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Progression of gut microbiome in preterm infants during the first three months

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The colonization and evolution of gut microbiota early in life play a vital role in shaping a healthy, robust immune system for infant health, whether in combating short-term illness or improving long-term health outcomes. Early-life clinical practices may interrupt or disrupt the normal colonization process of the infant gut microbiota, thereby increasing disease susceptibility. In this prospective cohort study, we analyzed the gut microbiota of 46 term and 23 preterm infants using 16S rRNA gene metagenomic sequencing. Fecal samples were collected at six timepoints during the first three months of life. Notably, gestational age was the main factor contributing to differences in the meconium microbial composition. Intriguingly, our study unveiled a more homogeneous microbial composition in preterm infants with more abundant *Bifidobacterium* from the postnatal age (PNA) of one month. Concurrently, the beneficial bacteria *Bifidobacterium* and *Lactobacillus* gradually increased, and the potentially pathogenic bacteria *Clostridium*, *Enterobacter*, *Enterococcus*, *Klebsiella*, and *Pseudomonas* gradually decreased. Furthermore, our study underscored a link between decreased microbial diversity of preterm infants and exclusive breastfeeding and antibiotic exposure. Moreover, preterm infants with patent ductus arteriosus (PDA) exhibited reduced microbial diversity but higher abundances of *Streptococcus oralis* and *Streptococcus mitis*.

Keywords Gut microbiome, NICU, 16S amplicon sequencing, Meconium, Patent ductus arteriosus (PDA), Preterm infants

Preterm birth and its associated complications are a leading cause of morbidity and mortality in children under the age of five worldwide[1]. As a result of their incomplete development, the majority of preterm infants require prolonged neonatal intensive care unit (NICU) admission following birth. Preterm infants with a lower birth weight tend to stay in the NICU for a prolonged duration[2,3]. Extended hospitalization affects the development and stability of the gut microbiota in preterm infants, hindering their ability to establish a gut microbiota resembling that of term infants[4]. Preterm infants receive a variety of treatments during their NICU stay, and the younger the gestational age, the more likely they are to receive antibiotics, as well as invasive procedures. This increases their risk for short-term complications related to the intestinal tract and lungs and may adversely affect long-term health[5]. Also, antibiotic treatment depletes gut commensal bacteria and creates a selective environment that promotes resistance, causing preterm infants to carry more antibiotic-resistance genes[6,7]. Neonatal respiratory distress syndrome, chronic lung disease, periventricular leukomalacia, PDA, retinopathy of prematurity (ROP), and necrotizing enterocolitis (NEC) are common complications of prematurity[8]. Infants diagnosed with preterm birth-related complications tend to have a lower microbial diversity[9].

Meconium is a distinctive substance composed of bile acids, pancreatic secretions, and epithelial cells that form before birth through the physiological mechanism of swallowing amniotic fluid in utero. Analysis of the infant meconium microbiome provides a better understanding of its potential role in promoting the development of the immune system, refining the intestinal mucosal barrier, and preventing pathogen invasion[10]. Research has indicated that preterm infants exhibit lower meconium diversity than term infants[11]. The establishment of the ecological niche of meconium microbiota is associated with microbial translocation in utero and is influenced by maternal factors during pregnancy[12].

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The gut microbiome of preterm infants consistently exhibits reduced microbial diversity compared to term infants, decreased colonization of beneficial gut bacteria, and increased abundance of potentially pathogenic bacteria[13–15]. Even at the age of 4, the low microbial diversity in the gut may persist[16]. Preterm infants are more often delivered by cesarean section, and cesarean section promotes colonization of common skin and environmental microorganisms[17]. The time window of gut microbial development in early life can be influenced by various factors, including maternal and infant factors during the prenatal, intrapartum, and postnatal phases. At the same time, gut microbiota disturbances can affect infants' immediate and future well-being, leading to disorders such as colic, NEC, obesity, diabetes mellitus, atopic symptoms, etc[18–21]. Thus, early life events provide a unique opportunity to regulate the infants' gut microbiota to safeguard microbial diversity and bolster immune resilience during critical developmental stages. Despite growing evidence of health risk association with the gut microbiome of children[22,23], limited studies have conducted frequent longitudinal sampling of preterm infants admitted to NICU, particularly in ethnically diverse regions of Southeast Asia, and have distinguished ethnicity-specific effects from other factors. This study aimed to determine differences in meconium microbial composition between term and preterm infants and to monitor the gut microbiota's acquisition and dynamic developmental trajectory in preterm infants admitted to NICU.

Results

Study cohort, demographic and clinical information

In total, 182 fecal samples from 46 term infants and 23 preterm infants in the first three months of life from 6 timepoints were analyzed. One meconium sample from a preterm infant (P16 M) and a fecal sample from a preterm infant of the PNA of one week (P48 w1) were excluded due to insufficient DNA. Demographics and features of the infants and their mothers are provided in Table 1. Gestational age, birth weight, mode of delivery, and maternal intrapartum antibiotic exposure significantly differed between term and preterm infants ($P < 0.05$). Preterm infants were more often delivered by cesarean section, and their mothers were more frequently exposed to intrapartum antibiotics. Infants did not differ in ethnicity or maternal diseases during pregnancy. However, mothers of preterm infants were more likely to have maternal chorioamnionitis, group B *Streptococcus* carriage, urinary tract infection, and hypertension during pregnancy.

Among 23 preterm infants, 8 (34.8%) experienced preterm premature rupture of membranes, 18 (78.3%) had respiratory distress syndrome (RDS), and other co-morbidities such as intraventricular hemorrhage (IVH)-6 (26.1%), bronchopulmonary dysplasia (BPD)-3 (13.0%), NEC-2 (8.7%), ROP-1 (4.3%), PDA-5 (21.7%), sepsis-9 (39.1%), and nosocomial pneumonia-6 (26.1%). The clinical signs of neonatal sepsis are frequently non-specific, and there is a lack of a globally agreed definition of neonatal sepsis[24]. In our study, sepsis was defined as either blood culture positive or culture negative, accompanied by symptoms and clinical signs. Presumed sepsis with high-risk factors was excluded from our analysis. Antibiotics used included intravenous penicillin, gentamicin, cefepime, cefotaxime, sulbactam-ampicillin, vancomycin, and meropenem for clinical sepsis episodes, either culture-proven or negative, as well as prophylaxis use for presumed sepsis. Since different infants were on different antibiotic regimens at different timepoints, we only counted the total duration of antibiotic use in the first three months of life. Preterm infants' mean hospitalization was 33.3 days (SD = 26.7), antibiotic use was 12.5 days (SD = 14.2), and other NICU practices such as parental nutrition − 6.5 days (SD = 11.2), ventilator − 12.3 days (SD = 21.3), invasive ventilator − 7.6 days (SD = 18.1), and peripherally inserted central catheter − 9.1 days (SD = 17.6). Breastfeeding rates were 13.0% ($n = 3/23$) in the first month, 43.5% ($n = 10/23$) in the second month and 39.1% ($n = 9/23$) in the third month (Supplementary Table 1).

