Journal of Microbiology (2022) Vol. 60, No. 10, pp. 1021–1031
DOI 10.1007/s12275-022-2180-2
eISSN 1976-3794
pISSN 1225-8873

Early gut microbiota in very low and extremely low birth weight preterm infants with feeding intolerance: a prospective case-control study[§]

Ling Liu[1,2], Dang Ao[2], Xiangsheng Cai[3], Peiyi Huang[2], Nali Cai[2], Shaozhu Lin[2], and Benqing Wu[1,3*]

[1]*Jinan University, Guangzhou, Guangdong 510000, P. R. China*
[2]*Department of Pediatrics, Affiliated Hospital of Guangdong Medical University, Zhanjiang, Guangdong 524000, P. R. China*
[3]*University of the Chinese Academy of Sciences - Shenzhen Hospital, Shenzhen, Guangdong 518000, P. R. China*

(Received Apr 25, 2022 / Revised Jun 30, 2022 / Accepted Jul 7, 2022)

The potential role of the gut microbiota in the pathogenesis of feeding intolerance (FI) remains unclear. Understanding the role of the gut microbiota could provide a new avenue for microbiota-targeted therapeutics. This study aimed to explore the associations between aberrant gut microbiota and FI in very low or extremely low birth weight (VLBW/ELBW) preterm infants. In this observational case-control study, VLBW/ELBW infants were divided into two groups: FI group and feeding tolerance (FT) group. 16S rRNA gene sequencing was performed to analyze the gut microbial diversity and composition of the infants. The differences in the gut microbiota of the two groups were compared. In total, 165 stool samples were obtained from 44 infants, among which, 31 developed FI and 13 served as controls. Alpha diversity was the highest in the meconium samples of the two groups. LEfSe analysis revealed that the abundances of Peptostreptococcaceae, Clostridiales and *Clostridia* in the FT group were significantly higher than in the FI group. At the phylum level, the FI group was dominated by Proteobacteria, and the FT group was dominated by Firmicutes. The meconium samples of the FI group had higher proportions of γ-proteobacteria and *Escherichia-Shigella* and a lower proportion of *Bacteroides* compared with the FT group. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis demonstrated that aberrant gut bacteria in the FI group were strongly associated with dysregulation of C5-Branched-dibasic-acid-metabolism, protein kinases, and sporulation. These findings reveal candidate microbial markers to prevent FI. Increased relative abundances of γ-proteobacteria and *Escherichia-Shigella* and decreased abundance of *Bacteroides* in meconium were associated with an increased risk of FI, while Peptostreptococcaceae, Clostridiales and *Clostridia* reduced the risk of FI in VLBW/ELBW infants.

*For correspondence. E-mail: wubenqing783@126.com
[§]Supplemental material for this article may be found at https://doi.org/10.1007/s12275-022-2180-2.

Keywords: gut microbiota, feeding intolerance, very low birth weight, extremely low birth weight, γ-proteobacteria, *Bacteroides, Escherichia-Shigella,* Peptostreptococcaceae, Clostridiales, *Clostridia*

Introduction

Preterm infants under 32 weeks of gestational age are prone to feeding intolerance (FI), which is a common cause of delayed enteral nutrition (Young *et al.*, 2017). Recent data indicates that FI occurs in around 30% of premature infants and 70% of very low birth weight (VLBW, < 1.5 kg) infants, with FI observed most commonly in the first four weeks of life (Arboleya *et al.*, 2017; Forsgren *et al.*, 2017; Morais *et al.*, 2019; El Manouni El Hassani *et al.*, 2021). FI presents as a series of symptoms and signs and is associated with extrauterine growth retardation (EUGR), higher morbidity and mortality, and longer ICU stay. In addition, FI complicates the nutritional management of the infant and prolongs the time of parenteral nutrition, which can lead to various complications, including cholestasis, liver and kidney damage, and catheter-related infections (Blaser *et al.*, 2014). As VLBW infants are affected by parenteral feeding, clarifying the pathogenetic mechanisms of FI and reducing the associated interruption to the feeding plan are key to achieving optimal nutrition for premature infants.

FI is a devastating condition in preterm infants; however, the onset of FI in premature infants is not yet fully understood and is likely multi-factorial. Changes to the gut microbiota early in life are critical to the maturation and regulation of intestinal motility, and the microbial communities present in the gut can impact the energy supply of intestinal epithelial cells among preterm infants and can even affect the maintenance of the barrier function of gut epithelial cells across the entire life span (The Human Microbiome Project Consortium, 2012; Yu *et al.*, 2016; Rao *et al.*, 2021). The gut microbiota of preterm infants differs substantially from that of normal term infants, exhibiting delayed colonization and low bacterial species diversity (Arboleya *et al.*, 2012). The aberrant gut microbiota may be responsible for increased intestinal permeability and aberrant motility, digestion, and absorption, resulting in translocation of bacteria to the bloodstream and an increased risk of FI in preterm infants (Campeotto *et al.*, 2009; Halpern and Denning, 2015). Yuan *et al.* (2019) investigated the gut microbial composition of preterm infants with FI and speculated that Klebsiella may be a potential biomarker for the diagnosis of FI. The aberrant gut microbiota of preterm infants was proposed to be a driving factor in FI in another study (Baldassarre *et al.*, 2019). These observations are consistent

with the long-standing belief that FI and gut bacteria might be causally associated. Thus, examination of the gut microbiota in preterm infants is critical to obtaining a more comprehensive understanding of the pathophysiology of FI.

To date, research on FI in preterm infants has mainly focused on retrospective analysis of possible risk factors for FI, the effects of prokinetic drugs and probiotics on the prevention and treatment of FI, and changes to the intestinal microbial composition of infants with FI (Qiao *et al.*, 2017; Yuan *et al.*, 2019; Navarro-Tapia *et al.*, 2020). However, the specific gut microbiota that are pathogenic or protect against FI in premature infants are yet to be determined. Aberrant gut microbiota established at a very early age may predict the occurrence of subsequent FI; however, this has yet to be confirmed empirically.

To this end, this study explored the relationships between the gut microbiota and FI in the highly unstable preterm infant gut. This prospective case-control study analyzed differences in the composition of the gut microbiota between VLBW/extremely low birth weight (ELBW, < 1 kg) infants who subsequently developed FI (FI group) and VLBW/ELBW infants that did not develop FI (control group) and evaluated the microbial communities that protected against FI. The primary objective of this study was to investigate the associations between aberrant gut microbiota composition and FI in preterm infants.

Materials and Methods

Study design and participants

This study was based on a cohort located in the west Guangdong area; a nested observational case-control study was performed within this cohort. The study sample was obtained from the Division of Neonatology of Guangdong Medical University between June and December 2020.

The inclusion criteria were as follows: (1) infants with a birth weight < 1.5 kg who were expected to survive beyond one week; (2) admitted to the neonatal intensive-care unit (NICU) of Guangdong Medical University Affiliated Hospital within the first 24 h after birth; (3) parents or their legal representative provided written informed consent. This study was approved by the Clinical Trial Ethics Committee of the Affiliated Hospital of Guangdong Medical University (approval number: PJ2020-044) and was performed strictly in accordance with the principle of informed consent. The Chinese clinical trial registration number was ChiCTR-2000038024. The guardians of each infant were informed of the purpose and the entire study protocol and provided written informed consent before study enrollment.

The exclusion criteria were as follows: (1) hospital stay less than 14 days; (2) presence of complex congenital heart disease, Down syndrome, genetic metabolic diseases, or other identified congenital anomalies; (3) diagnosed with surgical bowel diseases and/or structural bowel abnormalities and neonatal necrotizing enterocolitis (NEC). VLBW was defined as a birth weight of less than 1.5 kg, and ELBW was defined as a birth weight of less than 1 kg.

FI was diagnosed if any of the following symptoms occurred: (1) frequent vomiting (> 3 times/day); (2) unchanged or reduced feeding volume (lasting longer than three days); (3) gastric retention (more than 30% of the previous feeding volume); (4) abdominal distention; (5) "coffee ground" vomiting; (6) feeding volume < 8 ml/kg at each feed at the end of week two; and (7) fasting for more than two days (Qiao *et al.*, 2017). Research subjects who did not have the above symptoms were included in the FT group.

