



Prospective Study

Distinct early gut microbiota patterns by delivery mode within 100 hours of birth

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Abstract

BACKGROUND

Disruptions in the early-life gut microbiome have been reported to be associated with various health conditions. However, few studies have investigated the establishment of the gut microbiota in the immediate postnatal period.

AIM

To characterize the gut microbiota of neonates within the first 100 hours after birth.

METHODS

A total of 512 healthy neonates born at a tertiary hospital were enrolled in this study. Stool samples were collected between March 2024 and December 2024 and

categorized by delivery mode and stool collection time. Microbiota diversity and composition were assessed using full-length 16S rDNA sequencing. Stool samples from 140 neonates with predominant breastfeeding were analyzed.

RESULTS

Of the 140 neonates, 70 (50%) were female, 66 (47.1%) were delivered by vaginal delivery (VD), 74 (52.9%) were delivered by cesarean section (CS), and 87 (62.1%) were firstborn. The time of stool collection ranged from 13.2 minutes to 109.82 hours. A significant difference in the time of stool collection was observed between CS and VD neonates (43.0 hours; 95%CI: 35.8-50.1 *vs* 32.4 hours; 95%CI: 27.8-36.9; $P = 0.016$). Gut microbiota analysis revealed that CS was associated with higher alpha diversity (Chao1 and Shannon indices; $P < 0.001$) and beta diversity ($P < 0.001$). A reduction in the gut microbiota diversity was observed 48 hours after birth ($P < 0.001$). *Escherichia coli* predominated in the VD samples. *Bifidobacterium* species, including *Bifidobacterium longum*, *Bifidobacterium pseudocatenulatum*, and *Bifidobacterium bifidum*, emerged significantly 48 hours after birth. CS neonates exhibited a higher relative abundance of skin- and environment-associated taxa, including *Staphylococcus*, *Streptococcus*, and *Pseudomonas*, and markedly reduced colonization by *Bifidobacterium* species despite predominant breastfeeding.

CONCLUSION

Despite predominant breastfeeding, CS neonates showed reduced colonization by *Bifidobacterium* and a greater abundance of skin- and environment-associated taxa within the first 100 hours of life. These findings indicate that delivery mode can be a key determinant of early microbial assembly and highlight the need for targeted strategies to support the establishment of beneficial microbiota in CS-delivered infants.

Key Words: Gut microbiota; Neonate; Early life; Feeding; Normal labor; Cesarean section

Core Tip: This study characterizes the gut microbiota of healthy neonates within the first 100 hours after birth, which is a critical yet understudied window of microbial establishment. Neonates delivered by vaginal delivery showed early enrichment of *Bifidobacterium*, whereas those delivered by cesarean delivery exhibited a higher abundance of skin and environment-associated taxa and reduced colonization by *Bifidobacterium* despite predominant breastfeeding. These findings highlight the role of delivery mode as a key determinant of early microbial assembly and the need for targeted microbiota-supportive strategies for cesarean-born neonates.

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INTRODUCTION

The human gut microbiome plays a key role in shaping long-term health, particularly during the earliest stages of life. Disruptions in the gut microbiota within the first 1000 days of life have been reported to be associated with a wide range of conditions, including asthma[1,2], food allergies[3], obesity[4], functional gastrointestinal disorders, such as colic, regurgitation, and constipation[5], inflammatory bowel disease[6], type II diabetes[7], and susceptibility to frequent viral infections[8]. However, the high-resolution temporal dynamics of the microbiota during the immediate postnatal period (within the first 100 hours after birth), a critical developmental window, remain relatively poorly characterized.

Recent studies on the neonatal gut microbiome have shown that colonization begins much earlier than previously assumed, and low-diversity microbial communities can be detected shortly after birth[9-11]. Microbial load and diversity expand rapidly over the subsequent days and weeks, driven by key postnatal factors, such as delivery mode, feeding practices, environments, and antibiotic exposure[12,13]. These findings highlight early life as a dynamic and sensitive window during which microbial trajectories are determined. This period offers a unique opportunity for deeper exploration.

Among postnatal factors, delivery mode has been reported to strongly influence the initial gut microbial landscape[14-16]. Infants delivered by vaginal delivery (VD) are typically colonized by maternal vaginal and intestinal microbes, whereas those delivered by cesarean section (CS) are predominantly exposed to skin and environmental organisms[17]. These initial differences tend to diminish over time as diet, breastfeeding, and daily microbial exposures increase, thereby shaping gut colonization. However, whether microbial acquisition during this ultra-early period may have lasting effects on immune and metabolic development remains unclear[18]. Addressing this knowledge gap may help determine whether early deviations in the establishment of the gut microbiota, even if transient, have meaningful biological consequences.

Human milk oligosaccharides are a major driver of beneficial gut colonization, particularly by enriching *Bifidobacterium*, a genus associated with health-promoting effects[19,20]. However, whether breastfeeding can overcome

the microbiota-altering effects of CS, particularly during the earliest phase of life, remains unclear. Previous studies have often focused on later infancy[21-23], with limited data on microbial dynamics within the first 100 hours after birth.

Therefore, this study aimed to characterize the gut microbiota composition of neonates within the immediate postnatal period, with a focus on the combined effects of delivery mode, environment, and breastfeeding. We assessed microbial diversity and taxonomic composition at species-level resolution using full-length 16S rDNA sequencing *via* Oxford Nanopore Technologies, a third-generation long-read sequencing technique[24]. This approach enables a deeper understanding of early microbial colonization and may help identify specific bacterial species or strains with probiotic potential. The findings can provide valuable insights into the development of targeted probiotics to immediately restore or modulate the gut microbiota balance.

MATERIALS AND METHODS

Participants

This study included infants born at King Chulalongkorn Memorial Hospital, Bangkok, Thailand, who were enrolled in the “Prevalence of functional gastrointestinal disorders in infants and the effect of probiotics *Limosilactobacillus reuteri* DSM 17938: Randomized controlled trial study” (PROOF project). This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (No. 0855/66) and registered with clinicalTrials.gov (NCT06309199, first posted date 13/03/2024). Written informed consent was obtained from the enrolled fathers or mothers following detailed consultations.

