Ferretti et al., 2018; Korpela et al., 2018, 2020; Wampach et al., 2018; Shao et al., 2019; Mitchell et al., 2020). Our team has also proven experimentally that maternal fecal microbiota transplant immediately after birth restores the levels of *Bacteroides* in CSD infants (Korpela et al., 2020). Strain-level metagenome studies have shown that maternal strains are more likely to persist than other bacteria and that the retention of *Bacteroides* is higher in the infant's gut compared to other genera, terming them as "persisters" (Ferretti et al., 2018; Korpela et al., 2018; Lou et al., 2021; Wang et al., 2021). To investigate a possible role of the mother-infant transmission of *Bacteroides* in the present or depleted-Bacteroides groups, we studied the effect of maternal fecal microbiota composition. Fecal samples were collected from the mothers in the 2 weeks preceding delivery, and no differences in terms of fecal microbiota composition, either overall or within the composition or abundance of *Bacteroides* spp., could be detected between the two groups of mothers.

Conclusion

Our study explored the gut microbiota developmental trajectories in healthy infants from 3 weeks to 1 year of age. Our results confirm previous reports indicating depletion of *Bacteroides* in CSD infants but also in a significant proportion of VD infants. Despite screening of extensive metadata, the cause of the depletion in VD infants still remains unresolved and would require larger infant cohorts where particularly the effect of different breastfeeding patterns could be investigated.

Data availability statement

The sequence data that support the findings of this study are available under the BioProject ID: PRJEB52774. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethical Committee of the Hospital District of Helsinki and Uusimaa. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

AS, KK, K-LK, and WMdV designed and established the cohort. AS and WMdV managed the cohort. ED performed the sample collection, management, and DNA extraction. DM, AP, and AS planned and carried out the computational experiments, analyzed the data, and wrote the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.953475/full#supplementary-material

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Abundance of selected bacterial groups in healthy calves and calves developing diarrhea during the first week of life: Are there differences before the manifestation of clinical symptoms?

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Background: Diarrhea is still the most common and economically most significant disease of newborn calves.

Objective: Analysis of the development of selected bacterial groups in the feces of neonatal calves and its significance regarding diarrhea.

Animals: A total of 150 newborn Simmental calves reared in 13 Bavarian farms were included in the study.

Methods: Fecal samples of calves taken at 0/6/12/24/48/72/168 hours (h) since birth were analyzed qualitatively and quantitatively for aerobic and anaerobic bacteria, such as *Enterobacteriaceae*, *E. coli*, enterococci, and lactobacilli, using cultural, biochemical, and molecular-biological methods. Concurrently, the health status of the animals was recorded. The bacterial levels of healthy and diarrheic animals were compared using statistical methods. In addition, feces samples from calves that developed diarrhea were examined by ELISA for the presence of rotaviruses, coronaviruses, *E. coli* F5, and *Cryptosporidium (Cr.) parvum*.

Results: Fifty-seven out of 150 calves (37.3 %) that were examined developed diarrhea within the first week of life. In the feces of calves with diarrhea on day 1 of life, the levels of aerobes, *Enterobacteriaceae,* and *E. coli* were significantly increased ($p < 0.05$), while no significant differences in enterococci and lactobacilli were found. In animals with the onset of diarrhea on day 2 after birth, the load of lactobacilli was significantly reduced up to 24 h before the manifestation of clinical symptoms compared to healthy calves. For enterococci, this was only the case on the day of the onset of diarrhea. In addition, the ratios of aerobic and anaerobic bacteria, *Enterobacteriaceae* or *E. coli* to lactobacilli, of calves with diarrhea starting on day 2 after birth are

significantly higher than those of healthy calves. The detection frequency of specific pathogens in diarrheic calves increased over the first week of life.

Conclusion: The results suggest that the incidence of neonatal diarrhea in calves is favored by low levels of lactobacilli in the feces. From this, the hypothesis can be derived that, in addition to an optimal supply of colostrum, the earliest possible administration of lactobacilli might reduce neonatal diarrhea in calves. However, this must be verified in a subsequent feeding experiment.

KEYWORDS

diarrhea, microbiology, lactobacilli, dysbiosis, calves, newborn

Introduction

Diarrhea in newborn animals is a long-known health problem, possibly since humans started keeping domestic animals (Key et al., 2020). As early as 1793, Claß stated that "this evil happens frequently and many calves are lost" (Claß, 1793). Up to now, diarrhea is considered the most common and economically significant disease of young calves (Elze et al., 1994; Doll et al., 1995). A total of 5–10% of live-born calves die, and approximately half of all deaths among calves up to the first month of age are due to diarrhea (Kaske and Kunz, 2003; Brickell et al., 2009). The National Animal Health Monitoring System for U.S. dairy reported in 2014 that 56% of ill calves showed digestive disorders with a case fatality rate of 8.5%; most cases occurred in calves less than 1 month old (Urie et al., 2018).

Calf diarrhea is attributed to both non-infectious and infectious factors. Various enteric pathogens like bovine rotavirus (BRV), bovine coronavirus (BCoV), bovine viral diarrhea virus (BVDV), *Salmonella (S.) enterica*, *Escherichia (E.) coli*, *Clostridium (C.) perfringens*, and *Cryptosporidium (Cr.) parvum* along with newly emerging enteric pathogens such as bovine torovirus (BToV) and caliciviruses (bovine norovirus [BNoV] and Nebovirus) are known or considered to cause diarrhea (Cho and Yoon, 2014). In addition, factors such as housing type, colostrum intake, and hygienic conditions can be associated with field outbreaks and may favor the development of clinical symptoms and influence the severity and outcome of the disease (Lee et al., 2019).

Diarrhea is the most common clinical sign of gut dysbiosis and has been demonstrated in various animal species like dogs, cats, or horses (Guard et al., 2015; Suchodolski et al., 2015; Arroyo et al., 2020). Compared to healthy calves, feedlot cattle with hemorrhagic diarrhea showed significant increases in the relative abundance of *Clostridium*, *Blautia,* and *Escherichia*, and significant decreases in the relative abundance of *Flavobacterium*, *Oscillospira*, *Desulfonauticus*, *Ruminococcus*, *Thermodesulfovibrio*, and *Butyricimonas* (Zeineldin et al., 2018). Gomez et al. (2017) showed that the intestinal microbiota of healthy dairy calves appeared to be farm-specific, as were the changes during diarrhea. They suggested that dysbiosis can occur in diarrheic calves and is associated with changes in the predictive metagenomics function of the bacterial communities. In the present study, total aerobic and anaerobic colony forming units (cfu) in the fecal samples were determined to quantify the overall bacterial count development and the individual differences in the very first hours and days of life, as well as to see when the bacterial count plateau is normally reached. The selection of the further investigated bacterial groups was based on existing knowledge about the development of the microbiota according to which the intestinal tract of newborn calves is quickly colonized by *Enterobacteriaceae (E. coli*), clostridia, and enterococci. These bacteria are considered to predominate numerically during the first two days of the calves' lives. Furthermore, approximately 24 h after birth, lactobacilli establish themselves in the gastrointestinal tract and soon become a dominant species (Smith, 1965). Moreover, since lactobacilli are considered "beneficial microorganisms" (Pace et al., 2015), we wanted to investigate their development and impact on diarrhea in more detail.

It is not surprising that the fecal microbiota differs between healthy and diarrheic calves. However, hardly anything is known about the situation 1 or 2 days before clinical signs become manifest. Are there quantitative differences in selected bacterial groups in the feces of calves that stay healthy for the next few days versus those that develop diarrhea a day or two later? If that were the case, this might serve as a scientific basis for diarrhea prevention. To verify this hypothesis, we conducted a wide-ranging prospective study with newborn calves that combined clinical and extensive microbiological investigations during the first week of life.

Materials and methods

Animals

To have a representative number of diarrheic calves with a high probability of obtaining statistically sound results, 150 newborn animals (66 female and 84 male) of the Simmentaler breed were included in the study. To determine the number

of animals, the incidence data from the studies by Girnus (2004) and Reski-Weide (2013) were used. The calculation was carried out using the software G*Power 3.1.9.7 (Faul et al., 2007). The calves were reared in 13 Bavarian farms (cows/farm: 55–165). Calf management and feeding designs were very similar during the first week of life. At all farms, the calves were placed in so-called calf-igloos or weather-protected individual boxes in the outdoor area within 24 h after birth. The time until the first colostrum (fresh, hand-milked) intake and the amount of colostrum ingested were recorded for each calf (see Supplementary Table). The calves were fed milk from the mother cow, milked by hand, 3–4 times per day for the first 5 days of life, after which the calves received a milk replacer. The cows were not treated with antibiotics during the perinatal period. Farmers were also instructed not to feed a starter containing probiotics to avoid data bias. In case of diarrhea, only oral electrolytes were administered, if necessary.

Daily clinical examination of all calves was performed for at least 8 days. Among the common clinical parameters, particular attention was given to sensorium, sucking reflex, skin turgor, eyeball position, rectal temperature, heart rate, respiratory rate, appetite, and feces characteristics (Stöber, 2012). Particular attention was paid to the consistency of the feces. For the diagnosis of "diarrhea", the scoring system of the Clinic for Ruminants of the Veterinary Faculty of the University of Munich (Germany) was used (Prof. Wolfgang Klee, personal communication): feces that have the consistency of water or pea soup or that flow through spread fingers are classified as diarrhea.

Fecal samples were collected immediately after birth (0 h, meconium) and 6, 12, 24, 48, 72 (3 days), and 168 (7 days) h after birth for microbiological investigations. Animals showing diarrhea were excluded from data analysis from this point onward. Detailed data on the occurrence of diarrhea for each individual calf can be seen in Supplementary Table.

Sampling

Feces samples were obtained directly by the farmers after being instructed by the authors of the present study. The anal region of the calves was cleaned (Bode Baktolin Waschlotion, Paul Hartmann AG, Heidenheim a. d. Brenz, Germany) and disinfected (Safe Sept Hautdesinfektion Pumpspray, Henry Schein, Berlin, Germany). Then, an industrial-clean glove was put on and the feces were removed with a gloved finger directly from the calf's anus. The glove was pulled inside out, knotted, and placed in a freezer bag. This procedure should ensure that contamination is largely avoided. The samples were stored at 4–8°C for a maximum of 48 h until further investigation.

Microbiological examinations

The microbiological examinations were carried out as previously described (Schwaiger et al., 2020). Total aerobic and anaerobic cfu in the fecal samples were quantified by using the reference spatula method on Standard I Nutrient agar (Merck, Darmstadt, Germany) containing 7% defibrinated sheep blood (Fiebig, Idstein-Niederauroff, Germany) and Schaedler agar (Becton-Dickinson, Heidelberg, Germany) with 5% sheep blood and vitamin K (Fiebig, Idstein-Niederauroff, Germany; Merck, Darmstadt, Germany). This procedure ensures a detection limit of 10^2 living bacteria/g feces (Gedek, 1974). The criteria for counting the different bacterial groups were growth on selective agar plates as well as morphology and color of the colonies. In addition, representative colonies were analyzed by microscopical, biochemical, and/or MALDI-TOF methods (see below).

The number of *Enterobacteriaceae* was determined using Gassner agar (Merck, Darmstadt, Germany), lactobacilli using LAMVAB-agar (Hartemink and Rombouts, 1999), and enterococci using citrate azide tween carbonate (CATC) agar (VWR, Darmstadt, Germany).

Escherichia (*E.*) *coli* were isolated on Gassner agar and biochemically confirmed by the production of β-D-glucuronidase and by the criterion of metabolizing sorbitol (Fluorocult agar, Merck, Darmstadt, Germany). Suspicious lactose-positive *Enterobacteriaceae* colonies not matching these criteria were investigated by API 20E (Biomerieux, Nürtingen, Germany). Lactose- and indole-negative strains were tested with antiserum *Salmonella* Omnivalent (Sifin, Berlin, Germany) for agglutination.

Enterococcus (*Ent.*) *faecalis* and *Ent. faecium* were isolated on CATC-Agar (citrate azide tween carbonate agar; Sifin, Berlin, Germany) and biochemically confirmed by testing their metabolism of xylose, mannitol, arabinose, and sodium-pyruvate (Bejuk et al., 2000).

For the identification of *Clostridium* spp., bacteria growing under anaerobic conditions on Schaedler-agar were sub-cultured under both aerobic and anaerobic conditions to exclude facultative anaerobic bacteria. The remaining obligatory anaerobes were identified at the genus level as *Clostridium* spp. using micro-morphological (Gram +/−, rods, spores) and biochemical criteria (oxidase −, indole +/−).

Bacteria of the genus *Lactobacillus* on LAMVAB-agar were identified at the genus level by microscopic (Gram +, rod, no formation of spores) and biochemical criteria (catalase−, oxidase−, indole +/−). The determination of the species was carried out by using MALDI-TOF-MS (Bruker Microflex™ LT; Bruker, Billerica, USA). Briefly, spectra from each isolate were obtained in accordance with the manufacturer's instructions using fresh and pure cultures. A small amount of culture material was transferred with a toothpick onto a 96-spot polished steel target plate and overlaid with one microliter

TABLE 1 Detection of common pathogens in the feces of diarrheic calves ($n = 57$) using ELISA (BIO K 348) at different times after birth.

Onset of diarrhea		Number of calves harboring pathogens				
Day	Number of animals	BRV	BCoV	Cr	*E. coli* F5	BRV/Cr
1	6	–	–	1	–	–
2	16	–	1	5	–	–
3	5	–	1	2	–	1
4	3	1	–	1	–	–
5	5	2	2	–	–	1
6	14	5	–	6	–	3
7	8	2	3	2	–	1

BRV, bovine rotavirus; BCoV, bovine coronavirus; Cr, Cryptosporidium parvum.

of α-cyano-4-hydroxy-cinnamic acid. After drying at room temperature, species identification was performed using Bruker Microflex™ LT equipment (Bruker, Billerica, USA) and the Biotyper Real-Time Classification software v. 3.0 (Bruker Daltonics, Bremen, Germany).

In total, we examined 560 and 399 fecal samples from healthy and diarrheic calves, respectively, and more than 8,100 bacterial isolates were preserved for further identification.

In case of diarrhea, a digestive antigen sandwich enzyme-immunoassay (ELISA; BIO K 348, Bio-X Diagnostics, Rochefort, Belgium) was carried out according to the manufacturer's instructions to test for the presence of the most prevalent diarrhea pathogens BRV, BCoV, *E. coli* F5 (K99), and *Cr. parvum*.

Statistical analysis

All statistical analyses were performed using the 'R' software version 4.0.4 (http://www.r-project.org/). The cfu counts were log-transformed. The Wilcoxon rank-sum test was applied to compare the counts of bacterial groups of healthy and diarrheic calves. In addition, a Kolmogorov-Smirnov test was used to analyze the value distribution of the corresponding groups. The probability of the occurrence of diarrhea in the population of calves depending on the bacterial count at the species level was modeled by a logistic regression adjusting for the respective farm.

Results

General observations

Fifty-seven out of 150 calves (37.3 %) examined developed diarrhea within the first 7 days of life. The time of onset showed a bimodal distribution with peaks on days 2 and 6 (Table 1). Looking at the individual farms, the incidence of diarrhea was highly variable between 0 and 100% [in detail, from farm 1 to farm 13 (in %): 38/17/23/39/85/47/67/0/100/39/75/8, respectively]. For detailed information for each individual calf (see Supplementary Table).

The colostrum intake data are summarized in Figure 1. The colostrum was fed on average 1.7 h after birth. Calves that remained healthy in the first week received colostrum after an average of 1.5 h, while those that developed diarrhea ingesting it after 2 h on average ($p = 0.04$). All calves ingested an average of 1.78 ($\pm$0.72) liters (L) of colostrum. Calves that suffered from diarrhea during the first week consumed an average of 1.57 ($\pm$0.7) L, and those that remained healthy 1.92 ($\pm$0.7) L ($p = 0.006$). Detailed information on the time and volume of colostrum intake can be viewed in Supplementary Table for each individual calf.

Pathogens in the feces of calves with diarrhea

The results of the ELISA assays are summarized in Table 1. *Cr. parvum* ($n = 23$) was detected most frequently, followed by BRV ($n = 16$) and BCoV ($n = 7$). *E. coli* F5 was not found in the samples analyzed. *Cr. parvum* antigen was already detectable in one sample on the first day of life, while BCoV and BRV were only found on the second and third day, respectively. In addition, it was apparent that later diarrhea occurs more frequently within the first week of life, the more often pathogens can be assigned to clinical events by ELISA. Detailed information about the pathogens detected for each individual calf with diarrhea can be seen in Supplementary Table.

Quantity of selected bacterial groups in the feces of healthy and diarrheic calves

Based on the sampling design and the number of cases of diarrhea per day, the cases starting on days 1, 2, and 8 after birth were analyzed in more detail.

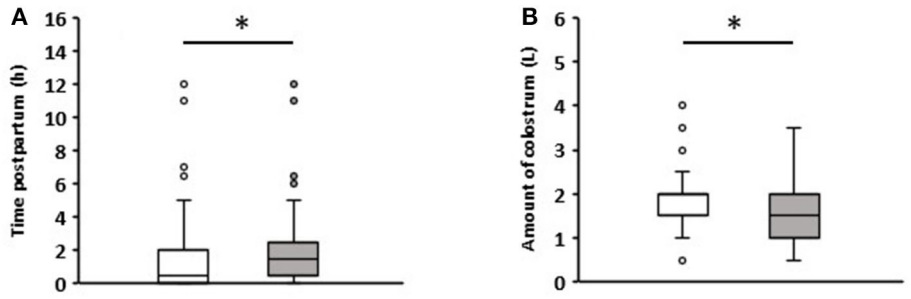

FIGURE 1
Comparison of colostrum intake data of newborn calves without (white boxes, $n = 81$) and with diarrhea (gray boxes, $n = 57$) in the first week of life. **(A)** Time to colostrum ingestion after birth. **(B)** Amount of colostrum ingested (Wilcoxon test, $*p < 0.05$).

Diarrhea on day 1

Six out of 150 calves suffered from diarrhea during the first 24 h after birth (Table 1). The quantitative analysis showed a significant increase in aerobic bacteria ($p = 0.029$), *Enterobacteriaceae* ($p = 0.013$), and *E. coli* ($p = 0.019$) in the feces of diarrheic calves (Figure 2). Interestingly, these results were already apparent 12 h after birth. No significant differences could be seen between healthy and diarrheic calves regarding anaerobic bacteria, lactobacilli, and enterococci. On day 2 of life, the counts of the bacterial groups analyzed did not differ between healthy and diarrheic calves. On average, both groups ingested almost the same amount of colostrum at the first feeding (healthy calves: 1.79 L; diarrheic calves: 1.6 L).

Diarrhea on day 2

As already mentioned, most diarrheal diseases ($n = 16$) occurred on day 2 after birth (Table 1). Interestingly, the group of calves with diarrhea on day 2 consumed significantly less colostrum on average than those that remained healthy (1.33 vs. 1.86 L; $p = 0.0025$). The results of the bacteriological investigation are shown in detail in Figure 3. The development of fecal bacterial counts of aerobes, anaerobes, *Enterobacteriaceae*, and *E. coli* was almost the same during the first 48 h after birth in both calves with and without diarrhea on day 2. However, 1 day after the onset of diarrhea, diarrheic calves had significantly increased levels of aerobic bacteria ($p = 0.000097$) and *Enterobacteriaceae* ($p = 0.002557$; Figures 3A,C). While a similar trend was observed for *E. coli* ($p = 0.0013$; Figure 3D), this was not the case for anaerobic bacteria.

When evaluating the development of lactobacilli, it was found that these counts were significantly reduced 24 h before the manifestation of diarrhea compared to calves that remained healthy ($p = 0.01$). This was also the case on days 2 and 3 after birth. With regard to the number of enterococci, a significant difference could only be found in the 48-h samples. It should be noted that, first, a growth reduction in enterococci and lactobacilli was observed, and then an increase in aerobic counts, *Enterobacteriaceae*, and *E. coli*.

In addition, we analyzed the ratio of fecal aerobic counts, anaerobic counts, *Enterobacteriaceae*, and *E. coli* in comparison to enterococci and lactobacilli of healthy and diarrheic calves during the first three days of life (Figure 4). In the case of lactobacilli, it was noticeable that the ratios obtained for animals suffering from diarrhea on day 2 were significantly higher than those obtained for healthy calves. This was evident as early as 24 h before the clinical manifestation of diarrhea. Similar results were also found for enterococci, albeit to a much lesser extent.

We also compared enterococci and lactobacilli of healthy calves from farms with diarrhea with the counts of calves from farms without diarrhea on day 2 during the first three days of life (Figure 5). As a result, the numbers of enterococci and lactobacilli were significantly reduced on days 2 and 3 in healthy calves from farms with diarrhea on day 2, although the numbers of enterococci and lactobacilli were almost the same in both groups 24 h after birth. When comparing the numbers of fecal enterococci and lactobacilli of healthy and diarrheic calves from farms with diarrheic calves on day 2, a similar result as shown in Figures 3E,F emerged: bacteria of the genus *Lactobacillus* was significantly reduced in the feces of calves with diarrhea (Figure 6). There was no correlation between the volume of colostrum consumption or the first feeding time and the concentration of lactobacilli in the feces (data not shown).

Diarrhea From day 3 to day 7

Only five calves developed diarrhea 3 days after birth (Table 1). No differences in the levels of the selected bacterial groups could be detected between healthy and diarrheic calves. Since no feces samples were collected between days 4 and 6, a detailed evaluation during this period is not possible. The colostrum intake of the diarrheic and healthy calves during this period did not differ significantly ($p = 0.112$). However, it is

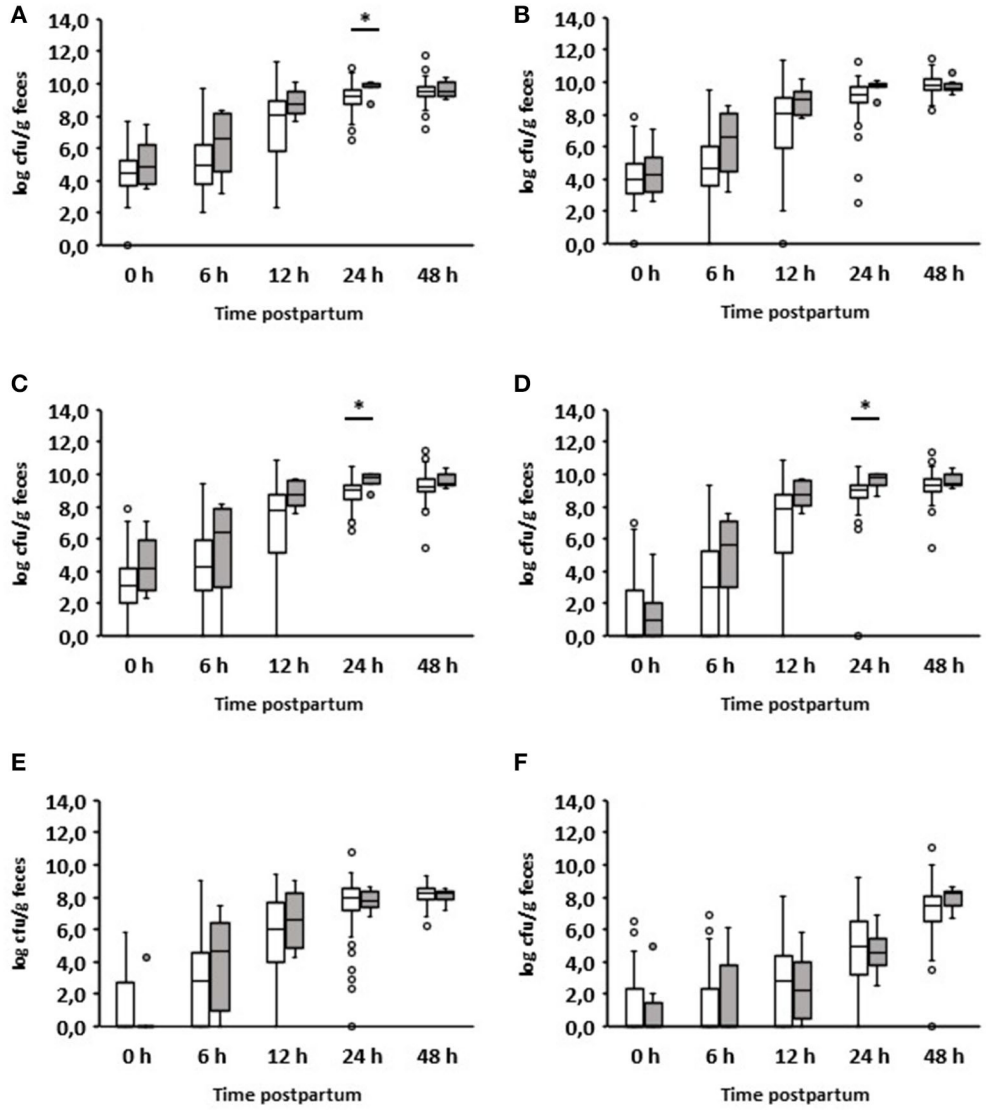

FIGURE 2
Fecal microbial contents of selected bacterial groups of healthy calves (white boxes, $n = 144$) and calves with diarrhea within 24 h after birth (gray boxes, $n = 6$). **(A)** Aerobic bacteria; **(B)** anaerobic bacteria; **(C)** *Enterobacteriaceae*; **(D)** *E. coli*; **(E)** *Enterococcus* spp.; **(F)** *Lactobacillus* spp. (Wilcoxon test, $*p < 0.05$).

notable that 14 cases of diarrhea occurred on day 6 after birth when BRV and/or Cr could be identified (Table 1).

Diarrhea on day 8

On day 8 after birth, eight of the 150 examined calves developed diarrhea (Supplementary Table). When fed for the first time, these eight calves ingested almost the same amount of colostrum as the animals that had remained healthy until that point (1.85 vs. 1.92 L, $p = 0.8962$). One day before, none of the numbers of aerobic bacteria, anaerobic bacteria, *Enterobacteriaceae*, *E. coli*, and lactobacilli changed in the feces in comparison to the fecal samples of animals that remained healthy. Only the number of enterococci was significantly reduced in the animals suffering from diarrhea the next day ($p < 0.05$; Figure 7).

Thirty-eight different species of Lactobacilli were detected in the feces of all calves during the whole investigation period. The most common species were identified as *L. brevis*, *L. fermentum*, *L. mucosae*, *L. murinus*, *L. parabuchneri*, *L. paracasei*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, and *L. salivarius*. The logit model showed that, with increasing concentration of *L. reuteri*, the probability of occurrence of diarrhea decreased; variable "a" was significant with $p = 0.036$.

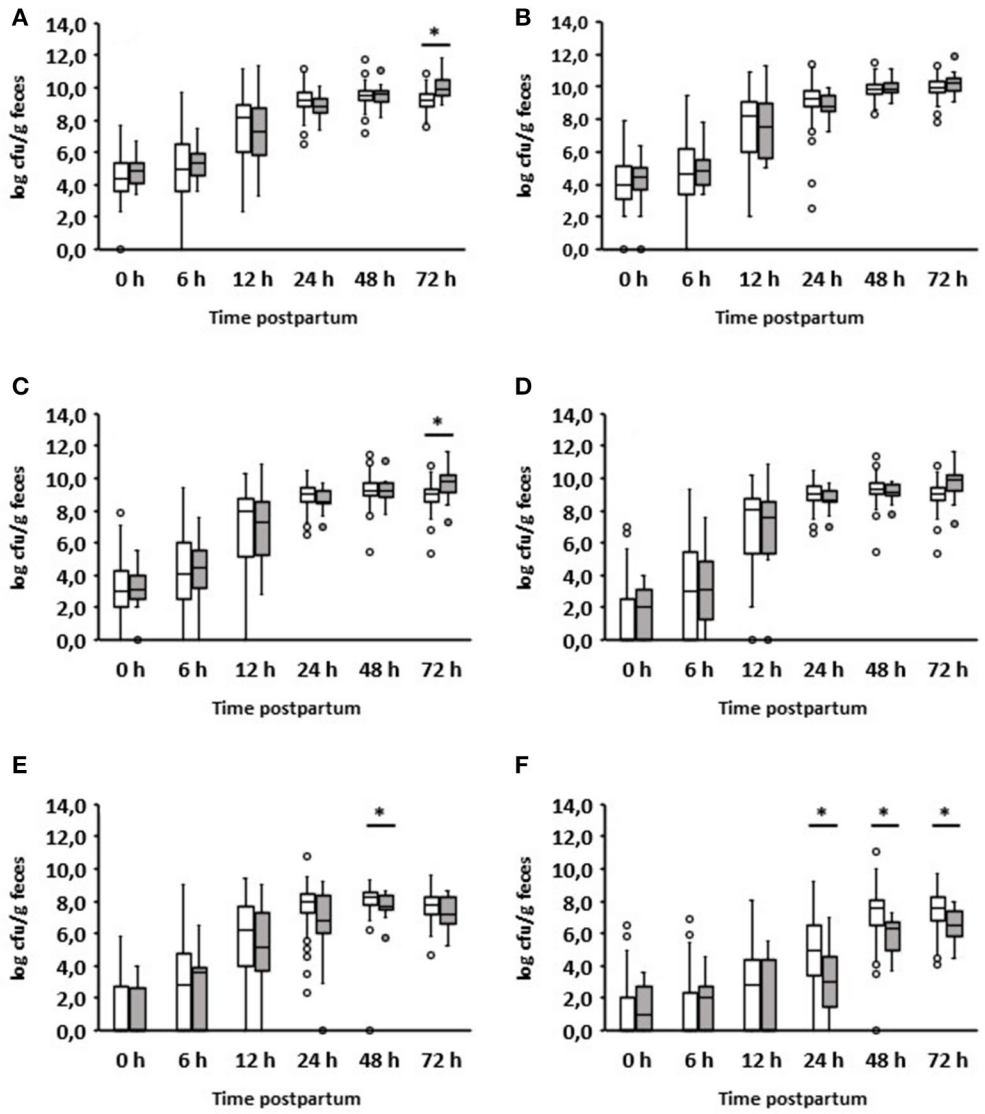

FIGURE 3
Development of the fecal microbial contents of selected bacterial groups during the first 72 h after birth. **(A)** Aerobic bacteria, **(B)** anaerobic bacteria, **(C)** *Enterobacteriaceae*, **(D)** *E. coli*, **(E)** *Enterococcus* spp., **(F)** *Lactobacillus* spp.; white boxes: values of healthy calves ($n = 134–128$); gray boxes: values of calves with diarrhea onset on day 2 ($n = 16$) (Wilcoxon test, $*p < 0.05$).

Discussion

Diarrhea in newborn calves is a multifactorial disease, and a variety of infectious and non-infectious criteria play a role in its development. In our study, about one-third of newborn calves showed diarrhea during the first 8 days of life. This incidence rate is comparable to that of previous investigations, which were conducted in the same geographic area and with the same cattle breed (Girnus, 2004; Reski-Weide, 2013). It is noteworthy that analysis of the frequency of diarrhea cases per day shows two peaks (day 2 and day 6 after birth), an observation that was already described by Bendali et al. (1999).