Meconium stage (M, within 72 h postnatal)

Meconium Microbiome comparison between term and preterm infants

The three most prevalent phyla were Proteobacteria (mean = term vs. preterm, 503.4E−3 vs. 451.6E-3), Firmicutes (322.2E−3 vs. 292.8E−3), and Actinobacteria (99.5E−3 vs. 106.1E−3) in the meconium of both term and preterm infants. (Fig. 1a). The top 20 genera of preterm infants represented a lower proportion of the overall diversity than term infants (Fig. 1b). From the differential abundance analysis, six phyla differed significantly (LDA > 3.0; Wilcoxon p value < 0.05) at the meconium stage. Term infants had higher abundances of Candidatus Saccharibacteria (15.9E−3, $p = 3.9E-3$), whereas preterm infants had higher abundances of Tenericutes (80.0E−3, $p = 2.0E-2$), Acidobacteria (33.3E−3, $p = 7.0E-4$), Cyanobacteria (12.4E−3, $p = 4.0E-4$), Planctomycetes (3.5E−3, $p = 5.0E-5$) and Chloroflexi (1.4E−3, $p = 1.0E-4$) (Fig. 1g). At the meconium stage, 6 and 22 genera were enriched in term and preterm infants, respectively (LDA > 3.0; Wilcoxon p value < 0.05). *Escherichia* (116.1E−3, $p = 3.0E-3$) was highly enriched in the term infants, whereas *Pseudomonas* (148.9E−3, $p = 2.0E-4$) dominated the preterm infants (Fig. 1h). Meanwhile, 17 and 28 species were enriched in term and preterm infants, respectively (LDA > 3.0; Wilcoxon p value < 0.05). *Escherichia coli* (1.4E−3, $p = 3.0E-3$) was significantly more abundant in the term infants, while *Pseudomonas aeruginosa* (2.9E−3, $p = 1.0E-4$) was significantly more abundant in the preterm infants (Fig. 1i).

A higher but statistically insignificant alpha diversity in meconium was observed in term vs. preterm infants (Fig. 1c,d; Shannon $p = 0.5$, Simpson $p = 0.2$). The Aitchison-based β diversity was visualized using t-SNE (Fig. 1e), and the statistical significance was calculated using PERMANOVA. Although no distinct clusters were observed between term and preterm infants at the meconium stage, the univariate PERMANOVA suggested significant separation in the meconium microbial composition between term and preterm infants (Supplementary Table 2; Group (Term and Preterm); $R^2 = 7.0E-2$, $P = 1.0E-3$).

Differences in metabolomic profiles at the meconium stage between term and preterm infants

The functional pathways were based on the MetaCyc database (MetaCyc: Metabolic Pathways From all Domains of Life). Applying LEfSe for differential abundant analysis, 19 and 14 functional pathways were enriched in

	Preterm (*N* = 23)	Term (*N* = 46)	*P* value
Number of meconium for 16S rRNA gene metagenomic sequencing	22	46	–
Mean gestational age, weeks (SD)	31.52 (3.06)	38.30 (0.84)	< 0.0001[a*]
Mean birth weight, g (SD)	1636.96 (558.77)	3057.07 (389.67)	< 0.0001[a*]
Mode of delivery:			
Cesarean section	19	23	0.0185[b*]
Vaginal delivery	4	23	
Gender:			
Male	15	20	0.1478[b]
Female	8	26	
Ethnicity:			
Malay	14	30	0.5853[b]
Chinese	4	10	
Indian	5	5	
Others	0	1	
Maternal intrapartum antibiotics:			
Yes	13	9	0.0046[b*]
No	10	37	
Maternal diseases during pregnancy:			
(1) Chorioamnionitis			
Yes	3	0	0.0603[b]
No	20	46	
(2) GBS carriage			
Yes	5	5	0.3974[b]
No	18	41	
(3) Urinary tract infection			
Yes	41	4	0.1160[b]
No	17	42	
(4) Hypertension			
Yes	5	3	0.1436[b]
No	18	43	
(5) Anemia			
Yes	7	13	> 0.9999[b]
No	16	33	

Table 1. Demographics and clinical characteristics of infants and their mothers. * *P* values < 0.05 were considered as significant. [a]*t*-test for continuous data; [b] Chi-square test for categorical data. *GBS* Group B Streptococcus

term and preterm infants, respectively (LDA > 3.0; Wilcoxon p value < 0.05). At birth, the protein synthesis, methylation of DNA or RNA, biosynthesis of phospholipids, amino acid metabolism, and carbohydrate metabolism were highly enriched in term infants. In contrast, in preterm infants, purine and/or pyrimidine deoxyribonucleoside or ribonucleoside degradation was significantly elevated (Fig. 1f).

Influence of prenatal and intrapartum factors on meconium

The different prenatal and intrapartum factors on meconium were evaluated using univariate PERMANOVA (Supplementary Table 2). Subsequently, significant factors were included in multivariate PERMANOVA. Multivariate PERMANOVA detected significant associations between birth weight (R^2 = 8.4E−2, p = 2.0E−3) and gestational age (R^2 = 7.5E−2, p = 4.8E−2) with the meconium microbial profile (Supplementary Table 4).

Among the 23 preterm infants, 14 (60.9%) were classified as "moderate to late preterm," and 9 (39.1%) as "extremely to very preterm." A high alpha diversity was recorded in the term infants, while the lowest alpha diversity was detected in the "extremely to very preterm" infants (Fig. 2a,b; Shannon p = 0.74, Simpson p = 0.36). Nevertheless, the differences did not achieve statistical significance. Beta diversity was visualized using t-SNE (Fig. 2c), but no distinct clusters were observed between different gestational ages at the meconium stage.

From the differential abundance analysis, compared with term infants, 27 and 24 species were enriched in "extremely to very preterm" infants and "moderate to late preterm" infants, respectively (LDA > 3.0; Wilcoxon p value < 0.05). *Pseudomonas aeruginosa* (p < 0.05) was significantly more abundant in both "extremely to very preterm" infants and "moderate to late preterm" infants (Fig. 2d,e).