The inclusion criteria were: (1) Healthy infants with a birth weight appropriate for gestational age; (2) Gestational age between 37 and 42 weeks; (3) Appearance, pulse, grimace, activity, and respiration score > 8 at 10 minutes; (4) Normal physical examination; and (5) No maternal history of probiotic use. All participants were randomized to receive probiotics or a placebo (sunflower oil), five drops daily for 56 days. Probiotic administration was initiated after discharge from the hospital. Caregivers were required to complete a daily diary for 2 months and attend face-to-face visits at participants' ages 1, 2, 4, and 12 months. Demographic data and participants' and parents' characteristics were recorded after birth and upon discharge from the hospital. The Rome IV diagnostic questionnaires were administered at 1, 2, 4, and 12 months to investigate functional gastrointestinal disorders and associated factors. Physical examinations and stool collection were performed by doctors and research nurses at each visit. The PROOF study has been conducted since March 14, 2024, and is still ongoing. The recruitment period for this study was between March 14, 2024, and December 25, 2024. Data on the targeted participants and their stool samples were extracted only from the first visit or after birth in infants who completed follow-up at birth, 1, 2, and 4 months.

Clinical and stool collection assessment

Data, including baseline characteristics, demographic data, socioeconomic status, maternal nutritional status, maternal underlying diseases, delivery mode, family history of allergic disease, family smoking, and feeding type, were obtained from maternal interviews and the electronic medical record system of the hospital.

Body mass index (BMI) was calculated and classified according to World Health Organization criteria into underweight (BMI < 18.5 kg/m²), normal weight (BMI 18.5–22.9 kg/m²), overweight (BMI 23–24.9 kg/m²), obesity class I (BMI 25–29.9 kg/m²), and obesity class II (BMI ≥ 30 kg/m²).

According to the hospital policy, all healthy neonates received their first feeding at approximately 6 hours of life and stayed with their mothers within 24 hours. Breastfeeding was initiated during this period, ensuring that most infants received breast milk within the first day of life. If breast milk was unavailable or breastfeeding was not initiated, infant formula was provided as an alternative.

Predominant breastfeeding was defined as receiving > 80% of the total feeds as breast milk rather than formula from birth until stool collection. Data on predominant breastfeeding were recorded based on maternal reports during the hospital stay. In addition to stool specimen collection, stool characteristics were analyzed, and the time of stool collection was recorded.

Sample collection and DNA extraction

Fecal samples were collected by two well-trained research nurses using aseptic techniques. Samples were obtained from diapers that were not contaminated with urine or directly *via* gentle rectal stimulation using a sterile cotton bud soaked in sterile water. The time of stool sample collection was recorded. Each sample was immediately transferred after collection into a sterile tube containing 2 mL of NAPSeq preservative solution (Bioentist, Thailand). The samples were rapidly frozen on-site using dry ice and transported to the laboratory on the same day. All samples were stored at 20 °C upon arrival until DNA extraction.

All procedures were performed using sterile consumables and under aseptic conditions by trained research staff to minimize contamination. Negative controls (extraction blanks) were included during sample processing and DNA extraction to monitor potential reagent or environmental contamination. Total DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions.

The full-length 16S rDNA sequencing is based on Oxford nanopore technologies

The bacteria full-length 16S rDNA (V1-V9 regions) was amplified using the primers modified from a previous publication [25], which comprised of the 3' specific target sequences (underlined) and 5' nanopore adaptors as follows: 27F 5'-

TTTCTGTTGGTGCTGATATTGCAGRGTTYGATYMTGGCTCAG-3' and 1492R 5'-ACTTGCCTGTCGCTCTATCTT-CCGGYTACCTTGTTACGACTT-3'. The polymerase chain reaction (PCR) reaction contained 50-100 ng of DNA template, 10 μ mol/L of each forward and reverse primer, 1X KOD One™ PCR master Mix (Toyobo Biotech, Japan), and nuclease-free water. The thermal condition for PCR was 98 °C for 2 minutes, 30 cycles of 98 °C for 10 seconds, 60 °C for 10 seconds, 68 °C for 10 seconds, and a final extension at 68 °C for 5 minutes. The PCR products were subsequently amplified by 5 cycles of barcode PCR (98 °C for 10 seconds, 60 °C for 10 seconds, 68 °C for 10 seconds) with PCR Barcoding Expansion 1-96 (EXPPBC096) kit (Oxford Nanopore Technologies, United Kingdom). The amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) and measured for quantity using a Qubit 4 fluorometer with a Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, United States). Then, the DNA library was equimolarly pooled (1 μ g in total 48 μ L) and size-selected using and purified using 0.5X Agencourt AMPure XP beads (Beckman Coulter, United States). After that, the purified DNA library was end-repaired and adaptor-ligated using a Ligation Sequencing Kit (SQK-LSK114) and sequenced on the R10.4.1 flow cell using the MinION Mk1C platform (Oxford Nanopore Technologies, United Kingdom). The negative controls (no-template reactions) and positive controls were included at every step to monitor contamination. Gel electrophoresis confirmed clear about 1.5 kb bands in all positive controls and samples but none in the negative controls, indicating successful amplification and absence of contamination (Supplementary Figure 1).

Data processing and bioinformatics analysis of gut microbiota

Raw reads (FAST5) were basecalled using Guppy v6.5.7 (Oxford Nanopore Technologies, United Kingdom) with a super-accuracy basecalling model and quality score threshold of > 15 [26]. The quality of the FASTQ sequences was initially assessed using MinIONQC. The FASTQ reads were demultiplexed and adaptor-trimmed using Porechop v0.2.4 (<https://github.com/rrwick/Porechop>). Filtered reads underwent clustering, polishing, and taxonomic identification using NanoCLUST pipeline, which is designed for Nanopore full-length 16S rRNA gene sequencing. Reads spanning the V1-V9 regions were clustered using an unsupervised approach based on k-mer composition similarity, followed by dimensionality reduction using uniform manifold approximation and projection and density-based clustering with Hierarchical Density-Based Spatial Clustering of Applications with Noise. For each cluster, a consensus sequence was generated and polished to reduce sequencing errors, and species-level taxonomic assignment was subsequently performed by comparison of the polished consensus sequences against reference sequences from the Ribosomal Database Project[27].