Not surprisingly, calf colostrum supply has a significant impact on diarrhea incidence—the earlier the calves were supplied with colostrum, and the higher the amount ingested, the lower the incidence of diarrhea. This fact has been repeatedly confirmed in numerous publications (Fallon and Harte, 1983; Donovan et al., 1998).

For reasons already discussed in our previous study (Schwaiger et al., 2020), we decided to apply cultivation techniques. Admittedly, it is known that a large proportion of intestinal bacteria cannot be detected using conventional bacteriology (Zoetendal and Mackie, 2005). However, these perhaps seemingly old-fashioned methods were better suited

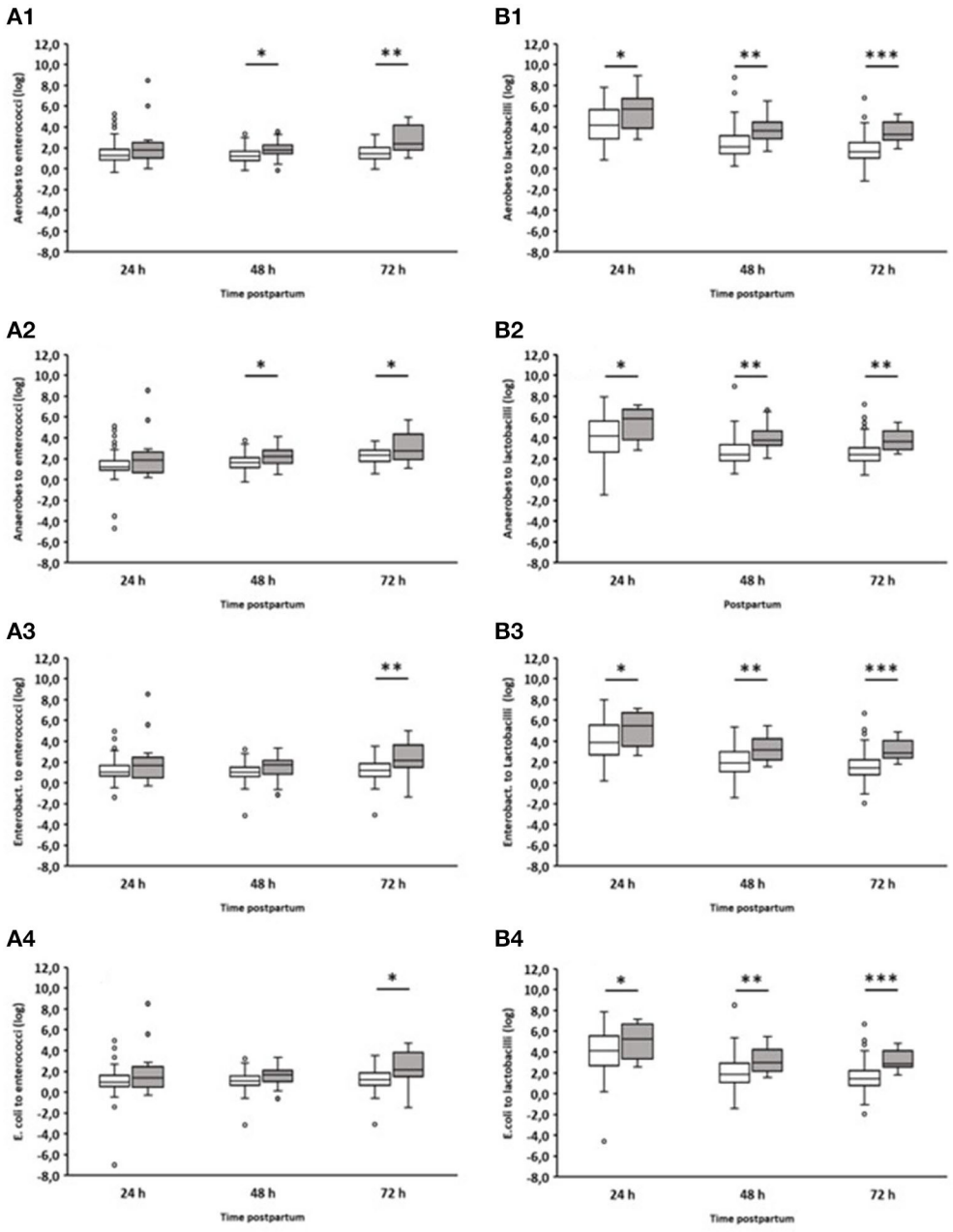

FIGURE 4
Ratio of counts (cfu/g feces) of specific bacterial groups to *Enterococcus* spp. or *Lactobacillus* spp. during the first 72 h after birth. **(A1)** Aerobic bacteria to enterococci; **(A2)** anaerobic bacteria to enterococci; **(A3)** *Enterobacteriaceae* to enterococci; **(A4)** *E. coli* to enterococci; **(B1)** Aerobic bacteria to lactobacilli; **(B2)** anaerobic bacteria to lactobacilli; **(B3)** *Enterobacteriaceae* to lactobacilli; **(B4)** *E. coli* to lactobacilli. White boxes: values of healthy calves ($n = 127–133$); gray boxes: values of calves with diarrhea onset on day 2 ($n = 16$; Wilcoxon test, $*p < 0.05$, $**p < 0.001$, $***p < 0.00001$).

for the purposes of the present study than next-generation sequencing (NGS) techniques because they allow absolute quantification of cell numbers instead of relative abundances. Additionally, all cultivated isolates can be differentiated down to species level, whereas the composition of the microbiota determined by NGS is characterized mostly at the phylum or genus level. To gain reliable new insights into the kinetics of the (cultivable) microbiota of newborn healthy and diarrheic calves down to the species level, cultural techniques were combined with MALDI-TOF.

Calves that developed diarrhea within the first day of life had significantly higher concentrations of aerobic bacteria,

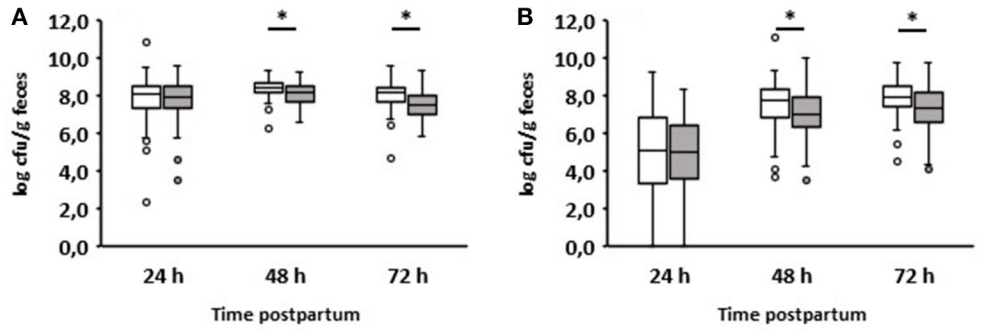

FIGURE 5
Counts of enterococci **(A)** and lactobacilli **(B)** in calves' feces. White boxes: feces of healthy calves from farms where no diarrhea occurred on day 2 of life ($n = 49$). Gray boxes: feces of healthy calves from farms where diarrhea occurred on day 2 of life ($n = 79$; Wilcoxon test, $*p < 0.05$).

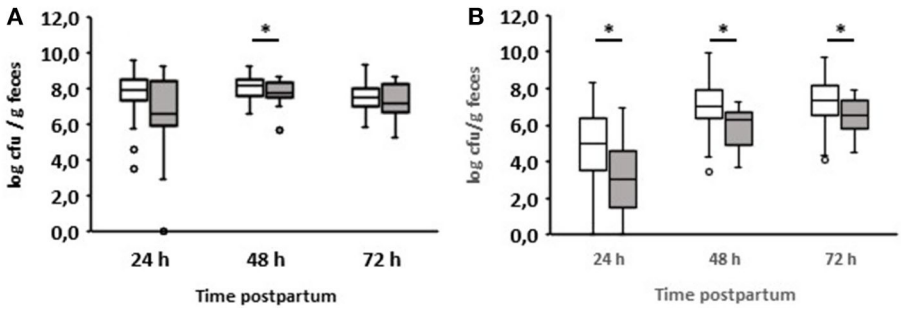

FIGURE 6
Counts of enterococci **(A)** and lactobacilli **(B)** in feces of calves from farms where diarrhea occurred on day 2 of life. White boxes: feces of healthy calves ($n = 79$). Gray boxes: feces of calves with diarrhea onset on day 2 of life ($n = 16$; Wilcoxon test, $*p < 0.05$).

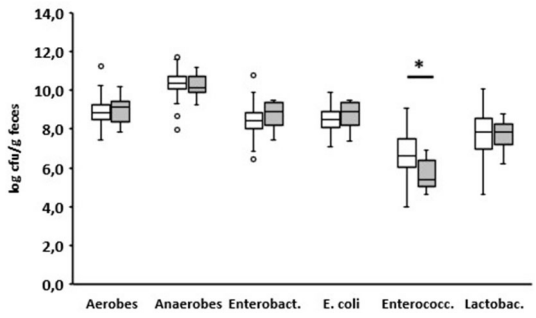

FIGURE 7
Fecal microbial contents of selected bacterial groups of healthy calves on day 7 after birth. White boxes: values of calves that remain healthy; gray boxes ($n = 101$): values of calves with diarrhea starting the next day ($n = 8$) (Enterobact., *Enterobacteriaceae*; Enterococc., *Enterococcus* spp.; Lactobac., *Lactobacillus* spp.; Wilcoxon test, $*p < 0.05$).

anaerobic bacteria, *Enterobacteriaceae*, and *E. coli* in the feces. The high levels of these bacterial groups may be related to poor hygiene during or immediately after birth. For example, Klein-Jöbstl et al. (2014) demonstrated that cleaning the calving area after each calving significantly reduces the odds of calf diarrhea on farms. Apart from *Cr. parvum*, we were unable to detect a specific diarrhea-associated pathogen in the feces of this group of calves by ELISA. Therefore, it is conceivable that a high bacterial load may have disturbed the intestinal microbiota, which is still very unstable at that time, resulting in diarrhea.

As already mentioned, we detected *Cr. parvum* antigen in the feces of one calf with the onset of diarrhea on day 1. Since the development cycle of *Cr. parvum* takes about 3–6 days (Rommel, 2000), it is rather unlikely that the diarrhea of this calf was caused by *Cr. parvum*. Instead, it seems more likely that the detected particles of *Cr. parvum* have passed through the gastrointestinal tract without going through a stage of development. This must also be considered about the occurrence of *Cr. parvum* on day 2 of life. Since this single-celled organism is transmitted by fecal contamination, the positive ELISA result might be used as an indicator of poor hygiene during the perinatal phase.

Evaluation of the data of calves that developed diarrhea on the second day after birth shows that 24 h before the onset of diarrhea the number of lactobacilli was significantly reduced compared to healthy calves. This fact was also found both on

the day on which diarrhea began and on the following day. Enterococci were significantly reduced only on the day diarrhea began. Interestingly, an increase in the numbers of aerobic bacteria, *Enterobacteriaceae*, and *E. coli* could only be observed one day after the onset of diarrhea.

The ratio of different bacterial taxa, especially Firmicutes to Bacteroidetes, is a frequently used parameter to describe gastrointestinal dysbiosis (Youmans et al., 2015; Bin et al., 2018). In fact, we could see a clear shift in the ratios of the numbers of aerobic bacteria, anaerobic bacteria, *Enterobacteriaceae,* and *E. coli* to lactobacilli. In the case of enterococci, this shift in the ratios was less pronounced (Figure 4). It is noteworthy that significant differences in the ratio of anaerobic bacteria to lactobacilli or enterococci were found between healthy calves and calves suffering from diarrhea, while this was not the case when comparing only the anaerobic bacterial counts (Figure 3). These results indicate that the proportions of aerobic bacteria, anaerobic bacteria, *Enterobacteriaceae*, and *E. coli* to lactobacilli have shifted to the disadvantage of the latter. The same is probably the case for enterococci, albeit to a lesser extent.

The reason why lactobacilli in calves that develop diarrhea do not increase as much as in animals that remain healthy cannot be clarified without further elaboration. As mentioned above, we were able to show the influence of colostrum supply on the incidence of diarrhea. However, there was no correlation between the amount of colostrum consumed or the first feeding time and the concentration of lactobacilli in the feces (data not shown). This agrees with the results of Fischer et al. (2018), who only found a relative reduction of lactobacilli in the colon when colostrum intake was delayed by 12 h. Since the components of the colostrum were not analyzed, we cannot make any statement about possible differences in the composition and the growth of lactobacilli.

An interesting aspect can be shown by comparing the fecal counts of enterococci and lactobacilli of healthy calves from farms with diarrhea with calves from farms without diarrhea on day 2 during the first 3 days of life (Figure 5): the numbers of enterococci and lactobacilli were significantly reduced on days 2 and 3 in healthy calves from farms with diarrhea on day 2, although the numbers of enterococci and lactobacilli were almost the same in both groups 24 h after birth. This discrepancy can result from different farm management systems. A so-called "farm effect" on the composition of the fecal microbiota has been observed by Weese and Jelinski (2017). However, as already mentioned, no major differences regarding the handling of newborn calves could be found. In addition, when comparing healthy and diarrheic calves from farms with diarrheic calves on day 2, bacteria of the genus *Lactobacillus* (and, to a lesser extent, also of the genus *Enterococcus*) were significantly reduced in the feces of calves with diarrhea (Figure 6). Recently, we showed that fecal samples from twin calves revealed higher similarity in single-strand conformation polymorphism profiles compared to their coresidents, indicating that the individual microbiota might be genetically or epigenetically influenced (Mayer et al., 2012).

The gut microbiota-modulating effects of enterococci and especially lactobacilli are discussed and demonstrated (Azad et al., 2018; Shin et al., 2019). It is well known that bacteria of both genera produce various compounds that inhibit the growth of bacteria. For example, enterocins were detected in cultures of *Ent. faecium, Ent. faecalis, Ent. Hirae*, and *Ent. durans* (Hernández-González et al., 2021), while strains of *L. reuteri* produce reuterin and reuterocycline (Talarico et al., 1988; Gänzle, 2004). In addition, growth-inhibition substances like organic acids, hydrogen peroxide, diacetyl, and ethanol are synthesized by lactobacilli (Millette et al., 2007; Vieco-Saiz et al., 2019). Because of this metabolic performance, and because of the competition for binding sites on the intestinal mucosa, enterococci and lactobacilli prevent the proliferation of bacteria and appear to be important for the development and stability of a eubiotic microbiota (Mokoena, 2017). The consequence of a decrease in enterococci and lactobacilli is an increase in the numbers of aerobic bacteria, *Enterobacteriaceae*, and *E. coli*.

L. reuteri, which is one of the *Lactobacillus* species predominant in calves (Schwaiger et al., 2020), seems to play a special role regarding their intestinal health. Delayed development of this species during the first 3 days of life favors the likelihood of diarrhea in the first week of life. The beneficial properties of *L. reuteri* are frequently discussed. In addition to the antibiotic effects mentioned above, inhibitory effects on viruses (especially rotavirus) as well as cryptosporidia are described (Alak et al., 1997; Glass et al., 2004; Seo et al., 2010; Ang et al., 2016). Our results contradict those of the recently published investigation by Slanzon et al. (2022). According to this study, *L. reuteri* occurs more frequently in the fecal microbiota of calves with gastrointestinal diseases. However, it should be noted that (1) calves of other breeds (Holstein, Jersey, Jersey-cross, and beef-cross vs. Simmentaler) and (2) a different age group (4–21 days vs. 1–7 days) were examined.

Looking at the counts of the selected bacterial groups on day 7 (Figure 5), a significant difference between calves that remained healthy and calves suffering from diarrhea the next day could only be found for enterococci. However, from day 5 of life, specific diarrhea pathogens (BVR, BCoV, and/or *Cr. parvum*) were detected in all calves with diarrhea (Table 1). It appears that from this point on, diarrhea is caused less by microbial dysbiosis than by specific pathogens. Surprisingly, we could not find any evidence for the presence of *E. coli* F5, a known pathogen in 2–3-day-old diarrheic calves. In contrast to Ewers et al. (2004) who tested individual isolates of *E. coli*, we used, in accordance with the manufacturer's instructions, feces as the test material, which may impair the sensitivity of the test system. In addition, it seems that the incidence of *E. coli* F5 in calves with diarrhea has decreased in recent years (Kolenda et al., 2015).

In conclusion, a restricted development of lactobacilli in the first days of life leads to a quantitative shift in the fecal

microbiota. This reflects an enteric dysbiosis that favors the onset of diarrhea in newborn calves. Specific pathogens as causative agents of diarrhea increase over the course of the first week of life. The number of lactobacilli is reduced about 24 h before the clinical manifestation of diarrhea on day 2 after birth. This fact suggests that the incidence of diarrhea in the first days of life might be reduced by administering lactobacilli (especially *L. reuteri*) as early as possible together with colostrum. This hypothesis must be verified using a targeted feeding experiment in an extensive follow-up study.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

KS: project coordination and support. JS: project implementation. CB: statistics. JB: project leader. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Ultra-early weaning alters growth performance, hematology parameters, and fecal microbiota in piglets with same genetic background

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Piglets with the same genetic background were used to investigate the effects of different lengths of suckling period on growth performance, hematology parameters, and fecal microbiota. All piglets were born by a sow (Landrace×Yorkshire). On day 14 postpartum, a total of 16 piglets [Duroc×(Landrace×Yorkshire)] with a similar initial body weight (2.48 ± 0.25 kg) were randomly assigned into two groups with four replicates per group, two pigs per replicate pen (one barrow and one gilt). On day 14 of age, experiment started, piglets from the first group were weaned (14W), whereas the others continued to receive milk until day 28 of age (28W). The experiment completed on day 70 of age, last 56 days. Growth performance parameters including body weight, average daily gain, feed intake, feed efficiency, and growth rate and hematology parameters including immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), albumin, globulin, and total protein were measured in this study. Additionally, a technique of 16S rRNA gene sequencing was used to analyze fecal microbiota for revealing how the changes in the lengths of suckling period on intestinal microbiota. We found that ultra-early weaning impaired growth performance of piglets, whose worse body weight, average daily gain, feed intake, feed efficiency, and growth rate were observed in 14W group at all measured timepoints in comparison with those in 28W group ($P < 0.05$). Moreover, higher contents of serum IgA ($P = 0.028$), IgG ($P = 0.041$), and IgM ($P = 0.047$), as well as lower contents of serum albumin ($P = 0.002$), albumin-to-globulin ratio ($P = 0.003$), and total protein ($P = 0.004$), were observed in 14W group in comparison with those in 28W group on day 28 of age, but not on day 70 of age. High-throughput pyrosequencing of 16S rRNA indicated that the intestinal microbiota richness in 14W group was lower than that in 28W group ($P < 0.05$); moreover, in comparison with 28W group at all sampling

timepoints, fecal microbiota in 14W group showed more beneficial bacteria and fewer pathogenic bacteria ($P < 0.05$). Therefore, we considered that ultra-early weaning had positive effects on immune status and fecal microbiota composition in piglets, but negative effects on growth performance and fecal microbiota abundance.

KEYWORDS

fecal microbiota, 16S, pig, immunity, ultra-early weaning, growth

Introduction

Breast-feed provides a possibility for the vertical transmission of pathogens from sows to offspring (Smith et al., 2008). Additionally, with the prolonging of lactation period, the yield and quality of milk from sows failed to provide adequate nutrients to support the large growth potential of offspring (Nuntapaitoon, 2022). Therefore, consistently intaking low-quality milk will limit the growth of piglets. On the contrary, with the growth of piglets, sows are reluctant to suckle the piglets, and they will limit the piglets getting in touch with the udder through frequent posture adjustments and even attack them (EFSA Panel on Animal Health and Welfare, 2022). Therefore, shortening the suckling period seems to have positive effects on the growth of piglets and the welfare of sows. Some studies reported that shortening the suckling period improved the reproductive performance and body conditions of sows (Spencer et al., 2003; Holman et al., 2021), as well as increased the economic value of piglets (Main et al., 2004, 2005). However, some studies reported that shortening suckling period negatively affected the growth performance (Ming et al., 2021), intestinal health (Cao et al., 2022), immune status (Tao et al., 2016), antioxidant capacity (Buchet et al., 2017), nutrient digestibility (Ming et al., 2021), and survival rate (Huting et al., 2019), as well as led to diarrhea, prolonged the required days to reach marketing weight, and increased feed cost (Smith et al., 2008; Massacci et al., 2018; Faccin et al., 2020a,b).

Recently, the relationship between gut microbes and productive performance of animals has received unprecedented attention (Isaacson and Kim, 2012). Studies on the effects of ultra-early weaning on intestinal microbiota of pigs are still limited.

Moreover, genetic background plays a key role in affecting the individual differences of animals (Champy et al., 2008). The same genetic background means the minimization of individual differences. Therefore, this study investigated the effects of ultra-early weaning on growth performance, hematology parameters, and fecal microbiota in piglets. According to the recommendation of Mabry et al. (1996) and Xue et al. (1997), piglets were weaned on day 14 or 28 of age in this study.

We hypothesized that shortening suckling period had negative effects on the growth performance and hematology parameters, moreover, led to the disorder in intestinal microbiota, and manifested in the increase of pathogenic bacteria and the decrease of beneficial bacteria. The objective of this study was to evaluate the effects of ultra-early weaning on growth performance, hematology parameters, and fecal microbiota in piglets.

Materials and methods

Experimental design, animals, and housing

A total of 16 14-day-old piglets [Duroc×(Landrace × Yorkshire)] with a similar initial body weight (2.48 ± 0.25 kg) were selected from the same sow (Landrace×Yorkshire) for ensuring the same genetic background. All piglets were randomly assigned into two groups with four replicates, two piglets per replicate pen (one barrow and one gilt). The experimental factor was the lengths of suckling period, of which piglets from the first group were weaned on day 14 of age (14W) and others were weaned on day 28 of age (28W). The experiment lasted to day 70 of age (56 days). In the group of 14W, piglets received creep feed during days 14 to 28 of age. On day 29 of age, all piglets were given the same feed, which was formulated to meet the recommendation of the National Research Council [NRC] (2012) and provided in a mashed form (**Table 1**). Experimental protocol (no. JMU00211232) and the process were approved and supervised by the Animal Care and Use Committee of Jinzhou Medical University (Jinzhou, China). The care and the treatment of the sows were according to the animal welfare legislation (Federation of Animal Science Societies, 2010).

Piglets did not receive creep feed during the suckling period. On the third after birth, piglets were subjected to routine management practices and received 1 ml of iron dextran (50 mg/kg). Male piglets were castrated.

All piglets were housed in an environmentally controlled nursery barn. The ambient temperature within the room was maintained at 30°C until day 35 of age and reduced by 1°C per week subsequently.

TABLE 1 Composition and nutrient levels of the experimental basal diet during post-weaning period (%, as-fed basis).

Ingredients, %	Days 14–28 of age[1]	Days 29–70 of age[2]
Corn	35.92	48.09
Puffed corn	18.00	15.00
Soybean meal	12.00	18.50
Fermented soybean meal	12.00	6.00
Whey protein	10.00	5.00
Fish meal	4.00	3.00
Corn starch	–	0.20
Spray-dried porcine plasma	3.00	–
Soy oil	2.20	1.08
Monocalcium phosphate	0.80	0.66
Limestone	0.60	0.90
Mineral and vitamin mixture[3]	0.40	–
Mineral and vitamin mixture[4]	–	0.50
Lysine	0.40	0.39
Salt	0.30	0.30
Threonine	0.15	0.16
Choline	0.10	–
Methionine	0.12	0.20
Tryptophan	0.01	0.02
Total	100.00	100.00
Analyzed composition,%		
Crude protein	20.77	18.88
Metabolizable energy, MJ/kg	14.83	14.61
Lysine	1.54	1.24
Threonine	1.01	0.73
Calcium	0.81	0.70
Methionine	0.45	0.36
Available phosphorus	0.37	0.34
Tryptophan	0.26	0.20
Crude fat	4.94	4.03
Crude fiber	5.56	5.32
Ash	2.18	2.47

[1]Dietary composition of piglets weaned on day 14 of age (ultra-early weaning group).
[2]Dietary composition of piglets in all groups.
[3]Provided per kg of complete diet: Zn 100 mg; Mn 4 mg; Fe 100 mg; Cu 100 mg; I 0.3 mg; Se 0.3 mg; vitamin A 14000 IU; vitamin D_3 4000 IU; vitamin E 4.7 mg; vitamin B_1 4 mg; vitamin B_2 10 mg; vitamin B_6 6 mg; vitamin B_{12} 0.04 mg; niacin 40 mg; pantothenic acid 20 mg; folic acid 2 mg; biotin 0.16 mg.
[4]Provided per kg of complete diet: Zn 80 mg; Mn 4 mg; Fe 100 mg; Cu 200 mg; I 0.14 mg; Se 0.25 mg; choline chloride 400 mg; vitamin A 10500 IU; vitamin D_3 3000 IU; vitamin E 22.51 IU; vitamin K_3 3 mg; vitamin B_1 3 mg; vitamin B_2 7.5 mg; vitamin B_6 4.5 mg; vitamin B_{12} 0.03 mg; niacin 30 mg; pantothenic acid 15 mg; folic acid 1.5 mg; biotin 0.12 mg.

The humidity was around 60%. Piglets had free access to feed and water.

Sampling and measurements

Growth performance

All piglets were weighed on days 14, 28, and 70 of age to calculate the average daily gain (ADG) and growth rate. Daily feed intake was recorded to measure the average daily feed intake (ADFI) based on the pen. Feed efficiency was calculated according to the values of ADG and ADFI.

Hematology parameters

All piglets were used for collecting blood *via* jugular venipuncture on days 28 and 70 of age. Blood samples (5 mL) were collected into vacuum tubes without anticoagulants (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). After collection, the blood samples were centrifuged (3500 × *g*) for 10 min at 4°C to extract the serum and then stored at −20°C until analysis. The contents of IgA, IgG, and IgM were measured by specific ELISA kit (Meimian Industrial Ltd., Co., Jiangsu, China). Additionally, the concentrations of albumin, globulin, and total protein were measured by a Beckman-CX4 automatic biochemical analyzer (Beckman Coulter, Inc., Brea, CA, USA).

Fecal microbiota analysis by 16S rRNA gene sequencing

Fresh stool samples were taken from 16 piglets. The specimens were kept in ice boxes until they arrived at the laboratory. A Magnetic Soil and Stool DNA Kit (cat# DP712, Tiangen Biotech Co., Ltd., Beijing, China) was used for extracting total DNA from 16 fecal samples (0.5 g). The concentration and purity of the extracted DNA were determined using a Qubit 2.0 spectrophotometer (Invitrogen, Carlsbad, CA, USA) and 1% (w/v) agarose gel electrophoresis. The quality of DNA was judged according to the results of agarose gel electrophoresis, and the result of "A" was considered high-quality DNA. The DNA samples were diluted with sterile water to a concentration of 1 ng/μL and stored at −20°C before analysis. Then, the V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with specific full-length universal forward (5′-ACTCCTACGGGAGGCAGCAG-3′) and reverse (5′-GGACTACHVGGGTWTCTAAT- 3′) primers. PCRs were performed in triplicate with each 20 μL reaction mixture containing 4 μL of 5 × FastPfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL FastPfu polymerase, and 10 ng of template DNA. The PCR conditions were 95°C for 3 min; 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, repeat for 27 cycles; and 72°C for 10 min. Subsequently, a Qiagen Gel Extraction Kit (cat# 28706, Qiagen, Germany) was used to further purify the PCR products. Simultaneously, the purity of the PCR mixture was evaluated using a Qubit 2.0 dsDNA HS Assay Kit (cat# Q32854, Invitrogen). The 16S rRNA gene sequencing was performed to analyze the fecal microbial community structures using the NovaSeq 6000 platform (Illumina, San Diego, CA, USA) in Novogene Bioinformatics Co., Ltd. (Tianjin, China).

Raw data were obtained by cutting low-quality reads using Cutadapt software version 1.9.1. Chimeric sequences were trimmed by alignment and detection. High-quality reads were clustered into operational taxonomic units (OTUs) at

97% sequence identity using Uparse v7.0.1001. The taxonomic assignment of the representative sequences was performed using QIIME v1.9.1. A rarefaction curve was plotted for each sample using R software (version 1.9.1) to determine the suitable sequencing depth that covers the extent of microbial diversity. The number of observed OTUs was used to calculate alpha-diversity, including observed species, Chao1, Ace, Shannon, and Simpson diversity indices, and beta-diversity, including Bray–Curtis and unweighted UniFrac. The calculation of construction of weighted pair-group method with arithmetic mean (UPGMA) trees was done using QIIME and R package software.

Statistical analysis

All data were examined for normality by Shapiro–Wilk test and QQ plots. Replicate served as the experimental unit. Student's *t*-test was used to analyze the data of hematology parameters as well as the alpha-diversity and beta-diversity from fecal microbiota by SPSS software (version 21.0). The results were presented as the means ± standard deviation. Spearman's analysis was used to evaluate the correlations between fecal microbiota and immunology parameters. Moreover, the growth performance parameters were analyzed by a MIXED procedure for repeated measurements at different sampling timepoints in which the statistical model accounted for the main effects of treatment, time, and their interaction. Tukey's *post-hoc* test was used to separate means among treatments. Variability in the data of growth performance was expressed as the standard error of means. A probability value below 0.05 was taken to denote statistical significance.

Results

Ultra-early weaning had negative effects on the growth performance of piglets, of which piglets in the group of 14W had lower body weight on days 28 (P = 0.027) and 70 (P = 0.001) of age, ADG during days 14–28 (P = 0.013), days 29–70

TABLE 2 Effects of ultra-early weaning on the growth performance in post-weaning piglets measured at different timepoints.

Items	28W[1]	14W[2]	SEM[3]	*P*-value		
				Time	Treatment	Time × treatment
Body weight, kg						
Day 14 of age	2.53	2.43	0.108		0.516	
Day 28 of age	6.09	5.29	0.205		0.027	
Day 70 of age	22.56	18.03	0.551		0.001	
Mean	10.39	8.58	0.164	<0.001	<0.001	<0.001
Average daily gain, g						
Days 14–28 of age	237.25	190.42	10.025		0.013	
Days 29–70 of age	401.55	310.88	12.101		0.001	
Days 14–70 of age	357.54	278.62	9.179		0.001	
Mean	332.12	259.97	5.020	<0.001	<0.001	0.043
Average daily feed intake, g						
Days 14–28 of age		232.09				
Days 29–70 of age	600.89	508.77	12.758		0.001	
Days 14–70 of age		370.43				
Feed efficiency[4]						
Days 14–28 of age		0.83				
Days 29–70 of age	0.67	0.61	0.017		0.028	
Days 14–70 of age		0.75				
Growth rate[5]						
Days 14–28 of age	2.42	2.18	0.075		0.020	
Days 29–70 of age	3.73	3.42	0.121		0.048	
Days 14–70 of age	8.99	7.44	0.337		0.006	
Mean	5.044	4.345	0.095	<0.001	<0.001	<0.001

[1]Piglets weaned on day 28 of age.
[2]Piglets weaned on day 14 of age.
[3]Standard error of means.
[4]Feed efficiency was calculated as the ratio of feed to gain.
[5]Growth rate was calculated as the ratio of final body weight to initial body weight.