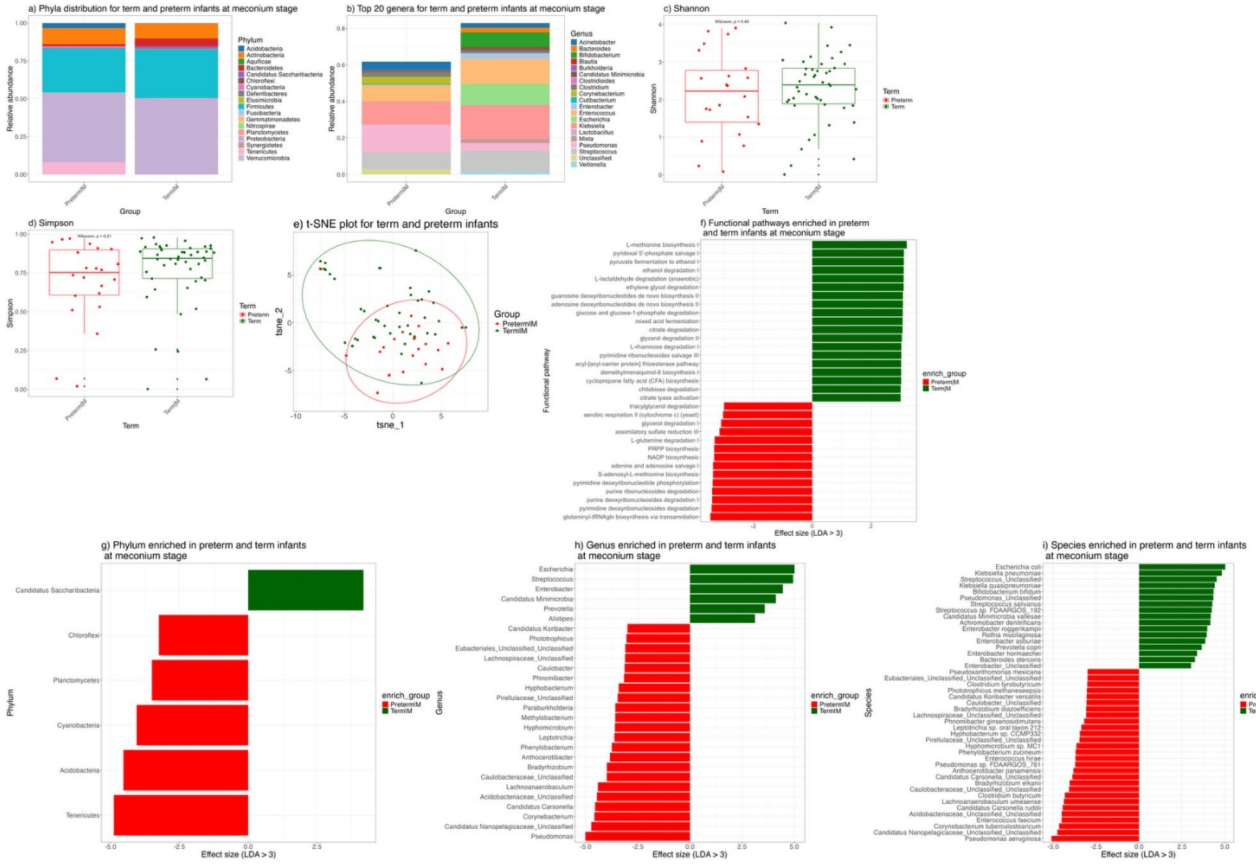

Fig. 1. Diversity of gut microbiome in term and preterm infants at the meconium stage. (**a**) Phyla distribution at the meconium stage showing higher Bacteroidetes abundance in terms, compared to Tenericutes and Acidobacteria predominance among preterms (**b**) Top 20 genera for term and preterm infants at the meconium stage showing a lower proportion of the overall diversity in preterms than terms (**c**), (**d**) α diversity distribution demonstrating higher but statistically insignificant α diversity in terms (**e**) t-SNE plot for term and preterm infants at the meconium stage suggested no distinct clusters were observed between term and preterm infants (**f**) Functional pathways (**g**) Phylum (**h**) Genus (**i**) Species enriched in preterm and term infants at the meconium stage (LDA > 3, *P* value < 0.05).

Gut microbial composition comparison of preterm infants at different timepoints (M, w1,w2,m1,m2,m3)

Between the PNA of one week and the PNA of three months, the most prevalent phyla were Proteobacteria, Firmicutes (w1 vs. m3 = 340.4E−3 vs. 349.1E−3), Actinobacteria, and Bacteroidetes. As gut microbes developed, Proteobacteria (w1 vs. m3 = 611.9E−3 vs. 110.1E−3) was gradually replaced by Actinobacteria (w1 vs. m3 = 41.4E−3 vs. 491.6E−3) and Bacteroidetes (w1 vs. m3 = 0.4E−3 vs. 34.8E−3). (Fig. 3a). At the genus level, *Blautia* (m1 = 15.7E−3) occupied a relatively higher abundance from the PNA of one month, *Bifidobacterium* (m3 = 442.4E−3) gained higher prevalence, while a reduction of *Klebsiella* (m3 = 18.3E−3) was apparent at the PNA of three months (Fig. 3b).

No significant changes in α diversity were detected from birth to the PNA of three months (Fig. 3c,d). Interestingly, from one month of PNA, a more homogeneous microbial composition with more abundant *Bifidobacterium breve*, *Bifidobacterium bifidum*, and *Bifidobacterium longum*, distinct from meconium to the PNA of two weeks was observed along the horizontal dimension of t-SNE (Fig. 3e,f). This change aligned with most infants being discharged from the hospital one month after birth.

Based on the linear graph, the relative abundance of the common gut colonizer followed a complex temporal trajectory (Fig. 4). From the PNA of one month to the PNA of three months, we observed a progressive decline in the abundance of *Clostridium* (m1 vs. m2 vs. m3 = 54.2E−3 vs. 29.0E−3 vs. 6.0 E−3), *Enterobacter* (m1 vs. m2 vs. m3 = 49.0E−3 vs. 36.7E−3 vs. 4.4E−3), *Enterococcus* (m1 vs. m2 vs. m3 = 156.8E−3 vs. 98.6E−3 vs. 79.2E−3), *Klebsiella* (m1 vs. m2 vs. m3 = 159.2E−3 vs. 117.0E−3 vs. 18.3E−3), and *Pseudomonas* (m1 vs. m2 vs. m3 = 4.7E−3 vs. 0.1E−3 vs. 0.1E−3), and a steady rise in the abundance of *Bifidobacterium* (m1 vs. m2 vs. m3 = 136.0E−3 vs. 348.0E−3 vs. 442.4E−3), and *Lactobacillus* (m1 vs. m2 vs. m3 = 13.5E−3 vs. 32.9E−3 vs. 42.7E−3). At the same time, *Bacteroides* (M vs. w1 vs. w2 vs. m1 vs. m2 vs. m3 = 1.8E−3 vs. 0.1E−3 vs. 0.1E−3 vs. 0.0 vs. 1.4E−3 vs. 17.4E−3) was found in the meconium of preterm infants, but it was almost undetectable in fecal samples until the PNA of two months.

