INTESTINAL MICROBIOTA IN THE PATHOGENESIS AND MANAGEMENT OF NECROTIZING ENTEROCOLITIS IN PRETERM INFANTS

EDITED BY: Guo Chunbao, Yingjie Lu and Yuan Shi
PUBLISHED IN: Frontiers in Pediatrics

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88974-665-1
DOI 10.3389/978-2-88974-665-1

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

INTESTINAL MICROBIOTA IN THE PATHOGENESIS AND MANAGEMENT OF NECROTIZING ENTEROCOLITIS IN PRETERM INFANTS

Topic Editors:
Guo Chunbao, Children's Hospital of Chongqing Medical University, China
Yingjie Lu, Harvard University, United States
Yuan Shi, Children's Hospital of Chongqing Medical University, China

Citation: Chunbao, G., Lu, Y., Shi, Y., eds. (2022). Intestinal Microbiota in the Pathogenesis and Management of Necrotizing Enterocolitis in Preterm Infants. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-665-1

Table of Contents

frontiers in Pediatrics

EDITORIAL
published: 17 February 2022
doi: 10.3389/fped.2022.837925

Editorial: Intestinal Microbiota in the Pathogenesis and Management of Necrotizing Enterocolitis in Preterm Infants

Chunbao Guo[1,2,3]* *and Yuan Shi*[2,3,4]*

[1] Department of Pediatric General Surgery, Children's Hospital of Chongqing Medical University, Chongqing, China, [2] Ministry of Education Key Laboratory of Child Development and Disorders, National Clinical Research Center for Child Health and Disorders, Children's Hospital of Chongqing Medical University, Chongqing, China, [3] China International Science and Technology Cooperation Base of Child Development and Critical Disorders, Chongqing Key Laboratory of Pediatrics, Children's Hospital of Chongqing Medical University, Chongqing, China, [4] Department of Neonatology, Chongqing Medical University, Chongqing, China

Keywords: necrotizing enterocolitis, intestinal microbiome, bioinformatic processing analysis, antibiotic management, probiotic prophylaxis, intrauterine hypoxia, gut microbiome dysbiosis

Editorial on the Research Topic

Intestinal Microbiota in the Pathogenesis and Management of Necrotizing Enterocolitis in Preterm Infants

Necrotizing enterocolitis (NEC) is the most fatal disease involving preterm infants, with substantial mortality (20–35%) as well as long-term morbidity in terms of gastrointestinal and neurodevelopmental symptoms. The incidence of NEC might be up to 10% for infants weighing <1,500 g (1). Approximately 10–100 trillion symbiotic bacteria constitute the human microbiome, most of which reside within the gastrointestinal tract. The neonatal period is essential for human microbiome development, which might contribute to many disease processes, including NEC (2).

Multiple studies have demonstrated a decrease in bacterial diversity and a Gammaproteobacteria bloom in the intestinal microbiota several weeks prior to the onset of NEC in preterm infants compared with term infants. Accordingly, the pathogenesis of NEC has been associated with microbial dysbiosis, which is characterized by an altered microbial community and microbial invasion of the gut mucosa with excessive inflammation, particularly in preterm infants (3). Environmental pressures should contribute to microbial dysbiosis in preterm infants, such as instrumentation, antibiotic stress, and neonatal intensive care unit (NICU) environments (4).

For necrotizing enterocolitis, the main therapeutic focus should be restoring the microbial balance to construct a healthy microbial community with optimization of beneficial organisms and pathogens (5). However, inconsistent results are usually reported, and they under power these concepts. For neonatal intestinal microbiome evaluation, technological advances have provided greater resolution and availability (6). With the current topic, we intend to provide more sound evidence for all areas of research involving the intestinal microbiome in the pathogenesis, diagnosis and management of NEC. Here, we published a special collection of 10 articles contributing to this concept.

Valuable insights into the intestinal microbiome for many disease processes, including NEC, are increasing (7). A clearer understanding of the healthy neonatal microbiome composition and the altered microbial community in preterm infants should be very important for its pathogenesis (8). First and foremost, a consistent pattern over time in microbiome composition with many different

Edited and reviewed by:
Andrew S. Day,
University of Otago, New Zealand

***Correspondence:**
Yuan Shi
shiyuan@hospital.cqmu.edu.cn
Chunbao Guo
guochunbao@hospital.cqmu.edu.cn;
guochunbao@foxmail.com

Specialty section:
This article was submitted to Pediatric Gastroenterology, Hepatology and Nutrition, a section of the journal Frontiers in Pediatrics

Received: *17 December 2021*
Accepted: *03 January 2022*
Published: *17 February 2022*

Citation:
Guo C and Shi Y (2022) Editorial: Intestinal Microbiota in the Pathogenesis and Management of Necrotizing Enterocolitis in Preterm Infants. Front. Pediatr. 10:837925. doi: 10.3389/fped.2022.837925

species has been presented in premature NEC infants. Here, in the current collection, several studies assessed the intestinal microbiome under various conditions utilizing 16S ribosomal ribonucleic acid (rRNA) sequence techniques among preterm infants.

Fu et al. emphasized the importance of the association between intestinal microbiota and NEC following bioinformatic processing analysis. They note that the microbial species varies following NEC occurrence and that multiple communities are critical for NEC development. NEC infants presented with a higher abundance of waterborne bacteria, which live in the environment. Li et al. found a significant difference in diversity of the intestinal flora after feeding intolerance recovery from 11 pairs of premature twins/triplets following the 16S rRNA gene sequencing. We find their approach applaudable and absolutely agree with the authors' point. Therefore, attention should be given to contamination during disease care and management within the NICU. They further proposed the prospect that in-depth investigation of microbial colonization could detect the mechanism of NEC.

The intestinal microbiome should be influenced by various factors in premature infants who experience NEC due to their disease severity. Recently, clinical research has confirmed that many prenatal and postnatal factors might adversely affect the gut microbial balance, including formula feeding, antibiotic management and mode of delivery (9). However, no randomized clinical trials (RCTs) are currently available to investigate the influence of various environmental and care conditions on the intestinal microbiome in premature infants. In this collection, three articles involve the association of gut microbial balance with family integrated care, intrauterine hypoxia and antibiotic regimens. Yang et al. present their prospective pilot study to evaluate the effect of family integrated care on intestinal flora diversity and found that short-term family integrated care did not significantly influence the establishment of gut microbial balance in premature NEC infants due to a small sample size. They recommend an adequately powered clinical trial to make a conclusion.

Intrauterine hypoxia is the most important cause of hypoxic-ischemic encephalopathy in infants, fetal intrauterine distress, growth restriction, and even fetal or neonatal death (10). Furthermore, intrauterine hypoxia could influence microbiota diversity formation, especially anaerobic bacterial colonization within the gastrointestinal tract. Sun et al. performed an experimental NEC study involving hypoxic conditions and indicated that intrauterine hypoxia could greatly promote the occurrence of NEC through disruption of the gut microbiota balance. Therefore, for infants born with intrauterine hypoxia, more caution should be taken for the monitoring of abdominal conditions, and careful feeding management should be required.

Studies have suggested that long-term antibiotic administration in infants could have a great impact on the composition of the normal intestinal microbial community, which could further interfere with metabolic and immune balance. The quick cessation of antibiotic management could restore microbiota diversity (11). In this study, Chang et al. further demonstrated that in VLBW preterm infants, antibiotic management had an essential influence on the structure of gut microbiota. Overabundance of Enterococcus could be present following usage of ampicillin and cefotaxime for prolonged periods. Unfortunately, despite previous exposure to antibiotics, various bacterial genera could still colonize the gastrointestinal tract, which might be harmful for preterm infants.

Han et al. found that infectious disease conditions were also related to the gut microbiota of neonates, such as pneumonia or meningitis. The initial gut microbiome is associated with its state following 1 week of antibiotic management. Under these conditions, antibiotic administration within 7 days had little effect on the composition of gut microbiota and community richness.

To construct the patient's own bacterial ecosystem with beneficial commensal bacteria, the use of prebiotic and/or probiotic administration has blossomed. Live microbiota (probiotics) could prevent gut microbiome dysbiosis through inhibition of pathogenic bacteria and promotion of beneficial bacterial colonization, and its use has been proven safe and effective in NEC management. To date, probiotic prophylaxis administration to preterm infants has been widely adopted in the NICU (12). However, probiotic utilization in preterm infants in China remains relatively limited. Furthermore, the administration protocols, combination strains and synergistic interactions might all lead to challenges in optimal probiotic selection for clinical usage. Meanwhile, food substrates within human milk, such as lactalbumin, lactoferrin inulin, or oligosaccharides, should be supplied to facilitate commensal flora reproduction and its activity.

The present research conducted by Juber et al. demonstrated that routine multispecies probiotic supplementation containing Bifidobacterium and Lactobacillus had a limited effect on the incidences of NEC and its mortality among infants with very low birth weight, which contrasts with multiple other studies, even with the exact same probiotic strain. Priyadarshi et al. found similar clinical outcomes over a 4-year period in preterm infants between the single- or two-strain probiotic prophylaxis strategy, including NEC incidence, feeding outcomes, and comorbidities. The different conclusions might have resulted from the limited sample size and relatively low baseline NEC incidences, which could not be defined with the threshold of probiotic efficacy. Further investigations with large-scale randomized controlled multicenter trials are needed to definitively determine the strain-specific effects in reducing NEC and mortality in preterm infants. A microbiome study of stool specimens should also be performed to determine the beneficial effects of intestinal colonization.

Understanding the exact mechanism of pathogenic bacterial strains in relation to NEC is critically important to produce and sustain a stable intestinal microbiota ecosystem (8). Cassir et al. report here a case of *Clostridium neonatale* bacteremia in a preterm neonate with NEC, suggesting its potential role in NEC pathogenesis. They emphasize that the sporulating form of *C. neonatale* could confer resistance to usual hospital environmental cleaning measures. The causal relationship between the isolation of *C. neonatale* and the occurrence of NEC should be further investigated, and it should be promising in diagnostic and therapeutic management.

Finally, the early diagnosis of NEC is challenging due to the non-specific clinical characteristics of more immature individuals. A more reliable earlier NEC diagnosis is critical for earlier intervention to arrest the disease (1). Hoffsten et al. investigated 189 early postnatal comprehensive biomarkers utilizing a proximity extension assay, including inflammatory, vascular proteomic, and oncological parameters. Altered gut microbiota is a risk factor for the development of NEC, and it is especially pronounced in premature NEC infants. Here, we infer that comprehensive analysis of the alterations in the intestinal microbial population might provide valuable diagnostic information to detect this disease early. However, due to the sophistication of bioinformatics analysis and different platforms in terms of data acquisition, variable results have been presented for intestinal microbiota analysis from study to study (3). Which select bacteria within the fecal sample are more appropriate for NEC detection should be further investigated.

Although the present evidence to guide microbiome management strategies remains controversial, the current collection may provide additional further understanding for optimization of the components of the microbiome in this field in preterm infants.

AUTHOR CONTRIBUTIONS

CG and YS designed and developed this Research Topic, contributed to the accompanying editorial, contributed to the current editorial, and approved the current submission in the present form. Both authors conferred great efforts to evaluate the published manuscripts.

ACKNOWLEDGMENTS

The authors thank all the doctors contribute their articles to the topic.

REFERENCES

1. Lin PW, Stoll BJ. Necrotising enterocolitis. *Lancet.* (2006) 368:1271–83. doi: 10.1016/S0140-6736(06)69525-1
2. Pammi M, Cope J, Tarr PI, Warner BB, Morrow AL, Mai V, et al. Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis. *Microbiome.* (2017) 5:31. doi: 10.1186/s40168-017-0248-8
3. Baranowski JR, Claud EC. Necrotizing enterocolitis and the preterm infant microbiome. *Adv Exp Med Biol.* (2019) 1125:25–36. doi: 10.1007/5584_2018_313
4. Cassir N, Simeoni U, La Scola B. Gut microbiota and the pathogenesis of necrotizing enterocolitis in preterm neonates. *Future Microbiol.* (2016) 11:273–92. doi: 10.2217/fmb.15.136
5. Neu J, Pammi M. Pathogenesis of NEC: Impact of an altered intestinal microbiome. *Semin Perinatol.* (2017) 41:29–35. doi: 10.1053/j.semperi.2016.09.015
6. Zhang X, Li L, Butcher J, Stintzi A, Figeys D. Advancing functional and translational microbiome research using meta-omics approaches. *Microbiome.* (2019) 7:154. doi: 10.1186/s40168-019-0767-6
7. Shreiner AB, Kao JY, Young VB. The gut microbiome in health and in disease. *Curr Opin Gastroenterol.* (2015) 31:69–75. doi: 10.1097/MOG.0000000000000139
8. Grady NG, Petrof EO, Claud EC. Microbial therapeutic interventions. *Semin Fetal Neonatal Med.* (2016) 21:418–23. doi: 10.1016/j.siny.2016.04.005
9. Rose AT, Patel RM. A critical analysis of risk factors for necrotizing enterocolitis. *Semin Fetal Neonatal Med.* (2018) 23:374–9. doi: 10.1016/j.siny.2018.07.005
10. Perger L, Mukhopadhyay D, Komidar L, Wiggins-Dohlvik K, Uddin MN, Beeram M. Maternal pre-eclampsia as a risk factor for necrotizing enterocolitis. *J Matern Fetal Neonatal Med.* (2016) 29:2098–103. doi: 10.3109/14767058.2015.1076386
11. Silverman MA, Konnikova L, Gerber JS. Impact of antibiotics on necrotizing enterocolitis and antibiotic-associated diarrhea. *Gastroenterol Clin North Am.* (2017) 46:61–76. doi: 10.1016/j.gtc.2016.09.010
12. Patel RM, Underwood MA. Probiotics and necrotizing enterocolitis. *Semin Pediatr Surg.* (2018) 27:39–46. doi: 10.1053/j.sempedsurg.2017.11.008

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

frontiers in Pediatrics

ORIGINAL RESEARCH
published: 10 March 2021
doi: 10.3389/fped.2021.648979

The Diversity of the Intestinal Flora Disturbed After Feeding Intolerance Recovery in Preterm Twins

Ying Li[1,2,3†], Chunhong Jia[2,3†], Xiaojun Lin[2†], Lili Lin[2], Lizhen Li[2], Xi Fan[2], Xiaoxia Huang[2], Zhanyuan Xu[2], Huixin Wang[2], Fan Wu[2,3] and Guosheng Liu[1*]*

[1] Department of Neonatology, The First Affiliated Hospital of Jinan University, Guangzhou, China, [2] Department of Pediatrics, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China, [3] Key Laboratory for Major Obstetric Disease of Guangdong Province, Guangzhou, China

Background: Feeding intolerance (FI) is a common condition in premature infants that results in growth retardation and even necrotizing enterocolitis. The gut microbiome is linked to FI occurrence; however, the outcome after FI recovery is unclear.

Methods: Fecal samples were collected from 11 pairs of premature twins/triplets for 16S rRNA gene sequencing. Initial fecal samples were collected shortly after admission, and then every other week until 7 weeks or discharge.

Results: After FI recovery, there was no significant difference in the β-diversity of the intestinal flora between the FI group and the feeding tolerance (FT) group. By contrast, there was a significant difference in the β-diversity. Proteobacteria was the predominant phylum in the microbiome of the FI group, whereas Firmicutes was the predominant phylum in the microbiome of the FT group. The predominant bacteria with LDA >4 between the two groups at 13–15 days after birth, 19–28 days after birth, and at discharge were different, with the proportions of Bacillus, Clostridium butyricum, and Clostridium being highest in the FT group and Firmicutes, unidentified_Clostridiales, and Proteobacteria being highest in the FI group. Similarly, there were significant differences in the relative abundances of KEGG pathways, such as fatty acid metabolism, DNA repair and recombination proteins, energy metabolism, and amino acid metabolism, between the two groups ($P < 0.01$).

Conclusions: There was a significant difference in diversity of the intestinal flora after feeding intolerance recovery. Feeding intolerance may disturb the succession of the intestinal bacterial community.

Keywords: premature infants, twins, triplets, feeding intolerance, microbiome

OPEN ACCESS

Edited by:
Yuan Shi,
Children's Hospital of Chongqing Medical University, China

Reviewed by:
Lu-Quan Li,
Chongqing Medical University, China
Xiaoqing Chen,
Nanjing Hospital Affiliated to Nanjing Medical University, China

***Correspondence:**
Guosheng Liu
tlgs@jnu.edu.cn
Fan Wu
gdwufan@126.com

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: *03 January 2021*
Accepted: *16 February 2021*
Published: *10 March 2021*

Citation:
Li Y, Jia C, Lin X, Lin L, Li L, Fan X, Huang X, Xu Z, Wang H, Wu F and Liu G (2021) The Diversity of the Intestinal Flora Disturbed After Feeding Intolerance Recovery in Preterm Twins. Front. Pediatr. 9:648979. doi: 10.3389/fped.2021.648979

INTRODUCTION

Feeding intolerance (FI), a common condition in premature infants, is caused by an underdeveloped gastrointestinal tract or a disruption in intestinal function. It leads to the slow progression to enteral feeding and prolongs the length of hospitalization. More importantly, FI can lead to neonatal necrotizing enterocolitis (NEC), which threats the life of preterm infants (1).

The human gut is the natural habitat for a large and dynamic bacterial community, and the importance of resident bacteria on a host's physiology and pathology is gradually being elucidated. Recent studies have reported that the status of the intestinal microbiome is closely associated with several diseases, including NEC, inflammatory bowel disease, obesity, cardiovascular disease, and cancer (2–4).

To date, few studies have examined the composition of the gut microbial community in premature infants with FI. It has been reported that the intestinal microbiome changes drastically in cases of FI. Specifically, the proportion of Proteobacteria increases, while that of Firmicutes decreases (5, 6). However, no causative agent has been identified for FI. Furthermore, the composition of the intestinal microbiome after FI recovery remains unknown. The main reason for this lies in the complexity of the gut microbiome, especially in preterm infants. At the perinatal stage, the composition of the gut microbial community can be altered by various factors such as the delivery mode, antibiotic exposure, gestational age at birth, route of feeding, and gender (7, 8). In addition, the microbial composition varies greatly in preterm infants (9, 10). Therefore, the search for the causative agent is complicated by differences in the gut microbiome across different infants (11).

A previous study has demonstrated that the development of the gut microbiome is similar between twins, even within the complex environment of neonatal intensive care (12). Studying twins may provide unique insights into the differences in the gut microbial community. In this study, premature twins/triplets were enrolled to explore the diversity of the gut microbiome in the event of FI. To eliminate birth canal contamination, we only included cesarean section cases. Our results indicate that the effects of FI on the intestinal flora in premature infants continues long after recovery.

MATERIALS AND METHODS

Subjects and Design

This study was conducted at the Third Affiliated Hospital of Guangzhou Medical University in Guangzhou, China. Fecal samples were collected from 11 pairs of premature twins/triplets for 16S rRNA gene sequencing from January 2020 to June 2020. Among these premature twins/triplets, eight pairs were discordant, that is, one infant exhibited clinical signs of FI, while the other infant did not. Initial fecal samples were collected in the first 2 days, and then every week until 7 weeks after birth or discharge. Good clinical practice (GCP) guidelines and regulations were followed for premature infants. Parents were well-informed about this study.

Diagnostic Criteria of FI

Premature infants diagnosed with FI presented with a gastric residual volume of more than 50% of the previous feeding volume, abdominal distension, gastric regurgitation and/or emesis. The disappearance of the aforementioned symptoms was defined as FI recovery (1, 13).

Exclusion and Inclusion Criteria

The inclusion criteria were as follows: (1) premature twins with a gestational age <34 weeks and a birth weight <2,000 g; (2) delivery in our hospital by cesarean section only; (3) premature infants were assigned to the FI group if they were diagnosed with FI and their condition improved to achieve full enteral feeding after treatment; and (4) premature infants were assigned to the FT group if they were not diagnosed with FI and achieved full enteral feeding during hospitalization.

The exclusion criteria were as follows: (1) infants who suffered from systemic inflammatory reaction, Crohn's disease, inflammatory bowel disease, congenital gastrointestinal anomalies, or necrotizing enterocolitis; (2) infants who had severe asphyxia, sepsis, coagulation disorders, or those who underwent abdominal surgery during hospitalization; (3) infants whose mother had chorioamnionitis or premature rupture of membranes, as well as perinatal medication and illness were unknown; (4) infants whose symptoms reappeared after FI recovery; and (5) infants who had not achieved full enteral feeding during hospitalization.

Fecal Samples Collection

Fecal samples were collected with disposable sterile fecal collection tubes from soiled patient diapers and delivered to the laboratory in an ice box. Fecal samples were collected from the first defecation until 7 weeks after birth or before discharge. All samples were stored at −80°C.

DNA Extraction, PCR Amplification, Library Preparation, and Sequencing

Total genomic DNA was extracted using the CTAB/SDS method. The concentration and purity of DNA were assessed on 1% agarose gels. 16S rRNA v4 genes were amplified using a specific primer pair (16S v4 515F-806R) with a barcode. All PCR reactions consisted of 15 μL of Phusion High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM each of forward and reverse primers, and 10 ng of template DNA. The thermal cycling conditions were pre-denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30s, and elongation at 72°C for 5 min. PCR products was purified with the Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's protocol. The library was assessed using the Qubit® 2.0 Fluorometer (Thermo Scientific) and the Agilent Bioanalyzer 2100. The library was sequenced using the Illumina NovaSeq platform and 250 bp paired-end reads were generated according to standard protocols by Novogene Co., Ltd. (Beijing, China).

Data Analysis

Paired-end reads derived from the original DNA fragments were merged using FLASH, which merged paired-end reads when there were overlaps between reads1 and reads2. Paired-end reads were assigned to each sample according to unique barcodes. Sequences were analyzed using QIIME software (Quantitative Insights Into Microbial Ecology), and in-house Perl scripts

were used to analyze α- (within samples) and β- (among samples) diversity. First, reads were filtered by QIIME quality filters. Thereafter, we used pick_de_novo_otus.py to identify the operational taxonomic units (OTUs) by generating an OTU table. Sequences with ≥97% similarity were assigned to the same OTU. We selected a representative sequence for each OTU and used the RDP classifier to annotate taxonomic information for each representative sequence. To compute the α-diversity, we rarified the OTU table and calculated three metrics: Chao1, observed species, and Shannon index. Rarefaction curves were generated based on these three metrics. QIIME calculated both weighted and unweighted unifrac, which are phylogenetic measures of β-diversity. We used unweighted unifrac for principal coordinate analysis (PCoA) and unweighted pair group method with arithmetic mean (UPGMA) clustering. PCoA generated principal coordinates and visualized them from complex, multidimensional data. To mine deeper data of the differences in microbial diversity between the samples, significance tests were conducted using the *t*-test, MetaStat, LEfSe, Anosim, and MRPP.

TABLE 1 | The demographic characteristics between FT group and FI group.

	FT group (*n* = 14)	FI group (*n* = 9)	*P-value*
Birth weight, g, median	1.38 ± 0.33	1.20 ± 0.28	0.181**
Gestational age, weeks, median	31.05 (28.60–33.10)	30.00 (28.60–31.50)	0.137***
Gender, M/F	8/6	5/4	0.094*
FGR (*n* %)	2(14.3%)	1(11.1%)	1.000*
Antenatal antibiotics (*n* %)	2(14.3%)	2(22.2%)	1.000*
Latamoxef, days, median	7.5 (0–27)	8.0 (0.0–11.0)	0.503***
Piperacillin, days, median	0(0.0–10.0)	0(0.0–9.0)	0.354***
Total parenteral feeding, days, median	0(0.0–0.0)	1(0.0–3.0)	<0.001***
Duration of NICU, days, median	34.0 (23.0–48.0)	49.0 (36.0–58.0)	0.020***

****Rank sum test was used to test the significance of difference in pairwise comparisons of the samples. **T-test was used to test the significance of difference in pairwise comparisons of the samples. *Chi square test was used to test the significance of difference in pairwise comparisons of the samples. The medians was described with interquartile interval (IQR).*

Statistical Analysis

All clinical data of the preterm infants were analyzed using SPSS 21.0 software. Continuous variables were reported as medians (interquartile interval, IQR), and categorical data were presented as ratios or percentages. A *t*-test and chi-square test were used to examine the significance of the differences in pairwise comparisons of the samples. Differences with $P < 0.05$ were considered statistically significant.

TABLE 2 | Basic information of twins and triplets.

Twins	GA (weeks)	BW (g)	MZ/ DZ	Diagnose	Antenatal antibiotics	Time FI diagnosed (*d*)	Time FI recovered (*d*)	Sample (*n*)
1A	29.1	980	MZ	FI	None	3	8	3
1B	29.1	1,380	MZ	FT	None		–	3
2A	30	760	MZ	FT	None		–	5
2B	30	1,185	MZ	FI	None	2	8	5
3A	28.6	1,330	DZ	FT	Cefuroxime		–	4
3B	28.6	1,350	DZ	FI	Cefuroxime	3	7	4
4A	30.2	960	DZ	FT	None		–	4
4B	30.2	1,440	DZ	FI	None	2	8	4
5B	30.6	1,600	DZ	FT	None		–	4
5C	30.6	1,655	DZ	FI	None	1	5	4
6A	33.1	1,490	DZ	FT	None		–	2
6B	33.1	1,600	DZ	FT	None		–	2
7A	32.1	1,540	MZ	FT	None		–	3
7B	32.1	1,800	MZ	FT	None		–	3
8A	31.5	1,380	DZ	FI	None	2	6	4
8B	31.5	1,315	DZ	FT	None		–	4
8C	31.5	760	DZ	FI	None	3	7	4
9A	29	1,135	DZ	FT	latamoxef	3	6	4
9B	29	1,045	DZ	FI	latamoxef		–	4
10A	33.1	1,706	MZ	FT	None		–	2
10B	33.1	1,750	MZ	FT	None		–	2
11A	29.3	1,040	MZ	FI	None	4	8	4
11B	29.3	1,000	MZ	FT	None		–	4

GA, gestational age; BW, birth weight; MZ, monozygotic; DZ, dizygotic; FI, feeding intolerance; FT, feeding tolerance.

RESULTS

Basic Information of Premature Twins

The 11 pairs of premature twins/triplets, including five pairs of monozygotic twins, three pairs of heterozygous twins, and three pairs of triplets, met the inclusion criteria. Among these premature twins/triplets, eight pairs were discordant, that is, one infant exhibited clinical signs of FI, while the other infant did not. The other three pairs exhibited no clinical signs of FI. Among the FI group the fecal flora from 5A to 9C failed to amplification. In summary, there were 14 cases in the FT group and nine cases in the FI group. All premature infants were fed with preterm formula after birth due to lack of breast milk. Only two pairs received antenatal antibiotics, and none of them received probiotics during hospitalization. Latamoxef was the most commonly prescribed antibiotic, and piperacillin was used occasionally, that is, if clinical conditions supported its use. The antibiotics were rarely used for more than 14 days. The demographic characteristics and the basic information of the twins and triplets is shown in **Tables 1**, **2**, respectively.

A total of 131 fecal samples, including 70 in the FT group and 61 in the FI group, were collected. Among them, 33 samples could not be amplified. A total of 82 fecal samples were analyzed, including 46 fecal samples from the FT group and 36 fecal samples from the FI group. The information of the fecal samples is shown in **Table 3** and **Supplementary Table 1**. The flowchart of our experiment is shown in **Figure 1**.

There Was No Significant Difference in the α-Diversity Between the FT Group and the FI Group After FI Recovery

The OTUs, rarefaction curve, and rank abundance curve are shown in **Supplementary Figures 1, 2**. At the phylum level, the main OTUs in the FT group were Firmicutes (93.63%), Actinobacteria (5.41%), and Proteobacteria (0.81%), whereas those in the FI group were Firmicutes (79.40%), Proteobacteria (18.19%), and Actinobacteria (2.24%), as shown in **Figure 2A**. At the genus level, the top six OTUs in the FT group were unidentified_ Clostridiales (40.32%), Enterococcus (24.88%), Streptococcus (10.18%), Clostridioides (6.65%), Staphylococcus (5.87%), and Bifidobacterium (4.82%), whereas those in the FI group were unidentified_Clostridiales (32.18%), Streptococcus (9.50%), Staphylococcus (9.44%), Enterococcus (8.90%), Paenibacillus (7.14%), and Kluyvera (4.74%), as shown in **Figure 2B**. The observed species was similar between two groups as shown in **Figure 2C**. Additionally, there was no difference in the alpha-diversity presented with Shannon index, Simpson's index and ACE index, respectively, as shown in **Figures 2D–F**.

TABLE 3 | The time of sample collection and antibiotics.

Samples collection time (day)								Latamoxef (day)	Piperacillin (day)	Diagnosis FI (day)	Duration FI (days)	TPN (days)
1A	**D3**	**D9**	**D14**	**D21**	**–**	**–**	**D44**	**D1–11**	**–**	**D3**	**5**	**1**
1B	D3	D9	D14	D21	–	–	D44	D1–11 D25–40	–	–	–	–
2A	D2	D9	D15	D21	D27	–	D40	D1–8	D21–28	–	–	–
2B	**D2**	**D9**	**D15**	**D21**	**D27**	**–**	**D40**	**D1–8**	**–**	**D2**	**6**	**2**
3A	D3	D7	D15	D21	D28	–	–	D1–9	–	–	–	–
3B	**D3**	**D7**	**D15**	**D21**	**D28**	**–**	**–**	**D1–9**	**–**	**D3**	**4**	**1**
4A	D2	D9	D14	D21	D28	–	–	D1–8	–	–	–	–
4B	**D2**	**D9**	**D14**	**D21**	**D28**	**–**	**–**	**D1–8**	**–**	**D2**	**6**	**3**
5B	D1	D7	D14	D21	D28	–	–	D1–5	D6–9	–	–	–
5C	**D1**	**D7**	**D14**	**D21**	**D28**	**–**	**–**	**D1–9**	**–**	**D1**	**4**	**0**
6A	D1	D7	D14	D21	–	–	–	D1–9	–	–	–	–
6B	D1	D7	D14	D21	–	–	–	D1–9	–	–	–	–
7A	D1	D7	D14	D21	D28	–	–	D1–7	–	–	–	–
7B	D1	D7	D14	D21	D28	–	–	D1–7	–	–	–	–
8A	**D1**	**D5**	**D12**	**D19**	**D31**	**D35**	**–**	**D1–4**	**–**	**D2**	**4**	**0**
8B	D1	D5	D12	D19	D31	D35	–	–	D1–10	–	–	–
8C	**D1**	**D5**	**D12**	**D19**	**D31**	**D35**	**–**	**–**	**D1–8**	**D3**	**4**	**1**
9A	D2	D7	D13	D21	D28	–	D47	D1–17	–	–	–	–
9B	**D2**	**D7**	**D13**	**D21**	**D28**	**–**	**D47**	**D1–10**	**–**	**D3**	**3**	**1**
10A	D1	D7	D14	–	–	–	–	–	D1–9	–	–	–
10B	D1	D7	D14	–	–	–	–	–	D1–9	–	–	–
11A	**D3**	**D8**	**D14**	**D21**	**D28**	**–**	**D40**	**D1–8**	**D41–49**	**D4**	**4**	**1**
11B	D3	D8	D14	D21	D28	–	D40	D1–7	D41–48	–	–	–

1A–11B represented all cases in study. 1A, 2B, 3B, 4B, 5C, 8A, 8C, 9B, and 11A were FI group with bold and the others were FT group. Total parenteral nutrition (TPN).

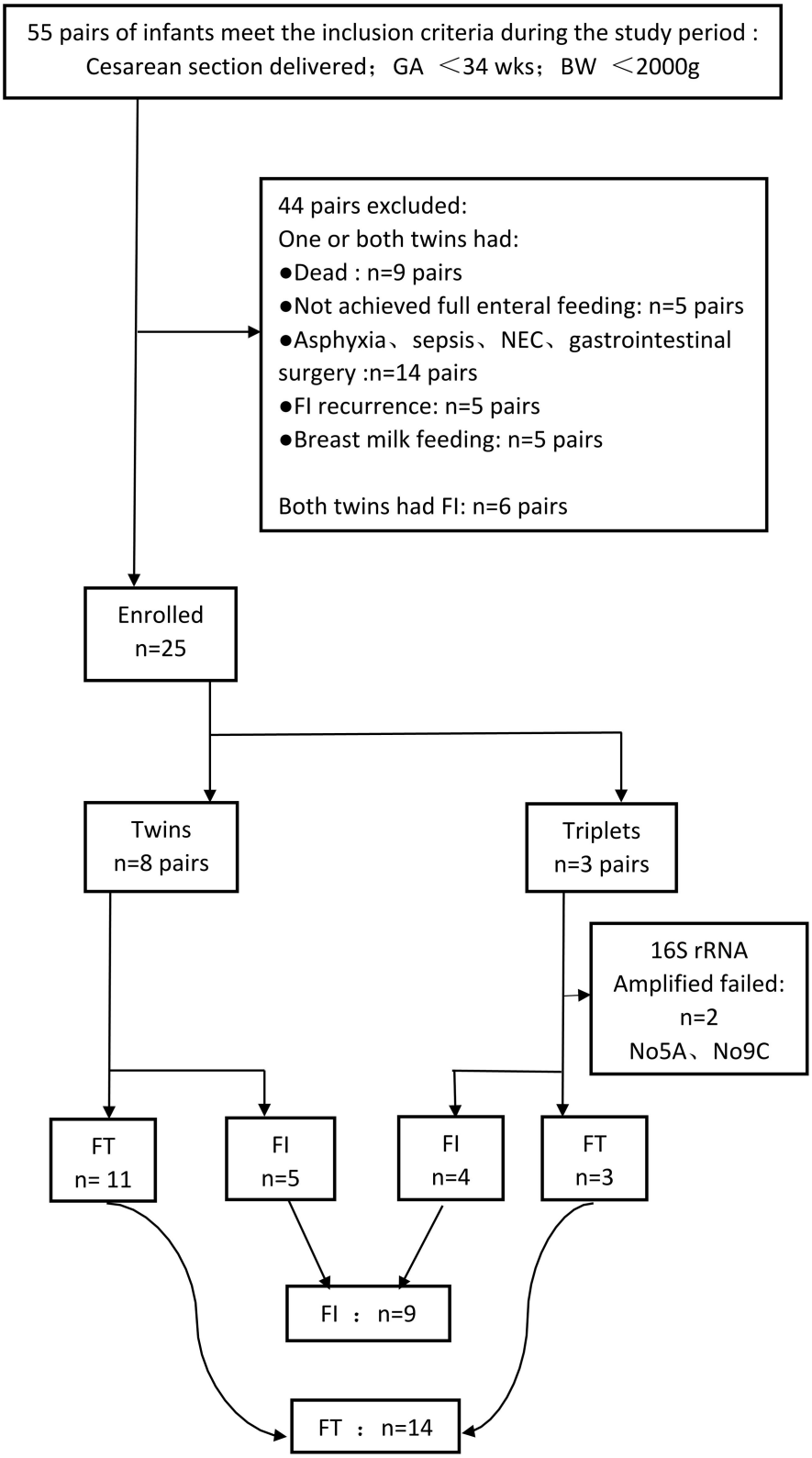

FIGURE 1 | Flowchart of our search.

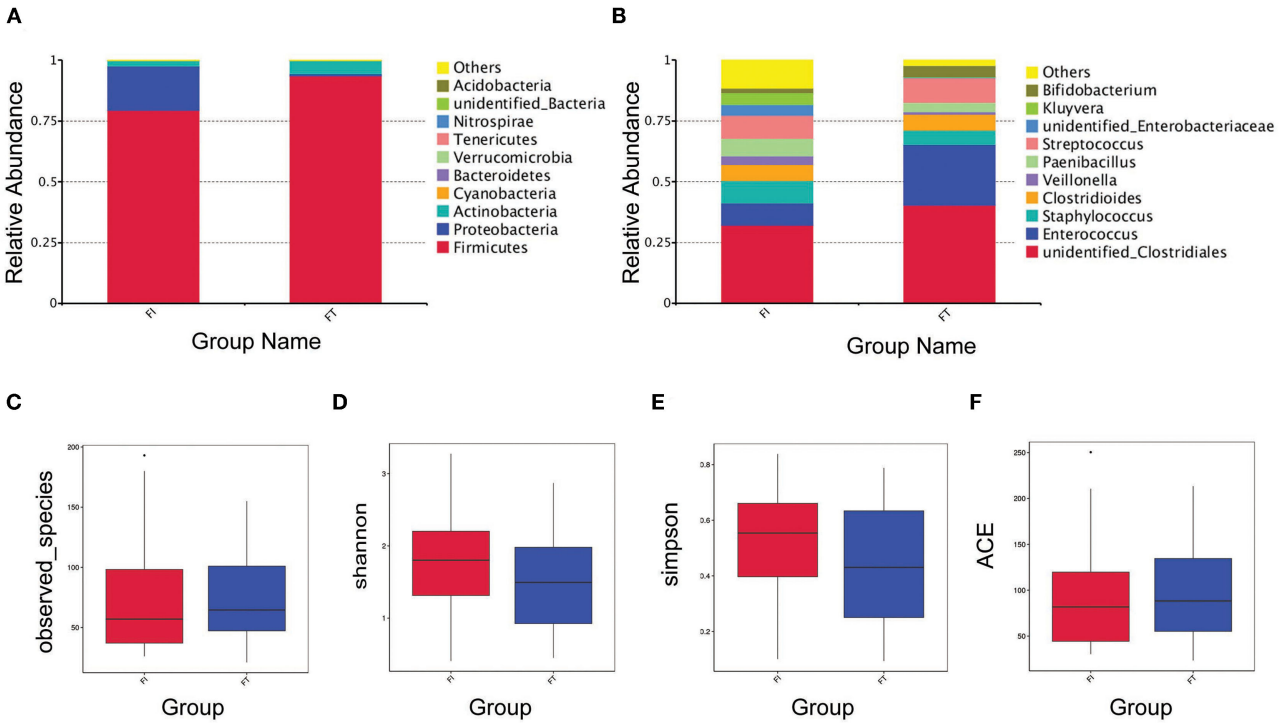

FIGURE 2 | Overview of the preterm infant gut microbiome and the α-Diversity. Relative abundance of bacteria at phylum level **(A)** and at genus level **(B)** between FT and FI groups. α-diversity analysis in the sample groups including observed-species **(C)**, Shannon index **(D)**, Simpson index **(E)** and ACE index **(F)**.

There Was a Significant Difference in the β-Diversity Between the FT Group and the FI Group

The box diagram based on unweighted and weighted UniFrac distance showed that there was a statistically significant difference in the β-diversity of intestinal flora between the FT group and the FI group after FI recovery ($P < 0.05$). PCoA analysis showed that similarities in the microbial community in the FT group were higher than those in the FI group (**Figure 3**). In particular, when the LDA score was set to >4, LEfSe analysis revealed that the predominant bacteria in the FI group were Proteobacteria, whereas those in the FT group were Firmicutes after FI recovery (**Figure 4** and **Supplementary Figure 3**). As mentioned above, not all the twins or triplets were discordant, which means one of the pair suffered FI and the other did not. In 11 pairs of premature twins/triplets, eight pairs of them were discordant. We further analyzed the only eight discordant pairs, as it was shown in the **Supplementary Figures 4, 5**. There was still significant differences in the β-diversity between two groups based on the eight discordant pairs.

The Developmental Trajectory of the Intestinal Flora Between the FT Group and the FI Group Was Different

To examine the developmental trajectory of the fecal microbiota after FI recovery, all samples were analyzed according to the collection time. The samples collected at 7–8 days after birth were grouped as time 1, 13–15 days after birth were grouped as time 2, 19–28 days after birth were grouped as time 3, and the remaining samples were grouped as time 4. LEfSe analysis revealed that the predominant bacteria with linear discriminant analysis (LDA) >4 in the FT group were Bacillus, Clostridium_Butyricum, and Clostridiales at time 2, time 3, and time 4, respectively. However, the predominant bacteria with LDA >4 in the FI group were Firmicutes, unidentified_Clostridiales, and γ-Proteobacteria at time 2, time 3, and time 4, respectively, as shown in **Figure 5**. Taking into account the differences in body weight and gestational age of subjects, stratification analysis was further conducted. According to weight or gestational age, it was found that there was still a statistically significant differences in the flora between FI and FT groups, as it was shown in the **Supplementary Figures 6, 7**. Because the samples are limited, the conclusion may be not sufficient.

Bacterial Functions Between the FT Group and the FI Group Was Different

Tax 4Fun was used to predict bacterial functions in the fecal samples. After FI recovery, the relative abundances of the KEGG pathways of fatty acid metabolism, DNA repair and recombination proteins, mitochondrial biogenesis, and terpenoid backbone biosynthesis in the FT group were significantly higher than those in the FI group ($P < 0.01$), whereas energy metabolism, amino acid metabolism, bacteria motility proteins,

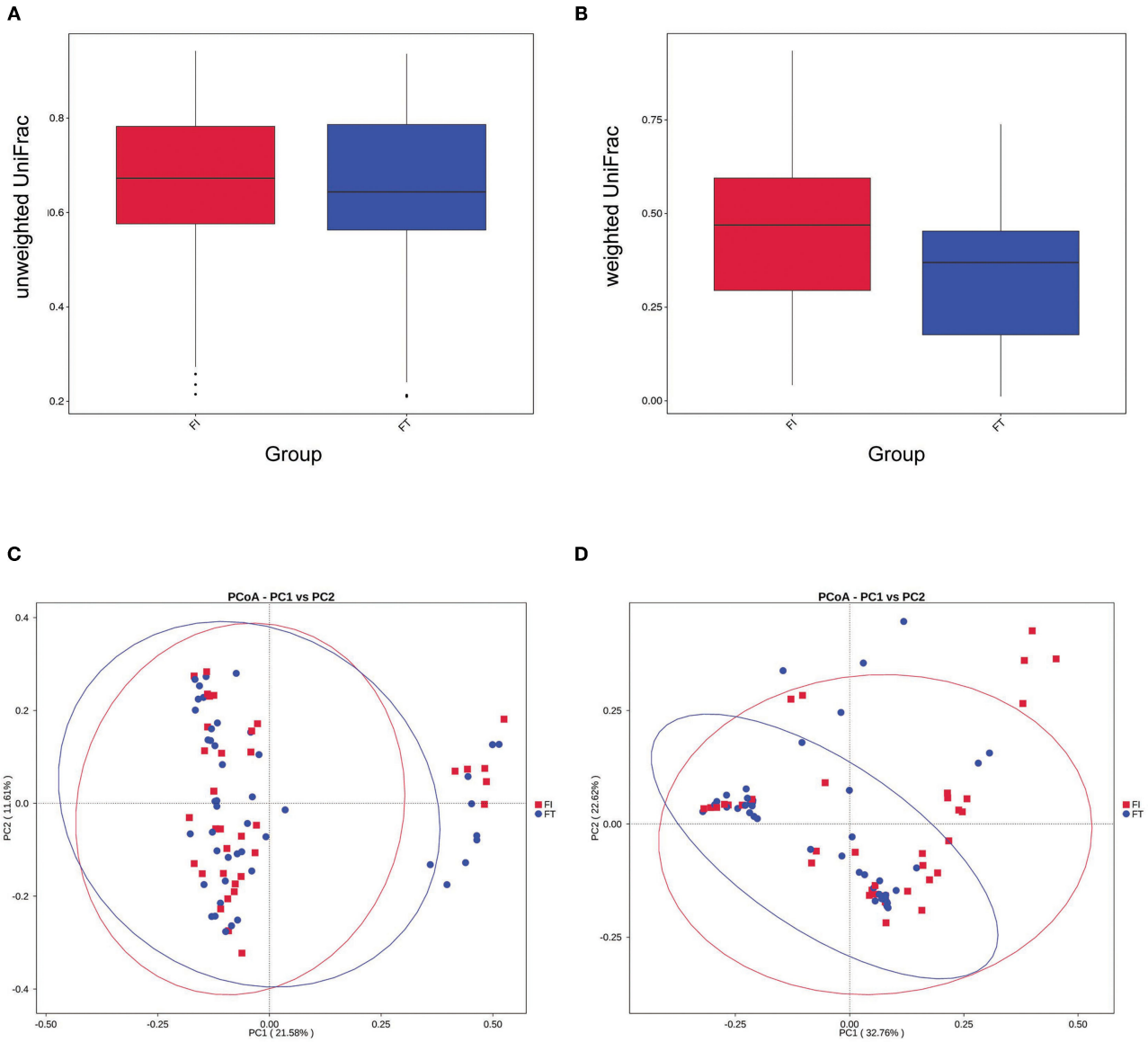

FIGURE 3 | β- diversity analysis. **(A)** Box diagram based on unweighted UniFrac beta diversity $P = 0.055$ (*t*-test) and $P = 0.040$ (two-wilcox). **(B)** Box diagram based on weighted UniFrac beta diversity $P < 0.001$. **(C)** PCoA analysis based on unweighted UniFrac distance. **(D)** PCoA analysis based on weighted UniFrac distance.

biofilm formation in *Escherichia coli*, cationic antimicrobial peptide resistance, and biofilm formation in *Vibrio cholerae* were significantly higher in the FI group than those in the FT group ($P < 0.01$), as shown in **Figure 6**.

DISCUSSION

The initial acquisition and early development of the intestinal microbiome during infancy are important to human health (14). It is now recognized that intestinal microbes are key modulators of disease status. Feeding intolerance (FI) is a common condition in preterm neonates, and it disrupts the homeostasis of the intestinal tract (15). However, the search for its causative agent during early life is complicated due to the mode of birth, antibiotic administration, environment, nutrition, and other individual differences (16). A previous study has demonstrated that microbiome development in preterm twins is similar (12). Thus, in this study, the gut microbiome of premature twins/triplets was examined, where one of the twins exhibited clinical signs of FI, while the other infant did not. To eliminate birth canal contamination, only cesarean section cases were included.

We found that the microbial β-diversity differed significantly between the FT group and the FI group after FI recovery. Enterobacteria from the Proteobacteria phylum were the predominant organisms in the FI group, whereas Enterococcus

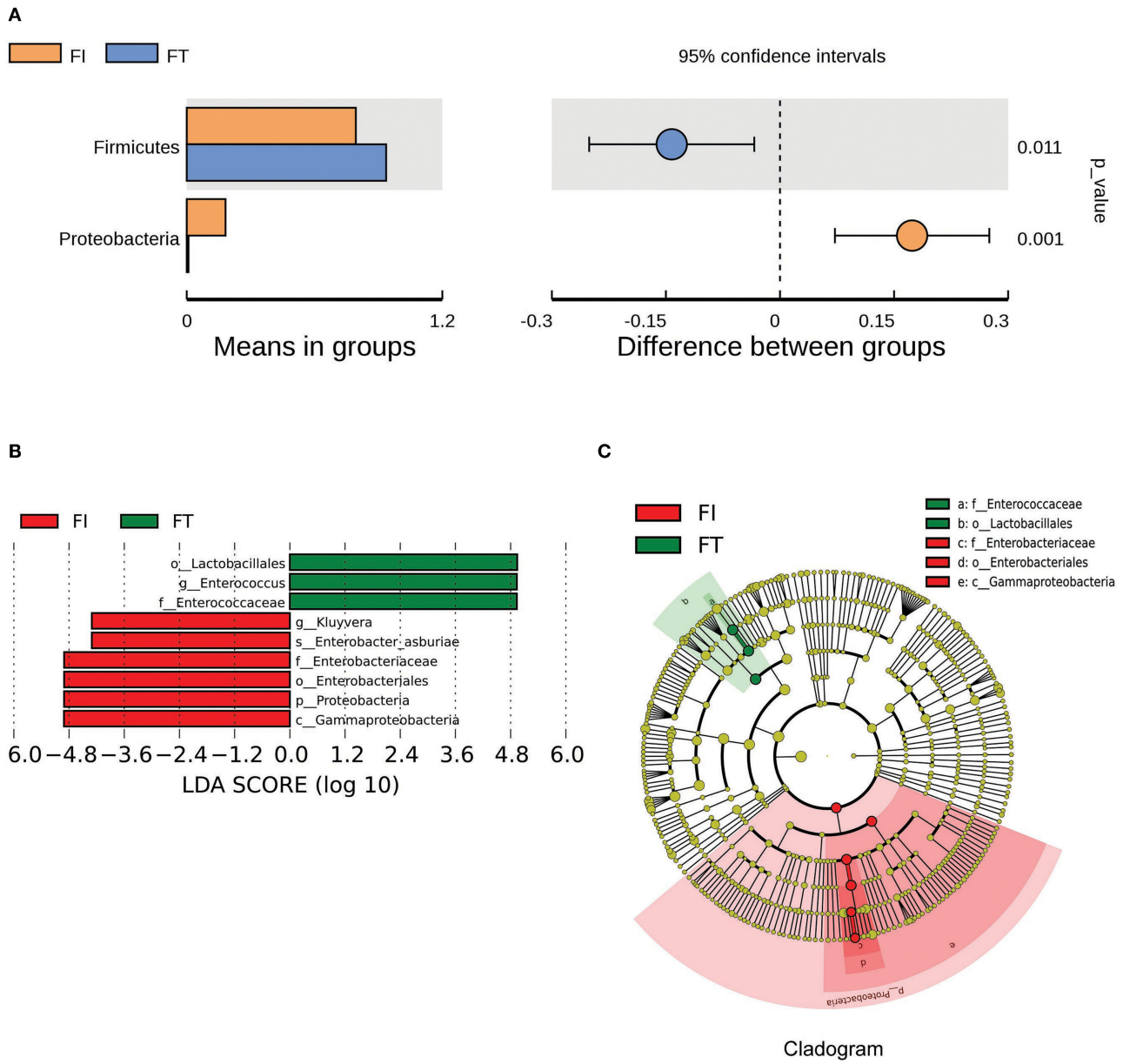

FIGURE 4 | The differential microbes between FI and FT groups. **(A)** Analysis of difference in microbes between two groups using *t*-test. **(B)** LDA value distribution. **(C)** Evolutionary cladistics (phylogenetic distribution).

from the Firmicutes phylum were the predominant organisms in the FT group. As previously reported, there was an increase in the relative abundance of Proteobacteria before and during the diagnosis of NEC and feeding intolerance. *Enterococcus faecalis* plays an immunomodulatory role by decreasing TGF-β and increasing IL-8 levels, and it also regulates the inflammatory response through TLR3, TLR4, TLR9, and TRAF6, which has a protective effect on the body. Our results showed that after FI recovery, the gut environment remained different from preterm infants without FI for at least 7 weeks after birth or discharge, which was also reflected in the different KEEG pathways between the two groups of premature infants. Our results were inconsistent with those of Yuan et al. who reported that Firmicutes was the predominant intestinal flora after FI recovery (6). We speculate the reason for this difference is as follows. Firstly, FI occurred in less than a week in our study, while it occurred at 3–18 d after birth in their study. Secondly, they collected stool samples 1 week after FI recovery, but we collected stool samples from the day of FI recovery to several weeks after recovery. As the development of the gut microbial environment has recently been shown to evolve in a pattern associated with PMA (postmenstrual age, that is, gestational age

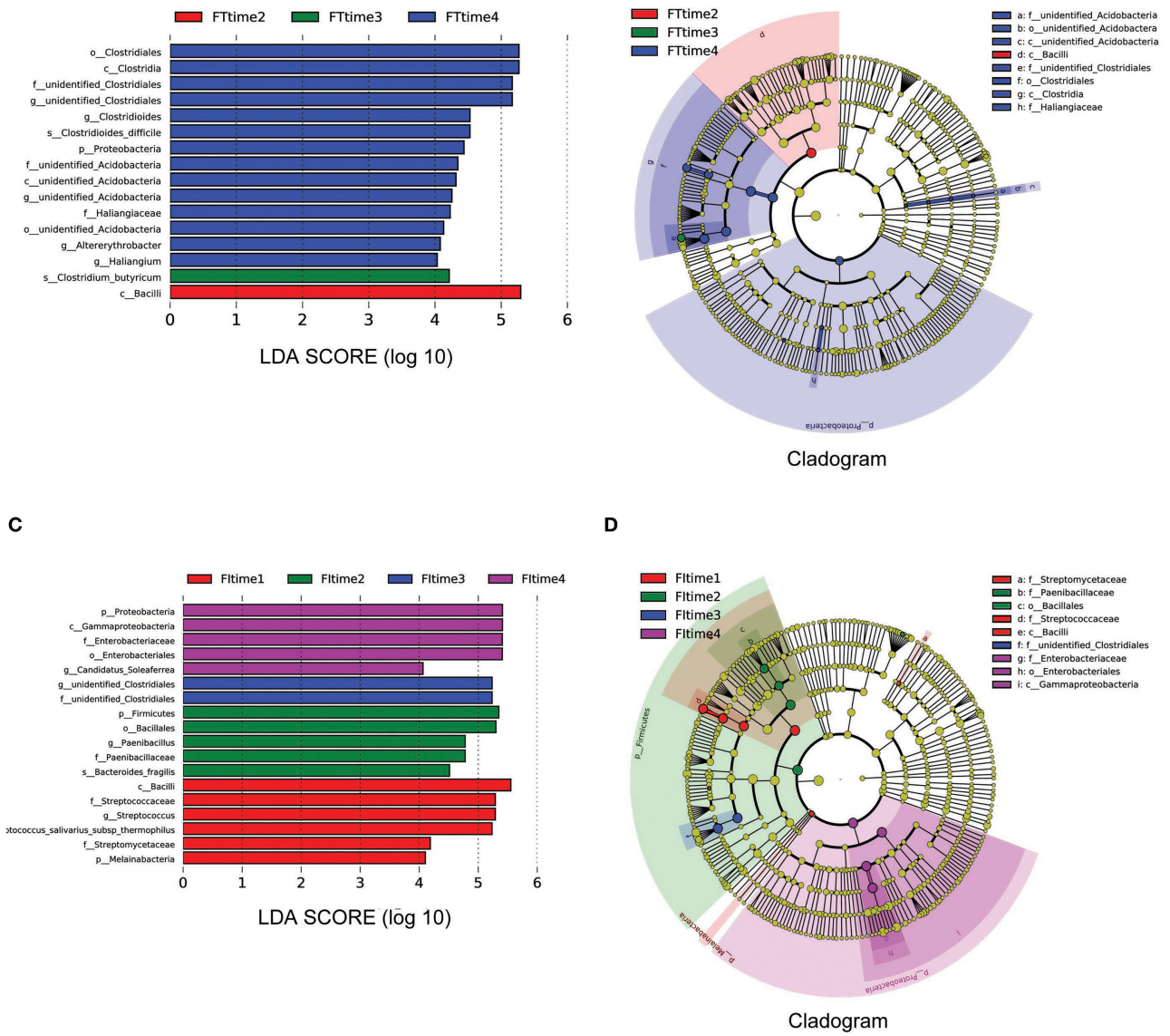

FIGURE 5 | LEfSe analysis based on the different time. LEfSe analysis **(A)** and Cladogram **(B)** in the FT groups over time. LEfSe analysis **(C)** and Cladogram **(D)** in the FT groups over time. Time 1: 7–8 days after birth, Time 2: 13–15 days after birth, Time 3: 19–28 days after birth, Time 4: until the time before discharge.

at birth plus week of life) (17), the different collection times of stool samples, which correspond to different PMAs, may have affected our results.

Our study also found that after FI recovery, the bacterial succession in preterm infants of the FI group was different from that of the FT group in the several weeks after birth. The fecal microbiota of FI infants was dominated by Bacilli at early PMA, followed by Clostridia and γ-Proteobacteria, while that of FT infants was dominated by Bacilli and Clostridia, with the latter class remaining unchanged until 7 weeks after birth before discharge. Grier et al. reported (18) an association between a diagnosis of NEC and microbial reverse transitions from γ-Proteobacteria to Bacilli, Clostridia, and γ-Proteobacteria or a delayed transition from Bacilli to Clostridia. Furthermore, previous studies have revealed that FI and NEC have similarities in clinical manifestations and intestinal flora changes (6, 19). We found that FI may affect the evolution of the gut microbiome in a manner similar to that of NEC. Therefore, enteral feeding of preterm infants that have recovered from FI should be monitored for several weeks after birth.

The accumulated evidence indicates that microbiota development is shaped by host biology and related to the environment. Previous studies have shown that the composition of the intestinal flora of preterm infants was affected by

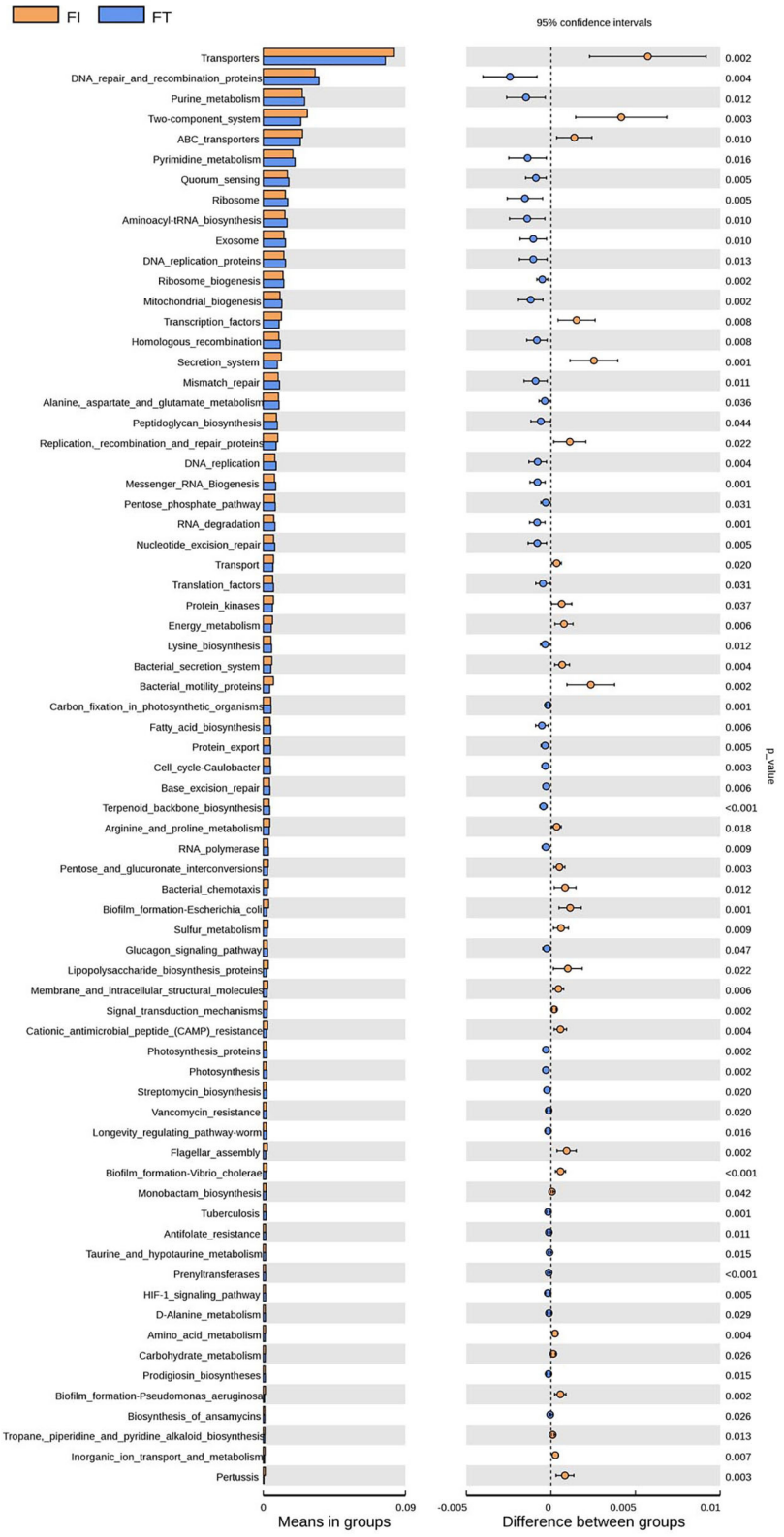

FIGURE 6 | KEGG pathway analysis between FI and FT groups.

many factors, such as maternal illness and nutritional status during pregnancy (20), delivery mode (21), feeding mode (22, 23), gestational age (24, 25), type and duration of antibiotic use (26, 27), intrauterine or nosocomial infection (28, 29), environment in NCU (30), use of probiotics and so on (31, 32). In our study, we choose those infants that were deliverd by cesarean, no breastmilk feeding, no NEC or sepsis, no probiotics and asphyxia, so we did not analyze those factors mentioned above. As previous studies have shown that the smaller the gestational age, the lower the diversity of intestinal flora, and the later the colonization of anaerobic bacteria such as bifidobacteria and lactic acid bacteria (24, 25). In this study, the range of the gestational age in eight pairs of infants discordant with FI were 28–32 weeks, and the range of the gestational age in three pairs of preterm infants without FI were 32–34 weeks, all of which are extremely premature infants. After preliminary stratified analysis according to gestational age, the main outcome is similar to the original results. Whether gestational age is an important factor that affects the composition of the intestinal flora after FI is cured is not clear, which need a larger sample to conclude. Previous studies have pointed out that the empirical use of antibiotics (including antenatal antibiotics and postnatal antibiotics) can lead to an decrease in Alpha diversity, delayed colonization of anaerobic bacteria, and the impact of antibiotics on the intestinal flora can last several months after the drug was stopped (33, 34). The pre-and post-natal antibiotics used in our study were Piperacillin/cephalosporin. Because there were only two pairs of infants (No 3 and No 5) had antenatal antibiotics, we did not analyze the effect of this factor on the intestinal microflora. In addition, although all preterm infants received either or both of these antibiotics mentioned above after birth, and there were differences in the type and duration of antibiotics usage among different pairs of infants, the type and duration of postnatal antibiotics used between the same pair of twins/triplets are relatively similar, so we did not analyze the intestinal flora of the two groups of preterm infants based on the duration and type of antibiotics under limited numbers.