To enhance data reliability, features detected in fewer than 10% of all samples were excluded prior to downstream analyses. Normalization was performed using total sum scaling to account for differences in sequencing depth across samples. relative abundance profiles were used for diversity and differential analyses. Alpha diversity (Chao1 and Shannon indices) and beta diversity (Bray-Curtis dissimilarity) were calculated and visualized using principal coordinate analysis (PCoA). Differentially abundant taxa were identified using the linear discriminant analysis effect size method, with statistical significance set at $P < 0.05$ and a linear discriminant analysis score > 2 . All downstream analyses were conducted using MicrobiomeAnalyst[28], and multiple group comparisons were additionally performed using Prism v10.1.1 (GraphPad Software, United States).

Statistical analysis for clinical data and gut microbiome data

Continuous variables are presented as mean (SD), mean (95%CI), or median (interquartile range), while categorical variables are expressed as n (%). For comparison between 2 groups, χ^2 or Fisher's exact test was used for categorical variables, and Student's t -test or Mann-Whitney U test was employed for continuous variables as appropriate. Values of $P < 0.05$ were considered statistically significant. Statistical analyses were carried out using STATA version 18.00 software.

In the gut microbiota analysis, differences between the two groups were assessed using the non-parametric Mann-Whitney test, and comparisons among more than two groups were conducted using the non-parametric Kruskal-Wallis method. PCoA based on Bray-Curtis distances was undertaken to visualize sample clustering patterns. Permutational Analysis of Variance (PERMANOVA) was used to assess differences in beta diversity. $P < 0.05$ was considered statistically significant. Bacterial abundances were analyzed at the phylum, genus, and species levels and presented as relative abundance. Only the top 10 bacterial genera and species were displayed, while those ranked 11th and beyond were grouped as 'others'. All statistical outputs, including pairwise comparisons and PERMANOVA results, are provided in Supplementary Table 1.

RESULTS

Participant characteristics

Stool samples were collected at multiple visits from 512 neonates. Among them, 172 neonates with complete serial samples (at birth, 1, 2, and 4 months) were included for DNA extraction. Of the 172 neonates, 30 were excluded due to poor DNA quality and 2 due to missing stool collection time. Finally, 140 neonates were included in the gut microbiota analysis.

Of the 140 neonates (50% female), 66 (47.1%) were delivered by VD, and 74 (52.9%) were delivered by CS. Most neonates (62.1%) were firstborn. The time of stool collection ranged from 13.2 minutes to 109.8 hours and was significantly longer in CS neonates than in VD neonates (43 hours; 95%CI: 35.8-50.1 *vs* 32.4 hours, 95%CI: 27.8-36.9; $P = 0.016$). Table 1 shows the demographic and socioeconomic data and family details.

Table 1 Baseline characteristics and family status of 140 predominantly breastfed neonates classified by mode of delivery, *n* (%)/mean (95%CI)

Characteristics	Total (<i>n</i> = 140)	Mode of delivery		<i>P</i> value
		Vagina delivery (<i>n</i> = 66)	Cesarean section (<i>n</i> = 74)	
Neonate (female)	70 (50.0)	32 (48.5)	38 (51.4)	0.735
Birth weight (g)	3080.60 (3022.88-3184.48)	3056.41 (2973.16-3138.84)	3103.80 (3023.77-3183.84)	0.428
Length (cm)	49.59 (49.29-49.88)	49.64 (49.14-50.15)	49.52 (49.28-49.87)	0.664
First child	87 (62.10)	40 (60.6)	47 (63.5)	0.723
Paternal age (years)	34.5 (33.4-35.6)	33.3 (31.6-35.6)	35.6 (34.1-37.0)	0.045 ^a
Paternal high education	89 (63.6)	35 (53.0)	54 (73.0)	0.014 ^a
Paternal low income	0	0	0	
Maternal age (years)	32.4 (31.5-33.2)	30.9 (29.6-32.3)	33.6 (32.6-34.6)	0.002 ^a
Maternal high education	107 (76.4)	46 (69.7)	61 (82.4)	0.076
Maternal low income	18 (12.9)	9 (13.6)	9 (11.2)	0.795
Maternal BMI				
Undernutrition	11 (7.9)	6 (9.1)	5 (6.8)	
Normal	60 (42.9)	26 (39.4)	34 (46.0)	
Overweight	18 (12.9)	6 (9.1)	12 (16.2)	0.513
Obesity class I	30 (21.4)	16 (24.2)	14 (18.9)	
Obesity class II	21 (15.0)	12 (18.1)	9 (12.2)	
Maternal allergic disease	6 (4.3)	4 (6.1)	2 (2.7)	0.327
Expanded family	77 (55.0)	36 (54.6)	41 (55.4)	0.919
Number in family	4.7 (4.4-5.0)	4.7 (4.3-5.2)	4.6 (4.2 – 5.1)	0.357
Family low income	18 (12.9)	9 (13.6)	9 (12.2)	0.795
Family history of allergy	47 (31.4)	22 (33.3)	25 (29.8)	0.956
Family history of asthma	9 (6.4)	7 (10.6)	2 (2.7)	0.057
Smoking in the family	35 (25.0)	23 (34.9)	12 (16.2)	0.011 ^a
Stool collection time	37.9 (33.6-50.1)	32.4 (27.8-36.9)	43.0 (35.8-50.1)	0.016 ^a
< 24 hours	54 (38.6)	23 (34.9)	31 (41.9)	
24-48 hours	48 (34.3)	30 (45.5)	18 (24.3)	0.023 ^a
> 48 hours	38 (27.14)	13 (19.7)	25 (33.8)	

^a*P* < 0.05.

BMI: Body mass index.

Neonates born by CS had older fathers (35.6 years; 95%CI: 34.1-37.0 *vs* 33.3 years; 95%CI: 31.6-35.1; *P* = 0.045), higher paternal education (73% *vs* 53%; *P* = 0.017), older mothers (33.6 years; 95%CI: 32.6-34.6 *vs* 30.9 years; 95%CI: 29.6-32.3; *P* = 0.002), and fewer family members who smoked (16.2% *vs* 34.9%; *P* = 0.011) than those born by VD. No significant differences in neonatal growth parameters, family structure, maternal nutritional or allergic status, economic status, feeding mode, or family history of allergy were observed between CS and VD neonates.