(P = 0.001), and days 14–70 (P = 0.001) of age, growth rate during days 14–28 (P = 0.020), days 29–70 (P = 0.048), and days 14–70 (P = 0.006) of age, ADFI during days 29–70 of age (P = 0.001), and feed efficiency during days 29–70 of age (P = 0.028) in comparison with those in the group of 28W (Table 2). Additionally, there was a significant time effect for body weight ($P < 0.001$), ADG ($P < 0.001$), and growth rate ($P < 0.001$). The mean value of body weight ($P < 0.001$), ADG ($P < 0.001$), and growth rate ($P < 0.001$) from the group of 14W was lower than that from the group of 28W. Interactions between time and treatment were also observed for body weight ($P < 0.001$), ADG (P = 0.043), and growth rate ($P < 0.001$).

On day 28 of age, piglets from the group of 14W had higher serum IgA (P = 0.028), IgG (P = 0.041), and IgM (P = 0.047) concentrations and lower serum albumin (P = 0.002) and total protein (P = 0.004) concentrations as well as albumin-to-globulin ratio (P = 0.003) than those from the group of 28W (Table 3).

As observed in Table 4, the results indicated that piglets from the group of 14W had lower Chao1 index (P = 0.002) and Ace index (P = 0.004) than those from 28W group on day 28 of age. However, the Shannon and Simpson diversity did not differ among the groups at different sampling timepoints. In addition, observed species (P = 0.008) in the group of 14W was lower than that in the group of 28W on day 28 of age (Table 4).

Spearman's correlations analysis between fecal microbiota and serum immunoglobulin parameters indicated that fecal microbiota in the level of genus were correlated with the serum immunoglobulin parameters (Table 5). Among them, on day 28 of age, the richness of *Prevotellaceae_NK3B31_group* was positively correlated with the concentrations of IgA (P = 0.005); the richness of *Prevotella* was positively correlated with the

TABLE 3 Effects of ultra-early weaning on the serum biochemical indicators in post-weaning piglets.

Items	28W[1]	14W[2]	*P*-value
Immunoglobulin A, mg/L			
Day 28 of age	684.44 ± 37.36	825.71 ± 49.83	0.028
Day 70 of age	768.57 ± 74.10	649.52 ± 54.74	0.139
Immunoglobulin G, g/L			
Day 28 of age	19.92 ± 1.09	22.97 ± 0.88	0.041
Day 70 of age	19.09 ± 0.48	17.84 ± 0.66	0.101
Immunoglobulin M, g/L			
Day 28 of age	14.40 ± 0.90	17.04 ± 1.04	0.047
Day 70 of age	13.76 ± 0.77	14.48 ± 0.28	0.282
Albumin, g/L			
Day 28 of age	36.23 ± 4.52	23.35 ± 1.76	0.002
Day 70 of age	32.30 ± 1.50	30.43 ± 2.29	0.220
Globulin, g/L			
Day 28 of age	16.55 ± 0.81	16.58 ± 1.14	0.973
Day 70 of age	18.33 ± 0.86	18.38 ± 1.76	0.961
Albumin to globulin ratio			
Day 28 of age	2.19 ± 0.30	1.41 ± 0.11	0.003
Day 70 of age	1.76 ± 0.11	1.67 ± 0.21	0.433
Total protein, g/L			
Day 28 of age	52.78 ± 4.16	40.18 ± 3.56	0.004
Day 70 of age	50.63 ± 1.86	48.80 ± 3.00	0.341

The results were presented as mean ± standard deviation.
[1]Piglets weaned on day 28 of age.
[2]Piglets weaned on day 14 of age.

TABLE 4 Summary of next generation sequencing data and effects of ultra-early weaning on diversity and abundance indexes at each sampling time in post-weaning piglets.

Alpha diversity indexes	28W[1]	14W[2]	*P*-value
Observed species			
Day 28 of age	754.33 ± 107.53	596.67 ± 45.56	0.008
Day 70 of age	759.33 ± 134.77	666.33 ± 99.52	0.204
Shannon index			
Day 28 of age	5.56 ± 0.61	5.01 ± 0.57	0.139
Day 70 of age	6.17 ± 0.20	6.26 ± 0.12	0.365
Simpson index			
Day 28 of age	0.92 ± 0.04	0.88 ± 0.06	0.155
Day 70 of age	0.95 ± 0.01	0.97 ± 0.01	0.130
Chao1 index			
Day 28 of age	919.79 ± 140.35	653.51 ± 74.67	0.002
Day 70 of age	768.13 ± 140.16	679.52 ± 101.57	0.238
ACE index			
Day 28 of age	916.83 ± 145.99	673.47 ± 72.69	0.004
Day 70 of age	787.97 ± 148.60	699.02 ± 104.76	0.258

The results were presented as mean ± standard deviation.
[1]Piglets weaned on day 28 of age.
[2]Piglets weaned on day 14 of age.

TABLE 5 Spearman's correlations analysis between fecal microbiota and serum immunoglobulin parameters.

Variables	IgA	IgG	IgM
Day 28 of age			
Prevotellaceae_NK3B31_group	0.943**	0.771	0.657
Christensenellaceae_R.7_group	−0.771	−0.600	−0.657
Prevotella	0.600	0.771	0.886**
Agathobacter	0.829*	0.657	0.486
Desulfovibrio	−0.771	−0.600	−0.657
Prevotellaceae_UCG.003	0.600	0.886**	0.714
Phascolarctobacterium	0.029	0.086	−0.029
Day 70 of age			
Prevotella	0.086	0.771	−0.406
Prevotellaceae_UCG.003	−0.543	−0.829*	0.406
Bacteroides	−0.371	−0.943**	0.464
Phascolarctobacterium	−0.486	−0.600	0.290
Solobacterium	−0.429	−1.000	0.232

IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M. *$P < 0.05$; **$P < 0.01$.

concentrations of IgM ($P = 0.019$); the richness of *Agathobacter* was positively correlated with the concentrations of IgA ($P = 0.042$); and the richness of *Prevotellaceae_UCG.003* was positively correlated with the concentrations of IgG ($P = 0.019$). On day 70 of age, the richness of *Prevotellaceae_UCG.003* ($P = 0.042$) and *Bacteroides* ($P = 0.005$) was negatively correlated with the concentrations of IgG.

The Venn diagram showed the distribution of bacterial unique OTUs among the groups based on the 16S rRNA gene sequencing analysis, and it visualized the distribution of shared and unique OTUs among the groups (the numbers within the Venn diagram represented the total number of OTUs in that community). Different colors represented different groups, and the number in the middle represented the number of OTUs shared by all groups. The distribution of fecal microbiota of piglets on day 28 of age is presented in Figure 1A, while that of piglets on day 70 of age is presented in Figure 1B. As shown in Figure 1A, 704 bacterial OTUs were shared among the groups, and the 587 unique OTUs in CA group (sample from piglets in 28W group on the timepoint of day 28 of age; 28W-28) and the 289 unique OTUs in TA group (sample from piglets in 14W group on the timepoint of day 28 of age; 14W-28) were observed. Additionally, as shown in Figure 1B, a total of 867 OTUs were identified by the Venn diagram as common to the treatments. The unique OTUs in CB group (sample from piglets in 28W group on the timepoint of day 70 of age; 28W-70) were 416, whereas those in TB group (sample from piglets in 14W group on the timepoint of day 70 of age; 14W-70) were 264.

The rank abundance (Figures 2A,D), rarefaction curves (Figures 2B,E), and species accumulation boxplot (Figures 2C,F) were adopted to access the richness of fecal bacteria community in each group and showed that the observed species gradually tend to be flat as the sample size increased, which indicated that the amount of data to be sequenced was reasonable, and the subsequent data and index analyses can be performed.

No statistical differences in beta-diversity indices (Bray–Curtis, Figures 3A,C; unweighted UniFrac, Figures 3B,D) have been observed based on the *t*-test at different sampling timepoints.

The unweighted pair-group method with arithmetic mean cluster tree based on the unweighted UniFrac distance was used to examine the similarity between samples at phylum level. On day 28 of age (Figure 4A), *Bacteroidota* and *Firmicutes* were predominated and the remaining bacterial sequences were mainly assigned to *Proteobacteria*, *Spirochaetota*, *unidentified_Bacteria*, *Actinobacteriota*, *Desulfobacterota*, *Euryarchaeota*, *Synergistota*, and *Acidobacteriota*. On day 70 of age (Figure 4B), *Firmicutes* and *Bacteroidota* were predominant and the remaining bacterial sequences were mainly assigned to *Spirochaetota*, *unidentified_Bacteria*, *Campilobacterota*, *Fibrobacterota*, *Proteobacteria*, *Actinobacteriota*, *Acidobacteriota*, and *Chloroflexi*.

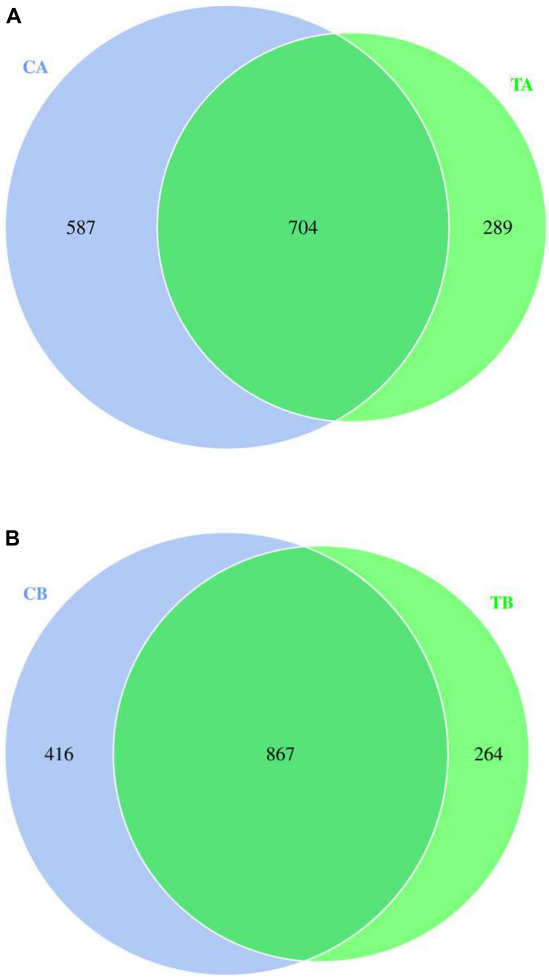

FIGURE 1
Venn graph for intestinal microbiota of piglets weaned on day 14 of age (14W) and day 28 of age (28W) at different sampling timepoints [day 28 of age **(A)**; day 70 of age **(B)**]. CA group was defined as the sample from piglets in 28W group at the timepoint of day 28 of age. TA group was defined as the sample from piglets in 14W group at the timepoint of day 28 of age **(A)**. CB group was defined as the sample from piglets in 28W group at the timepoint of day 70 of age. TB group was defined as the sample from piglets in 14W group at the timepoint of day 70 of age **(B)**.

The 10 most abundant bacteria of fecal microbiota at genus level are shown in Figure 5. As shown in Figure 5A, the predominant bacteria on day 28 of age were mainly involved in *Bacteroides*, *Treponema*, *UCG-002*, *Christensenellaceae_R-7_group*, *Escherichia–Shigella*, *Lactobacillus*, *Parabacteroides*, *Clostridium_sensu_stricto_1*, *Prevotellaceae_NK3B31_group*, and *Prevotella*. The top 10 predominant bacteria at day 70 of age were mainly involved in *Prevotellaceae_UCG-003*, *Prevotellaceae_NK3B31_group*, *Terrisporobacter*,

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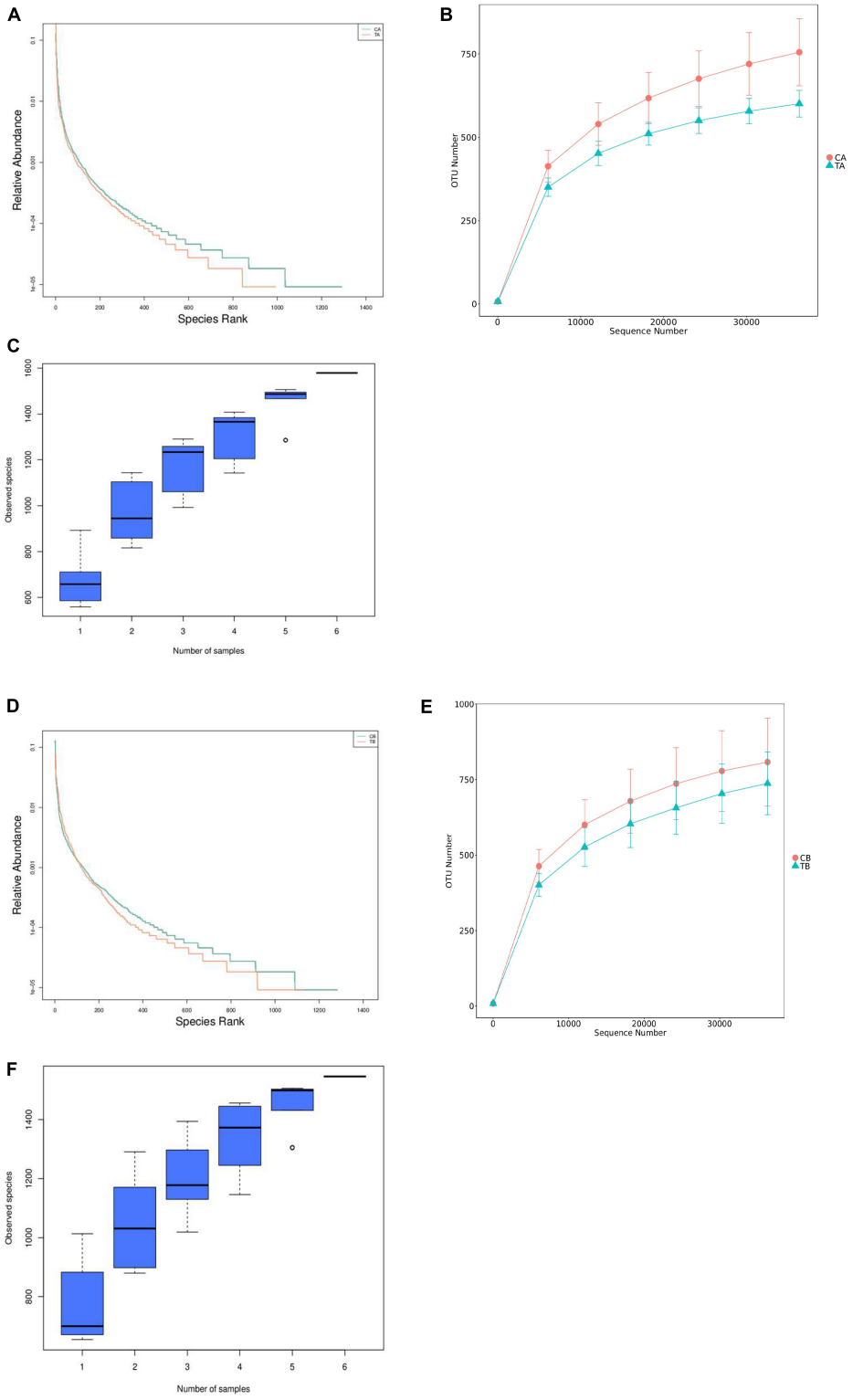

FIGURE 2
Rank abundance, rarefaction curves, and species accumulation boxplot for intestinal microbiota of piglets weaned on day 14 of age (14W) and day 28 of age (28W) at different sampling timepoints [day 28 of age **(A–C)**; day 70 of age **(D–F)**]. CA group was defined as the sample from piglets in 28W group at the timepoint of day 28 of age. TA group was defined as the sample from piglets in 14W group at the timepoint of day 28 of age **(A–C)**. CB group was defined as the sample from piglets in 28W group at the timepoint of day 70 of age **(D–F)**. TB group was defined as the sample from piglets in 14W group at the timepoint of day 70 of age.

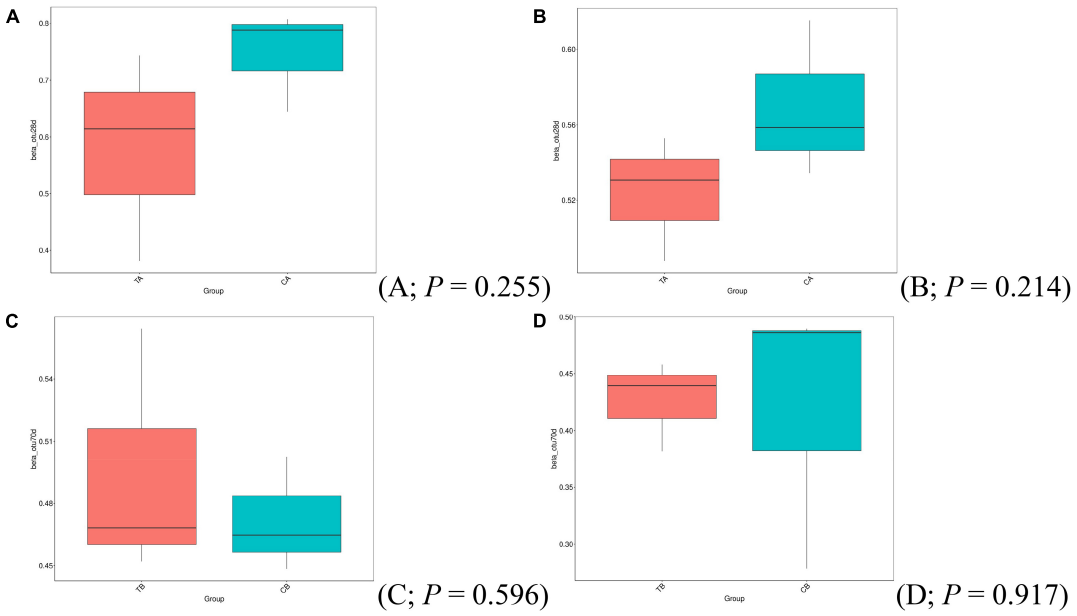

FIGURE 3
Beta-diversity analysis of Bray–Curtis **(A,C)** and unweighted UniFrac **(B,D)** using *t*-test for intestinal microbiota of piglets on day 14 of age (14W) and day 28 of age (28W) at different sampling timepoints [day 28 of age **(A,B)**; day 70 of age **(C,D)**]. CA group was defined as the sample from piglets in 28W group at the timepoint of day 28 of age. TA group was defined as the sample from piglets in 14W group at the timepoint of day 28 of age **(A,B)**. CB group was defined as the sample from piglets in 28W group at the timepoint of day 70 of age. TB group was defined as the sample from piglets in 14W group at the timepoint of day 70 of age **(C,D)**.

Alloprevotella, *Lactobacillus*, *Faecalibacterium*, *Treponema*, *Rikenellaceae_RC9_gut_group*, *Clostridium_sensu_stricto_1*, and *Prevotella* (**Figure 5B**).

Heatmap annotations are important components of a heatmap that show additional information associated with rows of columns. ComplexHeatmap provides very flexible support for setting annotations and defining new annotation graphics. On day 28 of age (**Figure 6A**), in comparison with TA group (14W-28), the richness of *Desulfovibrio* (P = 0.015) and *Christensenellaceae_R-7_group* (P = 0.044) was significantly upregulated and that of *Prevotellaceae_NK3B31_group* (P = 0.045), *Phascolarctobacterium* (P = 0.033), *Prevotella* (P = 0.045), *Prevotellaceae_UCG-003* (P = 0.013), and *Agathobacter* (P = 0.034) was significantly downregulated in the group of CA (28W-28). On day 70 of age (**Figure 6B**), the richness of *Solobacterium* (P = 0.049), *Bacteroides* (P = 0.015), *Prevotellaceae_UCG-003* (P = 0.033), and *Phascolarctobacterium* (P = 0.046) was significantly upregulated and that of *Prevotella* (P = 0.022) was significantly downregulated in the group of TB (14W-70) in comparison with those in the CB group (28W-70).

Discussion

The lengths of suckling period have been reported to affect the growth performance of piglets during post-weaning (Massacci et al., 2018; Faccin et al., 2020a,b). Collins et al. (2010) reported that piglets receiving 21-day suckling period had two times higher ADFI and weight gain than those receiving 13-day suckling period during the 11 days post-weaning. Faccin et al. (2020b) noted that the impairment of body weight and feed efficiency induced by weaning was ameliorated by prolonging the suckling period. Dunshea et al. (2002) found that piglets weaned on day 25 of age had a higher growth rate than those weaned on day 17 of age during 3 weeks post-weaning. In this study, we have also observed the worst growth performance of piglets in the ultra-early weaning group. Some studies attributed this growth performance impairment to the reduction in voluntary feed intake (Van der Meulen et al., 2010; Ming et al., 2021). Main et al. (2004) noted that prolonging suckling period would increase the acceptability of solid feed during post-weaning. The reduction in ADFI was also observed in this study; therefore, we speculated that the impairment of growth performance induced by shortening suckling period was related to the decrease in feed intake. Additionally, we observed that the growth performance impairment continued to day 70 of age, which means the growth impairment effect persisted throughout the overall experimental periods. Interactions between time and treatment were also observed for body weight, ADG, and growth rate in this study, which indicated that the impairment of growth performance was aggravated with the passage of time. Conversely, Partanen et al. (2007) reported that piglets allowed to suckle until day 36 of

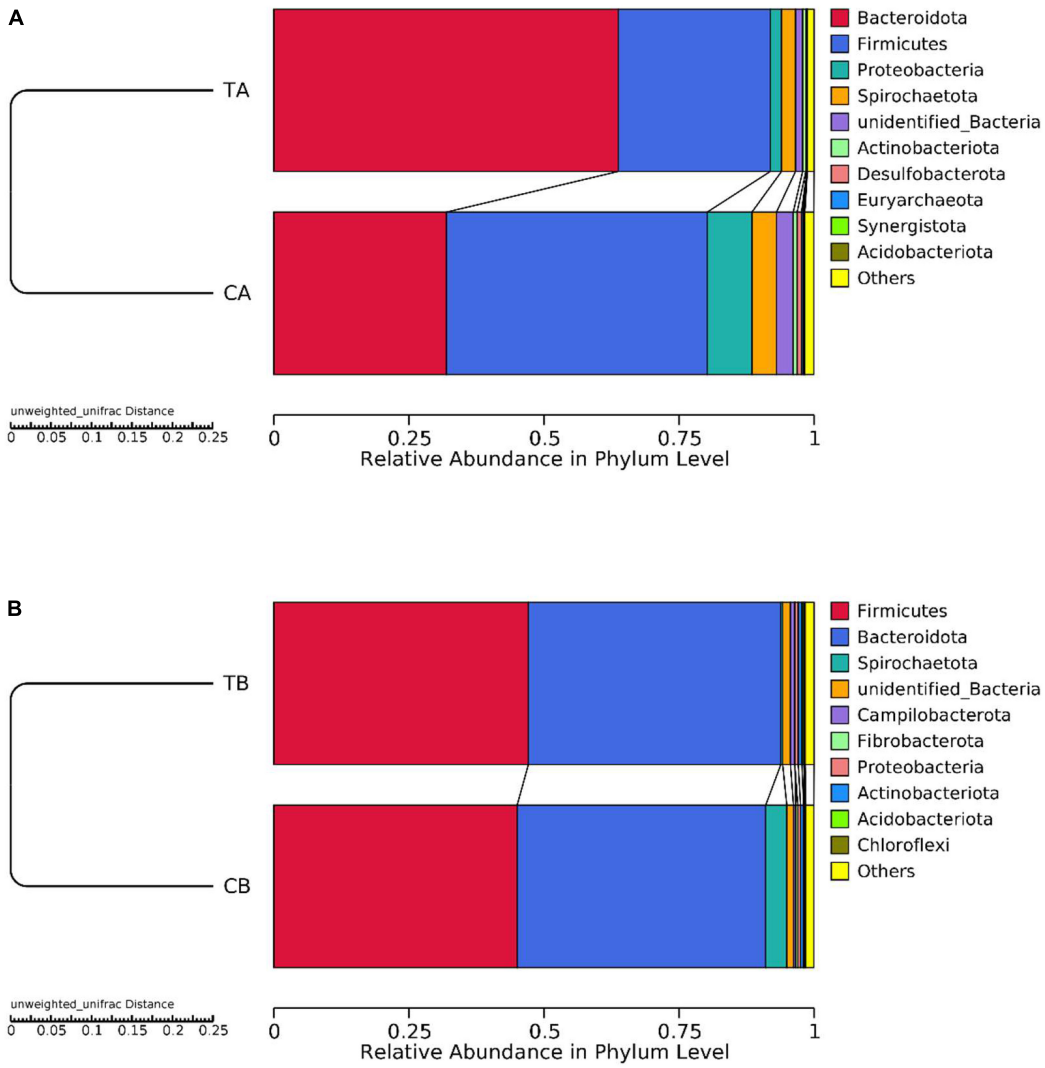

FIGURE 4
Taxonomic distribution at phylum level for intestinal microbiota of piglets weaned on day 14 of age (14W) and day 28 of age (28W) at different sampling timepoints [day 28 of age **(A)**; day 70 of age **(B)**]. The "others" represents the sum of the relative abundance except the top 10 in the figure. CA group was defined as the sample from piglets in 28W group at the timepoint of day 28 of age. TA group was defined as the sample from piglets in 14W group at the timepoint of day 28 of age **(A)**. CB group was defined as the sample from piglets in 28W group at the timepoint of day 70 of age. TB group was defined as the sample from piglets in 14W group at the timepoint of day 70 of age **(B)**.

age had a heavier body weight and a growth rate than those weaned on day 26 of age; however, the difference in body weight was diminished on day 49 of age. We have not observed the short-term growth impairment effect as similar to the above studies, which was probably due to the difference in genetic background. As the genetic background plays a key role in affecting individual differences (Alexander et al., 2008), this study was the first time to evaluate the effects of shortening suckling period on growth performance of piglets with the same genetic background. We considered that ultra-early weaning had negative effects on the growth performance of piglets, which was partially attributed to the reduction in voluntary feed intake.

Additionally, the composition of intestinal microbiota plays a close relationship with growth performance. Thousands of organism communities constitute the gut microbiome. The large diversity of the microbiota contributes to the development and metabolic needs of the host. Piglets have not established stable intestinal microbiota during the early life. It is reported that shortening suckling period of piglets would decrease the resistance to the pathogenic *Escherichia coli*, which was manifested in severe diarrhea, body weight reduction, and pathogen shedding (Wellock et al., 2008; McLamb et al., 2013). In addition, some studies indicated that shortening suckling periods of piglets would lead to the enrichment of harmful bacteria in the feces (Leliveld et al., 2013; Xu et al., 2014).

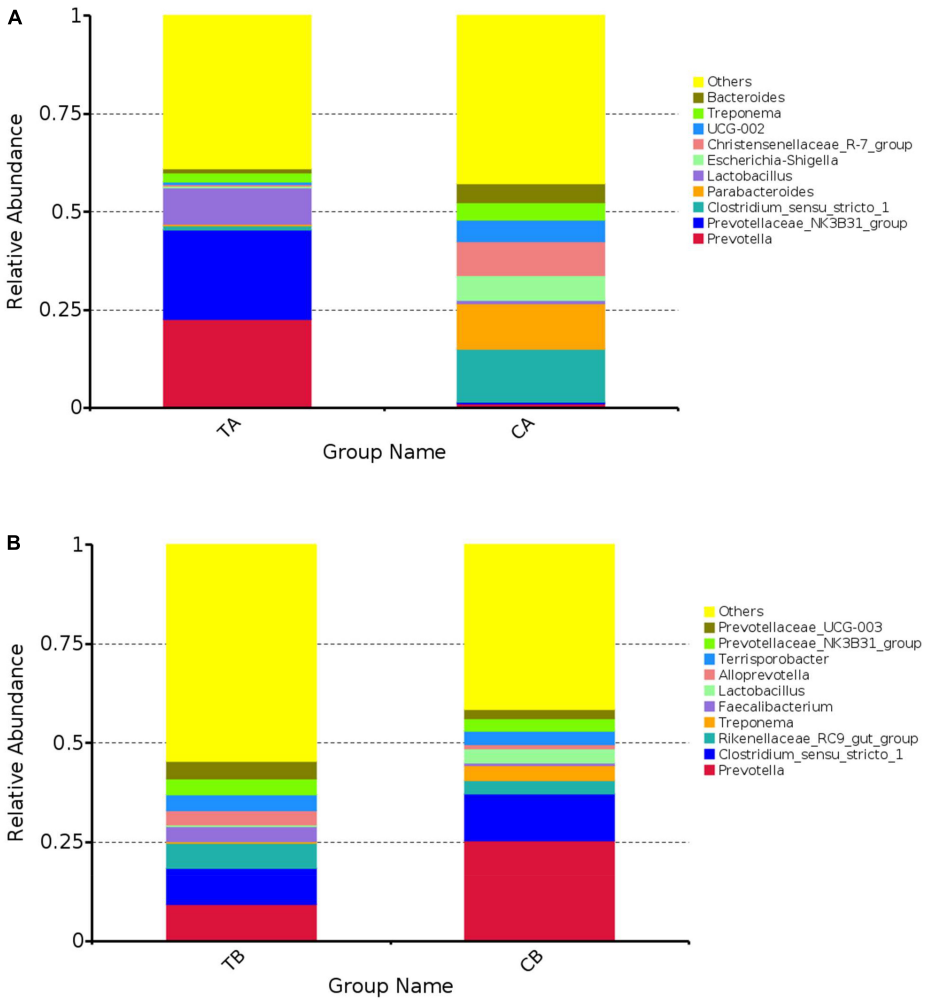

FIGURE 5
The 10 most abundant bacteria of gut microbiota at genus level for intestinal microbiota of piglets weaned on day 14 of age (14W) and day 28 of age (28W) at different sampling timepoints [day 28 of age **(A)**; day 70 of age **(B)**]. The horizontal axis is the groups. The vertical axis represents the relative abundance. The "others" represents the sum of the relative abundance except the top 10 in the figure. CA group was defined as the sample from piglets in 28W group at the timepoint of day 28 of age. TA group was defined as the sample from piglets in 14W group at the timepoint of day 28 of age **(A)**. CB group was defined as the sample from piglets in 28W group at the timepoint of day 70 of age. TB group was defined as the sample from piglets in 14W group at the timepoint of day 70 of age **(B)**.