There were several limitations in this study. Firstly, the sample size was relatively small. However, our study is the first longitudinal analysis of the development of the gut microbiome in premature twins/triplets, where one infant had FI and the other did not. Secondly, this was a single-center study, and it was difficult to eliminate inclusion bias. Thirdly, because most of our initially collected stool samples provided insufficient DNA for sequencing, we only analyzed the stool samples of infants that recovered from FI. Last but not least, we have not been able to compare the intestinal flora of different preterm infants according to the use of antibiotics, but we try to make up for this deficiency by selecting those premature twin/multiple fetuses with similar antibiotic usage. Regardless, the changes in the major bacterial phyla reported in this study are consistent with the current understanding of the effect of those microbes. In conclusion, we found that FI may disturb the diversity of intestinal flora even after FI recovery.

DATA AVAILABILITY STATEMENT

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. The data presented in the study are deposited in the (https://www.ncbi.nlm.nih.gov/sra/PRJNA698588, PRJNA698588) repository, accession number (SUB8976175).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the Third Affiliated Hospital of Guangzhou Medical University ([2020]009). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

FW and GL: conceptualization, project administration, formal analysis, and writing - review & editing. YL and CJ: writing -original draft, writing - review & editing, data curation, formal analysis, and methodology. XL, LLin, XF, and XH: methodology, investigation, validation, and formal analysis. HW and ZX: investigation, validation, and supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by National Natural Science Foundation of China (NSFC) (81741083 and 81801492), Natural Science Foundation of Guangdong Province (GDNSF) (2018A030310598), Medical Scientific Research Foundation of Guangdong Province (A2019102, A2019069), and General Guidance Project of Guangzhou Health Technology (20191A010064).

ACKNOWLEDGMENTS

We would like to thank all participating children and their parents. The International Science Editing Compuscript Ltd. provides language help.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.648979/full#supplementary-material

REFERENCES

1. Fanaro S. Feeding intolerance in the preterm infant. *Early Hum Dev.* (2013) 89(Suppl. 2):S13–20. doi: 10.1016/j.earlhumdev.2013.07.013

2. Althani AA, Marei HE, Hamdi WS, Nasrallah GK, El Zowalaty ME, Al Khodor S, et al. Human microbiome and its association with health and diseases. *J Cell Physiol.* (2016) 231:1688–94. doi: 10.1002/jcp.25284

3. Rios-Covian D, Ruas-Madiedo P, Margolles A, Gueimonde M, de Los Reyes-Gavilan CG, Salazar N. Intestinal short chain fatty acids and their link with diet and human health. *Front Microbiol.* (2016) 7:185. doi: 10.3389/fmicb.2016.00185
4. Budden KF, Gellatly SL, Wood DL, Cooper MA, Morrison M, Hugenholtz P, et al. Emerging pathogenic links between microbiota and the gut-lung axis. *Nat Rev Microbiol.* (2017) 15:55–63. doi: 10.1038/nrmicro.2016.142
5. Putignani L, Del Chierico F, Petrucca A, Vernocchi P, Dallapiccola B. The human gut microbiota: a dynamic interplay with the host from birth to senescence settled during childhood. *Pediatr Res.* (2014) 76:2–10. doi: 10.1038/pr.2014.49
6. Yuan Z, Yan J, Wen H, Deng X, Li X, Su S. Feeding intolerance alters the gut microbiota of preterm infants. *PLoS ONE.* (2019) 14:e0210609. doi: 10.1371/journal.pone.0210609
7. Lim ES, Zhou Y, Zhao G, Bauer IK, Droit L, Ndao IM, et al. Early life dynamics of the human gut virome and bacterial microbiome in infants. *Nat Med.* (2015) 21:1228–34. doi: 10.1038/nm.3950
8. Gensollen T, Iyer SS, Kasper DL, Blumberg RS. How colonization by microbiota in early life shapes the immune system. *Science.* (2016) 352:539–44. doi: 10.1126/science.aad9378
9. Dicksved J, Floistrup H, Bergstrom A, Rosenquist M, Pershagen G, Scheynius A, et al. Molecular fingerprinting of the fecal microbiota of children raised according to different lifestyles. *Appl Environ Microbiol.* (2007) 73:2284–9. doi: 10.1128/AEM.02223-06
10. Simioni J, Hutton EK, Gunn E, Holloway AC, Stearns JC, McDonald H, et al. A comparison of intestinal microbiota in a population of low-risk infants exposed and not exposed to intrapartum antibiotics: the Baby & Microbiota of the Intestine cohort study protocol. *BMC Pediatr.* (2016) 16:183. doi: 10.1186/s12887-016-0724-5
11. Stewart CJ, Marrs EC, Nelson A, Lanyon C, Perry JD, Embleton ND, et al. Development of the preterm gut microbiome in twins at risk of necrotising enterocolitis and sepsis. *PLoS ONE.* (2013) 8:e73465. doi: 10.1371/journal.pone.0073465
12. Moore TA, Wilson ME. Feeding intolerance: a concept analysis. *Adv Neonatal Care.* (2011) 11:149–54. doi: 10.1097/ANC.0b013e31821ba28e
13. Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics.* (2011) 27:2957–63. doi: 10.1093/bioinformatics/btr507
14. Lindberg TP, Caimano MJ, Hagadorn JI, Bennett EM, Maas K, Brownell EA, et al. Preterm infant gut microbial patterns related to the development of necrotizing enterocolitis. *J Matern Fetal Neonatal Med.* (2020) 33:349–58. doi: 10.1080/14767058.2018.1490719
15. Chong CYL, Bloomfield FH, O'Sullivan JM. Factors affecting gastrointestinal microbiome development in neonates. *Nutrients.* (2018) 10:274. doi: 10.3390/nu10030274
16. Pammi M, Cope J, Tarr PI, Warner BB, Morrow AL, Mai V, et al. Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis. *Microbiome.* (2017) 5:31. doi: 10.1186/s40168-017-0248-8
17. Grier A, Qiu X, Bandyopadhyay S, Holden-Wiltse J, Kessler HA, Gill AL, et al. Impact of prematurity and nutrition on the developing gut microbiome and preterm infant growth. *Microbiome.* (2017) 5:158. doi: 10.1186/s40168-017-0377-0
18. Elgin TG, Kern SL, McElroy SJ. Development of the neonatal intestinal microbiome and its association with necrotizing enterocolitis. *Clin Ther.* (2016) 38:706–15. doi: 10.1016/j.clinthera.2016.01.005
19. La Rosa PS, Warner BB, Zhou Y, Weinstock GM, Sodergren E, Hall-Moore CM, et al. Patterned progression of bacterial populations in the premature infant gut. *Proc Natl Acad Sci USA.* (2014) 111:12522–7. doi: 10.1073/pnas.1409497111
20. Chu DM, Meyer KM, Prince AL, Aagaard KM. Impact of maternal nutrition in pregnancy and lactation on offspring gut microbial composition and function. *Gut Microbes.* (2016) 7:459–70. doi: 10.1080/19490976.2016.1241357
21. Akagawa S, Tsuji S, Onuma C, Akagawa Y, Yamaguchi T, Yamagishi M, et al. Effect of delivery mode and nutrition on gut microbiota in neonates. *Ann Nutr Metab.* (2019) 74:132–9. doi: 10.1159/000496427
22. Cong X, Judge M, Xu W, Diallo A, Janton S, Brownell EA, et al. Influence of feeding type on gut microbiome development in hospitalized preterm infants. *Nur Res.* (2017) 66:123–33. doi: 10.1097/Nnr.0000000000000208
23. Lyons KE, Ryan CA, Dempsey EM, Ross RP, Stanton C. Breast milk, a source of beneficial microbes and associated benefits for infant health. *Nutrients.* (2020) 12:1039. doi: 10.3390/nu12041039
24. Chernikova DA, Madan JC, Housman ML, Zain-ul-Abideen M, Lundgren SN, Morrison HG, et al. The premature infant gut microbiome during the first 6 weeks of life differs based on gestational maturity at birth. *Pediatr Res.* (2018) 84:71–9. doi: 10.1038/s41390-018-0022-z
25. Yee AL, Miller E, Dishaw LJ, Gordon JM, Ji M, Dutra S, et al. Longitudinal microbiome composition and stability correlate with increased weight and length of very-low-birth-weight infants. *Msystems.* (2019) 4:e00229–18. doi: 10.1128/mSystems.00229-18
26. Greenwood C, Morrow AL, Lagomarcino AJ, Altaye M, Taft DH, Yu ZT, et al. Early empiric antibiotic use in preterm infants is associated with lower bacterial diversity and higher relative abundance of enterobacter. *J Pediatr.* (2014) 165:23–9. doi: 10.1016/j.jpeds.2014.01.010
27. Ruoss JL, Bazacliu C, Russell JT, Cruz D, Li N, Gurka MJ, et al. Routine early antibiotic use in symptomatic preterm neonates: a pilot randomized controlled trial. *J Pediatr.* (2021) 229:294–8 e293. doi: 10.1016/j.jpeds.2020.09.056
28. Mukhopadhyay S, Puopolo KM. Clinical and microbiologic characteristics of early-onset sepsis among very low birth weight infants: opportunities for antibiotic stewardship. *Pediatr Infect Dis J.* (2017) 36:477–81. doi: 10.1097/INF.0000000000001473
29. Raspini B, Porri D, De Giuseppe R, Chieppa M, Liso M, Cerbo RM, et al. Prenatal and postnatal determinants in shaping offspring's microbiome in the first 1000 days: study protocol and preliminary results at one month of life. *Ital J Pediatr.* (2020) 46:45. doi: 10.1186/s13052-020-0794-8
30. Brooks B, Olm MR, Firek BA, Baker R, Geller-McGrath D, Reimer SR, et al. The developing premature infant gut microbiome is a major factor shaping the microbiome of neonatal intensive care unit rooms. *Microbiome.* (2018) 6:112. doi: 10.1186/s40168-018-0493-5
31. Kaban RK, Wardhana BH, Rohsiswatmo R, Handryastuti S, Amelia N, Muktiarti D, et al. Lactobacillus reuteri DSM 17938 improves feeding intolerance in preterm infants. *Pediatr Gastroenterol Hepatol Nutr.* (2019) 22:545–53. doi: 10.5223/pghn.2019.22.6.545
32. Underwood MA. Probiotics and human milk oligosaccharides in premature infants. *Neoreviews.* (2019) 20:e1–11. doi: 10.1542/neo.20-1-e1
33. Zwittink RD, Renes IB, van Lingen RA, van Zoeren-Grobben D, Konstanti P, Norbruis OF, et al. Association between duration of intravenous antibiotic administration and early-life microbiota development in late-preterm infants. *Eur J Clin Microbiol Infect Dis.* (2018) 37:475–83. doi: 10.1007/s10096-018-3193-y
34. Rooney AM, Timberlake K, Brown KA, Bansal S, Tomlinson C, Lee KS, et al. Each additional day of antibiotics is associated with lower gut anaerobes in neonatal intensive care unit patients. *Clin Infect Dis.* (2020) 70:2553–60. doi: 10.1093/cid/ciz698

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

frontiers in Pediatrics

ORIGINAL RESEARCH
published: 26 April 2021
doi: 10.3389/fped.2021.675022

Intrauterine Hypoxia Changed the Colonization of the Gut Microbiota in Newborn Rats

*Yan Sun†, Lei Li†, Jiayu Song, Wei Mao, Kaihao Xiao and Chunming Jiang**

Department of Neonatology, The First Affiliated Hospital of Harbin Medical University, Harbin, China

Background: Accumulating evidence suggests a connection between the gut microbiota and neonatal diseases. Hypoxia may play an important role in the intestinal lesions in neonates.

Objective: This study aims to determine whether the gut microbiota differs between intrauterine hypoxic rats and healthy controls and to identify the factors that influence the changes in the gut microbiota.

Methods: We constructed an intrauterine hypoxia model in rats and collected the intestinal contents of intrauterine hypoxic newborn rats and normal newborn rats within 4 h and on the seventh day after birth. They were divided them into the intrauterine hypoxia first-day group (INH1), intrauterine hypoxia seventh-day group (INH7), normal first-day group (NOR1), and normal seventh-day group (NOR7). The contents of the intestines were sequenced with 16S rRNA sequencing, the sequencing results were analyzed for biological information, and the differences in the diversity, richness, and individual taxa among the groups were analyzed.

Results: The abundance of the gut microbiota of neonatal rats with intrauterine hypoxia was higher than that of the control group rats. Intrauterine hypoxia altered the structural composition of the gut microbiota in neonatal rats. The INH1 group showed increased species richness, phylogenetic diversity, and β-diversity, and altered relative abundance in several taxa compared to those in the control group. The differences in the microbiota among the four groups were significantly higher than those within the group, and the differences in the abundance and diversity of the INH7 and NOR7 groups decreased after 7 days of suckling. Functional analysis based on the Cluster of Orthologous Groups (COG) suggested that 23 functional COG categories. There was no significant difference in the functional categories between the hypoxia group and the normal group.

Conclusion: Intrauterine hypoxia changed the initial colonization of the gut microbiota in neonatal rats. It could increase the species richness and β-diversity of the gut microbiota, and altered relative abundances of several taxa.

Keywords: intrauterine hypoxia, gut microbiota, high-throughput sequencing, newborn rats, colonization, diversity

OPEN ACCESS

Edited by:
Yuan Shi,
Children's Hospital of Chongqing Medical University, China

Reviewed by:
Hongzhu Li,
Xiamen University, China
Hua Ben Zeng,
Army Medical University, China

****Correspondence:***
Chunming Jiang
chunming0451@163.com

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: *02 March 2021*
Accepted: *26 March 2021*
Published: *26 April 2021*

Citation:
Sun Y, Li L, Song J, Mao W, Xiao K and Jiang C (2021) Intrauterine Hypoxia Changed the Colonization of the Gut Microbiota in Newborn Rats. Front. Pediatr. 9:675022. doi: 10.3389/fped.2021.675022

INTRODUCTION

Intrauterine hypoxia is a prevalent problem in fetuses and neonates, and it is one of the important causes leading to fetal intrauterine distress, growth restriction, hypoxic-ischemic encephalopathy of neonates, and even fetal and neonatal death. During birth and thereafter, microbes colonize the gastrointestinal tract and forms a relatively stable microbial population (1–3). Due to the redistribution of blood in the body, the intestinal microenvironment of neonates with intrauterine hypoxia changes, resulting in an imbalance of the gut microbiota and subsequently in the production of a large number of toxic metabolites and an induced inflammatory response. Newborns are susceptible to immune dysfunction, and gut microbiota dysfunction may increase the risk of related diseases in adulthood.

Necrotizing enterocolitis (NEC) is a catastrophic disease that is a major cause of mortality in preterm infants who survive the first few days after birth (4). Necrotizing enterocolitis occurs in 7% of infants born at <1,500 g and up to 5% of those admitted to the neonatal intensive care unit (5–8). Necrotizing enterocolitis is associated with high mortality (15–30%) and long-term neurodevelopmental morbidity (5, 9). Premature birth, formula feeding, abnormal bacterial colonization, and intestinal barrier dysfunction have been identified as the main etiological factors that substantially potentiate the risk of NEC (4). Among these, the damage to the intestinal barrier is considered to be the most predominant cause of NEC in neonates. Maintaining the integrity of the intestinal barrier helps protect against NEC (10). Hypoxia is one of the factors known to damage the intestinal barrier (11). As one of the major pathological factors for infant intestines suffering from NEC (12), hypoxia induces cell death, and deterioration of the integrity of the intestinal barrier, thereby reducing the restorative ability of crypt stem cells (13). In addition, hypoxia can sensitize the immune response in the gut, and aggravates inflammatory reactions of the mucosal barrier to the bacterial invasion (14). In fact, the excessive inflammatory response represents a major pathogenesis of NEC in preterm infants (15).

Intestinal microecology is the most complex microecosystem in the human body. It not only is involved in the digestion of food, the absorption of nutrients, and the synthesis of vitamins but also plays an important role in immune regulation (16). Gut microbiota dysbiosis is closely associated with localized gastroenterologic disorders (17). It can even further affect the normal development of the nervous system and respiratory system through the brain–gut axis and lung–gut axis (18, 19), especially in the neonatal period. In early life, the gut microbiota is dynamic and its development is susceptible to host and environmental factors (20). Host–microbe interactions influence the intestinal tract and immune system (21). Hypoxia is a potential influencer of microbiota development, and hypoxia might interfere with microbiota development, especially with the colonization of anaerobic bacteria. However, there are limited studies on whether intrauterine hypoxia induces NEC through the disturbance of the gut microbiota. Therefore, additional studies are needed to clarify the relationship between intrauterine hypoxia and the gut microbiota.

MATERIALS AND METHODS

Animals Model

All procedures strictly adhered to the National Institutes of Research guidelines on the ethical use of animals and were under license from the local ethics review committee of First Affiliated Harbin Medical University. Wistar rats were supplied by the Animal Experiment Center of Harbin Medical University and were raised in a clean-grade experimental environment. Animals were housed under a 12-h light/dark cycle (22 ± 2°C) and fed with a standard chow diet and water. The cages and water bottles were sterilized every day. All endeavors were made to minimize the sufferings of the animals involved in the experiment. Female and male Wistar rats at the age of 3 months were mated in cages at a ratio male-to-female ratio of 1:2. The female rats were judged to be pregnant by checking whether there was a pregnancy plug in the vagina or whether there were sperm in a vaginal secretion smear of the female rats obtained the next morning, which was taken as the 0th day of pregnancy. The pregnant rats were kept in a room with a temperature of 21°C, a humidity of 60%, and 12-h light and dark cycle. One group of rats was stimulated with hypoxia after conception to construct an intrauterine hypoxia model as the experimental group, and the other group was treated as the control group without any intervention after conception. Intestinal contents of neonates with intrauterine hypoxia and normal neonates within 24 h and 7 days after birth were collected, respectively, and were divided into four groups: the first-day of intrauterine hypoxia group (INH1), the seventh-day of intrauterine hypoxia group (INH7), the first-day of normal group (NOR1), and the seventh-day of normal group (NOR7).

Construction of an Intrauterine Hypoxia Model

The pregnant rats in the hypoxia group were placed in a plexiglass cage from the 15th to the 21st day of gestation, and an oxygen analyzer (Pro OX120:BioSpherix, New York, NY) adjusted the nitrogen gas and air mixture flow and real-time monitoring of oxygen percentage in the organic glass cage, keeping the oxygen concentration at (10 + 1) %, which lasted for 4 h per day; at the same time, we added calcium chloride in the organic glass to absorb the excess carbon dioxide and water vapor in order to control the humidity, avoiding carbon dioxide retention, and acidosis. Before exposure to hypoxia, pregnant rats were intubated in the femoral artery with a catheter consisting of an arterial catheter, a connecting tube, and a three-way valve. After successful intubation, the incision was covered with gauze, and the rats were exposed to hypoxia. During hypoxia, blood was collected every hour by adjusting the three-way valve to connect it with a syringe, and the collected blood was immediately added to a blood gas detection chip (EG7+:Abbott, Lake Bluff, IL). The chip was inserted into a portable blood gas analyzer (I-STAT 200:Abbott), Oxygen partial pressure, blood oxygen saturation, and pH were measured. The arterial partial pressure

of oxygen of pregnant rats was maintained at 50–55 mmHg, and the blood oxygen saturation was maintained at 80–85% (22–24). The pregnant rats in the control group were kept at room temperature with an oxygen concentration of 21% until natural delivery.

Sample Collection

A total of 18 intestinal samples were collected from newborn rats throughout the study, including four from the first-day of the intrauterine hypoxia group (INH1), five from the seventh-day of the intrauterine hypoxia group (INH7), four from the first-day of normal group (NOR1), and five from the seventh-day of normal group (NOR7). DNA was extracted from all 18 samples and sequenced successfully for analysis in the bioinformatics analysis program. Four newborn rats in the experimental group and control group were randomly selected, within 4 h after birth (with no feeding) and euthanized by the decapitation method. In a sterile environment, the colon content samples of newborn rats were removed with a sterile scalpel and washed with sterile distilled water. The intestinal contents were dug out with a sterile scalpel for preservation in a freezer storage tube and immediately frozen at −80°C until further analysis. The remaining rats in both groups were fed their mothers' milk until the seventh day, and the intestinal contents were obtained by the same method.

DNA Extraction and PCR Amplification

Microbial DNA was extracted from the colon contents of newborn rats, and DNA quality was checked by 1% agarose gel electrophoresis. The V3–V4 regions of the bacterial ribosomal RNA gene were amplified, the specific primers with barcode were synthesized, the PCR products were recovered by excision from the gel using a gel recovery kit, eluted by Tris-HCL buffer, detected by 2% agarose electrophoresis, and denatured by sodium hydroxide; single-stranded DNA fragments were generated; and a Miseq library was constructed.

Double-end sequencing was carried out using a Miseq platform (Illumina, Inc., CA, USA). According to the overlap relation between PE reads, paired reads were merged into a sequence, and quality control and filtration were carried out for read quality and merging effects at the same time. Barcode and primer sequences at both ends of the sequence were used to distinguish samples to obtain effective sequences and correct sequence directions. Reads below 50 bp after quality control were filtered, and N-base reads were removed for data optimization.

Bioinformatics Analysis

Based on 97% similarity of optimized sequences, operational taxonomic units (OTUs) of non-repetitive sequences (excluding single sequences) were clustered to obtain an OTU abundance table. The OTUs were flattened based on the minimum number of sequences in a sample (34,479), and the OTUs with the total number of sequences annotated to the bacterial domain level were $\geq$20 to ensure the accuracy of subsequent data analysis. Alpha diversity indexes (Chao, ACE, Shannon, Simpson, coverage) were calculated to evaluate the richness, diversity, and coverage of the sample microbial community and to analyze the difference between the indexes. Principal coordinate analysis (PCoA) based on the binary Jaccard distance algorithm and non-metric multidimensional scaling analysis (NMDS analysis) based on the binary Jaccard distance algorithm were used to study the similarity or difference of sample community compositions and check whether the difference between groups was significant. LEfSe software was used to estimate the impact of the abundance of each species on the discriminant effect by linear discriminant analysis (LDA). Significant differences in COG categories in the four groups were determined using LEfSe analysis.

Statistical Analysis

Student's *t*-test was used to analyze the differences between alpha diversity indexes. The Adonis test was used to analyze the difference between groups based on PCoA results groups. An ANOSIM test was used to analyze the difference between groups based on NMDS. Continuous variables are described as the means ± standard deviations unless otherwise stated. The Wilcoxon rank-sum test was used for species difference analysis between groups, and differences were considered significant when $P < 0.05$.

RESULTS

Intrauterine Hypoxia Decreased the Birth Weights of Neonatal Rats

All pregnant rats gave birth naturally, and the body weights of newborn rats were measured within 2 h, which were accurate to 0.01 g. The mean gestation time of the hypoxia group and control group was 21.5 days (21–22 days), and the mean numbers of offspring were not different. Body weight loss is a common consequence of intrauterine hypoxia in newborns in the clinic (25, 26). **Figure 1** shows the body weight data from each group. On the first day, the body weights of the INH1 (4 cases) and NOR1 (4 cases) groups were 6.380 ± 0.203 and 7.675 ± 0.263 g, respectively. On the seventh day, the body weights of the INH7 ($n = 5$) and NOR7 ($n = 5$) groups were 11.660 ± 0.615 and 15.460 ± 0.799 g, respectively. After intrauterine hypoxia intervention, rats in the hypoxia group weighed significantly less than rats in the normal group ($p < 0.05$) (**Figure 1**). These findings demonstrate that Wistar rats presented with typical characteristics of IUGR.

Species Annotation and Evaluation

The total number of effective sequences and effective bases was 956,588 and 409,549,652 bp in the sequencing results, respectively. The average length of the sequences was 428.16. High-quality sequences with lengths in the range of 421–440 accounted for 97.28% of the total sequences. The mean of effective sequences of the intrauterine hypoxia group was 54,468, which was higher than the mean of 49,819 in the normal group (**Figure 2A**). The dilution curve of the alpha diversity index plateaued as the amount of data extracted increased, indicating that the sequencing data volume was reasonable (**Figure 2B**).

OTU Analysis Results

A total of 17 phyla, 30 classes, 72 orders, 110 families, 181 genera, and 222 species were measured in the four groups, with a total of 253 OTUs. In the INH1 group, there were 17 phyla, 29 classes,

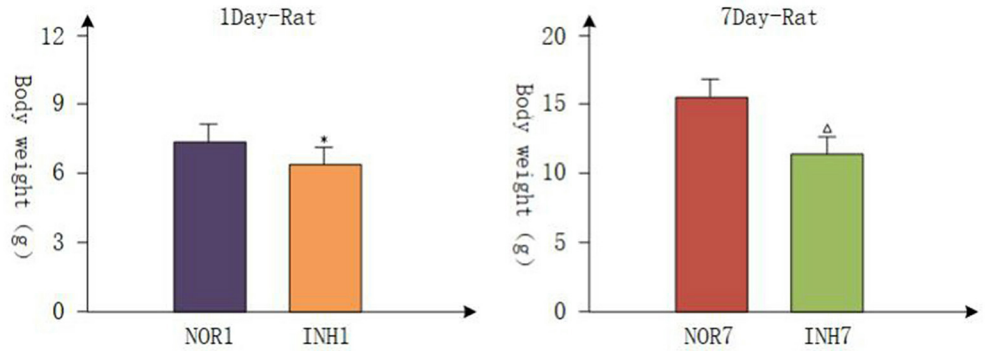

FIGURE 1 | NOR1 vs. INH1: *$P < 0.05$; NOR7 vs. INH7: Δ$P < 0.05$.

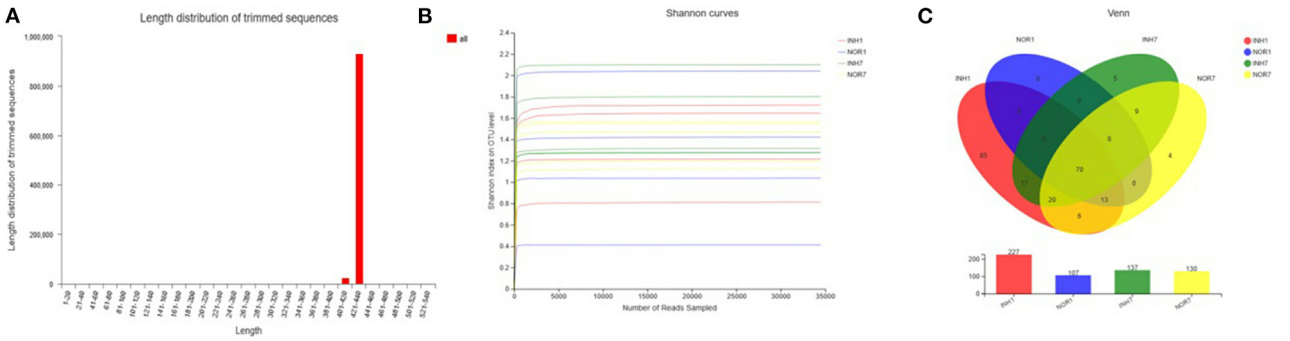

FIGURE 2 | (A) Sequence length distribution diagram. The X-axis represents the length interval, and the Y-axis represents the number of sequences. **(B)** Alpha-diversity Shannon index dilution curve, with the horizontal axis representing the amount of randomly selected sequencing data and the vertical axis representing the number of observed species. **(C)** A Venn diagram that can be used to count the number of common and unique species in multiple groups of samples, which can intuitively show the similarity and overlap of species composition in different environmental samples. Different colors represent different groups, overlapping numbers represent the number of species common to multiple groups, and non-overlapping numbers represent the number of species unique to the corresponding group.

69 orders, 100 families, 152 genera, 183 species, and 198 OTUs. In the INH7 group, there were 5 phyla, 12 classes, 23 orders, 39 families, 48 genera, 59 species, and 69 OTUs. In the NOR1 group, there were 4 phyla, 8 classes, 16 orders, 22 families, 26 genera, 34 species, and 39 OTUs. In the NOR7 group, there were 4 phyla, 8 classes, 17 orders, 29 families, 39 genera, 49 species, and 56 OTUs. The number of species in each classification level in the hypoxia groups was higher than that in the control groups. Among them, there were 85 unique OTUs in the INH1 group, 5 unique OTUs in the INH7 group, 0 unique OTUs in the NOR1 group, and 4 unique OTUs in the NOR7 group (**Figure 2C**).

Alpha Diversity Analysis

The mean values of the sobs, ACE, and Chao1 indexes reflecting community richness in the INH1 group were higher than those in the NOR1 group. The differences in the sobs and Chao indexes were significant ($P < 0.05$), as confirmed by Student's *t*-test. The mean values of the Shannon and Simpson indexes reflecting community diversity in the INH1 group were slightly higher than those in the NOR1 group ($P > 0.05$). Compared with those in the NOR7 group, the mean values of the sobs, ACE, Chao, and Simpson indexes in the INH7 group were slightly lower, while the mean values of the Shannon index were higher. There was no significant difference among the five indexes ($P > 0.05$). The mean value of the Shannon index of the INH1 group was slightly lower than that of the INH7 group, while the mean values of other indexes were higher than those of the INH7 group, and the difference in the Chao index was statistically significant ($P < 0.05$). There was no significant difference between the NOR1 group and the NOR7 group. The coverage indexes of all samples reflecting community coverage were 100%, indicating that the sequencing results could reflect the true microbial situation (**Figure 3**).

Analysis of Community Composition

The dominant microbiota constituents of the four samples at the phylum level were Firmicutes and Proteobacteria, followed by Actinomycetes and Bacteroidetes. The mean relative abundance of Firmicutes in the hypoxia groups was higher than that in the control groups, but the mean relative abundance of Proteobacteria was lower than that in the control groups. The difference was not statistically significant ($P > 0.05$) (**Figure 4A**). At the order level, the dominant groups were Lactobacillales and Enterobacteriales. In the INH1 group, the

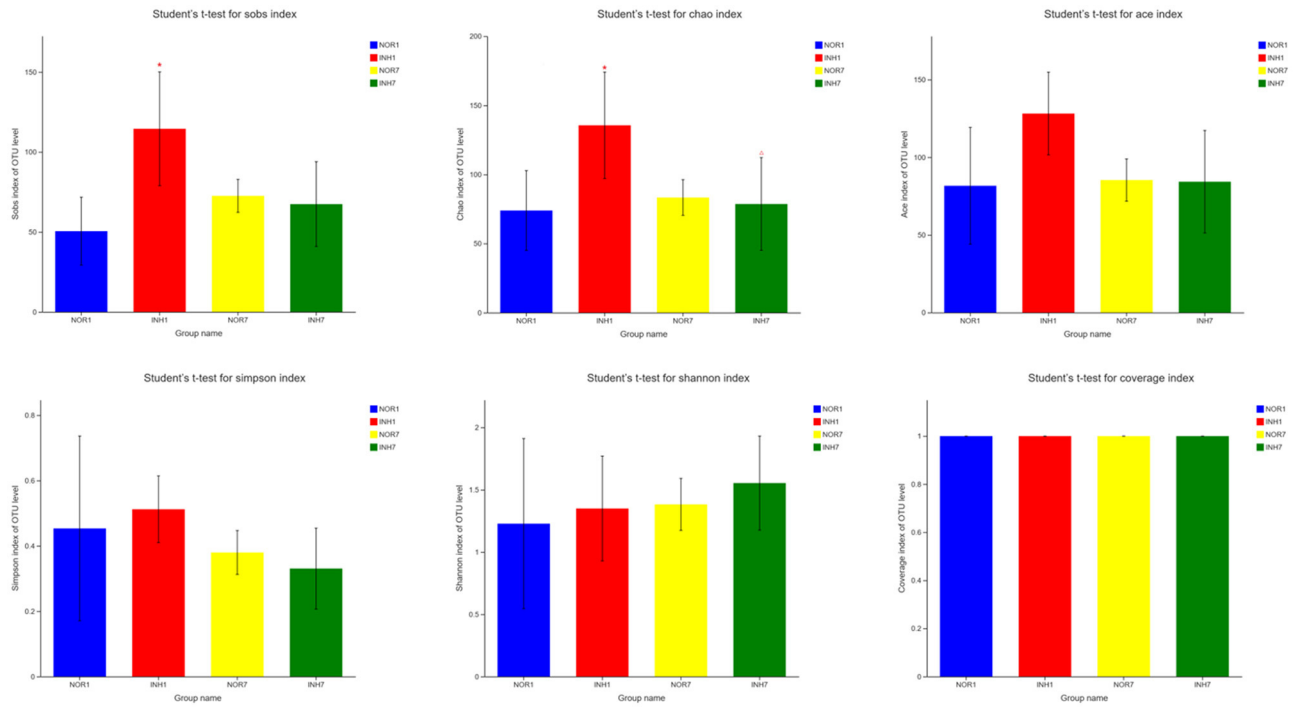

FIGURE 3 | Alpha diversity index test results. NOR1 vs. INH1: $^*P < 0.05$; INH1 vs. INH7: $\Delta P < 0.05$.

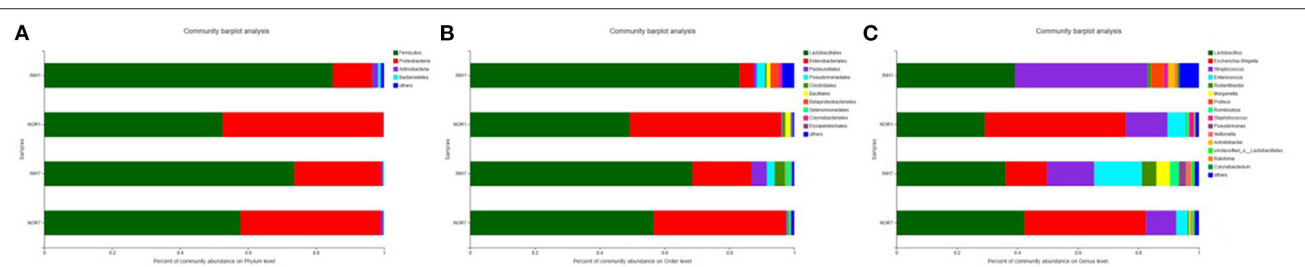

FIGURE 4 | (A) Bar plot of phylum-level community. **(B)** Bar plot of order-level community. **(C)** Bar plot of genus-level community. The vertical axis represents the sample name, the horizontal axis represents the proportion of species in the sample, the columns with different colors represent different species, and the length of the columns represents the proportion of the species.

abundance of Lactobacillales was significantly higher than that in NOR1, and the average relative abundance of Enterobacteriales was also significantly increased in the NOR1 group. In the INH7 group, the abundance of Enterobacteriales was lower than that in the INH1 group (**Figure 4B**). At the genus level, the dominant groups were Lactobacillus, Escherichia-Shigella, Streptococcus, and Enterococcus. In the INH1 group, the average relative abundances of Streptococcus and Lactobacillus were relatively high, and the abundances of Escherichia-Shigella and Enterococcus were significantly lower than those in the NOR1 group. In the INH7 group, the abundance of Escherichia-Shigella was lower than that in NOR7 but significantly increased compared with that in the INH1 group, and the average relative abundance of Enterococcus was also significantly increased compared with that in the INH1 group; its relative abundance was also higher than that in the NOR7 group (**Figure 4C**).

Analysis of Beta Diversity

Principal coordinate7 analysis was performed on the four groups of samples based on the binary Jaccard distance algorithm, namely, PCoA, to explore the similarity and difference in the community composition of the four groups. An Adonis test was used to examine the difference between groups. The results showed that the difference in the community composition of the four groups was greater than the difference within the group ($R^2 = 0.3238$), and the difference was statistically significant ($P < 0.05$) (**Figure 5A**). The INH1, NOR1, INH7, and NOR7 groups were subjected to NMDS analysis, namely, NMDS analysis, based on the binary Jaccard distance algorithm. ANOSIM was used for intergroup differences, showing that the differences in bacterial communities between groups were significant ($P < 0.05$) (**Figures 5B,C**).

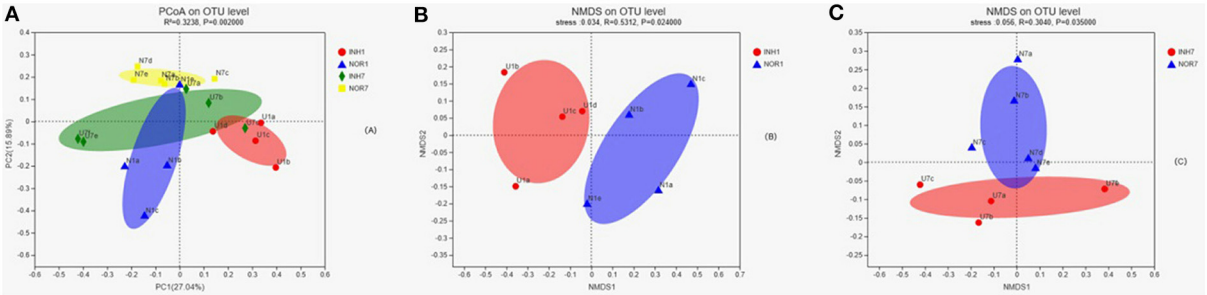

FIGURE 5 | (A) In PCoA, the X-axis and Y-axis represent the two selected principal coordinate axes, and the percentage represents the explanatory value of the principal coordinate axes to the difference in sample composition; the X-axis and Y-axis scale is a relative distance, with no practical significance. Points of different colors or shapes represent samples of different groups. The closer the two sample points are, the more similar the species composition of the two samples is. Panels **(B,C)** the results of NMDS analysis. Points with different colors or shapes represent samples of different groups. The closer the two sample points are, the more similar the species composition of the two samples is. Horizontal and vertical coordinates represent relative distances and have no practical significance. Stress is based on a test of the strengths and weaknesses of NMDS analysis results. It is generally believed that stress < 0.2 can be represented by a 2d NMDS point graph, which has certain explanatory significance. When stress < 0.1, it can be considered a good ranking; when stress < 0.05, it is well-represented.

Species Difference Analysis

The Wilcoxon rank-sum test was used to detect significant differences between groups at the phylum, order, and genus levels for the four groups. At the phylum level, intrauterine hypoxia modified the proportions of the two principal phyla (Proteobacteria and Firmicutes) of the gut microbiota with respect to those in the normal group, and the percentage of Firmicutes in the INH1 group reached 84.95%. The average relative abundance of Firmicutes in the two intrauterine hypoxia groups was higher than that in the control group. The dominant microbiota constituent of the INH1 group was Firmicutes, and the dominant microbiota constituent of the NOR1 group was Proteobacteria. Regarding the low-abundance phylum Patescibacteria, the INH1 and NOR1 groups exhibited a significant difference (**Figure 6A**). There was no statistically significant difference between the INH7 group and the NOR7 group (**Figure 6B**). The relative abundances of Firmicutes and Bacteroidetes and the ratio of Firmicutes to Bacteroidetes (F/B) of the INH7 group were higher than those of the INH1 group and higher than those of the NOR7 group. The ratio of Firmicutes to Bacteroidetes (F/B ratio) is a biomarker of dysbiosis (27). At the order level, the INH1 and NOR1 groups showed significantly different abundances of Flavobacteriales, which is a major taxonomic order observed in ambient water samples (**Figure 6C**). There were significant differences between the INH1 and INH7 groups in the abundances of three orders: Selenomonadales, Actinomycetales, and Coriobacteriales (**Figure 6D**). At the genus level, the dominant groups did not change: Lactobacillus, Escherichia-Shigella, Streptococcus, and Enterococcus. The INH1 and NOR1 groups showed significant differences in abundances of the genera Escherichia-Shigella, Ralstonia, and Pseudomonas ($p < 0.05$). In the NOR1 and NOR7 groups, Escherichia-Shigella showed clear enrichment (**Figure 6E**). The INH7 and NOR7 groups showed significant differences in the abundances of three genera: a high abundance of Escherichia-Shigella and a relatively low abundance of Veillonella and Actinomyces (**Figure 6F**). Streptococcus and Veillonella often coexist in the intestinal ecosystem. We noted that the Escherichia-Shigella abundance markedly decreased and that the Streptococcus abundance increased in the hypoxia groups.

Next, we used LEfSe multistage species difference discriminant analysis to detect species with significant differences in abundance in the four groups of samples and used LDA to evaluate the impact of each species on different effects. The results showed statistically significant differences among the following species: for the INH1 group: Ruminobacteriaceae, Delft bacteria, Flavobacteriales, Alteromonadales, Shewanellaceae, Muribaculaceae, etc.; for the INH7: unclassified Lactobacillales, Negativicutes, and Pseudomonadaceae; and for the NOR1 group: Enterobacteriales, Escherichia-Shigella. NOR7: Parasutterella, unclassified-Enterobacteriaceae, and Actinomycetales (**Figure 7A**). In the INH1 group, the enrichment of Muribaculaceae had the greatest impact on the difference between groups. Furthermore, Pseudomonadaceae had the most significant influence on the INH7 group. LEfSe analysis showed an increased distribution of Escherichia-Shigella in the NOR1 group. In the NOR7 group, Parasutterella and unclassified Enterobacteriaceae had the most significant influence on the difference between groups. Microbial community structures have not yet been well-grouped according to the different etiologies (**Figure 7B**).

COG Annotation and Analysis

We obtained COG family information through PICRUSt analysis to identify biologically significant differences in the four groups. The analysis provided insights into the functional properties of the gut microbiota. As shown in **Figure 8**, we found 23 different functional COG categories in the four groups. Of these categories, five functional COG categories were highly enriched in the NOR1 group, including unknown function [S], carbohydrate transport and metabolism [G], amino acid transport and metabolism [E], energy production and conversion [C], and translation, ribosomal structure, and biogenesis [J]. In contrast, the metagenomes of the INH1 group animals were enriched in unknown function [S]; translation, ribosomal structure, and biogenesis [J]; amino acid transport and metabolism [E];

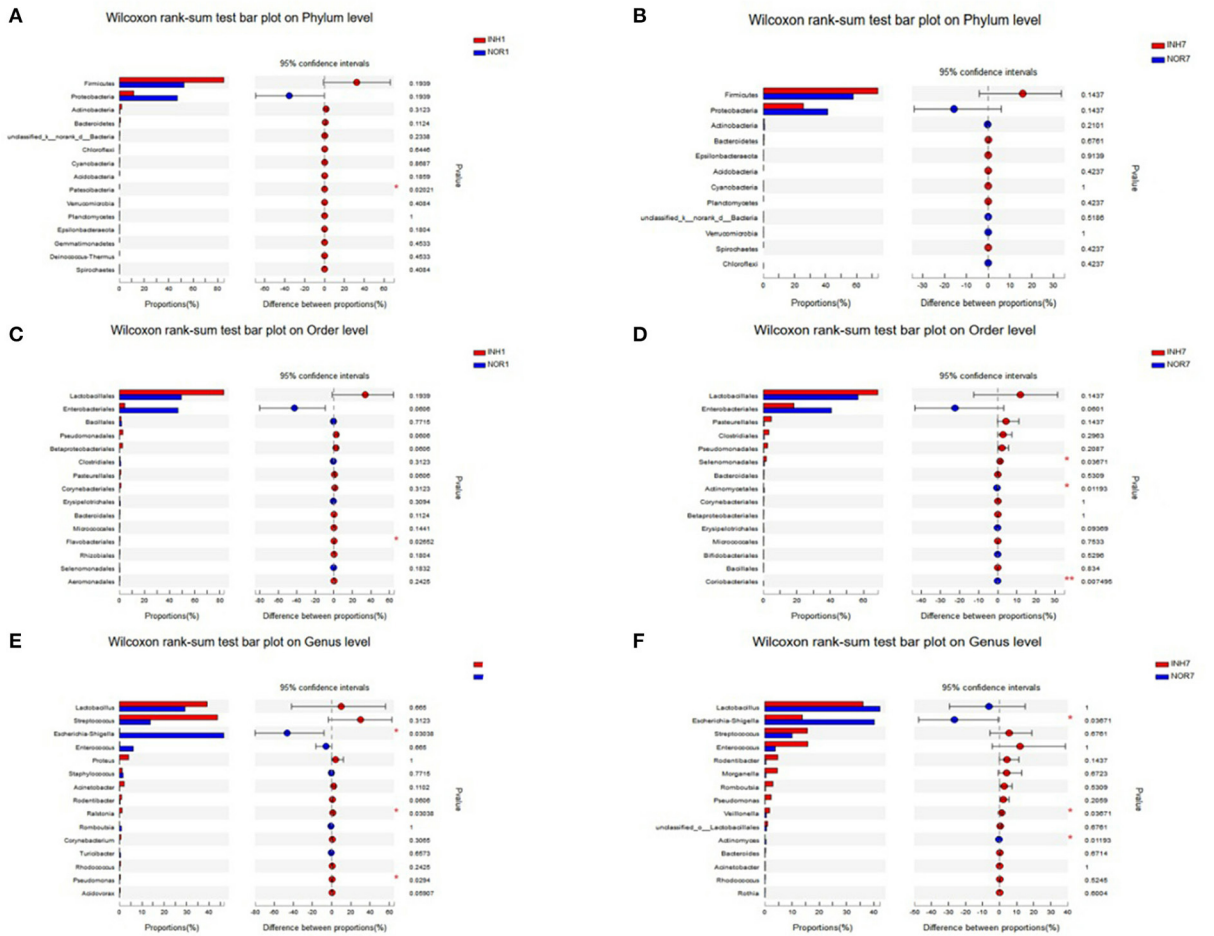

FIGURE 6 | The X-axis represents different groups, different colored boxes represent different groups, and the Y-axis represents the average relative abundance of a species in different groups. **(A,B)** Bar plot on Phylum level in different groups. **(C,D)** Bar plot on Order level in different groups. **(E,F)** Bar plot on Genus level in different groups.

carbohydrate transport and metabolism [G]; and transcription [K] (**Figure 8**). The metagenomes of the NOR7 group animals were enriched in unknown function [S]; carbohydrate transport and metabolism [G]; amino acid transport and metabolism [E]; translation, ribosomal structure, and biogenesis [J]; and inorganic ion transport and metabolism [P]. In the INH7 group, the enriched functional categories included unknown function [S]; carbohydrate transport and metabolism [G]; amino acid transport and metabolism [E]; translation, ribosomal structure, and biogenesis [J]; and transcription [K] (**Figure 8**). Notably, we found no significant difference in functional COG categories between the hypoxia group and the normal group.

DISCUSSION

Our results showed increased species richness and diversity of the gut microbiota on the INH1 in rats, and we identified several taxa that showed a significant difference in abundance between the hypoxia and control groups using *t*-tests and LEfSe analysis. These bacteria are closely linked to intestinal diseases.

The results show that no feeding newborn rats within 4 h after birth already contain diverse microbial communities, which is consistent with the latest viewpoint that initial microbiota colonization in the fetus before birth has already started (28, 29).

It is currently believed that an imbalance of the gut microbiota is associated with the occurrence of NEC, septicemia, bronchopulmonary dysplasia, and neurodevelopmental disorders (18, 19, 30, 31). Alpha diversity analysis was performed on the sequencing results for the gut microbiota of intrauterine hypoxia newborn rats, and the results showed that the diversity and richness of the gut microbiota in the first day of the intrauterine hypoxia group were higher than those of the control group, and the difference in richness was statistically significant. We can determine the reasons for this result from two aspects. First, pregnant rats were once fed milk containing Enterococcus carrying a gene marker, and Enterococcus containing the same gene marker was detected in fetal stool samples of their offspring (32), indicating that the gut microbiota of mothers is one

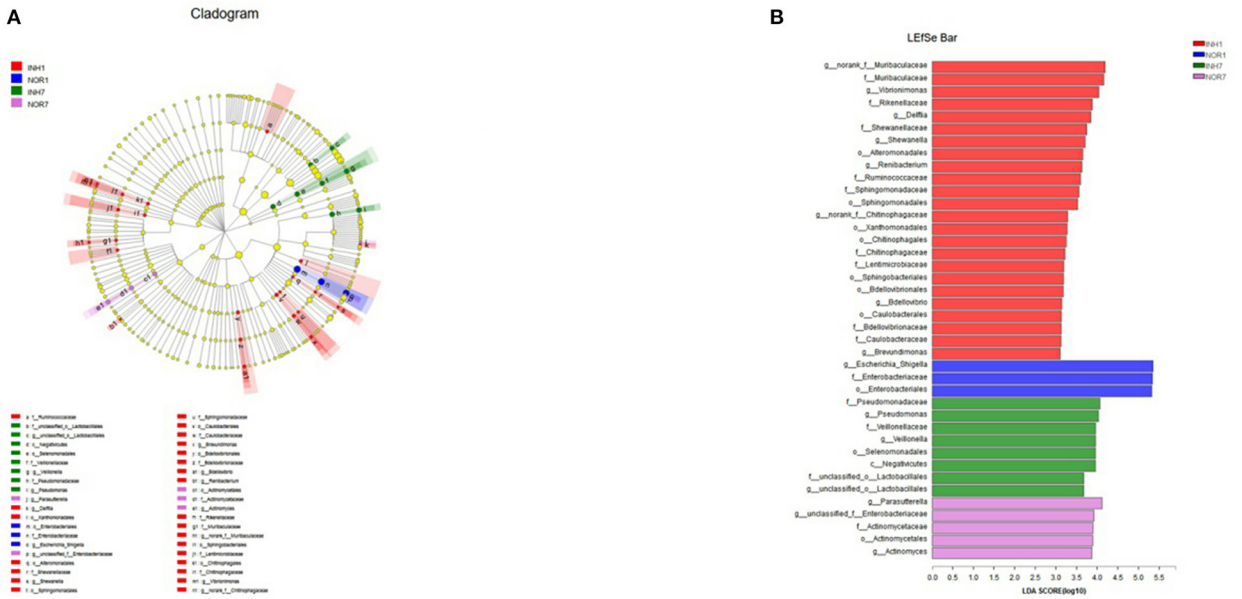

FIGURE 7 | (A) Different color nodes represent the microbial groups that are enriched significantly in the corresponding groups and have a significant influence on the difference between the groups. The light yellow nodes represent the microbial groups that have no significant difference in different groups or have no significant influence on the difference between groups. **(B)** The LDA discriminant histogram shows the counts of the microbial communities with significant effects in multiple groups based on the LDA score obtained through LDA analysis (linear regression analysis). The larger the LDA score is, the greater the influence of species abundance on the different effects.

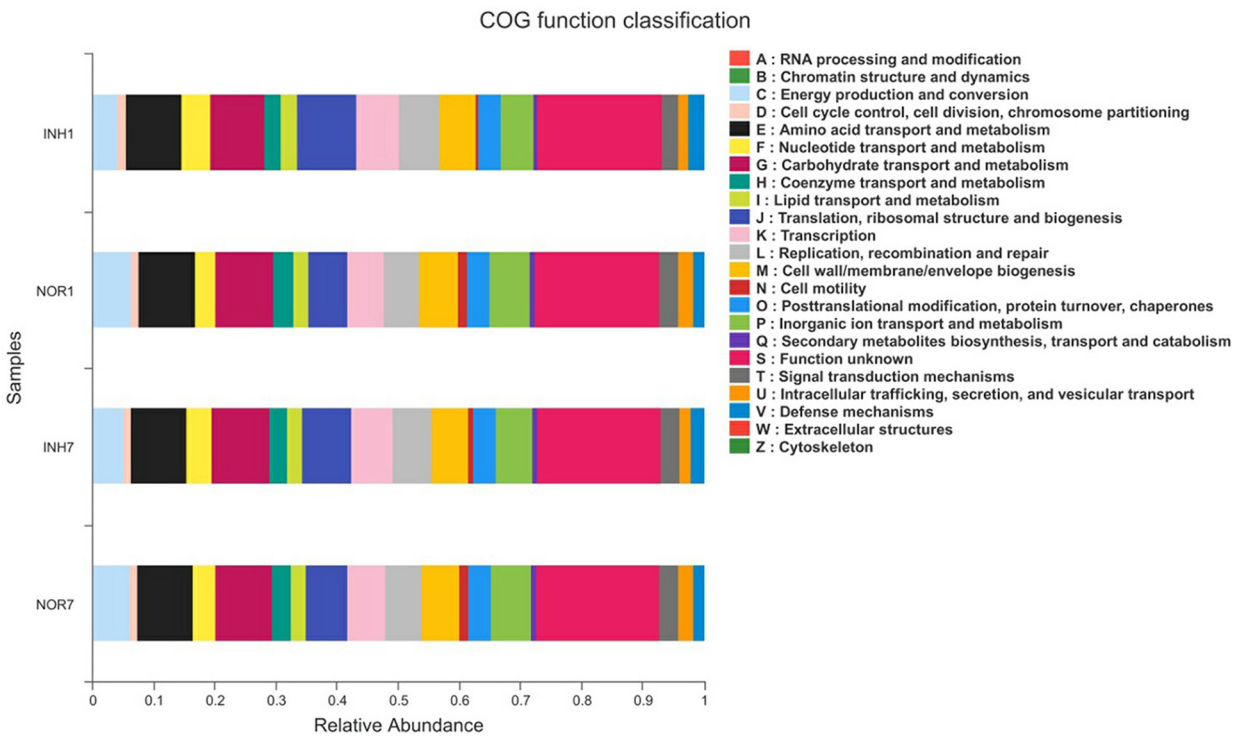

FIGURE 8 | The X-axis represents the average relative abundance of a function in different groups, and different colors represent different functions. The Y-axis represents different groups.

of the potential sources of the gut microbiota of newborns. In our study, the pregnant rats after hypoxia induction may have produced blood by the "diving reflex," diverting blood from the unimportant organs such as the bowel to vital organs (33), leading to intestinal mucosal damage and permeability enhancement and damaging the normal intestinal barrier protection function; intestinal bacteria are more likely to prompt matrix deposition through the intestinal epithelium, which involved in the vertical transfer of microorganisms in the womb, eventually transferring them to the newborn baby rat intestine. Thus, in the intestinal contents of the offspring, the observed abundance and diversity of the flora was higher than that in the control group animals. Additionally, studies have suggested that the neonatal gut microbiota is associated with swallowing amniotic fluid during fetal life (34, 35). A study on microbial molecular analysis of the amniotic fluid of premature infants found that the concentration of bacterial rDNA in the amniotic fluid was negatively correlated with the gestational age at delivery (36). Since gestational age is the best indicator to measure maturity (33), according to the results of the study, it can be speculated that the microbial community richness of the amniotic fluid is negatively correlated with fetal maturity, which could explain why on the first day of intrauterine hypoxia in this experimental group of newborn rats, the gut microbiota richness of the contents was higher than that in the control group because a change in richness is caused by a lack of oxygen with premature birth, as the system development is not mature.

Another interesting finding of this study was that after 7 days of suckling, the differences in the abundance and diversity of the INH7 and NOR7 group microbiota were not as significant as the differences between the two groups on the first day. The abundance of Streptococcus in the INH7 group was significantly lower than that in the INH1 group but still higher than that in the NOR7 group, while the abundance of Enterococcus and Escherichia increased significantly, and the common proportion of the three was close to that of Streptococcus in the INH1 group. Therefore, it can be inferred that there may be a competitive relationship among Streptococcus, Enterococcus, and Escherichia. The abundance and diversity of the microflora of neonatal rats with intrauterine hypoxia were closer to those of normal neonatal rats after suckling. We hypothesized that this was mainly due to a decrease in pathogenic bacteria with a higher abundance, which is consistent with previous studies showing that suckling can deplete harmful bacteria and enrich beneficial bacteria with a lower abundance. Suckling not only contributes to the formation of neonatal immune defense and reduces the colonization of pathogenic microorganisms but also induces the colonization of symbiotic organisms, thereby regulating intestinal inflammation to reduce intestinal damage and the risk of NEC (6, 37, 38). Breastfeeding is the most effective strategy to protect infants against NEC (6). Human milk oligosaccharides are non-digestible carbohydrates that are thought to influence the gastrointestinal microbiome and possibly protect against NEC (39, 40). In a single-center feeding study with a large sample size of 464 patients, the incidence of NEC in ELBW infants was found to be 1%, with only two deaths. This result was strongly associated with widespread breastfeeding and adherence to standardized feeding strategies (41). A retrospective study of breastfeeding in 550 very-low-birth-weight infants in the United States showed that continued breastfeeding significantly reduced the incidence of NEC in preterm infants (42).

Metagenomic sequencing has been widely applied in the comprehensive analysis of the relationships between microbial function and host physiology. We observed dynamic shifts in the compositions and functions of gut microbes and fecal metabolites in rats. The abundance of the INH1 group was higher than that of the NOR1 group in four functions, including chromatin structure and dynamics [B]; defense mechanisms [V]; cell motility [N]; and cytoskeleton [Z]. The data indicate that these molecular functions and biological processes might be implicated in the progression of various intrauterine hypoxia-induced complications. After 7 days, the functional differences between the two groups were narrowed, and we speculated that the gut microbiota of the rats after feeding tended to be consistent due to the influence of the diet and environment.

The results of species composition analysis showed that in the four groups of intestinal content samples, at the phylum level, the dominant gut microbiota constituents were Firmicutes and Proteobacteria, followed by Actinomycetes and Bacteroidetes, which was consistent with previous research results (43, 44). The mean relative abundance of Firmicutes in the two intrauterine hypoxia groups was higher than that in the two control groups, and the abundance proportion of Firmicutes in the INH1 group, in particular, reached 84.95%. The abundance of Proteobacteria in the NOR1 group was significantly higher than that in the INH1 group, and the ratio of Firmicutes to Bacteroidetes in the INH1 group was lower than that in the NOR1 group. After 7 days of dynamic observation, the gap between Firmicutes and Bacteroidetes abundances in the INH7 and NOR7 groups was narrowed, and the ratio of Firmicutes to Bacteroidetes in the INH7 group was higher than that in the INH1 group and that in the NOR7 group. Relevant studies have found that the ratio of Firmicutes to Bacteroidetes can affect the production of short-chain fatty acids. When the ratio increases, it can lead to a decrease in butyrate concentration in the body, promote the body's inflammatory response and destroy the integrity of the intestinal epithelial barrier, thus triggering an autoimmune response (45). Studies on inflammatory bowel disease, metabolic syndrome, and autism have found that the ratio of Firmicutes to Bacteroidetes has increased (46–48), suggesting that neonates with intrauterine hypoxia have an increased risk of these diseases in childhood and adulthood. At the genus level, the INH1 group microbiota was mainly composed of Streptococcus and Lactobacillus, among which a high proportion of Streptococcus may be one of the reasons for the susceptibility of neonates with intrauterine hypoxia to infection. The top three dominant strains of the NOR1 group were Escherichia, Lactobacillus, and Streptococcus, among which there were five species of the Escherichia genus. *Escherichia coli*, one of the common normal bacteria in the human intestinal tract, is involved in the synthesis of vitamin B and vitamin K. It is normally not pathogenic. Under certain conditions, some serotypes can cause gastrointestinal symptoms such as diarrhea and occasionally can also cause intestinal symptoms such as urinary tract infection. *Escherichia coli* is an important contributor to the initial colonization of the

gut microbiota in normal newborns and plays an important role in the later colonization of Bifidobacteria. Additionally, it has been found that oral non-pathogenic *E. coli* strains (O83 and Nissle 1917) can induce local mucosal and systemic immunity, resulting in increased specific antibody levels detected in neonatal feces, saliva, and serum (49) and reducing the possibility of neonatal infection. Therefore, the low immune function of neonates with intrauterine hypoxia may be related to the lack of *E. coli* in the initial colonizing community. In the INH1 group, the abundance of gram-negative bacilli, such as Acinetobacter, Ralstonia, Pseudomonas, and Proteus, was higher than that in the NOR1 group. This enrichment may lead to injury of intestinal epithelial cells and increased mucosal permeability, and a large amount of lipopolysaccharide (LPS) can subsequently enter the blood to activate TLR4, which leads to the emergence of NEC (50, 51). Intestinal hemodynamic disorder caused by intrauterine hypoxia is the main pathophysiological factor that leads to NEC. Current studies have shown that perinatal hypoxia and ischemia processes and inflammation are the origin points of fetal intestinal injury causing NEC, and "hypoxic-ischemic enterocolitis (HIEnt)" has been proposed to define and distinguish this unique clinical entity (52). These opportunistic pathogens also increase the risk of sepsis in newborns (53, 54). After a 7-day observation period of suckling, the abundance of Streptococcus in the INH7 group was significantly lower than that in the INH1 group but still higher than that in the NOR7 group, while that of Enterococcus and *E. coli* was significantly increased, and the proportion of the three was similar to that of Streptococcus in the INH1 group. Therefore, it can be speculated that there may be a competitive relationship among Streptococcus, Enterococcus, and *E. coli*. Lactobacillus was the dominant gut microbiota constituent in the four groups of samples, further proving that vertical transmission of the maternal vaginal microbiota is one of the main sources of initial colonization of the neonatal gut microbiota (55).