Clinical data

At enrollment, detailed data about the pregnancy and birth were collected from the medical records and via a questionnaire, including prenatal exposures, pregnancy complications, placental conditions, premature rupture of membranes, prenatal hormone use, and prenatal antibiotic use. The collected clinical data included sociodemographic information, mechanical ventilation time, vasoactive drugs, initial meconium time, enteral feeding time and feeding type, antibiotic exposure and duration, total number of days of hospitalization, and other outcomes related to the infant's preterm clinical evolution. These data were collected from the antenatal records, birth records, and maternal and infant charts.

Specimen collection and preservation

Sterile cotton swabs were used to collect the samples into sterile tubes. Approximately 0.2 g (the size of a soybean) of raw fecal material was obtained. Samples were collected at birth and every seven days for 28 days. Samples were immediately transferred to a refrigerator at -20°C and frozen until genetic analysis. The process of collecting and transporting samples was carried out in strict accordance with aseptic operating procedures. If the exclusion criteria were met, the collected samples were destroyed.

DNA extraction, PCR amplification, and sequencing

Microbial genomic DNA was extracted from the stool samples using a Zymo BIOMICSTM DNA Miniprep Kit (Zymo Research), according to the manufacturer's instructions. The DNA extract was checked on 1% agarose gel, and the DNA concentration and purity were determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher). The hypervariable (V4) region of the bacterial 16S rRNA gene was amplified with 519F:CAGCMGCCGCGGTAATWC and 806R: GGACTACVSGGGTATCTAAT primers using a PCR amplification kit (Vazyme Company).

PCR amplification of the 16S rRNA gene was performed as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturing at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 45 sec, single extension at 72°C for 10 min, and amplification completion at 4°C. The PCR product was extracted from 2% agarose gel and purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences), according to the manufacturer's instructions. DNA was quantified using a Quantus^{TM} Fluorometer (Promega). Purified amplicons were pooled in equimolar amounts and paired-end sequencing was performed on a NextSeq CN500 platform (Illumina company) for 150 bp (PE150). The forward and reverse primers were: 5′-ACACTCTTTCCCTAC

ACGAGGCTCTTCCGA-3′, 5′-GTGACTGGAGTTCAGA CGTGTGCTCTTCCGATC-3′. The full data sets have been submitted to the NCBI Sequence Read Archive (SRA) under accession numbers SRR2070780, SRR2070792, SRR2070796, and SRR2070799 (Bioproject: SRP059646).

Processing of sequencing data

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp (https://github.com/OpenGene/fastp), version 0.20.00 (Chen *et al.*, 2018), and assembled by FLASH (v1.2.11) (Magoč and Salzberg, 2011) according to the following criteria: (1) the 300 bp reads were truncated at any site receiving an average quality score of < 20 over a 50 bp sliding window; truncated reads shorter than 50 bp were discarded; reads containing ambiguous characters were also discarded; (2) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of the overlap region was 0.2; reads that could not be assembled were discarded; (3) samples were distinguished according to the barcode and primers, and the sequence direction was adjusted; exact barcode matching was performed and a mismatch of two nucleotides was allowed in primer matching.

Effective tags were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using UPARSE version 7.1 (Edgar, 2013); chimeric sequences were identified and removed. The taxonomic classification of each OTU representative 16S rRNA gene sequence was analyzed by the RDP Classifier algorithm based on the Silva (SSU128) database.

Analysis of microbiome data

Beta diversity involves a comparative analysis of differences between bacterial communities in two samples. Beta diversity was analyzed by principal components analysis (PCA), principal co-ordinates analysis (PCoA), and non-metric multidimensional scaling (NMDS). Multivariate statistical techniques, including PCA, PCoA, NMDS, ANOSIM, and ADONIS were performed using the vegan package. Microbial alpha diversity (Ace, Chao1, Shannon, Simpson), Bray-Curtis dissimilarity, and weighted and unweighted UniFrac distances were assessed in QIIME version 2.0. 16S rRNA gene prediction was performed using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) software.

Statistical analysis

All statistical analyses were performed using R (v3.5.3). For the demographic and delivery data of the infants and mo-

Table 1. Demographic characteristics and delivery data for study infants and mothers

Characteristics	FI group (n = 31)	FT group (n = 13)	$t/\chi^2/z$	*P*
Preterm neonates' characteristics				
Gestational age ($\bar{x} \pm s$, weeks)	30.0 ± 1.6	30.7 ± 1.7	-1.241	0.222
Birth weight ($\bar{x} \pm s$, kg)	1.23 ± 0.2	1.33 ± 0.18	-1.603	0.116
Male (n, %)	12 (38.7%)	9 (69.2%)	3.420	0.064
Cesarian section (n, %)	24 (77.4%)	8 (61.5%)	0.502	0.479
Singletons (n, %)	24 (77.4%)	10 (76.9%)	0.000	1.000
Fetal distress (n, %)	9 (29.0%)	3 (23.1%)	0.001	0.973
One min agar scores (IOR, scores)	8 (6, 9)	8 (7, 9)	-0.885	0.376
Initial enteral feeding time (IOR, days)	1 (0, 3)	0 (0, 1.5)	-1.490	0.136
Initial enteral feeding type is breast milk (n, %)	7 (22.6%)	1 (7.7%)	0.283	0.595
Initial meconium time (IOR, h)	8 (6, 16)	14 (6, 20.5)	-1.043	0.297
Feeding type mixed feeding	21 (67.7%)	10 (76.9%)		
Feeding type formula feeding	7 (22.6%)	2 (15.4%)	0.394	0.821
Feeding type breast feeding	3 (9.7%)	1 (7.7%)		
The time of mechanical ventilation (IOR, days)	12 (8, 25)	7 (2.5, 11)	-2.512	0.012**
The time of vasoactive drugs (IOR, days)	0 (0, 4)	0 (0, 0)	-2.175	0.030**
The time of antibiotics (IOR, days)	9 (5, 14)	6 (2, 8)	-2.058	0.040**
Probiotics use (n, %)	18 (58.1%)	10 (76.9%)	0.711	0.399
The time of probiotics (IOR, days)	4 (0, 10)	4 (1.5, 9)	-0.449	0.654
Pregnant mothers' characteristics				
Age (IOR, years old)	31 (28, 34)	33 (30, 37)	-1.550	0.121
Premature rupture of membranes (n, %)	9 (29.0%)	7 (53.8%)	1.483	0.223
The time of premature rupture of membranes > 18 h (n, %)	3 (9.7%)	5 (38.5%)	3.350	0.067
Placental abruption (n, %)	6 (46.2%)	4 (30.8%)	0.185	0.667
Use a full course of glucocorticoids (n, %)	21 (72.4%)	10 (76.9%)	0.000	1.000
Complications of pregnancy (n, %)	18 (58.1%)	5 (38.5%)	1.411	0.235
Chorioamnitis (n, %)	3 (9.7%)	1 (7.7%)	0.000	1.000
Use of antibiotics (n, %)	22 (70.9%)	10 (76.9%)	0.012	0.911
The time of antibiotics (IOR, days)	1 (0.3, 3.5)	1.5 (1, 3.75)	-0.553	0.580

**$P < 0.05$.

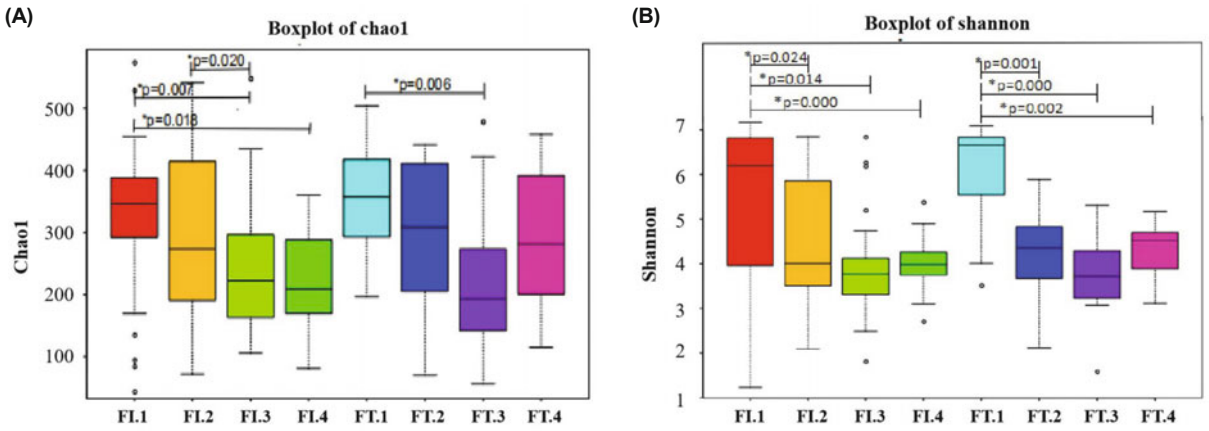

Fig. 1. Alpha diversity of the different groups. (A) The Chao1 index comparison among FI.1, FI.2, FI.3, FI.4, FT.1, FT.2, FT.3, and FT.4. (B) The Shannon index comparison among FI.1, FI.2, FI.3, FI.4, FT.1, FT.2, FT.3, and FT.4. The Kruskal-Wallis rank-sum test was used to test differences between the groups, followed by the Benjamini–Hochberg method to adjust the *P*-value. $P < 0.05$ was considered statistically significant.