Therefore, the duration of suckling period, also named weaning age, plays an important role in regulating the intestinal microbiota (Yang et al., 2018). Breast milk oligosaccharides have been shown to stimulate the growth of bifidobacteria and lactobacilli in the intestine of infants (Villares, 2008). Moreover, breast milk also presents various immunostimulatory factors, anti-inflammatory factors, and antimicrobial substances (Blewett et al., 2008). Therefore, the components presented in the milk will affect the intestinal microbiota to some extent (Schack-Nielsen and Michaelsen, 2007). The Chao1 index and Ace index are two indicators used to estimate species richness of intestinal microbiota. The Shannon and Simpson diversity values are indices used to estimate the microbial diversity in the samples. The bacterial community was analyzed following high-throughput pyrosequencing of 16S rRNA genes, and we found that ultra-early weaning led to a reduction in intestinal microbiota richness; however, this reduction effect was only observed on day 28 of age, but not on day 70 of age, which indicated that ultra-early weaning temporarily affected the richness of intestinal microbiota in piglets. This result was affirmed by the studies of Massacci et al. (2018) and Holman et al. (2021). Similarly, Massacci et al. (2018) observed a higher alpha-diversity in intestinal microbiota caused by prolonging suckling periods. Animals with richer microbiota are capable of increasing the resistance to enteric diseases during post-weaning period and possibly providing a competitive advantage to piglets (Massacci et al., 2018). In this study, the *Firmicutes* and *Bacteroidetes* were the dominant phyla in both timepoints,

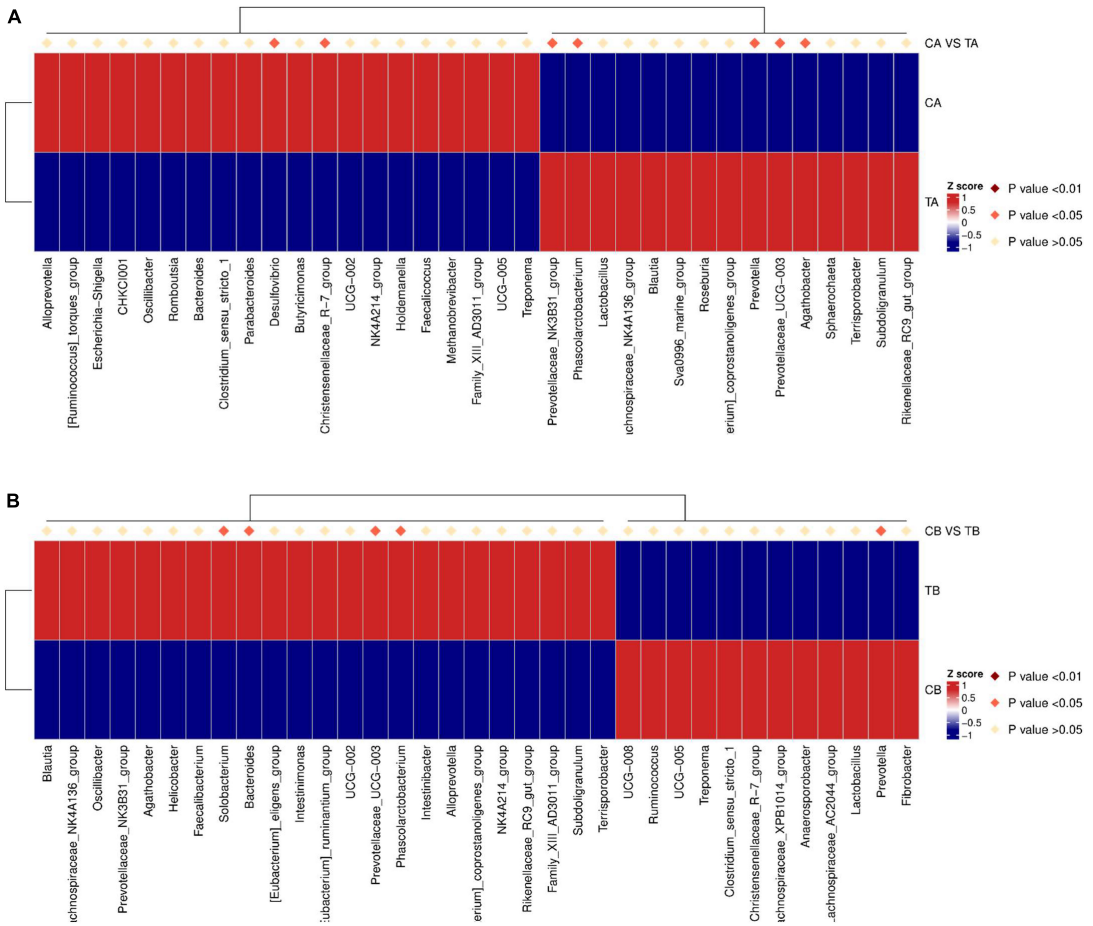

FIGURE 6
Top 35 species abundance clustering with ComplexHeatmap (genus level) for intestinal microbiota of piglets weaned on day 14 of age (14W) and day 28 of age (28W) at different sampling timepoints [day 28 of age **(A)**; day 70 of age **(B)**]. Underside represents the species annotation information. Upside represents the contrast between the groups. The corresponding value of the heat map is the Z value of the relative abundance of species in each row after the normalization treatment. CA group was defined as the sample from piglets in 28W group at the timepoint of day 28 of age. TA group was defined as the sample from piglets in 14W group at the timepoint of day 28 of age **(A)**. CB group was defined as the sample from piglets in 28W group at the timepoint of day 70 of age. TB group was defined as the sample from piglets in 14W group at the timepoint of day 70 of age **(B)**.

which was affirmed by the study of Yang et al. (2018). In addition, on day 28 of age, ultra-early weaning led to an increase in bacteria related to the production of short-chain fatty acids (SCFA), such as *Agathobacter* (Horvath et al., 2021), *Prevotellaceae_NK3B31_group* (Shang et al., 2021), *Prevotella* (Yang et al., 2018), and *Phascolarctobacterium* (Yang et al., 2018). The production of SCFA is important in energy homeostasis (Schwiertz et al., 2010; Blaut, 2015). In addition, ultra-early weaning led to an increase in intestinal *Prevotellaceae_UCG-003*, which is closely related to polysaccharide, protein, energy, and vitamin metabolism (Cui et al., 2022), and a decrease in *Desulfovibrio* and *Christensenellaceae_R.7_group*, which are the bacteria involved in inducing bowel disease (Marini et al., 2002; Mancabelli et al., 2017). On day 70 of age, ultra-early weaning led to an increase in *Bacteroides* (Yang et al., 2018) and *Phascolarctobacterium* (Yang et al., 2018), which are related to the production of SCFA, as well as *Prevotellaceae_UCG-003*, which is related to polysaccharide, protein, energy, and vitamin metabolism (Cui et al., 2022), but a decrease in *Prevotella*, which is involved in infections (Giri and Mangalam, 2019). Therefore, ultra-early weaning seems to increase beneficial bacteria and decrease harmful bacteria in the intestine and thus benefit establishing a healthy intestinal microbiota during the early life of piglets, which was affirmed by the study of Smith et al. (2008); shortening suckling period would decrease the risk of the vertical transmission of pathogens from sows to offspring.

On the contrary, the intestinal microbiota compositions are closely related to the immune status of the host. Most bacterial species are capable of inducing a strong host immunity response (Macpherson et al., 2005). Immunoglobulin is one of the important components in the immunity system, which mainly exists in the serum and intestinal mucosa. In this

study, we investigated the effects of ultra-early weaning on the contents of serum IgA, IgG, and IgM. We found that ultra-early weaning had positive effects on the immunological parameters on day 28 of age, but not on day 70 of age. Altering the lengths of suckling period has been demonstrated to affect the immune status of piglets (Cao et al., 2022). Salak-Johnson and Webb (2018) noted that piglets allowed 28-day suckling period had higher serum immunoglobulin contents than those weaned on day 14 of age. Some studies noted that shortening suckling period was capable of decreasing the serum IgA contents (Levast et al., 2010; Smith et al., 2010; Cao et al., 2022). Levast et al. (2010) demonstrated that the reduction in serum IgA levels induced by shortening suckling period was closely related to the intestinal environment. In this study, on day 28 of age, we found that the content of IgA was positively correlated with the abundance of *Prevotellaceae_NK3B31_group* and *Agathobacter*, that of IgG was positively correlated with the abundance of *Prevotellaceae_UCG.003*, and that of IgM was positively correlated with the abundance of *Prevotella*. However, no bacteria were positively correlated with the immunological parameters on day 70 of age. Therefore, we considered that the variation of intestinal microbiota composition induced by shortening suckling period would activate the immune system during the early life of piglets, which was manifested in the increase in serum immunoglobulin levels; however, this activation effect was not long term, but temporary.

Serum biochemical parameters including albumin, globulin, and total protein can be used as an indicator to indicate the situation of protein synthesis and nutritional status *in vivo* (Park and Kim, 2019). In this study, we observed low albumin and total protein contents as well as albumin-to-globulin ratio in the group of 14W in comparison with those in the group of 28W on day 28 of age, but not on day 70 of age. Similarly, Tao et al. (2016) and Hohenshell et al. (2000) reported that the effects of shortening suckling period on serum biochemical indicators were temporary and could be corrected to normal levels within some time post-weaning. This indicated that shortening suckling period will cause a malnutrition status for piglets, which was probably the reason for growth retardation as observed in this study. The malnutrition of piglets during early life may affect the development of other organs, which allows a severe challenge for the further growth of piglets.

Conclusion

This study demonstrated that shortening suckling period of piglets had a long-term effect on the impairment of growth performance, whereas it had a short-term effect on the increase in serum immunoglobulin parameters as well as the decrease in serum biochemical indicators and intestinal species abundance. Additionally, we observed that ultra-early weaning was capable of increasing the intestinal beneficial bacteria, but decreasing the pathogenic bacteria. Therefore, we considered that ultra-early weaning had positive effects on the immunity status and intestinal microbiota composition in piglets, but negative effects on the growth performance, nutritional status, and intestinal microbiota abundance. In the aspect of growth performance, the lower the weaning weight, the longer the time needed to reach marketing weight will be, which inevitably impaired profitability. However, we did observe an optimization of intestinal microbiota composition in piglets caused by shortening suckling period. Combining ultra-early weaning with other nutritional strategies may be an appropriate strategy to improve the overall post-weaning performance in piglets.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Experimental protocol and the process were approved and supervised by the Animal Care and Use Committee of Jinzhou Medical University (Jinzhou, China).

Author contributions

DD and CL were involved in writing – original draft, investigation, and writing – review and editing. SL, XF, and WX were involved in formal analysis and investigation. YC and DL were involved in conceptualization, methodology, supervision, and writing – review and editing. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Microbial regulation of offspring diseases mediated by maternal-associated microbial metabolites

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The microbiota plays a crucial role in individuals' early and long-term health. Previous studies indicated that the microbial regulation of health may start before birth. As the *in utero* environment is (nearly) sterile, the regulation is probably be originated from maternal microbiota and mediated by their metabolites transferred across the placenta. After the birth, various metabolites are continuously delivered to offspring through human milk feeding. Meanwhile, some components, for example, human milk oligosaccharides, in human milk can only be fermented by microbes, which brings beneficial effects on offspring health. Hence, we speculated that human milk-derived metabolites may also play roles in microbial regulation. However, reports between maternal-associated microbial metabolites and offspring diseases are still lacking and sparsely distributed in several fields. Also, the definition of the maternal-associated microbial metabolite is still unclear. Thus, it would be beneficial to comb through the current knowledge of these metabolites related to diseases for assisting our goals of early prediction, early diagnosis, early prevention, or early treatment through actions only on mothers. Therefore, this review aims to present studies showing how researchers came to the path of investigating these metabolites and then to present studies linking them to the development of offspring asthma, type 1 diabetes mellitus, food allergy, neonatal necrotizing enterocolitis, or autism spectrum disorder. Potential English articles were collected from PubMed by searching terms of disease(s), maternal, and a list of microbial metabolites. Articles published within 5 years were preferred.

KEYWORDS

short-chain fatty acids (SCFAs), tryptophan derivatives, indole derivatives, branched-chain fatty acids (BCFAs), succinate, bile acid derivatives, trimethylamine-*N*-oxide (TMAO), human milk oligosaccharides (HMOs)

Introduction

The microbiota plays a crucial role in individuals' early and long-term health *via* activating and developing the immune system, developing the central nervous system, and digesting and metabolizing food (Yao et al., 2021). When *in utero*, offspring are protected by maternal immunity and meanwhile they develop their own immunity. The initial development may be partly stimulated by maternal microbiota. Compared with the germ-free group, transiently gestational colonization maternal mice have given birth to germ-free neonates with altered intestinal mucosal innate immune composition and intestinal mucosal transcriptional signatures (Gomez de Agüero et al., 2016). Although the *in utero* environment is sterile or may carry a very tiny number of microbes, various microbial metabolites have been detected in animal umbilical cord blood, placenta, fetal intestine, fetal brain (Vuong et al., 2020; Bi et al., 2021), and human fetal intestine (Li et al., 2020), which sheds us a light that influences of maternal microbiota on fetal health development might be mediated by their metabolites. Macpherson et al. (2017) and Ganal-Vonarburg et al. (2020) have also supported the fact that the development of the fetal immune system was driven by microbial metabolites rather than live micro-organisms.

After birth, mothers can continuously strongly influence their offspring's health through human milk feeding. In the first 6 months, human milk, containing nutrients, antibodies, bioactive components, microbiota, metabolites, and human milk oligosaccharides (HMOs), fully covers an infant's needs for nutrition, energy, and protection (Singh et al., 2021). Also, bacterial metabolites in milk, such as short-chain fatty acids (SCFAs), indoles, 12, 13-dihydroxy-9Z-octadecenoic acid (12, 13-DiHOME), and methylamines, have been reported to impact offspring health (Gao et al., 2021; Stinson and Geddes, 2022). As the third most abundant solid component in human milk, HMOs are believed to provide nutrition not to infants but to infant microbiota (Sanchez et al., 2021). Lack of gut bifidobacteria or HMO-utilization genes was related to systemic inflammation in breastfed individuals that have been mitigated by the supplementation of *Bifidobacterium infantis* EVC001 fermenting HMOs into the metabolite indole-3-lactic acid (ILA) (Henrick et al., 2021). Conceptually, the HMOs-derived metabolites probably produced in the infant gut belong to infant metabolites, but HMOs intake by breastfed infants are from human milk, so they can also be considered maternal-associated microbiota metabolites. In general, we could say during pregnancy and lactation stage maternal-associated microbial metabolites may influence offspring health.

Previous studies collectively supported the hypothesis of Developmental Origins of Health and Disease (DOHaD) (Waterland and Michels, 2007). This hypothesis proposes that a critical window during prenatal and postnatal exists for stimuli to influence developmental pathways causing permanent changes in certain diseases' susceptibility. Since late-postnatal interventions or treatments cannot cure certain diseases but only reduce the syndromes, increasing researchers have speculated that their critical window may appear during very early life, i.e., prenatal stage and lactation stage. For example, supplementation with fish oil during pregnancy has reduced offspring asthma risk (Bisgaard et al., 2016). Current pieces of evidences also supported that maternal diet showed strong impacts on maternal gut microbial composition and further influenced fetal immune development, which was probably mediated by microbial metabolites (Gray et al., 2017; Alsharairi, 2020a).

These metabolites or milk-derived substrates could be detected in maternal blood or human milk, so they are promising indicators or biomarkers to guide us if maternal microbial intervention to regulate offspring diseases is in a good direction or at a sufficient level, which will assist our goal of early prediction, early diagnosis, early prevention, or early treatment through actions only on mothers. And the relevant metabolites from milk-derived substrates will improve our understanding of disease development. But reports between maternal-associated microbial metabolites and offspring diseases are still lacking and sparsely distributed in several fields. Also, the definition of the maternal-associated microbial metabolite is still unclear. Thus, it would be beneficial to comb through the current relevant knowledge for future investigations. We selected immune-mediated, metabolic, and neurodevelopmental diseases, namely asthma, type 1 diabetes mellitus (T1DM), food allergy (FA), neonatal necrotizing enterocolitis (NEC), and autism spectrum disorder (ASD). The exact causes of these diseases are still largely unknown, but both genetic and environmental factors, specifically microbiota, are believed as contributors to their development.

Therefore, this review aims to present studies showing how researchers came to the path of investigating maternal-associated microbial metabolites and then to present studies linking them to the development of offspring asthma, T1DM, FA, NEC, and ASD.

Search strategy

We identified potential relevant articles from PubMed utilizing the following terms: (asthma/type 1 diabetes/food allergy/necrotizing enterocolitis/autism) AND (maternal)

Abbreviations: AHR, aryl hydrocarbon receptor; ASD, autism spectrum disorder; BCFAs, branched-chain fatty acids; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; DOHaD, developmental origins of health and disease; DSLNT, disialyllacto-*N*-tetraose; FA, food allergy; HMOs, human milk oligosaccharides; ILA, indole-3-lactic acid; LGG, *Lacticaseibacillus rhamnosus* GG; NEC, neonatal necrotizing enterocolitis; SCFAs, short-chain fatty acids; TLR, toll-like receptor; TMAO, trimethylamine-*N*-oxide; Trp, tryptophan; T1DM, type 1 diabetes mellitus.

AND (microbiota/microbial/bacterial/fungal metabolite OR microbiota/microbial/bacterial/fungal molecule OR branched-chain fatty acids OR tryptophan OR aromatic amino acid OR other microbial metabolites from the list summarized by Nicholson et al. (2012), except vitamins, glucose, or urea in the list) NOT (review [Publication Type]) AND (English [Language]) (see **Supplementary material**). The inclusion criteria were original articles, written in English, and published in the last 5 years through 1 September 2022, including mainly animal and human studies. Articles not related to one of the five diseases, maternal, and microbial metabolites/milk-derived substrates were excluded. Additional articles were found from the reference lists of already retrieved publications.

Asthma

Asthma is one of the most common and non-communicable diseases affecting both children and adults with variable respiratory symptoms and variable airflow limitation, like cough, wheezing, shortness of breath, and chest tightness. The current treatment goals are only to minimize the symptom burden and the risk of adverse asthma outcomes (Ducharme et al., 2014; Papi et al., 2018).

Increasing studies have demonstrated that asthma is associated with the environmental indoor microbiome (Fu et al., 2020; Vandenborght et al., 2021) and human nasopharyngeal-, respiratory-, and gastrointestinal microbiome (Huang, 2015; Teo et al., 2015; Frati et al., 2019; Tang et al., 2021; Lee-Sarwar et al., 2022). However, the addition of antibiotics to the conventional treatment of oral corticosteroids has shown rather limited clinical benefits to control asthma exacerbations in both children and adults (Murray et al., 2021). Antibiotic exposure during childhood can reduce the microbial diversity of the host, increase the risk of asthma development, and prolong the symptoms (Toivonen et al., 2021; Kama et al., 2022). On the contrary, the intervention of probiotic *Lacticaseibacillus rhamnosus* GG (LGG) in infants with a high risk of asthma has gained beneficial effects, although the effects last temporarily (Durack et al., 2018). Recently, Alsharairi (2020b) has summarized that various probiotic strains of specific species of *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Enterococcus, Streptococcus, Blautia*, *Ruminococcus*, and *Faecalibacterium prausnitzii* could be strongly impacted by diet and may have the potential to reduce the risk of allergic asthma development. These studies imply that to reduce asthma risk, adding/increasing suitable probiotics or increasing microbial diversity appears more beneficial than erasing microbes without bias.

Recent studies have further indicated that offspring asthma was strongly associated with prenatal micro-organisms exposure. Maternal usage of antibiotics has a dose-related association with offspring asthma risk (Stokholm et al., 2014; Alhasan et al., 2020). Ingestion with probiotic *Bifidobacterium breve* M-16V during pregnancy changed the composition of fecal microbiota in neonates and protected the neonates against allergic airway inflammation accelerated by prenatal exposure to an air pollutant aerosol (Terada-Ikeda et al., 2020). In addition, maternal oral intake of an endotoxin-low lyophilized extract containing multiple toll-like receptor (TLR) ligands derived from a mixture of eight major respiratory tract bacterial pathogens has markedly reduced the susceptibility to allergic airway inflammatory disease in offspring (Mincham et al., 2018).

The above beneficial effects might not directly result from the microbes but be mediated by their microbial products, such as acetate. Acetate is one type of SCFA that is produced by the fermentation of dietary fiber or fermentable fiber, besides other types of fibers and carbohydrates, such as resistant starches or HMOs, collectively termed microbiota-accessible carbohydrates (Gray et al., 2017). The maternal gut microbiota-generated acetate was able to transfer to the fetus across the placenta and led the offspring being more resistant to asthma later in life (Thorburn et al., 2015). Acetate was capable of altering certain gene expressions in the fetal lung, such as the downregulation of gene *Nppa* expression, which suppressed the production of atrial natriuretic peptide, a molecule that participated in epithelial biology and immune regulation. They further demonstrated that maternal intake of a high-fiber diet or acetate protected offspring mice against induced allergic airway disease; however, this effect was failed to see when such intake was given to offspring after birth and throughout lactation. Indirectly, maternal gut bacteria-derived SCFAs, such as butyrate and propionate, separately have shown the ability to reduce stimulation of T lymphocytes *via* regulating dendritic cell function, and then generated a tolerogenic immune feature and enhanced a T-helper 1 (Th1) phenotype protective against asthma development, as asthma is linked to Th2 phenotype dominant (Singh et al., 2010; Singh et al., 2014; Gray et al., 2017). Furthermore, Alsharairi (2020b) has suggested that a very low-calorie ketogenic diet, i.e., an extremely low carbohydrate, high fat, and moderate protein diet, during pregnancy and lactation directly increased the abundance of SCFA-producing microbiota in maternal and infant gut and increased the amount of SCFAs in human milk *via* the entero-mammary pathway, which may contribute to an anti-inflammatory environment and may reduce the risk of infant asthma.

Also, supplementation with fish-oil-derived n-3 long-chain polyunsaturated fatty acids during pregnancy week 22–26 until one week after delivery has led to a 31% reduced risk of asthma in offspring at age of 5 years old (Bisgaard et al., 2016), which may be related to the elevated level of metabolite hydroxy-3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (hydroxy-CMPF) estimated at age 0 (Rago et al., 2019). The furan fatty acid metabolite CMPFs, a result of microbiome activity, are generally beneficial to human health (Xu et al., 2017) and can be vertically transferred to offspring (Olarini et al., 2022); however,

their clinical efficacy and mechanisms in reducing asthma still remain unclear.

Hence, exposure to microbial metabolites, such as acetate and hydroxy-CMPFs, during pregnancy and lactation may be associated with a reduction of offspring asthma risk, whereas more comprehensive studies are required.

Type 1 diabetes mellitus

Type 1 diabetes mellitus is an autoimmune disease that leads to the destruction of pancreatic β-cells that secret insulin (Lucier and Weinstock, 2022). The exact etiology is still largely unknown. Gene factors have well-explained that individuals with a family history of T1DM are more likely to develop T1DM (Ilonen et al., 2019). However, a large increase in the incidence of T1DM has appeared in children in genetically stable populations (Patterson et al., 2019), which supported that environmental factors may also play etiological roles in its development.

Among the environmental factors, microbiota exposure in recent decades has attracted increasing attention due to their capability to mature the immune system (Ilonen et al., 2019). In longitudinal studies, T1DM patients were associated with a decrease in microbial diversity along with an increased abundance of *Bacteroides, Bifidobacterium pseudocatenulatum, Roseburia hominis, Alistipes shahii*, *Parabacteroides*, *Blautia*, and *Ruminococcus* and with a decreased abundance of *Lactococcus* and *Akkermansia* (Dedrick et al., 2020). And *Bacteroides*-derived lipopolysaccharides have shown notably less ability to activate innate immunity and to elicit endotoxin tolerance than lipopolysaccharides from *Escherichia coli,* a common species in a population with a low incidence of T1DM (Vatanen et al., 2016).

Modification of gut microbiota or ingestion of microbial metabolites might be of interest to prevent T1DM. As an adjunct to insulin therapy, an intake of multispecies probiotics for 6 months has alleviated glycemic levels and inflammatory cytokines in T1DM patients aged 6–18 years old (Wang et al., 2022). The Environmental Determinants of Diabetes in the Young (TEDDY) study has demonstrated that a microbiome with more genes related to fermenting and biosynthesizing SCFAs, such as butyrate, showed protective effects on early-onset human T1DM (Vatanen et al., 2018). In addition, bacterial metabolite acetate or butyrate or consumption of acetate- and butyrate-yielding diet showed the ability to dampen T1DM progression in mouse models (Gao et al., 2009; Sun et al., 2015; Marino et al., 2017; Jacob et al., 2020). However, oral butyrate supplementation has failed to significantly affect innate or adaptive immunity in patients with long-standing T1DM, which may be attributed to differences in details of interventional design or physiology, pathology, and microbiology between humans and animals (de Groot et al., 2020).

Therefore, researchers have speculated that the microbial-related intervention may be better to be performed in early life or even during pregnancy before irreversible influences were formed. Early probiotic intervention only within the age of 27 days has exhibited a 60% reduction in the risk of islet autoimmunity in children in the group with the highest genetic risk of T1DM (Uusitalo et al., 2016). Adjunctive SCFAs, including formate, propionate, and butyrate, administration to maternal rats beginning before pregnancy combined with the administration to offspring after weaning successfully protected against the development of virus-induced T1DM in offspring, but administration to offspring beginning at weaning failed (Needell et al., 2017). Jia et al. (2020) have demonstrated that butyrate treatment during pregnancy and nursing dampened T1DM progression in the female mice offspring, which might be due to butyrate-induced inhibition of the activation of pancreatic dendritic cells in the offspring. Six-week feeding of 1% HMOs that was purified from pooled mature human milk from healthy donors has delayed and reduced T1DM incidence in non-obese diabetic mice and reduced the development of severe pancreatic insulitis in later life along with increased fecal SCFAs (Xiao et al., 2018). Moreover, several clinical studies have investigated whether maternal metabolites in cord blood played roles in offspring T1DM. Specifically, relating to microbial metabolites, lower levels of Trp and succinic acid in cord blood have been associated with later T1DM in the offspring (Oresic et al., 2008). Although the taurine/glycine-conjugated bile acid ratio and kynurenine/tryptophan ratio in cord blood have not been related to offspring T1DM (Vistnes et al., 2018; Tapia et al., 2021), the latter has been linked to carry the T1DM high-risk human leukocyte antigen genotype (heterozygous DQ2/DQ8) (Vistnes et al., 2018).

In summary, exposure to SCFAs, Trp, kynurenine, succinic acid before birth and HMOs-derived metabolites after birth may have influences on T1DM development in offspring.

Food allergy

Food allergy is defined as "an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food" (Boyce et al., 2010), which can trigger clinical symptoms ranging in severity from mild to life-threatening (Yu et al., 2016). The pathogenesis of FA is still not fully understood, but risk factors include increased hygiene, obesity, nutrition, timing and route of exposure to foods, and microbiome (Sicherer and Sampson, 2018; Childs et al., 2022).

The association between microbiota and FA was investigated only recently. Noval Rivas et al. (2013) first reported that the gut microbiota in mice with food allergy displayed a unique feature and may contribute to the pathogenesis of FA by influencing food antigen-specific regulatory T cells. Thereafter, increasing human cohorts have displayed the possible links between gut microbiota dysbiosis and the pathogenesis of FA (Lee et al., 2020). Probiotic LGG-supplemented formula has

been reported to improve tolerance acquisition in infants with cow's milk allergy, which might be attributed to the increased gut microbial diversity and increased microbial-derived butyrate (Berni Canani et al., 2016). In addition, findings of cohort studies have supported the association between low levels of fecal butyrate at 1 year of age and questionnaire-reported food allergy at 4 years and up to 6 years of age (Sandin et al., 2009; Roduit et al., 2019).

Human milk feeding possesses a long history of being related to reduce FA risk in the general population and high-risk children. But mother's milk with lower abundance, evenness, and a number of differential bacteria, less butyrate-producing bacteria, high levels of Trp/tyrosine/fatty acid metabolism in the predicted functional pathways of microbiota, or lower levels of butyrate have been related to infants with FA (Stinson et al., 2020; Wang et al., 2021). The concentration of butyrate in mature milk around 0.75 mM may show a protective effect against FA development (Paparo et al., 2021).

The presence of maternal gut commensal genus *Prevotella* may have a protective role in offspring FA development. A lower relative abundance of *Prevotella* has been detected in mothers' breastmilk of infants with FA (Wang et al., 2021). Moreover, Vuillermin et al. (2020) have suggested that maternal carriage during pregnancy rather than offspring carriage during the infancy of *Prevotella copri* has been positively associated with the absence of offspring FA. Although members of genus *Prevotella* are able to ferment dietary fiber into metabolite SCFA acetate and to ferment fat and fiber into SCFAs and succinate, they have only found an independent relationship between maternal high fat and fiber intake and either fecal succinate or lower risk of offspring FA. Therefore, they have deduced that the reduced risk of offspring FA was mediated not by microbial metabolite SCFA but by succinate during pregnancy. Succinate has been reported to enhance immunity (Rubic et al., 2008; Connors et al., 2018). However, carriage of *P. copri* or augmented fecal and serum succinate levels may associate with diseases, like rheumatoid arthritis, cardiovascular diseases, and inflammatory bowel diseases (Chu et al., 2021; Cui et al., 2021; Fremder et al., 2021). Future studies evaluating the influences of *P. copri* or succinate on offspring FA should also consider their impacts on other relevant diseases.

In summary, butyrate may be a crucial factor in human milk to reduce the risk of offspring FA, and maternal gut *P. copri*-derived succinate may also participate in the regulation of offspring FA risk.

Neonatal necrotizing enterocolitis

Neonatal necrotizing enterocolitis is one of the most common and devastating acquired diseases in neonates with a spectrum of various intestinal conditions (Neu and Walker, 2011). The pathophysiology is poorly understood; however, the pertinent factors may be prematurity, abnormal microbial colonization, and formula feeding (Alganabi et al., 2019; Quigley et al., 2019; Masi et al., 2021; Huang et al., 2022).

Consistent results have proven that human breast milk-fed infants developed far less NEC than exclusive formula-fed infants did (Miller et al., 2018). This beneficial effect could be derived from HMOs (Bode, 2012). Jantscher-Krenn et al. (2012) have demonstrated that a specific HMO type, disialyllacto-*N*-tetraose (DSLNT), exerted NEC-protective effects. Likewise, in a multicenter clinical cohort study, Autran et al. (2018) analyzed that NEC appeared in infants, who mostly received human milk with a significantly low concentration of DSLNT. Furthermore, Masi et al. (2021) have suggested that 241 nmol/ml could be an optimal threshold level for DSLNT to predict infants to develop NEC (91% accuracy) and to be healthy infants (86% accuracy). Also, intake of milk containing DSLNT below this threshold has exhibited abnormal microbiome development, i.e., with a low abundance of *Bifidobacterium longum* and a high abundance of *Enterobacter cloacae*, which indicated that DSLNT might be prebiotic for *B. longum*. Also, the usage of another two types of HMOs, 2′fucosyllactose and 6′-sialyllactose, alone or in combination showed the capability to inhibit TLR 4 signaling, thus protecting against mice or piglet NEC development (Sodhi et al., 2021). However, the identifications and roles of the above HMOs-derived metabolites in NEC prevention remain unclear.