In conclusion, intrauterine hypoxia affects the initial colonization of the gut microbiota in neonates to some extent, and neonates with intrauterine hypoxia have a higher risk of septicemia, NEC, infectious diseases, autoimmune diseases, inflammatory bowel disease, metabolic syndrome, and autism in childhood and adulthood than normal neonates. Although the microbial community of the INH7 group was significantly different from that of the INH1 group after suckling, it was still significantly different from that of the NOR7 group regarding the genera Escherichia-Shigella, Veillonella, and Actinomyces. In addition, the observation period of suckling in the present study was only 7 days, so the possibility of long-term influence cannot be ruled out.

In our study, the effect of intrauterine hypoxia on the intestinal microecology of neonatal rats in the early stage was investigated through high-throughput sequencing technology and a variety of bioinformatics analyses, providing a new idea for understanding the pathogenesis of related diseases of neonates with intrauterine hypoxia at birth, childhood, and adulthood. After abnormal colonization and proliferation of intestinal bacteria, the microbiota constituents participate in the pathophysiological process of NEC by generating metabolites and bacterial components and inducing an immune response. However, the specific mechanism by which gut microbiota dysregulation affects the development of NEC is still unclear and requires further research and exploration. The study of gut microbiota disorders provides a new idea for the early diagnosis of NEC, and microbiome optimization and correction of disorders provide a new strategy for the early prevention and treatment of NEC. As factors such as breastfeeding and intrauterine hypoxia are associated with NEC, further studies are needed to uncover the true contribution of these factors to microbiota development. Microbial dysbiosis preceding NEC in preterm infants is characterized by increased relative abundances of Proteobacteria and decreased relative abundances of Firmicutes and Bacteroidetes (56). Microbiome optimization may provide a novel strategy for preventing NEC.

Disturbances in microbiota development have been related to the development of disorders in the adult period. Our data indicate that intrauterine hypoxia could greatly influence health throughout life by interfering with gut microbiota development. In summary, intrauterine hypoxia may increase the risk of NEC by changing the colonization of the gut microbiota at birth. Babies born with intrauterine hypoxia require a more cautious feeding strategy and monitoring of abdominal conditions.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the SRA datebase repository, accession number (PRJNA706401).

ETHICS STATEMENT

The animal study was reviewed and approved by Local ethics review Committee of Harbin Medical University.

AUTHOR CONTRIBUTIONS

CJ designed the study. YS, LL, JS, WM, and KX performed the experiments and analyzed the data. YS and LL wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by Health Commission of Heilongjiang Province (2020-105).

ACKNOWLEDGMENTS

The authors thank the research team of Professor Yajun Zhao of Harbin Medical University for their instruction regarding the animal model.

REFERENCES

1. Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant gut microbiota. *PLoS Biol.* (2007) 5:e177. doi: 10.1371/journal.pbio.0050177
2. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, et al. Succession of microbial consortia in the developing infant gut microbiome. *Proc Natl Acad Sci USA*. (2011) 108 (Suppl 1):4578–85. doi: 10.1073/pnas.1000081107
3. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. *Nature*. (2012) 486:222–7. doi: 10.1038/nature11053
4. Neu J, Walker WA. Necrotizing enterocolitis. *N Engl J Med.* (2011) 364:255–64. doi: 10.1056/NEJMra1005408
5. Lin PW, Stoll BJ. Necrotising enterocolitis. *Lancet*. (2006) 368:1271–83. doi: 10.1016/S0140-6736(06)69525-1
6. Fanaroff AA, Stoll BJ, Wright LL, Carlo WA, Ehrenkranz RA, Stark AR, et al. Breast milk protects against the development of necrotizing enterocolitis through inhibition of Toll-like receptor 4 in the intestinal e pithelium via activation of the epidermal growth factor receptor. *Mucosal Immunol.* (2015) 8:1166–79. doi: 10.1038/mi.2015.30
7. Lemons JA, Bauer CR, Oh W, Korones SB, Papile LA, Stoll BJ, et al. Very low birth weight outcomes of the National Institute of Child health and human development neonatal research network, January 1995 through December 1996. NICHD Neonatal Research Network. *Pediatrics.* (2001) 107:E1. doi: 10.1542/peds.107.1.e1
8. Holman RC, Stoll BJ, Curns AT, Yorita KL, Steiner CA, Schonberger LB. Necrotising enterocolitis hospitalisations among neonates in the United States. *Paediatr Perinat Epidemiol.* (2006) 20:498–506. doi: 10.1111/j.1365-3016.2006.00756.x
9. Stoll BJ, Hansen NI, Adams-Chapman I, Fanaroff AA, Hintz SR, Vohr B, et al. National Institute of Child Health and Human Development Neonatal Research Network. Neurodevelopmental and growth impairment among extremely low-birth-weight infants with neonatal infection. *JAMA*. (2004) 292:2357–65. doi: 10.1001/jama.292.19.2357
10. Anand RJ, Leaphart CL, Mollen KP, Hackam DJ. The role of the intestinal barrier in the pathogenesis of necrotizing enterocolitis. *Shock*. (2007) 27:124–33. doi: 10.1097/01.shk.0000239774.02904.65
11. Manresa MC, Taylor CT. Hypoxia Inducible Factor (HIF) Hydroxylases as regulators of intestinal epithelial barrier function. *Cell Mol Gastroenterol Hepatol.* (2017) 3:303–15. doi: 10.1016/j.jcmgh.2017.02.004
12. Baregamian N, Rychahou PG, Hawkins HK, Evers BM, Chung DH. Phosphatidylinositol 3-kinase pathway regulates hypoxia-inducible factor-1 to protect from intestinal injury during necrotizing enterocolitis. *Surgery*. (2007) 142:295–302. doi: 10.1016/j.surg.2007.04.018
13. Chen Y, Lee SH, Tsai YH, Tseng SH. Ischemic preconditioning increased the intestinal stem cell activities in the intestinal crypts in mice. *J Surg Res.* (2014) 187:85–93. doi: 10.1016/j.jss.2013.10.001
14. Haddad JJ, Harb HL. Cytokines and the regulation of hypoxia-inducible factor (HIF)-1α [published correction appears in *Int Immunopharmacol.*]. *Int Immunopharmacol.* (2005) 5:461–83. doi: 10.1016/j.intimp.2004.11.009
15. Nanthakumar N, Meng D, Goldstein AM, Zhu W, Lu L, Uauy R, et al. The mechanism of excessive intestinal inflammation in necrotizing enterocolitis: an immature innate immune response. *PLoS ONE.* (2011) 6:e17776. doi: 10.1371/journal.pone.0017776
16. Kigbu A, Orimadegun, AE, Tongo OO, Odaibo GN, Olaleye DO, et al. Intestinal bacterial colonization in the first 2 weeks of life of Nigerian neonates using standard culture methods. *Front Pediatr.* (2016) 4:139. doi: 10.3389/fped.2016.00139
17. Lynch SV, Pedersen O. The human intestinal microbiome in health and disease. *N Engl J Med.* (2016) 375:2369–79. doi: 10.1056/NEJMra1600266
18. Dong T, Chen T, White RA, Wang X, Hu W, Liang Y, et al. Meconium microbiome associates with the development of neonatal jaundice. *Clin Transl Gastroenterol.* (2018) 9:182. doi: 10.1038/s41424-018-0048-x
19. Tirone C, Pezza L, Paladini A, Tana M, Aurilia C, Lio A, et al. Gut and lung microbiota in preterm infants: immunological modulation and implication in neonatal outcomes. *Front Immunol.* (2019) 10:2910. doi: 10.3389/fimmu.2019.02910
20. Rodriguez C, Taminiau B, Brévers B, Avesani V, Van. Broeck J, Leroux A, et al. Faecal microbiota characterisation of horses using 16 rDNA barcoded pyrosequencing, and carriage rate of *Clostridium difficile* at hospital admission. *BMC Microbiol.* (2015) 15:181. doi: 10.1186/s12866-015-0514-5
21. Martin R, Nauta AJ, Ben Amor K, Knippels LM, Knol J, Garssen J. Early life: gut microbiota and immune development in infancy. *Benef Microbes*. (2010) 1:367–82. doi: 10.3920/BM2010.0027
22. Chen F, Du S, Bian J, You ZB, Wu Y. Chronic hypoxia exposure during pregnancy is associated with a decreased active nursing activity in mother and an abnormal birth weight and postnatal growth in offspring of rats. *Horm Behav*. (2012) 61:504–11. doi: 10.1016/j.yhbeh.2012.01.009
23. Xue Q, Zhang L. Prenatal hypoxia causes a sex-dependent increase in heart susceptibility to ischemia and reperfusion injury in adult male offspring: role of protein kinase C epsilon. *J Pharmacol Exp Ther*. (2009) 330:624–32. doi: 10.1124/jpet.109.153239
24. Li G, Xiao Y, Estrella JL, Ducsay CA, Gilbert RD, et al. Effect of fetal hypoxia on heart susceptibility to ischemia and reperfusion injury in the adult rat. *J Soc Gynecol Investig.* (2003) 10:265–74. doi: 10.1016/s1071-5576(03)00074-1
25. Vrijens K, Tsamou M, Madhloum N, Gyselaers W, Nawrot TS. Placental hypoxia-regulating network in relation to birth weight and ponderal index: the ENVIRONAGE Birth Cohort Study. *J Transl Med.* (2018) 16:2. doi: 10.1186/s12967-017-1375-5
26. Vannucci RC. Hypoxic-ischemic encephalopathy. *Am J Perinatol*. (2000) 17:113–20. doi: 10.1055/s-2000-9293
27. Yang T, Santisteban MM, Rodriguez V, Li E, Ahmar N, Carvajal JM, et al. Gut dysbiosis is linked to hypertension. *Hypertension*. (2015) 65:1331–40. doi: 10.1161/HYPERTENSIONAHA.115.05315
28. Neu J. The microbiome during pregnancy and early postnatal life. *Semin Fetal Neonatal Med*. (2016) 21:373–9. doi: 10.1016/j.siny.2016.05.001
29. Satokari R, Grönroos T, Laitinen K, Salminen, S, Isolauri E. Bifidobacterium and Lactobacillus DNA in the human placenta. *Lett Appl Microbiol*. (2009) 48:8–12. doi: 10.1111/j.1472-765X.2008.02475.x
30. Lu J, Claud EC. Connection between gut microbiome and brain development in preterm infants. *Dev Psychobiol*. (2019) 61:739–51. doi: 10.1002/dev.21806
31. Neu J. Necrotizing enterocolitis. *World Rev Nutr Diet*. (2014) 110:253–63. doi: 10.1159/000358474
32. Jiménez E, Marín ML, Martín R, Odriozola JM, Olivares M, Xaus J, et al. Is meconium from healthy newborns actually sterile. *Res Microbiol*. (2008) 159:187–93. doi: 10.1016/j.resmic.2007.12.007
33. Regev RH, Reichman B. Prematurity and intrauterine growth retardation–double jeopardy. *Clin Perinatol.* (2004) 31:453–73. doi: 10.1016/j.clp.2004.04.017
34. Collado MC, Rautava S, Aakko J, Isolauri E, Salminen S. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. *Sci Rep.* (2016) 22 6:23129. doi: 10.1038/srep23129
35. Ardissone AN, de la Cruz DM, Davis-Richardson AG, Rechcigl KT, Li N, Drew JC, et al. Meconium microbiome analysis identifies bacteria correlated with premature birth. *PLoS ONE.* (2014) 9:e90784. doi: 10.1371/journal.pone.0090784
36. DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gotsch F, et al. Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. *PLoS ONE.* (2008) 3:e3056. doi: 10.1371/journal.pone.0003056
37. Sun J, Li Y, Pan X, Nguyen DN, Brunse A, Bojesen AM, et al. Human milk fortification with bovine colostrum is superior to formula-based fortifiers to prevent gut dysfunction, necrotizing enterocolitis, and systemic infection in preterm pigs. *J Parenter Enteral Nutr.* (2019) 43:252–62. doi: 10.1002/jpen.1422
38. Frost BL, Modi BP, Jaksic T, Caplan MS. New medical and surgical insights into neonatal necrotizing enterocolitis: a review. *JAMA Pediatr*. (2017) 171:83–8. doi: 10.1001/jamapediatrics.2016.2708
39. Ruiz-Palacios GM, Cervantes LE, Ramos P, Chavez-Munguia B, Newburg DS. *Campylobacter jejuni* binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. *J Biol Chem*. (2003) 278:14112–20. doi: 10.1074/jbc.M207744200

40. Newburg DS. Neonatal protection by an innate immune system of human milk consisting of oligosaccharides and glycans. *J Anim Sci*. (2009) 87:26–34. doi: 10.2527/jas.2008-1347
41. Murthy S, Parker PR, Gross SJ. Low rate of necrotizing enterocolitis in extremely low birth weight infants using a hospital-based preterm milk bank. *J Perinatol*. (2019) 39:108–14. doi: 10.1038/s41372-018-0235-3
42. Chowning R, Radmacher P, Lewis S, Serke L, Pettit N, Adamkin DH. A retrospective analysis of the effect of human milk on prevention of necrotizing enterocolitis and postnatal growth. *J Perinatol*. (2016) 36:221–4. doi: 10.1038/jp.2015.179
43. Milani C, Duranti S, Bottacini F, Casey E, Turroni F, Mahony J, et al. The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. *Microbiol Mol Biol Rev*. (2017) 81:e00036-e00017. doi: 10.1128/MMBR.00036-17
44. Del Chierico F, Vernocchi P, Petrucca A, Paci P, Fuentes S, Praticò G, et al. Phylogenetic and metabolic tracking of gut microbiota during perinatal development. *PLoS ONE*. (2015) 10:e0137347. doi: 10.1371/journal.pone.0137347
45. Segain JP, Raingeard de la Blétière D, Bourreille A, Leray V, Gervois N, Rosales C, et al. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. *Gut*. (2000) 47:397–403. doi: 10.1136/gut.47.3.397
46. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci USA*. (2007) 104:13780–5. doi: 10.1073/pnas.0706625104
47. Remely M, Aumueller E, Jahn D, Hippe B, Brath H, Haslberger AG. Microbiota and epigenetic regulation of inflammatory mediators in type 2 diabetes and obesity. *Benef Microbes*. (2014) 5:33–43. doi: 10.3920/BM2013.006
48. Strati F, Cavalieri D, Albanese D, De Felice C, Donati C, Hayek J, et al. New evidences on the altered gut microbiota in autism spectrum disorders. *Microbiome*. (2017) 5:24. doi: 10.1186/s40168-017-0242-1
49. Cukrowska B, LodInová-ZádnIková R, Enders C, Sonnenborn U, Schulze J, Tlaskalová-Hogenová H. Specific proliferative and antibody responses of premature infants to intestinal colonization with nonpathogenic probiotic *E. coli* strain Nissle 1917. *Scand J Immunol*. (2002) 55:204–9. doi: 10.1046/j.1365-3083.2002.01005.x
50. Sodhi CP, Neal MD, Siggers R, Sho S, Ma C, Branca MF, et al. Intestinal epithelial Toll-like receptor 4 regulates goblet cell development and is required for necrotizing enterocolitis in mice. *Gastroenterology*. (2012) 143:708.e5–18.e5. doi: 10.1053/j.gastro.2012.05.053
51. Carlisle EM, Poroyko V, Caplan MS, Alverdy JA, Liu D. Gram negative bacteria are associated with the early stages of necrotizing enterocolitis. *PLoS ONE*. (2011) 6:e18084. doi: 10.1371/journal.pone.0018084
52. Surmeli OO, Korkmaz A, Yigit S, Yurdakok M. Hypoxic-ischemic enterocolitis: a proposal of a new terminology for early NEC or NEC-like disease in preterm infants, a single-center prospective observational study. *Eur J Pediatr*. (2020) 179:561–70. doi: 10.1007/s00431-019-03539-w
53. Souza DC, Palmeiro JK, Maestri AC, Cogo LL, Rauen CH, Graaf ME, et al. *Ralstonia mannitolilytica* bacteremia in a neonatal intensive care unit. *Rev Soc Bras Med Trop*. (2018) 51:709–11. doi: 10.1590/0037-8682-0118-2018
54. Li JY, Chen SQ, Yan YY, Hu YY, Wei J, Wu QP, et al. Identification and antimicrobial resistance of pathogens in neonatal septicemia in China-a meta-analysis. *Int J Infect Dis*. (2018) 71:89–93. doi: 10.1016/j.ijid.2018.04.794
55. Jašarević E, Howerton CL, Howard CD, Bale TL. Alterations in the vaginal microbiome by maternal stress are associated with metabolic reprogramming of the offspring gut and brain. *Endocrinology*. (2015) 156:3265–76. doi: 10.1210/en.2015-1177
56. Mai V, Young CM, Ukhanova M, Wang X, Sun Y, Casella G, et al. Fecal microbiota in premature infants prior to necrotizing enterocolitis. *PLoS ONE*. (2011) 6:e20647. doi: 10.1371/journal.pone.0020647

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

frontiers in Pediatrics

ORIGINAL RESEARCH
published: 03 May 2021
doi: 10.3389/fped.2021.657349

Necrotizing Enterocolitis and Intestinal Microbiota: The Timing of Disease and Combined Effects of Multiple Species

Xin Fu[1†], *Siwen Li*[1†], *Yanfang Jiang*[2], *Xintong Hu*[2] *and Hui Wu*[1*]

[1] Department of Neonatology, The First Hospital of Jilin University, Changchun, China, [2] Gene Diagnosis Center, The First Hospital of Jilin University, Changchun, China

Background: The purpose of this study was to investigate the relationship between intestinal microbiota and necrotizing enterocolitis (NEC).

Methods: 16S rRNA gene sequencing was used to compare the microbial composition of feces. The first sample was collected within 48 h after birth, then once per week until the NEC diagnosis, and finally 1–2 weeks after treatment or 28 days after birth.

Results: The alpha diversity of the microbiota in the NEC group was higher than that in the control group. Beta diversity analysis showed that the control group had a higher similarity at the onset of NEC, while the NEC group was distributed in subgroups. Linear discriminant analysis effect size and taxonomic composition analyses indicated that the abundance of Bacteroides and Actinobacteria in NEC infants at birth was much higher than that in the control group, and this trend continued until NEC occurred. At this time, Rhizobiales, Dysgonomonas, Ochrobactrum, Ralstonia, Pelomonas, Acinetobacter, etc., were also more abundant in NEC infants. The upregulated different metabolic pathways in the NEC group were mainly concentrated on degradation/utilization/assimilation, biosynthesis, and generation of precursor metabolites and energy.

Conclusions:

1. The microbial community differs according to the time of NEC diagnosis (bounded by 20 days).
2. No single microorganism is related to NEC, and the combined effect of multiple species is of great significance in the occurrence of NEC. Premature infants are easily affected by bacteria living in the environment, and compared with ordinary premature infants, NEC infants have a higher abundance of waterborne bacteria. Therefore, attention should be paid to the contamination of water sources and various ventilator pipelines for premature infants hospitalized in the neonatal intensive care unit.
3. An in-depth study of the mode of microbial colonization in premature infants combined with the different functions of various metabolic pathways involved in different microorganisms may be able to identify the cause of NEC.

Keywords: necrotizing enterocolitis, intestinal microbiota, 16S rRNA, premature infants, newborn, risk factors

OPEN ACCESS

Edited by:
Yuan Shi,
Children's Hospital of Chongqing Medical University, China

Reviewed by:
Cheng Cai,
Shanghai Children's Hospital, China
Hua Wang,
Sichuan University, China
Yu He,
Children's Hospital of Chongqing Medical University, China

***Correspondence:**
Hui Wu
wuhui@jlu.edu.cn

[†] *These authors have contributed equally to this work and share first authorship*

Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: *22 January 2021*
Accepted: *22 March 2021*
Published: *03 May 2021*

Citation:
Fu X, Li S, Jiang Y, Hu X and Wu H (2021) Necrotizing Enterocolitis and Intestinal Microbiota: The Timing of Disease and Combined Effects of Multiple Species. Front. Pediatr. 9:657349. doi: 10.3389/fped.2021.657349

INTRODUCTION

Necrotizing enterocolitis (NEC) is one of the most common gastrointestinal emergencies in newborns and mostly occurs in premature infants born before 34 weeks of gestation. Abdominal distension, vomiting, diarrhea, hematochezia, shock, and multiple organ system failure in severe cases are the main clinical manifestations. In the United States, the incidence of proven or severe NEC (Bell stage II and III) is estimated to be ∼1–3 per 1,000 live births (1, 2).

As a typical intestinal infectious disease, in 1975, Sántulli et al. hypothesized that microbial imbalance in the digestive tract is involved in the pathogenesis of NEC, and this condition is dominated by bacterial disorders (3). This imbalance is manifested by the lack of colonization by certain normal bacteria in the intestine or the occurrence of abnormal colonization, which is regarded as a key risk factor in NEC (4). With the development of high-throughput sequencing, researchers can identify more abundant bacterial communities and have a deeper understanding of the relationship between NEC and gut microbes.

Current research not only focuses on the impact of individual microbes on the disease but also regards gut microbes as a whole. In addition to discovering the relationship between specific taxa such as Firmicutes, Proteobacteria, Klebsiella, and Actinomycetes and NEC, microbial diversity has been found to be associated with diseases. Because of the low diversity of intestinal microbes in early life, large individual differences, and diverse sequencing platforms, no specific species or uniform microbial characteristics have been confirmed as having a causal relationship with NEC. In the meantime, there are currently few studies on the flora characteristics of infants with NEC in Asia, especially in China. This study focused on infants from Jilin Province, China. In addition to analyzing the characteristics of intestinal flora during the occurrence of NEC, the meconium of infants was analyzed within 48 h after birth to explore the overall characteristics of the fecal flora of infants with NEC and its different flora. Understanding the intestinal microbiota can help us take measures to change the outcome of infants before the disease occurs.

MATERIALS AND METHODS

This study was conducted from February 2018 to April 2019 and approved by the Ethics Committee of the First Hospital of Jilin University (Ethics No. 2018-421).

Inclusion Criteria

1. Gestational age $\leq$ 33 weeks
2. Premature infants with incomplete meconium excretion (within 48 h after birth)

Exclusion Criteria

1. Congenital intestinal malformations and other serious malformations, such as fetal gastroschisis and intestinal atresia
2. Spontaneous intestinal perforation
3. Probiotic usage before the first stool specimen collection
4. Incomplete clinical data or failure to follow the procedures to store samples

Sample Collection Process

All fecal samples were collected within 48 h of birth of premature infants who met the inclusion criteria and then once a week. When an infant presented with NEC symptoms and was diagnosed with NEC (Bell stage II and III), they were classified into the NEC group, and stool samples were taken within 48 h. Next, stool samples were collected 1–2 weeks after treatment from premature infants with confirmed NEC. Premature infants without NEC would continue to provide weekly stool samples until they were discharged from the hospital. All infants left the study because of death and treatment abandonment. The samples were stored at −20°C within 24 h after collection and then transferred to −80°C for low-temperature storage until being sent to Shanghai Parsons Biotechnology Co., Ltd., for 16sRNA analysis.

Sample Size Selection and Control Group Matching

To exclude confounding factors, infants without NEC who matched the infants with NEC in gestational age, delivery mode, feeding patterns, antibiotic application time, and other diseases were enrolled as controls. In the early stage of the study, 10 samples (10 in the control group and 10 in the NEC group) were tested for intestinal flora analysis, and the mean value and standard deviation of the alpha diversity index were obtained. Then, the values were imported into MedCalc software for sample size calculation. The results showed that, with $\alpha = 0.05$, $\beta = 0.01$, 80% power, and 95% confidence level, the required sample size was 13 in the NEC group and 13 in the control group. We appropriately raised the sample number to 15 cases.

PCR Amplification and Illumina NovaSeq Sequencing

Total bacterial genomic DNA was extracted from fecal samples, and PCR amplification was performed on the V3–V4 region of the bacterial 16S rRNA gene. The forward primer was 338F (5′-ACTCCTACGGGAGGCAGCA-3′), and the reverse primer was 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The Illumina NovaSeqPE250 DNA library was selected as the sequencing platform to perform paired-end sequencing of community DNA fragments, and the original sequencing data were saved in FASTQ format (raw data about the study have been uploaded to NCBI, PRJNA694799).

Bioinformatic Processing

Sequence Data Analysis

Sequence data analysis was performed mainly with QIIME2 and R software (v3.2.0). The DADA2 algorithm was used to carry out depriming, quality filtering, denoising, splicing, and dechimerism; the algorithm no longer uses similarity clustering and only performs dereplication or an equivalent of 100% similarity clustering. After obtaining the amplicon sequence

variant abundance matrix based on the Greengenes database, the bacterial flora between groups was analyzed.

Overall Analysis of the Fecal Community

Alpha diversity analysis was used to show the richness and diversity of the community, and a rarefaction curve was used to judge whether the current sequencing depth of each sample was sufficient to reflect the microbial diversity and community abundance contained in the community sample. The R script was used to visualize the alpha diversity index values as box plots. Beta diversity analysis was performed mainly by using a two-dimensional sequence diagram of the non-metric multidimensional scaling (NMDS) analysis results to show the differences in microbial community composition of each sample and combined with ANOSIM to reflect the community differences between groups and within each group. At the same time, taxonomic composition analysis was carried out to analyze the trend of species change within each group.

Different Flora and Metabolic Analysis

Linear discriminant analysis effect size (LEfSe) analysis was used to obtain the different flora between the groups, and taxonomic composition analysis was combined to identify the different flora with higher relative abundance. Using the PICRUSt2 method, the MetaCyc database (https://metacyc.org/) was compared to obtain different metabolic pathways between groups to predict the function of the microbial community.

Statistical Method

SPSS 22 software was used to analyze the clinical data of all participants. The measurement data conforming to normal distribution were expressed as mean ± standard deviation ($X \pm SD$), and the comparison between groups was performed with *t*-test; a non-parametric test was used for abnormally distributed data. $P < 0.05$ indicated that the difference was statistically significant.

RESULTS

In this study, a total of 15 infants with NEC and 15 infants in the control group participated in the analysis. The specific screening process is shown in the flow diagram (**Figure 1**). Eight samples of infants were taken when NEC occurred. According to statistics, these eight samples were collected, on average, ∼1.25 days after the onset of NEC symptoms, and the NEC diagnosis time was 10–27 days after birth. After NEC treatment, the samples of six infants were collected. The control group stool samples were collected within 48 h of birth and the time close to the diagnosis in the NEC group. All infants in the NEC group were treated with antibiotics according to the severity of the disease, and three of them underwent enterostomy. All infants were breastfeeding priority, and some of the infants were given formula feeding due to insufficient breast milk. Feeding intolerance often occurs in premature infants, so some infants use probiotics. *Clostridium butyricum* and Bifidobacterium are commonly used in our center. To reduce the differences between groups, we also matched the types of probiotics used. The basic characteristics of the two groups are shown in **Table 1**.

Overall Characteristics of the Fecal Flora

The main purpose of the beta diversity analysis was to investigate the similarity of community structure among different samples. The NMDS of unweighted UniFrac distance was used. The closer (and farther) the distance between two points, the smaller (and greater) the difference in microbial communities between two samples. The Nbirth7 and Nbirth13 samples at birth had an outlier trend. After these two samples were excluded, the control group and the NEC group showed different aggregations at birth (**Figure 2A**), but the distribution of each sample was relatively scattered. The ANOSIM results suggest values of $R = 0.264285$ and $P < 0.003$. At the onset of NEC, there was obvious aggregation in the control group, while the NEC group was more dispersed and presented subgroups (**Figure 2B**). Combining the basic information of the specimens showed that the two discrete NEC groups were related to the time of the diagnosis of NEC: in one group, the NEC diagnosis time was more than 20 days, and in the other group, it was <20 days. Moreover, the ANOSIM results indicated values of $R = 0.812245$ and $P < 0.001$ upon NEC occurrence, indicating that the group classification was better. The stress values of the two NMDS results were 0.068 and 0.0285. It is generally believed that, when the value is <0.2, the NMDS analysis result is reliable.

The rarefaction curve tended to be flat after the sequencing depth reached 5,000, indicating that the sequencing results were sufficient to reflect the diversity in the sample (**Figure 2C**). The diversity index analysis showed that the Chao1 index of the NEC group at birth (**Figure 2D**) was higher than that of the control group ($P = 0.04$), but the Shannon index ($P = 0.19$), Good's coverage ($P = 0.17$), and Pielou's evenness index ($P = 0.3$) showed no significant difference, indicating that the number of microbes actually present in the NEC group was higher, but the uniformity was not different. Upon the occurrence of NEC (**Figure 2E**), the Chao1 index ($P = 0.01$), Shannon index ($P = 0.00081$), and Pielou's evenness index ($P = 0.001$) of the NEC group were higher, suggesting that, when NEC occurred in the infants, not only the intestinal microbial richness but also the evenness was higher than that in the control group. This study also performed a dynamic assessment of the stool of NEC infants (**Figure 2F**) and found that the microbial diversity of the NEC group did not change significantly over time.

Regardless of whether infants were from the NEC group or control group, the number of dominant species with high abundance in the intestinal flora decreased over time, and a stable flora dominated by fewer species was obtained. The histogram shows that the abundance of Enterobacteriaceae increased over time. The characteristics of the NEC group after treatment were similar to those of the control group (**Figure 3**).

Different Flora and Function Prediction

Through LEfSe analysis (logarithmic discriminant analysis threshold of 3), the difference between the NEC and control groups at each taxonomic level was obtained (**Supplementary Table 1**). While most microbes had low

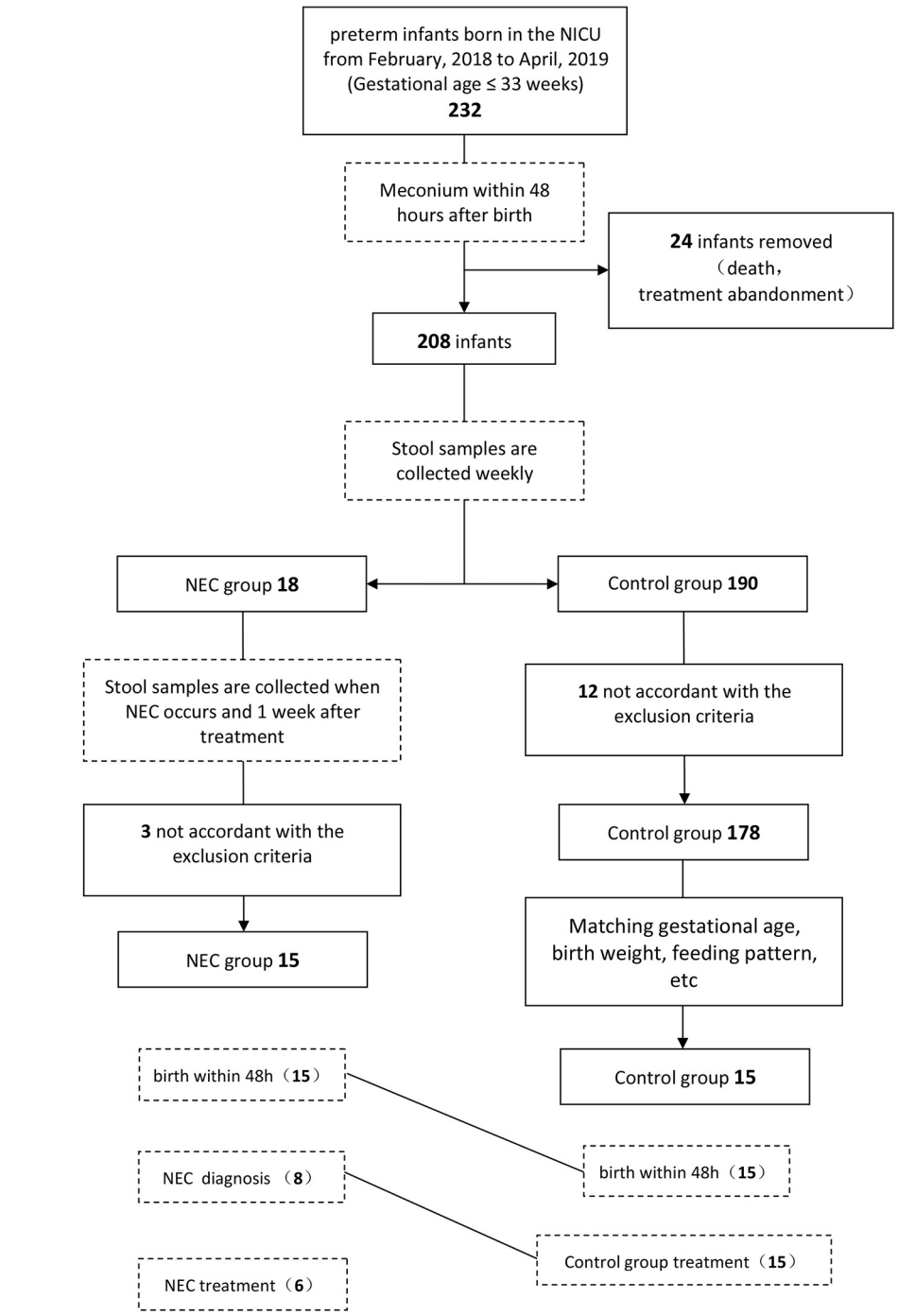

FIGURE 1 | Flow diagram.

relative abundance, this study analyzed various taxa with high abundance (**Figure 3**). The results showed that the abundance of Bacteroides and Actinobacteria in NEC infants at birth was much higher than that in the control group, and this trend continued until NEC occurred, with Rhizobiales at the family level and Dysgonomonas, Ochrobactrum, Ralstonia, Pelomonas, Acinetobacter, etc., at the genus level exhibiting the same characteristics. In addition, the NEC group had a

TABLE 1 | Infant characteristics and clinical information.

	Necrotizing enterocolitis (NEC) group ($n = 15$) Mean ± SD or *n* (%)	Control group ($n = 15$) Mean ± SD or *n* (%)	*P*-value
Gestational age (weeks)	30.2 ± 1.2	30.1 ± 1.9	0.92
Birth weight (kg)	1.46 ± 0.32	1.36 ± 0.37	0.41
Sex (male)	9 (60)	9 (60)	1.0
Delivery mode (vaginal)	7 (46.7)	8 (53.3)	1.0
Feeding pattern			
First stool (ambrosia/formula)	7/8	7/8	
Second stool (exclusive/mixed breastfeeding)	1/7	1/14	
Apgar score-−5 min	6.4 ± 1.7	6.3 ± 1.3	0.68
Probiotics	11 (73.3)	9 (60)	0.70
Clostridium butyricum	8	5	
Bifidobacterium	1	0	
Combined probiotics	2	4	
Use of antibiotics (days)	14.4 ± 6.8	9.7 ± 6.2	0.12
	Sulbenicillin, ceftriaxone, cefepime, meropenem, piperacillin-tazobactam	Sulbenicillin, cefoperazone, cefepime, vancomycin, piperacillin-tazobactam	
Premature rupture of membranes	2 (13.3)	5 (33.3)	0.39
Received surfactant	10 (66.7)	10 (66.7)	1.0
Received antenatal steroids	11 (73.3)	10 (66.7)	1.0
Received antenatal antibiotics	1 (6.7)	2 (13.3)	1.0
Maternal factors			
Gestational hypertension	5 (33.3)	4 (33.3)	1.0
Gestational diabetes	2 (13.3)	2 (13.3)	1.0

Mean ± SD, mean ± standard deviation.
The duration of antibiotic use in the NEC group was before NEC diagnosis.

higher abundance of Alphaproteobacteria, Betaproteobacteria, Sphingomonas, Lactobacillus, etc., when NEC occurred, while the control group had a significantly higher abundance of Gammaproteobacteria, Enterobacteriaceae, and Clostridiaceae.

PICRUSt2 analysis revealed the metabolic pathways involving microbes in all infant feces (**Figure 4A**), and the abscissa represents the number of functional pathways—pathways related to degradation/utilization/assimilation; biosynthesis plays a major role. Through comparison, the PWY-6210 and PWY-6505 pathways with statistically significant differences in the NEC group at birth were upregulated and involved in the degradation of L-tryptophan and aromatic compounds, and their species composition was mainly Ralstonia (**Figure 4B**). The two pathways also remained upregulated within 48 h of NEC diagnosis. At the time of NEC diagnosis, there were 50 upregulated different metabolic pathways in the NEC group (**Supplementary Table 2**). These pathways were mainly concentrated on degradation/utilization/assimilation, biosynthesis, and generation of precursor metabolites and energy, including the synthesis of sugar, enzyme cofactors, cell structure, fermentation of acetic acid and butyric acid, degradation of lysine and glutamic acid, etc. The species composition included Ralstonia, Dysgonomonas, Bacteroides, and Corynebacterium. At the same time, it was also found that the PWY-6478 pathway was mainly composed of two species, Dysgonomonas and Sediminibacterium, and these two species existed in the feces of infants with different NEC diagnosis times (**Figure 4C**).

DISCUSSION

NEC is a serious disease threatening the lives of newborns, but its cause is not truly understood. It is known that intestinal dysbacteriosis, which occurs before the disease appears, is one of the important factors leading to NEC (5). As a large organ formed by microbial aggregation, changes in the intestinal flora need to be further studied in combination with species and community.

The Overall Perspective of the Bacterial Community

A retrospective study from 2008 to 2015 found that the microbial diversity of most preterm infants with NEC was lower than that of other infants at the same gestational age (6). Moreover, some studies indicated that the Chao1 and Shannon indices were not significantly different between the NEC and control groups, which means that the lack of bacterial diversity was not related to the development of NEC (7–9). This study found that the NEC group had an advantage in the number of microbes at birth. In addition, the richness and evenness of microbes in the NEC group were higher than those in the control group when the disease occurred, which was different from the abovementioned

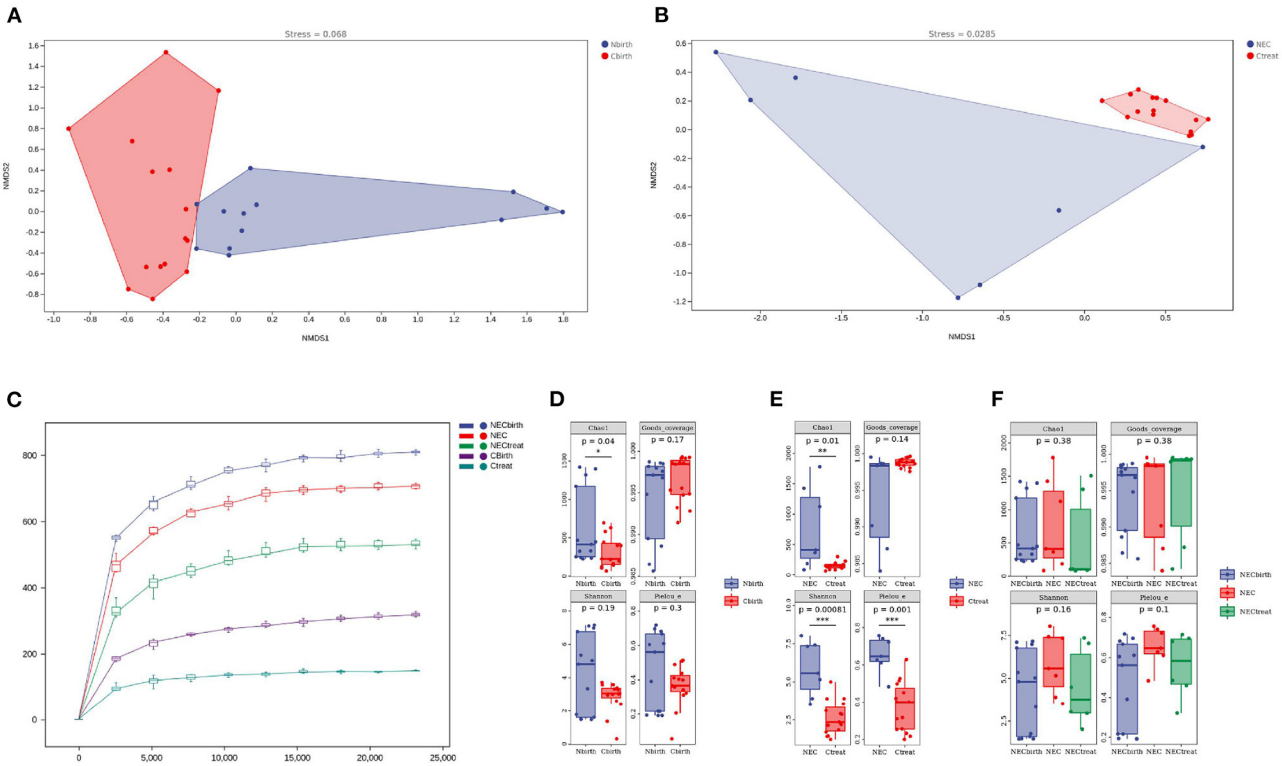

FIGURE 2 | Alpha and beta diversity analysis. **(A,B)** Beta diversity analysis: **(A)** at birth, **(B)** at necrotizing enterocolitis diagnosis. **(C)** Rarefaction curve. **(D–F)** Alpha diversity analysis. Chao 1 index is used to estimate the actual number of species in the community. Shannon diversity index comprehensively considered the richness and evenness of the community. Pielou's_e assesses the coverage of species in the community.

research results. The high microbial diversity of infants with NEC may be related to the greater differences between individuals in the NEC group, while the microbial communities in the control group were more similar. However, regardless of the level of diversity, it only provides different degrees of protection to pathogens and cannot directly induce NEC.

The beta diversity analysis suggested that a difference in the time of NEC diagnosis (with 20 days as the boundary) can also cause differences in the microbial communities of premature infants. Compared with the community at birth, the differences in the communities between the two groups upon the occurrence of NEC were more obvious and more representative. However, in this study, the number of sample cases when NEC occurs is small, and a larger-sample-size study is needed to prove this. A study that analyzed the intestinal flora of infants with early-onset and late-onset NEC (with 22 days as the boundary) indicated that the patterns of microbial progression and dominant colonizing microbes differed between the groups depending on when the infants were diagnosed with NEC (10). PICRUSt2 analysis also shows that early-onset NEC and late-onset NEC are controlled by different species in the PWY-6478 pathway, which further illustrates that the time of NEC occurrence may be related to the different compositions of intestinal microbes. Of course, this difference may also be related to the inconsistent rate of microbial progression caused by the age when the infants' feces were collected. More research is needed to clarify the characteristics of the microflora of infants at different growth stages. A 2014 study found that a variety of factors affect the intestinal flora, but they only affect the rate of progression, not the sequence of development (11). We need to understand how individual microbes change at different stages of the disease to prevent infants from progressing toward the disease through probiotics or other means in the early stage of the disease.

The Individual Microbes of the Bacterial Community

From a microscopic perspective, recent studies have shown that the main intestinal taxa in premature infants are Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, which play a key role in maintaining intestinal barrier function, increasing the expression of tight junctions, regulating mucin, and affecting biosynthesis. Moreover, the metabolites of these microbes provide energy for the proliferation of epithelial cells and stimulate the immune system (12, 13). The results suggest that the abundances of Bacteroidetes and Acinetobacter are significantly different between the time of birth and NEC onset. Although Bacteroidetes does not have the highest abundance, these species can change their surface structure to escape the host's immune response and produce lysozyme to mediate tissue destruction (14). In addition, in Bacteroides species, the nanH

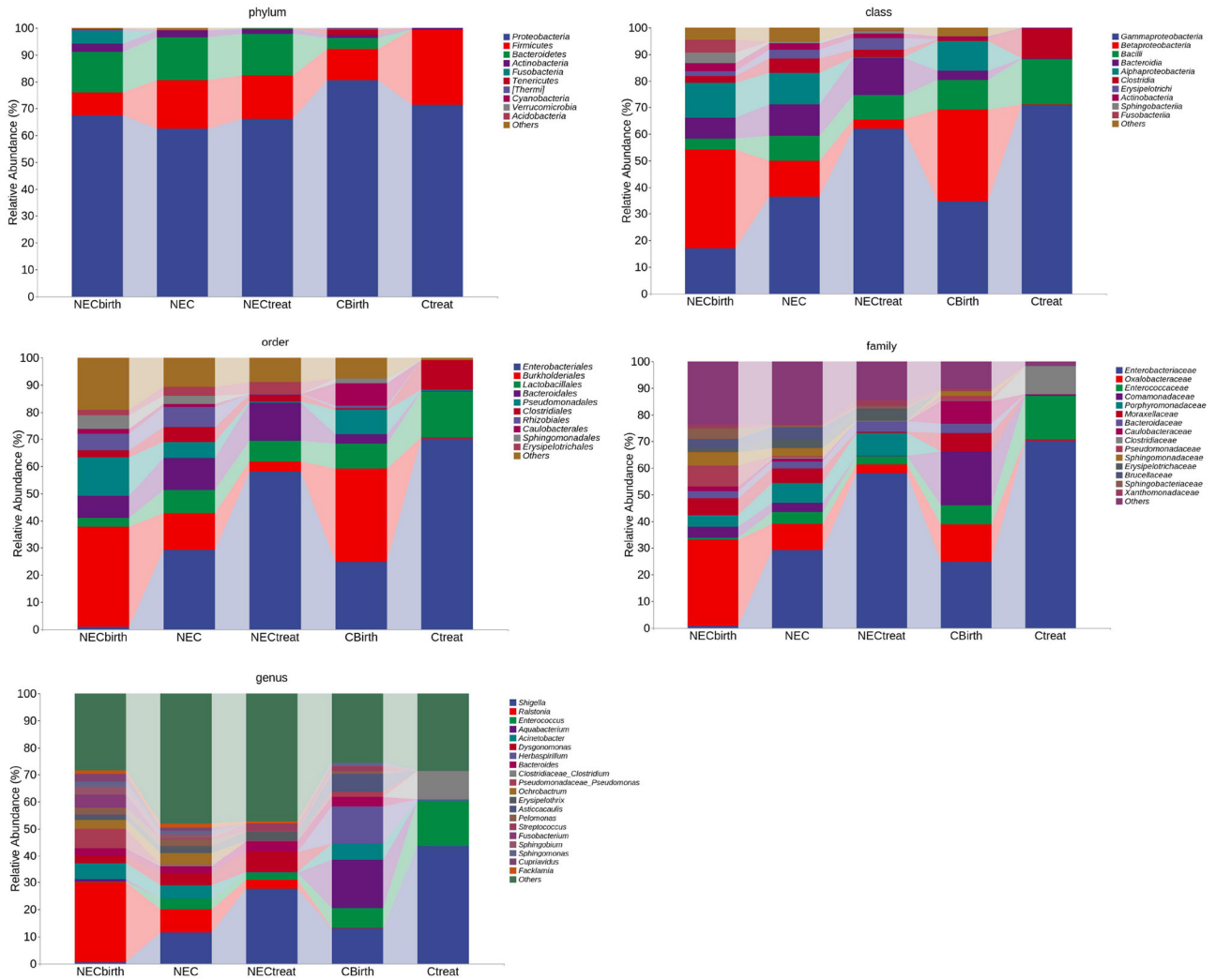

FIGURE 3 | Taxonomic composition analysis. This figure shows the bacteria at the top of each taxonomic level, the top 10 at the phylum, class, and order levels, the top 15 at the family level, and the top 20 at the genus level.

gene can produce neuraminidase to lyse mucin polysaccharides and enhance bacterial growth by producing available glucose. It can also influence the transfer and colonization of other bacterial communities through interleukin-36 (15). Bacteria influence the occurrence and development of disease even when they do not have a high relative abundance. Similarly, Acinetobacter species from Proteobacteria can adhere to human epithelial cells and produce enzymes that destroy tissue lipids. Identifying the characteristics of these microbes could help us to avoid the occurrence of diseases in a targeted manner or reduce the severity of diseases through treatment. In addition, studies have found that mixed infections caused by combinations of other bacteria with Acinetobacter are more toxic than only Acinetobacter infections (16). This suggests that a single strain with superior abundance is not the cause of NEC, and it might be the combined effect of various bacteria that causes NEC. We need to further study which species have higher toxicity and can cause disease and try to avoid the occurrence of combined effects. Acinetobacter is also linked to maternal digestive tract disease during pregnancy, which also reflects that infants' intestinal flora at birth is affected by their mothers (17). By controlling the mothers' disease development during pregnancy, the incidence of neonatal diseases could be reduced.

In addition to the mentioned microbes that mainly cause NEC *via* virulence, some environmental bacteria are more likely to have abundance advantages in the intestines of infants with NEC. For example, Dysgonomonas exists on environmental surfaces in medical institutions (18). Rhizobiales, Ralstonia, and Pelomonas can be detected on the surfaces of pipelines, such as those in hospital water sources and in-hospital ventilators, especially Ralstonia, which can survive even in disinfectants under low nutritional conditions (19–21). These environmentally derived bacteria exhibited colonization within 48 h after the birth of infants with NEC, indicating that premature infants are soon

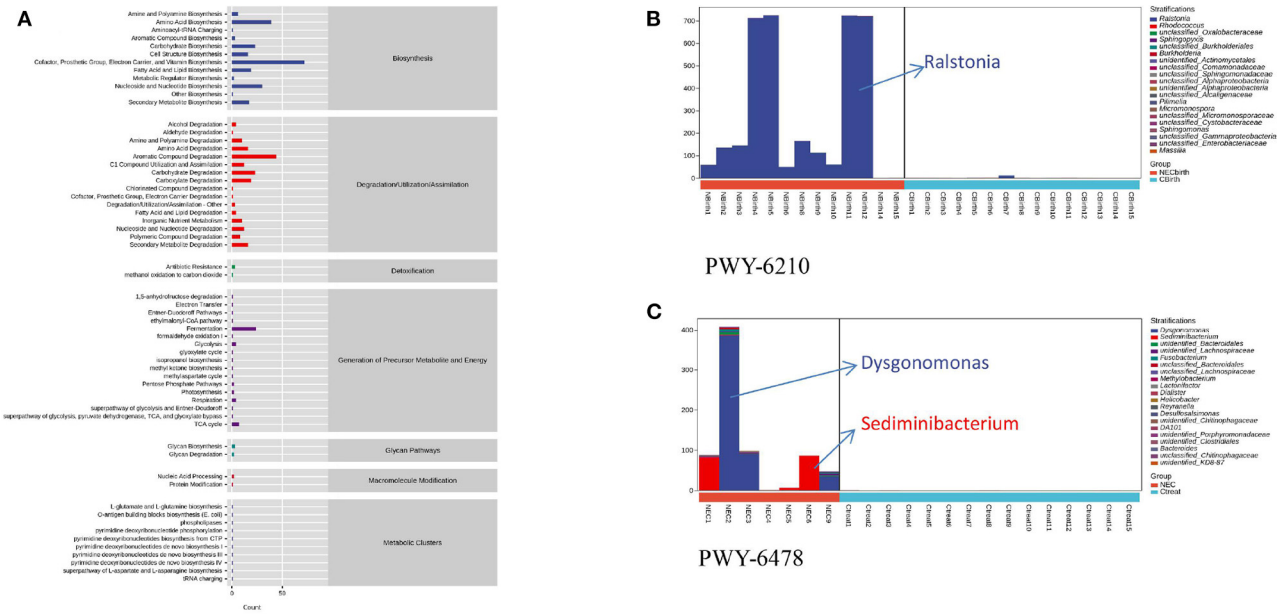

FIGURE 4 | Microbial function prediction. **(A)** Metabolic pathways involving microbes in all infant feces. **(B)** PWY-6210 pathway's species composition. **(C)** PWY-6478 pathway's composition of two species. NEC2, NEC3, and NEC9 are infants diagnosed with necrotizing enterocolitis within 20 days.

affected by the surrounding environment after leaving their mothers, such as in the operating room, in the neonatal intensive care unit (NICU) or *via* contact with the ventilator pipeline. When NEC occurs, the above-mentioned different flora still exists. Does this mean that the guts of infants with NEC are more likely to be colonized by waterborne bacteria? Targeting these waterborne bacteria or minimizing their exposure to premature babies could reduce the incidence of the disease.

A trend analysis of microbes indicated that the developmental trend in the NEC group after treatment was similar to that in the control group. The abundance of Alphaproteobacteria and Betaproteobacteria showed a downward trend, and Gammaproteobacteria showed an upward trend. It is reasonable to think that, when NEC occurs, the Alphaproteobacteria and Betaproteobacteria in the NEC group showed abundance advantages, which was consistent with the two bacteria being dominant in a large number of water sources and in biofilms, while the content of Gammaproteobacteria is relatively low (21, 22). On the other hand, this phenomenon is also associated with the various factors mentioned above that affect the intestinal flora but only influence the speed of progression, not the sequence of progression.

Functional prediction analysis also suggests that Ralstonia and Dysgonomonas, as the main species of many different metabolic pathways in NEC infants, are mainly involved in the degradation of various amino acids and aromatic compounds. Studies have shown that L-tryptophan participates in intestinal immune homeostasis and can play an anti-inflammatory effect in mammals (23). The L-tryptophan degradation pathway in NEC infants is upregulated both at birth and at the time of disease diagnosis, which reduces the anti-inflammatory effect of premature infants and makes them more prone to disease. Tryptophan is an essential amino acid that can only be ingested through food, and targeted supplementation with tryptophan may reduce the effect of microorganisms on immune homeostasis. In addition, some studies have found that aromatic amino acids can be used as substrates of metabolic pathways to affect intestinal permeability and systemic immunity (24). In-depth exploration of how the flora participates in various metabolic pathways in the human body can help us understand the occurrence of NEC.

There is also an interesting phenomenon that occurred here. Compared with other studies, some of our results are similar, such as Sphingomonas (25), and in some respects opposite, such as Gammaproteobacteria (18). Moreover, the specific microbe and species change trends found in infants with NEC vary from study to study (26–29). This difference may be due to a mixture of factors that alter the microbial community of newborns, such as gestational age, antibiotic treatment of infants and mothers, delivery mode, feeding pattern, and the use of probiotics (30). Additionally, different NICUs and different races will cause different results (31). No study has been able to completely eliminate the effects of these factors. Analysis of the selection criteria for the participants in each study showed that most of the studies excluded infants who received probiotics, and the selection of gestational age for infants varied from study to study. The different gene target regions (V1–V3 or V3–V5), different analysis platforms analyzed in 16sRNA sequencing technology, different NICU treatment plans, and environmental and ethnic differences—all of these factors—could have influenced the results of the study. Microorganisms carried in breast milk cannot be detected, which is an uncharted territory.

Limitations

Due to the particularity of the disease, only 15 premature infants with NEC were included in this study. The antibiotic utilization rate in premature infants was high, and it was difficult to acquire a sample before antibiotics were applied, resulting in influencing factors that could not be ruled out. This effect can only be reduced by matching the antibiotic application time of the two groups of children. Our study failed to use metagenomic sequencing technology to analyze the link between the intestinal flora and NEC, and more in-depth research is needed. In the comparison of the microbiomes of NEC patients, the inconsistency in age of onset of the disease is a confounding factor, and the relationship among antibiotics, delivery type (vaginal or cesarean section), diet, and infant age affects the rate of microbiota progression. Moreover, infants need to fast for a short duration after illness, which increases the difficulty of sample retention. This study was limited to a single research center, and more studies are needed to verify the conclusions.

Conclusions

1. Based on the time of NEC diagnosis (bounded by 20 days), the microbial community differs.
2. No single microorganism is related to NEC, and the combined effect of multiple species is of great significance in the occurrence of NEC. Premature infants are easily affected by bacteria living in the environment, and compared with ordinary premature infants, NEC infants have a higher abundance of waterborne bacteria, so attention should be paid to the contamination of water sources and various ventilator pipelines for premature infants hospitalized in the NICU.
3. An in-depth study of the mode of microbial colonization in premature infants, combined with the different functions of various metabolic pathways involved in different microorganisms, may be able to identify the cause of NEC.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the First Hospital of Jilin University (Ethics No. 2018-421). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

XF and SL contributed to the study design, data collection, data analysis, and manuscript writing. YJ an XH made contributions to the methodology and data interpretation. HW revised the manuscript and approved the final version. All the authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

FUNDING

This study was supported by the Jilin Province Health Technology Innovation Fund (Grant Number 2018J040). The funder of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report.