thers, Student's t tests or Mann-Whitney tests were used to identify statistically significant differences in continuous variables, and the χ^2 test or Fisher's exact test was used to identify differences in categorical variables. The Kruskal-Wallis test was used to examine differences between the FI and FT groups at different sampling times, followed by the Benjamini-Hochberg method to adjust the *P*-value. LefSe with an LDA cutoff of 2 was also applied to identify unique taxa or functions that differentiated the groups, in order to identify statistically significant biomarkers. Correlations between clinical indices and gut microbes were calculated by the Mantel test, using the R package "vegan". Spearman's correlations were also used to quantify the relationships between clinical indices and gut microbial composition using R (v 3.5.3). Canonical correlation analysis (CCA) using the R package "vegan" was also performed to determine which clinical indices were related to the gut microbial composition. LefSe and the Kruskal-Wallis test were used to explore the significant differences in the Kyoto Encyclopedia of Genes and Genomes (KEGG) functional prediction between the FI and FT groups. Differences at $P < 0.05$ were considered statistically significant.

Results

Demographic and maternal variables

From June 2020 to December 2020, 44 infants at the Affiliated Hospital of Guangdong Medical University met the inclusion criteria and were enrolled in the study. A total of 165 fecal samples collected from the infants during their NICU hospitalizations were included in this analysis (Supplementary data Table S1). There were 31 infants in the FI group (27 VLBW, 4 ELBW) and 13 in the FT group (12 VLBW, 1 ELBW). The

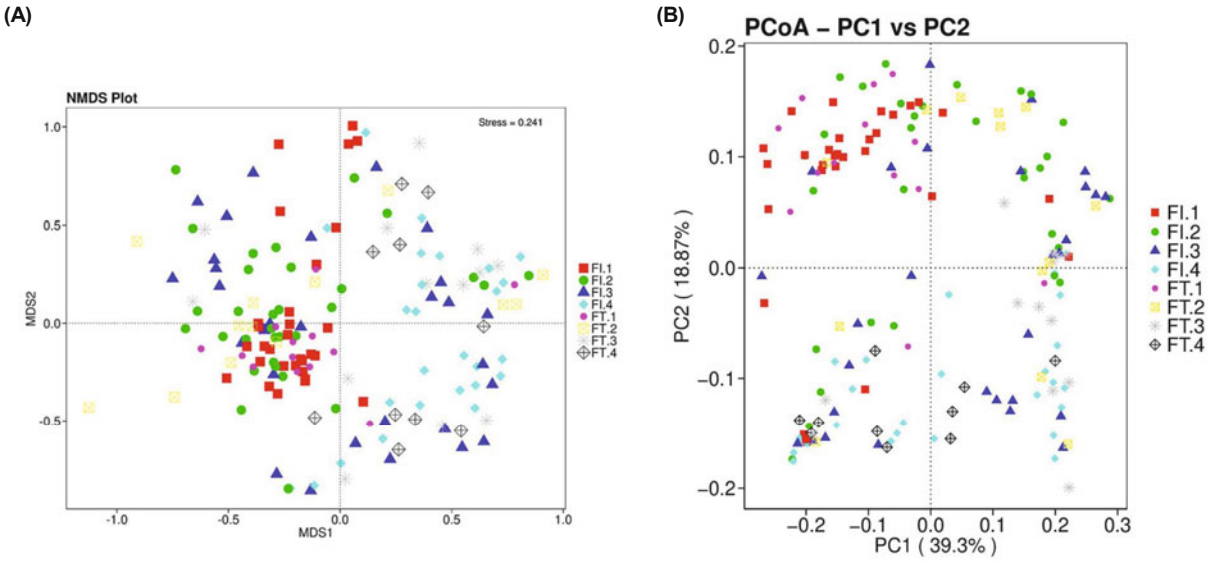

Fig. 2. Beta diversity in preterm infants. (A) NMDS. (B) Beta diversity was calculated using weighted UniFrac distances by PCoA; a symmetrical distribution of the fecal microbial community was observed among the samples.

two groups had similar baseline characteristics. Birth weight, gestational age at birth, gender, route of delivery, APGAR score, initial enteral feeding time, enteral feeding type, and initial meconium time of preterm infants in the FI group did not differ significantly from those of the FT group ($P > 0.05$). The time of mechanical ventilation, time of vasoactive drugs, and time of antibiotics differed significantly between the groups ($P < 0.05$). Three antibiotics (penicillin, cefuroxime, and ceftazidime) were used in early-onset sepsis. Meropenem and vancomycin were used in several preterm infants with late septicemia. There was no significant difference in the types of antibiotics between the two groups. There was no significant difference in probiotics use ($P > 0.05$). Most infants received mixed feeding (infant formula and some breast milk during the study period). The parenteral nutrition time, adequate feeding time, and length of stay in the FI group were significantly longer than those in the FT group ($P < 0.05$). The sociodemographic and clinical characteristics of mothers and preterm neonates are summarized in Table 1.

Data quality control

Samples obtained at the four time points in the FI group were named FI.1, FI.2, FI.3, FI.4; samples at the four time points in the FI group were named FT.1, FT.2, FT.3, FT.4. In total, 36,202 sequences of DNA were identified in this study over the observation period (Q20 92.9%; Q30 88.2%). The data quality standard was as follows: Q20 ≥ 80%, effective data volume ≥ 15,000. Q20 and Q30 refer to the percentage of bases whose base quality value of effective tags was greater than 20 (sequencing error rate less than 1%) and 30 (sequencing error rate less than 0.1%), respectively.

Analysis of gut microbiota diversity

Alpha diversity was evaluated using four metrics: ACE index, Chao1 index, Shannon index, and Simpson index. The alpha diversity indices varied over time in both the FI and FT groups, showing a similar trend. There were significant differences in the Chao1 index and Shannon index at the different sample collection times among the FI group. The alpha diversity of the meconium microbiota was at a higher level but showed a downward trend from day 7 to day 28 after birth (Fig. 1A and B). There were no significant differences in richness and diversity between the FI group and FT group at each time point.

Two analytical methods, i.e., NMDS (Fig. 2A) and PCoA (Fig. 2B), were used to compare the beta diversity of the two groups. The NMDS (Fig. 2A) and PCoA (Fig. 2B) analyses indicated that the stool microbiome of the FI.1 and FT.1 patients clustered separately from the FI.4 and FT.4 time points. Additionally, the gut bacterial community structure differences between the two groups were analyzed using ANOSIM and ADONIS analysis (Table 2). The ANOSIM and ADONIS analyses indicated that, in the FI group, the diversity of the gut microbiota composition significantly differed from that of the meconium (Table 2).

Analyses of fecal bacterial community abundance

The top 10 relative abundances of OTUs at the phylum level are shown. At the phylum level, Firmicutes, Proteobacteria, Actinobacteria, and Bacteroides accounted for more than 98% of the bacteria. Proteobacteria (red) was the most abundant phylum in the meconium of the FI and FT groups, and a gradual decrease in the fecal abundance was observed over time. Subsequently, the abundance of Firmicutes (blue) gradually increased and predominated in the FI.3, FI.4, FT.3, and FT.4 fecal samples (Fig. 3A). At the phylum level, Firmicutes and Proteobacteria were the most common phyla identified in the FI and FT groups; the FI group was dominated by Proteobacteria and the FT group was dominated by Firmicutes (Fig. 3B).