Analysis of gut microbiota profiles

Gut microbiota diversity: Rarefaction curves were generated before diversity analyses to assess sequencing depth across all samples. The curves reached a clear saturation plateau, indicating that the sequencing effort was sufficient to capture most microbial features within each sample (Supplementary Figure 2).

Gut microbiota diversity and composition were compared between CS and VD neonates. The CS group exhibited significantly higher alpha (Chao1 and Shannon indices, *P* < 0.001) and beta diversity (*P* < 0.001). Additionally, the relative abundance of several bacterial taxa differed by delivery mode.

Stool samples were further categorized by delivery mode and collection time (< 24 hours, 24-48 hours, and > 48 hours) to assess the effects of environmental exposure and early feeding. Alpha and beta diversity significantly declined in neonates from birth to > 48 hours in both delivery groups ($P < 0.001$). According to the Shannon index and beta diversity, significant reductions in diversity were observed between CS < 24 hours and CS > 48 hours, VD < 24 hours and VD 24-48 hours, and VD < 24 hours and VD > 48 hours ($P < 0.05$). Significant differences in the Chao1 index were observed between VD < 24 hours and VD > 48 hours ($P < 0.05$), but not between the CS subgroups.

Comparing delivery modes, Chao1 and Shannon indices were consistently lower in VD after 48 hours than in CS at all time points. Additionally, the Shannon index was significantly lower in VD 24-48 hours than in CS < 24 hours and VD > 48 hours than in CS < 24 hours ($P < 0.05$) (Figure 1).

Gut microbiota composition

Figure 2 shows the relative abundance of bacteria classified by delivery mode and stool collection time according to phylum, genus (top 10), and species (top 10). *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus gallolyticus*, *Acinetobacter baumannii*, *Lactococcus lactis*, *Methylobacterium radiotolerans*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Cupriavidus plantarum*, and *Pseudomonas aeruginosa* were the 10 most common bacterial compositions in the stool of 140 predominant breastfed neonates. *Escherichia/Shigella* and *Streptococcus* were predominant in VD neonates at birth. *Streptococcus* declined after 48 hours in VD neonates, being replaced by *Bifidobacterium* and *Klebsiella*, whereas *Streptococcus*, *Staphylococcus*, *Acinetobacter*, and *Pseudomonas* became more prominent in CS neonates after birth and reduced *Bifidobacterium* after 48 hours.

Significant gut microbiota composition

Significant bacterial biomarkers were identified across delivery modes and stool collection times (Figures 3 and 4). *Escherichia coli* was the most abundant species in the VD group, whereas *Bifidobacterium* species, particularly *Bifidobacterium longum*, *Bifidobacterium pseudocatenulatum*, and *Bifidobacterium bifidum*, were significantly enriched after 48 hours, reflecting the establishment of predominant breastfeeding.

Conversely, early colonization by *Bifidobacterium* was absent in CS-born neonates despite predominant breastfeeding. In these infants, *Novosphingobium aromaticivorans*, *Roseburia faecis*, *Gemmiger formicilis*, and *Methylobacterium tardum* were significantly enriched within 24 hours but became less abundant after that. Conversely, *Staphylococcus* (*Staphylococcus salivarius*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, and *Staphylococcus epidermidis*), *Streptococcus* (*Streptococcus salivarius* and *Streptococcus mitis*), *Pseudomonas aeruginosa*, *Novosphingobium aromaticivorans*, *Gemella haemolysans*, and *Cupriavidus plantarum* predominated later, indicating early environmental acquisition.

DISCUSSION

This study demonstrated significant associations between neonatal gut microbiota composition and key perinatal factors, including delivery mode, feeding, and stool collection timing. Distinct microbiota profiles between CS and VD neonates were observed using full-length 16S rDNA sequencing via Oxford nanopore technology in a relatively large cohort ($n = 140$). Both groups showed a decline in microbial diversity over time, reflecting the influence of postnatal factors, such as feeding.

In VD neonates, *Escherichia coli* was the dominant species at birth, whereas *Bifidobacterium* species, particularly *Bifidobacterium longum*, *Bifidobacterium pseudocatenulatum*, and *Bifidobacterium bifidum*, were significantly enriched after 48 hours. This may be due to exposure during VD and early breastfeeding. Conversely, *Bifidobacterium* was rarely detected in neonates with CS after 48 hours despite similar breastfeeding practices, indicating that CS disrupts early microbial seeding.

Gut microbiota colonization in neonates begins in utero and continues to evolve throughout the perinatal and postnatal periods. The establishment of gut flora is largely complete by the age of 3 years[29], and modification of gut microbiota before that time may impact immune system maturation and other health conditions[30]. Studies on gut microbiota dynamics in healthy neonates during the first few days of life are limited. However, this study provides valuable insights into the changes in this critical window, which may represent an opportunity to correct dysbiosis and reprogram the microbiota earlier. Although previous studies have reported the initial dominance of *Enterobacteriaceae*, *Bacteroides*, and *Enterococcus* in VD neonates and *Streptococcus* in CS neonates[17,31-33], this study expanded this understanding by demonstrating that *Escherichia/Shigella* and *Streptococcus* were predominant in VD neonates at birth. *Streptococcus* declined after 48 hours in VD neonates, being replaced by *Bifidobacterium*, whereas *Streptococcus* and *Staphylococcus* became more prominent in CS neonates after 24 hours of life. These temporal shifts highlight the need to consider sampling time when interpreting neonatal microbiota data.