ACKNOWLEDGMENTS

We thank the subjects and their families for their participation and the staff of the neonatal intensive care unit for their support in sample collection. We also thank our colleagues at the Gene Diagnosis Center for their help in sample storage.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.657349/full#supplementary-material

REFERENCES

1. Stoll BJ, Hansen NI, Bell EF, Walsh MC, Carlo WA, Shankaran S, et al. Trends in care practices, morbidity, and mortality of extremely preterm neonates, 1993-2012. *JAMA*. (2015) 314:1039–51. doi: 10.1001/jama.2015.10244
2. Holman RC, Stoll BJ, Curns AT, Yorita KL, Steiner CA, Schonberger LB. Necrotising enterocolitis hospitalisations among neonates in the United States. *Paediatr Perinat Epidemiol.* (2006) 20:498–506. doi: 10.1111/j.1365-3016.2006.00756.x
3. Sántulli TV, Schullinger JN, Heird WC, Gongaware RD, Wigger J, Barlow B, et al. Acute necrotizing enterocolitis in infancy: a review of 64 cases. *Pediatrics.* (1975) 55:376–87.
4. Patel RM, Denning PW. Intestinal microbiota and its relationship with necrotizing enterocolitis. *Pediatr Res.* (2015) 78:232–8. doi: 10.1038/pr.2015.97
5. Jacob JA. In infants with necrotizing enterocolitis, gut dysbiosis precedes disease. *JAMA.* (2016) 315:2264–5. doi: 10.1001/jama.2016.4341
6. Dollings MC, Brown L. An integrated review of intestinal microbiota in the very premature infant. *Neonatal Netw.* (2016) 35:204–16. doi: 10.1891/0730-0832.35.4.204
7. McMurtry VE, Gupta RW, Tran L, Blanchard EEt, Penn D, Taylor CM, et al. Bacterial diversity and Clostridia abundance decrease with increasing severity of necrotizing enterocolitis. *Microbiome.* (2015) 3:11. doi: 10.1186/s40168-015-0075-8
8. Normann E, Fahlén A, Engstrand L, Lilja HE. Intestinal microbial profiles in extremely preterm infants with and without necrotizing enterocolitis. *Acta Paediatr.* (2013) 102:129–36. doi: 10.1111/apa.12059
9. Sim K, Shaw AG, Randell P, Cox MJ, McClure ZE, Li MS, et al. Dysbiosis anticipating necrotizing enterocolitis in very premature infants. *Clin Infect Dis.* (2015) 60:389–97. doi: 10.1093/cid/ciu822

10. Zhou Y, Shan G, Sodergren E, Weinstock G, Walker WA, Gregory KE. Longitudinal analysis of the premature infant intestinal microbiome prior to necrotizing enterocolitis: a case-control study. *PLoS ONE.* (2015) 10:e0118632. doi: 10.1371/journal.pone.0118632
11. La Rosa PS, Warner BB, Zhou Y, Weinstock GM, Sodergren E, Hall-Moore CM, et al. Patterned progression of bacterial populations in the premature infant gut. *Proc Natl Acad Sci USA.* (2014) 111:12522–7. doi: 10.1073/pnas.1409497111
12. Purchiaroni F, Tortora A, Gabrielli M, Bertucci F, Gigante G, Ianiro G, et al. The role of intestinal microbiota and the immune system. *Eur Rev Med Pharmacol Sci.* (2013) 17:323–33.
13. Ashida H, Ogawa M, Kim M, Mimuro H, Sasakawa C. Bacteria and host interactions in the gut epithelial barrier. *Nat Chem Biol.* (2011) 8:36–45. doi: 10.1038/nchembio.741
14. Wexler HM. Bacteroides: the good, the bad, and the nitty-gritty. *Clin Microbiol Rev.* (2007) 20:593–621. doi: 10.1128/CMR.00008-07
15. Sequeira RP, McDonald JAK, Marchesi JR, Clarke TB. Commensal Bacteroidetes protect against Klebsiella pneumoniae colonization and transmission through IL-36 signalling. *Nat Microbiol.* (2020) 5:304–13. doi: 10.1038/s41564-019-0640-1
16. Bergogne-Bérézin E, Towner KJ. Acinetobacter spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clin Microbiol Rev.* (1996) 9:148–65. doi: 10.1128/CMR.9.2.148-165.1996
17. Jin M, Li D, Ji R, Liu W, Xu X, Li Y. Changes in intestinal microflora in digestive tract diseases during pregnancy. *Arch Gynecol Obstet.* (2020) 301:243–9. doi: 10.1007/s00404-019-05336-0
18. Lindberg TP, Caimano MJ, Hagadorn JI, Bennett EM, Maas K, Brownell EA, et al. Preterm infant gut microbial patterns related to the development of necrotizing enterocolitis. *J Maternal Fetal Neonatal Med.* (2020) 33:349–58. doi: 10.1080/14767058.2018.1490719
19. Bousbia S, Papazian L, Saux P, Forel JM, Auffray JP, Martin C, et al. Serologic prevalence of amoeba-associated microorganisms in intensive care unit pneumonia patients. *PLoS ONE.* (2013) 8:e58111. doi: 10.1371/journal.pone.0058111
20. Ryan MP, Adley CC. Ralstonia spp.: emerging global opportunistic pathogens. *Eur J Clin Microbiol Infect Dis.* (2014) 33:291–304. doi: 10.1007/s10096-013-1975-9
21. Farhat M, Alkharsah KR, Alkhamis FI, Bukharie HA. Metagenomic study on the composition of culturable and non-culturable bacteria in tap water and biofilms at intensive care units. *J Water Health.* (2019) 17:72–83. doi: 10.2166/wh.2018.213
22. Jin D, Kong X, Cui B, Jin S, Xie Y, Wang X, et al. Bacterial communities and potential waterborne pathogens within the typical urban surface waters. *Sci Rep.* (2018) 8:13368. doi: 10.1038/s41598-018-31706-w
23. Gao J, Xu K, Liu H, Liu G, Bai M, Peng C, et al. Impact of the gut microbiota on intestinal immunity mediated by tryptophan metabolism. *Front Cell Infect Microbiol.* (2018) 8:13. doi: 10.3389/fcimb.2018.00013
24. Dodd D, Spitzer MH, Van Treuren W, Merrill BD, Hryckowian AJ, Higginbottom SK, et al. A gut bacterial pathway metabolizes aromatic amino acids into nine circulating metabolites. *Nature.* (2017) 551:648–52. doi: 10.1038/nature24661
25. Stewart CJ, Nelson A, Scribbins D, Marrs EC, Lanyon C, Perry JD, et al. Bacterial and fungal viability in the preterm gut: NEC and sepsis. *Arch Dis Childhood Fetal Neonatal Ed.* (2013) 98:F298–303. doi: 10.1136/archdischild-2012-302119
26. Heida FH, van Zoonen A, Hulscher JBF, Te Kiefte BJC, Wessels R, Kooi EMW, et al. A necrotizing enterocolitis-associated gut microbiota is present in the meconium: results of a prospective study. *Clin Infect Dis.* (2016) 62:863–70. doi: 10.1093/cid/ciw016
27. Pammi M, Cope J, Tarr PI, Warner BB, Morrow AL, Mai V, et al. Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis. *Microbiome.* (2017) 5:31. doi: 10.1186/s40168-017-0248-8
28. Warner BB, Deych E, Zhou Y, Hall-Moore C, Weinstock GM, Sodergren E, et al. Gut bacteria dysbiosis and necrotising enterocolitis in very low birthweight infants: a prospective case-control study. *Lancet.* (2016) 387:1928–36. doi: 10.1016/S0140-6736(16)00081-7
29. Feng J, He Y, Liu D, Li L, Chen J, Yu J. The constitution and functional prediction of the microbiota in necrotizing enterocolitis with a gestational age of over 28 weeks. *Medicine.* (2019) 98:e17206. doi: 10.1097/MD.0000000000017206
30. Zou ZH, Liu D, Li HD, Zhu DP, He Y, Hou T, et al. Prenatal and postnatal antibiotic exposure influences the gut microbiota of preterm infants in neonatal intensive care units. *Ann Clin Microbiol Antimicrob.* (2018) 17:9. doi: 10.1186/s12941-018-0264-y
31. Stearns JC, Zulyniak MA, de Souza RJ, Campbell NC, Fontes M, Shaikh M, et al. Ethnic and diet-related differences in the healthy infant microbiome. *Genome Med.* (2017) 9:32. doi: 10.1186/s13073-017-0421-5

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

frontiers in Pediatrics

ORIGINAL RESEARCH
published: 31 May 2021
doi: 10.3389/fped.2021.651713

Impact of Early Empiric Antibiotic Regimens on the Gut Microbiota in Very Low Birth Weight Preterm Infants: An Observational Study

*Hung-Yang Chang[1,2], Jen-Shiu Chiang Chiau[3], Yu-Hsuan Ho[4], Jui-Hsing Chang[1,2], Kun-Nan Tsai[4], Chia-Yen Liu[4], Chyong-Hsin Hsu[1], Chia-Ying Lin[1], Mary Hsin-Ju Ko[1] and Hung-Chang Lee[1,2]**

[1] Department of Pediatrics, MacKay Children's Hospital, Taipei, Taiwan, [2] Department of Medicine, MacKay Medical College, New Taipei City, Taiwan, [3] Department of Medical Research, MacKay Memorial Hospital, Taipei, Taiwan, [4] Life Science, Delta Research Center, Delta Electronics Incorporation, Taipei, Taiwan

OPEN ACCESS

Edited by:
Guo Chunbao,
Children's Hospital of Chongqing Medical University, China

Reviewed by:
Silvia Salvatore,
University of Insubria, Italy
Zhenhua Guo,
Children's Hospital of Chongqing Medical University, China

***Correspondence:**
Hung-Chang Lee
8231boss@gmail.com

Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: 10 January 2021
Accepted: 06 May 2021
Published: 31 May 2021

Citation:
Chang H-Y, Chiang Chiau J-S, Ho Y-H, Chang J-H, Tsai K-N, Liu C-Y, Hsu C-H, Lin C-Y, Ko MH-J and Lee H-C (2021) Impact of Early Empiric Antibiotic Regimens on the Gut Microbiota in Very Low Birth Weight Preterm Infants: An Observational Study. Front. Pediatr. 9:651713. doi: 10.3389/fped.2021.651713

Frequent use of antibiotics in preterm infants disturbs their gut microbial balance. In this preliminary observational study, we investigated the effect of different antibiotic regimens, administered during the first week of life, on microbial composition and diversity in very low birth weight (VLBW) preterm infants. We performed fecal sampling of breastfed VLBW infants on days 7, 14, and 30. After excluding stool samples from infants who received probiotics or who were administered antibiotics beyond the age of 7 days, we compared gut microbiota profiles between infants receiving a combination of ampicillin and gentamicin for 3 days (AG group, $n = 10$) and those receiving a combination of ampicillin and cefotaxime for 7 days (AC group, $n = 14$) using 16S ribosomal DNA community profiling. We also assessed the changes over time in each group. Compared to the AG group, *Enterococcus* species were significantly more abundant in the AC group ($P = 0.002$), especially in 7-day samples (12.3 vs. 0.6%, respectively, $P = 0.032$). No difference was observed at phylum and genus level over time within each group. Species richness in the AC group decreased significantly in the 14-day ($P = 0.038$) and 30-day ($P = 0.03$) samples compared to that in the 7-day sample. The same was observed for microbial evenness; in contrast, no significant difference in Shannon index and beta-diversity was detected between the two groups. Controlling for relevant confounding variables did not change the results. In conclusion, different antibiotic regimens affect the early development of gut microbiota in VLBW preterm infants. Prolonged use of ampicillin and cefotaxime might result in overabundance of *Enterococcus*. However, given that no significant differences were observed in 1-month samples, bacterial genera appear to continue colonizing the gastrointestinal tract despite previous exposure to antibiotics. The clinical relevance of these findings should be elucidated by further studies.

Keywords: preterm, very low birth weight infants, antibiotics, microbiota, microbiome

INTRODUCTION

The gut microbiome plays a vital role in promoting overall health, and the perinatal period is considered critical for the development of infant gut microbiota. A healthy gut microbiota contributes significantly to the development of a healthy immune system, digestive functions, and neurological functions (1–3). The gut microbiota is highly dynamic during early infancy and is affected by several host and environmental factors, including gestational age, mode of delivery, type of feeding, administration of probiotics, living environment, and medical interventions (1–6). The onset and progression of several infectious diseases, chronic inflammatory bowel disease, allergies, obesity, and diabetes, are associated with disruption of the gut microbiota (2, 7).

The gut microbiota of preterm neonates, as compared to full-term neonates, is unique because it is characterized by delayed colonization of common bacterial genera and an abundance of pathogens (8, 9). Owing to concerns about a relatively immature immune system that may not elicit an optimal response to infection, preterm infants are generally prescribed empiric antibiotic therapy in neonatal intensive care units (NICUs) (10). Prolonged use or upgrade of antibiotics is very common following peripartum issues, such as prolonged rupture of membranes and suspected chorioamnionitis in mothers or non-specific symptoms and signs of infection in neonates. Previous studies showed that antibiotic overuse in early life could impair initial microbial colonization (11–14), which might contribute to subsequent adverse outcomes in preterm infants, such as late-onset sepsis (LOS), necrotizing enterocolitis (NEC), and even death (15–19).

Despite an increased understanding of how antibiotic treatment influences microbiota development in preterm infants, the impact of different antibiotic exposure on preterm microbial community is not well-studied. This preliminary observational study's primary goal was to investigate the effect of different antibiotic regimes, administered during the first week of life, on the development of gut microbiota in very low birth weight (VLBW, birth weight $<$1,500 g) preterm infants.

MATERIALS AND METHODS

Study Subjects

This prospective cohort observational study was conducted at the NICU of MacKay Children's Hospital from May 2017 until May 2018. The study was supported by a grant (MMH-106-69) from MacKay Memorial Hospital. Eligible subjects were VLBW preterm infants who received their mothers' milk. Exclusion criteria included infants with major congenital anomalies or malformations, those receiving nothing by mouth for $\geq$7 days or using probiotics during the study period, and those administered antibiotics after the age of 7 days. The study was approved by the Institutional Review Board of MacKay Memorial Hospital, and was performed in accordance with the 1964 Declaration of Helsinki and its later amendments (IRB number: 17MMHIS026e). Infants were recruited after written informed consent had been obtained from their parents.

Infants were given antibiotic treatment on clinical suspicion of a bacterial infection after analyzing blood cultures. Early empiric antibiotic use consisted of ampicillin plus gentamicin or ampicillin plus cefotaxime. Infants were divided into two groups: those who received 3 days of combination treatment with ampicillin plus gentamicin (AG group), and those who received 7 days of ampicillin plus cefotaxime (AC group). Treatment doses were based on existing guidelines and were adjusted according to patients' gestational age and birth weight. Antibiotic combinations and treatment duration were determined by the attending physicians based on the perceived risk of infection. Ampicillin and third-generation cephalosporins were administered to patients with high risk of infection over a period of 1 week as per our NICU protocols. Medical records were reviewed to identify pertinent infants and maternal factors, as well as feeding patterns.

Sample Collection and DNA Extraction

Freshly evacuated feces from soiled diapers were collected at 7, 14, and 30 days by medical staff. All samples were frozen at −80°C until further processing. For the extraction of fecal DNA, the QIAmp® DNA stool mini kit (Qiagen, Germany) was used according to the manufacturer's instructions.

PCR Amplification and Illumina MiSeq

The V3-V4 region of the 16S rRNA gene was amplified by PCR using universal primers (16S Amplicon PCR Forward Primer: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; 16S Amplicon PCR Reverse Primer: 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) (20). The PCR was performed in a total volume of 25 μL containing 0.2 μM of each primer and 12.5 μL of 2X KAPA HiFi HotStart ReadyMix (Roche, South Africa). PCR conditions were as follows: 95°C for 3 min; 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min in a Veriti® 96-well thermal cycler (Applied Biosystems, Singapore). The purification step was carried out using AMPure XP beads (Beckman Coulter, USA). Dual indices and Illumina sequencing adapters were attached to the PCR products using the Nextera XT Index Kit for each library. The treated products were quantified on a NanoDrop ND2000 spectrophotometer (Thermo Scientific, USA), Qubit dsDNA HS Assay Kit (Invitrogen, USA), and Labchip GX Touch 24 (Applied Biosystems, USA). Then, the amplicons were pooled together in equimolar quantities and paired-end sequenced (2 × 300 bp) on an Illumina MiSeq platform according to standard protocols.

Bioinformatics Analysis of Microbiota

To analyze bacterial communities, raw sequence reads were assessed for quality and then analyzed by QIIME (21). Each sample yielded more than 100,000 reads after pre-processing in USEARCH 6.1, which included a chimera check and merging of chimera-checked sequences. To pick and cluster the operational taxonomic units (OTUs) in each sample, we used the GreenGenes bacterial reference database with a threshold of 97% sequence identity, thus allowing for maximal taxonomic

TABLE 1 | Demographic and clinical characteristics of study subjects.

	AG group[a] ($N = 10$)	AC group[a] ($N = 14$)	*P*-value
Maternal antibiotics use	8	10	0.75
Prenatal steroid use	9	14	0.71
PROM >18 h	4	9	0.34
Cesarean delivery	7	12	0.36
Gestational age (week)[b]	29.0 (28.0–31.3)	29.0 (27.5–31.0)	0.74
Birth weight (gram)[b]	1,324 (1,223–1,405)	1,138 (846–1,388)	0.09
Male	6	6	0.42
SGA	4	10	0.13
Multiple births	4	1	0.06
APGAR score 5 mins <7	0	1	0.40
Start feeding (day)[b]	2.0 (2.0–3.3)	4.0 (2.0–7.0)	0.07
Reach full feeding (day)[b]	16.0 (10.8–24.5)	21.0 (14.8–29.5)	0.21
RDS need surfactant	5	8	0.49
Days on Oxygen (day)[b]	12.0 (4.3–49.3)	42.0 (3.5–70.5)	0.38
CLD	2	8	0.08
Any IVH	0	1	0.40
ROP need treatment	0	4	0.40
PDA need treatment	3	1	0.94
Total hospitalization days[b]	47.5 (40.8–63.5)	62.0 (44.0–76.0)	0.11

No cases of sepsis and necrotizing enterocolitis in both groups.
[a]The values represent the number of participants, unless otherwise specified.
[b]Values are expressed as median (IQR).
AG, ampicillin-gentamicin group; AC, ampicillin-cefotaxime group; PROM, preterm rupture of membranes; SGA, small for gestational age; RDS, respiratory distress syndrome; CLD, chronic lung disease; IVH, intraventricular hemorrhage; ROP, retinopathy of prematurity; PDA, patent ductus arteriosus.

specificity. Based on taxonomic analysis and species annotation, we generated relative abundance profiles of OTUs for all samples. Differences in community structure were analyzed at the phylum and genus levels; microbial richness and evenness were assessed by the Chao1 and Simpson's Evenness (Simpson's E) indices, respectively. The Shannon index was computed as a measure of alpha-diversity for each individual. To assess differences across groups (beta-diversity), we compared the AC and AG groups by principal component analysis (PCA) and visualized the results in R version 3.5 (https://www.r-project.org/).

Statistical Analyses

Patient characteristics were assessed using a two-sample *t*-test for continuous variables and Fisher's exact test for categorical variables. To determine significant differences across samples, the Kruskal-Wallis test was conducted for multiple comparisons followed by Dunn's *post-hoc* test using Benjamini-Hochberg for correction. Composition and alpha-diversity were compared between the two groups at three time points and over time within a group. Permutational multivariate analysis of variance (PERMANOVA) was performed to assess the effect of relevant confounding factors, including maternal antibiotics use, preterm rupture of membranes, birth weight, start of feeding, and chronic lung disease. Analysis of similarities (ANOSIM) was used for comparison of PCA results between groups. $P < 0.05$ was considered statistically significant.

RESULTS

Demographic Characteristics

Eighty-four breastfed VLBW infants were admitted to our NICU during the study period. After excluding infants who were administered probiotics ($n = 40$) or antibiotics after 1 week of age ($n = 20$), serial stool samples of the final cohort ($n = 24$) were analyzed. Based on exposure to different early postnatal antibiotic therapy, the 24 cases were divided into two groups: AG group ($n = 10$) and AC group ($n = 14$). The reasons for ampicillin-cefotaxime prescription included maternal chorioamnionitis ($n = 4$), increased levels of maternal C-reactive protein ($n = 5$), and infant leukopenia or leukocytosis ($n = 5$). Gestational age and birth weight were 30.0 ± 2.5 weeks and 1,286 ± 190 g in the AG group, and 28.9 ± 2.5 weeks and 1,099 ± 280 g in the AC group. Although there were no statistically significant differences among demographic and clinical characteristics between these two groups, infants in the AC group were more premature and carried a higher risk of infections than those in the AG group (**Table 1**).

Fecal Samples

Analysis of Microbiota Composition

Five stool samples (two 14-day samples in both groups, one 30-day sample in the AG group) could not be obtained due to absence of bowel movement at that time point. Valid sequence data (passing quality control) generated 67 samples. Overall microbiota composition of each group at phylum and genus level is illustrated in **Figure 1**.

Phylum Level

Detailed phylum-level analysis revealed no difference in the distribution of predominant phyla among both groups at any time. Although microbial abundance decreased with increasing age, *Firmicutes* was the most abundant phylum in both groups and remained dominant until day 30 (50.6% in the AG group and 62.7% in the AC group). In the AG group, the proportion of *Actinobacteria* increased over time (7.6, 17.1, and 32.7% at 7, 14, and 30 days, respectively); whereas that of *Proteobacteria* decreased (28.4, 17.5, and 16.7% at 7, 14, and 30 days, respectively). In contrast, in the AC group, the proportions of these two major bacterial phyla showed the opposite trend (*Actinobacteria*: 19.3, 9.0, and 10.9% at 7, 14, and 30 days, respectively; and *Proteobacteria*: 12.3, 27.2, and 26.3% at 7, 14, and 30 days, respectively). No statistically significant difference in the observed bacterial phyla was detected over time within any of the groups (all $P > 0.05$).

Genus Level

Except for *Enterococcus*, no statistical difference in bacterial composition at the genus level was observed between the AG and AC groups at any time or over time within any of the groups. Compared to the AG group, *Enterococcus* was significantly more abundant in the AC group ($P = 0.002$), especially in the 7-day sample (12.3 vs. 0.6%, respectively, $P = 0.032$). Such low abundance of *Enterococcus* in the AG group persisted even after controlling for relevant confounding factors. Although the relative abundance of each bacterium at genus level displayed no statistically significant difference over time within any group

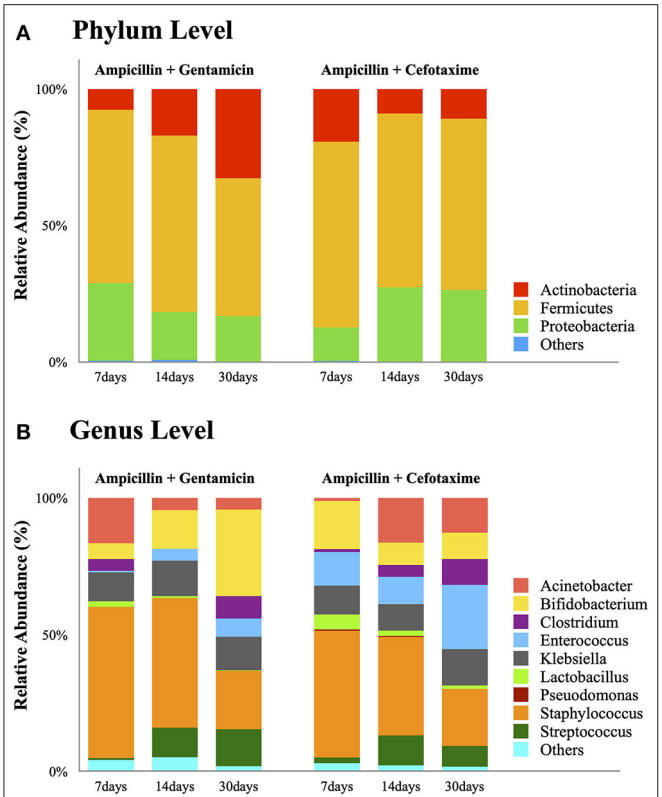

FIGURE 1 | Bar plots showing relative abundance of the most dominant bacterial communities in the AG and AC groups on days 7, 14, and 30. **(A)** Relative abundance at the phylum level. **(B)** Relative abundance at the genus level. AG, ampicillin-gentamicin group; AC, ampicillin-cefotaxime group.

(all $P > 0.05$), the proportion of *Enterococcus* increased in both groups (AG group: 0.6, 4.4, and 6.7% at 7, 14, and 30 days, respectively; AC group: 12.3, 13.1, and 23.5% at 7, 14, and 30 days, respectively). In contrast, the genus *Bifidobacterium* increased over time in the AG group (5.8, 14.1, and 31.6% at 7, 14, and 30 days, respectively), but decreased in the AC group (17.6, 8.1, and 9.7% at 7, 14, and 30 days, respectively). Indeed, *Bifidobacterium* became a dominant member of the community in the AG group at 30 days after birth (31.6%). Although the proportion decreased over time, *Staphylococcus* remained the top genus in both groups on days 7 and 14 (AG group: 55.3, 47.5, and 21.4% at 7, 14, and 30 days, respectively; AC group: 46.4, 36.0, and 20.9% at 7, 14, and 30 days, respectively). The abundance of *Lactobacillus* decreased over time (AG group: 2.1, 0.7, and 0.2% at 7, 14, and 30 days, respectively; AC group: 5.5, 2.0, and 1.3% at 7, 14, and 30 days, respectively); whereas that of *Streptococcus* increased (AG group: 0.7, 10.8, and 13.4% at 7, 14, and 30 days, respectively; AC group: 2.1, 10.5, and 10.9% at 7, 14, and 30 days, respectively). In comparison, *Klebsiella* remained around 10% in both groups at any time point.

Diversity Analysis of Gut Microbiota

Species richness (Chao1 index) in the AC group was found to be significantly lower at 14 days (175 vs. 375, $P = 0.038$) and 30 days (185 vs. 375, $P = 0.03$) compared to 7 days (**Figure 2A**). Microbial evenness (Simpson's E index) was significantly decreased in the AC group compared to that in the AG group (0.02 vs. 0.027, $P = 0.018$) on day 7 (**Figure 2B**). Moreover, in the AC group, it was lower in the 7-day sample than in the 14-day (0.02 vs. 0.057, $P = 0.042$) and 30-day samples (0.02 vs. 0.053, $P = 0.042$). Differences in richness and evenness were unchanged even after controlling for relevant confounding factors. No significant difference in alpha-diversity (Shannon index) between the two groups was observed over time (**Figure 2C**). Regarding beta-diversity, although most 7-day AG group samples were well-separated and positioned far from their corresponding AC group samples, microbial composition did not differ significantly between the two groups ($P = 0.131$) (**Figure 3A**). Samples corresponding to days 14 and 30 in these two groups clustered close to each other and were similar in their microbial composition ($P > 0.05$) (**Figures 3B,C**).

DISCUSSION

In the present study, we explored the changes in gut microbiota communities in VLBW preterm neonates, who were administered empirical antibiotic therapy. Among breastfed infants, we found a statistically significant decrease in microbial richness and evenness in those receiving ampicillin and cefotaxime over a period of 7 days compared to those receiving ampicillin and gentamicin over 3 days. Prolonged or intensive use of antibiotics increased the abundance of bacteria from the genus *Enterococcus*. This finding correlates with previous observations on differential gut microbiota colonization in VLBW preterm infants administered distinct antimicrobial agents (13, 14, 22, 23).

Antibiotics can alter the taxonomic, genomic, and functional features of gut microbiota. These dysbioses include loss of keystone taxa, reduced taxonomic diversity, metabolic changes, and growth of pathogenic organisms (24). Several factors must be considered when evaluating the effect of antibiotics on gut microbiota, including the spectrum of antibiotics, dose and duration of treatment, and the level of active antibiotics in the intestinal tract. The duration of antibiotic treatment is an important variable with a significant effect on the composition of gut microbiota and metabolites in preterm infants. Previous studies associated the prolonged use of antibiotics with reduced gut microbial diversity (13, 22, 25, 26). Others reported that exposure of preterm infants to different types of antibiotics might alter microbial composition (14, 23). The spectrum of cephalosporins is broader than that of penicillin, potentially leading to greater interference against the normal microflora (27). Gibson et al. reported that cefotaxime was associated with significantly reduced species richness, whereas gentamicin has no known effects in this respect (12). Previous findings also suggested that antibiotic regimens containing third-generation cephalosporins were more frequently associated with development of antibiotic resistance than regimens with aminoglycosides (11). In the present study, it remains to be determined if the observed gut microbial composition should be attributed to the different

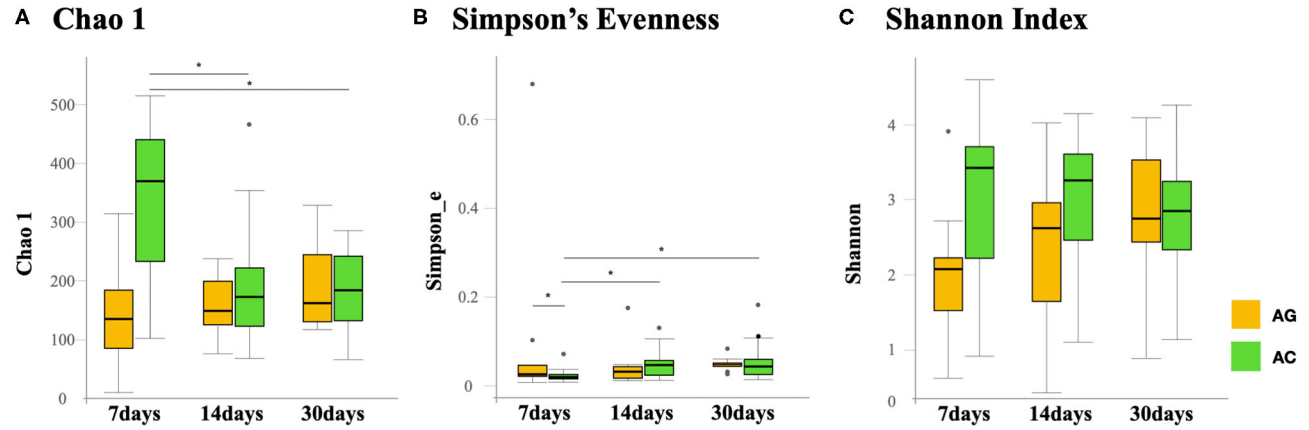

FIGURE 2 | Comparison of gut microbial alpha-diversity in the AG and AC groups on days 7, 14, and 30. **(A)** Richness was assessed using the Chao1 estimator. **(B)** Evenness was assessed using Simpson's Evenness index. **(C)** Diversity was assessed using the Shannon index. AG, ampicillin-gentamicin group; AC, ampicillin-cefotaxime group. Boxes represent the interquartile range, lines indicate medians, and whiskers indicate the range. *Significant differences ($P < 0.05$).

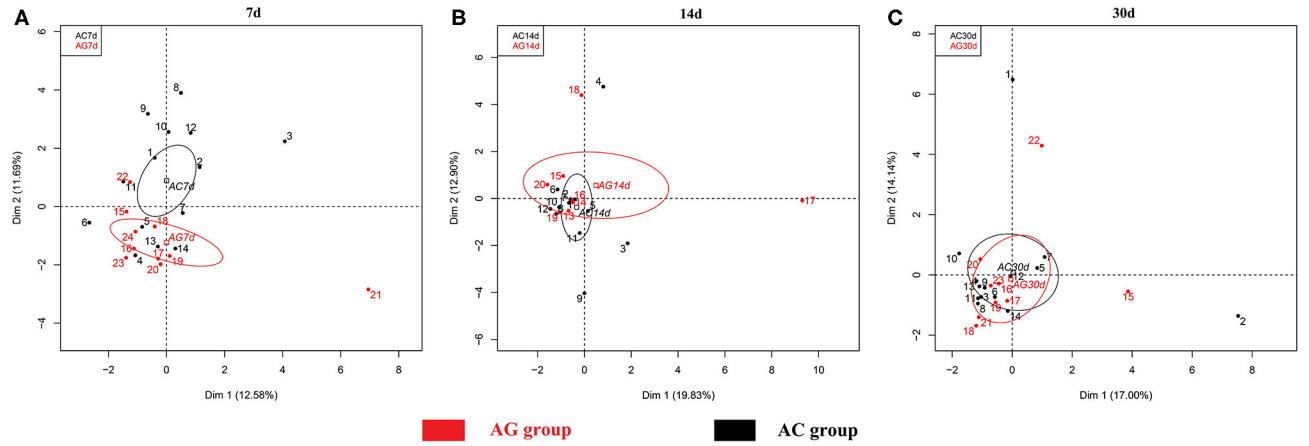

FIGURE 3 | Principal component analysis (PCA) of microbial communities in the AG and AC groups. **(A)** Day 7, **(B)** day 14, and **(C)** day 30. AG, ampicillin-gentamicin group; AC, ampicillin-cefotaxime group.

duration of antibiotic exposure or the administration of different combinations of antibiotics. Therefore, future studies on microbial composition should compare the outcomes of different antibiotic combinations with the same treatment duration.

Bacterial colonization in the intestine proceeds rapidly after birth, particularly with aerotolerant microbes (phase I bloom) (28, 29). Our study confirms that preterm birth and antibiotic exposure result in inadequate phase I colonization. *Firmicutes* were found to be dominant in the intestinal tract, corroborating previous results on preterm infants (30). In line with previous findings, our study also revealed that neonates exposed to ampicillin and cefotaxime harbored more *Proteobacteria* but fewer *Actinobacteria*, thus causing a shift in the composition of gut microbiota (31, 32). At the genus level, antibiotic treatment in the perinatal period negatively affected the abundance of protective commensal anaerobic bacteria such as *Bifidobacteria* (25, 31, 33, 34). As *Bifidobacteria* have been associated with expression of inflammatory response genes and stimulation of genes that promote integrity of the mucosal barrier, the risk of NEC in preterm infants increases with a decline in *Bifidobacteria*, resulting in an exaggerated inflammatory response (11, 17). Although the abundance of *Bifidobacteria* in this study did not change significantly, their percentage increased over time in the ampicillin-gentamicin group, while decreasing in the ampicillin-cefotaxime group. As shown in this study, prolonged use of antibiotics or third-generation cephalosporin treatment might result in excessive growth of *Enterococcus*. This observation is in line with the results of previous studies (14, 31, 35, 36). An increased abundance of not only *Enterococcus*, but also pathogenic bacteria, such as *Enterobacteriaceae*, *Streptococcus*, *Pseudomonas*, and *Clostridium difficile*, has been observed with perinatal antibiotic therapy (13, 14, 30, 31). These findings support

the hypothesis whereby antimicrobial agents selectively kill sensitive bacteria and allow rapid growth of antibiotic-resistant strains. According to a recent meta-analysis, neonates exposed to antibiotics carried a higher risk of developing antibiotic-resistant bacteria (11).

Our study did not address the association between differences in gut microbiota and clinical outcomes. It has been shown that short-term use of antibiotics might have a beneficial effect on reducing adverse outcomes in VLBW preterm infants (18, 37, 38). However, a study also highlighted the association between cefotaxime and adverse clinical outcomes (39). Here, such outcomes, including NEC and sepsis, were too few to determine associations with antibiotic exposure. Alterations in the composition of gut microbiota, fewer *Bifidobacteria*, decreased microbiota diversity, and overgrowth of pathogenic bacteria have been linked to NEC and LOS (40–44). Thus, altering microbiome composition through nutrient intervention including probiotics offers a promising strategy for the prevention of NEC and LOS (45, 46). Another unknown in our study is the recovery time for gut microbiota after antibiotic therapy is discontinued. The recovery rate depends on the duration of antibiotic treatment and the regimen applied. Short-term use of antibiotics may have only mild and temporary effects on gut microbiota; whereas infants exposed to antibiotics for a prolonged time harbor a persistently altered microbiota, which may continue up to the age of 3 months (25, 30). In our study, bacterial diversity at 1 month did not differ significantly between the two groups, which correlates with a previous study (22). This finding confirmed the notion that, although bacterial acquisition was perturbed following the use of antibiotics, microbiota continued to populate the gastrointestinal tract of neonates and eventually restored it to its pre-intervention equilibrium.

Among the strengths of our study is the use of serial fecal samples of preterm neonates to compare gut microbiota profiles. The study takes into account also the most significant factor associated with development of the microbiome community structure in early life, breastfeeding (2, 47–49). However, our study has also some limitations. We did not have a control group with no antibiotic exposure because all VLBW preterm infants in our NICU received empiric antibiotics at birth for presumed sepsis. Another limitation was the lack of stool samples collected immediately after birth. Exposure time to the two antibiotic combinations was different, which further limited the scope for comparison between the two groups. As a result, it is unclear, whether the significant findings of our study resulted from the different combination antibiotic therapy or the different duration exposure to the treatment. Moreover, the pre-existence of a higher risk for infection and more premature births in the AC group created a selection bias. We could not control all confounding factors that might have affected gut microbiota composition, such as maternal conditions and diet, gestational age, delivery mode, feeding types, other medications, and nutritional supplements. Further studies controlling these important confounding factors are required. Another important limitation of our study was a relatively small sample size, which might have led to study bias and insufficient power to detect any differences. Furthermore, we only included infants exposed to antibiotics in the first week of life and excluded those who received antibiotics afterward. The continuous use of antibiotics early in life may have a more severe and long-term impact on gut microbiota.

In summary, we were able to demonstrate that early administration of intravenous antibiotics to premature infants during the first postnatal week dramatically affected the development of neonatal intestinal microbiota. Different antibiotic regimens exert different effects on the evolution of gut microbiota in terms of composition, richness, and evenness. Prolonged use of third-generation cephalosporins strengthened the characteristic shift toward pathogenic bacteria, as indicated by excessive growth of *Enterococcus* in neonates treated with cefotaxime. However, these changes were no longer seen at 30 days of age, suggesting that new bacterial genera continued to populate the neonatal gastrointestinal tract despite initial antibiotic exposure. This also implies that rapid cessation of antibiotic treatment may allow for a faster recovery, whereas prolonged use of antibiotics further impairs recovery of the gut microbiota. Although the clinical and long-term health impact of these changes in microbiota remains to be determined, present findings strongly suggest the need to reduce unnecessary use of broad-spectrum antibiotics in preterm neonates. In the future, well-designed large-scale studies should focus on neonates subjected to the same antibiotic regimen, and compare the changes in their gut microbiome before and after antimicrobial therapy. This will help evaluate the effect of antibiotic exposure on gut microbiome metabolites, clinical outcomes, and long-term consequences.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI BioProject, accession no: PRJNA659014.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of MacKay Memorial Hospital according to the 1964 Declaration of Helsinki and its later amendments (IRB number: 17MMHIS026e). Written informed consent was obtained from the relevant individual(s), and/or minor(s)' legal guardian/next of kin to participate in this study.

DISCLOSURE

The Delta Electronics Incorporation was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

AUTHOR CONTRIBUTIONS

H-YC, J-SCC, and H-CL have prepared the project of this study. H-YC, J-HC, C-HH, and C-YLin performed participant recruitment. H-YC, J-SCC, Y-HH, K-NT, C-YLiu, and MK performed data collection and statistics. H-YC, J-SCC, Y-HH, MK, and H-CL have prepared the draft of manuscript. All authors revised and approved the final manuscript.

FUNDING

This study was supported by a grant (MMH-106-69) from MacKay Memorial Hospital.

ACKNOWLEDGMENTS

The authors are grateful to the staff of NICU for sample collection, and Mrs. Cheng-Yu Chang for valuable assistance.

REFERENCES

1. Collado MC, Cernada M, Neu J, Perez-Martinez G, Gormaz M, Vento M. Factors influencing gastrointestinal tract and microbiota immune interaction in preterm infants. *Pediatr Res.* (2015) 77:726–31. doi: 10.1038/pr.2015.54
2. Stewart CJ, Ajami NJ, O'Brien JL, Hutchinson DS, Smith DP, Wong MC, et al. Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature.* (2018) 562:583–8. doi: 10.1038/s41586-018-0617-x
3. Lu J, Claud EC. Connection between gut microbiome and brain development in preterm infants. *Dev Psychobiol.* (2019) 61:739–51. doi: 10.1002/dev.21806
4. Xu W, Judge MP, Maas K, Hussain N, McGrath JM, Henderson WA, et al. Systematic review of the effect of enteral feeding on gut microbiota in preterm infants. *J Obstet Gynecol Neonatal Nurs.* (2018) 47:451–63. doi: 10.1016/j.jogn.2017.08.009
5. Plummer EL, Bulach DM, Murray GL, Jacobs SE, Tabrizi SN, Garland SM, et al. Gut microbiota of preterm infants supplemented with probiotics: sub-study of the ProPrems trial. *BMC Microbiol.* (2018) 18:184. doi: 10.1186/s12866-018-1326-1
6. Wang Z, Neupane A, Vo R, White J, Wang X, Marzano SL. Comparing gut microbiome in mothers' own breast milk- and formula-fed moderate-late preterm infants. *Front Microbiol.* (2020) 11:891. doi: 10.3389/fmicb.2020.00891
7. Goulet O. Potential role of the intestinal microbiota in programming health and disease. *Nutr Rev.* (2015) 73(Suppl. 1):32–40. doi: 10.1093/nutrit/nuv039
8. Underwood MA, Sohn K. The microbiota of the extremely preterm infant. *Clin Perinatol.* (2017) 44:407–27. doi: 10.1016/j.clp.2017.01.005
9. Groer MW, Luciano AA, Dishaw LJ, Ashmeade TL, Miller E, Gilbert JA. Development of the preterm infant gut microbiome: a research priority. *Microbiome.* (2014) 2:38. doi: 10.1186/2049-2618-2-38
10. Puopolo KM, Benitz WE, Zaoutis TE, Committee on Fetus and Newborn, Committee on Infectious Diseases. Management of neonates born at </=34 6/7 weeks' gestation with suspected or proven early-onset bacterial sepsis. *Pediatrics.* (2018) 142:e20182896. doi: 10.1542/peds.2018-2896
11. Fjalstad JW, Esaiassen E, Juvet LK, van den Anker JN, Klingenberg C. Antibiotic therapy in neonates and impact on gut microbiota and antibiotic resistance development: a systematic review. *J Antimicrob Chemother.* (2018) 73:569–80. doi: 10.1093/jac/dkx426
12. Gibson MK, Wang B, Ahmadi S, Burnham CA, Tarr PI, Warner BB, et al. Developmental dynamics of the preterm infant gut microbiota and antibiotic resistome. *Nat Microbiol.* (2016) 1:16024. doi: 10.1038/nmicrobiol.2016.24
13. Greenwood C, Morrow AL, Lagomarcino AJ, Altaye M, Taft DH, Yu Z, et al. Early empiric antibiotic use in preterm infants is associated with lower bacterial diversity and higher relative abundance of Enterobacter. *J Pediatr.* (2014) 165:23–9. doi: 10.1016/j.jpeds.2014.01.010
14. Zhu D, Xiao S, Yu J, Ai Q, He Y, Cheng C, et al. Effects of one-week empirical antibiotic therapy on the early development of gut microbiota and metabolites in preterm infants. *Sci Rep.* (2017) 7:8025. doi: 10.1038/s41598-017-08530-9
15. Kuppala VS, Meinzen-Derr J, Morrow AL, Schibler KR. Prolonged initial empirical antibiotic treatment is associated with adverse outcomes in premature infants. *J Pediatr.* (2011) 159:720–5. doi: 10.1016/j.jpeds.2011.05.033
16. Alexander VN, Northrup V, Bizzarro MJ. Antibiotic exposure in the newborn intensive care unit and the risk of necrotizing enterocolitis. *J Pediatr.* (2011) 159:392–7. doi: 10.1016/j.jpeds.2011.02.035
17. Esaiassen E, Fjalstad JW, Juvet LK, van den Anker JN, Klingenberg C. Antibiotic exposure in neonates and early adverse outcomes: a systematic review and meta-analysis. *J Antimicrob Chemother.* (2017) 72:1858–70. doi: 10.1093/jac/dkx088
18. Ting JY, Roberts A, Sherlock R, Ojah C, Cieslak Z, Dunn M, et al. Duration of initial empirical antibiotic therapy and outcomes in very low birth weight infants. *Pediatrics.* (2019) 143:e20182286. doi: 10.1542/peds.2018-2286
19. Dobbler PT, Procianoy RS, Mai V, Silveira RC, Corso AL, Rojas BS, et al. Low microbial diversity and abnormal microbial succession is associated with necrotizing enterocolitis in preterm infants. *Front Microbiol.* (2017) 8:2243. doi: 10.3389/fmicb.2017.02243
20. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* (2013) 41:e1. doi: 10.1093/nar/gks808
21. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods.* (2010) 7:335–6. doi: 10.1038/nmeth.f.303
22. Dardas M, Gill SR, Grier A, Pryhuber GS, Gill AL, Lee YH, et al. The impact of postnatal antibiotics on the preterm intestinal microbiome. *Pediatr Res.* (2014) 76:150–8. doi: 10.1038/pr.2014.69
23. Gasparrini AJ, Crofts TS, Gibson MK, Tarr PI, Warner BB, Dantas G. Antibiotic perturbation of the preterm infant gut microbiome and resistome. *Gut Microbes.* (2016) 7:443–9. doi: 10.1080/19490976.2016.1218584
24. Vangay P, Ward T, Gerber JS, Knights D. Antibiotics, pediatric dysbiosis, and disease. *Cell Host Microbe.* (2015) 17:553–64. doi: 10.1016/j.chom.2015.04.006
25. Zwittink RD, Renes IB, van Lingen RA, van Zoeren-Grobben D, Konstanti P, Norbruis OF, et al. Association between duration of intravenous antibiotic administration and early-life microbiota development in late-preterm infants. *Eur J Clin Microbiol Infect Dis.* (2018) 37:475–83. doi: 10.1007/s10096-018-3193-y
26. Niu X, Daniel S, Kumar D, Ding EY, Savani RC, Koh AY, et al. Transient neonatal antibiotic exposure increases susceptibility to late-onset sepsis driven by microbiota-dependent suppression of type 3 innate lymphoid cells. *Sci Rep.* (2020) 10:12974. doi: 10.1038/s41598-020-69797-z
27. Sullivan A, Edlund C, Nord CE. Effect of antimicrobial agents on the ecological balance of human microflora. *Lancet Infect Dis.* (2001) 1:101–14. doi: 10.1016/S1473-3099(01)00066-4
28. Dogra S, Sakwinska O, Soh SE, Ngom-Bru C, Bruck WM, Berger B, et al. Rate of establishing the gut microbiota in infancy has consequences for future health. *Gut Microbes.* (2015) 6:321–5. doi: 10.1080/19490976.2015.1078051
29. Unger S, Stintzi A, Shah P, Mack D, O'Connor DL. Gut microbiota of the very-low-birth-weight infant. *Pediatr Res.* (2015) 77:205–13. doi: 10.1038/pr.2014.162
30. Arboleya S, Sanchez B, Milani C, Duranti S, Solis G, Fernandez N, et al. Intestinal microbiota development in preterm neonates and effect of perinatal antibiotics. *J Pediatr.* (2015) 166:538–44. doi: 10.1016/j.jpeds.2014.09.041
31. Tanaka S, Kobayashi T, Songjinda P, Tateyama A, Tsubouchi M, Kiyohara C, et al. Influence of antibiotic exposure in the early postnatal period on the

development of intestinal microbiota. *FEMS Immunol Med Microbiol.* (2009) 56:80–7. doi: 10.1111/j.1574-695X.2009.00553.x
32. Aloisio I, Quagliariello A, De Fanti S, Luiselli D, De Filippo C, Albanese D, et al. Evaluation of the effects of intrapartum antibiotic prophylaxis on newborn intestinal microbiota using a sequencing approach targeted to multi hypervariable 16S rDNA regions. *Appl Microbiol Biotechnol.* (2016) 100:5537–46. doi: 10.1007/s00253-016-7410-2
33. Korpela K, Salonen A, Virta LJ, Kekkonen RA, de Vos WM. Association of early-life antibiotic use and protective effects of breastfeeding: role of the intestinal microbiota. *JAMA Pediatr.* (2016) 170:750–7. doi: 10.1001/jamapediatrics.2016.0585
34. Zou ZH, Liu D, Li HD, Zhu DP, He Y, Hou T, et al. Prenatal and postnatal antibiotic exposure influences the gut microbiota of preterm infants in neonatal intensive care units. *Ann Clin Microbiol Antimicrob.* (2018) 17:9. doi: 10.1186/s12941-018-0264-y
35. Zwittink RD, van Zoeren-Grobben D, Renes IB, van Lingen RA, Norbruis OF, Martin R, et al. Dynamics of the bacterial gut microbiota in preterm and term infants after intravenous amoxicillin/ceftazidime treatment. *BMC Pediatr.* (2020) 20:195. doi: 10.1186/s12887-020-02067-z
36. Lu S, Huang Q, Wei B, Chen Y. Effects of beta-lactam antibiotics on gut microbiota colonization and metabolites in late preterm infants. *Curr Microbiol.* (2020) 77:3888–96. doi: 10.1007/s00284-020-02198-7
37. Berkhout DJC, Klaassen P, Niemarkt HJ, de Boode WP, Cossey V, van Goudoever JB, et al. Risk factors for necrotizing enterocolitis: a prospective multicenter case-control study. *Neonatology.* (2018) 114:277–84. doi: 10.1159/000489677
38. Li Y, Shen RL, Ayede AI, Berrington J, Bloomfield FH, Busari OO, et al. Early use of antibiotics is associated with a lower incidence of necrotizing enterocolitis in preterm, very low birth weight infants: the NEOMUNE-NeoNutriNet cohort study. *J Pediatr.* (2020) 227:128–34.e2. doi: 10.1016/j.jpeds.2020.06.032
39. Clark RH, Bloom BT, Spitzer AR, Gerstmann DR. Empiric use of ampicillin and cefotaxime, compared with ampicillin and gentamicin, for neonates at risk for sepsis is associated with an increased risk of neonatal death. *Pediatrics.* (2006) 117:67–74. doi: 10.1542/peds.2005-0179
40. Itani T, Ayoub Moubareck C, Melki I, Rousseau C, Mangin I, Butel MJ, et al. Preterm infants with necrotising enterocolitis demonstrate an unbalanced gut microbiota. *Acta Paediatr.* (2018) 107:40–7. doi: 10.1111/apa.14078
41. Young GR, Smith DL, Embleton ND, Berrington JE, Schwalbe EC, Cummings SP, et al. Reducing viability bias in analysis of gut microbiota in preterm infants at risk of NEC and sepsis. *Front Cell Infect Microbiol.* (2017) 7:237. doi: 10.3389/fcimb.2017.00237
42. Wang Y, Hoenig JD, Malin KJ, Qamar S, Petrof EO, Sun J, et al. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. ISME J. (2009) 3:944–54. doi: 10.1038/ismej.2009.37
43. Pammi M, Cope J, Tarr PI, Warner BB, Morrow AL, Mai V, et al. Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis. *Microbiome.* (2017) 5:31. doi: 10.1186/s40168-017-0248-8
44. Morrow AL, Lagomarcino AJ, Schibler KR, Taft DH, Yu Z, Wang B, et al. Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants. *Microbiome.* (2013) 1:13. doi: 10.1186/2049-2618-1-13
45. Chang HY, Chen JH, Chang JH, Lin HC, Lin CY, Peng CC. Multiple strains probiotics appear to be the most effective probiotics in the prevention of necrotizing enterocolitis and mortality: an updated meta-analysis. *PLoS ONE.* (2017) 12:e0171579. doi: 10.1371/journal.pone.0171579
46. Yeung CY, Chiang Chiau JS, Cheng ML, Chan WT, Chang SW, Chang YH, et al. Modulations of probiotics on gut microbiota in a 5-fluorouracil-induced mouse model of mucositis. *J Gastroenterol Hepatol.* (2019) 35:806–14. doi: 10.1111/jgh.14890
47. Lemas DJ, Yee S, Cacho N, Miller D, Cardel M, Gurka M, et al. Exploring the contribution of maternal antibiotics and breastfeeding to development of the infant microbiome and pediatric obesity. *Semin Fetal Neonatal Med.* (2016) 21:406–9. doi: 10.1016/j.siny.2016.04.013
48. Gregory KE, Samuel BS, Houghteling P, Shan G, Ausubel FM, Sadreyev RI, et al. Influence of maternal breast milk ingestion on acquisition of the intestinal microbiome in preterm infants. *Microbiome.* (2016) 4:68. doi: 10.1186/s40168-016-0214-x
49. Liu Y, Qin S, Song Y, Feng Y, Lv N, Xue Y, et al. The perturbation of infant gut microbiota caused by cesarean delivery is partially restored by exclusive breastfeeding. *Front Microbiol.* (2019) 10:598. doi: 10.3389/fmicb.2019.00598

Conflict of Interest: Y-HH, K-NT, and C-YLiu were employed by the company Delta Electronics Incorporation.

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

frontiers in Pediatrics

ORIGINAL RESEARCH
published: 30 August 2021
doi: 10.3389/fped.2021.729535

Clinical Outcomes of Single vs. Two-Strain Probiotic Prophylaxis for Prevention of Necrotizing Enterocolitis in Preterm Infants

Archana Priyadarshi[1,2]*, *Gemma Lowe*[1], *Vishal Saddi*[3], *Amit Trivedi*[2], *Melissa Luig*[1] and *Mark Tracy*[1]

[1] Westmead Hospital Neonatal Intensive Care Unit, Sydney, NSW, Australia, [2] Grace Centre for Newborn Intensive Care at The Children's Hospital at Westmead, Sydney, NSW, Australia, [3] Department of Pediatrics, Bankstown Hospital and Sydney Children's Hospital, Sydney, NSW, Australia

OPEN ACCESS

Edited by:
Yuan Shi,
Children's Hospital of Chongqing Medical University, China

Reviewed by:
Mark Alan Underwood,
University of California, Davis, United States
Scott Guthrie,
Monroe Carell Jr. Children's Hospital, United States

***Correspondence:**
Archana Priyadarshi
archana.priyadarshi@health.nsw.gov.au

Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: 23 June 2021
Accepted: 10 August 2021
Published: 30 August 2021

Citation:
Priyadarshi A, Lowe G, Saddi V, Trivedi A, Luig M and Tracy M (2021) Clinical Outcomes of Single vs. Two-Strain Probiotic Prophylaxis for Prevention of Necrotizing Enterocolitis in Preterm Infants. Front. Pediatr. 9:729535. doi: 10.3389/fped.2021.729535

Background: The administration of live microbiota (probiotic) via enteral route to preterm infants facilitates intestinal colonization with beneficial bacteria, resulting in competitive inhibition of the growth of pathogenic bacteria preventing gut microbiome dysbiosis. This dysbiosis is linked to the pathogenesis of necrotizing enterocolitis (NEC), an acquired multi-factorial intestinal disease characterized by microbial invasion of the gut mucosa, particularly affecting preterm infants. Probiotic prophylaxis reduces NEC; however, variations in strain-specific probiotic effects, differences in administration protocols, and synergistic interactions with the use of combination strains have all led to challenges in selecting the optimal probiotic for clinical use.

Aim: To compare any differences in NEC rates, feeding outcomes, co-morbidities in preterm infants receiving single or two-strain probiotics over a 4-year period. The two-strain probiotic prophylaxis was sequentially switched over after 2 years to the single strain probiotic within this 4-year study period, in similar cohort of preterm infants.

Methods: During two consecutive equal 2-year epochs, preterm infants (<32 weeks and or with birth weight <1,500 g) receiving two-strain (*Lactobacillus acidophilus* and *Bifidobacterium bifidum*) and single strain (Bifidobacterium breve M-16 V,) probiotic prophylaxis for prevention of NEC were included in this retrospective, observational study. The primary outcome included rates of NEC; secondary outcomes included prematurity related co-morbidities and feeding outcomes. Time to reach full enteral feeds was identified as the first day of introducing milk feeds at 150 ml/kg/day.

Results: There were 180 preterm infants in the two-strain, 196 in the single strain group from the two equal consecutive 2-year epochs. There were no differences in the NEC rates, feeding outcomes, all-cause morbidities except for differences in rates of retinopathy of prematurity.

Conclusion: In our intensive-care setting, clinical outcomes of single vs. two—strain probiotic prophylaxis for prevention of NEC were similar. Although our study demonstrates single strain probiotic may be equally effective than two-strain in the

prevention of NEC, small sample size and low baseline incidence of NEC in our unit were not sufficiently powered to compare single vs. two-strain probiotic prophylaxis in preventing NEC. Further clustered randomized controlled trials are required to study the effects of single vs. multi-strain probiotic products for NEC prevention in preterm infants.

Keywords: probiotic prophylaxis, preterms, necrotizing enterocolitis, single strain probiotic, two-strain probiotic

INTRODUCTION

Prophylactic probiotic supplementation is one extensively studied intervention in preventing Necrotizing enterocolitis (NEC), an acquired intestinal disease particularly affecting preterm infants characterized by intestinal inflammation, ischemia, necrosis, and disruption of bowel wall mucosal integrity (1). In NEC, microbial invasion of the mucosa and submucosa leads to gas production (pneumatosis intestinalis), and severe cases rapidly progress to intestinal gangrene, perforation, septicemia leading to complications such as short-gut syndrome, neurocognitive impairment, and even death (2). Multi-factorial etiology such as intestinal immaturity, inherent genetic predisposition, hypoxia-burden from cardio-respiratory disease, pathogenic microbial intestinal colonization, and poorly regulated immune responses have all been implicated leading to challenges in risk prediction and prevention of NEC (3). Preterm infants born < 32 weeks or those with birth weight <1,500 g are most at risk of NEC, and the use of exclusive human milk with avoidance of cow's milk-based formula has shown to reduce NEC (4–8). A major contributing factor in the etiology of NEC is thought to be dysbiosis of the gut microbiome (7, 8). A strategy to prevent this dysbiosis is to administer live microbiota supplements to preterm infants *via* enteral route to facilitate intestinal colonization with beneficial bacteria that competitively inhibit the growth of pathogenic bacteria, altering the risk of NEC and reducing the systemic inflammatory response associated with this condition (9–12). Furthermore, probiotics have antioxidant properties, improving feed tolerance (13).

The efficacy of different probiotic strains in preventing NEC has been described. However, due to variability of the strains used and differences in administration protocols, the clinical decision on the choice of probiotics remains challenging (14–16). Probiotic products containing more than one probiotic strain have been reported to be beneficial due to their synergistic effects (17, 18). Two recent network meta-analysis of clinical trials of probiotics in preterm infants have found more evidence to support benefit of multiple strain probiotics than single strain probiotics (19, 20).

Lactobacilli forms a minor component of the intestinal microbiota in preterm infants. Thus, two-strain probiotic (*Lactobacillus acidophilus* and *Bifidobacterium bifidum)* offers benefit as both strains produce lactic acid and short-chain fatty acids lowering intestinal pH, inhibiting the growth of *Escherichia coli*, a common organism implicated in the pathogenesis of NEC (21).

Overall, *Bifidobacterium* when administered to preterm infants has been associated with improved weight gain, decreased intestinal permeability, reduction in the abundance of potentially pathogenic bacteria colonizing the gut (17).

However, strain matters. A specific strain of *Bifidobacterium-Bifidobacterium breve* M-16V has been shown to result in significant reduction in established (stage II) NEC, albeit a well-designed randomized controlled trial of a different *Bifidobacterium breve* strain BBG-001, showed no benefit in NEC reduction (22–27).

This study compares the NEC rates, feeding outcomes, co-morbidities in a similar cohort of preterm infants switched over from two-strain to single probiotic strain prophylaxis for NEC prevention over two equal consecutive 2-year epochs.

MATERIALS AND METHODS

A retrospective, observational study in a tertiary neonatal intensive care unit (NICU) following routine clinical practice since 2014 to supplement probiotic prophylaxis to preterm infants after parental consent as a NEC prevention intervention. These probiotic products are imported and not manufactured in Australia; due to availability issues the two-strain probiotic was switched over to the single strain.

The study eligibility criteria included all infants admitted to our NICU from July 1, 2014–July 31, 2018 and those <32 weeks and or birth weight of <1,500 g, receiving supplemental probiotic prophylaxis until completion of corrected 34 weeks. Any infants with known chromosomal abnormality and significant congenital anomaly were not included in the study.

Of the total ($n = 675$), 47 receiving probiotics and Lactoferrin (as part of another study) were excluded due to potential influence of Lactoferrin on rates of sepsis. Amongst the remaining 628 infants, 272 received two-strain and 253 received single strain probiotic supplementation. Infants that were noted to have received both the probiotics during their admission, and those transferred to other special care nurseries were excluded ($n = 92$ in two-strain and $n = 57$ in single strain) **Figure 1**.

Epoch 1: 2 years (July 1, 2014 to August 31, 2016), eligible infants ($n = 180$) received two-strain *Bifidobacterium Bifidum* & *Lactobacillus acidophilus* (Infloran® SIT Laboratorio Farmaceutico, Mede, Italy); 250 mg capsules each containing 10^9 colony forming units (CFU) of *Lactobacillus acidophilus* 1×10^9 CFU (ATCC 4356) and *Bifidobacterium bifidum* 1×10^9 CFU (ATCC 15696).

Epoch 2: 2 years (September 1, 2016 to July 31, 2018) eligible infants (n=196) received single strain *Bifidobacterium breve* M-16 V (*Bifidobacterium breve* M-16V; Morinaga Milk Industry Co., Ltd, Japan 1×10^9 colony-forming unit).

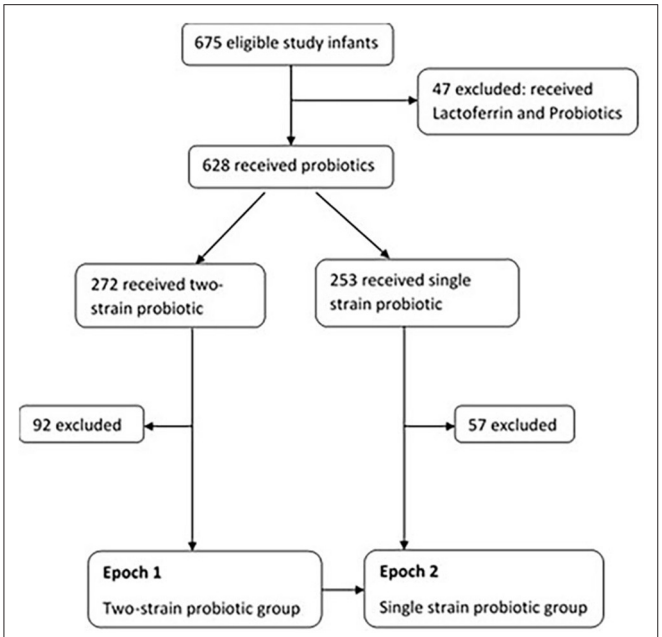

FIGURE 1 | Study design.

A 2-week transition period was allowed for infants receiving the two-strain probiotic to allow completion of the prophylaxis until corrected 34 weeks; infants that continued to then receive single strain, after having received the two-strain probiotic, beyond the transition period were excluded.