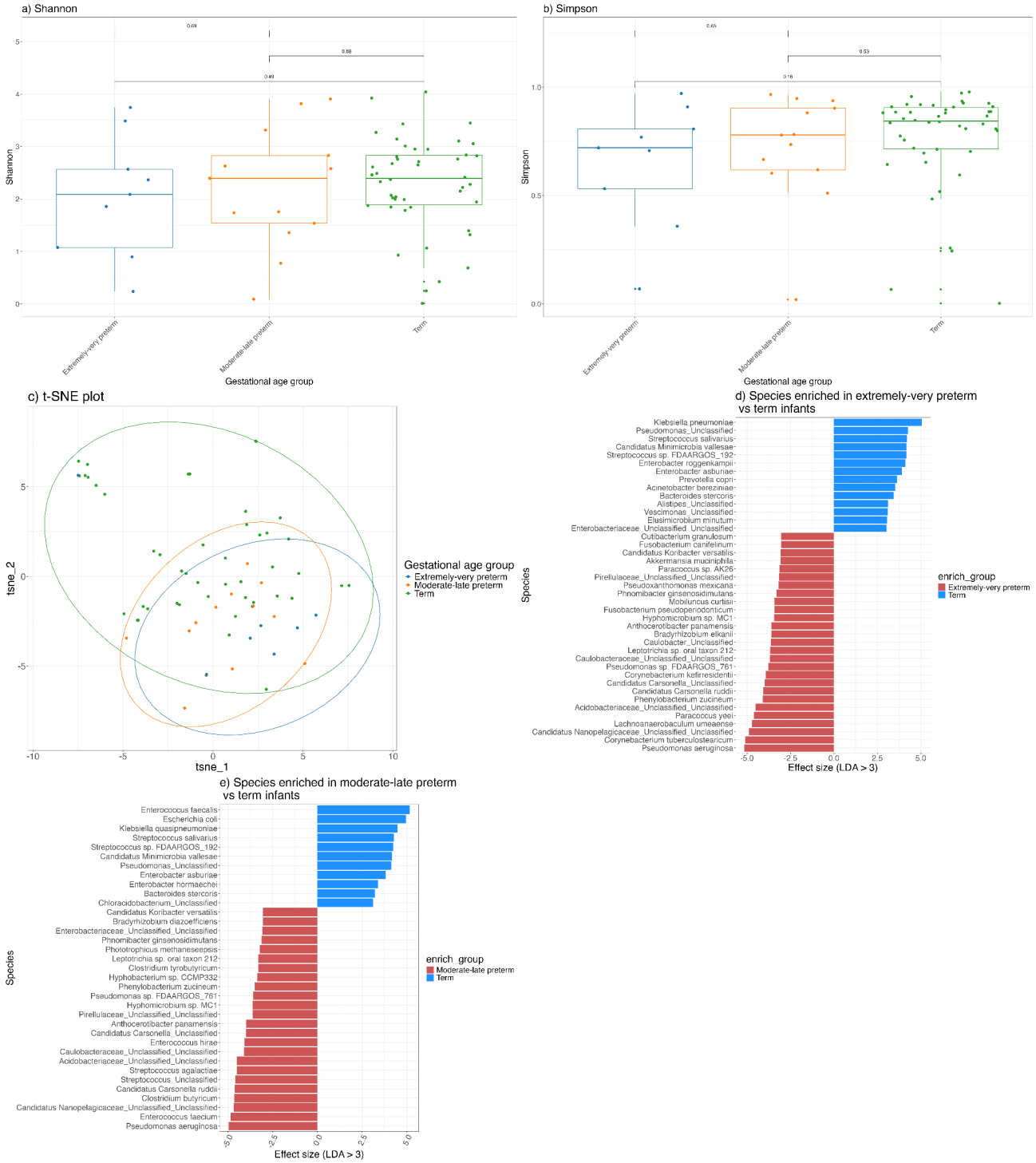

Fig. 2. Diversity of gut microbiome in infants of different gestational ages at the meconium stage. (**a**), (**b**) a trend of increasing but insignificant α diversity was detected across gestational age (**c**) t-SNE plot for infants of different gestational ages at the meconium stage (**d**), (**e**) differential abundant analysis of microbial taxa between term and preterm infants (LDA > 3, *P* value < 0.05).

Clinical covariates associated with the development of early gut microbial composition in NICU-exposed preterm infants

Univariate PERMANOVA was again conducted to identify prenatal and postnatal factors associated with gut microbial composition at the PNA of three months (Supplementary Table 3). The significant factors included in the multivariate PERMANOVA were total days on antibiotics ($R^2 = 9.4E{-}2$, $p = 1.0E{-}3$) and feeding methods ($R^2 = 1.2E{-}1$, $p = 2.5E{-}2$) (Supplementary Table 5). Since both total days on antibiotics and feeding methods were significantly associated with gut microbial composition, we attempted to analyze the relationship between

Fig. 3. Developmental trajectories of the gut microbiota in preterm infants during hospitalization. (**a**) Phyla distribution for preterm infants at different timepoints showing Proteobacteria was gradually replaced by Actinobacteria and Bacteroidetes (**b**) Top 20 genera for preterm infants at different timepoints showing *Blautia* occupied a relatively higher abundance from the postnatal age of one month, *Bifidobacterium* gained higher prevalence while a reduction of *Klebsiella* was apparent at the postnatal age of three months (**c**), (**d**) α diversity distribution demonstrating no significant changes from birth to the postnatal age of three months (**e**) t-SNE plot for preterm infants at different timepoints demonstrating a more homogeneous microbial composition starting the postnatal age of one month (**f**) LEfSe for stabilized (top right) and non-stabilized (middle) sample clusters based on t-SNE plot for preterm infants at different timepoints (LDA > 3, *P* value < 0.05), demonstrating stabilized clusters with more abundant *Bifidobacterium breve*, *Bifidobacterium bifidum*, and *Bifidobacterium longum*.