Although our data show increased alpha and beta diversity in CS neonates, it is necessary to consider that higher diversity does not necessarily indicate a healthier microbiome in this population. Furthermore, elevated diversity may reflect environmental exposure or instability rather than a beneficial state. Previous studies have shown that cesarean delivery is associated with reduced abundance of beneficial bacteria, such as *Bacteroides*, and increased presence of opportunistic or hospital-associated taxa, which could impact early immune development and metabolic programming [34,35]. Therefore, the observed higher diversity in neonates with CS should be interpreted with caution, as it may signal altered microbiota assembly rather than a protective effect. The significantly higher microbial diversity observed in CS neonates and the significantly different pathogens from VD, as determined by linear discriminant analysis effect size analysis, may reflect exposure to the hospital environment, as CS neonates are more likely to acquire hospital-associated

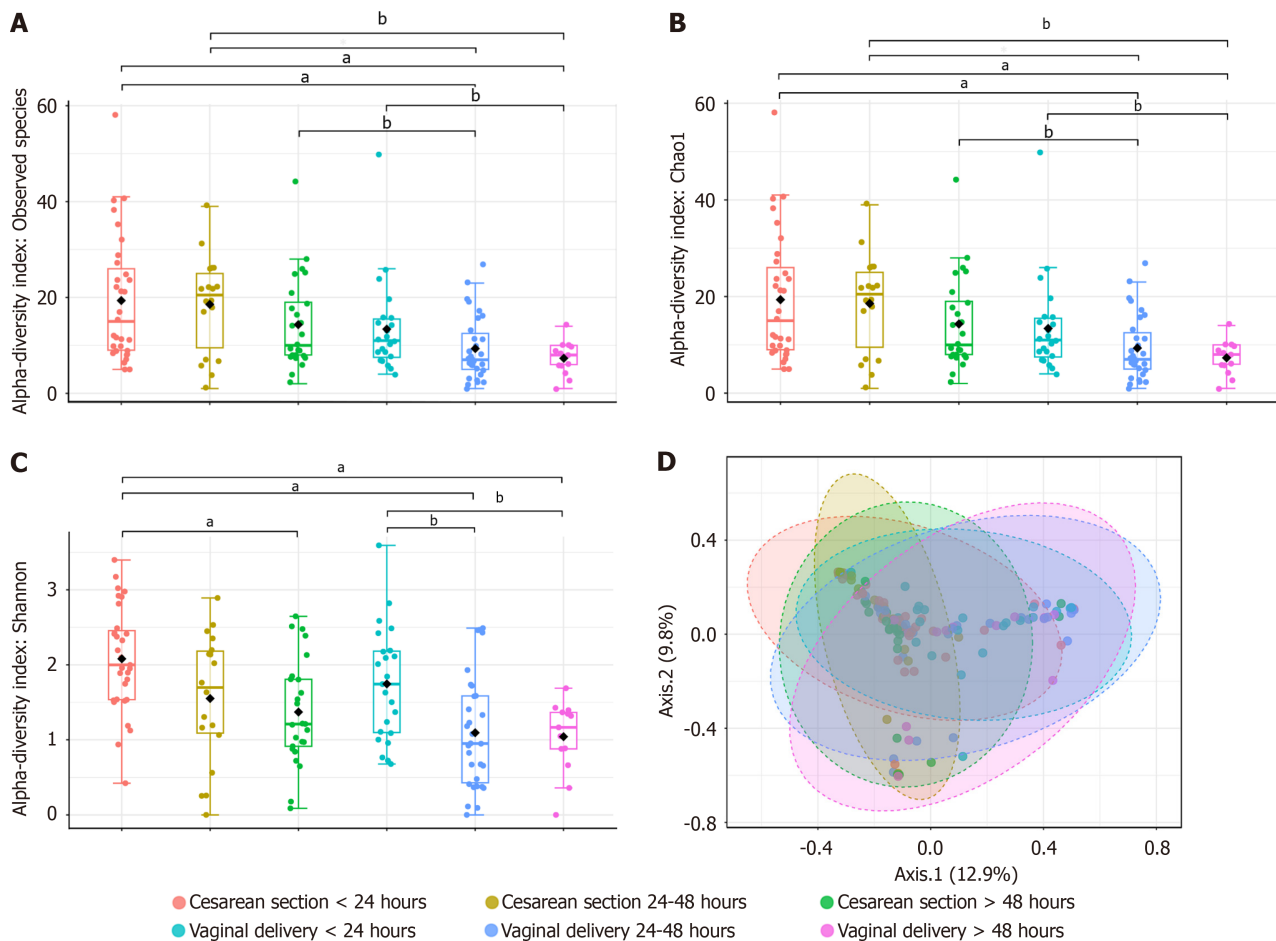


Figure 1 Bacterial diversities in the stool of predominant breastfed neonates according to mode of delivery and time of stool collection. A total of 140 samples were analyzed (cesarean section < 24 hours, $n = 31$; cesarean section 24-48 hours, $n = 18$; cesarean section > 48 hours, $n = 25$; vaginal delivery < 24 hours, $n = 23$; vaginal delivery 24-48 hours, $n = 30$; vaginal delivery > 48 hours, $n = 13$). A: Observed species; B: Alpha diversity based on species richness (Chao1 index); C: Alpha diversity based on species richness and evenness (Shannon index) were used to assess within-sample diversity; D: Beta diversity between samples was assessed using Bray-Curtis dissimilarity and visualized by principal coordinate analysis. Statistical differences in alpha diversity were evaluated using the Kruskal–Wallis test, while differences in beta diversity were assessed using PERMANOVA, $^aP < 0.001$; $^bP < 0.05$.

bacteria due to increased contact with healthcare personnel than those born vaginally[17].

Apart from the delivery mode and postnatal environment, prenatal factors also influence neonatal gut microbiota diversity and composition. In this study, differences in several prenatal factors, including parental age, parental educational level, maternal age, and smoking exposure in the family, were observed between the vaginal and cesarean delivery groups. Although parental and maternal age reached statistical significance in our cohort, the absolute differences were small and unlikely to be clinically meaningful. Additionally, higher paternal education did not translate into differences in socioeconomic status between the two groups, indicating a limited impact on microbiota-related outcomes in this context. Conversely, household smoking exposure appears to be a more biologically relevant factor. The higher prevalence of smoking exposure among infants born *via* VD raises the possibility that tobacco smoke may partly contribute to differences in gut microbial diversity. Accumulating evidence indicates that prenatal and early-life exposure to tobacco smoke is associated with reduced microbial diversity and altered gut microbial composition in infants, potentially through inflammatory, epigenetic, and environmental pathways affecting early microbial colonization and childhood obesity[36-38]. Therefore, although most baseline differences are unlikely to substantially confound our findings, smoking exposure warrants further consideration as a potential modifier of gut microbiota development and may explain why gut microbiota diversity is lower in neonates with normal labor than in CS neonates.