Table 2. The community structure differences between groups analyzed using Anosim and Adonis analysis

Analysis	Group	Analysis value	*P*
Anosim analysis	FI.1: FI.2	R = 0.055	0.017*
	FI.1: FI.3	R = 0.170	0.001**
	FI.1: FI.4	R = 0.389	0.001**
	FI.2: FI.3	R = 0.033	0.076
	FI.1: FT.1	R = -0.006	0.467
	FI.2: FT.2	R = 0.119	0.083
	FI.3: FT.3	R = 0.006	0.453
	FI.4: FT.4	R = -0.039	0.669
Adonis analysis	FI.1: FI.2	R2 = 0.038574;0.961426	0.01**
	FI.1: FI.3	R2 = 0.078734;0.921266	0.001**
	FI.1: FI.4	R2 = 0.144772;0.855228	0.001**
	FI.2: FI.3	R2 = 0.027831;0.972169	0.081
	FI.1: FT.1	R2 = 0.025275;0.974725	0.367
	FI.2: FT.2	R2 = 0.020427;0.979573	0.614
	FI.3: FT.3	R2 = 0.037021;0.962979	0.128
	FI.4: FT.4	R2 = 0.033551;0.966449	0.321

*$P < 0.05$; **$P < 0.01$.

At the class level, the relative abundance of γ-proteobacteria in the FI.1 group was higher than in the FT.1 group ($P = 0.01$). Similarly, the proportional abundance of bacterial class appeared different at baseline; the relative abundance of Bacteroides in the FI.1 group was significantly lower than that in the FT.1 group ($P = 0.03$) (Fig. 3C). The FI group had a higher abundance in γ-proteobacteria than the FT group, but there were no significant differences in the relative abundances of other microbial species at the class level (Fig. 3D).

The heatmap demonstrates that the proportional abundances of various genera were different at different sampling times. For example, *Ureaplasma* and *Pseudomonas* were more abundant in FI.1 and *Bacteroides*, digest *Streptococcus* and *Sphingomyces* were more abundant in FT.1 (Fig. 3E). There was a significantly higher proportion of *Escherichia-Shigella* and a significantly lower proportion of *Bacteroides* in FI.1 compared with FT.1 ($P = 0.002$ and $P = 0.021$, respectively).

The relative abundances of all fecal samples were compared between the FT and FI groups (Fig. 4A). A Kruskal-Wallis rank-sum test showed that Firmicutes were differentially enriched in the FT group (Fig. 4A). By contrast, Actinobacteria, *Bacilli*, *Escherichia-Shigella,* Proteobacteria, Bacteroides, and *Streptococcus*, which were enriched in the FI group, were reduced in the FI patients, but these differences were not statistically significant. The Unweighted Pair-group Method with Arithmetic Mean (UPGMA) cluster tree showed that the development of the gut microbiota of the FI group was

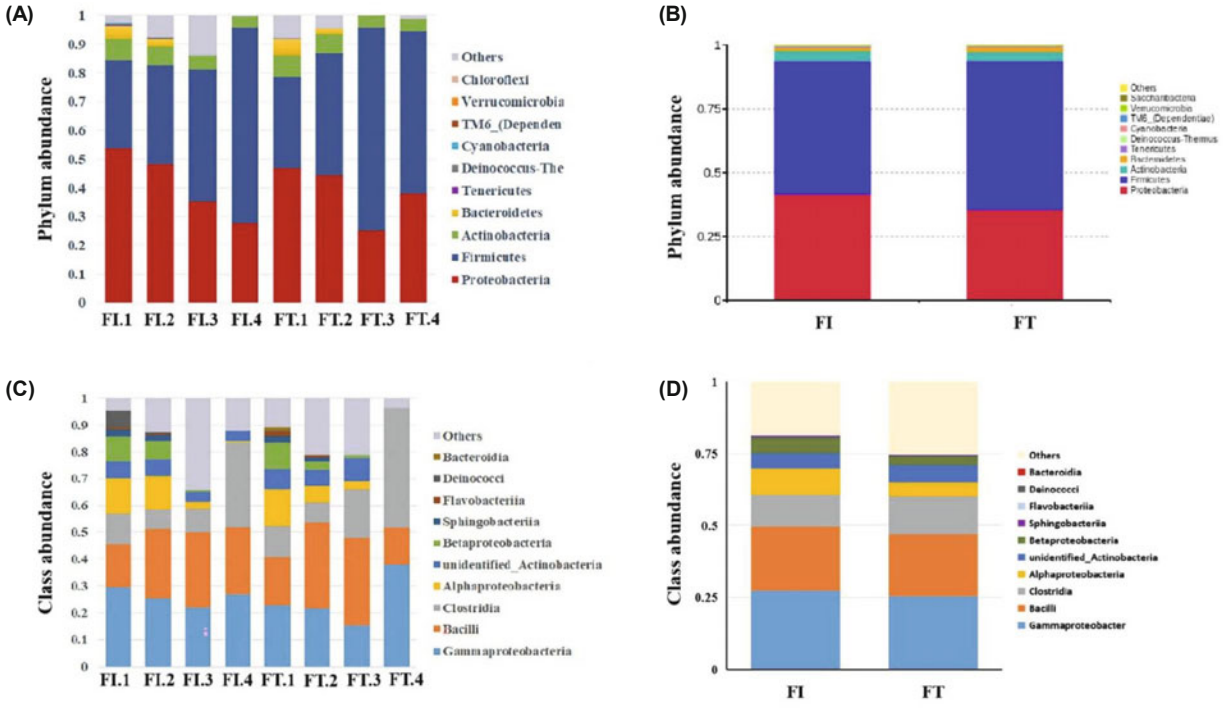

Fig. 3. The distribution of microbial groups in the different study groups. (A) The top 10 phyla at the phylum level are displayed in different colors for FI.1, FI.2, FI.3, FI.4, FT.1, FT.2, FT.3, and FT.4. The Kruskal-Wallis rank-sum test was used to analyze the differences between the FI and FT groups at different sampling times, and this was followed by the Benjamini–Hochberg method to adjust the *P*-value. (B) Barchart showing the relative abundances of the fecal microbiota at the phylum level in all samples for both the FI and FT groups. The Mann-Whitney test was used to examine differences between the groups. (C) The 10 most common gut microbiota at the class level varied over time for FI.1, FI.2, FI.3, FI.4, FT.1, FT.2, FT.3, and FT.4. The Kruskal-Wallis rank-sum test was used. (D) Barchart showing the relative abundances of bacteria at the class level in all the samples for both the FI and FT groups. The Mann-Whitney test was used. (E) Heatmap of the relative abundances of the 35 most abundant genera, and their corresponding phyla for FI.1, FI.2, FI.3, FI.4, FT.1, FT.2, FT.3, and FT.4. The warm color represents microbes with a high relative abundance in the sample group.

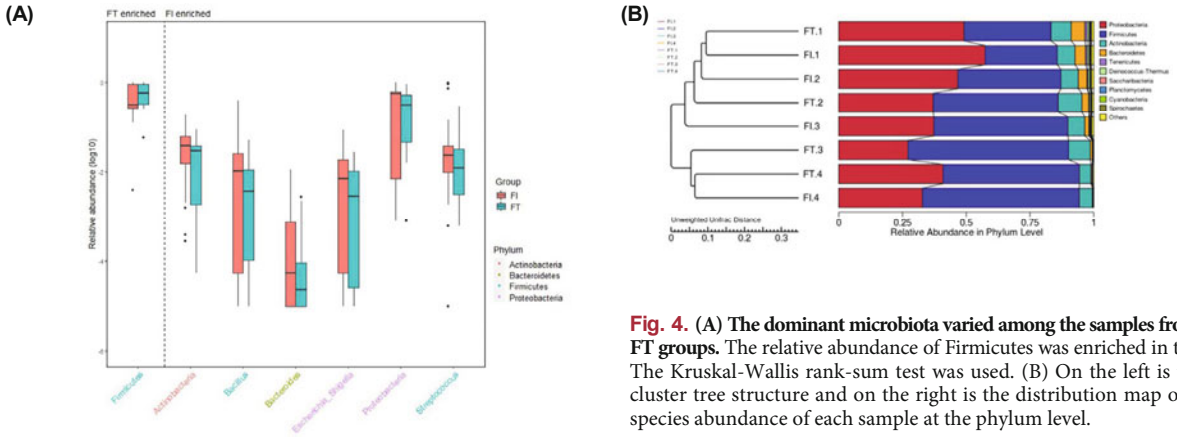

Fig. 4. (A) The dominant microbiota varied among the samples from the FI and FT groups. The relative abundance of Firmicutes was enriched in the FT group. The Kruskal-Wallis rank-sum test was used. (B) On the left is the UPGMA cluster tree structure and on the right is the distribution map of the relative species abundance of each sample at the phylum level.