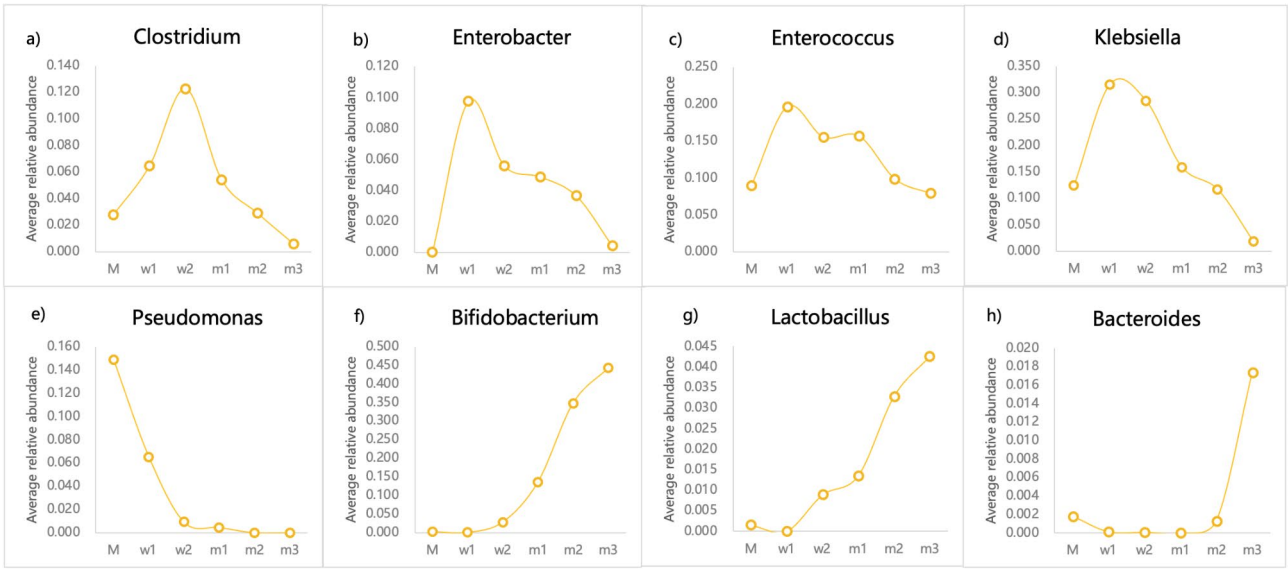

Fig. 4. Dynamics of the gut colonizer in preterm infants over six specific timepoints.

antibiotic use and gut microbiota. However, the data did not yield meaningful data due to large variations in antibiotic combinations used in preterm infants. Nevertheless, we further analyzed feeding methods and their relationship with gut microbial composition at the PNA of three months.

Feeding methods and gut microbiota of preterm infants

The proportion of preterm infants at the PNA of three months was almost equal for the different feeding methods (breastfed vs. formula-fed vs. mixed-fed = 39.1% vs. 30.4% vs. 30.4%) (Fig. 5a). Breastfed preterm infants had the lowest alpha diversity compared to formula-fed and mixed-fed infants at the PNA of three months (Fig. 5b; Shannon $p < 0.05$). β diversity was visualized using t-SNE (Fig. 5c), but no distinct clusters were observed between different feeding methods.

LEfSe was used for differential abundance analysis at the PNA of three months. Breastfed preterm infants had higher abundances of *Bifidobacterium breve* and *Streptococcus* sp. A12, whereas formula-fed preterm infants had higher abundances of unclassified species of Eubacteriales order (*Eubacteriales_Unclassified_Unclassified_Unclassified*), *Veillonella atypica*, and *Lacrimispora saccharolytica* (Fig. 5d).

Correlation of gut microbiota with preterm birth-related complications (IVH, BPD, NEC, RDS, ROP, PDA, Sepsis, Nosocomial pneumonia)

Preterm infants with preterm birth-related complications (IVH, BPD, NEC, RDS, ROP, PDA, nosocomial pneumonia) had lower α diversity at the PNA of three months, except sepsis (the Shannon diversity of whether diagnosed with sepsis was almost the same), but only BPD (Fig. 5e; Shannon $P = 3.1E{-}2$) and PDA (Fig. 5e; Shannon $P = 1.1E{-}2$) achieved statistical significance.

In contrast, the comparison of β-diversity revealed that only PDA was statistically significant (Supplementary Table 6; PERMANOVA $R^2 = 6.9E{-}2$, $p = 2.5E{-}2$). Notably, *Streptococcus oralis* and *Streptococcus mitis* were the dominant species that showed differential abundance in preterm infants with PDA (Fig. 5f).

Discussion

In our study, the most predominant phyla of meconium were Proteobacteria, Firmicutes, and Actinobacteria, in decreasing order of abundance, consistent with previous studies[25]. However, parallel studies reported differences in meconium's prevalence of dominant phyla (e.g., Firmicutes, Proteobacteria, and Bacteroides)[12,26–28]. The variation is likely attributed to the geographic location, ethnicity, and gestational age. Analysis of the meconium microbiome revealed significant differences between term and preterm infants. Notably, in addition to the common phyla of Proteobacteria, Firmicutes, Bacteroides, and Actinobacteria, we found a relative predominance of Tenericutes and Acidobacteria in the meconium of preterm infants and a lower abundance of Bacteroidetes. Many species in the Bacteroidetes and Firmicutes exert nutritional or pharmacological effects by degrading polysaccharides in food, including Starch, glycogen, cellulose, pectin, etc., which are generally unable to be degraded by host digestive enzymes[29], lower abundance of Bacteroidetes in preterm infants may be associated with impaired glucose metabolism in preterm infants[30,31]. Differences in the gut microbial composition at the phyla level may perform specific functions as part of the developing gut microecosystem, resulting in long-term impacts on the immune system or gut barrier function in preterm infants.

We found that the top 20 genera of preterm infants represented a lower proportion of overall diversity than term infants. This uneven structure of the meconium microbial composition of preterm infants may be related to the immature gut and immune function in preterm infants. The meconium of term infants was colonized by more