In this study, all stool samples were collected under standardized conditions before hospital discharge, minimizing environmental confounders. The observed narrowing of gut microbiota diversity over time may be due to the selective pressures of feeding. Although the neonates were predominantly breastfed, only VD neonates showed clear early colonization by *Bifidobacterium*, which underscores the critical role of VD in seeding beneficial microbes. The inability of the CS group to establish *Bifidobacterium* colonization may be due to disrupted maternal microbial transfer and perioperative antibiotic exposure. These factors contribute to early-life gut dysbiosis[18,39].

Importantly, this study employed full-length 16S rDNA sequencing *via* Oxford Nanopore Technologies, thereby enabling taxonomic resolution at the species level[40]. *Escherichia coli*, *Streptococcus gallolyticus*, and *Klebsiella variicola* were identified as dominant in VD neonates, species known to be part of the human gut microbiota[41] and likely acquired during vaginal birth. Conversely, *Novosphingobium aromaticivorans*, *Barnesiella intestinihominis*, *Pseudomonas aeruginosa*, and

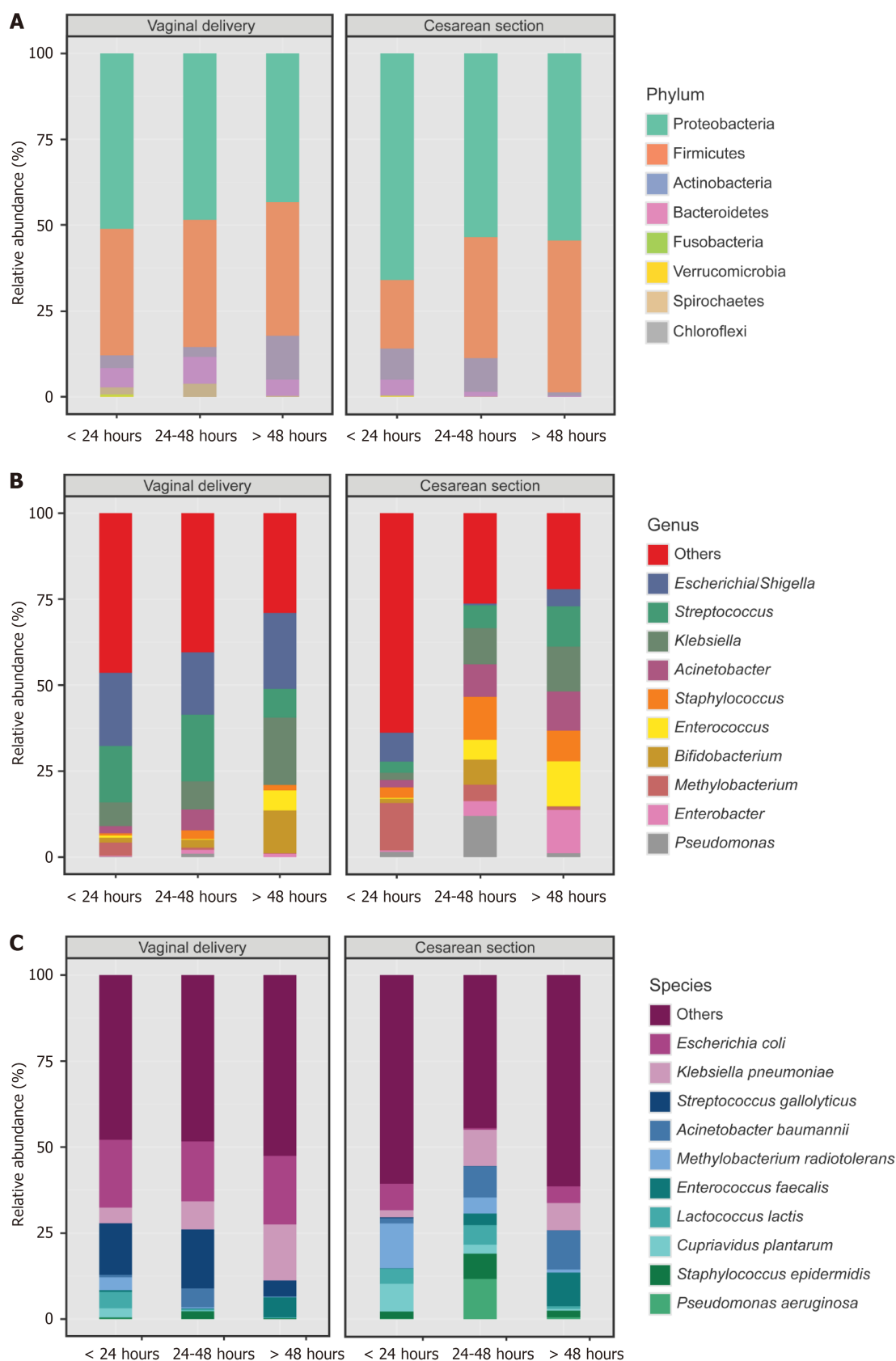


Figure 2 Differences in relative abundance of bacterial taxa in stool specimens of predominant breastfed neonates with different delivery modes and time of stool collection. A total of 140 samples were analyzed (cesarean section < 24 hours, $n = 31$; cesarean section 24-48 hours, $n = 18$; cesarean section > 48 hours, $n = 25$; vaginal delivery < 24 hours, $n = 23$; vaginal delivery 24-48 hours, $n = 30$; vaginal delivery > 48 hours, $n = 13$). Relative abundance profiles are shown at: A: Phylum level; B: Genus level (top 10 genera); C: Species level (top 10 species).