The primary outcome was rate of NEC between the two probiotic groups. Baseline NEC rates in our unit range from 2–6% in preterm infants < 28 weeks. The diagnosis of NEC was as per the case definition by the Australian and New Zealand Neonatal Network, ANZNN. (Diagnosis of NEC must have at least one of the following: (1). Diagnosis at surgery or post-mortem; (2). Radiological diagnosis, a clinical history plus: Pneumatosis intestinalis, or Portal vein gas, or persistent dilated loop on serial X-rays; (3). clinical diagnosis, a clinical history plus abdominal wall cellulitis, palpable abdominal mass).

Secondary outcomes included: feeding outcomes and all-cause morbidity until discharge or death during admission. The time to reach full enteral feeds was the first day of the infant receiving 150 ml/kg/day of enteral milk feeds. No changes in feeding practices, total parental nutrition use, respiratory management, or antibiotic treatment guidelines occurred during the study period.

PROBIOTIC PROTOCOL

Eligible infants received freshly reconstituted contents of the probiotic every day in sterile water via enteral route and continued until the completion of corrected age 34 weeks. The probiotics were commenced soon after birth (within 12–72 h) irrespective of the feeds, after obtaining parental consent.

The two-strain probiotic (each 250 mg capsule of two-strain containing *Lactobacillus acidophilus* [10^9 colony-forming units, NCDO 1748; National Collection of Dairy Organisms] and *Bifidobacterium bifidum* [10^9 colony-forming units, NCDO 2203; National Collection of Dairy Organisms, Reading, United Kingdom]; Laboratorio Farmaceutico, Italy), was administered in the dose of: infants with birth weight ≥ 1 kg: 1 capsule (250 mg) and ½ capsule (125 mg) to those with birthweight < 1 kg; in between feeds.

The single strain (each sachet with 1 gm dry powder containing *Bifidobacterium breve* M-16V 5×10^9 colony-forming units, Bifidobacteria B. breve M-16V® Morinaga) administered in the dose of 1 ml (2.5×10^9 colony-forming units) of the reconstituted solution daily *via* the enteral route, after dissolving the contents of one sachet in 2 ml of sterile water.

ETHICAL CONSIDERATIONS

Sydney Children's Hospitals Network Human Research Ethics Committee: 2019/ETH09863, approved the study along with a waiver of consent.

STATISTICAL CONSIDERATIONS

Data were analyzed using Stata (V.15 MP, StataCorp, College Station, Texas, USA). Categorical variables were expressed in frequency as a number of episodes (percentage). Continuous variables were expressed as mean with standard deviation for data with a normal distribution. The two-sample *t*-test with unequal variances or Mann–Whitney test was carried out to evaluate differences in continuous variables between the two study groups. The chi-squared test was used to study the differences in proportions in categorical variables. The Fisher exact test was used where cell numbers were small. Statistical significance was considered with a *p*-value was < 0.05. Given the potential for bias arising from analysis of the retrospective cohort, logistic regression analysis to adjust for confounding variables was not performed. A quality assurance chart "Proportion chart" with upper confidence interval was created identifying the NEC cases during each 6-month period during each epoch (**Figure 2**).

RESULTS

During the study period, 376 preterm infants were identified; 180 received two-strain and 196 received single strain probiotic. The mean gestational age (28 weeks) was similar in the two groups. The infants in two-strain group had statistically significant smaller mean birth weight (1,150 g vs. 1,230 g), were small for gestational age (36 vs. 17) and had an additional day with umbilical catheter (**Table 1**).

There was no difference in the mean timing of commencing trophic feeds (day 2) and volume of feeds achieved by day 5 of age. The single strain group were quicker to reach full feeds (13.2 vs. 15 days). The group that received two-strain probiotic had greater number of days receiving total parental nutrition peripherally (**Table 2**).

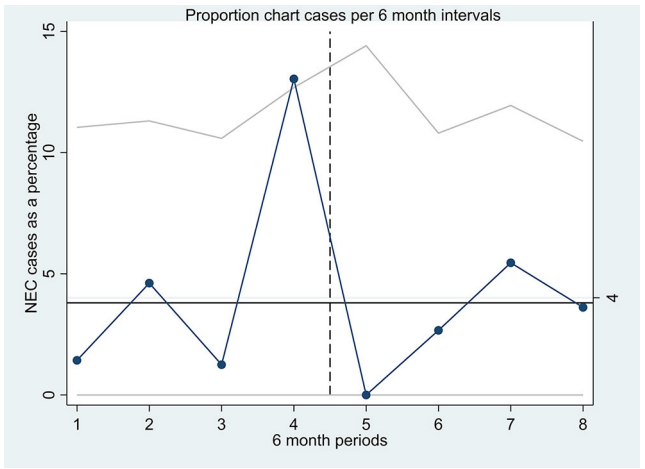

FIGURE 2 | A quality assurance proportion chart (P-chart) with upper confidence interval, each point on the P-chart represents the proportion of NEC cases (in percentage) of all the infants receiving probiotics in each 6-month period of the two epochs.

TABLE 1 | Clinical characteristics of preterm infants in the single vs. two-strain probiotic group.

	Two-strain ($n = 180$)	Single strain ($n = 196$)	*P*-value
Gestational age (weeks)	28.3 ± 2.5	28.6 ± 2.27	0.07
Birth weight (grams)	1,151 ± 337.5	1,237 ± 363	0.01
Male, *n* (%)	90 (50%)	111(56%)	0.20
Apgar 5 min	7 ± 1.69	7 ± 1.56	0.72
SGA*, *n* (%)	36 (20.1)	17 (8.6)	0.002
Mode of delivery			
Vaginal delivery, *n* (%)	66 (37%)	79 (40%)	
Caesarian section, *n* (%)	113 (63%)	117 (60%)	0.50
Antenatal steroids			
No antenatal steroids, *n* (%)	23 (13%)	18 (9%)	
Antenatal steroids <24 h, *n* (%)	50 (28%)	69 (35%)	
Antenatal steroids >48 h, *n* (%)	107 (59%)	109 (56%)	0.26
Abnormal umbilical artery Doppler flows, *n* (%)	4 (2%)	13 (6%)	0.04
Umbilical artery catheter days, mean (range)	1.96 (0–9)	0.92 (0–9)	0.0003
Umbilical venous catheter days, mean (range)	1.84 (0–8)	1.06 (0–9)	0.0002

SGA,small for gestational age.

There was no statistical difference in the number of cases with suspected or confirmed cases of NEC in the two groups. There were no atypical cases of NEC, that is, NEC prior to 10 days of life or after 36 weeks postmenstrual age. There was no difference in the number of sepsis episodes, total ventilation days, number of packed red blood cell transfusion treatments, rates of intra-ventricular hemorrhage, and hemodynamically significant patent ductus arteriosus in the two groups. Rates of retinopathy of prematurity differed significantly in the two groups (**Table 3**).

TABLE 2 | Feeding outcomes of preterm infants in single versus two-strain probiotic group.

	Two-strain ($n = 180$)	Single strain ($n = 196$)	*P*-value
Day of first feed commencement	2.28 (±1.66)	2.17 (±1.27)	0.44
Type of milk			
Expressed breast milk (EBM)	134 (74.5%)	155 (79%)	
Formula	38 (21%)	35 (18%)	
Both formula and EBM	8 (4.5%)	6 (3%)	0.53
Volume of starting trophic feeds mL (SD)	1.2 (±1.6)	1.5 (±3.5)	0.19
Volume of feeds achieved at 72 h ml	11.5 (±19)	10.9 (±18)	0.75
Volume of feeds achieved on day 5 (ml/kg/day)	27.2 (±35)	31.4 (±34)	0.23
Days to reach 120 ml/kg/day, ± SD	15.1 (±10)	13.2 (±8)	0.04
Days to reach 150 ml/kg/day, ± SD	17.4 (±11)	15.0 (±9)	0.06
Days to reach 24 kcal/30 ml feeds, ± SD	18.5 (±14.8)	16.9 (±15)	0.30
Number of days probiotic withheld, ± SD	1.9 (±3.8)	1.3 (±24)	0.048
Number of days of centrally administered TPN*, ± SD	14.6 (±13.5)	11.7 (±11.4)	0.02
Number of days of peripherally administered TPN*, ± SD	1.9 (±3.2)	3.4 (±3.3)	<0.0001
Days to reach birth weight, ± SD	11.6 (±5)	11.7 (±5)	0.78
Total weight gain before discharge (grams), ± SD	1,532 (±800)	1,555 (±920)	0.79
weight at discharge (grams), ± SD	2,640 (±668)	2,736 (±673)	0.16

Coagulase-negative Staphylococcus aureus was the most common organism identified during the sepsis episodes, there were no cases of probiotic-induced sepsis.

There were four deaths in the two-strain strain probiotic group and two in the single strain group. The causes of death included sepsis (four cases), a severe chronic lung disease with respiratory failure (one case), and congenital dyserythropoietic anemia (one case).

DISCUSSION

There was no difference in NEC rates, time to reach full enteral feeds, sepsis, all-cause morbidities except for retinopathy of prematurity (ROP) rates in our study of preterm infants receiving single or two-strain probiotic prophylaxis for NEC prevention during the two consecutive equal epochs. Prophylaxis options between single or two-strain probiotic choices were restricted due to the non-availability of the two-strain product in the second epoch, and thus single strain product was used. Lack of clarity on which probiotic product to choose from once both products were subsequently available led to the study question. Both the probiotics contained a strain of *Bifidobacterium*, but they were not the same species or strain. During the study period, infants achieved similar feeding outcomes, with no difference in NEC rates, and no adverse events from probiotic-induced sepsis. Thus, it is reasonable that further recommendation on the decision of probiotic product choice is guided by other relevant factors such

TABLE 3 | Comparison of the co-morbidities associated with prematurity in infants in the single versus two-strain probiotic group.

	Two-strain (*n* = 180)	Single strain (*n* = 196)	*P*-value
Early onset sepsis ≤ 7 days, *n* (%)	0	1 (0.5%)	1.0
Late onset sepsis > 7 days, *n* (%)			
1 episode	15(8.3%)	19(9.7%)	
≥ 2 episodes	0	3 (1.5%)	
Coagulase negative *Staphylococcus aureus*	11 cases	15 cases	
Escherichia coli	2 cases	0	
Bacillus cereus	0	1 case	
Enterococcus faecalis	1 case	2 cases	
Candida albicans	0 case	1 case	
Group B *Streptococcus*	0 case	1 case	
Lactobacillus lactus	1 case	0 case	
Invasive ventilation days, *n* (%)	2.73 (± 7.13)	1.67 (± 3.95)	0.08
CPAP days, *n (%)*	27.6 (± 29.9)	31.3 (± 51.18)	0.39
Retinopathy of prematurity (ROP) *n* (%)			
No ROP	121 (67%)	160 (82%)	0.003
Stage 1	17 (9.4%)	5 (2.5%)	
Stage 2	32 (17.8%)	21 (10.7%)	
Stage 3	10 (5.5%)	10 (5.1%)	
Treatment for ROP	10 (5.5%)	10 (5.1%)	
Number of blood transfusion episodes, *n* (%)			
None	81 (45%)	99 (50.5%)	
≥ 1	99 (55%)	97 (49.5%)	
Transfusions (mean) (SD)	2.3 (3.4)	1.7 (2.5)	0.05
Intraventricular hemorrhage (IVH), *n* (%)			
No IVH	172 (95.6%)	182 (95%)	0.21
Grade 1	0	2 (1%)	
Grade 2	5 (2.8%)	8 (4%)	
Grade 3	2 (1%)	0	
Grade 4	1 (0.5%)	4 (2%)	
Patent Ductus Arteriosus (PDA), n (%)			
None	119 (66%)	140 (71.4%)	0.27
Hemodynamically significant PDA	61 (34%)	56 (28%)	
NEC, *n* (%)			
None	169 (93.9)	188(95.9)	0.54
Stage 1: suspected NEC	4 (2.2%)	2 (1%)	
Stage 2: medical NEC	7(3.9%)	5 (2.6%)	
Surgical NEC	0	1 (0.5%)	
Death	4 (2%)	2 (1%)	-

as ease of administration, safe storage, and cost-effectiveness in the setting of our NICU.

The mean gestational age of all study infants was 28 weeks; however, two-strain probiotic group had smaller mean birthweight and more small-for-gestational age infants with resultant longer duration of umbilical catheter and centrally administered total parenteral nutrition (TPN) days compared to the single strain group. Despite these differences, there was no difference in their rates of NEC, feeding outcomes, prematurity related co-morbidities when receiving the single or two-strain probiotic. Given the uncontrolled nature of this retrospective cohort analysis, it was not appropriate to apply multivariate logistic regression to adjust for chance differences in these baseline characteristics.

Probiotics reduce time to achieve full enteral feeds and improve feed tolerance, particularly in exclusively breastfed infants (25, 26). In our study, most infants were exclusively breastfed (74.5 vs. 79%), and human milk, formula, mixed feeding (both human milk and formula) rates were similar in both groups. There was no difference in time to reach full enteral feeds or full fortification of feeds at 24 Kcal/30 ml by adding a human milk fortifier. The time to reach full enteral feeds ranged between 15 and 17 days in both the groups, comparable to other tertiary neonatal units with a low incidence of NEC (25). During this period, access to a donor milk bank was not available. All infants regained their birth weight on day 12 and achieved a similar weight gain of about 1,500 g upon discharge. Probiotic supplementation was withheld temporarily for 2 days and 1½ days in the two-strain and single strain groups respectively, due to feeding intolerance.

Probiotics reduce risk of late-onset sepsis and NEC (12, 28, 29). Potential mechanisms include increased barrier to bacterial migration across the intestinal mucosa, competitive exclusion of potential pathogens, modification of host responses to microbes, up-regulation of immune responses by augmentation of immunoglobulin A mucosal responses, enhancement of enteral nutrition (3, 12). There was no difference in the rates of NEC or late-onset sepsis in our groups. There was no difference in the use of antibiotics between the two groups, Coagulase-negative *Staphylococcus aureus* was the most common isolated organism on blood cultures amongst infants with sepsis, there were no cases of probiotic-related sepsis.

Probiotics have no reported benefit in the prevention of retinopathy of prematurity (ROP) (30, 31). In our single strain group, preterm infants had a favorable outcome for ROP with no ROP in 82% compared to 67% in the two-strain group. Between 2017 and 2018, several quality improvement projects for reducing ROP were implemented in our NICU, such as pulse-oxygen saturation target auditing, this coincided with the phase of switching over prophylaxis to the single strain. It is plausible that the difference in ROP rates amongst the two groups may be a chance finding due to its multi-factorial etiology linked to nutrition, oxygen administration, fluctuations in pulse oximetry oxygen saturations.

Its retrospective nature is a major limitation of our study. Firstly, the small sample size was not sufficiently powered to compare NEC rates. The overall low incidence of NEC (2–6% in preterm infants <28 weeks) in our unit will require a multi-centered randomized control trial of more than 1,000 preterm infants to prove the benefit of reducing NEC. We did not report on any product quality checks to assure the effectiveness of the probiotic product when administered. We did not establish evidence on intestinal colonization post-administration of probiotics. The concept of trans-colonization during the period of change from single to the two-strain probiotic was not investigated. Given the clear evidence of cross-contamination

with probiotic organisms, cluster randomized cross-over multi-center trials are required. The impact of antenatal administration of maternal antibiotics, and effects of probiotic volume on the gut osmotic load, stool microbiota were not included in our study. Finally, we did not include any long-term neurodevelopmental outcomes of infants receiving these probiotics.

In our study, strain-specific effects of probiotics did not differ in the prevention of NEC or severe stage of this disease. In addition, our study showed no differences in the clinical efficacy between the single or two-strain probiotic prophylaxis for NEC prevention in preterm infants with gestational age <32 weeks and or birth weight of <1,500 g. Based on the findings of our study, it is plausible that a single strain may be equally effective as a two-strain probiotic product. This calls for further investigations, as the impact of probiotics on incidence of NEC may not be strain-specific, and the mechanisms may be spread across the two products.

CONCLUSIONS

In our intensive-care setting, clinical outcomes of single vs. two-strain probiotic prophylaxis for prevention of NEC were similar. Although our study demonstrates single strain probiotic may be equally effective than two-strain in the prevention of NEC, small sample size and low baseline incidence of NEC in our unit were not sufficiently powered to compare single vs. two-strain probiotic prophylaxis in preventing NEC. Strain-specific effects need further exploration with cluster randomized cross-over multi-center trials adequately numbered to study the effects of different strains in NEC prevention along with a microbiome study of the stool specimens to determine the beneficial effects of intestinal colonization in improving feeding and NEC prevention in our preterm population.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Sydney Children's Hospitals Network Human Research Ethics Committee. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AP: data collection, manuscript drafting, critical review of the manuscript, and statistical analysis of the data. GL: data collection and manuscript drafting. VS, AT, and MT: statistical analysis and critical review of the manuscript. ML: critical review of the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We acknowledge and thank Gritta Kamarudin and Sophia Xu, Westmead Hospital Neonatal Intensive Care Pharmacists for their contribution in this research study.

REFERENCES

1. Eaton S, Rees CM, Hall NJ. Current research on the epidemiology, pathogenesis, and management of necrotizing enterocolitis. *Neonatology.* (2017) 111:423–30. doi: 10.1159/000458462
2. Neu J, Walker WA. Necrotizing enterocolitis. *N Engl J Med.* (2011) 364:255–64. doi: 10.1056/NEJMra1005408
3. Tanner SM, Berryhill TF, Ellenburg JL, Jilling T, Cleveland DS, Lorenz RG, et al. Pathogenesis of necrotizing enterocolitis: modeling the innate immune response. *Am J Pathol.* (2015) 185:4–16. doi: 10.1016/j.ajpath.2014.08.028
4. Tarnow-Mordi W, Soll RF. Probiotic supplementation in preterm infants: it is time to change practice. *J Pediatr.* (2014) 164:959–60. doi: 10.1016/j.jpeds.2013.12.050
5. Dermyshi E, Wang Y, Yan C, Hong W, Qiu G, Gong X, et al. The "golden age" of probiotics: a systematic review and meta-analysis of randomized and observational studies in preterm infants. *Neonatology.* (2017) 112:9–23. doi: 10.1159/000454668
6. Barrington KJ. Review. Probiotics prevented necrotising enterocolitis and reduced mortality in preterm neonates. *Arch Dis Child Educ Pract Ed.* (2011) 96:199. doi: 10.1016/S0140-6736(07)60748-X
7. Aceti A, Gori D, Barone G, Callegari ML, Mauro AD, Fantini MP et al. Probiotics for prevention of necrotizing enterocolitis in preterm infants: systematic review and meta-analysis. *Ital J Pediatr.* (2015) 41:89. doi: 10.1186/s13052-015-0199-2
8. Athalye-Jape G, Rao S, Patole S. Effects of probiotics on experimental necrotizing enterocolitis: a systematic review and meta-analysis. *Pediatr Res.* (2018) 83:16–22. doi: 10.1038/pr.2017.218
9. Wang Q, Dong J, Zhu Y. Probiotic supplement reduces risk of necrotizing enterocolitis and mortality in preterm very low-birth-weight infants: an updated meta-analysis of 20 randomized, controlled trials. *J Pediatr Surg.* (2012) 47:241–8. doi: 10.1016/j.jpedsurg.2011.09.064
10. Underwood M. Impact of Probiotics on Necrotizing Enterocolitis. *Semin Perinatol.* (2017) 41:41–51. doi: 10.1053/j.semperi.2016.09.017
11. Lin HC, Su BH, Chen AC, Lin TW, Tsai CH, Yeh TF, et al. Oral probiotics reduce the incidence and severity of necrotizing enterocolitis in very low birth weight infants. *Pediatrics.* (2005) 115:1–4. doi: 10.1542/peds.2004-1463
12. Jacobs SE, Tobin JM, Opie GF, Donath S, Tabrizi SN, Pirotta M. Probiotic effects on late-onset sepsis in very preterm infants: a randomized controlled trial. *Pediatrics.* (2013) 132:1055–62. doi: 10.1542/peds.2013-1339
13. Wang Y, Wu Y, Wang Y, Xu H, Mei X, Yu D, et al. Antioxidant properties of probiotic bacteria. *Nutrients.* (2017) 9:521. doi: 10.3390/nu9050521
14. Deshpande G, Rao S, Patole S, Bulsara M. Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. *Pediatrics.* (2010) 125:921–930. doi: 10.1542/peds.2009-1301
15. Hays S, Jacquot A, Gauthier H, Kempf C, Beissel A, Pidoux O, et al. Probiotics and growth in preterm infants: A randomized controlled trial, PREMAPRO study. *Clin Nutr.* (2016) 35:802–11. doi: 10.1016/j.clnu.2015.06.006
16. Underwood MA, Salzman NH, Bennett SH, Barman M, Mills DA, Marcobal A, et al. A randomized placebo-controlled comparison of 2 prebiotic/probiotic

combinations in preterm infants: impact on weight gain, intestinal microbiota, and fecal short-chain fatty acids. *J Pediatr Gastroenterol Nutr.* (2009) 48:216–25. doi: 10.1097/MPG.0b013e31818de195
17. Totsu S, Yamasaki C, Terahara M, Uchiyama A, Kusuda S. Bifidobacterium and enteral feeding in preterm infants: cluster-randomized trial. *Pediatr Int.* (2014) 56:714–9. doi: 10.1111/ped.12330
18. Hikaru U, Koichi S, Yayoi S, Hiromichi S, Hiroaki S, Yoshikazu O, et al. Bifidobacteria prevents preterm infants from developing infection sepsis. *Int J Probiotics Prebiotics.* (2010) 5:33–6.
19. Morgan RL, Preidis GA, Kashyap PC, Weizman AV, Sadeghirad B. McMaster probiotic, prebiotic, and synbiotic work group. probiotics reduce mortality and morbidity in preterm, low-birth-weight infants: a systematic review and network meta-analysis of randomized trials. *Gastroenterology.* (2020) 159:467–80. doi: 10.1053/j.gastro.2020.05.096
20. Chi C, Li C, Buys N, Wang W, Yin C, Sun J. Effects of probiotics in preterm infants: a network meta-analysis. *Pediatrics.* (2021) 147:e20200706. doi: 10.1542/peds.2020-0706
21. Deshpande GC, Rao SC, Keil AD, Patole SK. Evidence-based guidelines for use of probiotics in preterm neonates. *BMC Med.* (2011) 9:92. doi: 10.1186/1741-7015-9-92
22. Shiou S-R, Yu Y, Guo Y, He S-M, Mziray-Andrew CH, Hoenig J, et al. Synergistic protection of combined probiotic conditioned media against neonatal necrotizing enterocolitis-like intestinal injury. *PLoS ONE.* (2013) 8:e65108. doi: 10.1371/journal.pone.0065108
23. Athalye-Jape G, Rao S, Simmer K, Patole S. Bifidobacterium breve M-16V as a Probiotic for Preterm Infants. *J Parenter Enteral Nutr.* (2018) 42:677–88. doi: 10.1177/0148607117722749
24. Patole SK, Rao SC, Keil AD, Nathan EA, Doherty DA, Simmer KN. Benefits of *Bifidobacterium breve* M-16V supplementation in preterm neonates - a retrospective cohort study. *PLoS ONE.* (2016) 11: e0150775. doi: 10.1371/journal.pone.0150775
25. Aceti A, Gori D, Barone G, Callegari ML, Fantini MP, Indrio F, et al. Probiotics and time to achieve full enteral feeding in human milk-fed and formula-fed preterm infants: systematic review and meta-analysis. *Nutrients.* (2016) 8:471. doi: 10.3390/nu8080471
26. Athalye-Jape G, Deshpande G, Rao S, Patole S. Benefits of probiotics on enteral nutrition in preterm neonates: a systematic review. *Am J Clin Nutr.* (2014) 100:1508–19. doi: 10.3945/ajcn.114.092551
27. Costeloe K, Hardy P, Juszczak E, Wilks M, Millar MR. Probiotics in preterm infants study collaborative group. bifidobacterium breve BBG-001 in very preterm infants: a randomised controlled phase 3 trial. *Lancet.* (2016) 387:649–60. doi: 10.1016/S0140-6736(15)01027-2
28. Rojas MA, Lozano JM, Rojas MX, Rodriguez VA, Rondon MA, Bastidas JA. Prophylactic probiotics to prevent death and nosocomial infection in preterm infants. *Pediatrics.* (2012) 130:e1113–20. doi: 10.1542/peds.2011-3584
29. Woodman T, Strunk T, Patole S, Hartmann B, Simmer K, Currie A. Effects of lactoferrin on neonatal pathogens and *Bifidobacterium breve* in human breast milk. *PLoS ONE.* (2018) 13: e0201819. doi: 10.1371/journal.pone.0201819
30. Cavallaro G, Villamor-Martínez E, Filippi L, Mosca F, Villamor E. Probiotic supplementation in preterm infants does not affect the risk of retinopathy of prematurity: a meta-analysis of randomized controlled trials. *Sci Rep.* (2017) 7:13014. doi: 10.1038/s41598-017-13465-2
31. Saeidi R, Izanloo A. P449 Probiotics and retinopathy of premature: systemic review. *Arch Dis Child.* (2019) 104:A332. doi: 10.1136/archdischild-2019-epa.785

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pediatrics

ORIGINAL RESEARCH
published: 05 October 2021
doi: 10.3389/fped.2021.723617

One-Week Effects of Antibiotic Treatment on Gut Microbiota of Late Neonates With Pneumonia or Meningitis

Shujing Han[1†], Qiaoru Zhang[2†], Yijun Ding[2], Ping Chu[1], Jinjing Zhang[2], Jin Shi[1], Shengnan Jia[2], Caiyun Yang[2], Jie Lu[1] and Yajuan Wang[3*]*

[1] Beijing Key Laboratory for Pediatric Diseases of Otolaryngology, Head and Neck Surgery, MOE Key Laboratory of Major Diseases in Children, Beijing Pediatric Research Institute, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health (NCCH), Beijing, China, [2] Department of Neonatology, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health (NCCH), Beijing, China, [3] Department of Neonatology, Children's Hospital, Capital Institute of Pediatrics, Beijing, China

Background: The neonatal period is a critical period for the establishment of the intestinal microbial community. Antibiotics can change the composition of gut microbiota.

Methods: Fecal samples were collected from 14 patients with pneumonia and 14 patients with meningitis before and after antibiotic treatment, and fecal samples from five healthy neonates at the 14th and 21st days after birth were collected as well. DNA of fecal samples was extracted, and PCR amplification was performed targeting the V3–V4 variable region of 16S rDNA. After detection by high-throughput sequencing, OTU (operational taxonomic unit) clustering, species annotation, and α diversity analysis were calculated and analyzed statistically.

Results: In the healthy control group, the abundance of *Bifidobacterium* increased significantly from 16.75 to 40.42%, becoming the most dominant bacteria. The results of α diversity analysis suggested that the Sobs indexes of the gut microbiota in the pneumonia and meningitis groups were significantly lower than that in the healthy control group ($p < 0.05$). PCoA analysis showed that the gut microbiota of pneumonia and meningitis groups clustered distinctly with the control group (Adonis $p = 0.001$, $R^2 = 0.565$), and there was no significant change in the diversity of gut microbiota before and after the use of antibiotics.

Conclusions: The gut microbiota of neonates with infectious diseases were mainly related to the disease conditions. The initial state of neonatal gut microbiome determines its state after 1-week antibiotic treatment. Antibiotic application with 7 days had little effect on the community richness and some effect on the composition of gut microbiota of neonates with pneumonia or meningitis.

Keywords: gut microbiota, antibiotic, neonate, pneumonia, meningitis

OPEN ACCESS

Edited by:
Yuan Shi,
Children's Hospital of Chongqing Medical University, China

Reviewed by:
Lu-Quan Li,
Chongqing Medical University, China
Hui Wu,
First Affiliated Hospital of Jilin University, China
Xia Liu,
Baylor College of Medicine, United States

***Correspondence:**
Yajuan Wang
cxswyj@vip.sina.com
Jie Lu
lujiebch@163.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: *11 June 2021*
Accepted: *09 August 2021*
Published: *05 October 2021*

Citation:
Han S, Zhang Q, Ding Y, Chu P, Zhang J, Shi J, Jia S, Yang C, Lu J and Wang Y (2021) One-Week Effects of Antibiotic Treatment on Gut Microbiota of Late Neonates With Pneumonia or Meningitis. Front. Pediatr. 9:723617. doi: 10.3389/fped.2021.723617

INTRODUCTION

Human gut microbiota refers to the general term of microorganisms inhabiting the human digestive tract system, including bacteria, archaea, viruses, fungi, and protozoa. There are direct or indirect interactions between gut microbiota and the host to form a complex interaction network. This network constitutes a dynamic balance of the micro-ecosystem, which is closely related to human health and disease (1, 2). Current studies show that gut microbiota is closely associated with the occurrence and development of various chronic diseases, including diabetes, hypertension, cardiovascular diseases, brain diseases, and tumors (3). The early formation and development of this system is mainly regulated by human genetics and immunity, and the composition of microbial community is related to the habitat. In addition, diet, lifestyle, and medication (antibiotics) as well as probiotics and pre-biotics also play a key role in regulating microbiota, especially gut microbiota (1).

The infant period is a critical period for the establishment and formation of human normal gut microbiota. The establishment and formation of normal gut microbiota, the development and maturation of the immune system, and the formation of metabolic patterns occur simultaneously (4). During this period, the gut microbiota is in the stage of succession and easily influenced by external factors, especially antibiotics (5). Studies (6, 7) have shown that antibiotics have a great impact on the structure of neonatal intestinal microbial community, and the quick cessation of antibiotic treatment can restore the microbial community. The long-term use of antibiotics in infants could change the normal gut microbiota, further change the body's immune response and metabolic patterns, and has a long-term impact on the human body (8). Although more and more people know that antibiotic treatment can affect the development of neonatal microbiota, the vast majority of previous studies only focused on the use of antibiotics (8), and many studies did not refer to types of diseases suffered by newborns. Thus, the effect of antibiotic use on the gut microbiota of newborns in the treatment of diseases at different infection sites still remains unknown.

In the neonates, pneumonia is the most common infectious disease, while meningitis, especially severe meningitis, is the most severe form of infectious diseases in the neonates. In order to investigate the influences on intestinal flora of neonates by both disease condition and antibiotic treatment, we collected fecal samples of neonates with pneumonia or meningitis, before and after the use of antibiotics in neonatal intensive care unit (NICU). High-throughput sequencing was used to explore the difference of gut microbiota between healthy neonates and neonate patients with infectious diseases (pneumonia or meningitis) and the changes in gut microbiota in patients during the antibiotic treatment.

MATERIALS AND METHODS

Study Design

This prospective case-control study has been approved by the Institutional Review Board of Beijing Children's Hospital, Capital Medical University (Approval No.: 2015-36). Neonates with pneumonia or meningitis in the neonatal ward were included from January 2015 to May 2016. Meanwhile, healthy neonates in the same period were enrolled as the control group. Their general status, the mother's pregnancy, feeding, and other information were collected.

Patient Inclusion and Exclusion Criteria

All the patients and healthy neonates enrolled were 7- to 28-day-aged, full-term newborns with post-natal exclusive breastfeeding. All the parents of the participating children signed the informed consent form. The neonates in control group were healthy newborns born by the hospital staff in the same period without any antibiotic usage and infectious symptoms.

For diagnostic criteria of pneumonia, please refer to Reference (9). Bacterial pneumonia may be considered if one of the following criteria is met: (1) pulmonary signs: audible phlegm or rales; (2) chest radiograph: diffusely blurred images of the lungs, punctate, and patchy infiltrating images; (3) elevated blood inflammatory indexes (mainly leukocytes, neutrophils, CRP); and (4) bacterial culture of respiratory secretions was positive. Inclusion criteria for patients with pneumonia were (1) full term, >7 days of late neonates. (2) It met the diagnostic criteria of neonatal bacterial pneumonia. (3) Intravenous cefazoxime was used for anti-infection after admission, and the treatment course was 7 days. (4) No antibiotics were used before admission. Exclusion criteria for patients with pneumonia were (1) newborns who were not treated or did not receive regular treatment; (2) newborns with diarrheal diseases and other intestinal diseases, and other infections; (3) newborns who had malformation, congenital, or genetic metabolic diseases; (4) The stool specimen is not qualified.

Purulent meningitis diagnostic criteria were according to Reference (10). Inclusion criteria for patients with meningitis were (1) full term, >7 days of late neonates. (2) meet the diagnostic criteria of neonatal purulent meningitis and meet one of the following criteria: ① fever (body temperature $\geq$38.5°C); ② with convulsion, drowsiness, and other nervous system manifestations; ③ there are circulation instability and other manifestations of infection. (3) Intravenous meropenem was used for anti-infection after admission, and the treatment course was $\geq$7 days. (4) No antibiotics were used before admission. Exclusion criteria for patients with meningitis were (1) newborns who were not treated or did not receive regular treatment; (2) newborns with diarrhea and other intestinal diseases; (3) newborns who had malformation, congenital or genetic metabolic diseases; (4) The stool specimen is not qualified.

Specimen Collection

In the patient group, fecal samples were collected at two time points: (1) before the antibiotics were given and (2) the 7th day of antibiotic treatment. Healthy children' fecal samples were collected without urine, dust, and other pollution on their 14th and 21st day after birth. Fecal samples (>1 g) were put in the feces collection boxes, which had been sterilized before use. All samples were stored at −80°C.

High-Throughput 16S rRNA Amplicon Sequencing

DNA was extracted using a Stool Genomic DNA kit (Beijing ComWin Biotech Co., Beijing, China) following the instructions of the manufacturer. The DNA samples were then sent to Majorbio BioPharm Technology Co. Ltd. (Shanghai, China) for polymerase chain reaction (PCR) amplification, and Illumina high-throughput sequencing. The PCR amplification of the bacterial 16S rRNA gene amplicons (V3 + V4 regions) was performed using specific primers (338F-5′-barcode + ACTCCTACGGGAGGCAGCAG-3′; 806R-5′-GGACTACHVGGGTWTCTAAT-3′). The reaction was carried out with an initial melting step of 95°C for 3 min, followed by 27 cycles of 30 s at 95°C, 30 s at 55°C, and 45 s at 72°C, and a final elongation step of 10 min at 72°C. The 16S rDNA amplification products were electrophoresis in 2% agarose gel, and then detected and recorded on the MultiImager. The AxyPrepDNA gel recovery kit (Axygen, USA) was used to recover the PCR products. The DNA amplicons were used to conduct the high-throughput sequencing on an Illumina MiSeq platform.

Bioinformatic Analysis

Data analysis was conducted using the i-Sanger platform (http://www.i-sanger.com/) provided by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw data obtained by sequencing were split into samples according to the joint sequence. Then reads containing more than 10 low-quality reads, reads with joint contamination sequence exceeding 15 bp, and repeated reads were filtered out to obtain high-quality clean data and completed data filtering. Through the overlap relationship between reads, filtered reads were spliced into Tags and clustered into OTUs for species classification with 97% similarity using USEARCH (v7.0.1090). After initial OTU representative sequence was obtained, the chimera generated by PCR amplification was removed from the OTU representative sequence by UCHIME (v4.2.40). Finally, all Tags were compared back to the OTU representative sequence to get the final OTUs representative sequence of each sample by usearch_global.

The OTU representative sequences obtained previously were compared with the database Greengene (V201305) for species annotation using the RDP Classifier (v2.2), and the confidence threshold was set to 0.8. The relative abundance of each sample in phylum, class, order, family, genus, and species was obtained, and the bar plot in the R package was used to obtain the histogram of each sample. Five percent of the species merged into others. Biological diversity analysis was carried out based on the results of OTU analysis, and dilution curves were made according to the analysis results.

Statistical Analysis

The statistical analysis software SPSS 19.0 was used. Normal distribution of the variables was first tested. For two-group comparison and variables showing normal distribution, Student's *t*-test was used. The *p*-value was two-sided, and $p < 0.05$ was considered statistically significant.

RESULTS

General and Clinical Information

The patients with pneumonia or meningitis were diagnosed according to the diagnosis criteria mentioned above. The selection of antibiotics in the clinical consultation and treatment process is mostly based on clinical experience or pathogenic results. In order to reduce confounding factors, we only chose the pneumonia patients treated by cefazoxime and meningitis patients treated by meropenem. Finally, 14 patients with pneumonia, 14 patients with meningitis, and 5 healthy neonates as control were involved according to our inclusion criteria and exclusion criteria (**Figure 1**). **Table 1** shows the general and clinical examination information of all patients and healthy newborns.

Microbiota Community Structure of Newborns

Through optimization, a total of 2,538,884 high-quality sequences were obtained from 66 samples, with an average length of 447 bp and a length distribution between 420 and 460 bp. The total number of OTU representative sequence was 379. The sparse curve showed that the sequencing data were reasonable (**Supplementary Figure 1**).

The total number of OTUs in the healthy group (314) was much higher than that in the pneumonia group (138) and meningitis group (172). Alpha-diversity analysis (**Figure 2**) shows that both the Chao index and the Sobs index in control group were significantly higher than the patient group, indicating that the healthy neonates had a higher community richness and diversity. However, before and after the use of antibiotics, indexes of α-diversity were not significantly changed in both meningitis and pneumonia neonates, which means that bacteria population diversity was not significantly suppressed in individual newborns immediately after antibiotic administration.

The dominant bacteria of gut microbiota in both healthy and infected newborns were most commonly found in the following four phylum: *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* (**Figure 3A**). At the genus level (**Figure 3B**), the dominant bacteria of gut microbiota in our newborns were *Klebsiella*, *Escherichia–Shigella*, *Enterococcus*, and *Bifidobacterium.*

Changes in Gut Microbiota of Healthy Newborns

In the healthy control group, we clearly found that with age increase, *Actinobacteria* (from 20.68 to 43.67%) was increasing, while *Proteobacteria* (from 51.17 to 39.78%) and *Firmicutes* (from 22.45 to 14.04%) were decreasing (**Figure 4A**). At the genus level, the abundance of *Bifidobacterium* (belongs to *Actinobacteria*) increased significantly from 16.75to 40.42%, becoming the most dominant bacteria, and the abundances of *Klebsiella* (belongs to *Proteobacteria*), *Escherichia–Shigella* (belongs to *Proteobacteria*), *Veillonella* (belongs to *Firmicutes*), and *Bacteroides* (belongs to *Proteobacteria*) declined with growth of healthy newborns (**Figure 4B**). The rise and fall of the genus-level bacteria resulted

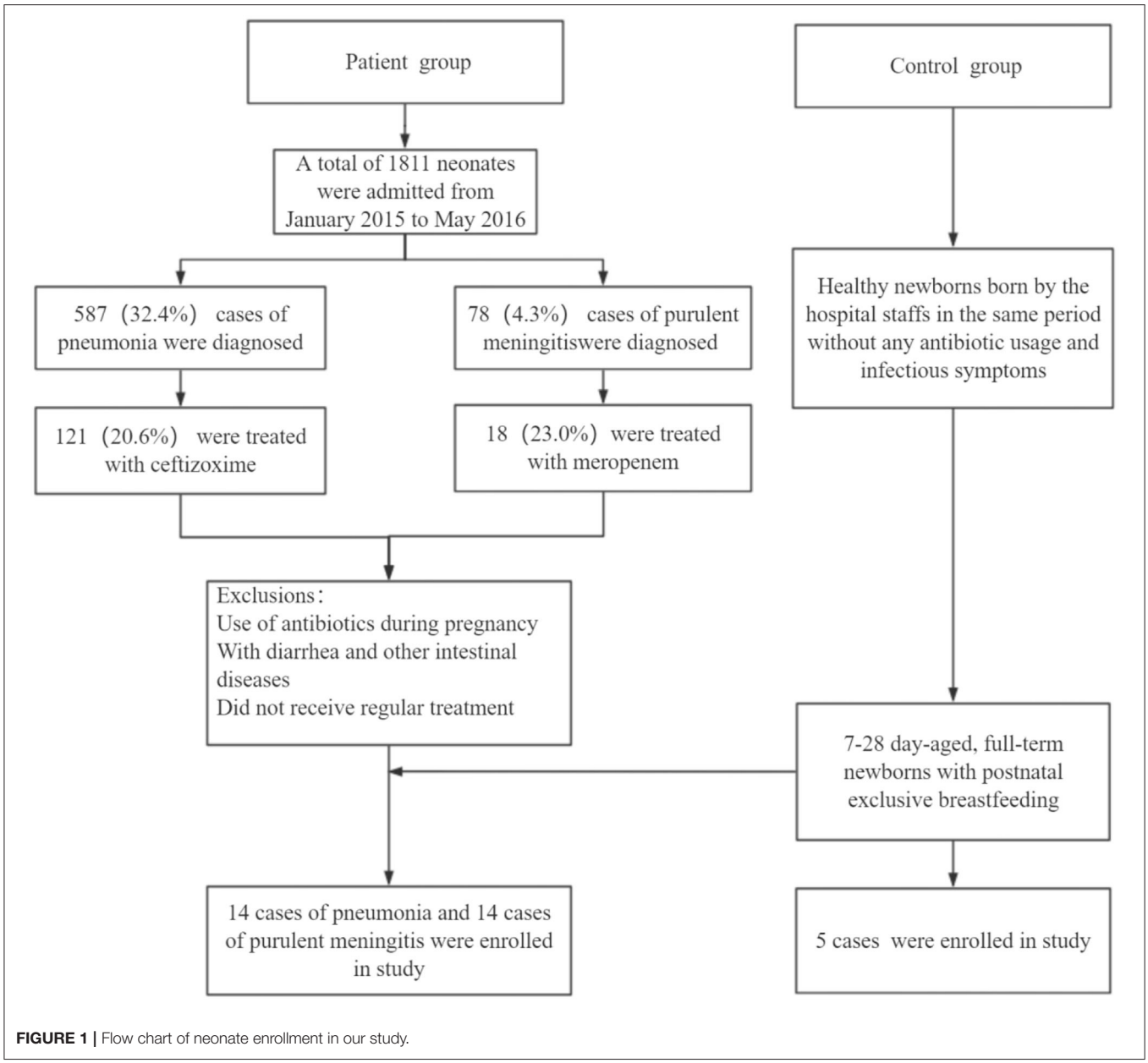

FIGURE 1 | Flow chart of neonate enrollment in our study.

in changes in the phylum level and also showed normal succession of bacteria in the intestines of healthy newborns.

Differences in Gut Microbiota Between Healthy Newborns and Patients

We first used principal coordinate analysis (PCoA) on unweighted UniFrac distances to examine the community structures of the gut microbiotas of the three groups. As shown in **Figure 5A**, the gut microbiota of the pneumonia and meningitis groups clustered distinctly with the control group (Adonis $p = 0.001$, $R^2 = 0.565$). After 7 days (the patients were treated by antibiotics and the healthy controls did not receive any intervention), the gut microbiota of two groups of patients still clustered distinctly with the control group (**Supplementary Figure 2**, Adonis $p = 0.027$, $R^2 = 0.146$).

At the genus level, *Enterococcus* (36.00%), *Escherichia–Shigella* (14.00%), and *Bifidobacterium* (12.93%) were the dominant bacteria in the pneumonia group, and *Escherichia–Shigella* (30.34%), *Klebsiella* (27.28%), and *Enterococcus* (6.34%) were the dominant bacteria in the meningitis group, while *Klebsiella* (34.12%), *Bifidobacterium* (16.75%), and *Escherichia–Shigella* (13.88%) were the dominant bacteria in the control group, but the differences between the groups of the patients and control group were not statistically significant.

LEfSe analysis can be used to analyze the differences in gut microbiota between groups and find out the microbial species that differ between groups, which is conducive to

TABLE 1 | General information and antibiotic treatment of newborns.

	Pneumonia group ($N = 14$)	Meningitis group ($N = 14$)	Control group ($N = 5$)	*p*-Value
Male (*n*)	8 (57.1%)	8 (57.1)	3 (60.0)	0.86
Age of admission (days)	16.18 ± 2.53	16.29 ± 1.97	14.00 ± 0.00	0.16
Birth weight (g)	3,415.36 ± 372.01	3,342.14 ± 568.78	3,470.00 ± 349.29	0.69
Gestational age (days)	275.79 ± 6.84	275.57 ± 7.26	274.4 ± 5.18	0.94
1 min Apgar scores ≤7	0	0	0	-
Delivery mode				0.10
Cesarean delivery	0	4	0	–
Vaginal delivery	14	10	5	–
Use of antibiotics during pregnancy	0	0	0	–
Manifestation				
Fever (≥38.5°C)	11 (78.6%)	14 (100%)	–	
Lung rales	14 (100%)	–	–	
NCPAP	8 (57.1%)	–	–	
Seizure	–	4 (28.6%)	–	
Irritability	–	7 (50%)	–	
Weak response	7 (50%)	11 (78.6%)	–	
Feeding difficulties	7 (50%)	9 (64.3%)	–	
CRT[e] > 2 s	-	11 (78.6%)	–	
Culture positive	2 (14.3%)	6 (42.9%)	–	
WBC[b] > 15×10^9/L	2 (14.3%)	3 (21.4%)	–	
CRP > 8 mg/L (n)[a]	5 (35.7%)	5 (35.7%)	–	
NEC[c] > 60%	2 (14.3%)	5 (35.7%)	–	
Patchy infiltrating images	14 (100%)			
CSF[d] > 21×10^6/L	–	14 (100%)	–	
CSF protein>1.2 g/L	–	9 (64.3%)	–	
CSF glucose <1.1–2.2 mmol/L	–	7 (50%)	–	
Antibiotic treatment	Ceftizoxime	Meropenem	–	

[a] *C-reactive protein.*
[b] *White blood cell count.*
[c] *Neutrophilic granulocyte percentage.*
[d] *Cerebro-spinal fluid cell count.*
[e] *Capillary refill time.*

the discovery of biomarkers. As shown in **Figure 5B**, relative abundance of *Bacillus*, *Anoxybacillus*, and *Erysipelatoclostridium* was significantly higher in the healthy group than in the meningitis or pneumonia group, while the relative abundance of *Lachnoclostridium* and *Rhodococcus* was significantly higher in the meningitis group, and relative abundance of *Saccharibacteria* was significantly higher in the pneumonia group. The LDA score threshold was 2.

We also compared the gut microbiota of two groups of patients before the use of antibiotics. The α-diversity analysis revealed that the Sobs and Chao index had no significant difference in community richness and diversity between pneumonia and meningitis group ($p = 0.057$, $p = 0.125$). PCoA analysis (**Figure 5A**) shows that the gut microbiota of pneumonia clustered distinctly with the meningitis groups, indicating that the species composition of gut microbiota from these two groups were quite different. At the genus level (**Figure 5C**), the abundance of *Enterococcus* (pneumonia 35.32%, meningitis 6.67%) and *Bifidobacterium* (pneumonia 13.49%, meningitis 1.64%) in pneumonia neonates was conspicuously higher than the meningitis groups ($p = 0.013$, $p = 0.030$).

Difference of Gut Microbiota Before and After the Use of Antibiotics

In the pneumonia group, community richness had no change before and after the use of antibiotics (**Figure 2**). Meanwhile, the PCoA plot (**Supplementary Figure 3**) also illustrated the gut microbiota before and after the use of antibiotics were not separated from each other. At the genus level, the abundance of *Bifidobacterium* was lower after 7-day antibiotic treatment (pneumonia 13.49%, pneumonia-antibiotic 2.47%, $p = 0.057$), but the difference was not statistically significant (**Supplementary Figure 3**).

In the meningitis group, community richness also had no change before and after the use of antibiotics (**Figure 2**). In addition, the PCoA plot (**Supplementary Figure 4**) also illustrated the gut microbiota before and after the use of antibiotics were not separated from each other. The abundance of *Bifidobacterium* had no difference before and after the antibiotic

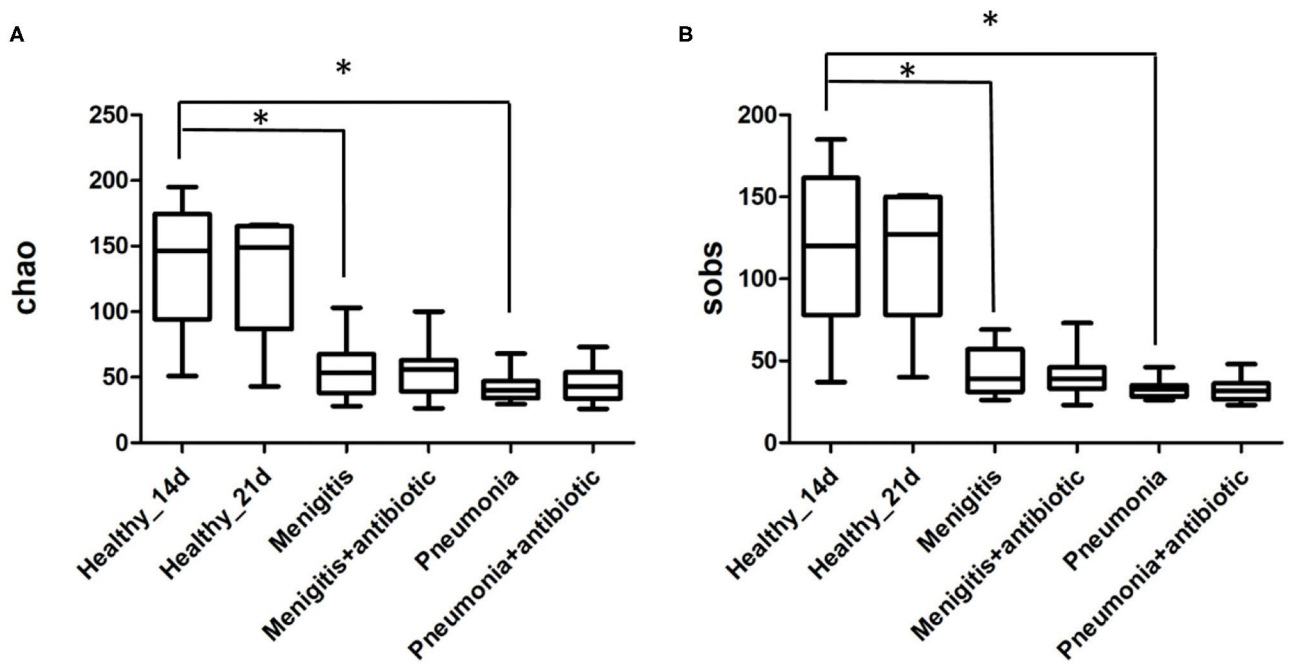

FIGURE 2 | Chao **(A)** and Sobs **(B)** indexes of α diversity analysis. *$p < 0.05$.

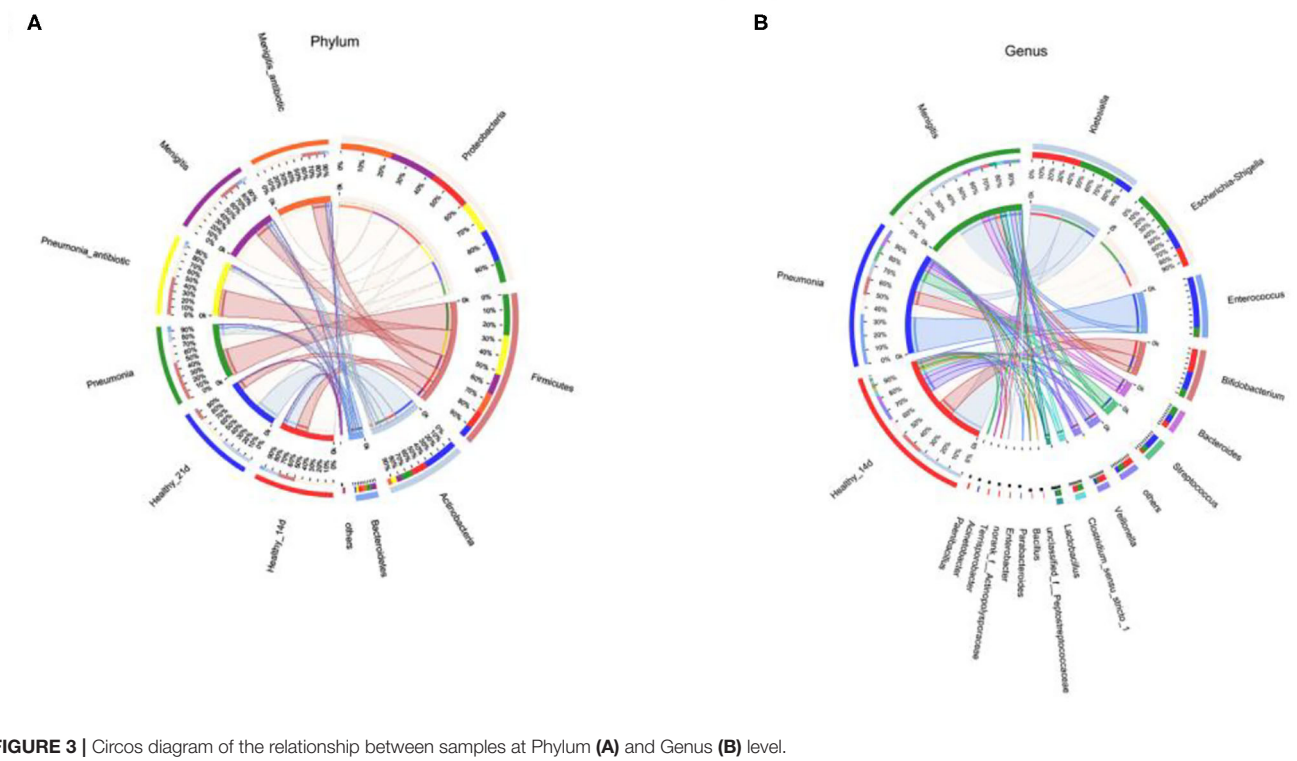

FIGURE 3 | Circos diagram of the relationship between samples at Phylum **(A)** and Genus **(B)** level.

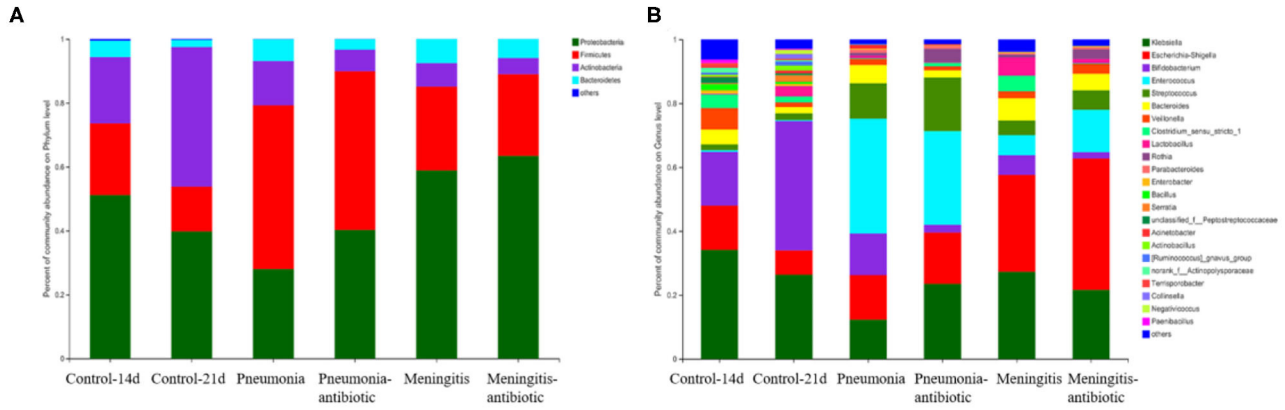

FIGURE 4 | Composition of gut microbiota in phylum **(A)** and genus **(B)** level.

treatment (meningitis 1.64%, meningitis-antibiotic 1.94%, $p = 0.824$) (**Supplementary Figure 4**).

DISCUSSION

Because the effect of antibiotic use on the gut microbiota of newborns in the treatment of different infectious diseases still remains unknown, we performed this study to investigate the influences on gut microbiota of neonates by disease condition and antibiotic treatment.

To reveal the influences on gut microbiota of neonates by disease condition, we compared the gut microbiota of neonates between the cases (patients with pneumonia or meningitis) and the healthy controls. The results showed that *Bifidobacteria* was the advantage bacterium in the control group, while *Enterococcus* and *Streptococcus* were the advantage bacterium in the pneumonia and meningitis group. The results of α diversity analysis suggested that the Sobs index of the gut microbiota in the pneumonia and meningitis groups was significantly lower than that in the healthy control group ($p < 0.05$). The above data suggested that before the use of antibiotic, the gut microbiota of late neonate with infectious diseases is already quite different from that of healthy neonate. A possible explanation of our result is that the gut microbiota might influence the immunity of the body, and further affect the susceptibility of the neonates to infectious diseases. A study on mice showed (11) that the composition of host intestinal microflora may affect the susceptibility of intestinal pathogens. Zeevi et al. (12) found that the disorder of the gut microbial metabolites could induce the increase production of interleukins and reduce the number of CD4 + CD25 + Foxp3 + cells, which led to the subclinical inflammation. The study of Schirmer et al. (13) expounded the relationship between the difference of gut microbiota and the difference of immune response in healthy individuals and discovered that gut microbiota could interfere with normal immune processes by affecting cytokines. In addition, *Bifidobacterium*, the advantage bacterium in healthy neonates, can prevent pathogenic bacteria from contacting intestinal epithelium, enhance the biological activity of phagocytes of the host, promote the secretion of IgA, enhance cellular immune function, and form intestinal mucosal barrier (14). *Bifidobacteria* can also secrete short-chain fatty acids to reduce the pH value of the intestinal tract and prevent abnormal fermentation of harmful bacteria (15–17).

For humans, antibiotic is a double-edged sword. With the wide application of antibiotics in infectious diseases, antibiotics play an important role in fighting bacterial infection and preventing the spread of pathogenic bacteria in the population and environment. However, the use of antibiotics also has negative effects on human health. Previous studies have shown that antibiotic exposure in childhood can increase the risk of obesity, diabetes, inflammatory bowel disease, asthma, allergy, and other diseases (1). Moreover, antibiotics can directly disrupt gut microbiome and change their composition in both children and adults, leading to a negative impact on the normal gut microbiota (6, 18). This was consistent with our findings. Under normal circumstances, with the increase in daily age, obligate anaerobe *Bifidobacterium* gradually becomes the dominant gut microbiota, but the gut microbiota structure changes under the influence of antibiotics, and the establishment of normal microecological environment in the intestinal tract is delayed, which might affect the colonization of obligate anaerobe and inhibit its growth (14, 19).

In the present study, the species composition of gut microbiota from pneumonia and meningitis groups were quite different, and the abundance of *Enterococcus* and *Bifidobacterium* in pneumonia neonates was conspicuously higher than the meningitis groups ($p < 0.05$), but the composition of gut microbiota had little difference before and after the 7-day antibiotic treatment in patients. Our results revealed that in the initial stage of infectious diseases, the gut microbiota of the patients in these two groups had already been quite different from each other, and the use of antibiotics had little effect on the gut microbiota of the patients, suggesting that the initial state of gut microbiome determined its state after antibiotic treatment. This was consistent with the previous research (20).