Branched-chain fatty acids (BCFAs) have been associated with reducing the risk of NEC development and improving intestinal disease conditions (Ran-Ressler et al., 2011; Ran-Ressler et al., 2013). BCFAs are mainly saturated fatty acids with one or more methyl branches on the carbon chain. They could be produced by gut microbes from fermenting branched-chain amino acids. They are major components of the cell membranes of various bacteria species, such as *Lactobacillus* and *Bifidobacterium*, and crucial microbial components in the gastrointestinal tract of neonates. Individuals are greatly exposed to BCFAs during their life *in utero* or breastfeeding period (Ran-Ressler et al., 2008; Jie et al., 2018). In the last month of gestation, the gastrointestinal tract of a full-term infant has been estimated to absorb and metabolize BCFAs from swallowing vernix caseosa (Ran-Ressler et al., 2008); however, the intestinal organs of preterm infants miss such exposure. In addition, preterm-infant human milk contained lower levels BCFAs than term-infant human milk did (Jie et al., 2018). Lack exposure of BCFAs might be a possible reason for a less matured gut in preterm infants.

The effects of SCFAs on NEC development are controversial. The establishment of normal microbiota is delayed in preterm infants, which may result in a deficiency of SCFAs in the gut and further impair the intestinal barrier function (Smith et al., 2013). A recent study has shown that fecal microbiota transplantation with samples from NEC patients resulted in NEC-like intestinal injury in germ-free mice, which may result from the transplanted less-butyrate-producing microbiota

downregulating anti-inflammatory regulatory T cells (He et al., 2021). Short-term intervention with probiotic bifidobacteria has reduced the dysbiosis of NEC in extremely preterm infants (gestational age < 28 weeks) and simultaneously increased levels of propionate and butyrate (Athalye-Jape et al., 2022). Also, butyrate in breastmilk has been reported to improve the fetal immature intestinal inflammatory response (Gao et al., 2021; Huang et al., 2022). The above studies have supported that SCFAs may be a new therapeutic agent for NEC. However, SCFAs have been conversely related to intestinal mucosal injury and played a role in the pathogenesis of NEC (Thymann et al., 2009; Roy et al., 2018). Four-week administration of *B. breve* has been effective to promote the establishment of normal intestinal microbial composition in extremely low-birth-weight infants, along with reduced production of butyric acid (Wang et al., 2007). Although the reasons for previous inconsistent results are unclear, the gestational age at birth, postnatal age, microbiota composition, and concentration of metabolites may influence the roles of SCFAs in the gastrointestinal tract. For example, a low concentration of butyrate (2 mM) has enhanced intestinal barrier function and decreased inulin permeability, whereas a high concentration of butyrate (8 mM) has caused severe intestinal epithelial cell apoptosis and disrupted the intestinal barrier (Peng et al., 2007).

Previous studies have mainly focused on investigations of NEC development and possible treatments after birth but until now without leading any effective way to cure it. Lu et al. (2021) have hypothesized that it may be possible to modulate the development of NEC *in utero*. Their mice study has indicated that maternal delivery of aryl hydrocarbon receptor (AHR) ligands, namely tested ligand indole-3-carbinole and "A18," to the fetus showed the capability to prevent the development of NEC by reducing TLR 4 signaling in the offspring gut. Indole and its derivates are natural AHR ligands and can be transformed into Trp by gut microbiota (Bosi et al., 2020). Through binding them, AHR could be activated and thereby enhance intestinal epithelial barrier function and regulate the immune response in the gut (Gasaly et al., 2021). Similarly, ILA, a metabolite produced by probiotic *B. infantis* fermenting human milk Trp, has also shown anti-inflammatory effects on intestinal epithelial cells by activating AHR (Meng et al., 2020). This anti-inflammatory feature of ILA was found only in the fetal mouse intestines, not in the mature ones. The underlined mechanism remains unclear. Moreover, a recent study has uncovered that not only Trp but also other aromatic amino acids can be metabolized by breastmilk-promoted *Bifidobacterium* species to respective aromatic lactic acids to further impact immune function in early life (Laursen et al., 2021).

In summary, maternal microbial-derived AHR ligands during pregnancy, butyrate, and BCFAs in milk, ILA derived from human milk Trp, and unknown metabolites derived from HMOs may have roles in NEC prevention.

Autism spectrum disorder

Autism spectrum disorder, also known as autism, is a highly heterogeneous neurodevelopmental disability that is characterized by persistent deficits in social communication and social interaction with the presence of restricted, repetitive patterns of behaviors, interests, or activities (American Psychiatric Association, 2013). Current medical treatments can only mitigate the associated symptoms or co-occurring diagnoses in a short term (Lord et al., 2020). The exact underlying mechanisms of ASD remain unclear, however, both genetic and environmental factors are believed as contributors to its development.

In recent years, the gut microbiota has shown communications with the brain to influence the development/syndromes of diseases like ASD *via* bidirectional brain-gut axis, including the enteric nervous system, immune system, the vagus nerve, aromatic amino acid Trp metabolism, and other various microbial byproducts (Cryan et al., 2019; Chernikova et al., 2021; Needham et al., 2021). Gut microbiota-derived metabolite 4-ethylphenylsulfate (4-EPS) has been indicated to cause ASD-related behavioral abnormalities in naïve wild-type mice (Hsiao et al., 2013; Santamaria et al., 2018). Hsiao et al. (2013) have further demonstrated that oral supplementation with *Bacteroides fragilis* restored the gastrointestinal microbial composition, enhanced the intestinal barrier integrity, reduced the intestinal permeability, improved the leakage of 4-EPS into the bloodstream, completely restored the level of serum metabolite 4-EPS, and finally reduced impairments in communicative, stereotypic, anxiety-like, and sensorimotor behaviors in maternal immune activation offspring who proved features of ASD.

Moreover, increasing reports have suggested that maternal factors, including overweight before pregnancy, prenatal high-fat diet, excessive gestational weight gain, maternal inflammation, and others, are associated with an increased likelihood of offspring ASD (Kim et al., 2017; Windham et al., 2019; Fernandes et al., 2021). Also, recent studies have indicated that maternal microbiota and the associated metabolites could influence fetal neurodevelopment *in utero*. For example, prenatal stress-caused reduction of maternal gut microbiota, including *Parasutterella excrementihominis* and *Bifidobacterium*, dysregulated the metabolic pathways of Trp, a well-studied precursor for multiple metabolites and showing crucial roles in proper immune- and neuro-development, reduced transportation of Trp and its derivative serotonin to fetus across the placenta, and thereby induced aberrant fetal neurodevelopment (Antonson et al., 2020; Chen et al., 2020; Galley et al., 2021). Also, maternal administration of kynurenine, another Trp derivative, has rapidly increased levels of kynurenine in fetal plasma and brain and caused offspring featured with ASD-like behavioral abnormalities (Murakami et al., 2021).

In addition, Vuong et al. (2020) have explored that even without environmental challenges maternal gut microbiota, particularly including *Clostridia*-dominant spore-forming bacteria, were able to restore the thalamocortical axonogenesis in fetuses of antibiotic-treated dams. This was probably mediated by maternal microbiota-derived or -modulated metabolites, such as trimethylamine-*N*-oxide (TMAO) and imidazole propionate, existing both in maternal blood and in the fetal brain. Also, low levels of fecal butyrate and accumulated propionic acid have been reported to be associated with ASD children (Liu et al., 2019; Cotrina et al., 2020). Prenatal administration of propionic acid may contribute to offspring ASD by altering development and behavior during adolescence (Foley et al., 2014). In a clinical screening, untargeted metabolomics in mid-pregnancy maternal serum, bile acid pathways have been associated with offspring ASD (Ritz et al., 2020).

In summary, during pregnancy maternal Trp derivatives, TMAO, imidazole propionate, propionic acid, and bile acid derivatives may participate in the regulation of fetal neurodevelopment diseases, like ASD.

Discussion

Micro-organisms and substrates are two basic factors for a microbial metabolite, so in the present review, maternal-associated microbial metabolites can be produced

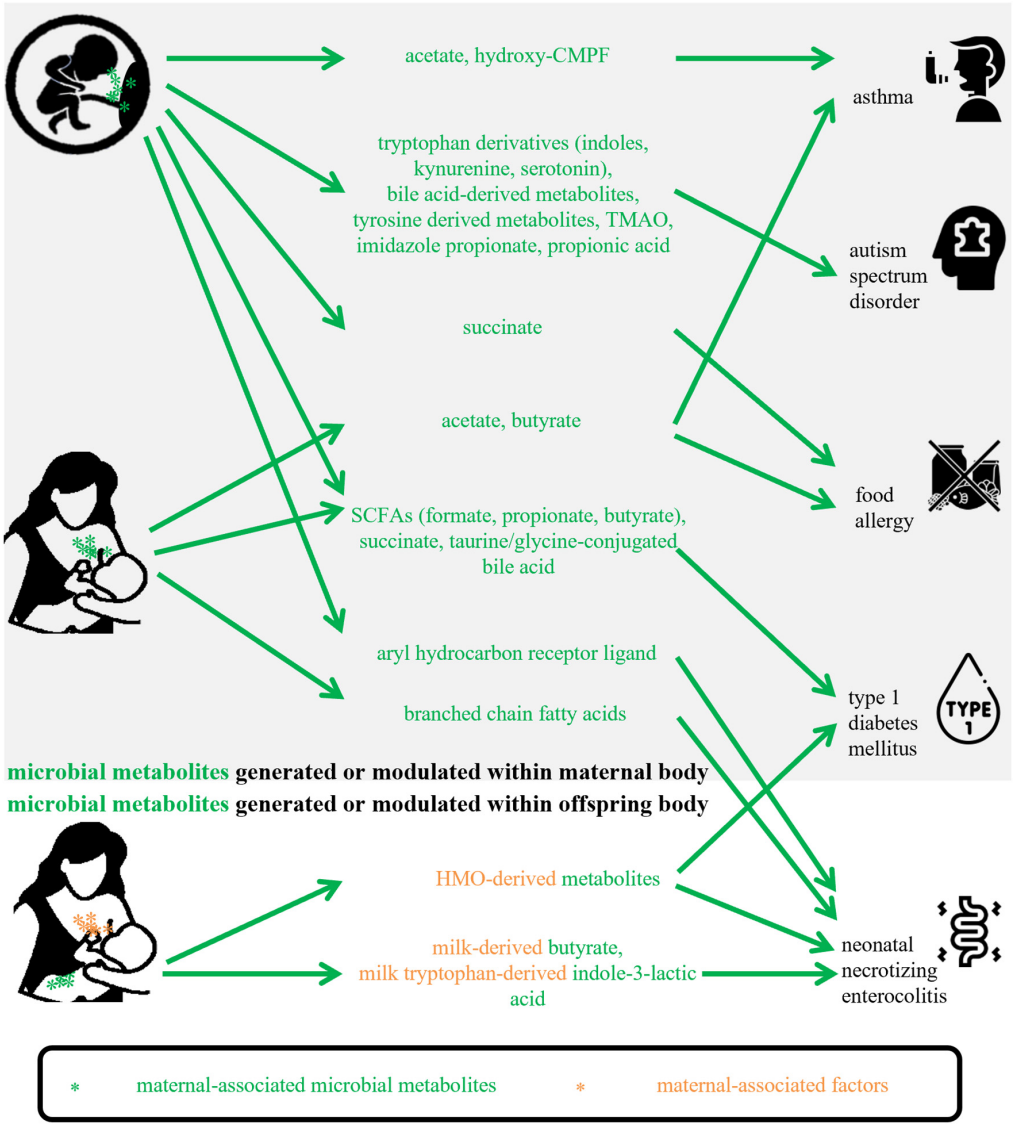

FIGURE 1
Maternal-associated microbial metabolites generated within maternal body or offspring body in this review. CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; TMAO, trimethylamine-N-oxide; SCFAs, short-chain fatty acids; HMO, human milk oligosaccharide.

TABLE 1 Main results of maternal-associated microbial metabolites and offspring diseases mentioned in this review.

Maternal-associated metabolites	Maternal-associated microbiota	Maternal-derived substrate or maternal diet	Study type	Main findings	References
Asthma					
Acetate	Maternal gut microbiome	High fiber diet	Mouse model	Placenta-transferred acetate changed the gene expression in the fetal lung that linking to asthma development. Maternal intake of high fiber diet or acetate protected offspring mice against induced allergic airway disease.	Thorburn et al., 2015
Hydroxy-3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (hydroxy-CMPF)	Not mentioned	Fish oil-derived n-3 long chain polyunsaturated fatty acids (n-3 LCPUFAs)	Cohort study	During pregnancy week 22–26 until 1 week after delivery supplementation with fish oil-derived n-3 LCPUFA reduced 31% risk of asthma in the offspring during the first 5 years of life, and higher level of hydroxy-CMPF in the offspring were estimated at age 0.	Bisgaard et al., 2016; Rago et al., 2019
Type 1 diabetes mellitus (T1DM)					
SCFAs (formate, propionate, butyrate)	Not mentioned	Adjunctive SCFAs	Rat model	Adjunctive SCFAs administration to maternal rats beginning prior to pregnancy combined with administration to offspring after weaning successfully protected against development of virus-induced T1DM in offspring, but administration to offspring beginning at weaning failed.	Needell et al., 2017
Butyrate	Not mentioned	Butyrate	Mouse model	Butyrate treatment during pregnancy and nursing dampened T1DM progression in the female mice offspring, which might be due to butyrate-induced inhibition of the activation of pancreatic dendritic cells in the offspring.	Jia et al., 2020
Succinic acid or succinate	Not mentioned	Tryptophan (Trp)	Cohort study	Lower levels of metabolites, including Trp, succinic acid, and creatinine, in cord blood were associated with later T1DM in the offspring.	Oresic et al., 2008
Taurine glycine-conjugated bile acid	Not mentioned	Bile acids	Cohort study	The taurine glycine-conjugated bile acid ratio in cord blood were not able to predict the development of T1DM in the offspring.	Tapia et al., 2021
Kynurenine (Kyn)	Not mentioned	Trp	Cohort study	Kyn Try ratio (KTR) in cord blood plasma at birth were not associated with offspring T1DM, but associated with carrying the T1DM high-risk human leucocyte antigen (HLA) genotype (heterozygous DQ2 DQ8).	Vistnes et al., 2018
SCFAs	Not mentioned	Human milk oligosaccharides (HMOs)	Mouse model	Six-week feeding of 1% HMOs, purified from pooled of mature human milk from healthy donors, delayed and reduced T1DM incidence in non-obese diabetic mice and reduced development of severe pancreatic insulitis in later life along with increased fecal SCFAs.	Xiao et al., 2018
Food allergy (FA)					
SCFAs, acetate, butyrate, formate	Not mentioned	Not mentioned	Cohort studies	SCFAs acetate, butyrate, and formate were able to be detected from human milk. And concentrations of acetate and butyrate were lower in atopic mothers than in non-atopic mothers.	Stinson et al., 2020

(Continued)

TABLE 1 (Continued)

Maternal-associated metabolites	Maternal-associated microbiota	Maternal-derived substrate or maternal diet	Study type	Main findings	References
Butyrate	Microbes from breastmilk	Not mentioned	Case-control study	Microbes from breastmilk showed protective effect against offspring FA partly by producing butyrate.	Wang et al., 2021
Butyrate	Not mentioned	Not mentioned	Cohort study and mouse model	Concentration of butyrate in mature milk around 0.75 mM may show protective effect against offspring FA development.	Paparo et al., 2021
Succinate, SCFAs	*Prevotella copri*	Diet high in fat and fiber	Cohort study	Maternal carriage of *P. copri* during pregnancy may decrease IgE-mediated FA in offspring, especially when women with a diet high in fat and fiber. The protective effect of *P. copri* may be mediated not by SCFAs, but by succinate.	Vuillermin et al., 2020
Neonatal necrotizing enterocolitis (NEC)					
Aryl hydrocarbon receptor (AHR) ligand	Not mentioned	Not mentioned	Mouse model	Maternal delivery of AHR ligands, namely ligand indole-3-carbinole and A18 in this study, to the fetus may prevent the development of NEC by reducing toll-like receptor 4 (TLR4) signaling in the offspring gut and independent of leukocyte activation.	Lu et al., 2021
Not mentioned	Not mentioned	HMO	Rat model	A specific HMO type, disialyllacto-N-tetraose (DSLNT), exerted the NEC-protective effects.	Jantscher-Krenn et al., 2012
Not mentioned	Not mentioned	HMO	Cohort study	NEC appeared in infants who mostly received human milk with significantly low concentration of DSLNT.	Autran et al., 2018
Not mentioned	*Bifidobacterium longum* and *Enterobacter cloacae*	HMO	Cohort study	241 nmol ml could be an optimal threshold level for DSLNT to predict infants to develop NEC (91% accuracy) and to be healthy infants (86% accuracy), and also infants obtained milk below this threshold exhibited abnormal microbiome development, i.e., with low abundance of *B. longum* and high abundance of *Enterobacter cloacae*, which indicated DSLNT might be a prebiotic for *B. longum*.	Masi et al., 2021
Not mentioned	Not mentioned	HMO	Mouse and piglet models	The usage of two types of HMOs 2′fucosyllactose and 6′-sialyllactose alone or combination showed capability to inhibit toll-like receptor four signaling, thus protecting against mice or piglet NEC development.	Sodhi et al., 2021
Branched-chain fatty acids (BCFAs)	Not mentioned	Not mentioned	Case-control study	Preterm-infant human milk contained lower levels BCFAs than term-infant human milk did.	Jie et al., 2018
Butyrate	Not mentioned	Breastmilk	*In vitro* and *in vivo* study	Butyrate, as a metabolite of breastmilk complex carbohydrates, improved the fetal immature intestinal inflammatory response induced by cytokine IL-1β.	Gao et al., 2021; Huang et al., 2022
Indole-3-lactic acid (ILA)	*Bifidobacterium infantis*	Milk Trp	*In vivo* study	ILA, a metabolite produced by probiotic *B. infantis* fermenting human milk Trp, has also shown anti-inflammatory effects on intestinal epithelial cells by activating AHR.	Meng et al., 2020

(Continued)

TABLE 1 (Continued)

Maternal-associated metabolites	Maternal-associated microbiota	Maternal-derived substrate or maternal diet	Study type	Main findings	References
Autism spectrum disorder (ASD)					
Serotonin	Not mentioned	Trp	Mouse model	Prenatal stress led to elevated Trp and serotonin in placenta.	Chen et al., 2020
Indoles, Kyn, serotonin	*Parasutterella* and *Bifidobacterium*	Trp	Mouse model	Reductions of Trp-associated microbes and concomitant dysregulation in Trp metabolic machinery in dam and offspring suggested that prenatal stress-induced Trp metabolic dysfunction may mediate aberrant fetal neurodevelopment.	Galley et al., 2021
Kyn	Not mentioned	Trp	Mouse model	Maternal administration of Kyn rapidly increased level of Kyn in fetal plasma and brain, and the offspring mice exhibited behavioral abnormalities similar to those observed in offspring of IL-17A-conditioned mice. IL-17A have been identified as a potential mediator to contribute to the development of ASD.	Murakami et al., 2021
Trimethylamine-*N*-oxide (TMAO) and imidazole propionate	*Clostridia*-dominant spore-forming bacteria	Not mentioned	Mouse model	Without environmental challenges maternal gut microbiota, particularly including *Clostridia*-dominant spore-forming bacteria, were able to restore the thalamocortical axonogenesis in fetus of antibiotic-treated dams	Vuong et al., 2020
Propionic acid (PPA)	Enteric bacteria	Not mentioned	Rat model	Prenatal administration of PPA may contribute to ASD in offspring, altering development and behavior during adolescence.	Foley et al., 2014
Bile acid-derived metabolites	Not mentioned	Bile acids	Case-control study	Bile acid pathways were related to maternal metabolite levels in mid-pregnancy serum associated with ASD in offspring.	Ritz et al., 2020
Not mentioned	*Parasutterella*	Trp, tyrosine, bile acids	Mouse model	Prenatal stressor-exposed mice lost multiple gut metabolic pathways. *Parasutterella* may mediate proper metabolic function in late pregnancy and even early immune development in the offspring.	Antonson et al., 2020

or modulated by maternal microbiota or can be fermented from maternal-derived substrates. Based on collected studies, they could involve metabolites that are generated or modulated (1) within the maternal body and transferred to offspring *via* the placental transportation or breastmilk feeding; (2) within the offspring body in the fermentation of substrates obtained from mothers. Figure 1 shows how the included maternal-associated metabolites in this review can be exposed to offspring. Compared with the metabolites in the former group, substrates-derived metabolites in the latter group and their possible roles were seldomly reported, however, the well-studied substrates, for example, HMOs, in the function of offspring health maintenance will provide a good background for future investigations of their metabolites. About the microbiota obtained from mothers *via* natural birth or human milk feeding, after exposure, they become infant early microbial colonizers, so this kind of metabolite was not included in the present study.

Table 1 shows the main findings of selected publications and lists the maternal-associated metabolites, microbiomes, and substrates or diets. The metabolites included SCFAs, BCFAs, succinate, Trp derivatives, indole derivatives, bile acids derivatives, hydroxy-CMPF, TMAO, and imidazole propionate. Within the limited number of studies, the potential mechanisms of these metabolites on offspring health maintenance may include (1) altering gene expressions encoding molecules that can participate in immune regulation; (2) activating AHR or succinate receptors; (3) enhancing intestinal epithelial barrier function; (4) inhibiting activation of dendritic cells.

Although plenty of studies have linked microbial SCFAs to several disease preventions or treatments, they were considered to relate to intestinal mucosal injury and contribute to the pathogenesis of NEC, which emphasizes the importance of concentration. It reminds researchers that metabolite concentration is a non-negligible detail when evaluating or interpreting the effects. Also, the relevant studies were mainly about bacterial metabolites and seldom involved fungal or other microbial metabolites.

Conclusion

Generally, previous researchers have first revealed the possible associations between changes in the microbiota, particularly in the gut, and risks of diverse diseases in the host, and following studies have moved it forward and indicated that the associations may be mediated by the microbial metabolites, especially during pregnancy and lactation maternal-associated microbial metabolites may have crucial roles in participating the microbial regulation of offspring diseases development.

However, studies relevant to the functions of maternal-associated metabolites are still scarce. Before we can carry out metabolite-associated early prediction, early diagnosis, early prevention, or early treatment through actions on mothers, high-quality animal and clinical trials are needed. Also, studies comprehensively evaluating the effects of altered maternal-associated metabolites on overall maternal and infant health maintenance are required.

Author contributions

All authors conceived, designed, drafted, and revised the manuscript and read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.955297/full#supplementary-material

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Relationship between maternal–infant gut microbiota and infant food allergy

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The gut microbiota plays a crucial role in food allergies. We sought to identify characteristics of the maternal gut microbiota in the third trimester and the infant gut microbiota in early life and the association of these microbiotas with infant food allergy. A total of 68 healthy pregnant women and their full-term newborns were selected from a cohort of 202 mother–infant pairs; among them, 24 infants had been diagnosed with food allergy within 1 year of age, whereas 44 infants were healthy without allergic symptoms. We collected 65 maternal fecal samples before delivery and 253 infant fecal samples at five time points following birth. Fecal samples were microbiologically analyzed using 16S rRNA gene sequencing. *Holdemania* abundance in the maternal gut microbiota in the third trimester was significantly higher in the non-allergy group than in the food allergy group ($P = 0.036$). In the infant gut microbiota, *Holdemania* was only found in meconium samples; its abundance did not differ significantly between the two groups. The change in the abundance of *Actinobacteria* over time differed between the non-allergy and food allergy groups (FA, $P = 0.013$; NA, $P = 9.8 \times 10^{-5}$), and the change in the abundance of *Firmicutes* over time differed significantly in the non-allergy group ($P = 0.023$). The abundances of genera *Anaerotruncus*, *Roseburia*, *Ruminococcus,* and *Erysipelotricaceae* were significantly different between the non-allergy and food allergy groups at different time points. Our results showed that maternal carriage of *Holdemania* during the third trimester strongly predicted the absence of food allergies in infants; there was no correlation between the presence of food allergies and the abundance of *Holdemania* in the infant gut microbiota. More dynamic fluctuations in phyla Actinobacteria and Firmicutes early in life protect against food allergy. Thus, the enrichment of the infant gut microbiota early in life with short-chain fatty acid-producing bacteria may be beneficial in preventing the development of food allergies in infants.

KEYWORDS

gut microbiota, early life, microbial establishment, infant, food allergy

Introduction

Food allergy refers to a specific immune response that can occur repeatedly after exposure to a specific food (NIAID-Sponsored Expert Panel et al., 2010). Food allergies, especially those in infants, can increase the risk of childhood allergic diseases and reduce the quality of life of the family (Vermeulen et al., 2018; Abrams et al., 2020); thus, food allergies have emerged as a major public health problem affecting children and adults. The prevalence of food allergies has increased in recent years, affecting ∼10% of the global population (Lopes and Sicherer, 2020).

The incidence and severity of food allergies have increased markedly with profound environmental and lifestyle changes, suggesting a link between an altered microbiota and the growing prevalence of allergic diseases. The hygiene hypothesis suggests that there might be a relationship between microbes and allergies (Strachan, 1989). Based on this, recent studies have proposed that antibiotic abuse, dietary changes, increased cesarean section rates, and formula feeding can alter the gut microbiota, causing an increase in the incidence of allergic diseases (Kim et al., 2019). Gut microbiota, especially in early life (0–6 months), is an important influential factor in immune and metabolic development and may have lasting consequences (Gensollen et al., 2016; Shu et al., 2019). Stable gut microbiota can promote the development of the host immune system and prevent food allergies (Chinthrajah et al., 2016; Gholizadeh et al., 2019). There is growing evidence that colonization of the gut microbiota begins in the fetus. Maternal intestinal, vaginal, and oral microbes may be the source of fetal gut microbiota, with many scholars believing that the maternal gut microbiota is the most important source (Thum et al., 2012; Hu et al., 2013; Walker et al., 2017). In a human study, pregnant women during the third trimester were administered *Lactobacillus rhamnosus*, which was discontinued at the end of pregnancy. *Lactobacillus rhamnosus* could be detected in the stool samples of babies delivered *via* vaginal delivery or cesarean section (Schultz et al., 2004), suggesting the gut microbiota in the third trimester of pregnancy influenced the colonization of the gut microbiota in infants.

To date, research on the relationship between the gut microbiota and infant disease has mainly focused on the gut microbiota during infancy; however, accumulating evidence shows that the maternal microbiome during pregnancy also plays a key role in preventing the development of an allergy-prone immune phenotype in the offspring (Gomez de Agüero et al., 2016; Vuillermin et al., 2017). Animal studies revealed that the maternal gut microbiota during pregnancy may influence the symptoms of allergic diseases in the offspring (Thorburn et al., 2015). Experiments in mice demonstrated that the maternal gut microbiota influenced neonatal adaptive immunity (Nyangahu et al., 2018). Additionally, experiments in germ-free mice have proved that the maternal gut microbiota during pregnancy affected the occurrence of allergic diseases in the offspring (Arrieta et al., 2015). Nonetheless, only one human study has found that maternal carriage of *Prevotella copri* during pregnancy reduced the risk of allergic disease in the offspring (Vuillermin et al., 2020). This suggests that human studies evaluating the relationship between maternal microbiota during pregnancy and the risk of allergic diseases in offspring are lacking.

In this study, we aimed to establish a cohort to collect and analyze the gut microbiota of pregnant women before delivery and of infants in the first 6 months after birth and investigate the relationship between maternal and infant gut microbiota and food allergy. We hypothesized that the maternal and infant gut microbiota play a role in the development of food allergy.

Materials and methods

Study design

This nested case–control study was conducted at the Peking University Third Hospital between February 2018 and May 2020. The study protocol was approved by the Medical Science Research Ethics Committee of Peking University Third Hospital, Peking, China (Approval No. M2018022).

Participants

A total of 202 healthy pregnant women who underwent regular prenatal check-ups were recruited. Only the pregnant women who had regular prenatal check-ups at our hospital and were deemed by the investigator to be in good physical and mental health based on their medical history and examination were eligible to participate. The exclusion criteria were as follows: (1) underlying diseases or pregnancy complications, (2) use of antibiotics or probiotics for 2 weeks before or after delivery, and (3) refusal to participate in the study. Written consent was obtained from all participants prior to inclusion in the study. Participants who met at least one of the following criteria during the study were excluded from further participation: (1) premature birth (before 37 weeks), (2) postmature birth (after 42 weeks), (3) unstable vital signs after birth, (4) congenital malformation(s) in the infant, and (5) used of antibiotics or probiotics in infants.

Determination of food allergy manifestations

Food allergy was diagnosed based on the following conditions: (1) one or more manifestations of poor sleep,

crying, anxiety, depression, rash, runny nose, sneezing, coughing, wheezing, vomiting, diarrhea, and blood in the stool; (2) disappearance or reduction of food allergy symptoms after discontinuation of the suspected allergenic food, or reappearance or aggravation of food allergy symptoms after the reintroduction of the suspected food; (3) one or more positive results in allergen-specific IgE, food challenge test, or skin punctum test. The infant was diagnosed with food allergy by the clinician if condition 1 was present and conditions 2 and/or 3 were met.

Clinical data and sample collection

After the establishment of the maternal–infant cohort, the health status of the mothers was investigated before delivery, and that of the infants was investigated every month after delivery until 1 year of age using a questionnaire-based survey. Stool samples were also collected. Maternal fecal samples were collected 1–2 weeks prior to the expected delivery date. Post-delivery, neonatal stool samples were collected from diapers at the following time points: first defecation; 3 days after birth; and 1, 3, and 6 months after birth. Trained professionals used sterile tubes with DNA stabilizers to collect stool samples during hospital or home visits. All samples were fully mixed with DNA stabilizer and stored at −80°C within 6 h after collection.

DNA extraction, PCR amplification, and high-throughput sequencing

DNA was extracted from each fecal sample using the modified protocol of the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). Briefly, 1 ml InhibitEX buffer and glass beads (0.5-mm diameter, Qiagen) were added to a 200 mg fecal sample. The mixture was homogenized and mixed at 60 Hz for 1 min (twice) using a homogenizer. DNA was purified in accordance with the manufacturer's instructions.