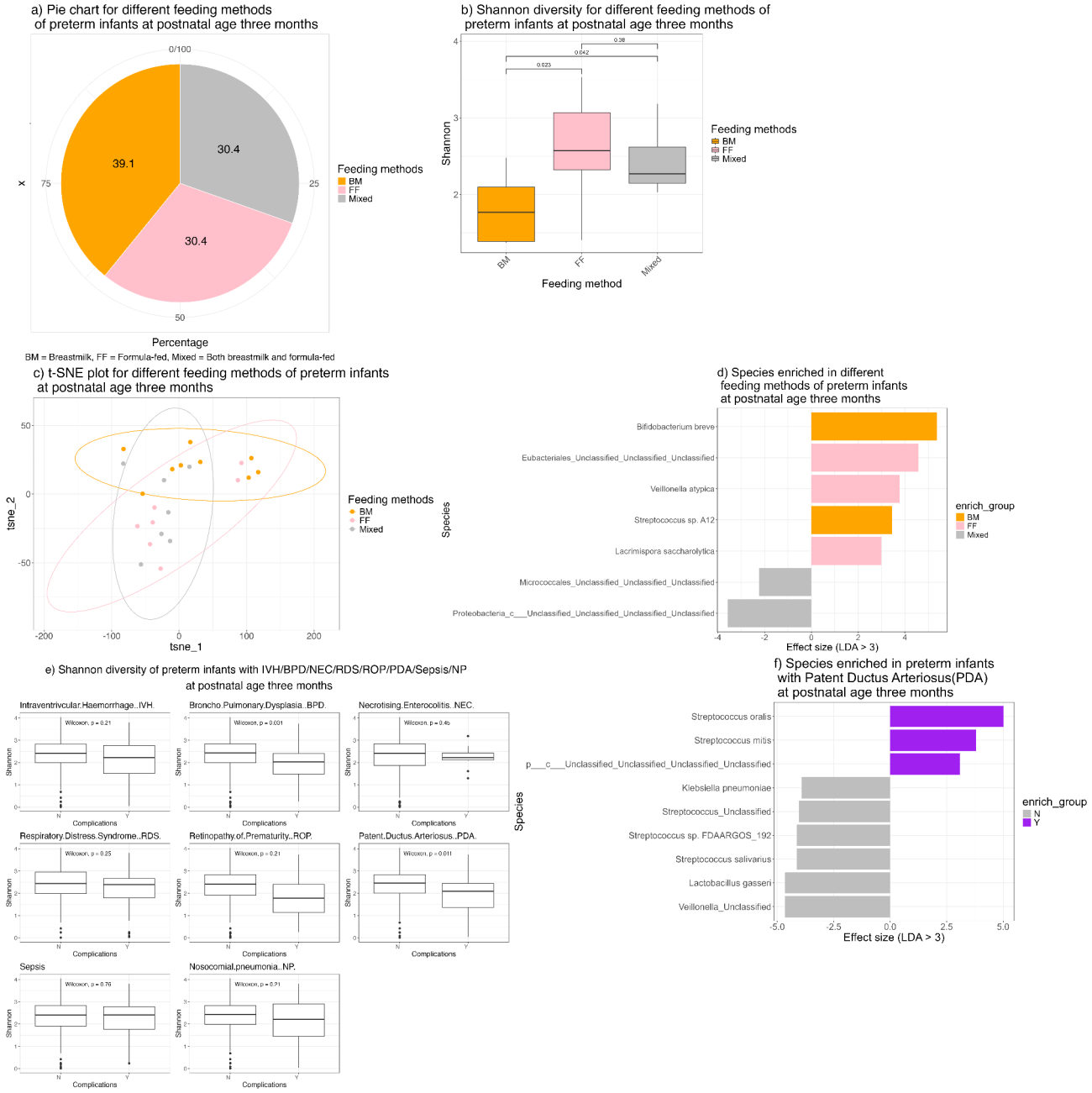

Fig. 5. Diversity of preterm infants with different feeding methods at the postnatal age of three months. (**a**) Pie chart for different feeding methods of preterm infants at the postnatal age of three months showing almost equal proportion for the different feeding methods (**b**) Shannon diversity for different feeding methods of preterm infants at the postnatal age of three months demonstrating breastfed preterm infants had the lowest alpha diversity compared to formula-fed and mixed-fed preterm infants (**c**) t-SNE plot for different feeding methods of preterm infants at the postnatal age of three months showing no distinct clusters were observed between different feeding methods (**d**) Species enriched in different feeding methods of preterm infants at the postnatal age of three months showing breastfed preterm infants had higher abundances of *Bifidobacterium breve* and *Streptococcus* sp. A12, whereas formula-fed preterm infants had higher abundances of unclassified species of Eubacteriales order (*Eubacteriales_Unclassified_Unclassified _Unclassified*), *Veillonella atypica* and *Lacrimispora saccharolytica* (**e**) Shannon diversity of preterm infants with IVH/BPD/NEC/RDS/ROP/PDA/ Sepsis/Nosocomial pneumonia at the postnatal age of three months demonstrating lower α diversity of preterm infants with preterm birth-related complications (IVH, BPD, NEC, RDS, ROP, PDA, nosocomial pneumonia), but only BPD and PDA achieved statistical significance (**f**) Species enriched in preterm infants with PDA at the postnatal age of three months showing *Streptococcus oralis* and *Streptococcus mitis* were the dominant species that were differentially abundant in preterm infants with PDA.

bacteria from the digestive tract (e.g., *Enterococcus*, *Escherichia*, *Bifidobacterium*, *Bacteroides*, and *Clostridiodes*). In comparison, preterm infants were colonized by more bacteria from the skin and hospital environment (e.g., *Corynebacterium*, *Cutibacterium*, and *Acinetobacter*). Preterm infants in our study were predominantly delivered via cesarean section as well as were more exposed to maternal intrapartum antibiotics compared to term infants. Studies found that the gut microbiota of infants born by cesarean section is similar to that of maternal skin and hospital environment[17], that a reduction in *Bacteroides* is a specific indicator of cesarean section delivery[32], and that infants with intrapartum antibiotic exposure exhibit a diminished abundance of *Bacteroides*[33]. This might contribute to the differences in the meconium microbial composition between term and preterm infants in our cohort. Similar to the findings of Chernikova et al. (2018)[15], a higher but statistically insignificant alpha diversity in meconium was observed in term vs. preterm infants. These findings are consistent with previous studies[34], that preterm birth disrupted normal gut colonization, reducing diversity and altering the microbial composition.

As compared to preterm infants, the functional pathways of term infants were more enriched in L-methionine biosynthesis I, pyridoxal 5'-phosphate salvage I, pyruvate fermentation to ethanol I, and L-lactaldehyde degradation (anaerobic). While comparing with term infants at the meconium stage, the functional pathways of preterm infants were more enriched in glutaminyl-tRNA^{gln} biosynthesis via transamidation, pyrimidine deoxyribonucleosides degradation, purine deoxyribonucleosides degradation I, and purine ribonucleosides degradation. *Escherichia coli* played an important role in amino acid metabolism[35]. Functional pathway indicated that term infants had increased L-methionine biosynthesis at birth, potentially associated with *Escherichia* (*Escherichia coli*). Some species of *Streptococcus* generated lactic acid during fermentation, which promoted gut health[36]. The increased prevalence of L-lactaldehyde degradation (anaerobic) in the functional pathway of term infants may be associated with *Streptococcus* (*Streptococcus unclassified* and *Streptococcus salivarius*). These functional pathways may correlate with a more stable microecological environment in term infants. While, the elevation of purine and/or pyrimidine deoxyribonucleoside or ribonucleoside in preterm infants may be related to elevated potential pathogen bacteria, like *Pseudomonas* (*Pseudomonas aeruginosa*) *and Corynebacterium* (*Corynebacterium tuberculostearicum*), some of which participate in cellular metabolic regulation[37]. The proliferation of pathogenic microbes and the decrease of beneficial microbes in preterm infants resulted in distinct functional pathways compared to term infants.

Furthermore, our study identified gestational age as the best explanatory variable contributing to differences in the initial microbial colonization profiles of meconium. This finding aligned with the positive correlation between gestational age and birth weight, highlighting the impact of early life events on gut microbiota development. Notably, the meconium microbiota in "extremely to very preterm" infants may contribute to delayed growth and development, and impaired intestinal immune and metabolic functions[38]. To the best of our knowledge, the study of microbiota in meconium is extremely rare in Malaysia. In this study, we did not detect significant differences in alpha and beta diversity reported elsewhere[38,39]. The lack of statistical significance might be attributed to the low sample size, but a trend of increasing alpha diversity with gestational age was observed.