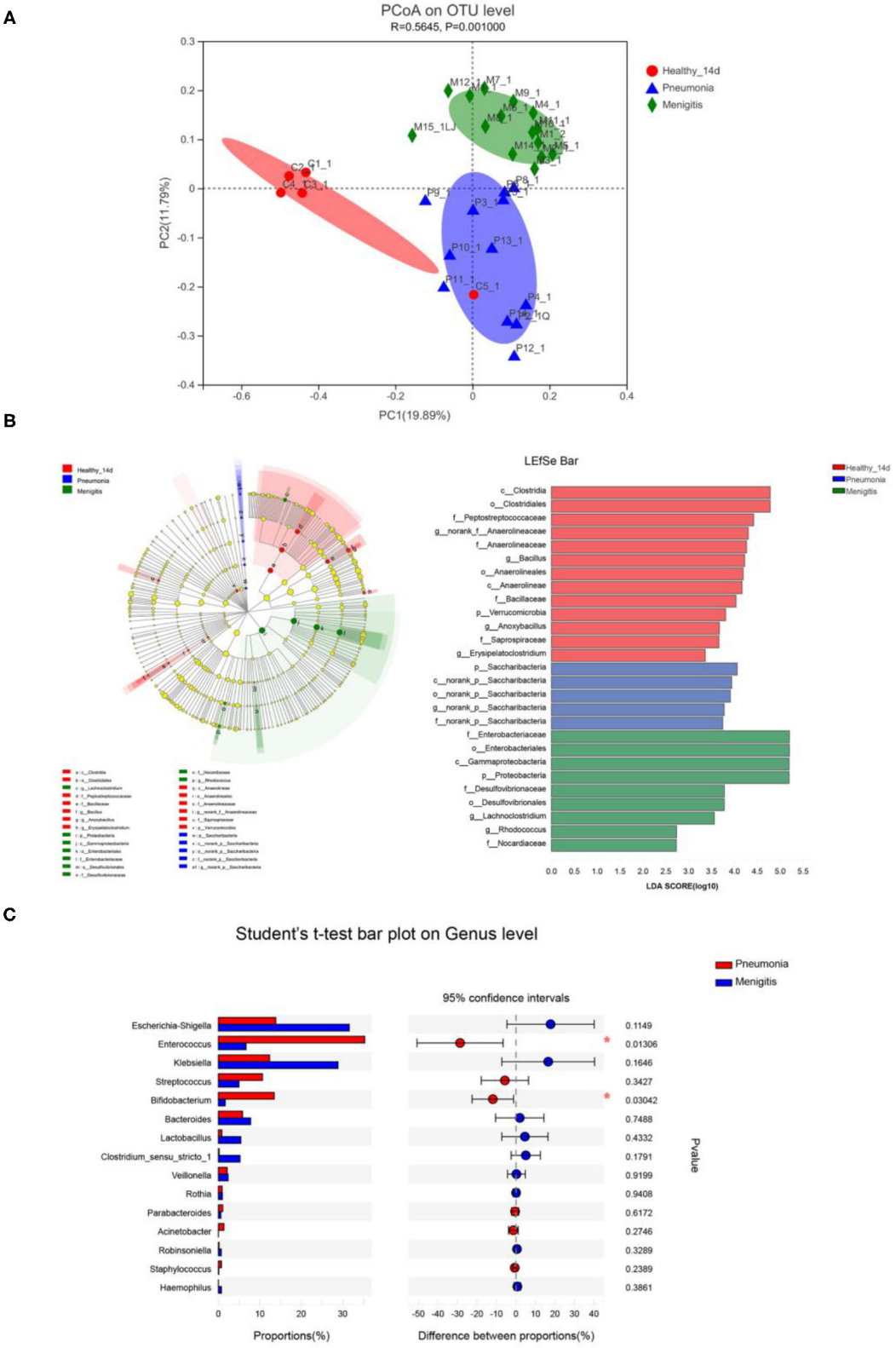

FIGURE 5 | Differences in gut microbiota between healthy newborns and patients before the use of antibiotics. **(A)** Principal coordinate analysis (PCoA) among healthy group and patient groups. **(B)** LEfSe analysis and LDA score among healthy group and patient group. **(C)** Relative abundance of significantly different genera between pneumonia group and meningitis group. *$p < 0.05$.

Raymond et al. (20) exposed 18 young healthy volunteers to a 7-day course of antibiotic, and the results indicated that inter-individual variability at the species level was greater than the effect of the antibiotic. For most participants, it was found that the dominant taxa were not perceptibly affected by the antibiotic.

Our study has some limitations. First, the sample size was small, especially in the healthy control group. Although there were differences in the results of multiple bacteria, and the *p*-values were all high in this study, it may be caused by the small sample size. Second, in addition to antibiotic treatment condition, other extrinsic and intrinsic factors, such as genetic background, pregnancy and childbirth condition, diet of nursing mothers, could also influence the gut microbiota of neonates. Due to the small sample size, we did not consider these influence factors in this study. More verification by expanding the samples is needed to confirm our findings in the future.

CONCLUSION

The gut microbiota of neonates with infectious diseases were mainly related to the disease conditions. The initial state of the neonatal gut microbiome determines its state after 1-week antibiotic treatment. Antibiotic application within 7 days had little effect on the community richness and some effect on the composition of gut microbiota of neonates with pneumonia or meningitis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: http://db.cngb.org/cnsa/project/CNP0001982/reviewlink/.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Beijing Children's Hospital, Capital Medical University (Approval No. 2015-36). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JL and YW contributed to conception and design of the study. QZ, YD, JZ, JS, SJ, and CY collected cases and did the experiments. SH and PC performed the statistical analysis. SH and QZ wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (81872676), Beijing Natural Science Foundation (7192063), and Special Fund of the Pediatric Medical Coordinated Development Center of Beijing Municipal Administration of Hospitals (XTYB201806).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.723617/full#supplementary-material

Supplementary Figure 1 | The sparse curve of healthy group and patient group.

Supplementary Figure 2 | PCoA analysis among healthy group and patient groups after 7 days antibiotics treatment in patient groups.

Supplementary Figure 3 | Difference of gut microbiota before and after the use of antibiotics in pneumonia group. **(A)** PCoA analysis before and after the use of antibiotics. **(B)** Relative abundance of significantly different genera before and after the use of antibiotics.

Supplementary Figure 4 | Difference of gut microbiota before and after the use of antibiotics in meningitis group. **(A)** PCoA analysis before and after the use of antibiotics. **(B)** Relative abundance of significantly different genera before and after the use of antibiotics.

REFERENCES

1. Gilbert JA, Blaser MJ, Caporaso JG, Jansson JK, Lynch SV, Knight R. Current understanding of the human microbiome. *Nat Med*. (2018) 24:392–400. doi: 10.1038/nm.4517
2. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, et al. Host-gut microbiota metabolic interactions. *Science*. (2012) 336:1262–7. doi: 10.1126/science.1223813
3. Lynch SV, Pedersen O. The human intestinal microbiome in health and disease. *N Engl J Med*. (2016) 375:2369–79. doi: 10.1056/NEJMra1600266
4. Kundu P, Blacher E, Elinav E, Pettersson S. Our gut microbiome: the evolving inner self. *Cell*. (2017) 171:1481–93. doi: 10.1016/j.cell.2017.11.024
5. Moeller, Andrew H. The shrinking human gut microbiome. *Curr Opin Microbiol*. (2017) 38:30–5. doi: 10.1016/j.mib.2017.04.002
6. Fouhy F, Guinane CM, Hussey S, Wall R, Ryan CA, Dempsey EM, et al. High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. *Antimicrob Agents Chemother*. (2012) 56:5811–20. doi: 10.1128/AAC.00789-12
7. Zwittink RD, Renes IB, Lingen RA, van Zoeren-Grobben D, Konstanti P, Norbruis OF, et al. Association between duration of intravenous antibiotic administration and early-life microbiota development in late-preterm infants. *Eur J Clin Microbiol Infect Dis*. (2018) 37:475–83. doi: 10.1007/s10096-018-3193-y
8. Zimmermann P, Curtis N. Effect of intrapartum antibiotics on the intestinal microbiota of infants: a systematic review. *Arch Dis Child Fetal Neonatal Ed*. (2020) 105:201–8. doi: 10.1136/archdischild-2018-316659
9. Duke, T. Neonatal pneumonia in developing countries. *Arch Dis Child Fetal Neonatal Ed*. (2005) 90:211–9. doi: 10.1136/adc.2003.048108
10. Cao Y, Cheng G, Hou X, Jiang L, Liu J, Liu L, et al. [Etiology, diagnosis and treatment of neonatal bacterial meningitis]. *Chin J Perinat Med*. (2016) 19:881–4. doi: 10.3760/cma.j.issn.1007-9408.2016.12.001
11. Blaser MJ. Antibiotic use and its consequences for the normal microbiome. *Science*. (2016) 352:544–5. doi: 10.1126/science.aad9358

12. Zeevi D, Korem T, Segal E. Talking about cross-talk: the immune system and the microbiome. *Genome Biol*. (2016) 17:50. doi: 10.1186/s13059-016-0921-4
13. Schirmer M, Smeekens S, Vlamakis H, Jaeger M, Oosting M, Franzosa EA, et al. Linking the human gut microbiome to inflammatory cytokine production capacity. *Cell*. (2016) 167:1125–36.e8. doi: 10.1016/j.cell.2016.10.020
14. Bäckhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, et al. Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe*. (2015) 17:690–703. doi: 10.1016/j.chom.2015.04.004
15. Warner BB, Deych E, Zhou Y, Hall-Moore C, Weinstock GM, Sodergren E, et al. Gut bacteria dysbiosis and necrotising enterocolitis in very low birthweight infants: a prospective case-control study. *Lancet*. (2016) 387:1928–36. doi: 10.1016/S0140-6736(16)00081-7
16. Wardwell LH, Huttenhower C, Garrett WS. Current concepts of the intestinal microbiota and the pathogenesis of infection. *Curr Infect Dis Rep*. (2011) 13:28–34. doi: 10.1007/s11908-010-0147-7
17. Bokulich NA, Chung J, Battaglia T, Henderson N, Jay M, Li H, et al. Antibiotics, birth mode, and diet shape microbiome maturation during early life. *Sci Transl Med*. (2016) 8:343ra82. doi: 10.1126/scitranslmed.aad7121
18. Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc Natl Acad Sci USA*. (2011) 108(Suppl. 1):4554–61. doi: 10.1073/pnas.1000087107
19. Bashan A, Gibson TE, Friedman J, Carey VJ, Weiss ST, Hohmann E, et al. Universality of human microbial dynamics. *Nature*. (2016) 534:259–62. doi: 10.1038/nature18301
20. Raymond F, Ouameur AA, M Déraspe, Iqbal N, Gingras N, Dridi B, et al. The initial state of the human gut microbiome determines its reshaping by antibiotics. *ISME J*. (2016) 10:707–20. doi: 10.1038/ismej.2015.148

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pediatrics

ORIGINAL RESEARCH
published: 22 October 2021
doi: 10.3389/fped.2021.755437

Early Postnatal Comprehensive Biomarkers Cannot Identify Extremely Preterm Infants at Risk of Developing Necrotizing Enterocolitis

Alice Hoffsten[1], Laszlo Markasz[1,2], Helene Engstrand Lilja[1,3], Karl Wilhelm Olsson[1,4] and Richard Sindelar[1,2]*

[1] Department of Women's and Children's Health, Uppsala University, Uppsala, Sweden, [2] Neonatal Intensive Care Unit, University Children's Hospital, Uppsala, Sweden, [3] Section of Pediatric Surgery, University Children's Hospital, Uppsala, Sweden, [4] Department of Surgical Sciences, Uppsala University, Uppsala, Sweden

Background: Necrotizing enterocolitis (NEC) is a fatal disease where current diagnostic tools are insufficient for preventing NEC. Early predictive biomarkers could be beneficial in identifying infants at high risk of developing NEC.

Objective: To explore early biomarkers for predicting NEC in extremely preterm infants (EPIs).

Methods: Blood samples were collected on day 2 (median 1.7; range 1.5–2.0) from 40 EPI (median 25 gestational weeks; range 22–27): 11 developed NEC and 29 did not (controls). In each infant, 189 inflammatory, oncological, and vascular proteomic biomarkers were quantified through Proximity Extension Assay. Biomarker expression and clinical data were compared between the NEC group and Controls. Based on biomarker differences, controls were sorted automatically into three subgroups (1, 2, and 3) by a two-dimensional hierarchical clustering analysis.

Results: None of the biomarkers differed in expression between all controls and the NEC group. Two biomarkers were higher in Control 1, and 16 biomarkers were lower in Control group 2 compared with the NEC group. No biomarker distinguished Control 3 from the NEC group. Perinatal data were similar in the whole population.

Conclusions: Early postnatal comprehensive biomarkers do not identify EPIs at risk of developing NEC in our study. Future studies of predictors of NEC should include sequential analysis of comprehensive proteomic markers in large cohorts.

Keywords: necrotizing enterocolitis, biomarker, preterm infant, cluster analysis, serum

OPEN ACCESS

Edited by:
Yuan Shi,
Children's Hospital of Chongqing Medical University, China

Reviewed by:
Lu-Quan Li,
Chongqing Medical University, China
Shan He,
The First People's Hospital of Yunnan Province, China

****Correspondence:***
Alice Hoffsten
alice.hoffsten@kbh.uu.se

Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: *08 August 2021*
Accepted: *16 September 2021*
Published: *22 October 2021*

Citation:
Hoffsten A, Markasz L, Lilja HE, Olsson KW and Sindelar R (2021) Early Postnatal Comprehensive Biomarkers Cannot Identify Extremely Preterm Infants at Risk of Developing Necrotizing Enterocolitis. Front. Pediatr. 9:755437. doi: 10.3389/fped.2021.755437

INTRODUCTION

Even though mortality rates among preterm infants have halved during the past two decades (1), the aim to reduce it further continues. Recent studies (2–5) have found an upsurge of necrotizing enterocolitis (NEC) as cause of death. The increase of mortality from NEC can be due to successful care of other early illnesses, allowing the preterm infants to survive long enough to be susceptible to NEC (6, 7).

Since the first description of NEC (8), decades have been devoted to understand its pathogenesis and etiology. The recent knowledge on NEC suggests that mechanisms are multifactorial with both prenatal and postnatal factors. Current consensus proposes that NEC is an inflammatory disease, where injuries to the intestinal wall barrier lead to bacterial invasion and necrosis (6). The immaturity of the preterm gut barrier and the developing, over-reactive immune system enhance the response and destruction (9). Exaggerated mucosal inflammation and necrosis may be amplified by abnormal microcirculation (10). Postnatally, diet (11, 12) and epidermal growth factors (13) will affect intestinal maturation and can thus have an impact on the susceptibility of NEC. Furthermore, low birth weight (BW), being small for gestational age (SGA), anemia, and increased immaturity are all recognized risk factors of NEC (14, 15).

Besides substantial mortality, NEC is associated with longer hospital stays (16), impaired neurodevelopment (17), and morbidity due to short bowel syndrome following lifesaving surgical interventions (18).

Diagnosis of NEC is based on the modified Bell's staging criteria, consisting of radiographic, clinical, and laboratory findings (19, 20). Early diagnosis is more challenging in more immature infants, due to non-specific clinical and radiographic signs (21). Desolately, it is also in the most premature newborns that the mortality and incidence of NEC are the largest (22). It is therefore of great interest to find a more reliable and earlier diagnosis of NEC in these individuals. This would enable earlier intervention and thus reduce progression, morbidity, and mortality of NEC. A precise diagnosis will also decrease over-treatment (23).

Plasma proteins have been proven useful in identifying diseases in extremely preterm newborns, for instance, in bronchopulmonary disease (BPD), patent ductus arteriosus (PDA), and retinopathy of prematurity (ROP) (23–26). Many attempts have been made to identify biomarkers in serum, stool, and urine for early diagnosis of NEC, but the clinical relevance of these findings still remains low. Individual inflammatory biomarkers are usually non-specific, reflect general inflammation rather than specifically NEC, and are detected at later stages of NEC (24–26). Thus, they do not facilitate an earlier diagnosis. Some biomarkers require the infant to pass stool, which is not always possible in advanced NEC (25, 27). Inter-individual and intra-individual variations have also been obstacles to exploring useful biomarkers (28). Other difficulties for finding predictive biomarkers in NEC could be that the correlation between biomarkers and disease is not as linear, for instance, between vascular endothelial growth factor (VEGF) and ROP, and that NEC probably is multifactorial. The individuals at risk of developing NEC may also display heterogeneity and have different risks. Therefore, exploring combinations of comprehensive biomarkers in infants at the highest risk of developing NEC may be useful.

The aim was to prospectively study early comprehensive biomarkers in serum from extremely preterm infants who might or might not develop NEC. Biomarker patterns in healthy infants are compared with patterns in those who later developed NEC. Besides finding useful potential early biomarkers to improve current diagnostic tools in this high-risk population, this could also yield valuable information about the pathophysiology of NEC.

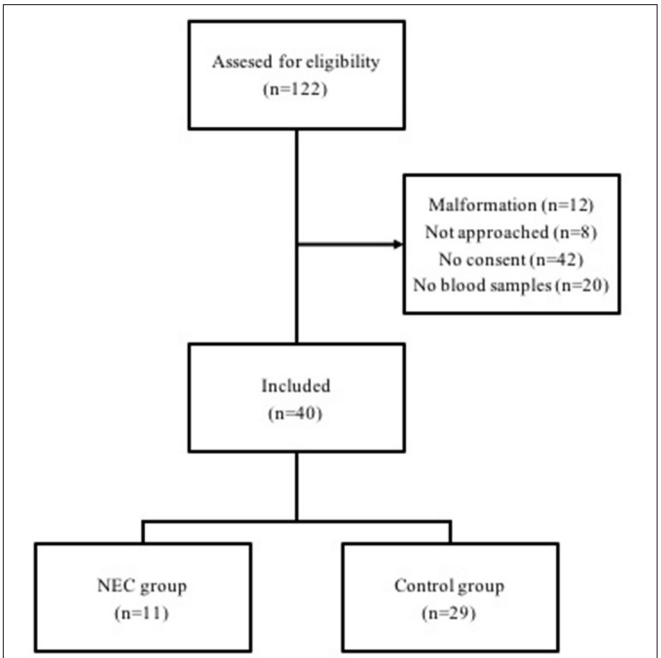

FIGURE 1 | Flowchart of included infants ($n = 40$) born between 22 and 27 weeks of gestational age, and reasons for exclusion.

METHODS AND MATERIALS

Study Setting and Participants

Infants born in November 2012–May 2015 at Uppsala University Children's Hospital before 28 weeks of gestation were prospectively included. Those with major congenital anomalies or heart defects were excluded. The population has previously been studied in the DAPPR-cohort (*Ductus Arteriosus and Pulmonary circulation in PReterm infants*) (29) and have been approved for study by the Regional Ethical Review Board in Uppsala, D:nr 2011/046. Out of 122 infants born during this period, 40 completed the full blood sampling needed in this study to obtain a level of significance of 5% and a power of at least 0.80 in this multi-parametrical study (**Figure 1**). All infants were included after informed and written consent from the parents was obtained. Eventually, 11 developed NEC and 29 controls did not. Background data from SNQ for the 122 individuals born during the period were used to compare background data in the study population, to ensure a reliable representation of the study period.

TABLE 1 | All quantified biomarkers ($n = 202^*$).

4E-BP1	CCL28	CXCL10	ESM-1	hGDNF	IL-1ra	LEP	MMP-7	PTPN22	TM	VE-statin
ADA	CCL4	CXCL11	EZR	HGF	IL-2	LIF	MPO	PTX3	TNF	VIM
AGRP	CD244	CXCL13	FABP4	hK11	IL-20	LIF-R	MYD88	RAGE	TNFB	
AM	CD40	CXCL16	FADD	HSP 27	IL-20RA	LITAF	NEMO	REG-4	TNF-R1	
AR	CD40-L	CXCL5	FAS	ICOSLG	IL-22 RA1	LOX-1	NRTN	REN	TNF-R2	
ARTN	CD5	CXCL6	FasL	IFN-gamma	IL-24	LYN	NT-3	RETN	TNFRSF4	
AXIN1	CD6	CXCL9	FGF-19	IL-1 alpha	IL27-A	mAmP	NT-pro-BNP	SCF	TNFRSF9	
BAFF	CD69	Dkk-1	FGF-21	IL-10	IL-2RB	MB	NTRK3	SELE	TNFSF14	
BDNF	CDCP1	DNER	FGF-23	IL-10RA	IL-33	MCP-1	OPG	SIRT2	t-PA	
Beta-NGF	CDH3	ECP	FGF-5	IL-10RB	IL-4	MCP-2	OSM	SLAMF1	TRAIL	
BNP	CDKN1A	EGF	Flt3L	IL-12	IL-5	MCP-3	PAPPA	SPON1	TRAIL-R2	
CA-125	CEA	EGFR	FR-alpha	IL-12B	IL-6	MCP-4	PAR-1	SRC	TRANCE	
CAIX	CHI3L1	eIF-4B	FS	IL-13	IL-6RA	MIA	PARK7	ST1A1	TR-AP	
CASP-3	CSF-1	EMMPRIN	FUR	IL-15RA	IL-7	MIC-A	PDGF subunit B	ST2	TSLP	
CASP-8	CST5	EN-RAGE	GAL	IL-16	IL-8	MIP-1 alpha	PD-L1	STAMPB	TWEAK	
CCL11	CSTB	Ep-CAM	Gal-3	IL-17A	ILT-3	MK	PECAM-1	TF	uPA	
CCL19	CTSD	EPO	GDF-15	IL-17C	ITGA1	MMP-1	PIGF	TGF-alpha	U-PAR	
CCL20	CTSL1	ErbB2/HER2	GH	IL-17RB	ITGB1BP2	MMP-10	PRL	THPO	VEGF-A	
CCL23	CX3CL1	ErbB3/HER3	HB-EGF	IL-18	KLK6	MMP-12	PRSS8	TIE2	VEGF-D	
CCL25	CXCL1	ErbB4/HER4	HE4	IL-18R1	LAP TGF-beta-1	MMP-3	PSGL-1	TIM	VEGFR-2	

**The 13 biomarkers excluded due to analytical error are shaded.*

Data Extraction and Study Variables

For each individual, clinical and laboratory parameters were studied. This included diagnosis of NEC defined as Bell stage ≥ IIa (19), as well as other associated diseases, such as PDA, intraventricular hemorrhage (IVH), bronchopulmonary dysplasia (BPD), respiratory distress syndrome (RDS), ROP, and infection/septicemia. Clinical features also included administration of prenatal steroids, preeclampsia, chorioamnionitis, delivery mode, twin birth, APGAR score, GA, BW, respiratory illnes, onset of illness, and mortality.

Proximity Extension Assay (PEA)

Blood samples were collected from the umbilical arterial catheter on day 2 (median 1.7; range 1.5–2.0) after birth. The samples were centrifuged for 7 min at 2,400 × *g*, after which the serum was extracted to be stored at −80°C while awaiting analysis. A total of 202 biochemical markers (**Table 1**) were quantified in each individual with proximity extension assay (PEA), Olink, with the Proseek Multiplex 96 × 96 CVD I, Oncology I, and Inflammation I biomarker panels. PEA is suitable for serum analysis of preterms since it requires small blood volumes (1 μl) (30). Thirteen biomarkers were excluded due to analytical error (marked in **Table 1**). The final number of biomarkers in this study was 189.

Clustering and Identification of Control Groups

Automatic cluster analysis of biomarker levels was performed with Cluster 3.0 (31), in which all biomarker levels were weighed equally. The two-dimensioned hierarchical multivariate analysis outlines the Euclidean distance between two factors or groups. Results from the cluster analysis were displayed as a map of color pixels with Java Treeview (32).

The controls were divided automatically into three subgroups by the clustering program (Control 1, Control 2, and Control 3) according to their biomarker expression patterns. After this, biomarker levels were statistically compared between the control groups, to ensure statistical support for the identified groups. New clustering was performed to explore how the NEC group clusters with the controls. A multivariate logistic regression to verify correlation found in the cluster analysis was not possible due to the amount of variables.

Statistical Analysis

Statistical analysis was performed in Excel Version 15.27 (161010) and SPSS (1.0.0. 1447 64-bit edition). A *p*-value was considered statistically significant when <0.05. All tests of significance were two-tailed. The expression level of each biomarker was compared with a Student's *t*-test. After this, a Benjamini–Hochberg analysis was performed to reduce the risk for false-positive results. The q-value for this explorative study was set at 0.1. Background data within the study population ($n = 40$) were compared in the NEC group ($n = 11$) and the control group ($n = 29$) with Student's *t*-test for parametric data and the Mann–Whitney U for non-parametric data. Pearson's correlation was used to determine whether a biomarker level correlated to GA or BW. Background data from SNQ

for the individuals born during the period (n = 122) were compared with data from the study population (n = 40) by chi-square test for nonparametric values and Student's t-test for parametric values.

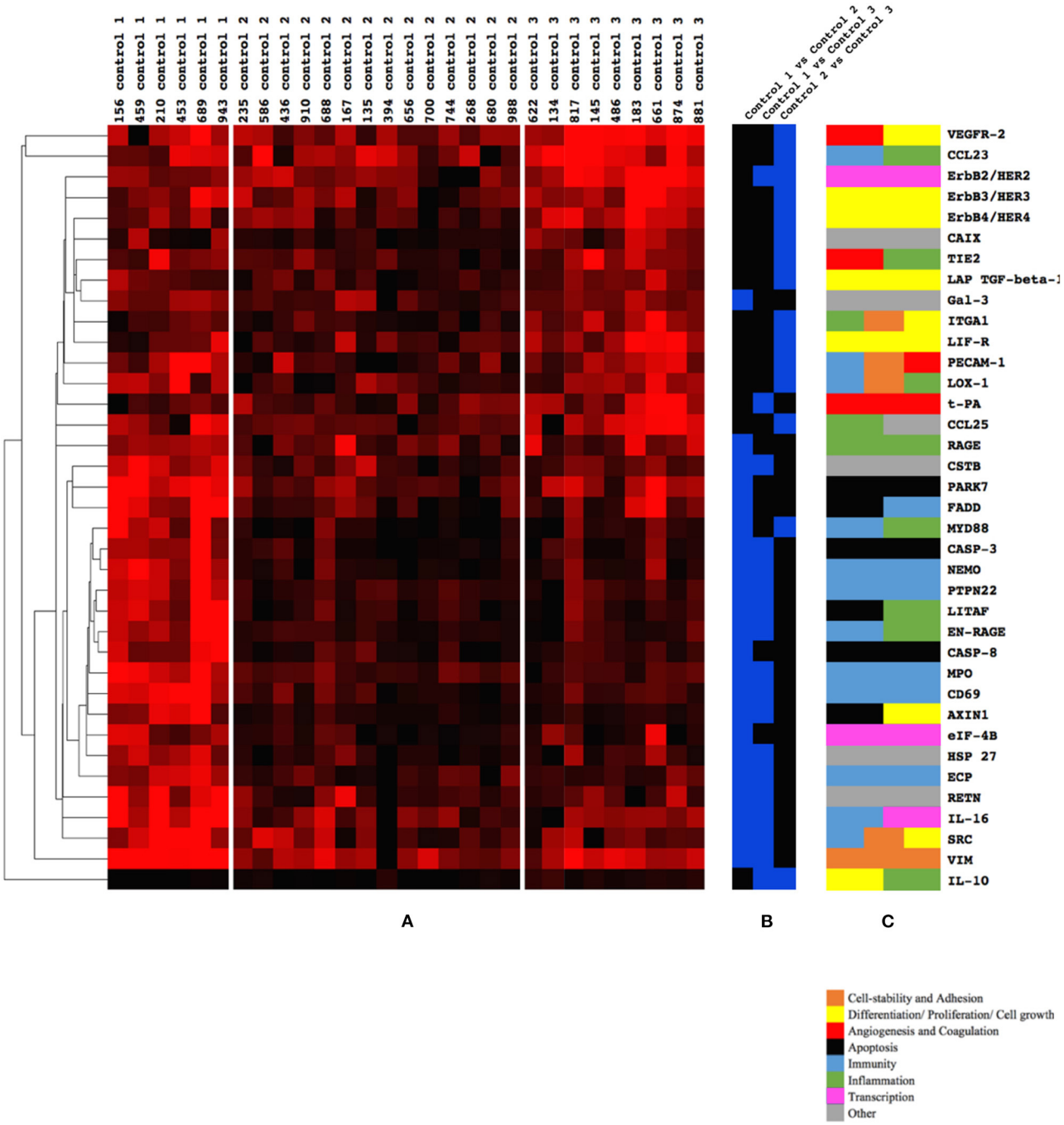

FIGURE 2 | (A–C) Biomarkers (n = 37) that differed between the three Control groups, 1, 2, and 3 (blue), and their physiological function (brown, yellow, red, black, light blue, green, pink, and gray). **(A)** Three control subgroups could be identified by clustering of all biomarkers (n = 189). Significantly different biomarkers (n = 37) between the three control groups (1, 2, and 3) are presented. Higher intensity of red color indicates a higher expression of a given biomarker. **(B)** The comparison in which the biomarker differed in a comparison. Blue color indicates a significant difference of expression between control groups listed at the top of the column. Note that 23 biomarkers differed between Controls 1 and 2; 16 between Controls 1 and 3; and only 14 between Controls 2 and 3. **(C)** The function/functions of each biomarker as defined by www.humanproteinatlas.org (brown, yellow, red, black, light blue, green, pink, and gray).

RESULTS

No statistical difference was found in biomarker expression levels ($n = 189$) when comparing the NEC group with all controls: however, heterogeneities in controls could be further studied (see in **Appendix 1**).

Identification of Control Groups Through Cluster Analysis

Two-dimensional hierarchical clustering of biomarkers was performed including only controls to explore subgroups with unique biomarker profiles. The clustering software identified three control groups: Control 1, Control 2, and Control 3. Each control group had a unique pattern of biomarker expression. After statistical analysis, 37 biomarkers were identified to differentiate between any combinations of the control groups (**Figure 2**). Thus, each control group was characterized by an individual biomarker expression pattern. The expression pattern of the control groups, the differences in the expression levels between the groups, and the biological function of the presented biomarkers are shown in **Figure 2**. In **Appendix 1**, all biomarkers ($n = 189$) are clustered in all individuals ($n = 40$), where most of the NEC patients form a cluster with infants from Control 3.

Biomarkers of Significance

Eighteen biomarkers differed when comparing the NEC group with any combination of the three control groups (**Figure 3**). Two biomarkers differed when comparing NEC with Control 1, six biomarkers differed when comparing the NEC group with Control 2, and no biomarker ($n = 189$) differed in expression when comparing the NEC group with Control 3. This seems to be visually confirmed when all controls were clustered together with NEC patients, as most of the NEC patients appeared in the same cluster as Control 3 (**Appendix 2**). The direction (+/–) of differences in biomarker expression between NEC patients and control groups are displayed in **Figure 3B**. **Figure 3C** shows the differences of the given biomarker between the control groups.

Clinical Characteristics of the Study Population

Prenatal steroids were administered to the all individuals ($n = 40$). Incidence of the comorbidities RDS, IVH, BPD, infection, ROP, and persistent pulmonary hypertension of the newborn (PPHN) was compared in *NEC group vs. all controls* as well as *NEC group vs. control groups 1, 2, and 3*; however, no difference in incidence was found (**Appendix 3**). Twin pregnancy, preeclampsia, chorioamnionitis, and APGAR score (at 1, 5, and 10 min) did not differ between the NEC group, all controls, and Control groups 1, 2, and 3 (**Appendix 3**). A cluster

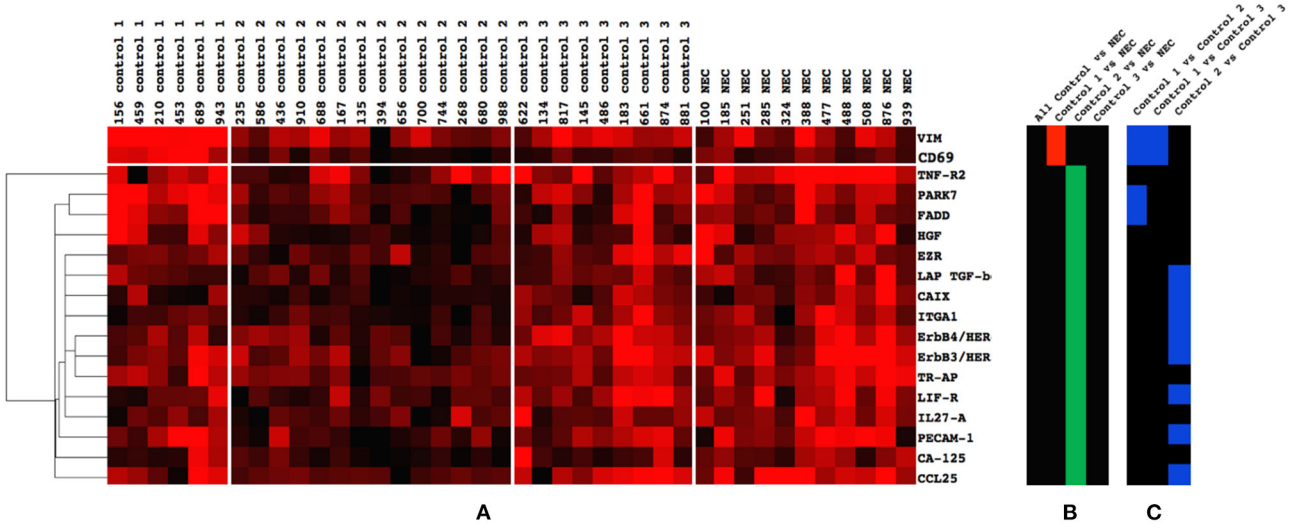

FIGURE 3 | (A–C) Biomarkers ($n = 18$) that differed between Controls 1, 2, and 3 and the necrotizing enterocolitis (NEC) group. **(A)** Cluster of biomarkers ($n = 18$) that exhibited significant difference in expression when the NEC group was compared with all controls or separate control groups (1, 2, and 3). **(B)** Significant difference between controls and NEC. A red color is an indication that the mean expression level of the given protein was lower in NEC patients as compared with the control group. A green color signifies that the mean expression in the NEC patients was higher than in the control group. Note the higher expression of two biomarkers in Control 1 compared with the NEC group (*red*) and the lower expression of 16 biomarkers in Control 2 compared with the NEC group (green). Note also that no biomarker differed between all controls and the NEC group (black), nor between Control 3 and the NEC group (black). **(C)** Comparison of the 18 NEC specific biomarkers between Controls 1, 2, and 3. A blue color indicates a difference in expression between the two control groups listed at the top of the column. Note that four biomarkers differed between Controls 1 and 2 (blue), two biomarkers between Controls 1 and 3 (blue), and eight biomarkers between Controls 2 and 3 (blue). Only two biomarkers differed between all three control groups (*VIM* and *CD69*). The biological function according to www.humanproteinatlas.org of the six factors TNF-R2, HGF, EZR, TR-AP, IL-27A, and CCL25 are as follows: TNF-R2, apoptosis; HGF, differentiation/proliferation/cell growth; EZR, cell stability and adhesion; TR-AP, other (blood pressure regulation); IL-27A, immunity; and CCL25, inflammation and immunity.

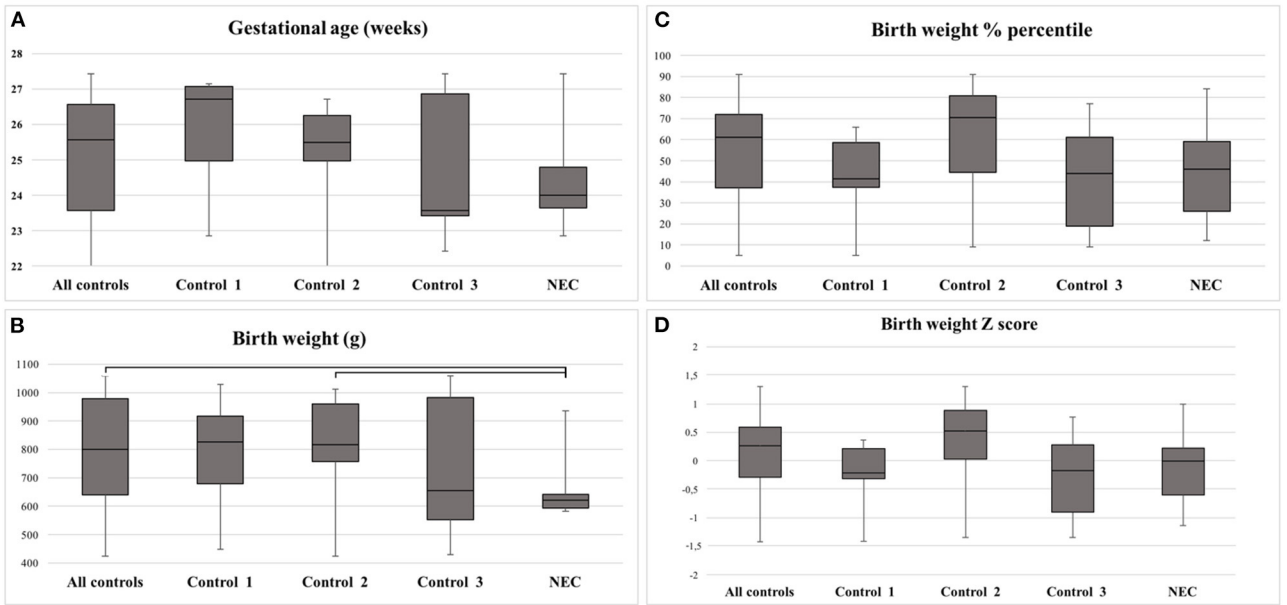

FIGURE 4 | (A–D) Clinical data for the study population and detected differences. **(A)** Gestational age (GA) in weeks and days. **(B)** Birth weight (BW) in grams. **(C)** BW in percentiles. **(D)** BW in Z score. Note that BW differed between all controls and the necrotizing enterocolitis (NEC) group, as well as between Control 2 and the NEC group. Bars depict significant differences. Statistical calculations displayed in **Table 2**.

analysis of clinical parameters along with significant biomarkers is displayed in **Appendix 2**.

The ratio of NEC in the individuals born during the study period ($n = 122$) was 16.4%, similar to that of the study group ($n = 40$, 27.5%, $p = 0.211$). The mortality during the 2 years was 25.4%, and in the study population, 17.5% ($p = 0.412$). The controls in the study population ($n = 29$) who did decease ($n = 3$) lived at least 3 months.

Median GA of all individuals was 25 weeks (range 22–27). No difference was found in GA between the groups (**Figure 4A**; **Table 2**). The BW was lower in NEC as compared with all controls ($p = 0.023$), or with Control 2 ($p = 0.026$) (**Figure 4B**; **Table 2**). Differences disappeared when the BW was adjusted to BW percentile (BW%) and BW Z score (**Figures 4C,D**; **Table 2**). None of the infants were growth restricted, as BW Z score was >-2 SD (**Figure 4D**). Visually, there was a tendency toward lower median GA, BW%, and BW Z score in Control 3 as compared with the NEC group (**Figures 4A,C,D**); however, this could not be statistically confirmed (**Table 2**; Control 3 vs. NEC). The median GA of Control 1 appears to be higher than the rest of the study population (**Figure 4A**).

The median time from birth until diagnosis of NEC was 9 days (range 2–18). The mean GA of those developing NEC earlier than 9 days was 24.4 weeks, which was not higher (p = 0.882) than the mean GA of those who developed NEC later (mean GA 23.8).

Correlation Analysis

A negative correlation was found between BW and expression of hepatocyte growth factor (HGF), ErbB3/HER3, and Erb4/HER4.

TABLE 2 | Detected statistical differences in parameters presented in **Figures 4A–D**.

p-value	GA	BW	BW%	BW Z score
All controls vs. NEC	0.116	**0.023**	0.451	0.434
Control 1 vs. NEC	0.122	0.209	0.817	0.713
Control 2 vs. NEC	0.139	**0.026**	0.139	0.075
Control 3 vs. NEC	0.630	0.407	0.912	0.085
Control 1 vs. Control 2	0.502	0.973	0.151	0.085
Control 1 vs. Control 3	0.318	0.680	0.910	0.953
Control 2 vs. Control 3	0.567	0.503	0.165	0.071

GA, BW, BW%, and BW Z score were compared between NEC, all controls, and Control groups 1–3. Detected differences by clustering were analyzed by two-sided paired Student's t-test and corrected with Benjamini–Hochberg for multiple comparisons. A p-value of <0.05 (presented in bold) was considered significant.
GA, gestational age; BW, birth weight; NEC, necrotizing enterocolitis.

For the rest of the biomarkers, no correlation between biomarker expression and BW or GA could be confirmed (**Table 3**).

Selected Analysis of Biomarkers Previously Studied in Necrotizing Enterocolitis and Colitis

A literature search in PubMed of the 18 biomarkers that differed between the control groups and the NEC group (**Figure 2**) showed that TNF-R2, HGF, and tartrate-resistant acid phosphatase 5 (TR-AP) have previously been described in relation to NEC. Fas-associated protein with death domain (FADD) and PARK7 have been reported in relation to colitis.

TABLE 3 | Correlations between biomarkers ($n = 18$) and GA or BW.

	GA		BW	
	Pearson's coefficient	*p*-value	*Pearson coefficient*	*p*-value
VIM	0.203	0.216	0.101	0.539
CD69	0.177	0.266	0.065	0.713
TNF-R2	0.080	0.623	0.165	0.294
PARK7	0.090	0.566	−0.082	0.610
FADD	0.069	0.668	−0.084	0.623
HGF	−0.291	0.059	**−0.500**	**0.001**
TR-AP	−0.235	0.153	−0.311	**0.051**
EZR	−0.090	0.566	−0.242	0.136
LAP TGF-beta-1	−0.042	0.806	−0.202	0.216
CAIX	−0.088	0.566	−0.213	0.566
ITGA1	−0.087	0.566	−0.146	0.356
ErbB3/HER3	−0.270	0.092	**−0.359**	**0.022**
ErbB4/HER4	**−0.402**	**0.011**	**−0.480**	**0.001**
TR-AP	−0.235	0.153	−0.311	**0.052**
LIF-R	−0.127	0.424	−0.104	0.540
IL27-A	**0.337**	**0.031**	0.224	0.173
PECAM-1	−0.061	0.713	−0.072	0.668
CA-125	−0.139	0.389	−0.167	0.278
CCL25	−0.202	0.216	−0.225	0.153

Each significantly different biomarker ($n = 18$) between Controls 1 and 2 and NEC was analyzed with Pearson's correlation to see if the biomarker level correlated to gestational age or birth weight. Significant correlations ($p < 0.05$; presented in bold) were found between GA and ErbB4/HER4 or IL27-A (blue); BW and HGF, ErbB3/HER3, or ErbB4/HER4 (gray), but with a relatively low correlation coefficient of ≤0.50.
GA, gestational age; BW, birth weight; NEC, necrotizing enterocolitis.

Besides the expression of these five proteins, **Figure 5** includes vimentin (VIM) and CD69, which were elevated in Control 1 as compared with all other groups. The levels of FADD, TNF-R2, HGF, TR-AP, and PARK7 were elevated in NEC compared with Control 2 (**Figure 5**).

DISCUSSION

In this study of 40 extremely preterm infants, 189 biomarkers with functions mostly in inflammation, proliferation, and vascularization were quantified at day 2 after birth in an effort to find potential early risks of emerging NEC. Eleven of 40 infants later developed NEC.

Our main finding is that no biomarker ($n = 189$) differed in expression when comparing infants who later developed NEC with all controls. Furthermore, all infants ($n = 40$) had comparable clinical perinatal history. This suggests that on day 2, the individuals in this high-risk group overall exhibit the same starting point in regard to inflammation, vascularization, and possibly in the risk of NEC development. This opens up for postnatal factors influencing which individuals go on to develop NEC and that prophylactic treatment and close monitoring very well can be beneficial in reducing NEC incidence.

The subdivision of the controls may be important to investigate variation of NEC risk shortly after birth. If a blood test taken at day 2 reveals biomarker patterns incongruent with those found in NEC, this could be an indication of lower risk of developing NEC. Contrariwise, a group of individuals exhibiting similar patterns as the NEC group could be suspected to have an increased risk of developing NEC. The latter group could benefit from close observation and prophylactic treatment.

Control 3 stands out as particularly interesting to compare with the NEC group. Besides similar clinical and perinatal parameters, not a single biomarker ($n = 189$) differed in expression when comparing Control 3 with those who later developed NEC. Control 3 even exhibited a visual trend toward lower median GA and adjusted BW than the NEC group. Both factors would essentially make Control 3 more prone to develop NEC (15) than the NEC group itself. Our data suggest that at day 2 after birth, Control 3 may exhibit the same risk of developing NEC as the individuals that later did develop NEC. Based on these observations, it seems feasible to hypothesize that postnatal, rather than perinatal, factors determine which individuals are at the highest risk of developing NEC in this cohort of extremely preterm infants.

Several biomarkers ($n = 18$) differed when comparing the NEC group with Control 1 and 2. Two of them were higher in Control 1 as compared with NEC, Control 2, and Control 3, namely, VIM and CD69.

VIM, a type III intermediate filament, is a component in the cytoskeleton (33). In rat models with inflammatory bowel disease (IBD), VIM expression was increased (34). Knock-out of VIM in mouse models with induced IBD has considerably less inflammation that in those with VIM (35). In our study, VIM is lower in NEC patients than Control 1, which is incongruent with the function of VIM.

CD69 expression indicates leucocyte activation and is an early marker of inflammation (36). CD69 has been found to be upregulated after intestinal bacterial exposure (37) and downregulated in murine models with severe anemia (38). Furthermore, CD69 is thought to reduce tissue damage from ischemia, by reducing endothelial activation (39) and has increased expression in blood cells after intake of probiotics in healthy adults (40). It also plays a role in immunosuppressive regulatory cells, through promotion of IL-10 production (41). We found lower levels of CD69 in NEC patients compared with Control 1, which is not conflicting in with the functions described above.

In a risk evaluation, we observed that a simultaneously higher expression of VIM and CD69 on day 2 indicated a lower risk for NEC. There was a visual tendency toward higher GA in Control 1, which could affect biomarker levels. However, in the correlation analysis of VIM and CD69 with clinical data, expression levels could not be linked to GA and BW.

The homogeneity in clinical characteristics of the patients both with and without NEC signifies a suitable basis for analysis, since it minimizes the risk of confounding factors influencing biomarker concentrations. The inverse correlations between NEC and GA and/or BW are well-known major risk factors for NEC (15). In the present study, we found only a tendency for such a correlation, probably because the study population consisted of

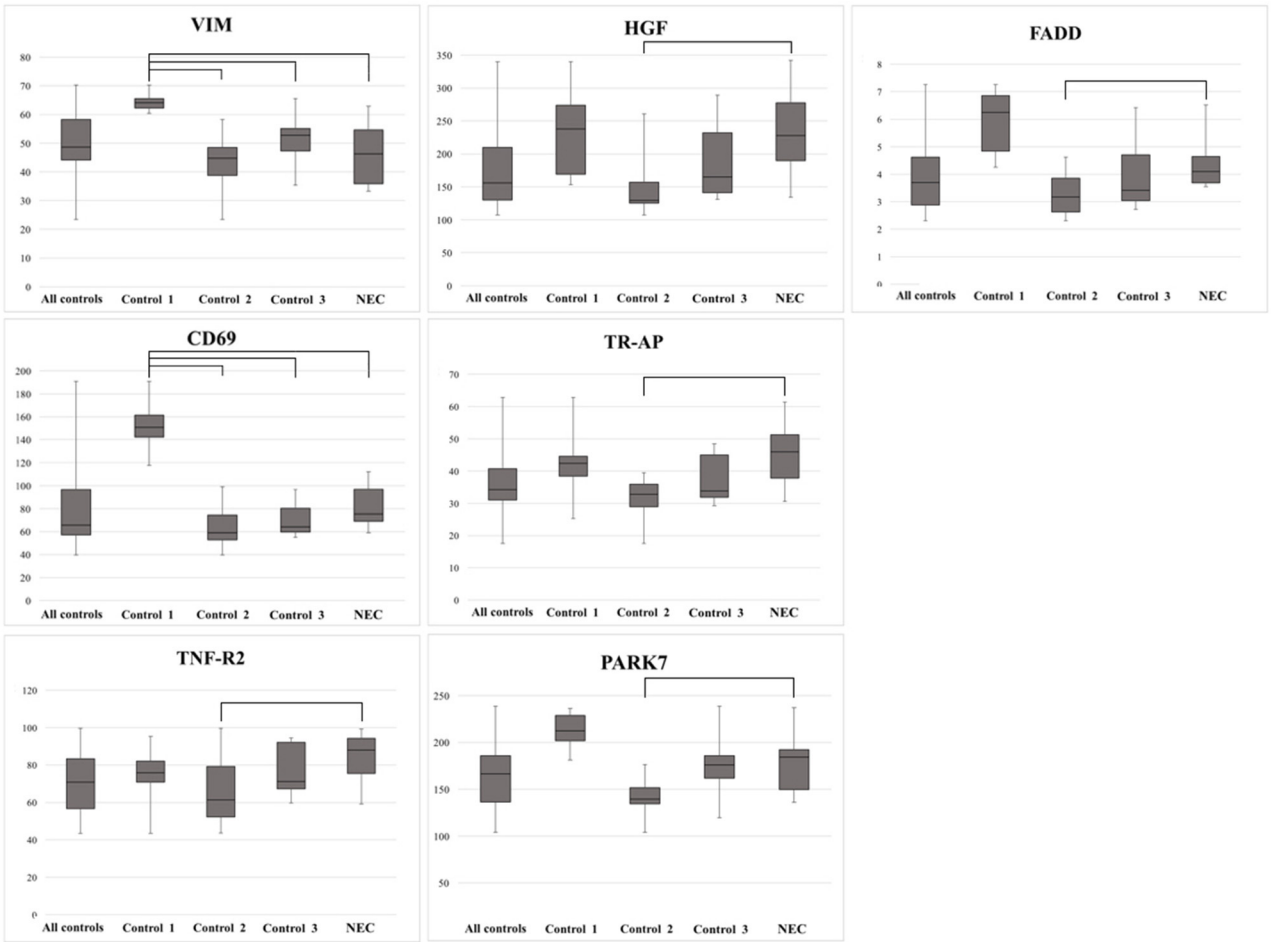

FIGURE 5 | Biomarkers previously reported linked to necrotizing enterocolitis (NEC) ($n = 7$), and with the significant difference between Controls 1, 2, and 3 and the NEC group. Note the higher expression of VIM and CD69 in Control 1 compared with the NEC group, and the lower expression of TNF-R2, PARK7, FADD, HGF, and TR-AP in Control 2 compared with the NEC group. Bars depict significant differences.

extremely preterm infants, thus focusing on individuals already at the highest risk of developing NEC.

Sixteen biomarkers were lower in Control 2 as compared with the NEC group. Twelve of these also differed in expression when comparing the three control groups, while six biomarkers (Ca-125, IL27A, TR-AP, EZR, HGF, and TNF-R2) did not. Since these six biomarkers do not differ when comparing NEC with Control 3, they could be what indicates a high risk of developing NEC. Some of these proteins have previously been linked to colitis.

We found that TNF-R2 was lower in a group of controls, which is in accordance with previous NEC studies (42, 43). Tumor necrosis factor has pleiotropic effects with both pro- and anti-inflammatory effects (44). TNF-R2 has been postulated to be a pro-inflammatory mediator in the pathophysiology of NEC (42). Increased TNF-R2 signaling in mice has been found to induce intestine barrier loss, resulting in colitis (45, 46). TPN nutrition further contributes to TNF dysregulation of the epithelial barrier function in mouse models (45). The increased level of TNF-R2 found in the NEC group could be an indication of a predisposed compromised intestinal barrier.

HGF regulates cell proliferation, cell survival, and angiogenesis (47), which are especially important in enterocytes (48). In our study, we found that HGF expression was inversely correlated to BW and a tendency toward it being inversely correlated to GA. Inverse correlation of GA and HGF expression has been found previously (49) and would be in line with the fact that increased prematurity increases risk for NEC (15). Protein levels are higher in the second trimester as compared with levels found in urine from newborns (50). Although HGF correlation to BW in preterm has not been fully studied, HGF has been described to be a biomarker for being SGA (51). Being SGA is a risk factor for NEC (15). In contrast, it has been found that fetal swallowing of amniotic fluid containing HGF decreases NEC incidence in rats (52) and that induced colitis yields greater damage in HGF-deficient mice (53). To summarize, high HGF could be an indication of increased immaturity and being SGA.

TR-AP is a serum marker for activated macrophages and chronic inflammation and is being explored for diagnosis of chronic inflammatory diseases (54). TR-AP-positive macrophages reside in the lamina propria of the healthy colon, and a histopathological increase of TR-AP expression has been found in colitis-induced rats (55). NEC has to our knowledge not previously been linked to TR-AP. The elevated TR-AP expression in the NEC group could signify increased inflammatory activity.

Leukemia inhibitory factor receptor (LIF-R), carbonic anhydrase IX (CAIX), integrin alpha (ITGA), and their potential links to NEC and colitis have, to our knowledge, not been reported. Although no link has previously been made between FADD and NEC, FADD has been found to prevent intestinal inflammation (56), and knock-out of FADD has been shown to induce colitis (57). Parkinson's disease protein 7 (PARK7) deficiency leads to increased apoptosis in colitis and has been proposed as a therapeutic target for colitis (58) but has never been linked to NEC.

There are previous studies on the link between NEC/colitis and expression of ezrin (EZR) (59, 60), tumor growth factor beta 1 (TGF-β1) (42), VIM (34, 35), IL-10 (61), epidermal growth factor receptor 3 (ErbB3/HER3) (62), epidermal growth factor receptor 4 (ErbB4/HER4) (63, 64), platelet endothelial cell adhesion molecule 1 (PECAM-1) (65, 66), carcinoma antigen 125 (CA-125) (67, 68), and chemokine ligand 25 (CCL25) (69, 70). These findings are not congruent with the direction of expression level (+/−) in NEC patients compared with controls in this study. However, this analysis is not a study of biomarker level in stated NEC but an attempt to determine levels before onset of fulminant NEC. To this date, few data are available for such comparison.

Strengths and Limitations

The reason for not finding differences in biomarkers between NEC and all controls could be that day 2 after birth is too early to detect relevant biomarkers. Given the single blood sampling, it was not possible to consider the chance of biomarker levels being influenced by diurnal variation, or whether day 2 after birth is the optimal time to find valuable biomarkers. Further studies with sequential blood sampling would be beneficial to take these factors into account.

A strength of this study is its benchmarking of biomarker patterns along with perinatal clinical data to explore risk of NEC development. The results from this study can be used for comparison in future studies. The single, early blood sampling illustrates the starting point of extremely preterm infants before being influenced by postnatal factors. While we hypothesize that postnatal factors influence why Control group 3 did not develop NEC, it was not possible to verify this in this study due to the data not being collected.

The reason for the relatively high NEC incidence in the study group (27%) as compared with national data on extremely preterm infants (9%) (71) could be the fact that survival until day 2 was necessary to be included in the study, since this was the time of blood sampling. This excluded infants who died from early causes of death, such as asphyxia, respiratory conditions, IVH, congenital anomalies, and early infections (72–75). The relatively high incidence of NEC could also be ascribed to the low GA in the entire study group.

CONCLUSION

In this study of extremely preterm infants, the expression of early comprehensive biomarkers ($n = 189$) at day 2 of life could not distinguish those who later developed NEC from all controls. Thus, the study could not identify biomarkers that can be used to select infants at high risk of developing NEC when comparing the NEC group with all controls. After subdivision of controls into three groups, simultaneously elevated VIM and CD69, or simultaneously lower expression of TNF-R2, PARK7, FADD, HGF, and TR-AP, could be regarded as a lower risk for developing NEC in some of the infants. Known risk factors of NEC were not higher in individuals who later developed NEC, which suggests that postnatal factors influence NEC development.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Etikprövningsmyndigheten, Uppsala, Sweden. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

AH performed collection of data, statistical analysis, wrote and edited the manuscript. LM performed data collection analysis, statistical analysis as well as writing and revising of the manuscript. HL partook in data analysis and in writing and revising the manuscript. KO set up the study, performed data collection, statistical analysis, and revised the manuscript. RS set up the study, performed data collection, data analysis, and writing and revising of the manuscript. The manuscript has been read and approved for submission by all authors. All authors approve this version to be published.