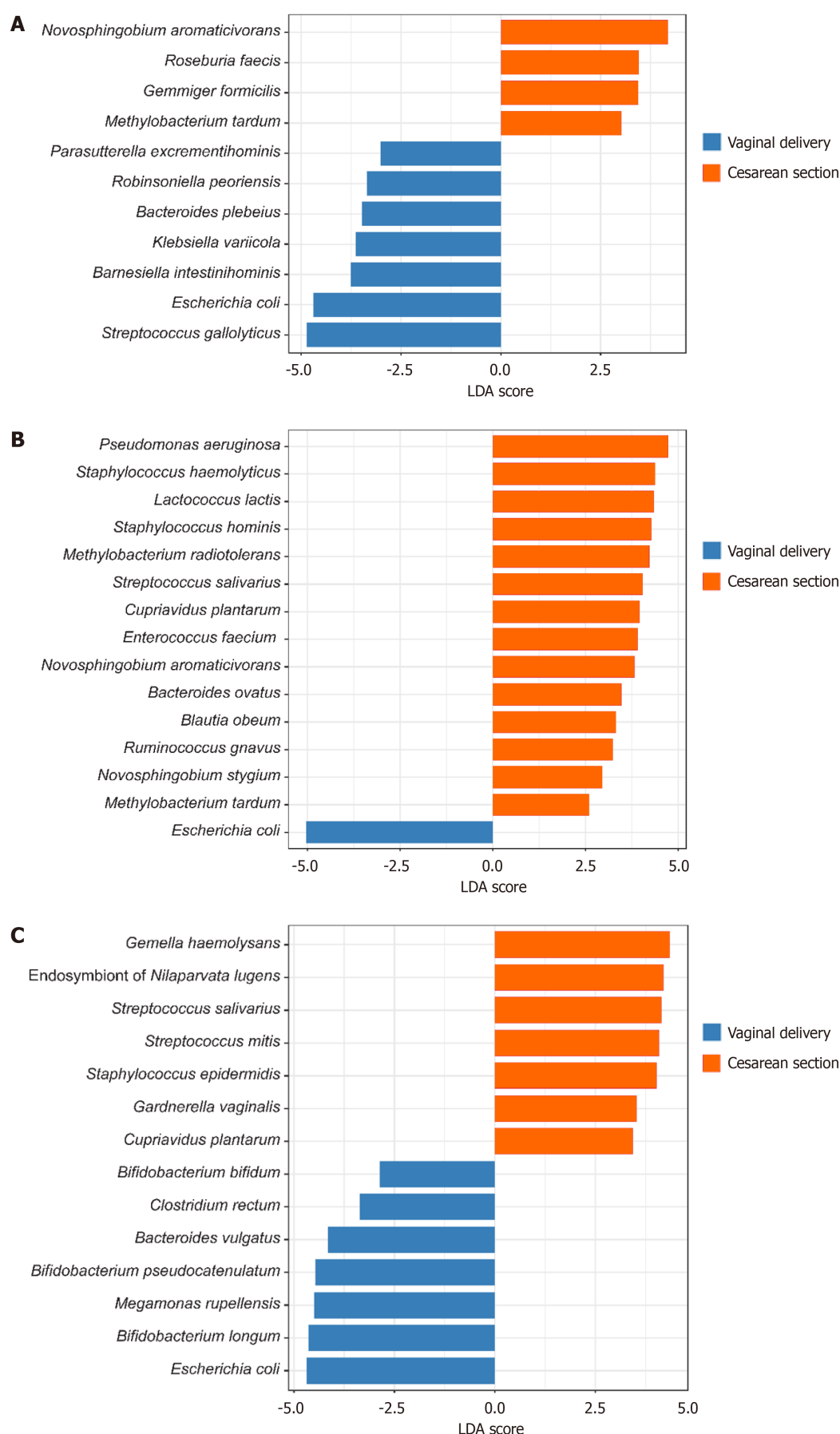


Figure 3 Differentially abundant bacterial taxa identified by linear discriminant analysis effect size analysis according to mode of delivery and time of stool collection. Linear discriminant analysis effect size was used to identify bacterial taxa with LDA scores > 2.0 and $P < 0.05$ (Kruskal–Wallis test)

between delivery modes. A total of 140 samples were included (cesarean section < 24 hours, $n = 31$; cesarean section 24-48 hours, $n = 18$; cesarean section > 48 hours, $n = 25$; vaginal delivery < 24 hours, $n = 23$; vaginal delivery 24-48 hours, $n = 30$; vaginal delivery > 48 hours, $n = 13$). Analyses were performed separately for samples collected at: A: < 24 hours; B: 24-48 hours; C: > 48 hours after birth.