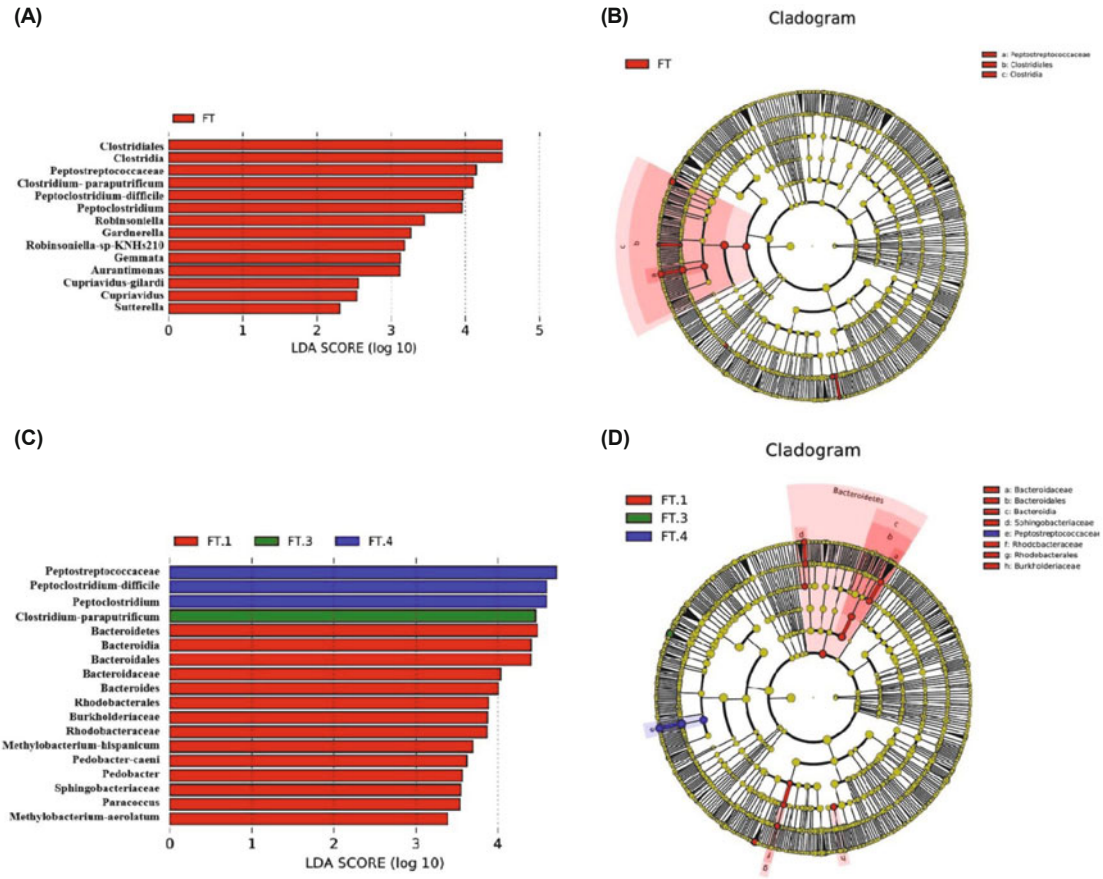

Fig. 5. Different structures of the gut microbiota according to LEfSE analysis. (A) LDA value distribution histogram between the FI and FT groups. (B) Cladogram of differentially abundant taxa, from the phylum level down to the species level, between the FI and FT groups. (C) LDA value distribution histogram among FI.1, FI.2, FI.3, FI.4, FT.1, FT.2, FT.3, and FT.4. The microbes with an LDA score higher than 2 are displayed. (D) Cladogram of differentially abundant taxa, from the phylum level down to the species level, among FI.1, FI.2, FI.3, FI.4, FT.1, FT.2, FT.3, and FT.4. LEfSe analysis with the Kruskal-Wallis sum-rank test was used to distinguish between FI.1, FI.2, FI.3, FI.4, FT.1, FT.2, FT.3, and FT.4. The microbes with an LDA score higher than 2 are displayed.

slower than that of the FT group, but the development of the gut microbiota in the FI group had essentially caught up with that of the FT group on day 28 (Fig. 4B).

Analysis of differential species at the genus level

The abundances of Peptostreptococcaceae, Clostridiales, *Clostridia*, and *Clostridium-paraputrificum* (LDA score higher than 4) in the FT group were significantly higher than those of the FI group (Fig. 5A). The cladogram representing the fecal microbial structure revealed that Peptostreptococcaceae, Clostridiales, and *Clostridia* exhibited the greatest differences between the FI and FT groups (Fig. 5B). As shown in Fig. 5C and D, the LEfSe results indicated that the meconium of the FT group had high levels of Bacteroidetes, bacteroidia, bacteroidales, and bacteroidaceae as compared to the FI group, and a higher level of clostridium paraputrificum was observed at three weeks post-birth. Peptostreptococcaceae predominated at one-month post-birth. This initial colonization of the gut microbiota appears critical to the development of FI.

Table 3. Correlation analysis of environmental factors and species richness

Environmental factors	r	*P*
Postnatal days	0.1535	0.001
Gestational age	0.0418	0.092
Birth weight	0.0053	0.437
Delivery mode	0.0343	0.097
Feeding way	-0.0059	0.578
Antibiotic use	-0.105	0.595
Probiotics use	0.0466	0.105

r is the correlation coefficient. If the value of r is larger, the correlation between the environmental factors and species abundance information is greater.
$P < 0.05$ indicates statistical significance.

Correlation analysis between the intestinal microenvironment and clinical indices

As shown in Table 3, the r value of postnatal days was the largest, indicating that postnatal days had the greatest impact on the structure of the gut microbiota. Figure 6A demonstrates that postnatal days was positively correlated with community and species distributions. Spearman's correlation analysis revealed that postnatal days and *Escherichia-Shigella* were negatively correlated (Fig. 6B).

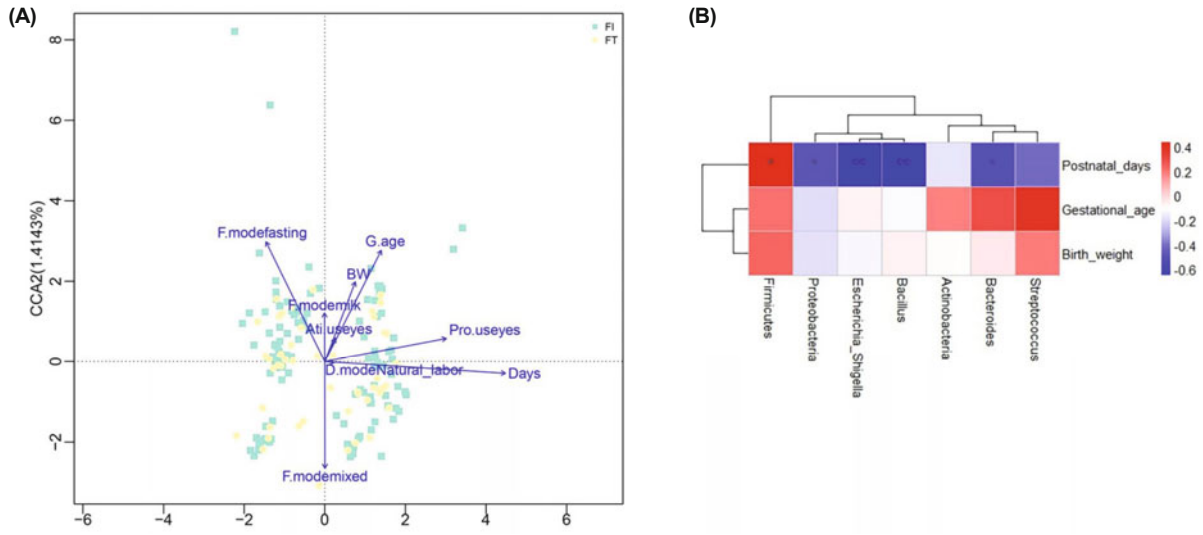

Fig. 6. (A) CCA analysis showing the correlations between the clinical indices and the gut microbiota in all samples from the FI and FT groups. (B) Spearman correlation analysis was used to examine the associations between the clinical indices (gestational age, birth weight, and postnatal days) and the gut microbiota of all stool samples. *$P < 0.05$, **$P < 0.01$.