FUNDING

This study was funded by H.K.H. Kronprinsessan Lovisas Förening för Barnasjukvård (Grant Number 2018-00459).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.755437/full#supplementary-material

REFERENCES

1. World Health Organisation. GHO | By category | Nu*mber of deaths (thousands) - Data by WHO Region*. WHO (2018). Available online at: http://apps.who.int/gho/data/view.main.CM1300N?lang=en (accessed October 3, 2020).
2. Berrington JE, Hearn RI, Bythell M, Wright C, Embleton ND. Deaths in preterm infants: changing pathology over 2 decades. *J Pediatr.* (2012) 160:49–53.e1. doi: 10.1016/j.jpeds.2011.06.046
3. Norman M, HAllberg B, Abrahamsson T, Björklund LJ, Domellöf M, Farooqi A, et al. Association between year of birth and 1-year survival among extremely preterm infants in Sweden during 2004-2007 and 2014-2016. *JAMA*. (2019) 321:1188–99. doi: 10.1001/jama.2019.2021
4. Stoll BJ, Hansen NI, Bell EF, Walsh MC, Carlo WA, Shankaran S, et al. Trends in care practices, morbidity, and mortality of extremely preterm neonates, 1993–2012. *JAMA*. (2015) 314:1039–51. doi: 10.1001/jama.2015.10244
5. Hoffsten A, Markasz L, Ericson K, Nelin LD, Sindelar R. The value of autopsy in preterm infants at a Swedish tertiary neonatal intensive care unit 2002–2018. *Sci Rep*. (2021) 11:14156. doi: 10.1038/s41598-021-93358-7
6. Rasiah V, Yajamanyam PK, Ewer AK. Necrotizing enterocolitis: current perspectives. *RRN*. (2014) 2014:31–42. doi: 10.2147/RRN.S36576
7. Travers CP, Clark RH, Spitzer AR, Das A, Gerite TJ, Carlo WA. Exposure to any antenatal corticosteroids and outcomes in preterm infants by gestational age: prospective cohort study. *BMJ*. (2017) 356:j1039. doi: 10.1097/01.aoa.0000527027.63123.44
8. Mizrahi A, Barlow O, Berdon W, Blanc WA, Silverman WA. Necrotizing enterocolitis in premature infants. *J Pediatr*. (1965) 66:697–705. doi: 10.1016/S0022-3476(65)80003-8
9. Javid PJ, Riggle KM, Smith C. Necrotizing enterocolitis and short bowel syndrome. In: Gleason CA, Juul SE, editors. *Avery's Diseases of the Newborn*. Seattle, WA: Elsevier. (2017). p. 1090–7.
10. Watkins DJ, Besner GE. The role of the intestinal microcirculation in necrotizing enterocolitis. *Semin Pediatr Surg*. (2013) 22:83–7. doi: 10.1053/j.sempedsurg.2013.01.004
11. Colomé G, Sierra C, Blasco J, García MV, Velverde E, Sánchez E. Intestinal permeability in different feedings in infancy. *Acta Paediatr*. (2007) 96:69–72. doi: 10.1111/j.1651-2227.2007.00030.x
12. Weaver LT, Laker MF, Nelson R, Lucas A. Milk feeding and changes in intestinal permeability and morphology in the newborn. *J Pediatr Gastroenterol Nutr*. (1987) 6:351–8. doi: 10.1097/00005176-198705000-00008
13. Dvorak B. Milk epidermal growth factor and gut protection. *J Pediatr*. (2010) 156:S31–5. doi: 10.1016/j.jpeds.2009.11.018
14. Patel RM, Knezevic A, Shenci N, Hinkes M, Keene S, Roback KA, et al. Association of red blood cell transfusion, anemia, and necrotizing enterocolitis in very low-birth-weight infants. *JAMA*. (2016) 315:889–97. doi: 10.1001/jama.2016.1204
15. Samuels N, van de Graaf RA, de Jonge RCJ, Reiss IKM, Vermeulen MJ. Risk factors for necrotizing enterocolitis in neonates: a systematic review of prognostic studies. *BMC Pediatr*. (2017) 17:105. doi: 10.1186/s12887-017-0847-3
16. Bisquera JA, Cooper TR, Berseth CL. Impact of necrotizing enterocolitis on length of stay and hospital charges in very low birth weight infants. *Pediatrics*. (2002) 109:423–428. doi: 10.1542/peds.109.3.423
17. Brunse A, Abbaspour A, Sangild PT. Brain barrier disruption and region-specific neuronal degeneration during necrotizing enterocolitis in preterm pigs. *Dev Neurosci*. (2018) 40:198–208. doi: 10.1159/000488979
18. Rich BS, Dolgin SE. Necrotizing enterocolitis. *Pediatr Rev*. (2017) 38:552–9. doi: 10.1542/pir.2017-0002
19. Bell MJ, Ternberg JL, Feigin RD, Keating JP, Marshall R, Barton L, et al. Neonatal necrotizing enterocolitis. Therapeutic decisions based upon clinical staging. *Ann. Surg*. (1978) 187:1–7. doi: 10.1097/00000658-197801000-00001
20. Walsh MC, Kliegman RM. Necrotizing enterocolitis: treatment based on staging criteria. *Pediatr Clin North Am*. (1986) 33:179–201. doi: 10.1016/S0031-3955(16)34975-6
21. Palleri E, Aghamn I, Bexelius TS, Bartocci M, Wester T. The effect of gestational age on clinical and radiological presentation of necrotizing enterocolitis. *J Pediatr Surg*. (2018) 53:1660–4. doi: 10.1016/j.jpedsurg.2017.09.018
22. Lin PW, Stoll BJ. Necrotising enterocolitis. *Lancet*. (2006) 368:1271–83. doi: 10.1016/S0140-6736(06)69525-1
23. Nantais-Smith L, Kadrofske M. Noninvasive biomarkers of necrotizing enterocolitis. *J Perin Neonatal Nurs*. (2015) 29:69–80. doi: 10.1097/JPN.0000000000000082
24. Aydemir O, Aydemir C, Sarkabadayi YU, Canpolat FE, Erdeve O, Biyyikli Z, et al. Fecal calprotectin levels are increased in infants with necrotizing enterocolitis. *J Maternal Fetal Neonatal Med*. (2012) 25:2237–41. doi: 10.3109/14767058.2012.684172
25. Garg BD, Sharma D, Bansal A. Biomarkers of necrotizing enterocolitis: a review of literature. *J Maternal Fetal Neonatal Med*. (2018) 31:3051–64. doi: 10.1080/14767058.2017.1361925
26. Terrin G, Stronati L, Cucchiara S, De Curtis M. Serum markers of necrotizing enterocolitis: a systematic review. *J Pediatr Gastroenterol Nutr*. (2017) 65:e120. doi: 10.1097/MPG.0000000000001588
27. Josefsson S, Bunn SK, Domellöf M. Fecal calprotectin in very low birth weight infants. *J Pediatr Gastroenterol Nutr*. (2007) 44:407–13. doi: 10.1097/MPG.0b013e3180320643
28. Däbritz J, Jenke A, Wirth S, Foell D. Fecal phagocyte-specific S100A12 for diagnosing necrotizing enterocolitis. *J Pediatr*. (2012) 161:1059–64. doi: 10.1016/j.jpeds.2012.06.003
29. Olsson KW, Larsson A, Jonzon A, Sindelar R. Exploration of potential biochemical markers for persistence of patent ductus arteriosus in preterm infants at 22-27 weeks' gestation. *Pediatr Res*. (2019) 86:333–8. doi: 10.1038/s41390-018-0182-x
30. Olink. *Proximity Extension Assay (PEA) Technology*. Olink (2020). Available online at: https://www.olink.com/data-you-can-trust/technology/ (accessed April 9, 2020).
31. de Hoon MJL, Imoto S, Nolan J, Miyano S. Open source clustering software. *Bioinformatics*. (2004) 20:1453–4. doi: 10.1093/bioinformatics/bth078
32. Saldanha AJ. Java Treeview–extensible visualization of microarray data. *Bioinformatics*. (2004) 20:3246–8. doi: 10.1093/bioinformatics/bth349
33. The Human Protein Atlas Project. *Tissue expression of VIM - Summary - The Human Protein Atlas. The Human Protein Atlas.* Available online at: https://www.proteinatlas.org/ENSG00000026025-VIM/tissue (accessed October 23, 2020).
34. Boros É, Csatári M, Varga C, Bálint B, Nagy I. Specific gene- and microRNA-expression pattern contributes to the epithelial to mesenchymal transition in a rat model of experimental colitis. *Mediators Inflamm*. (2017) 2017:5257378. doi: 10.1155/2017/5257378
35. Mor-Vaknin N, Legendre M, Yu Y, Serezani CHC, Garg SK, Jatzek A, et al. Murine colitis is mediated by vimentin. *Sci Rep*. (2013) 3:1045. doi: 10.1038/srep01045
36. Laguna T, Notario L, Pippa R, Fontela M, Vázquez B, Maicas M, et al. New insights on the transcriptional regulation of CD69 gene through a potent enhancer located in the conserved non-coding sequence 2. *Mol Immunol*. (2015) 66:171–9. doi: 10.1016/j.molimm.2015.02.031
37. Ge Y, Gong M, Colliou N, Zadeh M, Li J, Jones DP, et al. Neonatal intestinal immune regulation by the commensal bacterium, P. UF1. *Mucosal Immunol*. (2019) 12:434–44. doi: 10.1038/s41385-018-0125-1
38. Arthur CM, Nalbant D, Feldman HA, Saeedi BJ, Matthews J, Kamili NA, et al. Anemia induces gut inflammation and injury in an animal model of preterm infants. *Transfusion*. (2019) 59:1233–45. doi: 10.1111/trf.15254
39. Brait VH, Miró-Mur F, Pérez-de-Puig I, Notario L, Hurtado B, Pedragosa J, et al. CD69 plays a beneficial role in ischemic stroke by dampening endothelial activation. *Circ Res*. (2019) 124:279–91. doi: 10.1161/CIRCRESAHA.118.313818
40. Harbige LS, Pinto E, Allgrove J, Thomas LV. Immune Response of healthy adults to the ingested probiotic lactobacillus casei Shirota. *Scand J Immunol*. (2016) 84:353–64. doi: 10.1111/sji.12495
41. Yu L, Yang F, Zhang F, Guo D, Li L, Wang J, et al. CD69 enhances immunosuppressive function of regulatory T-cells and attenuates colitis by prompting IL-10 production. *Cell Death Dis*. (2018) 9:905. doi: 10.1038/s41419-018-0927-9
42. Cho SX, Berger PJ, Nold-Petry CA, Nold MF. The immunological landscape in necrotising enterocolitis. *Expert Rev Mol Med*. (2016) 18:e12. doi: 10.1017/erm.2016.13

43. Olsson KW, Sindelar R. Early Biochemical markers associated with development of necrotizing enterocolitis. *Pediatr Neonat Individualized Med.* (2017) 6:e060244. doi: 10.7363/060244
44. Ye LL, Wei XS, Zhang M, Niu YR, Zhou Q. The significance of tumor necrosis factor receptor type II in CD8+ regulatory t cells and CD8+ effector T cells. *Front Immunol.* (2018) 9:583. doi: 10.3389/fimmu.2018.00583
45. Feng Y, Teitelbaum DH. Tumour necrosis factor-α-induced loss of intestinal barrier function requires TNFR1 and TNFR2 signalling in a mouse model of total parenteral nutrition. *J Physiol.* (2013) 591:3709–23. doi: 10.1113/jphysiol.2013.253518
46. Su L, Nalle SC, Shen L, Turner ES, Singh G, Breskin LA, et al. TNFR2 activates MLCK-dependent tight junction dysregulation to cause apoptosis-mediated barrier loss and experimental colitis. *Gastroenterology.* (2013) 145:407–15. doi: 10.1053/j.gastro.2013.04.011
47. Boros P, Miller CM. Hepatocyte growth factor: a multifunctional cytokine. *Lancet.* (1995) 345:293–5. doi: 10.1016/S0140-6736(95)90279-1
48. MohanKumar K, Namachivayam K, Ho TTB, Torres BA, Ohls RK, Maheshwari A. Cytokines and growth factors in the developing intestine and during necrotizing enterocolitis. *Semin Perinatol.* (2017) 41:52–60. doi: 10.1053/j.semperi.2016.09.018
49. Jimenez-Gomez G, Benavente-Fernandez I, Lubian-Lopez SP, Matias-Vega M, Lechuga-Campoy JL, Saez-Benito A, et al. Hepatocyte growth factor as an indicator of neonatal maturity. *J Pediatr Endocrinol Metab.* (2013) 26:709–14. doi: 10.1515/jpem-2012-0303
50. Kurauchi O, Itakura A, Ando H, Kuno N, Mizutani S, Tomoda Y. The concentration of hepatocyte growth factor (HGF) in human amniotic fluid at second trimester: relation to fetal birth weight. *Horm Metab Res.* (1995) 27:335–8. doi: 10.1055/s-2007-979973
51. Tjoa ML, Mulders MAM, van Vugt JMG, Blankenstein MA, Oudejans CBM, van Wijk IJ. Plasma hepatocyte growth factor as a marker for small-for-gestational age fetuses. *Eur J Obstetr Gynecol Reprod Biol.* (2003) 110:20–5. doi: 10.1016/S0301-2115(03)00083-6
52. Jain SK, Baggerman EW, MohanKumar K, Namachivayam K, Jagadeeswaran R, Reyes VE, et al. Amniotic fluid-borne hepatocyte growth factor protects rat pups against experimental necrotizing enterocolitis. *Am J Physiol Gastrointest Liver Physiol.* (2014) 306:G361–9. doi: 10.1152/ajpgi.00272.2013
53. Itoh H, Naganuma S, Takeda N, Miyata S, Uchinokura S, Fukushima T, et al. Regeneration of injured intestinal mucosa is impaired in hepatocyte growth factor activator-deficient mice. *Gastroenterology.* (2004) 127:1423–35. doi: 10.1053/j.gastro.2004.08.027
54. Janckila AJ, Yam LT. Biology and clinical significance of tartrate-resistant acid phosphatases: new perspectives on an old enzyme. *Calcif Tissue Int.* (2009) 85:465–83. doi: 10.1007/s00223-009-9309-8
55. Lång P, Lange S, Delbro D, Andersson G. Induction and cellular expression of tartrate resistant acid phosphatase during dextran sodium sulphate induced colitis in rats. *Histochem Cell Biol.* (2009) 132:599–612. doi: 10.1007/s00418-009-0647-4
56. Schwarzer R, Jiao H, Wachsmuth L, Tresch A, Pasparakis M. FADD and caspase-8 regulate gut homeostasis and inflammation by controlling MLKL- and GSDMD-mediated death of intestinal epithelial cells. *Immunity.* (2020) 52:978–93.e6. doi: 10.1016/j.immuni.2020.04.002
57. Welz PS, Wullaert A, Vlantis K, Kondylis V, Fernández-Majada V, Ermolaeva M, et al. FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation. *Nature.* (2011) 477:330–4. doi: 10.1038/nature10273
58. Zhang J, Xu M, Zhou W, Li D, Zhang H, Chen Y, et al. Deficiency in the anti-apoptotic protein DJ-1 promotes intestinal epithelial cell apoptosis and aggravates inflammatory bowel disease via p53. *J Biol Chem.* (2020) 295:4237–51. doi: 10.1074/jbc.RA119.010143
59. Murchie R, Guo CH, Persaud A, Muise A, Rotin D. Protein tyrosine phosphatase σ targets apical junction complex proteins in the intestine and regulates epithelial permeability. *Proc Natl Acad Sci USA.* (2014) 111:693–8. doi: 10.1073/pnas.1315017111
60. Solaymani-Mohammadi S, Singer SM. Regulation of intestinal epithelial cell cytoskeletal remodeling by cellular immunity following gut infection. *Mucosal Immunol.* (2013) 6:369–78. doi: 10.1038/mi.2012.80
61. Edelson MB, Bagwell CE, Rozycki HJ. Circulating pro- and counterinflammatory cytokine levels and severity in necrotizing enterocolitis. *Pediatrics.* (1999) 103:766–71. doi: 10.1542/peds.103.4.766
62. Almohazey D, Lo YH, Vossler CV, Simmons AJ, Hsieh JJ, Bukar EB, et al. The ErbB3 receptor tyrosine kinase negatively regulates Paneth cells by PI3K-dependent suppression of Atoh1. *Cell Death Differ.* (2017) 24:855–65. doi: 10.1038/cdd.2017.27
63. Frey MR, Brent Polk D. ErbB receptors and their growth factor ligands in pediatric intestinal inflammation. *Pediatr Res.* (2014) 75:127–32. doi: 10.1038/pr.2013.210
64. McElroy SJ, Castle SL, Bernard JK, Almohazey D, Hunter CJ, Bell BA, et al. The ErbB4 ligand neuregulin-4 protects against experimental necrotizing enterocolitis. *Am J Pathol.* (2014) 184:2768–78. doi: 10.1016/j.ajpath.2014.06.015
65. Sugimoto N, Rui T, Yang M, Bharwani S, Handa O, Yoshida N, et al. Points of control exerted along the macrophage-endothelial cell-polymorphonuclear neutrophil axis by PECAM-1 in the innate immune response of acute colonic inflammation. *J Immunol.* (2008) 181:2145–54. doi: 10.4049/jimmunol.181.3.2145
66. Rijcken E, Menningen RB, Schaefer SD, Laukoetter MG, Anthoni C, Spiegel HU, et al. PECAM-1 (CD 31) mediates transendothelial leukocyte migration in experimental colitis. *Am J Physiol Gastrointest Liver Physiol.* (2007) 293:G446–52. doi: 10.1152/ajpgi.00097.2007
67. Ataseven H, Oztürk ZA, Arhan M, Yüksel O, Köklü S, Ibiş M, et al. Cancer antigen 125 levels in inflammatory bowel diseases. *J Clin Lab Anal.* (2009) 23:244–8. doi: 10.1002/jcla.20323
68. Yamamoto-Furusho JK, Ascaño-Gutiérrez I, Furuzawa-Carballeda J, Fonseca-Camarillo G. Differential expression of MUC12, MUC16, and MUC20 in patients with active and remission ulcerative colitis. *Mediators Inflamm.* (2015) 2015:659018. doi: 10.1155/2015/659018
69. Egan CE, Sodhi CP, Good M, Lin J, Jia H, Yamagichi Y, et al. Toll-like receptor 4–mediated lymphocyte influx induces neonatal necrotizing enterocolitis. *J Clin Invest.* (2016) 126:495–508. doi: 10.1172/JCI83356
70. Trivedi PJ, Bruns T, Ward S, Mai M, Schmidt C, Hirschfield GM, et al. Intestinal CCL25 expression is increased in colitis and correlates with inflammatory activity. *J Autoimmun.* (2016) 68:98–104. doi: 10.1016/j.jaut.2016.01.001
71. SNQ Report Group. *Årsrapport 2018, Yearly Report 2018.* (2019). Vol. 103. Available online at: https://www.medscinet.com/PNQ/uploads/website/arsrapporter/Neonatalregistrets%20%C3%85rsrapport%202018._3.0.pdf (accessed September 30, 2020).
72. Avery ME. Surface properties in relation to atelectasis and hyaline membrane disease. *Arch Pediatr Adolesc Med.* (1959) 97:517. doi: 10.1001/archpedi.1959.02070010519001
73. Ballabh P. Intraventricular hemorrhage in premature infants: mechanism of disease. *Pediatr Res.* (2010) 67:1–8. doi: 10.1203/PDR.0b013e3181c1b176
74. Bancalari E, Claure N, Sosenko IRS. Bronchopulmonary dysplasia: changes in pathogenesis, epidemiology and definition. *Semin Fetal Neonatal Med.* (2003) 8:63–71. doi: 10.1016/S1084-2756(02)00192-6
75. Håkansson S, Lilja M, Jacobsson B, Källén K. Reduced incidence of neonatal early-onset group B streptococcal infection after promulgation of guidelines for risk-based intrapartum antibiotic prophylaxis in Sweden: analysis of a national population-based cohort. *Acta Obstet Gynecol Scand.* (2017) 96:1475–1483. doi: 10.1111/aogs.13211

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

frontiers in Pediatrics

ORIGINAL RESEARCH
published: 28 October 2021
doi: 10.3389/fped.2021.757299

Routine Administration of a Multispecies Probiotic Containing *Bifidobacterium* and *Lactobacillus* to Very Low Birth Weight Infants Had No Significant Impact on the Incidence of Necrotizing Enterocolitis

Brian A. Juber[1,2], *Timothy J. Boly*[2], *Graeme J. Pitcher*[3] *and Steven J. McElroy*[4]*

[1] Department of Pediatrics, University of Nebraska Medical Center, Omaha, NE, United States, [2] Stead Family Department of Pediatrics, University of Iowa, Iowa City, IA, United States, [3] Division of Pediatric Surgery, Department of Surgery, University of Iowa, Iowa City, IA, United States, [4] Department Pediatrics, University of California, Davis, Davis, CA, United States

OPEN ACCESS

Edited by:
Yuan Shi,
Children's Hospital of Chongqing Medical University, China

Reviewed by:
Janet Elizabeth Berrington, Newcastle upon Tyne Hospitals NHS Foundation Trust, United Kingdom
Lu-Quan Li, Chongqing Medical University, China

***Correspondence:**
Steven J. McElroy
sjmcelroy@ucdavis.edu

Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: 11 August 2021
Accepted: 11 October 2021
Published: 28 October 2021

Citation:
Juber BA, Boly TJ, Pitcher GJ and McElroy SJ (2021) Routine Administration of a Multispecies Probiotic Containing Bifidobacterium and Lactobacillus to Very Low Birth Weight Infants Had No Significant Impact on the Incidence of Necrotizing Enterocolitis. Front. Pediatr. 9:757299. doi: 10.3389/fped.2021.757299

Background: Necrotizing enterocolitis (NEC) is the leading cause of gastrointestinal morbidity in preterm infants, and prevention and treatment strategies have remained largely unchanged over the past several decades. As understanding of the microbiome has increased, probiotics have been hypothesized as a possible strategy for decreasing rates of NEC, and several studies have noted significant decreases in rates of NEC after initiation of probiotics in preterm infants. However, a recent AAP report cited caution on the use of probiotic use in part because studies of probiotic use in ELBW infants are lacking. As our unit began routine use of probiotics for all infants <33 weeks in 2015 and we are a leading institution for intact survival of ELBW infants, we attempted to answer if probiotic use can impact the rate of NEC in VLBW and ELBW infants.

Methods: We conducted a single-center retrospective chart review of infants with modified Bell's stage ≥2a NEC for the 4 years prior to and 5 years after initiation of a protocol involving routine supplementation of a multispecies probiotic to premature infants at the University of Iowa, Stead Family Children's Hospital. The primary outcome measures were rates of modified Bell's stage ≥2a NEC and all-cause pre-discharge mortality at our institution before and after initiation of routine probiotic supplementation in 2015.

Results: In our institution, neither the rates of modified Bell's stage ≥2a NEC, nor the rates of all-cause mortality were significantly altered in very low birth weight (VLBW) infants by the initiation of routine probiotic use (NEC rates pre-probiotic 2.1% vs. post-probiotic 1.5%; all-cause mortality rates pre-probiotic 8.4% vs. post-probiotic 7.4%). Characteristics of our two cohorts were overall similar except for a significantly lower 5-minute APGAR score in infants in the post-probiotic epoch (pre-probiotic 8 vs. post-probiotic 6 $p = 0.0316$), and significantly more infants in the post-probiotic epoch received probiotics (pre-probiotics 0% vs. post-probiotics 65%; $p < 0.0001$). Similarly, probiotic use had no impact on the incidence of NEC when we restricted our data to only

extremely low birth weight (ELBW) infants (pre-probiotics 1.6% vs post-probiotics 4.1%). When we restricted our analysis to only inborn infants, probiotics still had no impact on NEC rates in VLBW infants (1.5% pre- and 1.1% post-probiotic, $p = 0.61$) or ELBW infants (2% pre- and 2.1% post-probiotic, $p = 0.99$)

Conclusions: Contrary to other studies, we found no significant difference in rates of modified Bell's stage ≥2a NEC or all-cause pre-discharge mortality in VLBW infants following routine administration of a multispecies probiotic supplement.

Keywords: necrotizing enterocolitis (NEC), bifidobacteria, probiotics, VLBW (very low birth weight), ELBW (extreme low birth weight infants)

INTRODUCTION

Necrotizing enterocolitis (NEC) is the leading cause of gastrointestinal morbidity and mortality in premature infants, with ∼6–9% of very low birth weight (VLBW) infants developing NEC (1–3). Mortality rates are typically 20–30% and can be as high as 40% in the extremely low birth weight (ELBW) population of infants (4). Despite intensive research into pathophysiology and novel therapeutics, medical therapies have largely remained the same over the past several decades, consisting of nasogastric drainage, bowel rest, broad-spectrum intravenous antibiotics, and parenteral fluids and nutrition (5). In severe cases, surgery may be required for perforation or signs of bowel necrosis, but the optimal modality of surgical management remains unclear (6, 7).

Both the etiology and pathophysiology of NEC are diverse and multifactorial. Recently, microbiome studies in preterm infants have yielded valuable insights into its contributions to many disease processes, including NEC. The human microbiome consists of 10–100 trillion symbiotic bacteria, most of which are harbored in the gastrointestinal tract (8). The establishment and composition of the neonatal microbiome, especially in preterm infants, is adversely affected by many prenatal and postnatal factors, including mode of delivery, breast milk vs. formula feeding, and both duration and type of antibiotic exposure (9–14). Distinct differences in microbiome composition have been noted in premature infants compared to term infants (15). These alterations are especially pronounced in premature infants with NEC, implicating a perturbed and non-diverse microbiome as a risk factor for later development of NEC (3, 16–19).

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host, and they are often used to diversify the microbiome in both the pediatric and adult populations (20). Multiple studies and meta-analyses have demonstrated that administration of probiotics to preterm infants in the neonatal intensive care unit (NICU) is likely safe and may decrease the risk of NEC and mortality (21–26). However, despite widespread use in other developed countries, the practice of administering probiotics to preterm infants remains relatively limited and controversial in the United States (27). A recent survey of NICUs in the United States found that despite a lack of evidence-based guidelines, ∼1 in 10 NICUs give VLBW infants probiotic supplements either routinely or to select infants as a part of their clinical practice (28). Part of this controversy stems from the fact that probiotics are considered a dietary supplement by the United States Food & Drug Administration (FDA), meaning that probiotics are not required to meet the same stringent regulatory standards as medications that are marketed as a drug to treat a certain disorder. Consequently, there is marked heterogeneity in commercially available probiotic products. Since the effects of probiotics are highly strain specific (29), it is difficult to compile aggregate data from different prospective studies and clinical trials. Moreover, it has also been noted that the contents of commercially available probiotic preparations often do not match what is listed on the label (30). Given the lack of definitive evidence for efficacy, conflicting data on safety, and potential for harm in a highly vulnerable population of patients, a recently published American Academy of Pediatrics (AAP) clinical report was unable to support the routine, universal use of probiotics in preterm infants, particularly ELBW infants (31).

Multiple formulations of probiotics exist, with *Bifidobacterium* and *Lactobacillus* as the most common strains used (32). *Bifidobacterium* is a genus of gram-positive anaerobic bacteria that was first isolated from the stool of breastfed infants in 1899, and it has been associated with a reduction in NEC in preterm infants (33, 34). Similarly, *Lactobacillus* is a genus of gram positive facultative anaerobic bacteria which has also been demonstrated to have multiple positive microbiological and immunological effects on the host (35). Each genus confers specific benefits, but recently published population-based research suggests that a combination of *Bifidobacterium* and *Lactobacillus* is safe and bestows the most benefit to the neonatal population (26). An additional meta-analysis published several years earlier also demonstrated there was a highly significant reduced risk of NEC and mortality when a multiple strain probiotic was used, which was not present with use of single strain probiotic formulations (24).

In 2015, our institution introduced a protocol for routine probiotic supplementation in premature infants. This decision was supported by previously published studies demonstrating a significant decrease in NEC with the multispecies probiotic consisting of four *Bifidobacterium* species and *Lactobacillus rhamnosus* (22, 23). However, there is currently a paucity of literature examining probiotic use in ELBW infants. Because of our institution's high rate of neurodevelopmentally intact survival for VLBW and ELBW infants (36) as well as our anecdotally low baseline rate of NEC (2–4%), we performed a

retrospective cohort study examining rates of NEC in VLBW infants before and after initiation of routine supplementation of a multispecies probiotic. We also performed a subgroup analysis of rates of NEC in ELBW infants born <1,000 grams. We hypothesized that we would see lower rates of NEC and all-cause mortality in the post-probiotic epoch compared to the pre-probiotic epoch in both the VLBW and ELBW cohorts.

METHODS

Our Context, Study Design and Selection of Study Population

The Stead Family Children's Hospital at the University of Iowa has an 87-bed level 4 NICU that has all subspecialty medical and surgical services and acts as both an inborn birth center and a referral center for Iowa and the surrounding states. The NICU admits an average of 153 VLBW and 71 ELBW infants per year. Our unit is known for its traditionally low NEC rate and its high rates of intact survival at the limits of viability (36). This was a single-center retrospective cohort study primarily focusing on preterm VLBW infants for the 4 years preceding and 5 years following implementation of a protocol for routine supplementation of a multispecies probiotic. The study was approved by the University of Iowa Institutional Review Board (IRB#201410743).

To develop our dataset, all infants with pediatric surgery consults were identified in the 4 years preceding and 5 years following the initiation of probiotics at our institution. Per unit protocol, all infants who have concern for NEC (medical or surgical) have a pediatric surgical consult placed. Infants were excluded due to major congenital abnormalities (congenital diaphragmatic hernia [CDH], gastroschisis, or omphalocele), anatomic obstruction of the gastrointestinal tract (intestinal atresia, malrotation, or Hirschsprung disease), inguinal hernia repair, G-tube placement, or peritoneal dialysis catheter placement.

Data Collection and Identification of Infants With NEC

Infants with pediatric surgical consults during the years 2011–2019 who did not have the above exclusion criteria had data collected regarding baseline characteristics, feeding practices, risk factors for intestinal illness, and markers of illness severity. Chart review and data collection was conducted by two individuals who were also part of the consensus group. Study data were collected and managed using REDCap (Research Electronic Data Capture) electronic data capture tools at the University of Iowa (37, 38).

Within this cohort, a diagnosis of modified Bell's stage ≥2a NEC, spontaneous intestinal perforation (SIP), or neither condition was determined by consensus of a group consisting of pediatric surgeons and neonatologists. Consensus on a diagnosis was defined as agreement of at least 3 out of the 4 group members. We used the modified Bell's classification stage ≥2a to classify cases of NEC (39). Infants who did not meet these criteria in general had a single episode of bilious emesis with a negative workup, or had a concern for NEC (i.e., bloody stools, abdominal distention, abdominal discoloration, or increasing gastric residuals) but did not meet consensus criteria for Bell's stage ≥2a NEC. We then separated infants into the pre-probiotic and post-probiotic cohorts based on date of birth. Because routine probiotic supplementation began in 2015, 2011–2014 was defined as the pre-probiotic epoch and 2015–2019 was defined as the post-probiotic epoch.

Probiotic Protocol

Beginning in 2015, our institution began routine administration of Ultimate Flora Baby Probiotic® (www.renewlife.com), a multispecies probiotic formulation consisting of four *Bifidobacterium* species (*Bifidobacterium breve*, *bifidum*, *infantis*, and *longum*) plus *Lactobacillus rhamnosus* GG at a concentration of 2×10^9 colony forming units per 0.5 g. The probiotic was prepared daily by our pharmacy under conditions that minimize the risk of contamination by adding 0.5 g of probiotic to 2.5 mL of 2.5% dextrose in sterile water. This formulation is then administered to eligible infants via enteral gavage once daily prior to an enteral feed. Infants eligible for probiotic supplementation were at least 3 days old, born at <33 0/7 weeks gestational age, with a corrected post-menstrual age (PMA) of at least 24 0/7 weeks, who were also receiving intake of at least 6 mL of enteral feedings per day. The probiotic is routinely held during periods of NPO status and is otherwise continued daily until the infant reaches a corrected PMA of 36 0/7 weeks, at which point it is discontinued.

Statistical Analysis

Parametric *t*-testing was performed for continuous variables and chi-square testing was performed for categorical variables as appropriate using GraphPad Prism v9. Statistical significance was defined as a $p < 0.05$, a non-significant trend was defined as a *p*-value more than 0.05 but <0.1, and a non-significant (NS) finding was defined as a $p > 0.1$. All error values reported are standard error of the mean.

RESULTS

Identification of Infants With NEC

A total of 950 infants were identified with pediatric surgical consults between the years of 2011–2019 (**Figure 1**). Of those, 657 were excluded from analysis due to the presence of major congenital abnormalities (CDH, gastroschisis, or omphalocele), anatomic obstruction of the gastrointestinal tract (intestinal atresia, malrotation, or Hirschsprung disease), or consult only for inguinal hernia repair or gastrostomy tube or peritoneal dialysis catheter placement, leaving a total of 293 infants remaining for data extraction from electronic medical records. Following data extraction, consensus was obtained from a group of neonatologists and pediatric surgeons on cases of modified Bell's stage ≥2a NEC, resulting in a total of 37 cases of NEC between 2011 and 2019. There were 14 cases of NEC in the pre-probiotic epoch (2011 and 2014) and 23 cases of NEC in the post-probiotic epoch (2015 and 2019). The other 256 infants in the set were identified as having a SIP (20 infants) or having some other diagnosis (236 infants).

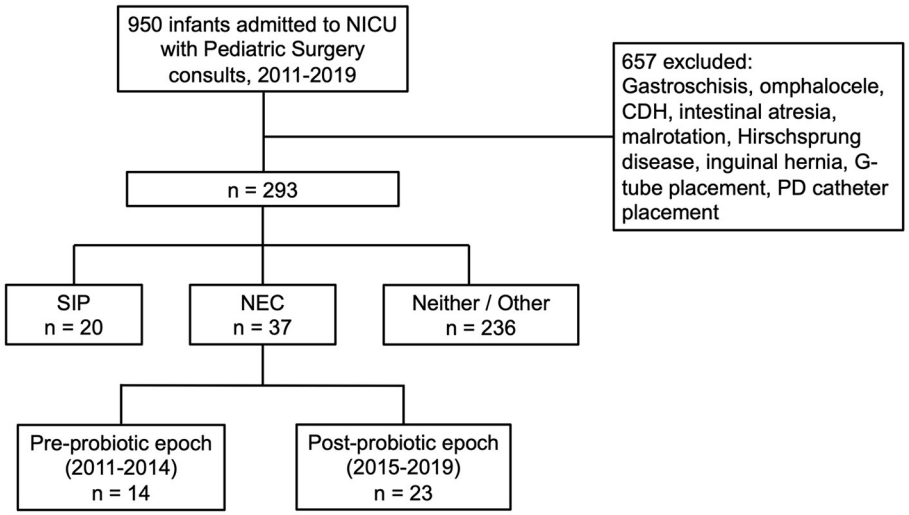

FIGURE 1 | Flow diagram of patients included in the study. From an original sample of 950 pediatric surgery consults from 2011 to 2019, through consensus of a group of neonatologists and pediatric surgeons, we identified 14 cases of NEC in the pre-probiotic epoch (2011–2014) and 23 cases of NEC in the post-probiotic epoch (2015–2019).

Baseline Characteristics of Mothers and Infants

Data were collected regarding gestational age, birth weight, sex, race, birth location, maternal parity, prolonged rupture of membranes (more than 18 h), antenatal betamethasone administration (at least 1 dose), mode of delivery (vaginal birth vs. Cesarean section), small for gestational age (SGA, defined as birth weight <10th percentile on Fenton growth curve), maternal Medicaid status (as a marker for maternal socioeconomic status), and receipt of antibiotics immediately upon NICU admission for each case of NEC in the pre-probiotic and post-probiotic epochs (**Table 1**). Maternal characteristics were similar between the two groups with no significant differences. Infant characteristics were overall similar between both epochs. There were no significant differences in gestational age, birth weight, sex, race, SGA status, or rate of antibiotic use amongst the infants. We did find that infants with NEC in the post-probiotic epoch had a significantly lower 5 min APGAR score compared to infants with NEC in the pre-probiotic epoch (mean APGAR score 6.3 vs. 7.8; $p = 0.0316$).

Feeding Practices

Data were collected regarding day of life (DOL) enteral feeds initiated, DOL feeds reached 40 mL/kg/day (feed volume in our unit when feeds are fortified), DOL feeds first fortified to 24 kcal/oz, feeding amount at time of fortification, type of feeding (exclusive breast milk, exclusive formula, or mix), amount of enteral intake on day of diagnosis and for 3 days prior to diagnosis, and average total fluid intake (parenteral + enteral) for 3 days prior to diagnosis and through day of diagnosis for each case of NEC in the pre-probiotic and post-probiotic epochs (**Table 2**). There were no significant differences in feeding practices between the two epochs.

Probiotic Use

Data were collected regarding probiotic use and DOL probiotics were started (if applicable) for each case of NEC in the pre-probiotic and post-probiotic epochs (**Table 2**). Overall, within the cohort of NEC cases during the post-probiotic epoch, 15/23 (65%) infants received probiotics at some point in their course, more than the infants with NEC during the pre-probiotic cohort (0/14, 0%). Within the VLBW cohort in the post-probiotic epoch, 15/16 (94%) received probiotics, and 13/14 (93%) of ELBW infants received probiotics compared to 0% for both groups in the pre-probiotic epoch. Of the infants with NEC in the post-probiotic epoch who received probiotics, probiotics were started on average on DOL 15. To understand the impact of outborn birth, a secondary analysis done that was restricted to only infants who were inborn. In this secondary analysis, more infants in the post-probiotic epoch received probiotics compared to the pre-probiotic epoch (67% vs. 0%) and there was a near-significant delay of 3 weeks in probiotic initiation in outborn infants compared to inborn infants (probiotics initiated on DOL 28 vs. DOL 7, respectively; $p = 0.0596$).

Rates of NEC for VLBW and ELBW Infants

Between 2011 and 2019 at our institution, 1.7% of infants with birth weight <1,500 grams, 3.0% of infants with birth weight <1,000 grams, and 3.0% of infants with birth weight <750 grams developed modified Bell's stage ≥2a NEC (**Figure 2**). There were not statistically significant differences between the NEC rates based on weight.

During the pre-probiotic epoch (2011 and 2014), 8 VLBW infants developed NEC, corresponding to an average NEC incidence of 1.5% (**Table 3A**, **Figure 3A**). Rates of NEC by year during the pre-probiotic epoch ranged from 0.8 to 2.1%. During the post-probiotic epoch (2015 and 2019), 16 VLBW

TABLE 1 | Baseline characteristics of infants diagnosed with NEC and their mothers before and after initiation of probiotics in 2015.

	Pre-probiotic epoch (2011–2015) *n* = 14	Post-probiotic epoch (2015–2020) *n* = 23	Significance
Gestational age	30.6 (±1.2)	28.7 (±1.1)	NS
Birth weight (g)	1,628 (±240)	1,332 (±216)	NS
Sex			NS
Male (%)	7 (50)	15 (65)	
Female (%)	7 (50)	8 (35)	
Race			NS
White (%)	10 (71)	15 (65)	
Black (%)	2 (14)	3 (13)	
Other (%)	2 (14)	5 (22)	
Birth location			$p = 0.074$
Inborn (%)	12 (86)	12 (52)	
Outborn (%)	2 (14)	11 (48)	
Maternal parity	2.3 (±0.6)	1.9 (±0.2)	NS
Rupture of membranes >18 h (%)	2 (14)	6 (26)	NS
Antenatal steroids, at least 1 dose (%)	11 (79)	19 (83)	NS
Mode of delivery			NS
Vaginal (%)	10 (71)	12 (52)	
Cesarean (%)	4 (29)	11 (48)	
5-min APGAR score	7.8 (±0.5)	6.3 (±0.4)	$p = 0.0316$
Small for gestational age (%)	0 (0)	4 (17)	NS
Maternal Medicaid status (%)	9 (64)	9 (39)	NS
Antibiotics started at birth (%)	13 (93)	22 (96)	NS

Infants in the post-probiotic epoch had a significantly lower 5-min APGAR score compared to those in the pre-probiotic epoch. Otherwise, there were no statistically significant differences between the two epochs.

TABLE 2 | Practices for feeding and probiotic use for infants diagnosed with NEC before and after initiation of probiotic supplementation in 2015.

	Pre-probiotic epoch (2011–2015) *n* = 14	Post-probiotic epoch (2015–2020) *n* = 23	Significance
DOL feeds initiated	1.9 (±0.6)	1.8 (±0.7)	NS
DOL feeds reached 40 mL/kg/day	7.0 (±2.1)	11.6 (±3.5)	NS
Type of feeding			NS
Breast milk and/or donor milk (%)	7 (50)	13 (59)	
Breast milk + preterm formula (%)	6 (43)	8 (36)	
Preterm formula (%)	1 (7)	1 (5)	
DOL feeds fortified	8.1 (±1.9)	12.8 (±3.2)	NS
Feeding amount at fortification (mL/kg/day)	61.9 (±9.6)	56.2 (±10.0)	NS
Average enteral intake on day of diagnosis (mL/kg/day)	98.4 (±12.6)	85.4 (±14.0)	NS
Enteral intake 1 day prior to diagnosis (mL/kg/day)	94.3 (±12.0)	79.9 (±14.8)	NS
Enteral intake 2 days prior to diagnosis (mL/kg/day)	94.9 (±11.7)	77.7 (±15.3)	NS
Enteral intake 3 days prior to diagnosis (mL/kg/day)	86.8 (±11.7)	73.1 (±15.3)	NS
Average total fluid intake for 3 days prior to diagnosis (mL/kg/day)	129.4 (±3.0)	138.3 (±10.7)	NS
Probiotics administered (%)	0 (0)	15 (65)	$p < 0.0001$
DOL probiotics started	N/A	15.0 (±5.3)	N/A

There were no significant differences in feeding practices in the post-probiotic epoch compared to the pre-probiotic epoch. Significantly more infants in the post-probiotic epoch received probiotics.

infants developed Bell's stage ≥2 NEC, corresponding to an average NEC incidence of 2.1%. Rates of NEC by year during the post-probiotic epoch ranged from 0.7 to 3.9%. There was not a statistically significant difference in rates of NEC in the VLBW population between the pre-probiotic and post-probiotic epochs (**Figure 3A**). When a subgroup analysis was performed on only inborn VLBW infants, 8 infants developed NEC in the pre-probiotic epoch (1.5% average incidence with a yearly variance ranging from 0.8 to 2.1%.) and 8 infants developed NEC in the post-probiotic epoch (1.1% average incidence with a yearly variance ranging from 0.7 to 1.4%). These differences were not significantly different (**Table 3B**, **Figure 3C**).

During the pre-probiotic epoch, 4 ELBW infants developed Bell's stage ≥2 NEC (1.6% average incidence with yearly variance from 1.5 to 1.6%), and 14 infants developed NEC in the post-probiotic epoch (4.1% incidence with yearly variance from 1.6 to 6.7%). These differences were not statistically different (**Table 3A**, **Figure 3A**). When a subgroup analysis was performed on only inborn ELBW infants, 5 infants developed Bell's stage ≥2 NEC in the pre-probiotic epoch (2.0% average incidence with a yearly variance ranging from 1.5 to 3.2%.) and 7 infants developed NEC in the post-probiotic epoch (2.1% average incidence with a yearly variance ranging from 1.5 to 2.7%). These differences were not significantly different (**Table 3B**, **Figure 3C**).

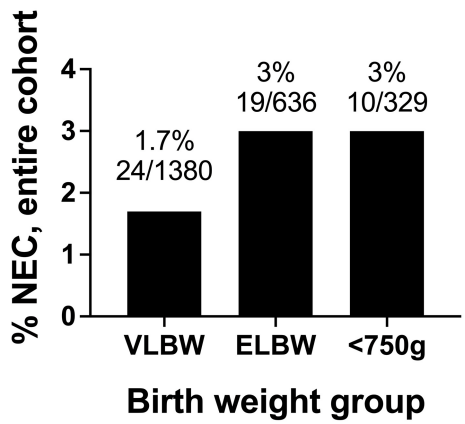

FIGURE 2 | Rates of modified Bell's stage ≥2a NEC from 2011 to 2019 based stratified by birth weight. In our cohort, the incidence of NEC was 1.7% in VLBW infants, 3% in ELBW infants, and 3% in infants with birthweights <750 grams.

TABLE 3 | Incidence of Bell's stage ≥2 NEC and all-cause pre-discharge mortality for infants before and after initiation of probiotic supplementation in 2015.

	Pre-probiotic epoch (2011–2014)	Post-probiotic epoch (2015–2019)	Significance
A: Inborn and outborn infants			
NEC cases (Bell's stage 2+), VLBW <1,500 g (%; range)	8 (1.5; 0.8–2.1)	16 (2.1; 0.7–3.9)	NS
NEC cases (Bell's stage 2+), ELBW <1,000 g (%; range)	4 (1.6; 1.5–1.6)	14 (4.1; 1.6–6.7)	$p = 0.090$
All-cause pre-discharge mortality (%; range)	43 (7.4; 5.3–9.9)	67 (8.4; 5.2–11.5)	NS
B: Inborn infants only			
NEC cases (Bell's stage 2+), VLBW <1,500 g (%; range)	8 (1.5; 0.8–2.1)	8 (1.1; 0.7–1.4)	NS
NEC cases (Bell's stage 2+), ELBW <1,000 g (%; range)	5 (2.0; 1.5–3.2)	7 (2.1; 1.5–2.7)	NS

Within the VLBW population, there were no significant differences in NEC or all-cause mortality (A). Upon a sub-group analysis of ELBW infants, a there was a trend toward a higher rate of Bell's >2a NEC in the post-probiotic epoch compared to the pre-probiotic epoch, but this did not reach statistical significance. There remained no statistically significant difference between the two epochs when only inborn infants were analyzed (B).

Rates of All-Cause Mortality for VLBW Infants

During the pre-probiotic epoch, there were 43 VLBW deaths from all-cause mortality, corresponding to an average all-cause mortality incidence of 7.4%. VLBW mortality rates by year during the pre-probiotic epoch ranged from 5.3 to 9.9%. During the post-probiotic epoch, there were 67 VLBW deaths from all-cause mortality, corresponding to an average all-cause mortality incidence of 8.4%. VLBW mortality rates by year during the post-probiotic epoch ranged from 5.2 to 11.5%. There was no significant difference between VLBW mortality rates between the pre-probiotic and post-probiotic epochs (**Table 3**, **Figure 3B**).

Risk Factors for Intestinal Illness

Data were collected regarding weight at diagnosis, DOL at diagnosis, PMA at diagnosis, clinical and culture positive early onset sepsis (EOS) and late onset sepsis (LOS), ratio of total stools to DOL of diagnosis, ratio of total number of glycerin suppositories received to DOL of diagnosis, rate of hemodynamically significant patent ductus arteriosus (hsPDA), modality of hsPDA treatment, manipulation of hsPDA within 3 days of diagnosis, and hydrocortisone or dexamethasone exposure within 3 days of diagnosis (**Table 4**). hsPDA was defined as a PDA that necessitated medical or surgical treatment (2011–2017) or by Iowa PDA score (2018–2019) (41). Stool and glycerin ratios were obtained by counting the total number of stools passed and the total number of glycerin suppositories received prior to the day of diagnosis and dividing by the number of days of life at time of diagnosis.

Risk factors for intestinal illness were overall similar between the infants with NEC in the pre-probiotic epoch compared to the post-probiotic epoch. There were non-significant trends toward a higher proportion of infants in the post-probiotic epoch compared to the pre-probiotic epoch who had clinical or culture positive EOS (68% vs. 36%) and who were diagnosed with a hsPDA (55% vs. 21%). However, modality of treatment and the proportion of infants who had manipulation of the hsPDA within

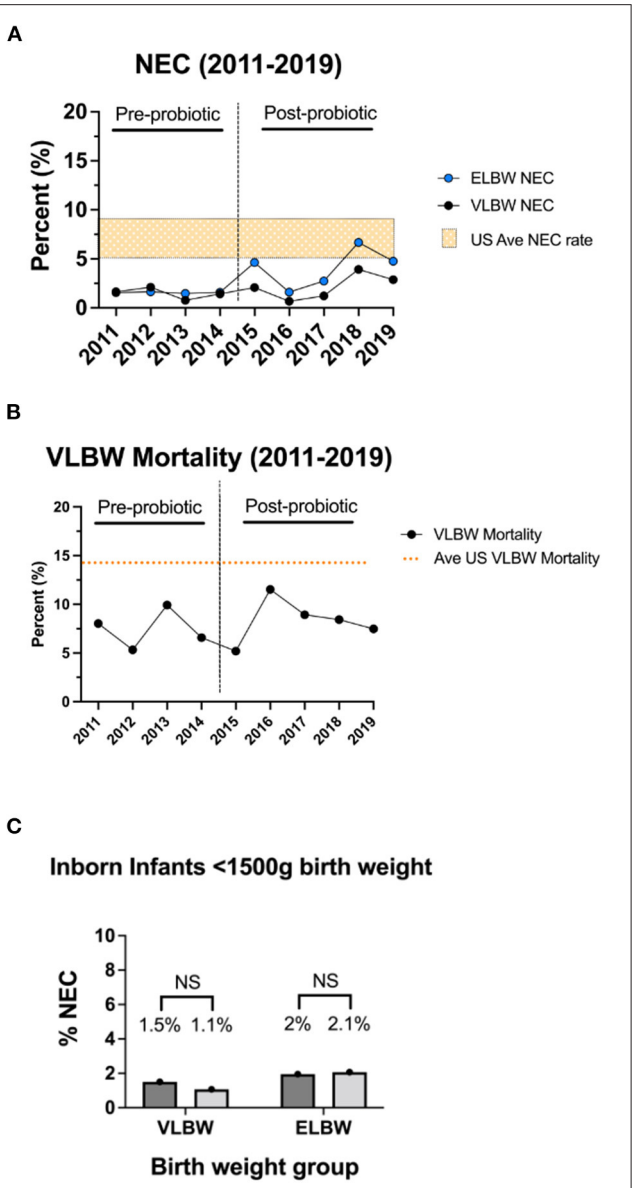

FIGURE 3 | Rates of modified Bell's stage ≥2a NEC and all-cause mortality by year. Rates of modified Bell's stage ≥2a NEC **(A)** and all-cause pre-discharge mortality **(B)** for infants in the pre-probiotic and post-probiotic epochs. For the VLBW population, routine administration of probiotics was not associated with a significant difference in rates of NEC or all-cause mortality. Rates are compared to US average data denoted by orange bar (NEC range, **A**) or orange line (VLBW mortality rate, **B**) (3, 40). Sub-group NEC analysis of ELBW infants demonstrated a trend toward a higher rate of NEC in the post-probiotic epoch, but this did not reach statistical significance. **(C)** Rates of NEC in VLBW and ELBW inborn only infants pre- and post-probiotic initiation showing no difference between the inborn cohorts.

TABLE 4 | Risk factors for intestinal illness in infants diagnosed with NEC before and after initiation of probiotic supplementation in 2015.

	Pre-probiotic epoch (2011–2015) $n = 14$	Post-probiotic epoch (2015–2020) $n = 23$	Significance
Weight at diagnosis (g)	1,778 (±237)	1,687 (±216)	NS
DOL at diagnosis	15.4 (±2.3)	21.9 (±4.4)	NS
PMA at diagnosis	32.8 (±1.1)	32.5 (±1.1)	NS
Early onset sepsis (clinical + culture positive) (%)	5 (36)	15 (68)	$p = 0.087$
Late onset sepsis (clinical + culture positive) (%)	9 (64)	14 (64)	NS
Total stools/day of life at diagnosis	2.4 (±0.3)	1.7 (±0.4)	NS
Total glycerins given/day of life at diagnosis	0.07 (±0.03)	0.08 (±0.03)	NS
hsPDA (%)	3 (21)	12 (55)	$p = 0.083$
Method of hsPDA treatment			NS
Acetaminophen (%)	1 (33)	5 (28)	
Ibuprofen (%)	1 (33)	1 (6)	
Indomethacin (%)	0 (0)	7 (39)	
Ligation (%)	1 (33)	3 (17)	
Catheter closure (%)	0 (33)	2 (11)	
Hydrocortisone or dexamethasone within 3 days of diagnosis (%)	0 (0)	4 (19)	NS

There were no significant differences between infants diagnosed with NEC in the post-probiotic epoch compared to the pre-probiotic epoch. There was a trend toward a higher rate of early onset sepsis and a higher percentage of infants with a diagnosis of hemodynamically significant patent ductus arteriosus (hsPDA) in the post-probiotic epoch compared to the pre-probiotic epoch, but these trends did not reach statistical significance.

TABLE 5 | Markers of illness severity in infants diagnosed with NEC before and after initiation of probiotics of probiotic supplementation in 2015.

	Pre-probiotic epoch (2011–2015) $n = 14$	Post-probiotic epoch (2015–2020) $n = 23$	Significance
WBC count on day of diagnosis	12.5 (±1.8)	12.8 (±2.3)	NS
WBC count 1 day after diagnosis	9.9 (±1.5)	11.2 (±2.8)	NS
Platelet count on day of diagnosis	324 (±28)	320 (±45)	NS
Platelet count 1 day after diagnosis	301 (±43)	264 (±52)	NS
CRP on day of diagnosis	1.3 (±1.0)	2.9 (±1.1)	NS
CRP 1 day after diagnosis	5.1 (±2.2)	5.5 (±1.1)	NS
Surgical management			
Surgery required (%)	6 (43)	12 (55)	NS
Drain placement only (%)	0 (0)	0 (0)	NS
Exploratory laparotomy (%)	4 (29)	9 (41)	
Both drain + laparotomy (%)	2 (14)	3 (14)	
Bowel resection (%)	7 (50)	10 (45)	NS
Days receiving TPN prior to discharge	39.0 (±6.3)	44.0 (±8.0)	NS

There were no significant differences in markers of illness severity in infants diagnosed with NEC in the post-probiotic epoch vs. in the pre-probiotic epoch.

3 days of diagnosis of NEC was unchanged between the two groups. When analysis was restricted to inborn only infants, there was a significant increase in rate of diagnosis of hsPDA in the infants with NEC in the post-probiotic epoch compared to the pre-probiotic epoch (67% vs. 17%; $p = 0.0361$).

Markers of Illness Severity

Data were collected regarding laboratory values (white blood cell [WBC] count, absolute neutrophil count [ANC], platelet count, and C-reactive protein [CRP]) on day of diagnosis and 1 day following diagnosis, need for inotrope or pressor therapy within 3 days of diagnosis, need for surgical management and type of surgical management if needed, total antibiotic days for the most common anti-microbial agents used in the first 30 days of life, and total days of total parenteral nutrition (TPN) for each case of NEC in the pre-probiotic and post-probiotic epochs (**Table 5**). Markers of illness severity were not significantly different between the infants with NEC in the pre-probiotic epoch and post-probiotic epoch.

DISCUSSION

We performed a single-center retrospective cohort study examining rates of modified Bell's stage ≥2a NEC and all-cause pre-discharge mortality before and after initiation of routine supplementation of a multispecies probiotic supplementation in 2015. We found no difference in rates of NEC or all-cause mortality in VLBW infants in the post-probiotic epoch (2015–2019) compared to the pre-probiotic epoch (2011–2014), which was inconsistent with our initial hypothesis. When examining only ELBW infants, while there was a trend toward a higher rate of NEC in the post-probiotic epoch, this difference was not significant. Compared to infants diagnosed with NEC in the pre-probiotic epoch, infants with NEC in the post-probiotic epoch did have a modestly significant lower 5 min APGAR score (6.3 vs. 7.8; $p = 0.0316$) as well as an increased rate of probiotic use (65% vs. 0%; $p < 0.0001$). The current estimate of the incidence of NEC in VLBW infants is ∼6–7% (2). This study makes it clear that the rate of NEC at our institution is well below average, as through consensus with our surgery colleagues, over a 9 year period, we have identified rates of NEC in VLBW infants of 0.7–3.9%. There are several possible factors that are contributing to our lack of efficacy for probiotic use to decrease our NEC incidence. First, NEC is a multifactorial disease and while probiotics have been shown to lower NEC rates, no study has taken NEC incidence to zero. Thus, it is possible that we did not see a decrease in the rate of NEC in the post-probiotic epoch because our institution may have a NEC rate lower than the threshold that can be impacted by probiotic use. Our overall average rate of NEC during both epochs combined (2011–2019) was 1.9% which is significantly below the national average and below the NEC rate in most probiotic publications (2, 42). Furthermore, if the rate of NEC in the year 2018 (3.9%) is removed as an outlier, NEC rates at our institution then range between 0.7 and 2.9%, with an overall NEC rate of 1.6% from 2011 to 2019. Other studies, some of which used the exact same formulation of probiotic that is used

at our institution, have shown a significant decrease in NEC rate with probiotic use; however, the baseline rates of NEC in these studies prior to the intervention were appreciably higher than baseline NEC rates at our institution, ranging from 4.4–9.8% (22, 23, 25). While these studies found significant decreases in NEC following probiotic use, the post-probiotic NEC rate ranged from 2.0 to 5.4%, which is the approximate average rate of NEC at our institution regardless of probiotic use (22, 23, 25).

Secondly, our institutional feeding guidelines, especially in the ELBW population, favor routinely slow and cautious feeding volume advances, rarely exceeding increases of more than 10–15 mL/kg/day. As rapid feeding advancements are considered a risk factor for NEC, this may play a role in our low incidence. Lastly, we have extremely high rates of maternal and donor breast milk use, with subsequent low use of bovine-origin formula as the base feed which has been shown to be associated with lower rates of development of NEC (43, 44).

Interestingly, in the sub-group analysis of only ELBW infants, there was a non-significant trend toward higher rates of NEC in the post-probiotic epoch compared to the pre-probiotic epoch (4.1% vs. 1.6%). While this did not reach statistical significance, a 2.5-fold increase may be of clinical significance. However, it is important to note that there was an increase in practice heterogeneity during this epoch as we had a large turnover of both neonatologists and nurses during this time frame, which were unrelated to the initiation of probiotic supplementation, but may have confounded our results.

Following our institution's initiation of routine probiotic supplementation, 65% of the infants who went on to develop NEC received probiotics at some point in their course, an increase from the pre-probiotic epoch (0%). There were several cases of NEC in term infants in the post-probiotic epoch, and since these infants do not receive probiotic supplementation routinely at our institution, the rate of probiotic use in the NEC population was lower than expected. When we only examined VLBW infants with NEC in the post-probiotic epoch, we found that the rate of probiotic use increased to 94%, and when we further restricted this analysis to the ELBW population, who are at highest risk for development of NEC, we found that the rate of probiotic use was 93%. There was a single infant who fell into both the VLBW and ELBW categories who did not receive probiotics. This infant was outborn and developed NEC at the hospital of birth; following transport to our facility the infant was critically unwell and passed away several weeks later due to complications from NEC and was never stable enough to receive probiotic supplementation. The rate of probiotic use was even more pronounced when analysis was restricted to inborn only VLBW infants, all of whom (100%) received probiotic supplementation at an average of 7 days of life.

The analysis of our data found a modestly significant lower average 5-min APGAR score in the infants with NEC in post-probiotic epoch compared to the pre-probiotic epoch (6 vs. 8). While a drop in APGAR score from 8 to 6 may be interpreted by some providers as substantial, we believe this is unlikely to be clinically significant. First, although many studies have attempted to link APGAR scores with long-term metrics, these studies were both unsuccessful and attempted to causally link APGAR scores with a long-term deficit such as lower intelligence or neurodevelopmental impairment (45). Second, there is significant inter-user variability in assigning APGAR scores (46) and this variability in and of itself may at least partially explain the difference we saw between the two groups. Third, although a lower 5 min APGAR score could indicate that the infants with NEC in the post-probiotic epoch were more ill at baseline, and therefore possibly at increased risk for complications such as NEC, we examined multiple other metrics of illness severity (**Table 5**) and found no significant differences between the infants with NEC in the two epochs. Similarly, the lower APGAR score could also be related to the non-significant trend toward a higher percentage of outborn infants who went on to develop NEC in the post-probiotic epoch compared to the pre-probiotic epoch (48% vs. 14%), since resuscitation of high risk and critically ill infants is often more difficult in lower-resource settings. Finally, APGAR scores are assigned in the minutes after an infant is born and therefore would not have been affected by later supplementation with probiotics, which did not occur until an average of DOL 7 for inborn infants and DOL 28 for outborn infants. There was also a trend toward a higher rate of EOS in infants with NEC in the post-probiotic epoch compared to the pre-probiotic epoch, which again may indicate that the infants with NEC in the post-probiotic epoch were more ill at baseline, but none of these cases were culture positive, and this did not reach statistical significance, making its actual effect on our study unclear.

Lastly, we noted a higher proportion of infants with NEC in the post-probiotic epoch compared to the pre-probiotic epoch who were diagnosed with hsPDA (55% vs. 21%). This finding is most likely due to diagnostic bias. In 2018, during the post-probiotic epoch, our institution implemented a neonatologist-performed targeted neonatal echocardiography (TnECHO) protocol for screening all infants born <27 weeks for hsPDA in the first 18–24 h of life. Prior to this, echocardiograms were only obtained in the postnatal period if there was clinical suspicion for hsPDA as opposed to universal screening. Because of this universal screening TnECHO protocol for hsPDA in premature infants at our institution, more cases of hsPDA were likely discovered and treated, contributing to this trend. When we restricted our analysis to inborn only infants, who were subject to this protocol, the proportion of infants with hsPDA who developed NEC was significantly higher in the post-probiotic epoch compared to the pre-probiotic epoch, confirming that these findings are likely due to diagnostic bias.

This study has several limitations. First, this study is retrospective in nature and prone to many confounding variables and biases for which we were not able to completely control. There continues to be considerable controversy regarding probiotic supplementation in preterm infants, and retrospective data is not nearly as convincing or definitive as randomized trials and prospective studies. Although some small single center trials and prospective studies involving probiotic supplementation have been successfully completed showing a positive benefit to risk ratio, the collective data is still mixed on whether probiotics provide definitive benefit to premature infants and currently the AAP does not recommend universal probiotic

supplementation to premature infants (31). Another limitation of this study is that it only includes data from a single center where approximately one third of infants are outborn and spend various periods of time at lower level NICUs prior to transfer to our institution, where there is substantial variation in clinical care from what we provide. However, as discussed above, even when analysis was restricted to inborn only infants, there still was not a significant reduction in our rate of NEC in the post-probiotic.

In conclusion, this study demonstrates that at our institution, routine supplementation of a multispecies probiotic had no effect on rates of modified Bell's stage ≥2a NEC or all-cause mortality. This contrasts with multiple other studies in the literature, some using the exact same probiotic strain, where there were significant reductions in these metrics. We hypothesize that these findings were likely due to a low baseline rate of NEC at our institution which fell below the threshold of probiotic efficacy. Additional large scale randomized control trials and prospective studies, in addition to improved standardization and regulation of probiotic strains by the FDA, are needed in order to definitively determine whether probiotics provide a conclusive benefit and importantly which strains and preparations are the most effective in reducing NEC and mortality in preterm infants.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Iowa Institutional Review Board (IRB#201410743). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

BJ and SM: conceptualization, manuscript preparation (original draft), statistical analysis, and funding. BJ, TB, and SM: methodology. BJ and TB: data collection. BJ, TB, GP, and SM: consensus on NEC cases and manuscript preparation (review). SM: supervision and project administration. All authors contributed to the article and approved the submitted version.

FUNDING

BJ was supported by grant funding NIH/NIAID T32AI007260. SM was supported by the Stead Family Department of Pediatrics.

ACKNOWLEDGMENTS

The authors would like to recognize and thank Dr. Erica Carlisle and Chelsea Pangburn, RN for their assistance reaching consensus on cases of NEC, as well as coordination of the study.