The V3-V4 region of the bacterial 16S ribosomal RNA genes was amplified by PCR using barcoded primers 341F 5′-CCTACGGGRSGCAGCAG-3′ and 806R 5′-GGACTACVVGGGTATCTAATC-3′ (Wang and Qian, 2009). PCRs were performed in a volume of 30 μl containing 15 μl 2 × KAPA Library Amplification ReadyMix, 1 μl each primer (10 μM), 50 ng template DNA, and ddH_2O (Pereira et al., 2017).

Amplicons were extracted from 2% agarose gels, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions, and quantified using Qubit®2.0 (Invitrogen, Waltham, MA USA). All quantified amplicons were pooled to equalize the concentrations for sequencing using the Illumina MiSeq PE250 (Illumina, Inc., San Diego, CA, USA). The paired-end reads (∼250 bp in length) were overlapped at their 3′ ends for concatenation into original longer tags using PANDAseq (https://github.com/neufeld/pandaseq, version 2.9).

16S RRNA gene sequencing and statistical analysis

The lengths and average base quality of the assembled tags, trimmed barcodes, and primers were checked. 16S tags were restricted between 220 bp and 500 bp such that the average Phred score of the bases was not lower than 20 (Q20) and not higher than three ambiguous N. The copy number of the tags was enumerated, and the redundancy of repeated tags was removed. Only tags with a frequency >1, which tend to be more reliable, were clustered into operational taxonomic units (OTUs). OTUs were clustered with 97% similarity using UPARSE, and chimeric sequences were identified and removed using Usearch (Edgar, 2013). Representative OTU sequences were compared with those of the 16S rRNA genes of known species using the RDP method for classification (Cole et al., 2014).

Alpha diversity was evaluated using Chao1, Shannon, Simpson, and Observed_species indices, and the value of the alpha diversity was calculated using the QIIME (V1.9.1) software. Beta diversity was calculated using the QIIME (V1.9.1) software and an iterative algorithm with weighted and unweighted species richness information. Gplots, vegan, and ade4 packages in R were used to analyze bacterial community composition and heatmap clustering at the genus level, and the results of multivariate ANOVA based on dissimilarities (Adonis) and principal coordinates analysis (PCoA), respectively. The linear discriminant analysis effect size (LEfSe) software was used to perform LEfSe to identify potential microbial biomarkers (Segata et al., 2011).

Statistical analysis

Statistical analyses were performed using SPSS version 25.0 (IBM, Armonk, NY, USA). Data are presented as the mean ± standard deviation, and the chi-square test of independent samples was used to analyze the differences in basic data and influencing factors between the non-allergy and allergy groups. The Wilcoxon test was used to assess the differences between the two groups at one time point. The Kruskal–Wallis test was used for analyzing the differences among periods in the non-allergy and food allergy groups. Differences were considered significant at $P < 0.05$.

TABLE 1 Clinical data of maternal participants.

Clinical data	Food allergy ($n = 24$)	Non-allergy ($n = 44$)	P^b
Age (year)[a]	31.91 ± 2.63	32.68 ± 3.47	0.52
Primigravida			0.24
Yes, *n* (%)	20 (83.3)	31 (70.5)	
No, *n* (%)	4 (16.7)	13 (29.5)	
Han ethnicity			1.00
Yes, *n* (%)	23 (95.8)	42 (95.5)	
No, *n* (%)	1 (4.2)	2 (4.5)	
Allergic symptoms			0.10
Yes, *n* (%)	17(75.0)	22 (50.0)	
No, *n* (%)	7 (25.0)	22 (50.0)	

[a]Data are presented as the mean ± standard deviation.
[b]$P < 0.05$, indicates statistical significance.

TABLE 2 Clinical data of infant participants.

Clinical data	Food allergy ($n = 24$)	Non-allergy ($n = 44$)	P^b
Gestational age at delivery (weeks)[a]	39.6 ± 0.89	39.9 ± 0.83	0.58
Birth weight (g)[a]	3.40 ± 0.17	3.52 ± 0.35	0.47
Height (cm)[a]	50.64 ± 2.19	50.71 ± 3.42	0.93
Sex			0.976
Male, *n* (%)	13 (54.2)	24 (54.5)	
Female, *n* (%)	11 (45.8)	20 (45.5)	
Mode of delivery			0.69
Vaginal delivery, *n* (%)	18 (75)	31 (70.5)	
Cesarean, *n* (%)	6 (25)	13 (29.5)	
Time for complementary food			0.341
<6 months, *n* (%)	11 (45.8)	15 (34.1)	
>6 months, *n* (%)	13 (54.2)	29 (65.9)	
Contact with pets			0.196
Yes, *n* (%)	7 (29.2)	7 (15.9)	
No, *n* (%)	17 (70.8)	37 (84.1)	
Use of antibiotics			0.699
Yes, *n* (%)	5 (20.8)	11 (25)	
No, *n* (%)	19 (79.2)	33 (75)	
Siblings			0.821
Yes, *n* (%)	7 (29.2)	14 (31.8)	
No, *n* (%)	17 (70.8)	30 (68.2)	
Feeding patterns			0.539
Breastfeeding, *n* (%)	16 (66.7)	26 (59.1)	
Mixed feeding, *n* (%)	8 (33.3)	18 (40.9)	
Duration of breastfeeding			0.889
<6 months, *n* (%)	3 (12.5)	5 (11.4)	
>6 months, *n* (%)	21 (87.5)	39 (88.6)	

[a]Data are presented as the mean ± standard deviation.
[b]$P < 0.05$, indicates statistical significance.

Results

Study participants

Of the 202 mother–infant pairs, 135 who met the criteria and were followed up regularly were included in the study cohort. They were divided into the food allergy group (FA group; $n = 24$) and the non-allergy group (NA group; $n = 44$) based on whether the infants had food allergies at 1 year of age (Supplementary Figure 1). No significant differences in maternal age, primigravida, ethnicity, and allergy symptoms were observed between the two groups (Table 1). No significant differences in the factors influencing the gut microbiota, such as delivery mode, contact with pets, number of siblings, and antibiotic consumption by the infant within 6 months of birth, and feeding factors, including breastfeeding, milk powder feeding, and time of complementary feeding, were observed between the two groups (Table 2).

Reads and OTUs

In total, 318 stool samples were collected, and the number of samples collected at different times is detailed in Supplementary Table 1. Overall, 9,020,380 high-quality reads were obtained from 253 infant samples (35,653.68±1,928.69 high-quality reads per sample, which were clustered into 2,232 OTUs). Additionally, 2,329,028 reads were obtained from 65 maternal samples (35,831±1,758.6 high-quality reads per sample, which were clustered into 1,018 OTUs).

Differences in the change of alpha diversity of fecal microbiota in NA and FA groups

In the maternal samples, although the microbial diversity in the FA group was lower than that in the NA group, neither of these indices were significantly different between the two groups (Figure 1). In the infant samples, alpha diversity indices varied over time in both the NA and FA groups and demonstrated a similar trend. In day 1 samples (meconium), richness and diversity were at a higher level than in other periods but exhibited a downward trend from day 3 to 1 month after birth. Then they continued to increase until 6 months

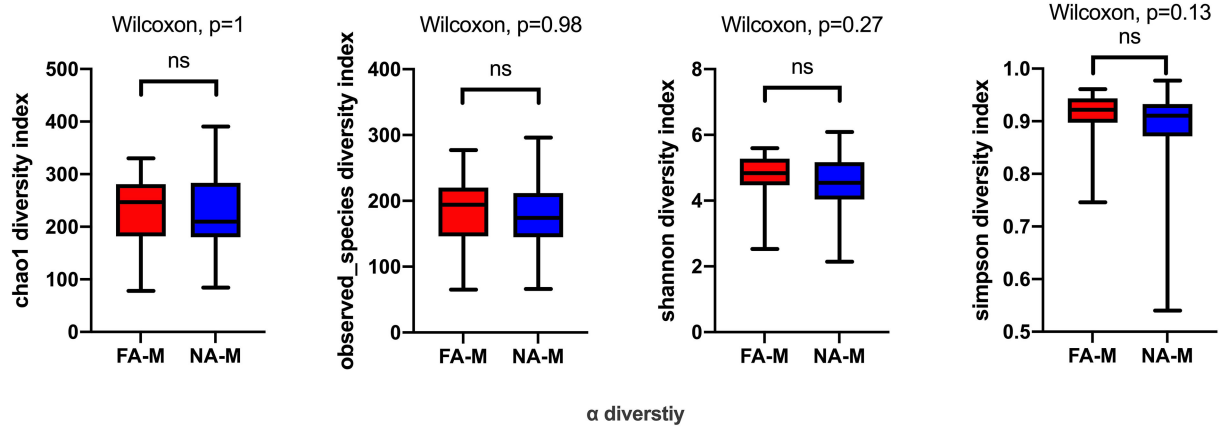

FIGURE 1
Differences in the alpha diversity of maternal samples between the non-allergy (NA) and food allergy (FA) groups, as measured by different measure indices. Chao1 and Observed_species indices reflect species richness, and Shannon or Simpson indices reflect species diversity. ns, not significant.

after birth, but they did not return to their levels on day 1 (Figure 2).

A longitudinal comparison of the difference among different periods revealed that the NA and FA groups have similar Chao1 indices. Significant differences were observed between 1 day and other periods and between 1 month and 6 months. With respect to the Simpson, Shannon, and Observed_species indices, a comparison of every time point with day 1 showed significant differences at more time points in the NA group than in the FA group (Supplementary Figure 2). A comparison of the difference between the two groups in the same period revealed that richness and diversity in the NA group were higher than those in the FA group on day 1, day 3, 1 month, and 3 months, and there was a difference between the two groups at 1 month. At the 6-month time point, richness and diversity in the NA group were slightly lower than those in the FA group without statistical significance (Figure 3).

Differences in the change of beta diversity of fecal microbiota in NA and FA groups

Principal coordinates analysis revealed that the maternal samples between the two groups were close and the Adonis test revealed a P-value > 0.05, i.e., the differences were not significant (Supplementary Figure 3A). In the infant samples, a comparison of the differences in the same group at different periods showed similar results, with day 1 samples in both groups significantly clustered away from samples at other periods in the same group; the Adonis test revealed that the difference was significant (Figure 4). Comparison of the difference between the two groups in the same period showed that the NA and FA samples were tightly clustered and could not be distinguished, and the Adonis test revealed that the five indices between the two groups did not exhibit any significant differences at any time point (Supplementary Figures 3B–F).

The composition of fecal microbiota at the phylum level varied over time in the two groups

In all samples, the gut microbiota at the phylum level was mainly enriched in Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes. The relative abundance of Bacteroidetes (FA 46.29%, NA 51.94%) and Firmicutes (FA 42.09%, NA 38.44%) were higher in the maternal samples, and the FA and NA groups exhibited a similar relative abundance of these bacteria. The relative abundance of Proteobacteria and Firmicutes was higher in the infant samples, and the abundance of each phylum changed over time (Figure 5). The relative abundance of Proteobacteria and Bacteroidetes in the FA and NA groups did not differ over time; however, there was a difference in the abundance of Actinobacteria (at various time points in the FA and NA groups) (FA, $P = 0.013$; NA, $P = 9.8 \times 10^{-5}$) and Firmicutes (at various time points in the NA group) ($P = 0.023$). Actinobacteria abundance was similar on day 1 (FA, 2.97%; NA, 4.81%) and day 3 (FA, 10.62%; NA, 11.32%) and gradually increased at 1 month (FA, 12.57%; NA, 18.43%) and 3 months (FA, 18.36%; NA, 27.74%). However, at 6 months (FA 19.32%, NA 16.96%), Actinobacteria abundance in the NA group suddenly decreased, whereas that in the FA group continued to increase steadily. Firmicutes abundance in both groups presented a similar trend from day 1 to 3 months, gradually increasing from day 1 (FA, 31.46%; NA, 33.90%) to day 3 (FA,

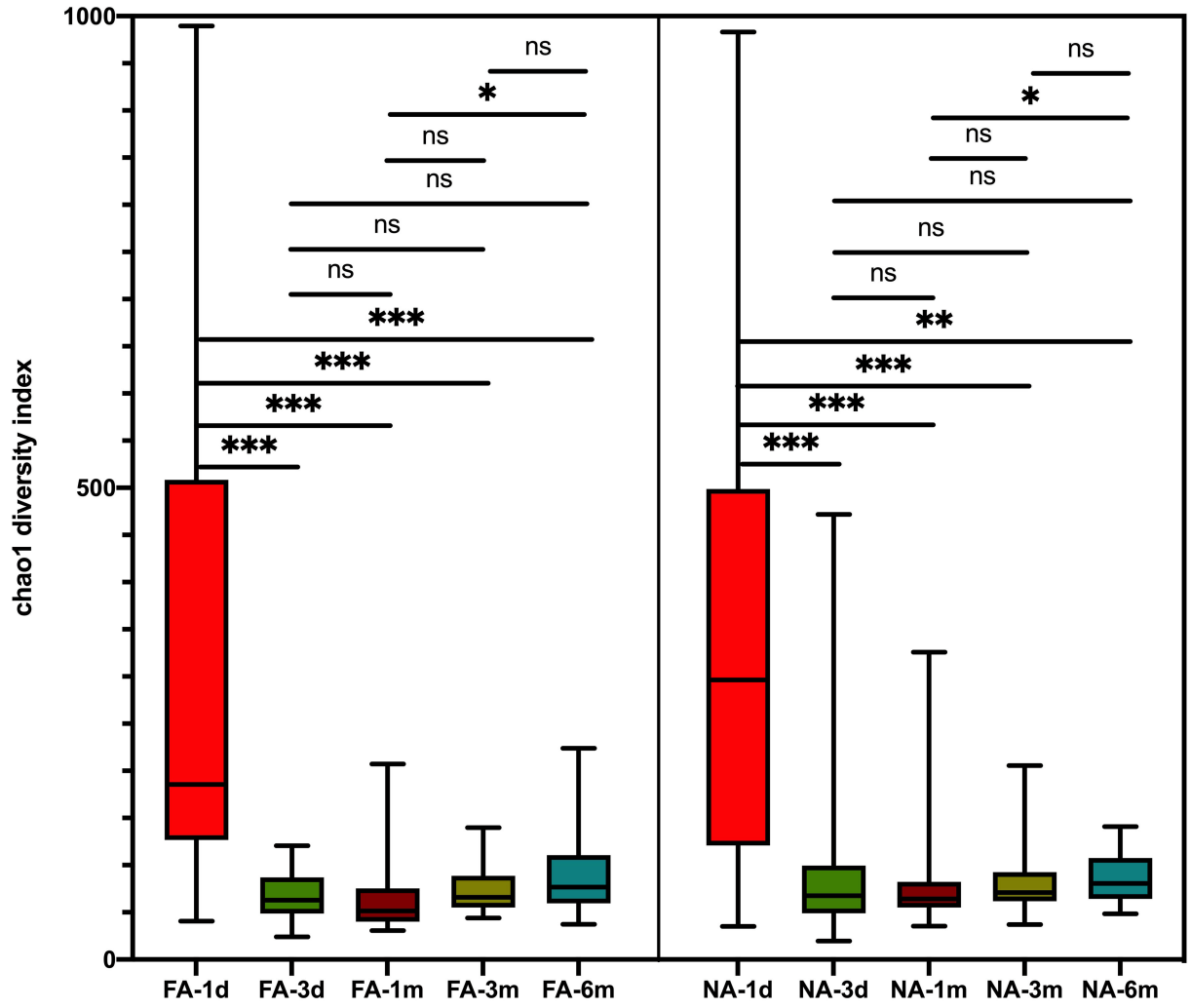

FIGURE 2
Differences in variation over time of alpha diversity between the non-allergy (NA) and food allergy (FA) groups using the Chao1 index. The Wilcoxon test was used to analyze differences among time groups in the non-allergy and food allergy groups, respectively, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.

45.26%; NA, 44.83%). After reaching the highest value on day 3, Firmicutes abundance exhibited a decreasing trend (1 month [FA, 35.43%; NA, 34.49%) and 3 months (FA, 28.90%; NA, 20.59%)]. At 6 months (FA, 21.27%; NA, 25.45%), Firmicutes abundance in the NA group suddenly increased; however, that of Firmicutes in the FA group continued to decrease steadily (Figure 6).

The composition of fecal microbiota at the genus level varied over time in the two groups

In the maternal samples, at the genus level, among the top 20 species, *Bacteroides* accounted for the highest proportion (FA, 35.28%; NA, 37.88%), which was higher in the NA group than in the FA group, followed by *Prevotella* (FA, 6.41%; NA, 12.59%), whose proportion was higher in the NA group, and *Escherichia/Shigella* (FA, 5.56%; NA, 3.94%), whose proportion was higher in the FA group. The proportions of *Clostridium XIVa* (FA, 4.04%; NA, 1.96%), *Faecalibacterium* (FA, 4.11%; NA, 3.37%), *Parabacteroides* (FA, 4.63%; NA, 2.25%), and *Ruminococcus* (FA, 2.83%; NA, 1.89%) were higher in the FA group than in the NA group, whereas those of *Roseburia* (FA, 2.90%; NA, 3.86%), *Dialister* (FA, 1.90%; NA, 2.16%), and *Megamonas* (FA, 0.14%; NA, 1.59%) were higher in the NA group (Figure 7A). Further analysis of the abundance of the main maternal gut microbiota in the offspring's gut microbiota revealed that the proportions of *Bacteroides* and *Escherichia/Shigella* exhibited the same trend between the two

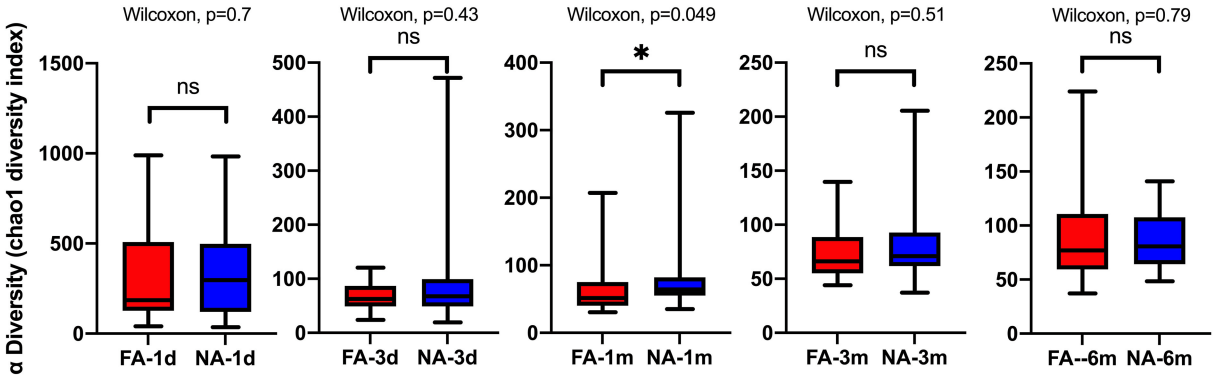

FIGURE 3
Differences in the same-time alpha diversity between non-allergy (NA) and food allergy (FA) groups using the Chao1 index. The Wilcoxon test was used to assess differences between two groups at one time point. *$P < 0.05$; ns, not significant.

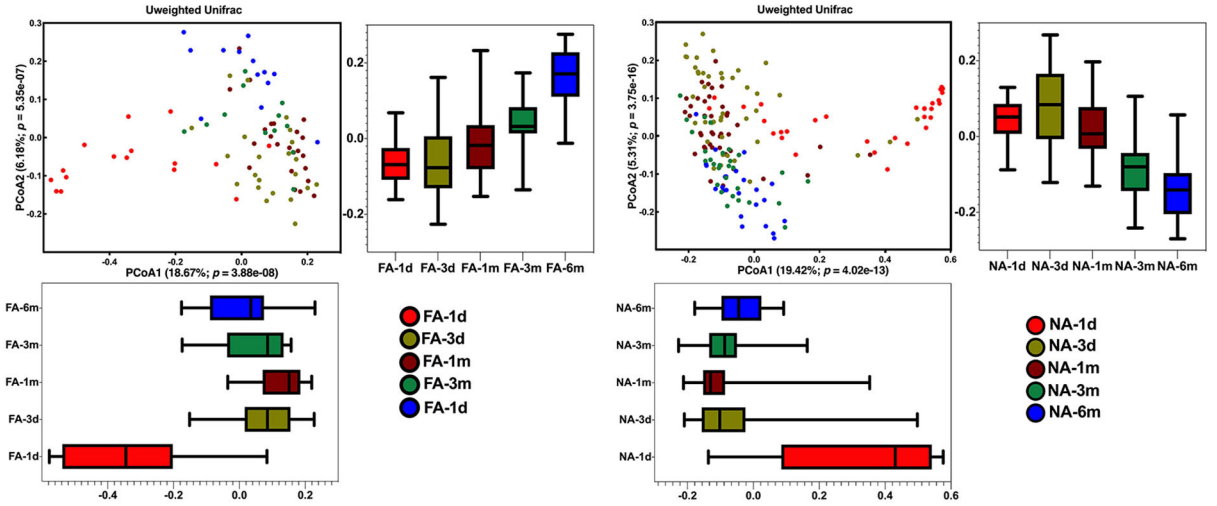

FIGURE 4
Differences in beta diversity over time between non-allergy (NA) and food allergy (FA) groups, as determined using principal coordinates analysis (PCoA) combined with Adonis analysis. $P < 0.05$ indicates a statistical significance.

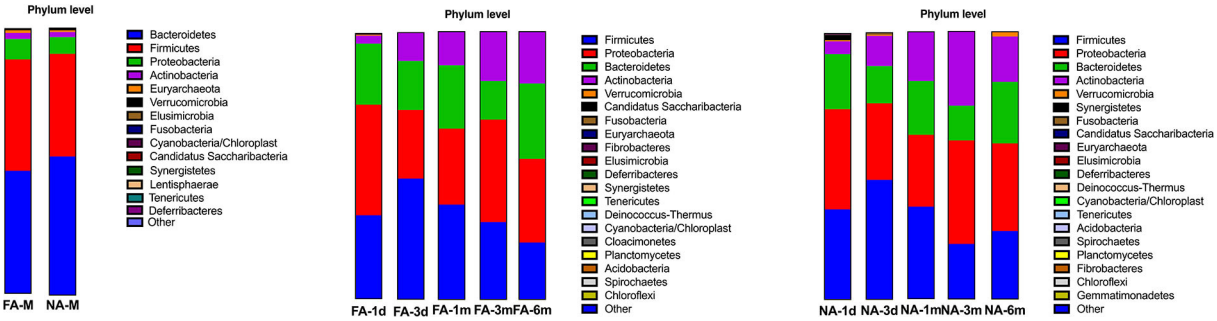

FIGURE 5
Bacterial phyla in the maternal and infant cohorts in the non-allergy (NA) and food allergy (FA) groups.

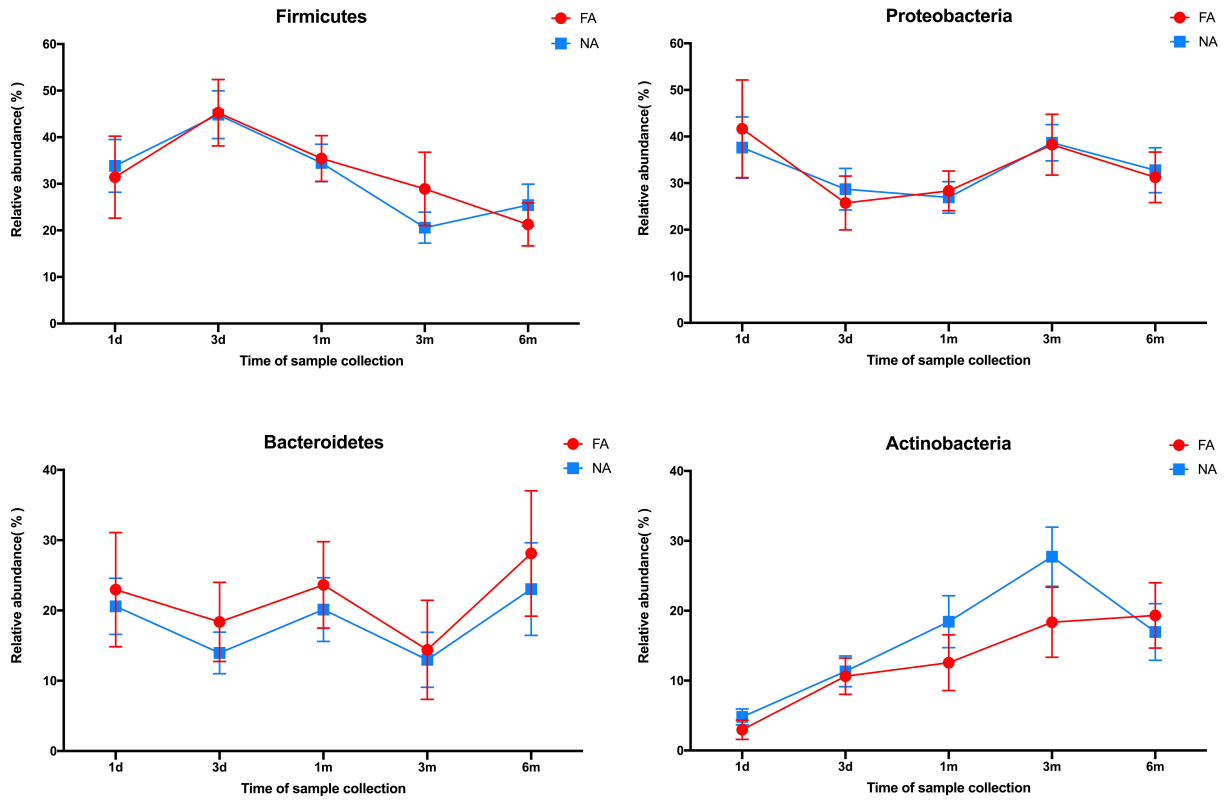

FIGURE 6
Differences in the relative abundance of major phyla over time between the non-allergy (NA) and food allergy (FA) groups.

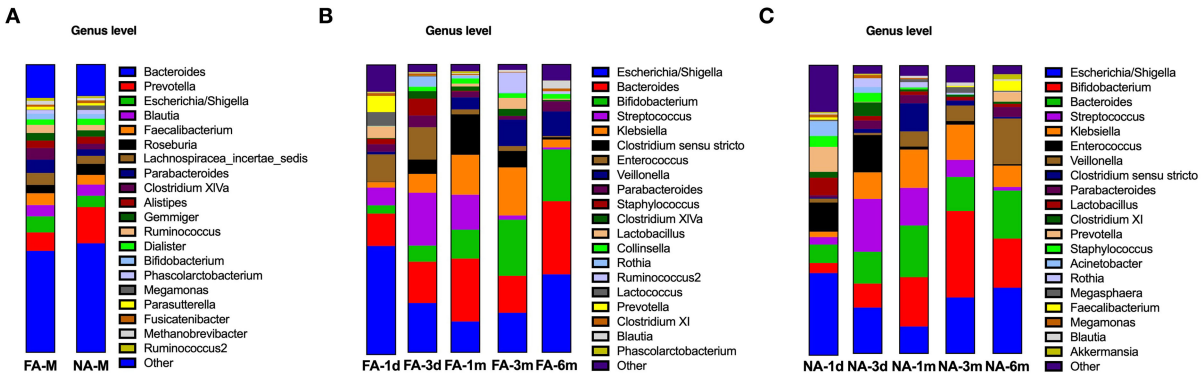

FIGURE 7
Bacterial genera in maternal **(A)** and infant **(B,C)** participants in the non-allergy (NA) and food allergy (FA) groups.

groups. The abundance of *Prevotella* in the infant gut microbiota was high only on day 1 (FA, 5.58%; NA, 8.62%), low at other periods, and increased again at 6 months in the NA group (3.29%) (Figure 8A).

In the infant samples, taxonomy-based analysis revealed that the fecal microbiota in the early life (0–6 months) primarily comprised species belonging to the genera *Bacteroides* and *Escherichia/Shigella*, followed by *Bifidobacterium* and *Streptococcus*. The abundance of each genus at different periods after birth varied with respect to increase and decrease; compared with the FA group, variations in the NA group had different trends (Figures 7B,C).

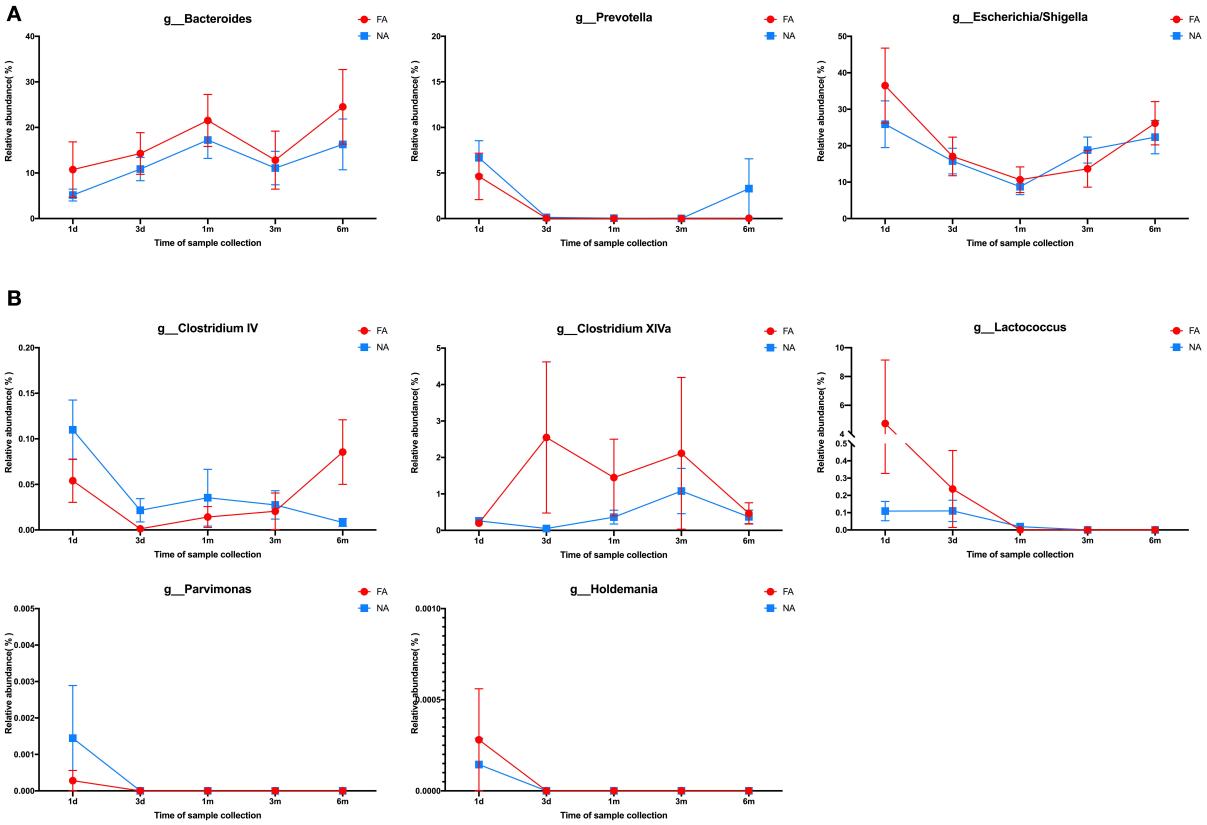

FIGURE 8
(A) Changes in the maternal main gut microbiota in late pregnancy compared with the infant gut microbiota over time. **(B)** Changes in the maternal differential gut microbiota in late pregnancy compared with infant gut microbiota over time.