Prediction of gut microbiota function

The results revealed that several KEGG pathways exhibited significant discriminatory function between the FI and FT groups (Fig. 7A). Sporulation was enriched in the FT group. C5-Branched-dibasic-acid-metabolism and the protein kinases pathway were highly enriched in the FI group. Amino-acid-metabolism, nucleotide-metabolism, sulfur-relay-system, transcription-related-proteins, nitrotoluene-degradation, and cholerae-pathogenic-cycle were significantly enriched in FT.4 compared to the other groups (Fig. 7B).

Discussion

The aims of this study were to provide new insight into the state of the gut microbiome before the development of FI, to determine whether an aberrant gut microbiota serves as an important pathogenic factor, and to identify microbiota-associated biomarkers for FI. This prospective study examined the association between the colonization of pathogenic intestinal microbes and the occurrence of FI in VLBW/ELBW infants. Additionally, potentially protective bacteria against FI were examined. The findings revealed that increased amounts of γ-proteobacteria and *Escherichia-Shigella* in meconium were positively associated with the occurrence of FI, while an increased relative abundance of *Bacteroides* was found to reduce the risk of FI. In recent years, an aberrant gut microbiota has been found to be associated with metabolic disorders such as diabetes, metabolic syndrome, non-alcoholic fatty liver disease, and steatohepatitis, and even psychiatric disorders such as depression (Biedermann and Rogler, 2015). The colonization of aberrant bacteria in the gut microbiota of preterm infants, compared to that of healthy term infants, could be due to intestinal inflammation or antibiotic treatment disrupting anaerobiosis (Litvak *et al.*, 2017), the delayed introduction of human milk, a high rate of cesarean delivery, and/or total parenteral nutrition (Navarro-Tapia *et al.*, 2020). Studies have shown that short-chain fatty acids (SCFAs) produced by *Bacteroides* can regulate the immune response and energy metabolism and provide energy for intestinal epithelial cells (Ichikawa *et al.*, 2002). The dominance of obligate anaerobic bacteria in the intestines ensures the production of related metabolites to maintain intestinal homeostasis. The cla-

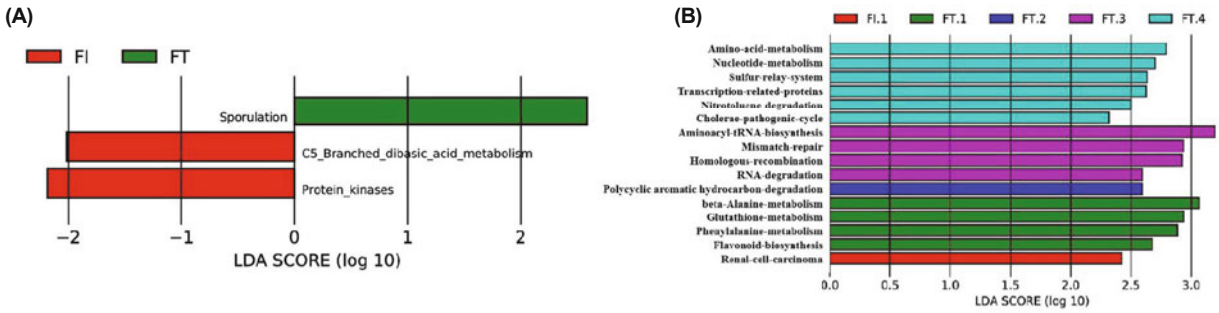

Fig. 7. Prediction of gut microbiota function by KEGG analysis.

dogram representing the fecal microbial structure revealed that Peptostreptococcaceae, Clostridiales, and *Clostridia* were more abundant in the FT group of preterm infants than the FI group. The Peptostreptococcaceae and Clostridiaceae families are known to contain spore-forming bacteria (Browne *et al.*, 2021). *Clostridia,* which are anaerobic and spore-forming Gram-positive bacteria, play a critical role in maintaining the hypoxic environment in the intestine, providing colonization resistance to enteric pathogens (Diallo *et al.*, 2021).

A total of 44 subjects (39 VLBW and 5 ELBW) were included in this study, and the incidence of FI among VLEW infants was 69% while the incidence of FI among ELBW infants was 80%. The prevalence of FI (n = 31 studies) in previous studies varies remarkably from 2 to 75% (Blaser *et al.*, 2014). This variation may be due to inconsistent diagnostic criteria. The risk of FI in preterm infants is high, and younger gestational age and lower birth weight are risk factors for FI.

This study revealed that the meconium of ELBW infants initially exhibited a high level of facultative anaerobes (Proteobacteria) with a major shift from aerobic bacteria and facultative anaerobes to obligate anaerobes (Firmicutes) around two weeks after birth; this coincides with rapid morphological and functional gut maturation. Aberrant gastrointestinal colonization in the F1 group appeared distinguishable only in meconium samples. In the past, it was believed that the intestinal tract of the fetus in utero was sterile, and the gut microbiota began to colonize after being exposed to the extrauterine environment. However, meconium is now known to contain microbial rDNA, suggesting that infants are colonized before birth (Moles *et al.*, 2013). Collado *et al.* (2016) found that the gastrointestinal microbiota detected in neonatal meconium was highly similar to that in the maternal placenta and amniotic fluid. The authors speculated that the fetal intestinal microbiome is derived from swallowed amniotic fluid in utero, and this may play a role in the inflammatory response which triggers preterm birth (Ardissone *et al.*, 2014). Increasing evidence indicates that microbial gut colonization already begins before delivery (Jiménez *et al.*, 2008; Ardissone *et al.*, 2014; Chong *et al.*, 2021). Interestingly, the microbiota of the neonatal gut may reflect the transfer of specific bacterial groups from the mother's microbiota to the newborn infant, which could be the true origin of the mammalian gut microbiota. The gut microbiota of preterm infants is more variable in its composition and less stable over time compared with that of an adult, and is significantly affected by environmental factors including the mode of delivery, feeding type, antibiotics, and the introduction of solid food (Unger *et al.*, 2015). In this study, there were no differences in environmental factors between the two groups, so the trajectory of microbiome development was similar between the FT and FI groups.

The alpha diversity of the microbial community was highest in the meconium samples from each group (FI.1 and FT.1). Differences in the gut microbiota between the FI and FT groups were only found in the meconium samples. Therefore, the initial colonization of the gut microbiota plays a critical role in the development of FI. Preterm infants have an underdeveloped immune system and aberrant colonization, possibly resulting in low diversity (Dahl *et al.*, 2018).

FI is often the early manifestation of NEC. The pathogenesis of NEC remains unclear, but an aberrant gut microbiota in premature infants might play a role in the underlying pathogenesis of NEC (Pammi *et al.*, 2017). Evidence suggests that aberrant maternal gut microbiota composition could act as a trigger for preterm birth (Baldassarre *et al.*, 2019). Warner *et al.* (2016) found that the gut microbiota was aberrant before the occurrence of NEC, and was mainly characterized by an increase in the proportion of γ-proteobacteria and a decrease in obligate anaerobes (such as *Clostridium*). Heida *et al.* (2016) found that the abundance of *Clostridium perfringens* in the meconium of the NEC group was higher than that in the control group.