Although most studies indicated that the gut microbiota of preterm infants predominantly consisted of Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes, different patterns of microbial compositional development of preterm infants over time were observed. As gut microbes developed, Proteobacteria was gradually replaced by Actinobacteria and Bacteroidetes[40]. Bacteroidetes were consistently at low abundance[13], while Actinobacteria were consistently at high prevalence in early life[41]. Previous studies have found that *Bacteroides* were enriched in most vaginally delivered infants but reduced in cesarean section infants. In our cohort, 82.6% of the preterm infants were delivered via cesarean section, and like the findings of Mitchell et al.[42], we demonstrated that cesarean section preterm infants were born with the colonization of *Bacteroides*, but almost no *Bacteroides* was detected until the PNA of two months. The gut microbiota of preterm infants was strongly age-dependent. Some genera, such as *Blautia*, were virtually absent at early timepoints but became present in relatively high abundance from one month onwards. Over time, the microbiota progressed into a stable structure with *Bifidobacterium* as the predominant bacteria, while a reduction of *Klebsiella* was apparent.

Most studies have found that the diversity of gut microbes in preterm infants increased along with the PNA. Nevertheless, our study, like that of de Muinck and Trosvik[43] found a non-linear progression of gut microbes in preterm infants, as well as a more homogeneous microbial composition with a higher abundance of *Bifidobacterium breve*, *Bifidobacterium bifidum*, and *Bifidobacterium longum* in our cohort from the PNA of one month to the PNA of three months. At the same time, this trend toward a more homogeneous microbial composition may be associated with increased breastfeeding rates in our cohort of preterm infants after discharge from the hospital between the PNA of one month and the PNA of three months. As with most studies, the specific developmental trajectories from the PNA of one month to the PNA of three months further confirmed a gradual increase in the colonization of preterm infants of the beneficial bacteria *Bifidobacterium* and *Lactobacillus*, and a gradual decrease of the potential pathogenic bacteria *Clostridium*, *Enterobacter*, *Enterococcus*, *Klebsiella* and *Pseudomonas*[11].

Studies have shown that microbial diversity was lower in exclusively breastfed infants[44,45]. In contrast, formula-fed infants had a more diverse gut microbiota resembling a more mature gut microbiota[45]. This is consistent with our finding that breastfed preterm infants had the lowest α diversity compared to formula-fed and mixed-fed, allowing for a relatively higher abundance of beneficial bacteria. Our cohort of breast-fed infants had higher abundances of *Bifidobacterium breve* and *Streptococcus* sp. A12. Kordy et al.[46] found *Bifidobacteria breve* in the rectum of a mother of a cesarean section infant, breast milk, and fecal from the infant. This finding suggested a potential in vivo hematogenous transfer pathway for entero-lactotransfer in breastfed infants, indicating that gut microbes can be established in infants by sharing the mother's microbiome during breastfeeding. Ruiz-Ojeda et al.[47] also found higher *Bifidobacterium breve* in breastfed, two-month-old infants. Fehr et al.[48] demonstrated that *Streptococcus* spp. and *Veillonella dispar* co-existed in mother's milk and infant feces and that the relative abundance of both genera was positively correlated between the abundance in breast milk and infant feces.

Antibiotics are essential medicines for perinatal healthcare and the treatment of newborns, especially preterm infants, and the survival rate during the perinatal period often depends on effective antibiotic treatment. Nevertheless, the use of antibiotics inevitably affects the host's gut microbiota by reducing diversity[49,50] and promoting the proliferation of antibiotic-resistance genes[51]. The mean total duration of antibiotic use for the preterm cohort throughout their NICU stay was 12.5 days (SD = 14.2). Reyman et al.[50] revealed that different antibiotic regimens influenced gut microbes differently. However, the information on the antibiotic used was non-retrievable, so the association of microbial composition with the specific antibiotic types was impossible. As per the findings of Zwittink et al.[52], we found that the duration of antibiotic treatment was the primary determinant of gut microbial composition, with infants showing a high degree of variability between and within infants across different durations of antibiotic treatment. In the study by Zwittink et al.[52], infants who received prolonged antibiotic treatment were mainly manifested by a decrease in *Bifidobacterium* and an increase in *Enterococcus*.

We detected a lower microbial diversity in preterm infants diagnosed with diseases (IVH, BPD, NEC, RDS, ROP, PDA, Nosocomial pneumonia). However, only differences in PDA and gut microbiota were significantly associated in our cohort, although associations between gut microbiota and NEC and sepsis have been reported in other cohorts[21,53]. Our cohort of PDA infants had decreased α diversity of gut microbiota, while *Streptococcus oralis* and *Streptococcus mitis* were the domain species that were differentially abundant in preterm infants with PDA. Among the five preterm infants with PDA, the duct sizes ranged from 0.8 mm to 3.7 mm, and three of them had shunting across the duct, leading to hemodynamic disturbance, i.e., hemodynamically significant PDA (hsPDA). Ventricular septal defect, atrial septal defect, and PDA are the three most common types of congenital heart disease. Gut microbiota abnormalities may be associated with hemodynamic abnormalities in preterm infants with hsPDA. Infants with hsPDA have an aortopulmonary shunt and may have systemic hypoperfusion, which causes altered intestinal hemodynamics and disturbances in peritoneal or mesenteric blood flow[54]. To our knowledge, there are no studies on the gut microbiota of infants with PDA. However, studies by Zhang et al.[55] and Huang et al.[56] found that the composition of the gut microbiota was significantly disturbed in infants with critical congenital heart disease compared to non-cardiac infants. In our study, the prevalence of *Streptococcus oralis* and *Streptococcus mitis* was significantly elevated in infants with PDA; this may indicate a potential association between specific species and clinical outcomes. This finding highlights a potential link between cardiovascular health and gut microbiota in preterm infants, and there is a need to further investigate the underlying mechanisms of this link.

This study has several limitations. First, the lack of detailed information on the specific types of antibiotics administered to preterm infants constrained our ability to analyze the association between gut microbiota and particular antibiotic regimens. Second, because of the relatively small sample size, $n = 23$ (five late preterm, nine moderate preterm, six very preterm, and three extremely preterm), we reclassified the samples into "extremely to very preterm," "moderate to late preterm," which limited our capacity to elucidate the relationship between variations in more detailed gestational age categories and differences in the meconium microbiota. At the same time, the meconium samples in our study were collected either as first-pass meconium or within 72 h postnatal. Nevertheless, our results on the composition of meconium microbiota were consistent with previously published studies of larger cohorts[25]. Therefore, we believe that these samples are representative of the gut microbial composition of the studied infants. Finally, while we identified a significant association between PDA and gut microbiota within a longitudinally sampled cohort, the generalizability of these findings requires validation in larger, more diverse cohorts. Expanding the sample size and breadth of future studies will enhance statistical power and facilitate a better understanding of the associations between individual developmental trajectories and relevant health outcomes.