The abundances of these four dominant bacterial genera varied with time and exhibited a similar trend between the NA and FA groups. *Bacteroides* had the highest proportion, and its abundance gradually increased within 1 month after birth (FA, 21.9%; NA, 17.9%), decreased at 3 months (FA, 12.9%; NA, 11.8%), and increased again at 6 months (FA, 25.4%; NA, 16.7%). The proportion of *Escherichia/Shigella* was 28.3% and 37.5% on day 1, gradually decreased to 9.3% and 10.7% at 1 month, and gradually increased to 22.8% and 27.1% at 6 months in the NA and FA groups, respectively. The proportion of *Streptococcus* in the NA group (2.6%) was lower than that in the FA group (6.1%) on day 1 and reached its highest value on day 3 with similar values in the NA (18.358%) and FA groups (18.364%). Subsequently, *Streptococcus* proportion decreased gradually and was higher at 1 month (FA, 12.13%; NA, 13.09%), 3 months (FA, 1.49%; NA, 5.91%), and 6 months (FA, 0.73%; NA, 1.26%) in the NA group than in the FA group. The proportion of *Bifidobacterium* increased gradually over time, which was different from that observed for other dominant bacterial genera. The *Bifidobacterium* proportion was the lowest on day 1 and was higher in the NA group (3.5%) than that in the FA group (2.9%). It increased gradually and reached its highest value on day 3 (FA, 5.61%; NA, 8.33%) and at 1 month (FA, 10.04%; NA, 17.17%), and was higher in the NA group (30%) than in the FA group (19.5%). At 6 months, the *Bifidobacterium* proportion returned to the same level (FA, 17.98%; NA, 17.08%).

Comparison of gut microbiota between the NA and FA groups

LEfSe analysis was used to identify the communities or species that had a significant influence on sample division, as presented in Figure 9. There were no significant differences between the two groups at the same time points. At the genus level, significant differences in relative abundance between the two groups at the same time point are listed in Table 3.

In the maternal samples, the relative abundance of taxa belonging to the genera *Clostridium IV* ($P = 0.032$), *Clostridium XlVa* ($P = 0.013$), *Lactococcus* ($P = 0.033$), and *Parvimonas* ($P = 0.018$) in the FA group was higher than that in the NA group. The

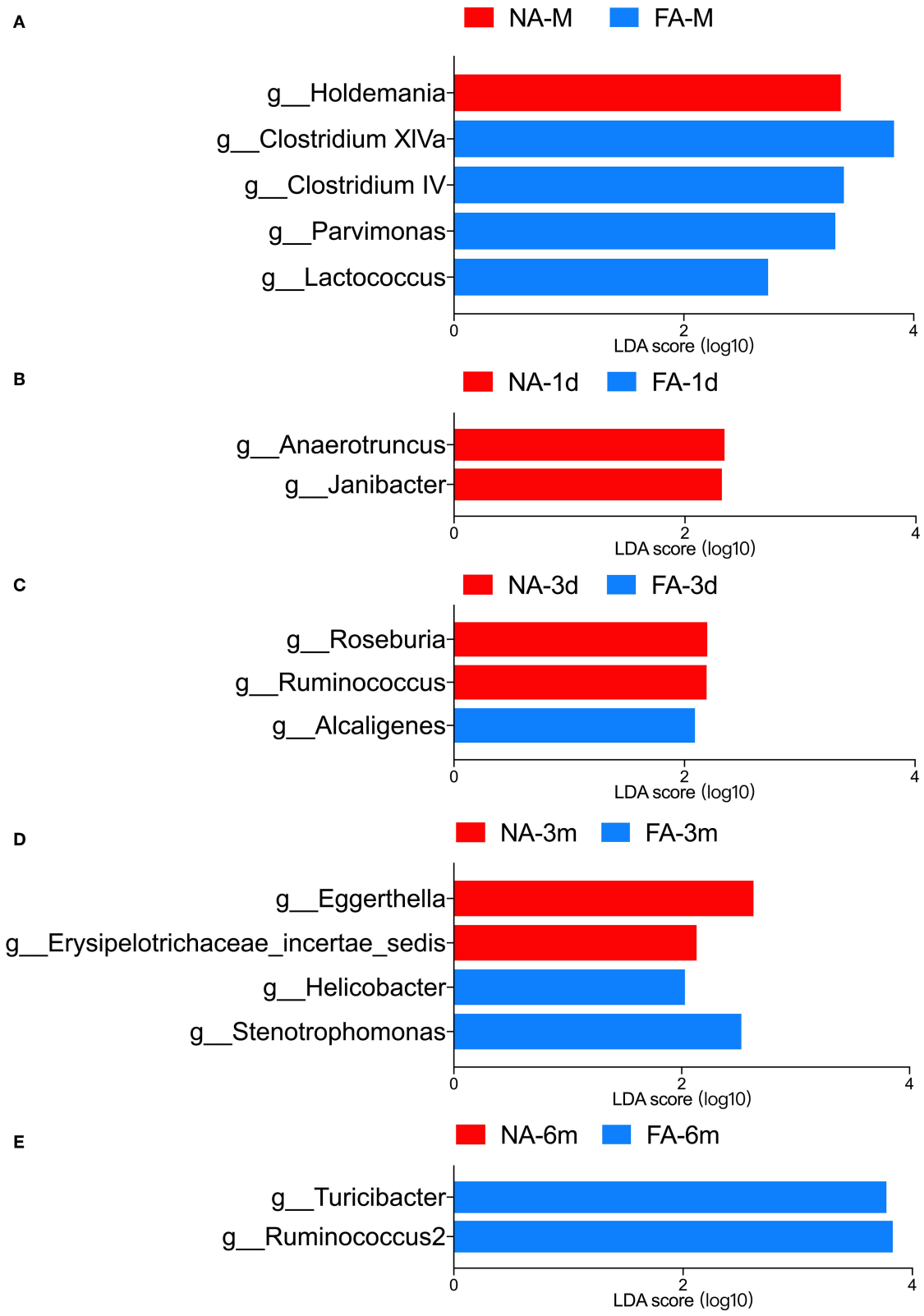

FIGURE 9
LEfSe analysis of maternal **(A)** and infant gut microbiota at different timepoints **(B–E)** was compared between the non-allergy group (NA) and food allergy (FA) groups. Only the genera with linear discriminant analysis (LDA) scores > 2 and $P < 0.05$ are presented.

relative abundance of *Holdemania* ($P = 0.036$) in the FA group was lower than that in the NA group. However, in the infant samples, *Holdemania* was observed only in the day-1 samples (meconium) rather than in samples of other periods, and its abundance did not differ between the two groups. Similarly, the relative abundance of *Lactococcus* in the day-1 samples was higher in the FA group than in the NA group. There was a significant decrease in abundance over time in the FA group, and in the 1, 3, and 6-month samples, the abundance of *Lactococcus* was higher in the NA group; however, there was no significant difference between the two groups. Further analysis of the changes in the different infant gut microbiota revealed that *Parvimonas* and *Holdemania* were only observed in meconium microbiota, while *Lactococcus* was only observed on day 1, day 3, and 1 month; however, there was no significant difference between the two groups. The relative abundance of *Clostridium XlVa* was the lowest on day 1, which increased and decreased over time. Altogether, the relative abundance of *Clostridium XlVa* in the FA group was greater than that in the NA group; however, there was no significant difference between the two groups at each time point. For the genus *Clostridium IV*, the relative abundance in the FA group was higher than that in the NA group at all time points except at 6 months (Figure 8B).

Comparison of the infant samples of two groups at the same time points revealed that in 1-day samples (meconium), the relative abundance of taxa belonging to the genera *Anaerotruncus* ($P = 0.029$) and *Janibacter* ($P = 0.018$) in the healthy group was higher than that in the diseased group, whereas the relative abundance of the genera *Acidaminococcus* ($P = 0.015$) and Hydrogenophaga ($P = 0.020$) in the NA group was lower than that in the FA group. In the 3-day samples, the relative abundance of the genera *Roseburia* ($P = 0.040$) and *Ruminococcus* ($P = 0.039$) in the NA group was higher than that in the FA group, and the relative abundance of genus *Alcaligenes* ($P = 0.016$) in the NA group was lower than that in the FA group. However, at 1 month, no significant difference was observed between both groups. In 3-month samples, the relative abundance of the genera *Atopobium* ($P = 0.016$), *Eggerthella* ($P = 0.021$), and *Erysipelotrichaceae* ($P = 0.049$) in the NA group was higher than that in the FA group. In 6-month samples, the relative abundance of the genus Ruminococcus2 ($P = 0.0016$) in the NA group was significantly lower than that in the FA group.

TABLE 3 Comparison of gut microbiota at the genus level between the non-allergy and food allergy groups.

Participants/ Time		Genus	Food allergy (mean)	Non-allergy (mean)	P^a
Mother		*Clostridium IV*	7.98E-03	4.48E-03	0.032
		Clostridium XlVa	3.41E-02	1.74E-02	0.013
		Holdemania	7.80E-06	2.45E-05	0.036
		Lactococcus	8.77E-05	1.39E-05	0.033
		Parvimonas	2.73E-05	0	0.018
Infant	**Day 1**	*Acidaminococcus*	1.12E-05	0	0.015
		Anaerotruncus	1.12E-05	4.55E-04	0.029
		Hydrogenophaga	5.32E-05	1.45E-06	0.020
		Janibacter	1.57E-04	5.09E-04	0.018
	Day 3	*Alcaligenes*	6.11E-05	0	0.016
		Roseburia	6.93E-05	3.43E-04	0.040
		Ruminococcus	1.83E-05	1.34E-04	0.039
	3 months	*Atopobium*	5.23E-05	1.89E-04	0.016
		Eggerthella	9.71E-05	9.51E-04	0.021
		Erysipelotrichaceae	0	2.98E-04	0.049
		Helicobacter	3.74E-05	3.09E-06	0.0075
		Parascardovia	2.24E-05	0	0.029
		Stenotrophomonas	8.59E-05	1.55E-06	0.034
	6 months	*Ruminococcus2*	7.76E-03	9.34E-06	0.0016
		Turicibacter	9.65E-04	0	0.017

Values are expressed as the mean of the relative abundance. Only the genera with significant differences are listed.
[a]P <0.05 indicates statistical significance.

Discussion

Accumulating evidence has demonstrated that the infant gut microbiota can influence the development of food allergies. Additionally, the influence of the maternal gut microbiota in the late trimester on the development of allergic diseases in the offspring has been proposed recently. Therefore, in this prospective study of 68 pairs of mothers and infants in China, we sought to investigate the relationship between food allergy and gut microbiota in the third trimester and early infancy by collecting and analyzing stool samples from mothers within 1–2 weeks before delivery and infants at five time points.

In this study, based on questionnaire-based survey results, the history of food allergy in the mother increased the risk of food allergy in the offspring. The maternal fecal microbiota investigated before delivery exhibited no difference in alpha diversity and beta diversity between the NA and FA groups. This suggests that the gut microbiota of healthy pregnant women in the third trimester of pregnancy is similar in species composition and abundance. However, we identified some bacterial genera whose relative abundance differed significantly between the two groups. The relative abundance of *Holdemania* was significantly higher in the NA group than in the FA group. *Holdemania* is a gram-positive anaerobic genus belonging to the family *Erysipelotrichaceae* and is involved in the catabolism of mucin, which can promote intestinal barrier damage and trigger a systemic inflammatory response after entering the intestinal mucosa (Raimondi et al., 2021). It was associated with diet during pregnancy i.e., high fiber intake was associated with a higher abundance of *Holdemania* (Gomez-Arango et al., 2018).

The abundance of *Holdemania* also positively correlated with total polyunsaturated fatty acids (PUFAs) and both ω-3 and ω-6 PUFAs (Barrett et al., 2018). Meta-analysis results suggested the benefits of increased ω-3 PUFAs in the maternal diet and outcomes of childhood allergic diseases, including food allergy (Best et al., 2016). Thus, the abundance of *Holdemania* in the maternal feces during pregnancy is potentially an important predictor of food allergies in the offspring. The relative abundance of this bacterium in the infant gut microbiota was very low, suggesting that its effect on food allergy in the offspring is not correlated with its content in the infant gut microbiota. We also detected *Holdmania* only on day 1 in the infant gut microbiota, albeit not at other times. There may be a correlation between the gut microbiota of infants (meconium) and that of mothers in the third trimester.

By contrast, in the maternal samples, the relative abundance of *Clostridium IV*, *Clostridium XIVa*, and *Lactococcus* in the FA group was high. These *Clostridium* spp. have rarely been mentioned in studies on maternal gut microbiota and offspring food allergy; however, a concordance was observed in infant gut microbiota and allergic disease studies (Vael et al., 2011; Nylund et al., 2013; Chen et al., 2016). Importantly, the abundance of *Clostridium XIVa* and *Lactococcus* in the gut microbiota of infants with food allergies was low (Atarashi et al., 2011; Savage et al., 2018). *Lactococcus* is a probiotic that helps prevent the development of food allergies (Frossard et al., 2007; Shin et al., 2018), which is contrary to our maternal gut microbiota findings. In the infant gut microbiota, the relative abundance of this bacterium was considerably low after 1 month, and there was no significant difference between the two groups at each time point, suggesting that the effect of *Lactococcus* on infant food allergy may play a central role in the colonization of the infant's intestine.

The infant gut microbiota changes dynamically early in life. Our study revealed that gut microbiota richness in both the NA and FA groups was the highest on day 1 after birth, gradually declined thereafter, reached the lowest level at 1 month after birth, and gradually increased, with significant differences between 1 day and the other time points, suggesting that the meconium microbiota of infants may be affected by the gut microbiota in the third trimester of pregnancy. Comparison of the fecal microbiota of infants between the two groups revealed a significant difference in alpha diversity at 1 month; however, there was no difference in beta diversity at any time point. These results indicate that the gut microbiota in infants may be reconstructed after birth. This reconstruction process is divided into two stages, i.e., birth to 1 month and 1-6 months.

The gut microbiota of healthy infants also underwent dynamic reconstruction, as evidenced by changes at the phylum level. In our study, the relative abundance of Actinobacteria was similar between the two groups during birth until 1 month, with that of the NA group being slightly higher than that of the FA group. However, from 1 to 6 months, this difference in the relative abundance of Actinobacteria between the two groups gradually increased. At 6 months, Actinobacteria abundance suddenly decreased in the NA group; no such trends were observed in the FA group. Similarly, the variation trend and relative abundance of Firmicutes were similar during birth until 1 month. At 1-6 months, the difference between the two groups gradually increased. At 6 months, abundance suddenly increased in the NA group, and no such fluctuation was detected in the FA group. These results suggest that the abundance of Actinobacteria and Firmicutes fluctuated more dynamically early in life (6 months after birth), and these fluctuations may be beneficial for the prevention of food allergy. This is similar to some of the findings reported in another study (Shen et al., 2019).

We observed some differences between the NA and FA groups at the genus level. The relative abundance of *Anaerotruncus* on day 1, *Roseburia* and *Ruminococcus* on day 3, and *Erysipelotrichaceae* at 3 months were significantly higher in the NA group than in the FA group, suggesting that these genera may have a protective effect on infant food allergy. *Anaerotruncus*, belonging to the Ruminococcaceae family that includes short-chain fatty acid (SCFA)-producing bacteria (Yan et al., 2019), confers protective effects against food allergy (Shu et al., 2019). *Roseburia* is a butyrate-generating microorganism (Duncan et al., 2004; Zhuang et al., 2019), and it has been considered a potential indicator of intestinal health (Tamanai-Shacoori et al., 2017); a link has been established between *Roseburia* and intestinal diseases, including inflammatory bowel disease (Kellermayer, 2019; Luo et al., 2019; Yang et al., 2020), IBS (Chassard et al., 2012), and colon cancer (Wang et al., 2012). *Roseburia* has also been reported to strengthen intestinal barrier function and enhance Treg population expansion (Patterson et al., 2017; Yan et al., 2019), which may be beneficial with respect to food allergies. The genus *Ruminococcus* has been implicated in mediating protection from asthma through the production of SCFAs and volatile substances with the ability to reduce T-helper cell type 2-mediated allergic airway inflammation (Ege, 2017; Zhuang et al., 2019), and it has also been suggested that it may be involved in the development of ovalbumin tolerance (Xu et al., 2022). *Erysipelotrichaceae* has also been reported to be a key butyrate-producing bacterium (Estaki et al., 2016), which is reported to be more abundant in the gut microbiota during the first month of life in infants with IgE-mediated food allergy (Joseph et al., 2022). We observed that these bacteria have one thing in common: They are SCFA-producing bacteria. Recently, SCFAs, the main class of gut microbiota-derived metabolites, have been proposed to have beneficial effects with respect to food allergy. Some studies suggest that SCFA butyrate directly affects mast cells by epigenetically regulating FcεRI-mediated signaling molecules (Folkerts et al., 2018, 2020; Wang et al., 2018). Thus, by directly inhibiting IgE-mediated mast cell degranulation and allergen-induced histamine release, microbial SCFAs, such as butyrate, may exhibit therapeutic benefits with

respect to human food allergies. One study reported that children with a milk allergy had lower levels of butyrate in their feces than healthy controls (Berni Canani et al., 2018). Altogether, these results establish an important role for dietary fiber and SCFAs in reinforcing the integrity of the epithelial barrier, oral tolerance, and protection against food allergy (Luu et al., 2020). Interestingly, in this study, we observed that the abundance of SCFA-producing bacteria in the feces of non-allergic infants was significantly higher than that in infants with food allergies. This suggests that a high abundance of SCFA-producing bacteria in the gut may have a protective effect with respect to food allergies, possibly *via* increased concentration of SCFAs, especially butyric acid, in the gut. However, it is necessary to further study the specific mechanisms by which gut microbiota influences the development of food allergies using mouse models.

We observed that *Prevotella* was the second dominant bacterial genus in the maternal gut microbiota. However, in the infant gut microbiota, the relative abundance of *Prevotella* was higher only on the first day after birth and lower at later time points, suggesting that the maternal gut microbiota in the third trimester of pregnancy partly affects the meconium microbiota of the offspring. Nonetheless, with the reconstruction of gut microbiota in the offspring, this effect was not retained. An increase in *Prevotella* abundance in the maternal gut microbiota during pregnancy had a protective effect on food allergy, which is unrelated to *Prevotella* abundance in the gut microbiota of the offspring (Vuillermin et al., 2020). In our study, the relative abundance of *Prevotella* in the maternal gut microbiota in the NA group was higher than that in the FA group, albeit there was no significant difference between the two groups. In both groups, the presence of *Holdemania*—associated with food allergy in the offspring—was not related to the abundance of this bacteria in the infant gut microbiota. We can further speculate that the effect of the maternal gut microbiota on food allergy in the offspring is not only mediated by regulating the changes in the infant gut microbiota but rather through metabolites.

This study had certain limitations. First, to analyze the relationship between the maternal (during pregnancy) and infant intestinal microbiota, we compared changes in the main flora and differential bacteria between the maternal (during pregnancy) and infant intestinal flora only. Second, both milk microbiota and saliva microbiota may affect the composition of the gut microbiota (Pannaraj et al., 2017; Reddel et al., 2022); however, we did not collect milk and saliva samples, which may explain the discrepancies between our results and the results of previous studies.

Conclusion

In this study, maternal carriage of *Holdemania* during the third trimester strongly predicts the absence of food allergies in the offspring. However, it was not observed to be significantly correlated with the distribution of the gut microbiota in the progeny. The maternal gut microbiota in the third trimester of pregnancy was correlated with the infant gut microbiota on day 1 after birth (meconium). However, this effect was not retained post-reconstruction of the infant gut microbiota after birth, suggesting that the effect of maternal gut microbiota on food allergy in the offspring may not be primarily mediated through the regulation of changes in the infant gut microbiota. The infant gut microbiota undergoes dynamic changes early in life. The abundance of phyla Actinobacteria and Firmicutes fluctuates more dynamically during the early period of life, which may be beneficial for the prevention of food allergies. SCFA-producing bacteria, especially butyric acid-producing bacteria, including *Anaerotruncus*, *Roseburia*, *Ruminococcus*, and *Erysipelotricaceae*, may have a protective effect against food allergy. Nonetheless, the specific mechanism responsible for this phenomenon needs to be studied further.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI - PRJNA848136.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Ethics Committee of Peking University Third Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

ZL, YX, and SW conceived the study. YW, LL, YX, and ZL recruited participants. SW, RZ, XL, YG, and ND collected samples. SW wrote the manuscript and prepared the figures. All authors provided critical intellectual content and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.933152/full#supplementary-material

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A health-promoting role of exclusive breastfeeding on infants through restoring delivery mode-induced gut microbiota perturbations

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The establishment of human gut microbiota in early life is closely associated with both short- and long-term infant health. Delivery mode and feeding pattern are two important determinants of infant gut microbiota. In this longitudinal cohort study, we examined the interplay between the delivery mode and feeding pattern on the dynamics of infant gut microbiota from 6 weeks to 6 months post-delivery in 139 infants. We also assessed the relationship between infant respiratory infection susceptibility and gut microbial changes associated with delivery mode and feeding pattern. At 6 weeks postpartum, the composition and structure of gut microbiota of cesarean section-delivered (CSD) infants differed from those of vaginally delivered (VD) infants, with decreased *Bacteroides* and *Escherichia-Shigella* and increased *Klebsiella*, *Veillonella,* and *Enterococcus*. At 6 months postpartum, these delivery mode-induced microbial shifts were restored by exclusive breastfeeding, resulting in similar gut microbial profiles between VD and CSD infants who were exclusively breastfed ($P = 0.57$) and more variable gut microbial profiles between VD and CSD infants who were mixed fed ($P < 0.001$). We identified that the VD-associated genera were enriched in healthy infants, while the CSD-associated genera were enriched in infants who suffered from respiratory infections. Our findings indicate that exclusive breastfeeding may play a health-promoting role by reducing infant respiratory infection susceptibility through the restoration of gut microbiota perturbations caused by cesarean section.

KEYWORDS

gut microbiota, early life, delivery mode, exclusive breastfeeding, disease susceptibility

Introduction

The gut microbiota is regarded as a complex and dynamic organ that interacts with the host metabolic pathways, immune responses, and developmental processes, influencing long-term host health (Tamburini et al., 2016; Robertson et al., 2019; Brodin, 2022). During the first years of life, the human gut microbiota develops toward an adult-like community by 2–3 years old, exhibiting a highly stage-specific progression (Stewart et al., 2018). The maturation of gut microbiota is shaped by numerous perinatal factors, including delivery

mode (Reyman et al., 2019; Shao et al., 2019; Song et al., 2021), feeding pattern (Stewart et al., 2018; Fehr et al., 2020), antibiotic exposure (Bokulich et al., 2016), and gestational age (Fouhy et al., 2019). Recent longitudinal cohort studies have proven that delivery mode is a major determinant of gut microbiota during the first weeks of life, with cesarean section delivery (CSD) disrupting the natural transmission of gut microbiota from mothers to offspring (Shao et al., 2019). Specifically, the enrichment of *Bifidobacterium*, *Escherichia*, *Bacteroides,* and *Parabacteroides* in vaginally delivered (VD) infants can promote human milk oligosaccharide (HMO) utilization (Wang et al., 2019) and immune stimulation in early life (Jakobsson et al., 2014; Wampach et al., 2018). In contrast, the gut microbiota of infants delivered by cesarean section is dominated by *Enterococcus*, *Staphylococcus*, *Streptococcus,* and *Klebsiella*, which are associated with the hospital environment (Lax et al., 2017).

A growing body of evidence shows that cesarean section delivery is associated with adverse effects on infant and child immune development (Pattaroni et al., 2018), resulting in higher rates of asthma (Roduit et al., 2009; Tang et al., 2021) and respiratory infections (RIs) as morbidities (Bosch et al., 2016; Baumfeld et al., 2018; Reyman et al., 2019). A reduction in health-promoting *Bifidobacterium* and an enrichment in potentially pathogenic *Enterococcus* and *Klebsiella* in cesarean section-delivered infants have been linked with more RIs during the first year of life (Reyman et al., 2019), indicating a mediating role of gut microbial changes in the cesarean delivery-induced disease susceptibility.

The effect of the delivery mode gradually diminishes by 6 months of life (Hill et al., 2017) or earlier (Reyman et al., 2019), indicating that the gut microbiota could recover from the state associated with cesarean section delivery. Our previous study demonstrated that shifts in infant gut microbiota associated with cesarean section delivery were alleviated by exclusive breastfeeding (EBF) in the first several weeks of life, suggesting an interactive impact on feeding pattern and the delivery mode of gut microbiota (Liu et al., 2019). Additionally, prolonged breastfeeding duration influenced the gut microbiota of CSD infants but not VD infants at 24 weeks of age, indicating that CSD infants may benefit from breastmilk by obtaining specific bacteria, initially lacking due to delivery mode (Hill et al., 2017). The gut microbiota of exclusively breastfed and formula-fed infants remain distinct (Backhed et al., 2015b), even when specific components are added to the formula to promote breastfed-like microbial communities (Baumann-Dudenhoeffer et al., 2018), demonstrating that the nutritional and immune benefits of breastfeeding were indispensable (Gopalakrishna and Hand, 2020; Gridneva et al., 2021; Donald et al., 2022).

However, few studies have explored the relationship between infant respiratory health and gut microbiota shifts associated with delivery mode and feeding pattern. In this prospective cohort study containing 139 infants, we investigated the health-promoting role of exclusive breastfeeding on infant gut microbiota shifts induced by cesarean section, which were associated with reduced respiratory infection susceptibility during the first months of life.

Materials and methods

Ethics

Written informed consent was obtained from the legal guardian for the publication of any potentially identifiable data included in this article.

Study population

This ongoing prospective cohort study has been conducted at Peking University First Hospital since October 2017, which recruited 139 infants at 6 weeks postpartum, and 72.7% (101/139) of them completed the 6-month follow-up. This study was approved by the Institutional Ethics Committee of Peking University First Hospital (V2.0/201504.20), and all the participants or legal guardians provided written informed consent.

Clinical information

Prenatal and perinatal information was obtained from electronic medical records and questionnaire surveys (Supplementary Table 1), including maternal age, gravidity, parity, pre-pregnancy body mass index (BMI), delivery mode, gestational age, infant sex, birth weight, feeding patterns, and the occurrence of respiratory infection (RI) events during the first 6 months of age. Considering the confounding effect of delivery mode, we also recorded the type (labored or elective) and cause of cesarean section. EBF is defined as feeding with breast milk exclusively after birth. MF is defined as feeding with a mixture of varying proportions of breast milk and formula milk. RI events are defined as the occurrence of the following mother-reported symptoms: pneumonia, bronchitis, or fever (>38°C) accompanied by snuffling, sneezing, coughing, or wheezing.

Sample collection, DNA extraction, and 16s rRNA gene sequencing

A total of 139 infants were collected at 6 weeks, with 101 infants longitudinally collected at 6 months. Fresh stool samples were self-collected at home, according to the standardized protocol, as described in a previous study (Liu et al., 2019). All stool samples were frozen at −80°C within 2 h. DNA was extracted with the QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany), following the manufacturer's protocols. The V3-V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) with 341 forward primers (5′ CCTACGGGNBGCASCAG) and 805 reverse primers (5′ GACTACNVGGGTATCTAATCC). PCR was performed in a 25-μl volume with 1 U of HiFi HotStart DNA Polymerase (KK2502, Kapa Biosystems, Cape Town, South Africa), 12.5 ng of template DNA, and 5 μM of forward and reverse primers with the following amplification program: initial denaturation at 95°C for 3 min; 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; and final extension

at 72°C for 5 min and hold at 4°C. AMPure XP beads (A63882, Beckman) were used to purify the 16S V3-V4 amplicons away from the free primers and primer dimers.

For Illumina sequencing adapter attachment, PCR was performed for a second time with Illumina sequencing primers under the same conditions as the first time, only with seven cycles and an annealing step increased to 1 min. DNA libraries were quantified with a NanoDrop 2000 system and purified with AMPure XP beads before sequencing on the Illumina HiSeq 2500 platform. Fast Length Adjustment of Short Reads (FLASH) was used to merge paired-end reads from sequencing (Magoc and Salzberg, 2011). Low-quality reads were filtered with the FASTQ quality filter (-p 90 -q 25 -Q33) using the FASTX Toolkit 0.0.14, and chimeric reads were removed by USEARCH 64-bit v8.0.1517. The number of reads for each sample was normalized based on the smallest sample size by random subtraction.

16s rRNA data processing and statistical analyses

The 16s rRNA sequencing data were processed by Quantitative Insights Into Microbial Ecology (QIIME). Operational taxonomic units (OTUs) were aligned by the UCLUST algorithm with 97% identity and taxonomically classified using the SILVA 16S rRNA database v128. The bacterial compositions were visualized with a heatmap, which was generated via Seaborn, a Python data visualization library. Alpha diversity was evaluated by Shannon and Simpson indexes. Beta diversity was calculated based on weighted UniFrac and Bray–Curtis distance matrices and visualized by principal coordinates analysis (PCoA) of weighted UniFrac and Bray–Curtis distance matrices, in 1,000 permutations, and statistical comparisons of groups were calculated by multivariate permutational analysis of variance (PERMANOVA) methods using the Adonis function in the R package "vegan." Metric variables are shown as the mean ± SD or median (interquartile range) and compared with Student's *t*-test or Mann–Whitney *U*-test, according to the normality of the data distribution. The Chi-squared and Fisher's exact tests were used to compare the proportions of analyses. The significance was set at a *P*-value of <0.05. GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA) was used for statistical and graphical preparation.

Results

Population characteristics

In this longitudinal study, stool samples were collected from each individual at ~6 weeks postpartum (6W, $n = 139$) and 6 months postpartum (6M, $n = 101$), and some of the individuals had been included in our previous study (Liu et al., 2019). Among 139 infants, 93 (66.9%) infants were born by vaginal delivery, and 46 infants were born by cesarean section delivery. Among 101 infants, 71 (70.1%) infants were born by vaginal delivery, and 30 infants were born by cesarean section delivery. Basic characteristics at 6W postpartum and 6M postpartum stratified by delivery mode, are shown in Table 1. Clinical variables were similar between the VD and CSD groups, with the exception of gestational age ($P < 0.001$ at 6W; $P = 0.03$ at 6M), which was intrinsically related to the delivery mode. Notably, VD infants were more likely to receive exclusive breastfeeding; this difference persisted to 6M, although significance was not reached. The occurrence rate of RI over the first 6 months was significantly higher in CSD infants than in VD infants ($P = 0.02$).