Moles *et al.* (2013) reported that samples from infants that required mechanical ventilation showed a higher frequency of Serratia. However, in the current study, there was no difference in the abundance of Serratia between the FI and FT groups. In the study by Piñeiro-Ramos *et al.* (2020), which examined the impact of donor breast milk on the gut microbiota of VLBW infants, the average time of mechanical ventilation was about 15 days. In the current study, the FI group had an average time of mechanical ventilation of 12 days, while the FT group had an average time of mechanical ventilation of 8 days. Although the difference between the two groups was statistically significant, the total mechanical ventilation time was relatively short. The duration of vasoactive drugs was 0–4 days in the FI group and 0 days in the FT group, with no significant clinical difference. An effect of vasoactive drugs on intestinal flora has not been reported in the relevant literature. There was no significant difference in the types of antibiotics between the two groups. The average duration of antibiotic treatment was 9 days in the FI group and 6 days in the FT group. The main antibiotics used were penicillin, cefuroxime, and ceftazidime. Meropenem and vancomycin were used in several preterm infants with late septicemia. Most VLBW infants received broad-spectrum antibiotics during their early postnatal course, possibly resulting in inadequate phase I colonization with apparent inverse correlations between the number of days of antibiotics in the first month postnatally and both microbial diversity as well as the total bacterial load in the stool (Gewolb *et al.*, 1999). Early studies suggested that the colonization of beneficial bacteria such as *Lactobacillus* is specifically affected, and that antibiotic usage promotes a bloom of *Staphylococcus* (Westerbeek *et al.*, 2006). However, the current study did not find an increase in *Staphylococcus*. There was no significant difference in the richness and evenness of the two groups at one-month postnatal.

These findings suggest that aberrant bacterial colonization may play a role in the pathogenesis of FI. Therefore, the use of probiotics may serve to restore or provide necessary symbiotic strains, thus strengthening the intestinal wall barrier, regulating the immune response, and competitively inhibiting pathogens (Moles *et al.*, 2015). A systematic review by Athalye-Jape *et al.* (2014) demonstrated that the administration of probiotic strains had a beneficial effect on enteral nutrition-related outcomes, reduced the incidence of FI, and shortened the hospital stay of children. Havranek *et al.* (2013) reported that probiotic treatment can significantly improve postprandial superior mesenteric arterial blood flow. This

is another possible mechanism by which probiotics may improve feeding problems, as an increase in postprandial mesenteric blood flow is important for food digestion. However, due to the significant heterogeneity in the clinical trials in terms of the selected probiotic species and dosage regimens, there is still insufficient data on the efficacy of specific probiotic strains. Further studies are needed to determine the best probiotic bacteria strains and useful dosage regimens.

The results of the present study revealed a strong positive association between postnatal days and early gut microbiota diversity. A recent study found a strong correlation between gestational age and the gut microbiota (Morais *et al.*, 2020). However, the results of the current study indicated that gestational age was not an important factor in bacterial diversity. Finally, we performed KEGG analysis to determine the functional differences between the FI and FT groups. Sporulation was enriched in the FT group. Comparatively, the FI group showed two significantly enriched pathways, C5-branched dibasic acid metabolism and protein kinases. Browne *et al.* (2021) found that loss of sporulation may be associated with different evolutionary trajectories centered on host adaptation, such as genome reduction and specialized metabolic capabilities. Moreover, Zenda *et al.* (2018) reported that the C5-branched dibasic acid metabolism pathway plays a critical role in cell fate transition during protoplast reprogramming into stem cells.

Affected by the pandemic of COVID-19, time for collecting specimens in this study was relatively short. Our study was limited by relatively small sample size and was conducted in a single center. Therefore, multi-center, larger sample size studies are needed to more fully validate an potential role of the gut microbiome in FI, and demonstrate the microbial markers of FI for early prediction.

Conclusion

In summary, this study attempted to identify protective microbial markers against FI and verify the association between an intestinal microecological imbalance and the occurrence of FI. The findings indicated that increased relative abundances of γ-proteobacteria and *Escherichia-Shigella,* and decreased abundance of *Bacteroides* in meconium samples are associated with an increased risk for FI, while Peptostreptococcaceae, Clostridiales, and *Clostridia* reduce the risk of FI in VLBW/ELBW infants. This is consistent with the mounting evidence supporting the relationship between an aberrant gut microbiota and FI. These findings support the critical role of initial colonization of the gut microbiota in the development of FI.

Acknowledgements

This study was sponsored by grants from the Affiliated Hospital of Guangdong Medical University Clinical Research Program (LCYJ2020B004). Sequencing of the 16S rRNA gene was performed by the Advanced Biomedical Laboratory.

This study was supported by Shenzhen Guangming District Soft Science Project Fund (grant.no.2021R01055).

Conflict of Interest

The authors have no conflict of interest to report.

Ethical Statement

This study was approved by the Clinical Trial Ethics Committee of the Affiliated Hospital of Guangdong Medical University (approval number: PJ2020-044) and was performed strictly in accordance with the principle of informed consent. The Chinese clinical trial registration number was ChiCTR-2000038024. The guardians of each infant were informed of the purpose and the entire study protocol and provided written informed consent before study enrollment.

References

Arboleya, S., Binetti, A., Salazar, N., Fernandez, N., Solis, G., Hernandez-Barranco, A., Margolles, A., de Los Reyes-Gavilan, C.G., and Gueimonde, M. 2012. Establishment and development of intestinal microbiota in preterm neonates. *FEMS Microbiol. Ecol.* **79**, 763–772.

Arboleya, S., Martinez-Camblor, P., Solís, G., Suárez, M., Fernández, N., de Los Reyes-Gavilan, C.G., and Gueimonde, M. 2017. Intestinal microbiota and weight-gain in preterm neonates. *Front. Microbiol.* **8**, 183.

Ardissone, A.N., de la Cruz, D.M., Davis-Richardson, A.G., Rechcigl, K.T., Li, N., Drew, J.C., Murgas-Torrazza, R., Sharma, R., Hudak, M.L., Triplett, E.W., *et al.* 2014. Meconium microbiome analysis identifies bacteria correlated with premature birth. *PLoS ONE* **9**, e90784.

Athalye-Jape, G., Deshpande, G., Rao, S., and Patole, S. 2014. Benefits of probiotics on enteral nutrition in preterm neonates: a systematic review. *Am. J. Clin. Nutr.* **100**, 1508–1519.

Baldassarre, M.E., Di Mauro, A., Capozza, M., Rizzo, V., Schettini, F., Panza, R., and Laforgia, N. 2019. Dysbiosis and prematurity: is there a role for probiotics? *Nutrients* **11**, 1273.

Biedermann, L. and Rogler, G. 2015. The intestinal microbiota: its role in health and disease. *Eur. J. Pediatr.* **174**, 151–167.

Blaser, A.R., Starkopf, J., Kirsimagi, Ü., and Deane, A.M. 2014. Definition, prevalence, and outcome of feeding intolerance in intensive care: a systematic review and meta-analysis. *Acta Anaesthesiol. Scand.* **58**, 914–922.

Browne, H.P., Almeida, A., Kumar, N., Vervier, K., Adoum, A.T., Viciani, E., Dawson, N.J.R., Forster, S.C., Cormie, C., Goulding, D., *et al.* 2021. Host adaptation in gut Firmicutes is associated with sporulation loss and altered transmission cycle. *Genome Biol.* **22**, 204.

Campeotto, F., Baldassarre, M., Butel, M.J., Viallon, V., Nganzali, F., Soulaines, P., Kalach, N., Lapillonne, A., Laforgia, N., Moriette, G., *et al.* 2009. Fecal calprotectin: cutoff values for identifying intestinal distress in preterm infants. *J. Pediatr. Gastroenterol. Nutr.* **48**, 507–510.

Chen, S., Zhou, Y., Chen, Y., and Gu, J. 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **34**, i884–i890.

Chong, C.Y.L., Vatanen, T., Alexander, T., Bloomfield, F.H., and O'Sullivan, J.M. 2021. Factors associated with the microbiome in moderate-late preterm babies: a cohort study from the DIAMOND randomized controlled trial. *Front. Cell. Infect. Microbiol.* **11**, 595323.

Collado, M.C., Rautava, S., Aakko, J., Isolauri, E., and Salminen, S. 2016. Human gut colonisation may be initiated *in utero* by distinct microbial communities in the placenta and amniotic fluid.

Sci. Rep. **6**, 23129.