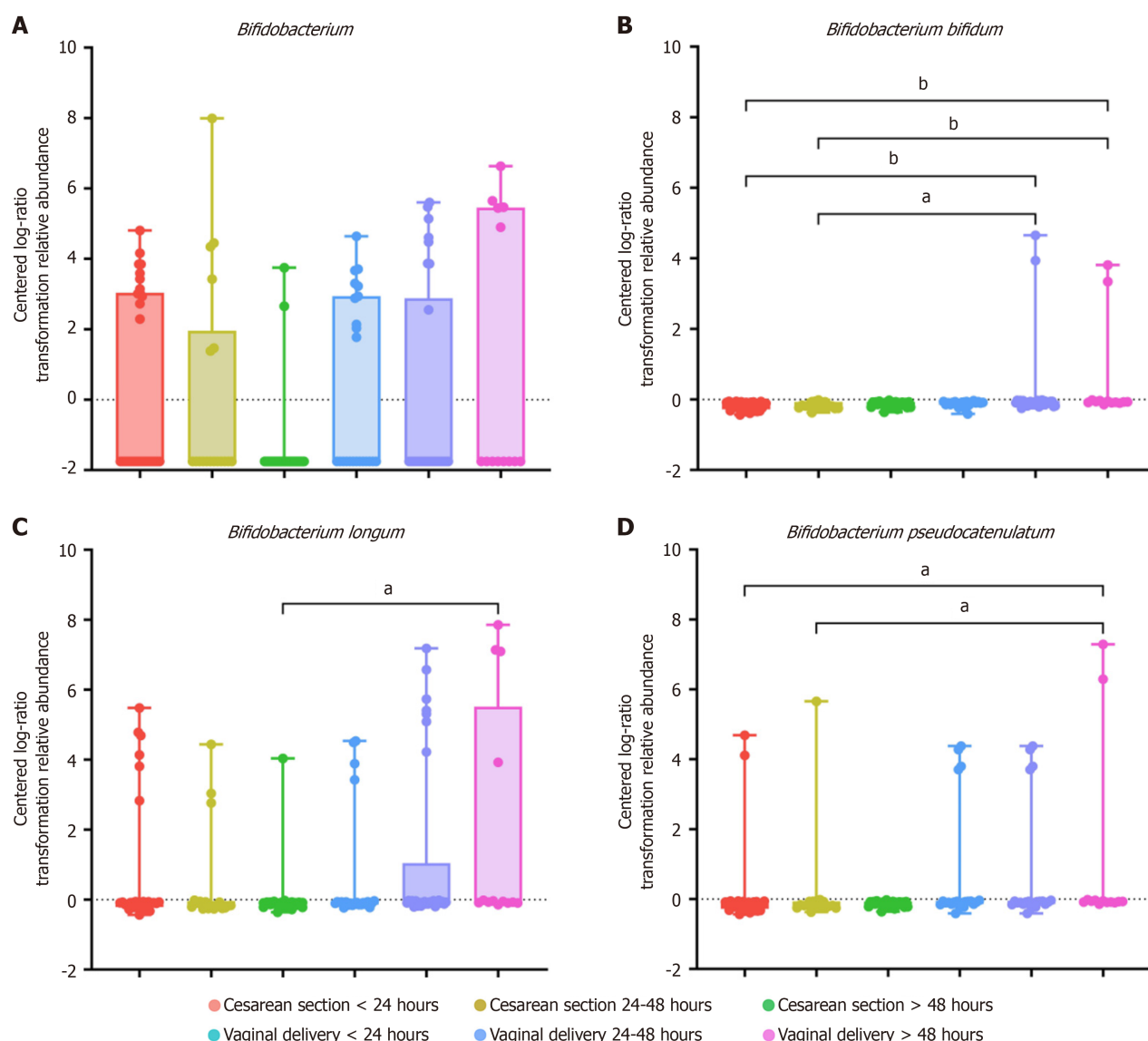


Figure 4 Centered log-ratio transformed relative abundance of *Bifidobacterium* taxa significantly enriched by linear discriminant analysis effect size analysis. Box-and-whisker plots show the centered log-ratio-transformed relative abundance of: A: *Bifidobacterium* (genus); B: *Bifidobacterium bifidum*; C: *Bifidobacterium longum*; D: *Bifidobacterium pseudocatenulatum* across different time points (< 24 hours, 24-48 hours, and > 48 hours). A total of 140 samples were analyzed (cesarean section < 24 hours, $n = 31$; cesarean section 24-48 hours, $n = 18$; cesarean section > 48 hours, $n = 25$; vaginal delivery < 24 hours, $n = 23$; vaginal delivery 24-48 hours, $n = 30$; vaginal delivery > 48 hours, $n = 13$). Boxes represent the median and interquartile range, with whiskers indicating the minimum and maximum values. Statistical differences among time-point groups were evaluated using the Kruskal–Wallis test, followed by Dunn's *post hoc* test with multiple-testing correction. Adjusted P values are indicated in the plots, ^a $P < 0.05$; ^b $P < 0.01$.

Gemella haemolysans predominated in CS, indicating contamination of hospital environments, including operating rooms, antibiotic use before CS, and postpartum care[42]. Further studies are needed to explore the relationship between microbiome species and long-term health to clarify how these bacterial species may contribute to the development of health conditions.

This study has several strengths, including the application of 16S *rDNA* sequencing by Oxford nanopore technology, which enhances the identification of bacterial species in a relatively large study population of neonates in Thailand. Stool collection timing before hospital discharge likely provides a representative snapshot of the gut microbiota at different points after birth. Additionally, mothers in the VD and CS groups breastfed their neonates, highlighting the role of breastfeeding in shaping the gut microbiota. However, this study examined the microbiota at single time points, limiting the ability to assess dynamic changes in individual gut microbiota over time and to evaluate the diagnostic value of gut microbiota in different groups. In this study, 140 stool samples were analyzed due to logistical and financial constraints.

However, this sample size may limit the statistical power of some subgroup comparisons. Additionally, all samples were from a single medical center in Thailand, and some significant demographic differences were observed between the CS and VD groups, including parental age, education, and family smoking, which may limit the generalizability of the results. Therefore, caution should be exercised when interpreting the results. Further larger multicenter studies with diverse populations and longitudinal designs are needed to validate these findings and their clinical implications.

CONCLUSION

This study highlighted the significant impact of delivery mode and early feeding practices on the neonatal gut microbiota composition. CS and VD were associated with distinct gut microbiota profiles, with CS exhibiting a higher diversity of hospital-associated bacteria. Although both groups were predominantly breastfed, differences in the establishment of key bacterial genera, such as *Bifidobacterium*, were observed only in VD neonates. Despite the limited ability to track changes in individual microbiota over time, the study's findings provide valuable insights into early microbial dynamics in neonates. Species-level identification of dominant and deficient microbes enables the development of strain-specific probiotic interventions tailored to support the establishment of healthy microbiota in CS-born neonates and improve long-term health outcomes.

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FOOTNOTES

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Author contributions: Sintusek P conceptualized and designed the study, designed the data collection instruments, collected data, carried out the initial analyses, drafted the initial manuscript, and critically reviewed the manuscript; Payungporn S conceptualized the study, designed and contributed to the experiments, confirmed the validity of the data, and critically reviewed and revised the manuscript; Klomkliew P, Visedthorn S, and Phutthawong K conducted the experiments, analyzed the data, interpreted the results, and reviewed the manuscripts; Noicharoen T, Soontornsook A, and Tran DL designed, collected the data, and reviewed the manuscript; all authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

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Data sharing statement: The data, analytic methods, and study materials from this study will be made available to other researchers upon reasonable request to the corresponding author. Sequencing data have been deposited in Sequence Read Archive under accession number PRJNA1265435, and metadata will be shared in de-identified form to protect participant confidentiality. To request access to the data, please contact Suthida Visedthorn (tangsuthida.chula@gmail.com). A temporary access link will be generated upon request; note that the link will expire shortly after its creation.

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