The most predominant phyla in meconium were Proteobacteria, Firmicutes, and Actinobacteria, which were thought to be the early gut colonizers of infants, possibly reflecting microbial exposure in utero[57]. Compared to preterm infants, the meconium microbiota of term infants showed higher diversity and differentially enriched taxa. The gut microbiota of preterm infants predominantly consisted of Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. As gut microbes developed, Proteobacteria was gradually replaced by Actinobacteria and Bacteroidetes. The gut microbiota of preterm infants was strongly age-dependent and progressively developed into a more homogeneous gut microbial structure with *Bifidobacterium* as the predominant bacteria. At the same time, a decrease in *Klebsiella* was apparent. Divergences in gut microbiota may significantly influence functional pathways at birth. The taxonomic composition of the meconium microbiota of infants was primarily associated with gestational age. Additionally, variations in the gut microbiota of preterm infants at the PNA of three months were linked to individualized treatments, including feeding methods and the duration of antibiotic therapy. Also, we found lower microbial diversity in preterm infants diagnosed with preterm birth-related complications, especially the association between PDA and gut microbiota. Preterm infants with PDA had a higher abundance of *Streptococcus oralis* and *Streptococcus mitis*, which, to the best of our knowledge, has not been reported before, highlighting the role of gut microbiota in disease. Our study further refines the association between PDA, an early-life congenital heart disease, and the gut microbiota as a clinical non-invasive diagnostic biomarker of congenital heart disease, providing a basis for developing non-invasive diagnostic tests and personalized intervention strategies, thereby improving clinical outcomes.

Materials and methods

Study cohort

The study was approved by the Medical Research Ethics Committee, Universiti Malaya Medical Center (UMREC) (MRECID.NO: 20211020-10700) and adheres to the International Conference on Harmonization - Guidelines for Good Clinical Practice (ICH-GCP) and the Declaration of Helsinki. Written informed consent was obtained from all the parents of the infants enrolled in this study.

Term infants were recruited as controls from May 2022 to November 2022 in the postpartum ward of the Universiti Malaya Medical Center (UMMC). Preterm infants (gestational age < 37 weeks) were recruited in the NICU of the UMMC.

Infants with severe or lethal congenital malformations (especially congenital intestinal malformations) or who required immediate surgery were excluded from the study. Clinical information on mothers and infants was obtained from the electronic medical record system. Information on the feeding of preterm infants after discharge from the hospital until the end of the study was collected through a questionnaire.

Sample collection

Fecal samples were collected at six specific timepoints: within 72 h postnatal (M), the PNA of one week (w1), the PNA of two weeks (w2), the PNA of one month (m1), the PNA of two months (m2), and the PNA of three months (m3). Fecal samples from preterm infants during hospitalization were obtained by nurses as part of routine NICU care and stored at -80 °C. Out-of-hospital, fresh samples were collected from diapers by the infants' parents and stored immediately at -20 °C. It was then transported to the hospital in ice packs or dry ice and kept at -80 °C until analysis. A total of 184 fecal samples were collected for 16S rRNA gene metagenomic analysis.

DNA extraction and 16S rRNA gene metagenomic sequencing

DNA was extracted from 220 mg of feces per sample using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The library (14 pM) was prepared using the Zymo Quick-16S NGS Library Prep Kit following the manufacturer's instructions. PCR amplification of the 16S rRNA V3-V4 regions was performed using primers V3-V4F (5′- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG -3′) and V3-V4R (5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C -3′)[58], with annealing temperature of 55 °C. Sequencing was conducted using an Illumina MiSeq platform employing V3 chemistry, resulting in 2 × 250 bp paired-end reads.

Quality filtration and bioinformatics analysis of gut Microbiome data

The raw sequence data yielded a mean of 119,162 (± 3415.9) reads per sample. R package DADA2 version 1.20.0[59] was used to remove primers, denoise, and merge the reads. As a result, a mean of 92,771 (± 8527.4) merged reads per sample was obtained and exported into Pathway Prediction by Phylogenetic Placement (PAPRICA) pipeline version 0.7.2[60] for taxonomy and functional pathway annotation. In brief, the sequences were aligned and placed into a phylogenetic tree created from 16S to 23S rRNA gene reads comprising all completed bacterial and archaeal genomes from the RefSeq database. The RefSeq database used in our analysis comprised 11,305 and 346 bacterial and archaeal genomes, respectively. Phylogenetic groupings, or "edges," were inferred based on the placement of the sequences in the phylogenetic tree. An edge can refer to either the terminal node of the tree or a path connecting two nodes. Unique numbers were assigned to each edge, and the number of consensus sequences in the same edge was recorded into the abundance table for further downstream analyses.

The downstream data analysis was performed using RStudio version 4.3.0. The taxa were filtered based on the threshold abundance value of 0.0001 (0.01%) using R package phyloseq version 1.36.0 for further analysis. The phyla and top 20 genera abundances and compositions across different groups were visualized using ggplot2 version 3.4.2. The α-diversity measures, including Shannon (*H*) and Simpson (1/D)'s diversities between term and preterm groups, were performed and the significance differences between the groups were inferred using the Wilcoxon rank sum test using the in-built function in R package phyloseq version 1.36.0. *P* value of < 0.05 was regarded as significant. For β-diversity, term and preterm groups clustering was visualized with t-Distributed Stochastic Neighbor Embedding (t-SNE). The significant associations between different factors with the gut microbiome profile were analyzed using Permutational Multivariate Analysis of Variance (PERMANOVA) with 999 permutations implemented in the adonis2 function of R package vegan version 2.6-4. Linear discriminant analysis effect size (LEfSe) from R package microbiomeMarker version 1.3.3 was conducted to identify the differentially abundant features between groups using the Kruskal–Wallis rank sum test and a linear discriminant analysis (LDA) to estimate their effect size. Features were deemed statistically significant with a *P* value of < 0.05 and an LDA score > 3.0 for taxa and functional pathways, respectively. The correlation matrix between multiple demographic variables and clinical parameters using the Spearman correlation method was generated using the R package corrplot version 0.92.

Data availability

The 16S amplicon sequences were deposited in GenBank under BioProject number PRJNA1108502, available at https://www.ncbi.nlm.nih.gov/sra/PRJNA1108502.

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Author contributions
C.S.J.T., A.A.K., K.Y.Toh, and Y.M.C. conceived and designed the experiments. F.F.L. recruited the participants, collected the samples and clinical data, did laboratory work, bioinformatics, and statistical analysis, and wrote the manuscript. S.L.H. performed the bioinformatic and statistical analysis and edited the manuscript. K.Y.Toh and L.W.Z.L. conducted the laboratory experiments and funded this study. C.S.J.T. and K.Y.Toh obtained the funding and critically reviewed the manuscript. Y.M.C., Y.Q.L., C.W.C., and A.A.K. reviewed and edited the manuscript. All authors have agreed to submit the manuscript, have read and approved the final draft, and take full responsibility for its content, including the accuracy of the data and its statistical analysis.

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Declarations

Competing interests
The authors declare no competing interests.

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