Dahl, C., Stigum, H., Valeur, J., Iszatt, N., Lenters, V., Peddada, S., Bjørnholt, J.V., Midtvedt, T., Mandal, S., and Eggesbø, M. 2018. Preterm infants have distinct microbiomes not explained by mode of delivery, breastfeeding duration or antibiotic exposure. *Int. J. Epidemiol.* **47**, 1658–1669.

Diallo, M., Kengen, S.W.M., and López-Contreras, A.M. 2021. Sporulation in solventogenic and acetogenic clostridia. *Appl. Microbiol. Biotechnol.* **105**, 3533–3557.

Edgar, R.C. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* **10**, 996–998.

El Manouni El Hassani, S., Niemarkt, H.J., Berkhout, D.J.C., Peeters, C.F.W., Hulzebos, C.V., van Kaam, A.H., Kramer, B.W., van Lingen, R.A., Jenken, F., de Boode, W.P., *et al.* 2021. Profound pathogen-specific alterations in intestinal microbiota composition precede late onset sepsis in preterm infants: a longitudinal multicenter case-control study. *Clin. Infect. Dis.* **73**, e224–e232.

Forsgren, M., Isolauri, E., Salminen, S., and Rautava, S. 2017. Late preterm birth has direct and indirect effects on infant gut microbiota development during the first six months of life. *Acta Paediatr.* **106**, 1103–1109.

Gewolb, I.H., Schwalbe, R.S., Taciak, V.L., Harrison, T.S., and Panigrahi, P. 1999. Stool microflora in extremely low birthweight infants. *Arch. Dis. Child. Fetal Neonatal Ed.* **80**, F167–F173.

Halpern, M.D. and Denning, P.W. 2015. The role of intestinal epithelial barrier function in the development of NEC. *Tissue Barriers* **3**, e1000707.

Havranek, T., Al-Hosni, M., and Armbrecht, E. 2013. Probiotics supplementation increases intestinal blood flow velocity in extremely low birth weight preterm infants. *J. Perinatol.* **33**, 40–44.

Heida, F.H., van Zoonen, A.G.J.F., Hulscher, J.B.F., Te Kiefte, B.J.C., Wessels, R., Kooi, E.M.W., Bos, A.F., Harmsen, H.J.M., and de Goffau, M.C. 2016. A necrotizing enterocolitis-associated gut microbiota is present in the meconium: results of a prospective study. *Clin. Infect. Dis.* **62**, 863–870.

Ichikawa, H., Shineha, R., Satomi, S., and Sakata, T. 2002. Gastric or rectal instillation of short-chain fatty acids stimulates epithelial cell proliferation of small and large intestine in rats. *Dig. Dis. Sci.* **47**, 1141–1146.

Jiménez, E., Marín, M.L., Martín, R., Odriozola, J.M., Olivares, M., Xaus, J., Fernández, L., and Rodríguez, J.M. 2008. Is meconium from healthy newborns actually sterile? *Res. Microbiol.* **159**, 187–193.

Litvak, Y., Byndloss, M.X., Tsolis, R.M., and Bäumler, A.J. 2017. Dysbiotic Proteobacteria expansion: a microbial signature of epithelial dysfunction. *Curr. Opin. Microbiol.* **39**, 1–6.

Magoč, T. and Salzberg, S.L. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **27**, 2957–2963.

Moles, L., Escribano, E., de Andrés, J., Montes, M.T., Rodríguez, J.M., Jiménez, E., Sáenz de Pipaón, M., and Espinosa-Martos, I. 2015. Administration of *Bifidobacterium breve* PS12929 and *Lactobacillus salivarius* PS12934, two strains isolated from human milk, to very low and extremely low birth weight preterm infants: a pilot study. *J. Immunol. Res.* **2015**, 538171.

Moles, L., Gómez, M., Heilig, H., Bustos, G., Fuentes, S., de Vos, W., Fernández, L., Rodríguez, J.M., and Jiménez, E. 2013. Bacterial diversity in meconium of preterm neonates and evolution of their fecal microbiota during the first month of life. *PLoS ONE* **8**, e66986.

Morais, J., Marques, C., Teixeira, D., Durão, C., Faria, A., Brito, S., Cardoso, M., Macedo, I., Pereira, E., Tome, T., *et al.* 2020. Extremely preterm neonates have more *Lactobacillus* in meconium than very preterm neonates - the *in utero* microbial colonization hypothesis. *Gut Microbes* **12**, 1785804.

Morais, J., Marques, C., Teixeira, D., Durão, C., Faria, A., Brito, S., Cardoso, M., Macedo, I., Tomé, T., and Calhau, C. 2019. FEEDMI: a study protocol to determine the influence of infant-feeding on very-preterm-infant's gut microbiota. *Neonatology* **116**, 179–184.

Navarro-Tapia, E., Sebastiani, G., Sailer, S., Toledano, L.A., Serra-Delgado, M., García-Algar, O., and Andreu-Fernández, V. 2020. Probiotic supplementation during the perinatal and infant period: effects on gut dysbiosis and disease. *Nutrients* **12**, 2243.

Pammi, M., Cope, J., Tarr, P.I., Warner, B.B., Morrow, A.L., Mai, V., Gregory, K.E., Kroll, J.S., McMurtry, V., Ferris, M.J., *et al.* 2017. Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis. *Microbiome* **5**, 31.

Piñeiro-Ramos, J.D., Parra-Llorca, A., Ten-Doménech, I., Gormaz, M., Ramón-Beltrán, A., Cernada, M., Quintás, G., Collado, M.C., Kuligowski, J., and Vento, M. 2020. Effect of donor human milk on host-gut microbiota and metabolic interactions in preterm infants. *Clin. Nutr.* **40**, 1296–1309.

Qiao, L.X., Zhu, W.Y., Zhang, H.Y., and Wang, H. 2017. Effect of early administration of probiotics on gut microflora and feeding in pre-term infants: a randomized controlled trial. *J. Matern. Fetal Neonatal. Med.* **30**, 13–16.

Rao, C., Coyte, K.Z., Bainter, W., Geha, R.S., Martin, C.R., and Rakoff-Nahoum, S. 2021. Multi-kingdom ecological drivers of microbiota assembly in preterm infants. *Nature* **591**, 633–638.

The Human Microbiome Project Consortium. 2012. Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207–214.

Unger, S., Stintzi, A., Shah, P., Mack, D., and O'Connor, D.L. 2015. Gut microbiota of the very-low-birth-weight infant. *Pediatr. Res.* **77**, 205–213.

Warner, B.B., Deych, E., Zhou, Y., Hall-Moore, C., Weinstock, G.M., Sodergren, E., Shaikh, N., Hoffmann, J.A., Linneman, L.A., Hamvas, A., *et al.* 2016. Gut bacteria dysbiosis and necrotising enterocolitis in very low birthweight infants: a prospective case-control study. *Lancet* **387**, 1928–1936.

Westerbeek, E.A., van den Berg, A., Lafeber, H.N., Knol, J., Fetter, W.P.F., and van Elburg, R.M. 2006. The intestinal bacterial colonisation in preterm infants: a review of the literature. *Clin. Nutr.* **25**, 361–368.

Young, G.R., Smith, D.L., Embleton, N.D., Berrington, J.E., Schwalbe, E.C., Cummings, S.P., van der Gast, C.J., and Lanyon, C. 2017. Reducing viability bias in analysis of gut microbiota in preterm infants at risk of NEC and sepsis. *Front. Cell. Infect. Microbiol.* **7**, 237.

Yu, Y., Lu, L., Sun, J., Petrof, E.O., and Claud, E.C. 2016. Preterm infant gut microbiota affects intestinal epithelial development in a humanized microbiome gnotobiotic mouse model. *Am. J. Physiol. Gastrointest. Liver Physiol.* **311**, G521–532.

Yuan, Z., Yan, J., Wen, H., Deng, X., Li, X., and Su, S. 2019. Feeding intolerance alters the gut microbiota of preterm infants. *PLoS ONE* **14**, e0210609.

Zenda, T., Liu, S., Wang, X., Jin, H., Liu, G., and Duan, H. 2018. Comparative proteomic and physiological analyses of two divergent maize inbred lines provide more insights into drought-stress tolerance mechanisms. *Int. J. Mol. Sci.* **19**, 3225.