Infant gut microbiota clusters according to age

In total, 38,955,382 bacterial reads were identified in 240 fecal samples, which were annotated into 654 OTUs distributed over 8 bacterial phyla. Firmicutes (43.9%) was the most abundant phylum, followed by Actinobacteria (21.9%), Proteobacteria (21.2%), and Bacteroidetes (12.6%).

We first conducted a chronological comparison of infant gut microbial composition and community structure between 6 weeks and 6 months of age. No significant difference was found in alpha diversity between the 6W and 6M groups, according to either the Shannon index (Figure 1A, $P = 0.41$) or the Simpson index (Figure 1B, $P = 0.43$). Beta diversity was assessed using Bray–Curtis distance matrices, and infant gut microbiota structure remained markedly distinct between 6W and 6M (Figure 1C, Adonis, $P < 0.001$, $R^2 = 0.058$). The gut microbiota of infants converged toward a tighter cluster at 6M compared to 6W. This observation was further consolidated by a comparison of the weighted UniFrac within-group distance (Figure 1D, $P < 0.0001$), suggesting that infants shared a more homogeneous microbial community with aged.

A heatmap of the top 15 genera based on an average relative abundance of >1% was used to identify age-associated patterns in infant gut microbiota. The heatmap revealed that *Clostridium sensu stricto1*, *Klebsiella*, and *Streptococcus* (highlighted on the left side of the heatmap), which are associated with the hospital environment (Lax et al., 2017), are present at higher relative abundances at 6W ($P < 0.05$, $P < 0.001$, and $P < 0.001$, respectively). In contrast, *Bifidobacterium*, *Bacteroides*, *Escherichia-Shigella*, and *Veillonella* (highlighted on the right side of the heatmap) were enriched at 6M ($P < 0.05$, $P = 0.11$, $P < 0.001$, and $P < 0.001$, respectively, Figure 1E).

The effect of delivery mode on infant gut microbiota dissipates with age in a feeding pattern-dependent manner

We further investigated the impact of delivery mode on the infant gut microbiota, stratified by age. At 6W, a significant difference was found in the gut microbial community structure between VD and CSD infants (Figure 2A, Adonis, $P = 0.037$, $R^2 = 0.012$). However, this difference dissipated at 6M (Figure 2B, Adonis, $P = 0.215$, $R^2 = 0.012$). These results are consistent with previous studies (Stewart et al., 2018; Wampach et al., 2018; Reyman et al., 2019; Shao et al., 2019), demonstrating that delivery

TABLE 1 Infants' clinical parameters stratified by delivery mode at 6W and 6M.

Characteristics	6W			6M		
	Mean ± SD, median (IQR) or *n* (%)			Mean ± SD, median (IQR) or *n* (%)		
	VD (*N* = 93)	CSD (*N* = 46)	*P*-value	VD (*N* = 71)	CSD (*N* = 30)	*P*-value
Maternal age (years)	31.67 ± 3.77	32.72 ± 3.87	0.10	31.41 ± 3.85	32.90 ± 4.29	0.08
Gestational age (weeks)	39.22 ± 1.26	38.41 ± 1.24	***<0.001***	39.14 ± 1.25	38.7 ± 1.09	***0.03***
Maternal pre-pregnancy BMI	22.19 ± 3.27	23.19 ± 3.12	0.08	22.08 ± 3.11	23.37 ± 3.25	0.06
Gravity	2 (1, 2)	2 (1, 3)	0.09	1 (1, 2)	2 (1, 3)	0.11
Parity	1 (1, 2)	1 (1, 2)	0.19	1 (1, 1)	1 (1, 2)	0.29
Sampling time (days)	48 (44, 55)	49 (43.75, 58.75)	0.48	208 (199, 219)	202 (187, 217)	0.15
Birth weight (g)	3,302 ± 382.2	3,296 ± 469.5	0.94	3,280 ± 366.3	3,377 ± 468.9	0.27
Gender			0.15			0.19
Male	54 (58.06)	20 (43.48)		42 (59.15)	13 (43.33)	
Female	39 (41.94)	26 (56.52)		29 (40.85)	17 (56.67)	
Feeding patterns			0.06			0.1
EBF	68 (73.12)	26 (56.52)		27 (38.03)	6 (20)	
MF	25 (26.88)	20 (43.48)		44 (61.97)	24 (80)	
RIs	-	-		9 (12.68)	10 (33.33)	***0.02***

Clinical characteristics stratified by delivery mode. Continuous variables are shown as mean ± SD (normally distributed) or median with IQR (non-normally distributed); categorical variables are shown in absolute numbers with percentages (%). The *P*-value of variables is determined by Student's *t*-test, the Mann–Whitney *U*-test, or Fisher's exact test, and the significance is in bold and italicized.

6W, 6 weeks postpartum; 6M, 6 months postpartum; VD, vaginally delivered; CSD, cesarean section delivered; SD, standard deviation; IQR, interquartile range; EBF, exclusive breastfeeding; MF, mixed feeding; RIs, respiratory infections.

mode is a major determinant of gut microbiota in early life but with a gradually diminished effect throughout infancy.

Considering the interplay between breastfeeding and delivery mode in relation to the infant gut microbiota (Hill et al., 2017; Liu et al., 2019), we hypothesized that the impact of delivery mode would be influenced by feeding patterns. To test this hypothesis, we conducted a stratified analysis based on samples collected at 6M separately ($n = 101$). The 101 infants were divided into two groups according to feeding patterns: exclusive breastfeeding ($n = 33$) and mixed feeding ($n = 68$) groups. As expected, no significant difference in the gut microbiota structure was observed between VD ($n = 27$) and CSD ($n = 6$) infants in the breastfeeding groups (Figure 2C, Adonis, $P = 0.57$, $R^2 = 0.05$). However, in the mixed feeding groups, a stronger significant difference in gut microbiota structure was observed between VD ($n = 44$) and CSD ($n = 24$) infants, with a higher R^2 value of 0.11 and a *P*-value of <0.001 (Figure 2D). This suggested that breastfeeding may alleviate the disturbance of gut microbiota in CSD infants.

Next, we aimed to identify specific taxa responsible for this feeding pattern-dependent change. The average relative abundances of the top 10 genera were compared between VD and CSD infants via a cross-sectional analysis. At 6W, all VD infants ($n = 93$) were enriched in *Bacteroides* and *Escherichia-Shigella* (Figure 3A, $P < 0.01$ and $P < 0.05$, respectively), while CSD infants ($n = 46$) were depleted of these two commensal genera and enriched in *Klebsiella*, *Veillonella,* and *Enterococcus* (Figure 3A, $P < 0.05$ for all). These findings are in agreement with recent observations in other cohort studies (Backhed et al., 2015a; Reyman et al., 2019; Shao et al., 2019). At 6M, the relative abundances of the top 10 genera were comparable between VD and CSD infants in the breastfeeding groups (Figure 3B), whereas the relative abundances of the aforementioned discriminative taxa remained different between VD and CSD infants in the mixed-feeding groups, with a lower relative abundance of *Bacteroides* (Figure 3C, $P < 0.01$) and a higher abundance of *Klebsiella*, *Veillonella,* and *Streptococcus* in CSD infants ($P < 0.05$ for all), while the relative abundances of *Escherichia-Shigella* were comparable between VD and CSD infants ($P = 0.6$).

Impact of delivery mode-induced microbiota changes on infant health

Since previous studies have reported an association between delivery mode and susceptibility to respiratory diseases (Baumfeld et al., 2018), which might be mediated by gut microbiota composition (Reyman et al., 2019), we further investigated the relationship between delivery mode and infant RI status, according to parental self-reporting over the first 6 months. Indeed, the incidence of RI was significantly higher in CSD infants (9/71) than in VD infants (10/30, 12.7 vs. 33.3%, $P = 0.02$), prompting our hypothesis that delivery mode-induced gut microbial changes are associated with the RI status. Consequently, all infants at 6 months postpartum ($n = 101$) were divided into two groups based on the occurrence of at least one RI event: the RI group ($n = 19$) and the non-RI group ($n = 82$). The relative abundances of the five delivery mode-associated bacterial taxa (*Bacteroides*, *Escherichia-Shigella, Klebsiella*, *Veillonella,* and *Enterococcus*) were

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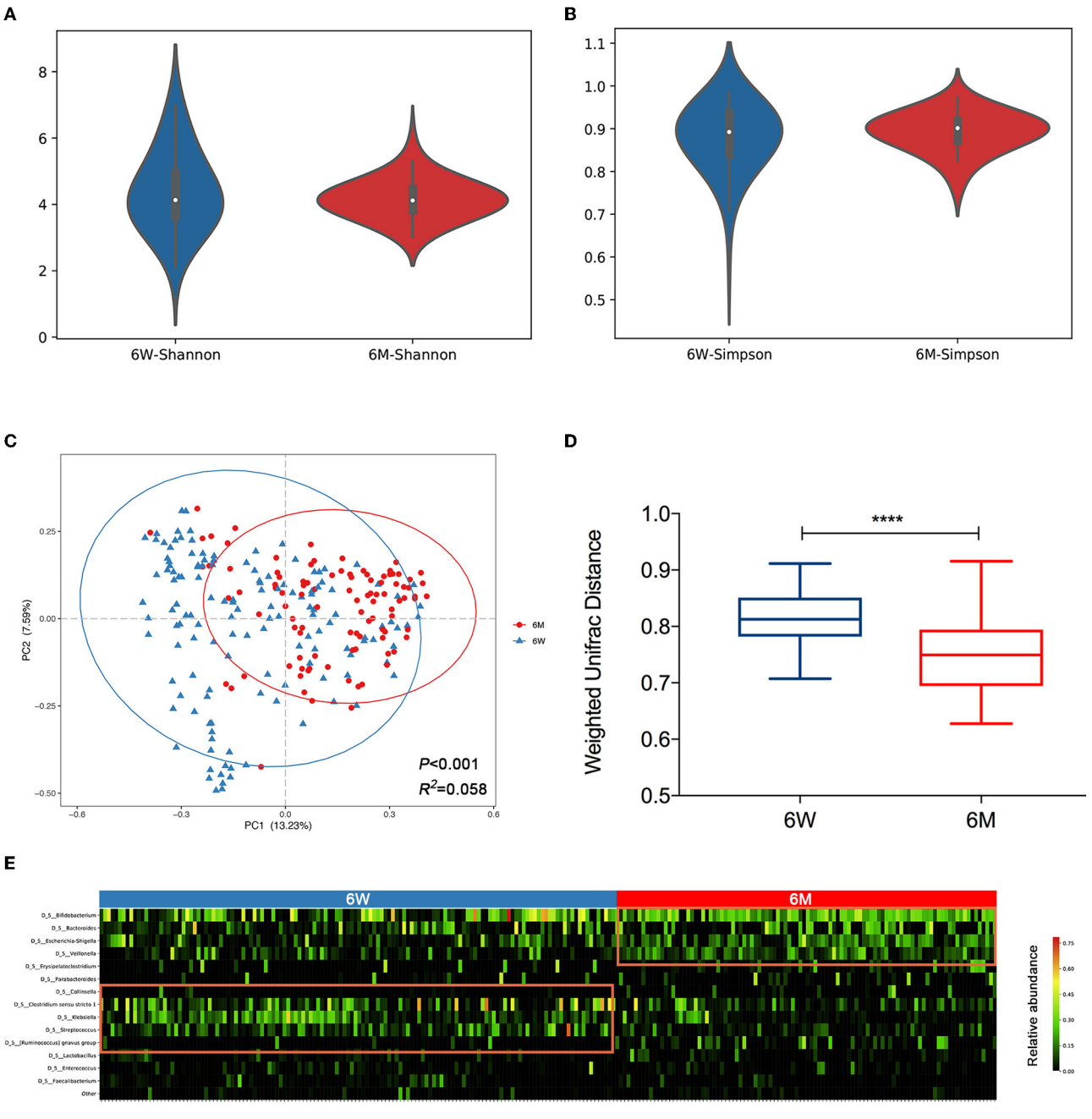

FIGURE 1
The distinct composition and community structure of gut microbiota in 6-week-old and 6-month-old infants. Alpha diversity is shown as the Shannon index **(A)** and Simpson index **(B)**. **(C)** Principal coordinates analysis (PCoA) based on Bray–Curtis distance is shown along the first two principal coordinate (PC) axes with Adonis *p*-value and effect sizes (R^2). **(D)** Comparison of the weighted UniFrac within-group distance of infants. The shorter distance indicated greater similarity in microbial community composition. Boxplots were shown as medians with range. **(E)** A heatmap of the top 15 bacterial OTUs (average relative abundance >1%) clustered according to age. Values range from low (black) to high (red). A significant difference was determined by Student's *t*-test ($^{****}p < 0.0001$). 6W, 6-weeks-old infants; 6M, 6-months-old infants.

compared between the RI and non-RI groups. As expected, the VD-associated *Escherichia-Shigella* was more abundant in the non-RI group (Figure 4, $P < 0.0001$), whereas CSD-associated *Klebsiella* was enriched in the RI group ($P < 0.001$). Additionally, *Bacteroides*, enriched in VD infants, was more abundant in the non-RI group ($P = 0.32$), while *Veillonella* and *Enterococcus*, enriched in CSD infants, were more abundant in the RI group ($P = 0.15$, $P = 0.78$, respectively), although the difference did not reach the statistical significance.

Considering the mediating role of the feeding pattern on the relationship between delivery mode and gut microbiota, we further categorized infants ($n = 101$) into four groups based on the combination of delivery mode and feeding pattern: vaginally delivered and exclusively breastfed (VB, $n = 27$), vaginally delivered

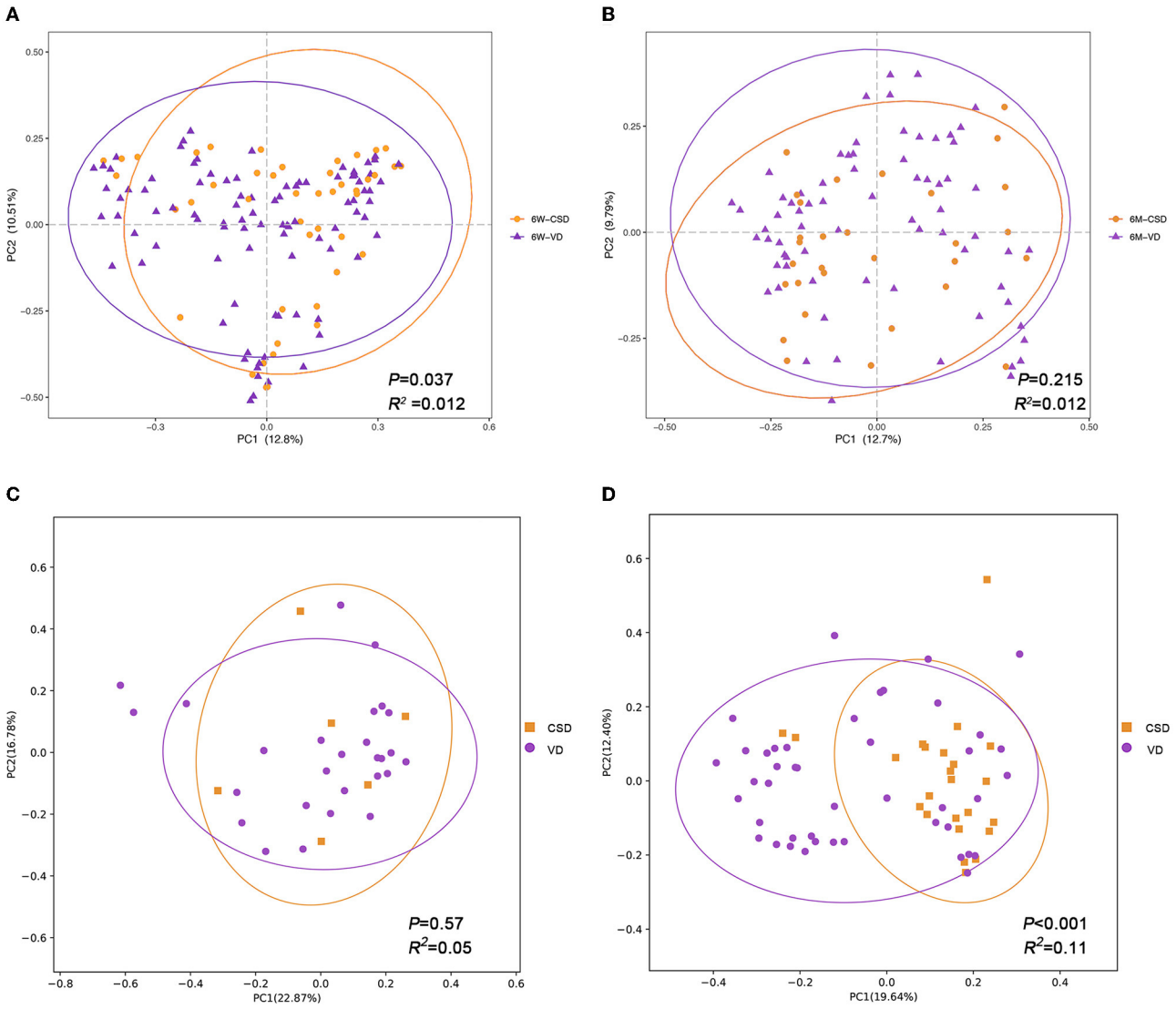

FIGURE 2
The impact of delivery mode on infant gut microbiota is gradually diminished and influenced by exclusive breastfeeding. Principal coordinates analysis (PCoA) plots of infant samples stratified according to the delivery mode, based on Bray–Curtis distance, are shown along the first two principal coordinate (PC) axes with Adonis *p*-value and effect sizes (R^2). **(A)** Infants aged 6 weeks. **(B)** Infants aged 6 months. **(C)** Infants who were exclusively breastfed during 6 months. **(D)** Infants who were mixed fed during 6 months. VD, vaginally delivered; CSD, cesarean section delivered.

and mixed fed (VM, $n = 44$), cesarean delivered and exclusively breastfed (CB, $n = 6$), and cesarean delivered and mixed fed (CM, $n = 24$). We defined the VB group as the healthy reference group and found that the incidence of RIs of the CB group (1/6, 16.7%) was similar to that of the VB group (5/27, 18.5%), which was much lower than that of the CM group (9/24, 36%). Although the difference did not reach statistical significance ($P = 0.26$), this result indicated that exclusive breastfeeding may play a health-promoting role by rectifying the gut microbial composition induced by the cesarean section delivery.

Discussion

Gut microbiota establishment in early life is essential to host health and disease susceptibility, which is greatly influenced by two perinatal factors, delivery mode and feeding pattern. However, the knowledge of the relationship between infant health and gut microbiota shifts associated with delivery mode and feeding pattern is limited. In this longitudinal study of 139 infants, we observed that the perturbations of infant gut microbiota caused by cesarean section were associated with higher risks of respiratory infection in the first months of life. These delivery mode-induced microbiota shifts were alleviated by exclusive breastfeeding, resulting in reduced respiratory infection susceptibility. Our study indicates a health-promoting role of exclusive breastfeeding on infants through restoring the delivery mode-induced gut microbial perturbations.

In recent decades, the rates of cesarean section have steadily increased worldwide, reaching 32.4% in the US and 34.9% in China (Li et al., 2017). This rise has been linked to a higher risk of metabolic and immune disorders in CSD offspring in the short

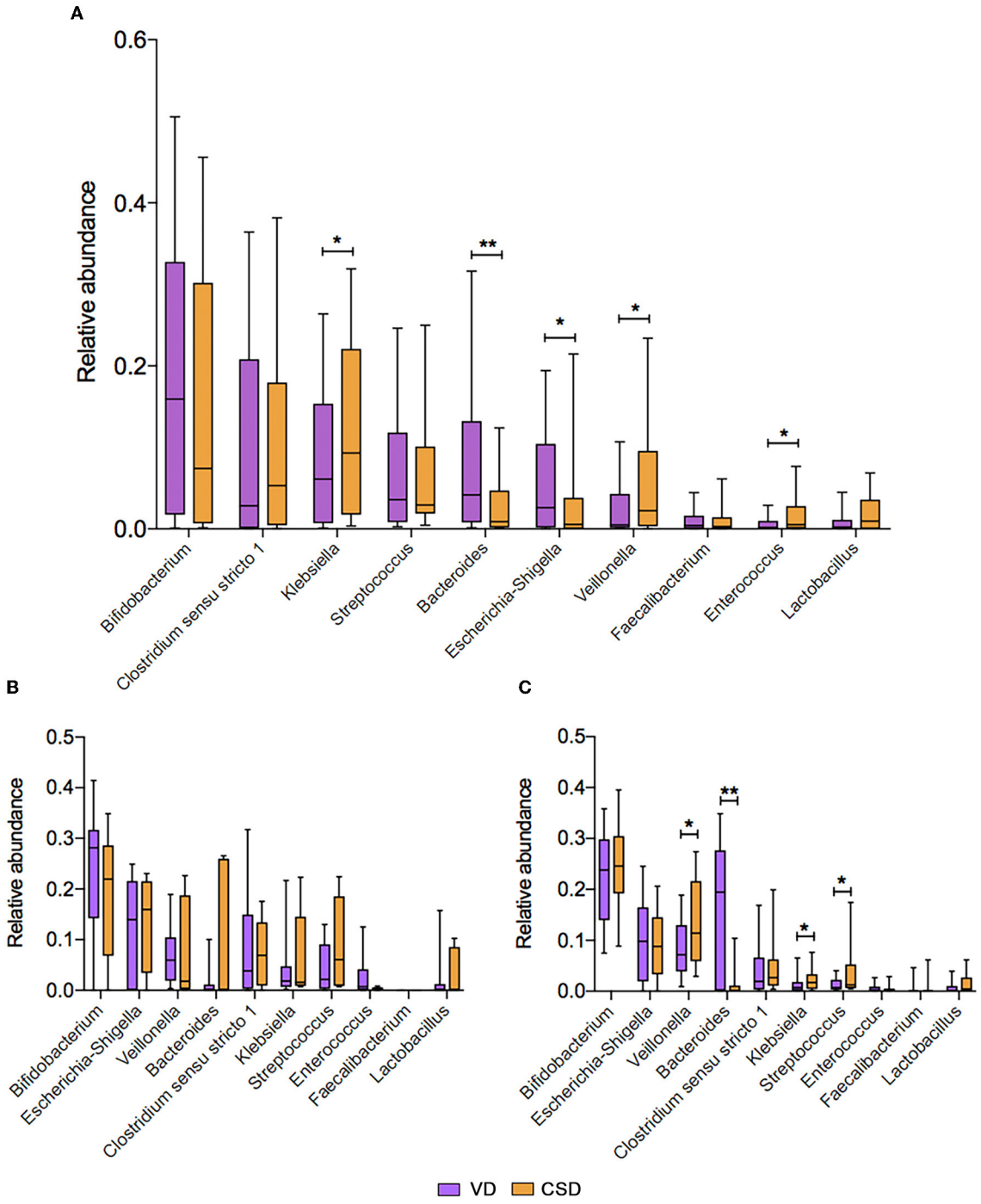

FIGURE 3
The mean relative abundance of the top 10 genera associated with the delivery mode. **(A)** The comparison of mean relative abundance of the top 10 genera between VD and CSD infants at P6W. The comparison of mean relative abundance of the top 10 genera between VD and CSD infants who were exclusively breastfed **(B)** and mixed fed **(C)** at P6M. A significant difference was determined by Student's *t*-test or the Mann–Whitney *U*-test ($^{*}p < 0.05$; $^{**}p < 0.01$). VD, vaginally delivered; CSD, cesarean section delivered.

and long terms (Huh et al., 2012; Sevelsted et al., 2015). Although the exact mechanism remains poorly understood, increasing evidence has demonstrated that gut microbiota might mediate the association between cesarean section delivery and susceptibility to obesity (Tun et al., 2018), diabetes mellitus (Andersen et al., 2020; Chavarro et al., 2020), asthma (Stokholm et al., 2020), respiratory tract infections (Reyman et al., 2019), and chronic immune issues (Wampach et al., 2018). Consistent with these findings, we observed a higher incidence of RIs during the first 6 months of life in CSD infants compared with VD infants. This outcome was related to low *Escherichia-Shigella* and high *Klebsiella* profiles in the intestine caused by cesarean section delivery. In addition to these

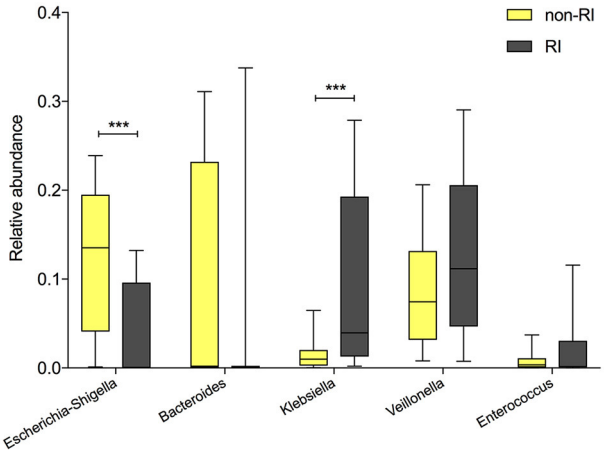

FIGURE 4
The comparison of the relative abundance of delivery mode-associated bacterial taxa between the RI and non-RI groups. A significant difference was determined by Student's *t*-test or the Mann–Whitney *U*-test ($^{***}p < 0.001$). RI, respiratory infection.

two bacterial taxa, a decreased relative abundance of *Bacteroides* and the enrichment of *Veillonella* and *Enterococcus* were observed in our CSD infants, which aligns with previous studies (Reyman et al., 2019; Shao et al., 2019).

The lack of specific bacterial taxa in CSD infants has been shown to disturb the maturation of the infant intestine and immune systems (Jakobsson et al., 2014; Tamburini et al., 2016). For example, the low *Bacteroides* profile in CSD infants, considering a signature of disturbed gut microbiota development in early life (Shao et al., 2019), is related to a reduction in lipopolysaccharide (LPS) exposure, thus decreasing the stimulation of primary human immune cells and exerting a long-term impact on immune-mediated diseases (Jenmalm, 2011; Jakobsson et al., 2014; Wampach et al., 2018). *Klebsiella*, regarded as an opportunistic pathogen, is a common cause of hospital infection, and an increased ratio of *Klebsiella*/*Bifidobacterium* in early life is related to the latter development of pediatric allergies (Low et al., 2017). Combining our observation, the enrichment of *Klebsiella* may be involved not only in the risk of immunological disorders but also in the risk of infectious diseases in CSD offspring.

Consistent with previous studies (Hill et al., 2017; Reyman et al., 2019; Shao et al., 2019), we found that the effect of delivery mode on microbial profiles gradually decreased with infant age. Unique to our study, we further determined whether this dynamic trajectory was affected by feeding patterns, which was prompted by our previous observation that microbial alterations caused by cesarean section delivery could be rectified by EBF in a single cross-sectional analysis at 6 weeks of life (Liu et al., 2019). As expected, the dynamic effect of delivery mode on infant gut microbiota was dependent on the feeding pattern, demonstrating that the microbial compositional disturbance associated with cesarean section delivery could be corrected by EBF through increasing or decreasing specific bacterial taxa but not by MF. Taking *Klebsiella* as an example, the difference in relative abundance between VD and CSD infants at 6 weeks was rectified by EBF lasting for 6 months of life but exacerbated by MF.

A recent systematic review assessed that the relative abundances of particular gut-commensal bacteria genera were associated with childhood respiratory infection (Alcazar et al., 2022), suggesting that gut microbiota might be a determinant of childhood respiratory disease. In this study, we further compared the incidence of RIs among VB, CB, and CM infants, taking VB infants as a healthy reference. Although no significant difference was found, we observed a trend toward a higher incidence of RIs in CM infants than in CB infants. In terms of gut microbiota, our results provide evidence to support the previous viewpoint that prolonged EBF reduces the risk of infectious diseases in infancy (Duijts et al., 2010; Brodin, 2022), especially in CSD infants. However, the proportion and duration of EBF were significantly lower in CSD infants (Vestermark et al., 1991; Dewey et al., 2003).

Breast milk and its components exert an important influence on the nature of early-life immune responses during microbial colonization. Although artificial oligosaccharides are added to infant formula milk to mimic the composition of human breast milk, a significant difference in gut microbial composition is still observed between EBF and MF infants (Azad et al., 2013; Stewart et al., 2018). Presumably, IgA antibodies and maternal antibodies in breast milk help tip the balance toward tolerance and immune-microbe mutualism while providing passive immunity to defend against invasive bacteria (Brodin, 2022). In addition to the different components between breast milk and formula milk, the breast milk microbiota also plays an important role (Le Doare et al., 2018). The abundance of specific microbial strains of *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, and *Staphylococcus* species in the infant's intestine increased with the proportion of daily breast milk intake in a dose-dependent manner (Pannaraj et al., 2017), strongly indicating the transfer of microbes from breast milk to the infant intestine (Martin et al., 2012). Furthermore, HMOs in breast milk are metabolized by microbiota, leading to the production of metabolites such as indole-3-lactic acid, which likely mediates some of the beneficial effects of breast milk early in life (Henrick et al., 2021). Taken together, these results highlight the importance of EBF, especially for CSD infants, and provide a potential reference for optimizing formula milk by adding specific bacterial taxa, resulting in a gut microbial profile resembling that of EBF infants.

The strengths of our study included the longitudinal sampling, which enabled us to assess the dynamic effect of delivery mode on infant gut microbiota. In addition, a mixed feeding regimen was included in our study, which was often introduced when mothers return to work or prepare to wean in China, while most previous studies usually focused on the comparison of gut microbiota in EBF and exclusively formula-fed infants (Azad et al., 2013; Gomez-Llorente et al., 2013; Bergstrom et al., 2014). Finally, we highlighted the association between infant clinical consequences and delivery mode-induced gut microbial perturbations. However, our study has some limitations. The incidence of respiratory infection was determined by the self-report of the mother, which may be biased by factitious factors. To investigate the relationship between health outcomes and delivery mode, detailed information about clinical phenotype was lacking.

Conclusion

In conclusion, we assessed the dynamic impact of delivery mode on infant gut microbiota and attenuated it in a feeding pattern-dependent manner during the first months of life. The perturbation of infant gut microbiota caused by cesarean section was restored by exclusive breastfeeding, resulting in reduced respiratory infection susceptibility in the first years of life. Our study suggests a health-promoting role of exclusive breastfeeding on infants by restoring the delivery mode-induced gut microbial perturbations.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The studies involving human participants were reviewed and approved by Peking University First Hospital (V2.0/201504.20). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

YL, JM, and HY conceived the study design. YL, SQ, YF, and SW were responsible for the recruitment and collection of samples. NL, FL, and BZ were responsible for the laboratory assays. YL performed the data analysis and completed the initial manuscript. HY revised the manuscript. All authors have read and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1163269/full#supplementary-material

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