REFERENCES

1. Patel RM, Kandefer S, Walsh MC, Bell EF, Carlo WA, Laptook AR, et al. Causes and timing of death in extremely premature infants from 2000 through 2011. *N Engl J Med.* (2015) 372:331–40. doi: 10.1056/NEJMoa1403489
2. Alsaied A, Islam N, Thalib L. Global incidence of necrotizing enterocolitis: a systematic review and meta-analysis. *BMC Pediatr.* (2020) 20:344. doi: 10.1186/s12887-020-02231-5
3. Duchon J, Barbian ME, Denning PW. Necrotizing enterocolitis. *Clin Perinatol.* (2021) 48:229–50. doi: 10.1016/j.clp.2021.03.002
4. Fitzgibbons SC, Ching Y, Yu D, Carpenter J, Kenny M, Weldon C, et al. Mortality of necrotizing enterocolitis expressed by birth weight categories. *J Pediatr Surg.* (2009) 44:1075–76. doi: 10.1016/j.jpedsurg.2009.02.013
5. Neu J, Walker WA. Necrotizing enterocolitis. *N Engl J Med.* (2011) 364:255–64. doi: 10.1056/NEJMra1005408
6. Rees CM, Eaton S, Kiely EM, Wade AM, Mchugh K, Pierro A. Peritoneal drainage or laparotomy for neonatal bowel perforation? A randomized controlled trial. *Ann Surg.* (2008) 248:44–51. doi: 10.1097/SLA.0b013e318176bf81
7. Sola JE, Tepas JJ III, Koniaris LG. Peritoneal drainage versus laparotomy for necrotizing enterocolitis and intestinal perforation: a meta-analysis. *J Surg Res.* (2010) 161:95–100. doi: 10.1016/j.jss.2009.05.007
8. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. *Nature.* (2007) 449:804–10. doi: 10.1038/nature06244
9. Grönlund MM, Lehtonen OP, Eerola E, Kero P. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. *J Pediatr Gastroenterol Nutr.* (1999) 28:19–25. doi: 10.1097/00005176-199901000-00007
10. Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, Wagendorp AA, Klijn N, Bindels JG, et al. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr.* (2000) 30:61–7. doi: 10.1097/00005176-200001000-00019
11. Jakobsson HE, Jernberg C, Andersson AF, Sjölund-Karlsson M, Jansson JK, Engstrand L. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS ONE.* (2010) 5:e9836. doi: 10.1371/journal.pone.0009836
12. Alexander VN, Northrup V, Bizzarro MJ. Antibiotic exposure in the newborn intensive care unit and the risk of necrotizing enterocolitis. *J Pediatr.* (2011) 159:392–7. doi: 10.1016/j.jpeds.2011.02.035
13. Torrazza RM, Neu J. The altered gut microbiome and necrotizing enterocolitis. *Clin Perinatol.* (2013) 40:93–108. doi: 10.1016/j.clp.2012.12.009
14. Shao Y, Forster SC, Tsaliki E, Vervier K, Strang A, Simpson N, et al. Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. *Nature.* (2019) 574:117–21. doi: 10.1038/s41586-019-1560-1
15. Elgin TG, Kern SL, Mcelroy SJ. Development of the neonatal intestinal microbiome and its association with necrotizing enterocolitis. *Clin Ther.* (2016) 38:706–15. doi: 10.1016/j.clinthera.2016.01.005
16. Wang Y, Hoenig JD, Malin KJ, Qamar S, Petrof EO, Sun J, et al. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. *ISME J.* (2009) 3:944–54. doi: 10.1038/ismej.2009.37
17. Mai V, Young CM, Ukhanova M, Wang X, Sun Y, Casella G, et al. Fecal microbiota in premature infants prior to necrotizing enterocolitis. *PLoS ONE.* (2011) 6:e20647. doi: 10.1371/journal.pone.0020647
18. Morrow AL, Lagomarcino AJ, Schibler KR, Taft DH, Yu Z, Wang B, et al. Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants. *Microbiome.* (2013) 1:13. doi: 10.1186/2049-2618-1-13
19. Denning NL, Prince JM. Neonatal intestinal dysbiosis in necrotizing enterocolitis. *Mol Med.* (2018) 24:4. doi: 10.1186/s10020-018-0002-0

20. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol.* (2014) 11:506–14. doi: 10.1038/nrgastro.2014.66
21. Wang Q, Dong J, Zhu Y. Probiotic supplement reduces risk of necrotizing enterocolitis and mortality in preterm very low-birth-weight infants: an updated meta-analysis of 20 randomized, controlled trials. *J Pediatr Surg.* (2012) 47:241–8. doi: 10.1016/j.jpedsurg.2011.09.064
22. Jacobs SE, Tobin JM, Opie GF, Donath S, Tabrizi SN, Pirotta M, et al. Probiotic effects on late-onset sepsis in very preterm infants: a randomized controlled trial. *Pediatrics.* (2013) 132:1055–62. doi: 10.1542/peds.2013-1339
23. Janvier A, Malo J, Barrington KJ. Cohort study of probiotics in a North American neonatal intensive care unit. *J Pediatr.* (2014) 164:980–5. doi: 10.1016/j.jpeds.2013.11.025
24. Chang HY, Chen JH, Chang JH, Lin HC, Lin CY, Peng CC. Multiple strains probiotics appear to be the most effective probiotics in the prevention of necrotizing enterocolitis and mortality: an updated meta-analysis. *PLoS ONE.* (2017) 12:e0171579. doi: 10.1371/journal.pone.0171579
25. Robertson C, Savva GM, Clapuci R, Jones J, Maimouni H, Brown E, et al. Incidence of necrotising enterocolitis before and after introducing routine prophylactic *Lactobacillus* and *Bifidobacterium* probiotics. *Arch Dis Child Fetal Neonatal Ed.* (2020) 105:380–6. doi: 10.1136/archdischild-2019-317346
26. Chi C, Li C, Buys N, Wang W, Yin C, Sun J. Effects of probiotics in preterm infants: a network meta-analysis. *Pediatrics.* (2021) 147:e20200706. doi: 10.1542/peds.2020-0706
27. Kutylowksi J, Yahia N. Types, frequency, duration, and dosage of probiotics to prevent necrotizing enterocolitis in preterm infants among countries. *Adv Neonatal Care.* (2019) 19:188–97. doi: 10.1097/ANC.0000000000000605
28. Viswanathan S, Lau C, Akbari H, Hoyen C, Walsh MC. Survey and evidence based review of probiotics used in very low birth weight preterm infants within the United States. *J Perinatol.* (2016) 36:1106–11. doi: 10.1038/jp.2016.144
29. Szajewska H, Van Goudoever JB. To give or not to give probiotics to preterm infants. *Am J Clin Nutr.* (2014) 100:1411–2. doi: 10.3945/ajcn.114.099978
30. Lewis ZT, Shani G, Masarweh CF, Popovic M, Frese SA, Sela DA, et al. Validating bifidobacterial species and subspecies identity in commercial probiotic products. *Pediatr Res.* (2016) 79:445–52. doi: 10.1038/pr.2015.244
31. Poindexter B. Use of probiotics in preterm infants. *Pediatrics.* (2021) 47:e2021051485. doi: 10.1542/peds.2021-051485
32. Londono Calle YC, Diehl-Jones W, Heaman M, Chiu A, Friel J. Probiotic protocols used in neonatal intensive care units in the USA and Canada. *Paediatr Child Health.* (2019) 24:419–20. doi: 10.1093/pch/pxz077
33. O'callaghan A, Van Sinderen D. Bifidobacteria and their role as members of the human gut microbiota. *Front Microbiol.* (2016) 7:925. doi: 10.3389/fmicb.2016.00925
34. Patole SK, Rao SC, Keil AD, Nathan EA, Doherty DA, Simmer KN. Benefits of *Bifidobacterium breve* M-16V supplementation in preterm neonates - a retrospective cohort study. *PLoS ONE.* (2016) 11:e0150775. doi: 10.1371/journal.pone.0150775
35. Zhang Z, Lv J, Pan L, Zhang Y. Roles and applications of probiotic Lactobacillus strains. *Appl Microbiol Biotechnol.* (2018) 102:8135–43. doi: 10.1007/s00253-018-9217-9
36. Watkins PL, Dagle JM, Bell EF, Colaizy TT. Outcomes at 18 to 22 months of corrected age for infants born at 22 to 25 weeks of gestation in a center practicing active management. *J Pediatr.* (2020) 217:52–8.e51. doi: 10.1016/j.jpeds.2019.08.028
37. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)–a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform.* (2009) 42:377–81. doi: 10.1016/j.jbi.2008.08.010
38. Harris PA, Taylor R, Minor BL, Elliott V, Fernandez M, O'neal L, et al. The REDCap consortium: building an international community of software platform partners. *J Biomed Inform.* (2019) 95:103208. doi: 10.1016/j.jbi.2019.103208
39. Walsh MC, Kliegman RM. Necrotizing enterocolitis: treatment based on staging criteria. *Pediatr Clin North Am.* (1986) 33:179–201. doi: 10.1016/S0031-3955(16)34975-6
40. Horbar JD, Carpenter JH, Badger GJ, Kenny MJ, Soll RF, Morrow KA, et al. Mortality and neonatal morbidity among infants 501 to 1500 grams from 2000 to 2009. *Pediatrics.* (2012) 129:1019–26. doi: 10.1542/peds.2011-3028
41. Rios DR, Martins FF, El-Khuffash A, Weisz DE, Giesinger RE, Mcnamara PJ. Early role of the atrial-level communication in premature infants with patent ductus arteriosus. *J Am Soc Echocardiogr.* (2021) 34:423–32.e421. doi: 10.1016/j.echo.2020.11.008
42. Patel RM, Underwood MA. Probiotics and necrotizing enterocolitis. *Semin Pediatr Surg.* (2018) 27:39–46. doi: 10.1053/j.sempedsurg.2017.11.008
43. Battersby C, Longford N, Mandalia S, Costeloe K, Modi N. Incidence and enteral feed antecedents of severe neonatal necrotising enterocolitis across neonatal networks in England, 2012-13: a whole-population surveillance study. *Lancet Gastroenterol Hepatol.* (2017) 2:43–51. doi: 10.1016/S2468-1253(16)30117-0
44. Berkhout DJC, Klaassen P, Niemarkt HJ, De Boode WP, Cossey V, Van Goudoever JB, et al. Risk factors for necrotizing enterocolitis: a prospective multicenter case-control study. *Neonatology.* (2018) 114:277–84. doi: 10.1159/000489677
45. Montgomery KS. Apgar scores: examining the long-term significance. *J Perinat Educ.* (2000) 9:5–9. doi: 10.1624/105812400X87716
46. O'donnell CP, Kamlin CO, Davis PG, Carlin JB, Morley CJ. Interobserver variability of the 5-minute Apgar score. *J Pediatr.* (2006) 149:486–9. doi: 10.1016/j.jpeds.2006.05.040

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

frontiers in Pediatrics

ORIGINAL RESEARCH
published: 26 November 2021
doi: 10.3389/fped.2021.678254

Influence of Family Integrated Care on the Intestinal Microbiome of Preterm Infants With Necrotizing Enterocolitis and Enterostomy: A Preliminary Study

Mengyang Yang, Juan Du, Qin Yang, Wenyan Dou, Min Jiang† and Mingyan Hei*†*

Department of Neonatology, Neonatal Center, Beijing Children's Hospital, Capital Medical University, National Center of Children's Health, Beijing, China

The aim of this study was to investigate the influence of family integrated care (FICare) on the intestinal microbiome of preterm infants with necrotizing enterocolitis and enterostomy. This was a prospective pilot study at Beijing Children's Hospital. Premature infants with an enterostomy who met the enrollment criteria were divided into the 2-week FICare and non-FICare groups (non-randomly). We collected their fecal samples and subjected the intestinal microbiomes to 16S rRNA gene sequencing. Operational taxonomic units (OTU) were analyzed to assess the intestinal microbiome richness, and we then carried out α-diversity, β-diversity, and species clustering analyses and a linear discriminant analysis (LDA) effect size (LEfSe) analysis to identify the differences in the microbial communities between the two groups. There were 12 patients enrolled in the study (FICare, $n = 7$; non-FICare, $n = 5$). There were no significant between-group differences in demographic characteristics, or in the relative abundances of phyla and genera. The major bacterial phyla were Proteobacteria, Firmicutes, and Actinobacteria, and *Serratia*, *Enterococcus*, *Cronobacter*, and *Bifidobacterium* dominated at the genus level. The α-diversity analysis indicated that the intestinal flora was more diverse in the non-FICare group than the FICare group ($p < 0.05$). However, most of the other indicators did not suggest a difference between the two groups. There was a high proportion of shared OTUs between the two groups, and the PCoA and clustering analyses indicated that the two groups were difficult to distinguish, indicating that the intestinal microbiomes were relatively similar between the groups. In summary, short-term FICare had no significant positive effect on the establishment of intestinal flora diversity in premature infants with necrotizing enterocolitis and enterostomy. The trial was registered in the Chinese Clinical Trial Registry (ChiCTR-OPN-17011801).

Keywords: intestinal microbiome, family integrated care, preterm infants, necrotizing enterocolitis, enterostomy

OPEN ACCESS

Edited by:
Yuan Shi,
Children's Hospital of Chongqing Medical University, China

Reviewed by:
Janneke Dekker,
Leiden University, Netherlands
Hui Wu,
First Affiliated Hospital of Jilin University, China
Xiaoqing Chen,
Nanjing Hospital Affiliated to Nanjing Medical University, China

****Correspondence:***
Mingyan Hei
heimingyan@bch.com.cn
Min Jiang
jiangmin7496@sina.com

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: *09 March 2021*
Accepted: *28 October 2021*
Published: *26 November 2021*

Citation:
Yang M, Du J, Yang Q, Dou W, Jiang M and Hei M (2021) Influence of Family Integrated Care on the Intestinal Microbiome of Preterm Infants With Necrotizing Enterocolitis and Enterostomy: A Preliminary Study. Front. Pediatr. 9:678254. doi: 10.3389/fped.2021.678254

INTRODUCTION

The gut microbiota is a major factor influencing both health and disease (1). The composition and development of the microbiota during early life affects health into adulthood (2). Infants who need intensive care are usually nursed in high-sanitary incubators, receive antibiotics, have restricted breastmilk intake, and have limited contact with the mother's skin. These factors all affect the development of the gut microbiota (3). How to improve infants' intestinal flora in the neonatal intensive care unit (NICU) is a current research focus.

From 2014 to 2017, our research group conducted a multicenter prospective cluster-randomized controlled trial on the effect of family integrated care (FICare) on premature infants in China (4), and a study based on our series of FICare trial showed that FICare improved the richness and diversity of the intestinal microbiome in premature infants who didn't undergo surgery in the NICU and helped to establish a microbiome that was similar to that in term infants in the NICU who were breastfed and did not undergo surgery (5). As a novel model of NICU care, FICare allows the infant's parents to participate in the care of the infant in the NICU. A previous study showed that FICare resulted in a 25% increase in weight gain, an 80% increase in the rate of breastfeeding, a 25% decrease in parental stress, and a significant reduction in nosocomial infections and critical incident reports compared to the results in matched controls (6). FICare provides more mother-to-child contact and more breastfeeding opportunities for premature babies in their early lives, which could improve the intestinal microbiome of premature infants in the NICU.

Infants with necrotizing enterocolitis (NEC), spontaneous intestinal perforation, or intestinal atresia commonly require abdominal surgery and the creation of a small bowel enterostomy (7). These children face complex detrimental environmental factors, such as the interruption of the intestine and intraluminal contact with air via a stoma (8), so it is more difficult for normal intestinal flora to become established. There are few studies on intestinal flora issues in infants with enterostomy (9). To investigate the influence of FICare on the intestinal microbiome of newborn infants with NEC and enterostomy in the NICU, we collected fecal samples from neonates who underwent enterostomy owing to NEC, and subjected their intestinal microbiota to high-throughput sequencing.

METHODS

Ethical Approval

This prospective study was performed at Beijing Children's Hospital from January 2018 to January 2020. The trial was registered in the Chinese Clinical Trial Registry (ChiCTR-OPN-17011801) in 2017. The study was conducted according to the principles of the Declaration of Helsinki and its revisions, and ethical approval was obtained from the Medical Ethics Committee of Beijing Children's Hospital (#2017-106). All parents of the infants provided signed informed consent before participation.

Infants

Premature infants who were diagnosed with NEC and underwent enterostomy followed by a stay in the NICU at Beijing Children's Hospital were divided into the FICare group (case group) and the non-FICare group (control group). The trial inclusion criteria were as follows: (a) gestational age >28 and <35 weeks; (b) has begun enteral feeding and continuous increase in milk supply for >24 h; (c) stable vital signs (defined as not requiring any ventilation support nor inotropic agent, and the patients' HR/BP/R/SpO2 are in normal range) for >48 h; (d) the first diagnosis was neonatal necrotizing enterocolitis (NEC); and (e) with jejunostomy, ileostomy, or colostomy. The exclusion criteria were as follows: (a) need for invasive ventilation; (b) major congenital anomaly or metabolic disease; (c) family unwilling to commit to staying in the hospital for more than 6 hours per day;(d) lack of consent; (e) birth weight (BW) < 400 g; (f) parental social factors or language barriers, that affect the treatment of the infant; (g) possible discharge within 2 weeks; and (h) oral intestinal probiotics taken after birth. Among the 34 FICare patients with enterostomy, 7 patients were finally included in the FICare group and 5 patients were in the non-FICare group. The detailed enrollment flowchart was in **Figure 1**. We collected data on the infant characteristics (sex, gestational age, BW, age at the start of enteral feeding, age at NEC diagnosis, and age at enterostomy, rate of breastfeeding, duration of supplemental oxygen use, duration of antibiotic use, top three diagnoses except NEC, weight at discharge, and 5-min Apgar score) and maternal characteristics (age, antenatal steroid use, prolonged rupture of membranes >18 h, delivery mode, and major maternal medical complications) (**Table 1**).

FICare Implementation and Sample Collection

After the enterostomy surgery was finished, the newborns were admitted to the NICU. After their vital signs stabilized and antibiotics had been finished for 1 week, they were divided into the two groups. We had established a professional FICare team and transformed the wards accordingly. Only the mothers of FICare newborns were allowed to enter the NICU for direct infant care, but the education and coaching sessions were open to both parents. Site training included bundles of written protocols, printed parent education materials, printed staff training materials, an onsite FICare training workshop and hands-on training, and an all-site training meeting in Beijing. A total of 13 non-invasive care skills were taught to the parents by the FICare team, which included the six-step washing technique, positioning of newborn babies, diaper use and estimation of urine output, umbilical cord care, oral care, and kangaroo skin contact. Our team also guided the parents on recording the baby's general condition, body temperature, heart rate, weight, urine output, milk intake, vitality and mood. The NICU team provided practical support and the FICare implementation policy details (10). The FICare newborns were given FICare for 2 weeks. The parents in the non-FICare group did not receive the FICare training and their infants were provided with standard care.

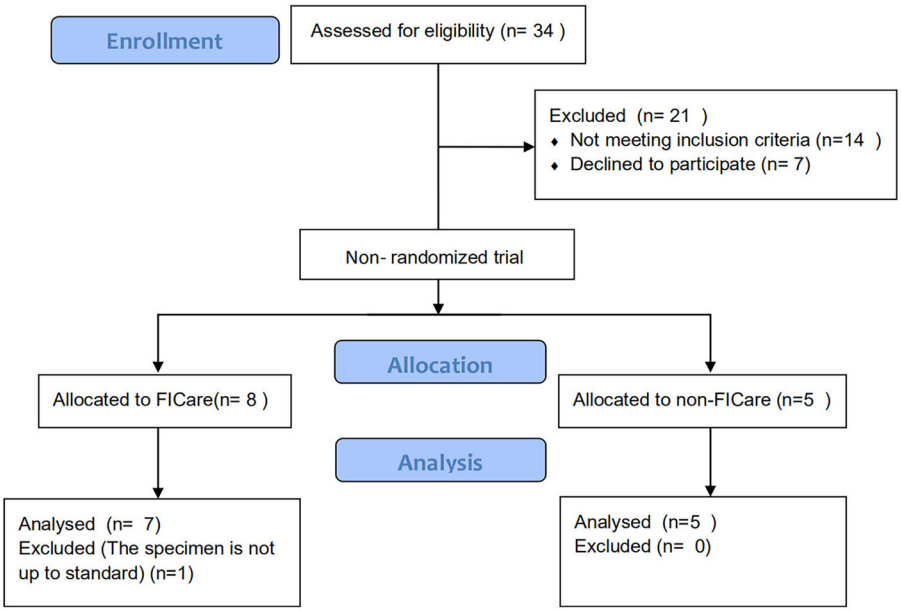

FIGURE 1 | Flowchart of patient enrollment.

After 2 weeks of FICare, fresh feces were collected from the infants in the FICare group over the following week (≥2 times, with intervals >24 h). Non-FICare group samples were collected at corresponding time points. A total of 38 samples were collected from the 12 premature NEC infants with enterostomy. Each tube containing a fecal sample was sealed with a sealing membrane and rapidly stored in a −80°C freezer for later analysis. All fecal samples were sent to a testing company for 16S rRNA high-throughput sequencing of the intestinal flora.

Data Processing and Analysis

After extracting the total DNA from the samples, we amplified the V4 region of the 16S rRNA gene by using polymerase chain reaction (PCR) with a 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primer pair, which had sequencing adapters attached to the ends. After amplification, the PCR products were subjected to purification, quantification, and homogenization to construct the sequencing library. Purified amplicons were pooled using equimolar concentrations and paired-end sequenced (2 × 251 bp) on an Illumina MiSeq platform (Illumina, San Diego, CA, USA)

MiSeq Reporter software v2.5.1.3 (Illumina, San Diego, CA, USA) with default parameters was used to split the raw sequencing data according to each sample's sequences, and to remove the sub and low-quality sequences. We obtained the V4 region assembly sequences after quality control and sequence splicing. i.e., the raw tags. To obtain high-quality tags, tags <225 or >280 bp were filtered out and chimeric sequences were removed using UCHIME v4.2 (11) (http://drive5.com/uchime).

After the Tags were optimized, an operational taxonomic unit (OTU) analysis was used to cluster the sequences at the 97% similarity level using UPARSE (singleton OTUs with an abundance of 1 in a single sample, which may represent very rare OTUs or may be caused by sequencing errors, were filtered out) (12). The species were annotated and a taxonomic analysis was carried out using the Silva 16S rRNA gene database, employing a confidence threshold of 80% (13). We used a Venn diagram to visualize the number of shared and unique OTUs in the two groups. Thereafter, based on the representative sequences in each OTU, α-diversity, β-diversity, and species clustering analyses were carried out. Regarding the α-diversity analysis, the species abundance indices (ACE and Chao), Simpson index, and Shannon index were used to evaluate the species abundances and diversity of the microbial communities. Regarding the β-diversity analysis, species diversity between samples was calculated using weighted and unweighted UniFrac approaches. Mothur software was used to conduct these analyses (14). Moreover, principal coordinate analysis (PCoA) results were plotted using the PCA command in Mothur, which was used to perform principal component analysis of the microbial flora in each sample to assess the similarity between the samples. Correlation analysis results were visualized using heatmaps. Furthermore, linear discriminant analysis (LDA) effect size (LEfSe) analysis (also known as biomarker analysis), which analyzes significant differences between groups, was used to estimate the impact of the abundance of each component (i.e., taxonomic group) on the difference between the two groups (15).

Statistical Analysis

Continuous data with normal distribution, we presented the data with mean (SD), and for data with non-normal distribution, we presented the data with median (IQR) are expressed as mean ± standard deviation (SD). If the data were normally distributed,

TABLE 1 | Baseline data: Infant demographics and maternal characteristics.

Characteristic	FICare (7)	Non-FICare (5)	*p* value
Male sex, *n* (%)	4 (57)	3 (60)	0.56
Gestational age, week, median (IQR)	31 (29.6–31.6)	32 (29.7–32.3)	0.45
Birth weight, g, mean (SD)	1510 (448.8)	1614 (502.2)	0.72
Singleton, n (%)	3 (43)	3 (60)	1
Cesarean delivery, *n* (%)	3 (43)	4 (80)	0.29
Apgar <7 at 5 min, *n* (%)	1 (14.3)	1 (20)	1
Prolonged rupture of membranes >18 h, n (%)	1 (14.3)	2 (40)	0.52
Maternal age, year, mean (SD)	30.3 (4.8)	29.2 (4.2)	0.69
Complete antenatal corticosteroids, *n* (%)	2 (29)	2 (40)	1
Breastfeeding, *n* (%)	6 (86)	2 (40)	0.20
Maternal complications, *n* (%)			
Hypertension	2 (29)	0	0.03
Gestational diabetes	0	1 (20)	0.42
Thyroid dysfunction	0	0	
(Suspected) sepsis	0	2	0.15
Main diagnosis of infants (except NEC and prematurity), *n* (%)			
Respiratory distress syndrome	7 (100)	5 (100)	-
Perinatal asphyxia	3 (43)	1 (20)	0.58
(Suspected) sepsis	0	1 (20)	0.42
Hypoglycemia	0	0	
Pneumonia	2 (29)	1 (20)	1
Age at the start of enteral feeding, d, median(IQR)	2 (2, 4)	2 (2, 8)	0.39
Age at NEC diagnosis, d, mean (SD)	14 (4)	12.6 (8.8)	0.72
Age at enterostomy, d, mean (SD)	13.3 (6.5)	17 (8.2)	0.40
Duration of supplemental oxygen, d, mean (SD)	28.4 (19.5)	17.8 (11.4)	0.30
Duration of antibiotic use, d, mean (SD)	17.6 (6.8)	10.8 (3.6)	0.07
Weight at discharge, g, mean (SD)	2155 (88.5)	2295 (178.6)	0.10

the two groups were compared using the independent-samples *t*-test. If the data were not normally distributed, the two groups were compared using the Mann–Whitney U test. Categorical data are expressed as frequency (%), and the chi-square test was used to compare the two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Participants

There were 12 infants who met our eligibility criteria and were enrolled in the study, comprising 7 in the FICare group and 5 in the non-FICare group. The demographic and clinical information on the newborns in the two groups is shown in **Table 1**. The majority of premature infants included in this study were born in our hospital, with a mean gestational age of about 30 weeks and a mean BW of about 1,500 g. There were no significant differences in demographic or clinical characteristics such as the proportion of male infants and the proportion with Apgar score <7 at 5 min. There was a significant difference in maternal hypertension, but not in the other complications. The age at NEC diagnosis was about 12 days for both groups (12 ± 5.3 vs. 12.6 ± 8.8, $P = 0.72$). In addition to the diagnosis of NEC, all the FICare and non-FICare preterm infants had a diagnosis of respiratory distress syndrome, while 43 and 20% of the FICare and non-FICare groups, respectively, had perinatal asphyxia ($p = 0.576$). The FICare group had a longer duration of supplemental oxygen use than the non-FICare group (28.4 ± 19.5 vs. 17.8 ± 11.4), a longer duration of antibiotic use (17.6 ± 6.8 vs. 10.8 ± 3.6), a younger age at enterostomy (15.3 ± 4.9 vs. 17 ± 8.2), and a higher breastfeeding rate (86 vs. 40%), but none of the differences were significant.

Relative Composition analysis

We collected 20 samples from the FICare group and 18 samples from the non-FICare group. Among them, 3 samples in the FICare group did not meet the required standards and were discarded. After processing, a total of 1,571,424 high-quality Tags were obtained from the 35 samples, with a mean of 44,897 per sample.

The 35 samples produced 119 OTUs. The minimum number of high-quality Tags in these samples was 11,347. The shared and distinct OTUs in the two groups were plotted in a Venn diagram (**Figure 2A**). The 50 OTUs were shared by the two groups The FICare group had 16 unique OTUs, while the non-FICare group had 53 unique OTUs. The abundance of OTUs preliminarily indicated the species richness of the samples. The results showed that the richness and diversity of intestinal flora was higher in the non-FICare group than the FICare group, but there were many shared OTUs in the groups.

PCoA is a flexible tool for analyzing microbiota composition data (16) that allows the overall effects of different treatments or environments on microbiota composition to be assessed. In PCoA plots, the dispersion or aggregation can reflect the differences in the gut microbiota in the two groups being compared based on the OTU composition (97% similarity) of the various samples. The results revealed no differences in microbiota composition between the two groups (**Figure 2B**).

The intestinal flora structure and relative bacterial abundances in the fecal samples are shown at the phylum level (**Figures 3A,C**) and genus level (**Figures 3B,D**). At the phylum level, the major bacteria in both groups were Proteobacteria, Firmicutes, and Actinobacteria. The most abundant phylum in both groups was Proteobacteria. There were no significant differences in relative abundance between the two groups at phylum level ($p > 0.05$) (**Table 2**). The proportion of Proteobacteria was higher in the FICare group (60.9 ± 9.68%) than the non-FICare group (48.41 ± 8.97%), and Proteobacteria and Firmicutes also accounted a higher proportion in the FICare group (90.9%) than in the non-FICare group (87.4%). The mean number of intestinal bacterial genera per infant in the FICare and non-FICare group was 13.6 ± 6.7 and 11.6 ± 5.9, respectively, but the difference was not significant ($p = 0.35$). At the genus level, *Serratia*, *Enterococcus*,

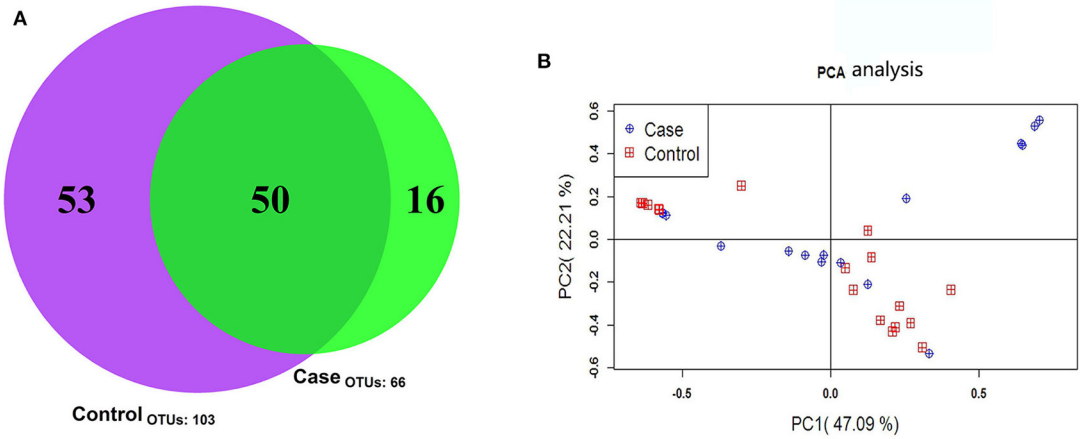

FIGURE 2 | (A) OTU Venn diagram **(B)** principal component analysis score plots of fecal microbial composition within the case and control group. The first and second component are shown on the x- and y-axis.

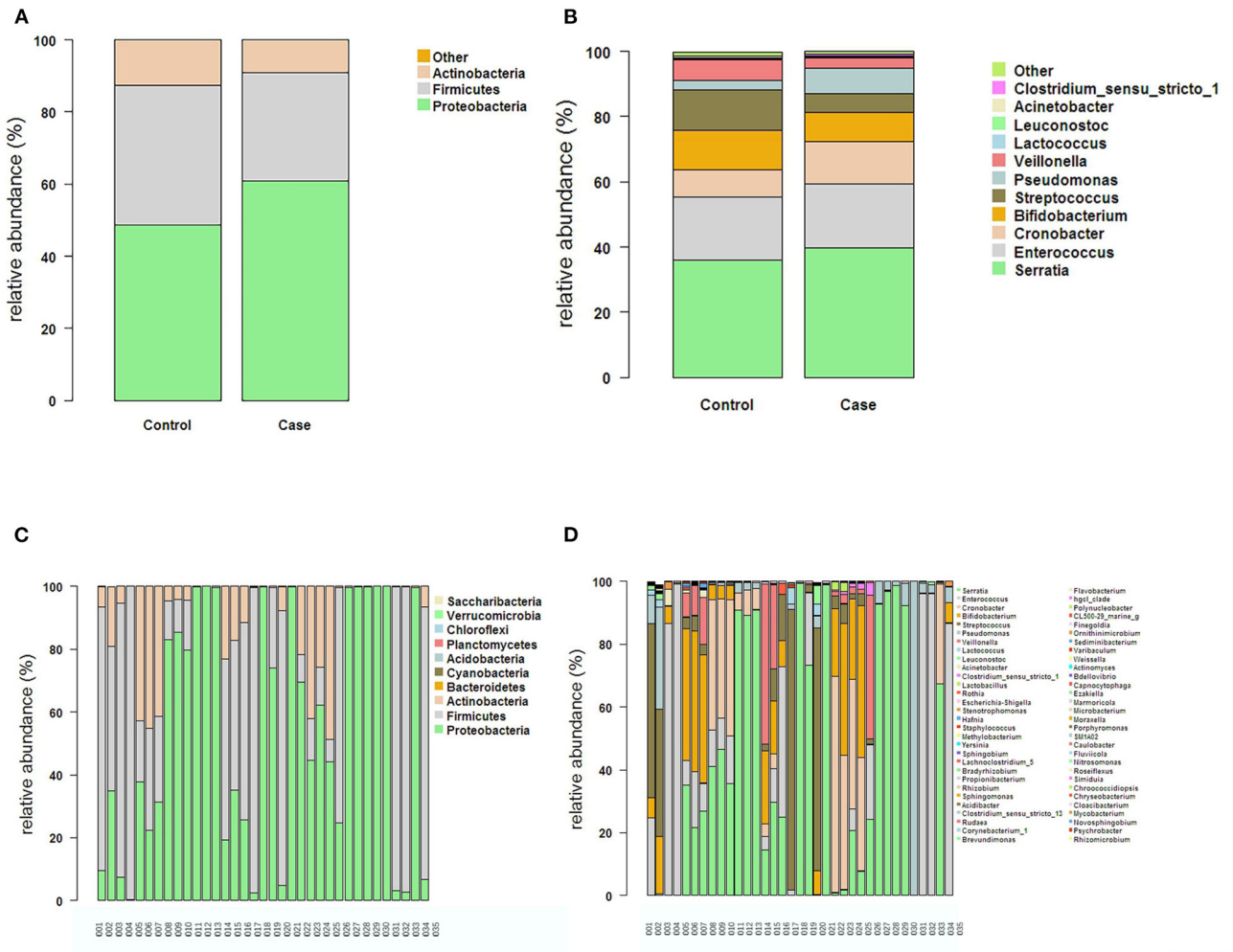

FIGURE 3 | The structure and relative abundance of the flora in the two groups at the Phylum level **(A)** and the genus level **(B)**. The structure and relative abundance in each sample at the phylum level **(C)** and the genus level **(D)**.

Cronobacter, and *Bifidobacterium* were dominant, accounting for >90% of the total. There were no significant differences in relative abundance at the genus level ($p > 0.05$) (**Table 3**). The proportion of *Bifidobacterium* was higher in the non-FICare group than the FICare group (5.05 vs. 0.05%), but the difference was not significant ($p = 0.195$).

After assessing the between-group differences in microbial abundances using statistical tests, a false discovery rate (FDR) analysis was used to further evaluate the significance of the differences, so as to identify the bacteria underlying the composition differences between the two groups. We attempted to identify the significant differences between the groups at both the phylum and genus levels. No significant differences were found between the two groups at the phylum level. At the genus level, there were 7 genera with significant differences in abundance between the two groups based on the p values ($p < 0.05$) (**Table 4**). Based on the FDR analysis, four bacteria were found to exhibit significant between-group differences in the proportions based on the q values ($q < 0.05$), comprising *Clostridium_sensu_stricto_13*, *Rudaea*, *Hafnia*, and *Clostridium_sensu_stricto_1*. However, the proportions of these four bacteria in the two groups were all <1%.

LEfSe Analysis

LEfSe analysis can be used to demonstrate different gut microbiota structures between two groups and aid in identifying significantly differential biomarkers between groups (15). LEfSe uses LDA to estimate the influence of the abundance of each component (i.e., taxonomic group) on the difference between groups. The figure generated in the LEfSe analysis (**Figure 4A**) shows the taxonomic groups with the largest differences between the two groups at various levels. The histogram (**Figure 4B**) shows the differences in 8 phylotypes between the two groups. At the genus level, the LEfSe analysis (**Table 5**) showed that 4 bacteria could be used as biomarkers to distinguish the two groups. In the FICare group, Clostridia (class level) could be used as a biomarker, which is consistent with the FDR analysis results (*Clostridium_sensu_stricto_1* belongs to Clostridia). While in the non-FICare group, *Enterobacteriaceae*, *Moraxellaceae*, and *Staphylococcaceae* (different families) could be used as biomarkers. Which has two more bacteria than the FDR analysis (*Hafnia* belongs to *Enterobacteriaceae*).

Species Clustering Analysis

Figure 5 shows a heatmap of the species clustering analysis results at the genus level (top 20 species), which reflects the similarities and differences in community composition among the samples. In the dendrogram above the heatmap, the shorter the branch length, the more similar the species composition and abundance between samples. The heatmap indicates the species abundances in the various samples and the similarities in species-specific abundances in the various samples. There was little difference between the FICare and non-FICare groups, but there were differences to a certain extent in the composition within the FICare and non-FICare group, respectively.

β-Diversity Analysis

The β-diversity of the gut microbiota in the two groups was assessed, which involved comparing species diversity between

TABLE 2 | Proportions of intestinal flora phyla in fecal samples in the two groups.

Group	Proteobacteria, mean ± SD	Firmicutes, mean ± SD	Actinobacteria, mean ± SD
FICare	60.92 ± 9.67	30.02 ± 9.65	9.06 ± 3.8
Non-FICare	48.57 ± 8.95	38.86 ± 8.30	12.54 ± 3.71
p value	0.33	0.46	0.53

TABLE 4 | False discovery rate (FDR) analysis of differences in abundances between the two groups.

Genus	Non-FICare		FICare		*p* value	*q* value
	Mean	SD	Mean	SD		
Clostridium_sensu_stricto_13	0.000069	0.000048	0	0	0.000131	0.003825
Rudaea	0.000069	0.000069	0	0	0.000131	0.003825
Hafnia	0.001783	0.000987	0	0	0.002997	0.043758
Clostridium_sensu_stricto_1	0	0	0.005179	0.002587	0.002997	0.043758
Yersinia	0.000304	0.000154	0.000005	0.000005	0.004995	0.058344
Acinetobacter	0.005427	0.00308	0.000223	0.000109	0.007992	0.077792
Staphylococcus	0.001439	0.000565	0.000135	0.000098	0.011988	0.100019

TABLE 3 | Proportions of intestinal flora genera in fecal samples in the two groups.

Group	*Serratia*, mean ± SD	*Enterococcus*, mean ± SD	*Cronobacter*, mean ± SD	*Bifidobacterium**	*Pseudomonas**	*Streptococcus**	*Veillonella**
FICare (7)	39.71 ± 10.52	19.61 ± 8.69	13.02 ± 5.31	0.05 (0,14.61)	1.3 (0.095,4.415)	0.24 (0,2.985)	0.01 (0.005,1.35)
Non -FICare (5)	35.91 ± 8.16	19.28 ± 6.90	8.43 ± 3.59	5.05 (0.025,19.5825)	0.28 (0.0175,2.0375)	1.215 (0.1,11.215)	0.005 (0,8.0925)
p value	0.777	0.976	0.474	0.195	0.134	0.134	0.443

**Expressed as median (lower quartile, upper quartile) and analyzed using non-parametric tests.*

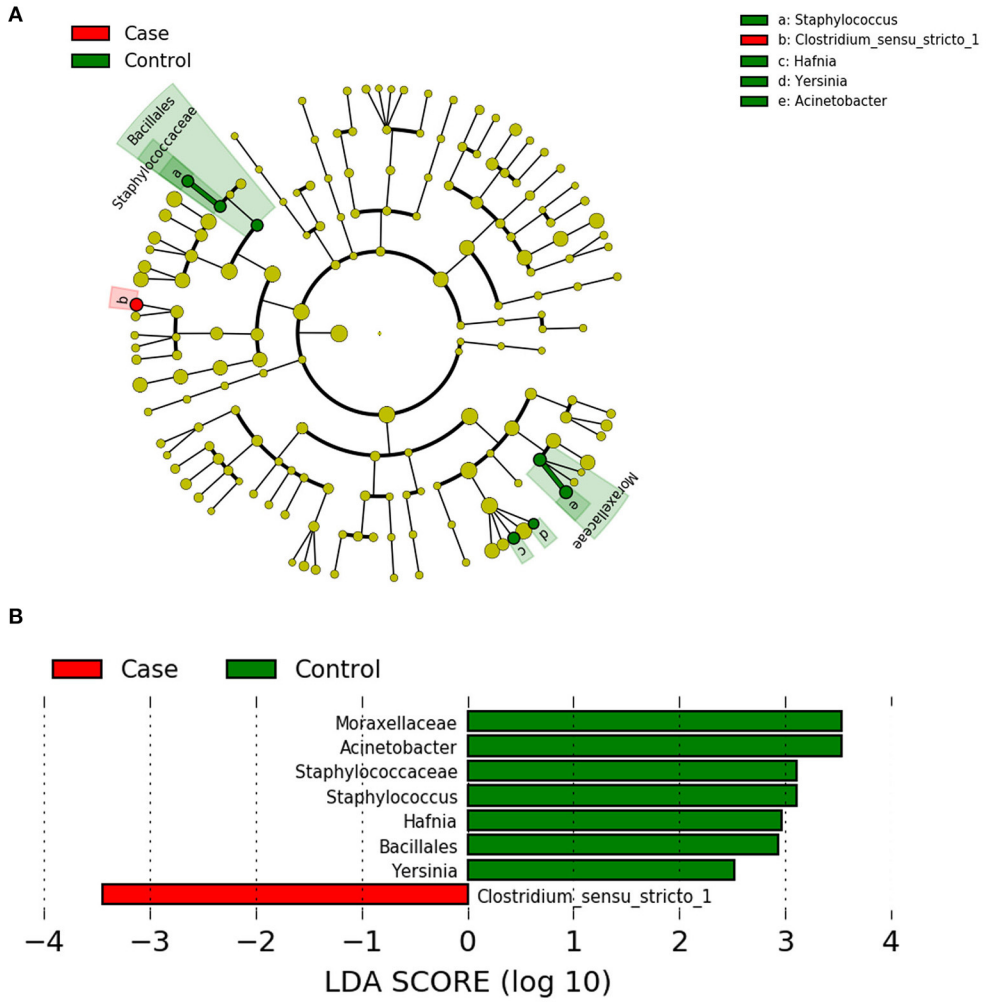

FIGURE 4 | (A) LEfSe cladogram, the diameter of each circle is proportional to the abundance. **(B)** Histogram of the LDA scores for diferentially abundant features among groups.

each pair of samples. UniFrac uses phylogenetic information to compare community differences between samples, which takes into account the evolutionary distance between species, the difference is represented by the 0-1 distance value, the larger the value, the greater the difference between samples; samples from the same environment are more likely to concentrate at the same node in the evolutionary tree, that is, the branch length between them is short and the similarity is high, so the results can be interpreted as an index of β-diversity.

The clustered UniFrac results (**Figure 6**), included weighted UniFrac, which takes into account sequence abundance) and unweighted UniFrac results, which only considers whether the sequence is present in the community, not the abundance of the sequence. In the unweighted UniFrac heatmap, many values were close to 1, which indicates that there was a certain difference between the pairs of samples. There may be great differences in microbial community structure between different samples from the same individual. In the weighted UniFrac heatmap, there were many lower values, the results revealed that species similarity was high between the pairs of samples.

Based on the β-diversity results, we carried out dendrogram clustering analyses and calculated the distances between the samples to assess the similarity of species composition. The results are shown in **Figure 7**. The shorter the branch length in the dendrogram, the closer the samples, indicating similar species composition. The results confirmed that the two groups were similar with small differences.

α-Diversity Analysis

The α-diversity can reflect the species diversity within a single group. Observed OTUs, Chao, and abundance-based coverage estimator (ACE), which reflect species richness, did not significantly differ between groups (**Table 6**). The rarefaction curves, with distinct asymptotes, are presented in **Figure 8**, which indicates near-complete sampling of the microbial communities. Good's coverage was 99.96 ± 0.02 for the observed OTUs.

TABLE 5 | LDA effect sizes.

Feature	Logarithm value	Group	LDA score	*p* value
Bacteria.Firmicutes.Bacilli.Bacillales	3.2233	Non-FICare	2.9327	0.0283
Bacteria.Firmicutes.Bacilli.Bacillales.Staphylococcaceae	3.1580	Non-FICare	3.1052	0.0088
Bacteria.Firmicutes.Bacilli.Bacillales.Staphylococcaceae.Staphylococcus	3.1580	Non-FICare	3.1033	0.0088
Bacteria.Firmicutes.Clostridia.Clostridiales.Clostridiaceae_1.Clostridium_sensu_stricto_1	3.7140	FICare	3.4468	0.0067
Bacteria.Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae.Hafnia	3.2512	Non-FICare	2.9684	0.0023
Bacteria.Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae.Yersinia	2.4929	Non-FICare	2.5225	0.0077
Bacteria.Proteobacteria.Gammaproteobacteria.Pseudomonadales.Moraxellaceae	3.7364	Non-FICare	3.5304	0.0229
Bacteria.Proteobacteria.Gammaproteobacteria.Pseudomonadales.Moraxellaceae.Acinetobacter	3.7351	Non-FICare	3.5293	0.0239

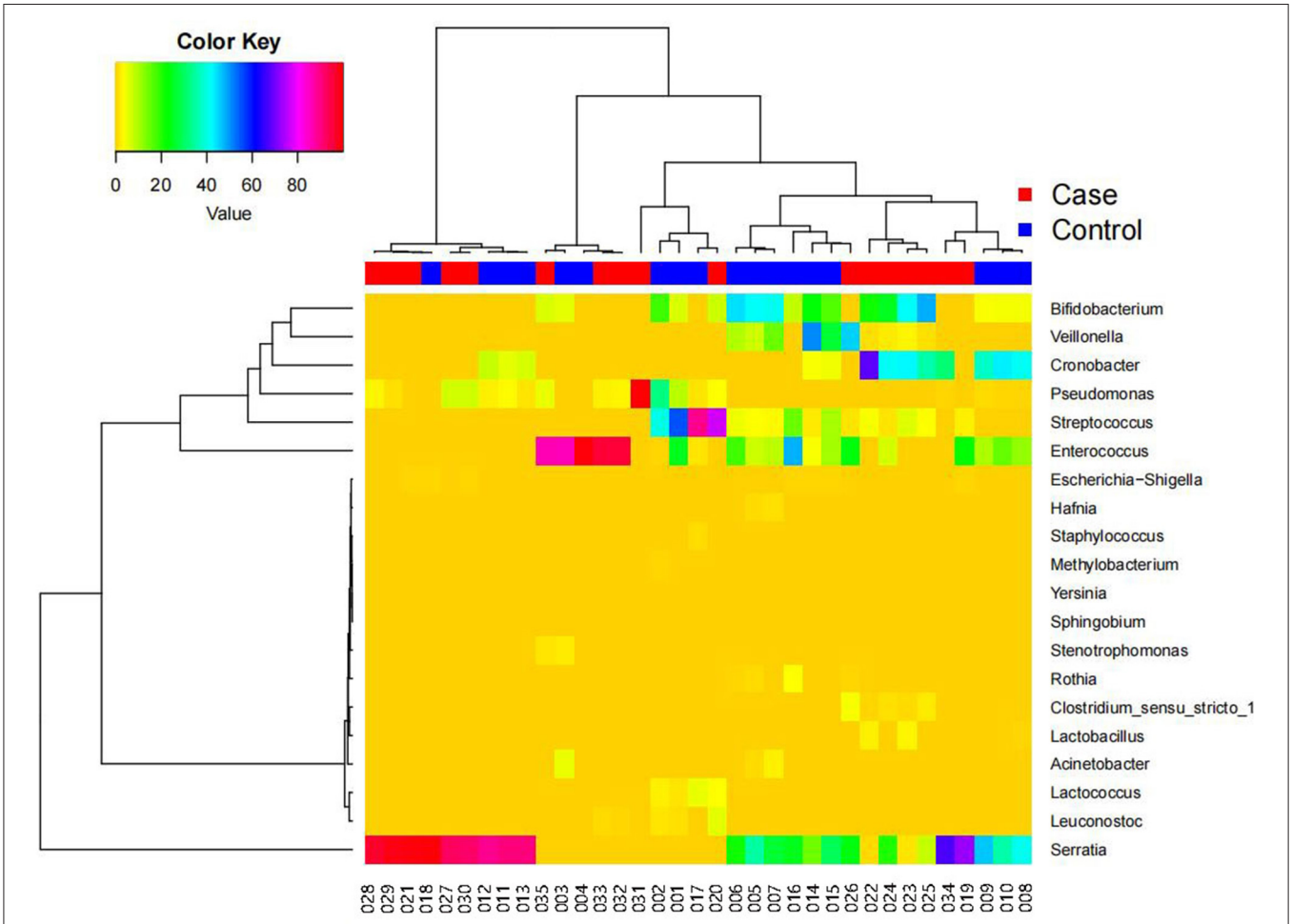

FIGURE 5 | The heat map between bacterial community in two groups at the genus level.

Shannon index and Simpson index reflect the diversity of microorganisms in each sample, which is affected by species richness and evenness. The Shannon index is more sensitive to the richness of the community and the rare OTU, and is more suitable for complex communities; while the Simpson index is more sensitive to the uniformity and the dominant OTU in the community, and is more suitable for simple communities. Shannon index in the non-FICare group was significantly higher than that in the FICare group (0.99 ± 0.57 vs. 0.62 ± 0.20, P =0.045) (**Table 6**), indicating that species richness in the non-FICare group was higher and also consistent with LEfSe results. Simpson index was higher in the FICare group (0.52 ± 0.28 vs. 0.70 ± 0.26, *P* =0.045) (**Table 6**). Both of them indicated a significantly greater diversity in the non-FICare group.

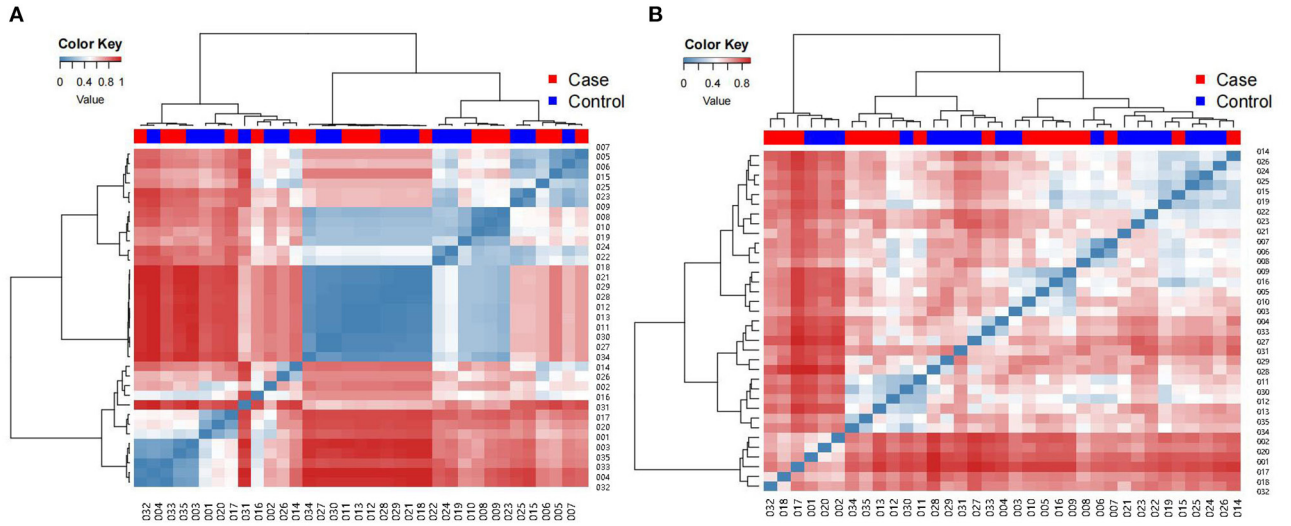

FIGURE 6 | Beta diversity heat map **(A)** is weighted, **(B)** is unweighted.

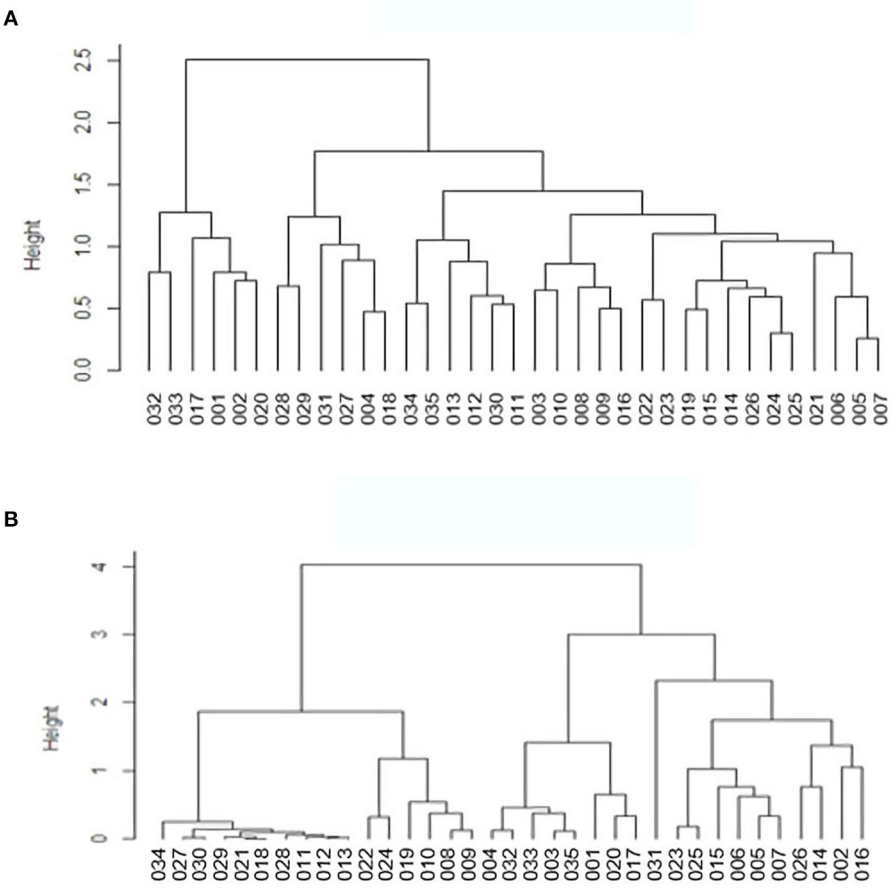

FIGURE 7 | Cluster analysis diagram **(A)** is unweighted, **(B)** is weighted.

DISCUSSION

In this prospective study on the influence of 2 weeks of FICare on the intestinal flora of preterm NEC infants with enterostomy, there was a significant difference in α-diversity between the FICare and non-FICare groups, with higher α-diversity in the non-FICare group. The other indicators did not exhibit significant differences between the two groups. There was a high proportion of shared OTUs in the two groups, and the PCA and clustering analyses could not significantly distinguish the two groups. These findings might have indicated that the intestinal microbes of the newborns in the two groups were relatively similar. However, though the differences were not statistically significant, they could be clinically relevant and influence the outcomes, because the sample size was too small.

FICare (17) is considered a highly comprehensive parent-involved care model for NICU care, and it can promote parental empowerment, learning, shared decision making, and positive parent–infant caregiving experiences. Emerging evidence (18–20) indicates that FICare is a promising intervention for infants and families that could improve outcomes for preterm infants and provide effective parental support. Our previous multicenter prospective cluster-randomized controlled trial demonstrated that FICare (4) is feasible in China and positively influenced the duration of hospitalization, cost of care, duration of supplemental oxygen use, infection, antibiotic use, infant growth, and breastfeeding rate. More research on the effectiveness of FICare is currently underway (21, 22). However, there have been few studies on the care of infants undergoing surgery (23) and there has been no research on whether FICare can improve the gut microbiota of these infants. One study found that two-weeks' FICare can improve the intestinal flora diversity of non-surgical preterm NICU infants (5). Unlike that study, our study indicates that short-term FICare did not exhibit clinical efficacy regarding the intestinal flora diversity for preterm NEC infants with enterostomy in the NICU. The reasons may include the long time required for intestinal flora establishment in premature neonates (24) and the more severe disease that newborns undergoing surgery often have, which necessitates more time for gut flora restoration, so changes may take longer than the 2 weeks in our study. There remains a lack of knowledge about optimum care for preterm infants with enterostomy. As the enrolled neonates had severe health conditions, which often changed rapidly, they required more high-quality care to protect their fragile intestinal flora. Inexperienced parents usually cannot rapidly identify these changes, so they require longer training and education in order

TABLE 6 | Alpha diversity statistical results.

Index	Non-FICare, mean ± SD	FICare, mean ± SD	*p* value (KW)
Good's coverage	0.9997	0.9996	-
Observed OTUs	18.94 ± 13.07	14.82 ± 8.62	0.305
Chao	21.79 ± 14.52	20.78 ± 13.52	0.882
ACE	26.60 ± 15.24	22.92 ± 12.17	0.843
Simpson	0.52 ± 0.28	0.70 ± 0.26	0.045
Shannon	0.99 ± 0.57	0.62 ± 0.20	0.045

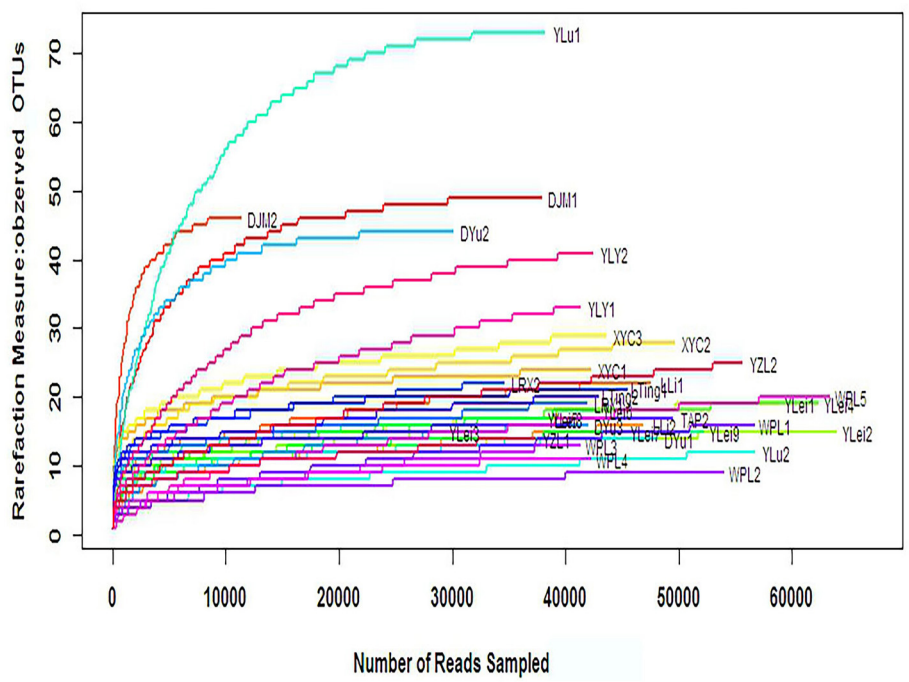

FIGURE 8 | The rarefaction curve of samples.

to provide appropriate care. This may have contributed to the slightly better diversity in the non-FICare group than in the FICare group. Nevertheless, most infants undergoing surgery also need long-term out-of-hospital care, and it is better for their parents to adapt to the care role early. FICare allowed close contact between the mother and infant, and provided time and space for the mother to breastfeed the neonate in the NICU. The influence of moving from the NICU to home on the intestinal flora can be reduced by allowing long-term mother-to-child contact in the NICU. Due to changes in the environment, handling, feeding, and treatment regimens, the change in the care model, could also bring about multiple effects that could enhance the microbial transmission to neonates. How these factors affect the development of the intestinal flora needs further research. Moreover, we should also pay attention to the longer-term health management of these infants after discharge. Long-term follow-up and investigation of other types of indicators (feeding, growth and development, defecation, and other diseases) and the detailed changes in the intestinal flora are needed to fully assess the effectiveness of FICare. Based on the effects of FICare demonstrated in our previous research (4, 5), it is worthwhile to further research whether FICare is beneficial.

In healthy neonates or preterm infants with mild disease, the intestinal flora is dominated by the genus *Lactobacillus* in the phylum *Firmicutes* (25). Research on mouse models of acute traumatic brain injury indicated that *Bifidobacterium* and *Lactobacillus* improved the electrophysiological peristalsis function of small intestinal smooth muscle during acute traumatic brain injury via the PKC/MLCK/MLC signaling pathway (26), which improved intestinal function. However, data on the extent to which surgical interruption of the intestine affects the developing intestinal microbiota are rare (9). In our study of NEC infants with enterostomy, the intestinal flora was dominated by Proteobacteria, which is one of the biggest bacterial phyla and comprises Gram-negative bacilli, including many pathogenic bacteria. At the genus level, *Serratia*, *Enterococcus*, and *Cronobacter* accounted for the majority of the bacteria. This is partly consistent with a study by Younge et al. (27), who observed a relative increase in many genera in the Enterobacteriaceae family (including *Serratia*, *Escherichia*, *Pantoea*, and *Citrobacter*) in infants with enterostomy. Bacteria in this family can induce potent host inflammatory responses and are frequent invasive pathogens in premature infants and infants with short bowel syndrome (28–30). Several studies have reported that the high level of oxygen in the newborn gastrointestinal (GI) tract favors facultative anaerobes (e.g., *Enterobacteriaceae*, *Enterococcus*, and *Streptococcus*) (31, 32). Theoretically, the basic conditions of the infants, antibiotic use, and the surrounding environment may underlie any differences in the neonatal intestinal flora between the FICare and non-FICare groups. The causality and effect size of various factors need to be assessed in the future. The enrolled newborns may need a longer time to develop normal intestinal flora. Whether oral probiotics, such as *Bifidobacterium* and *Lactobacillus*, can improve their intestinal flora also needs more research.

The intestinal microbiome has an essential role in intestinal function, including nutrient absorption, metabolism, maintenance of barrier integrity, and protection against infection (33, 34). Caporaso et al. (35) found that the human microbiome exhibited a phenomenon known as adaptation, involving changes in response to environmental factors (such as diet, intestinal transit time, and drug use) followed by changes back to the original composition. Research on laboratory rats has shown that the living environment has a profound effect on the development of the immune system and changed the nature and distribution of immune cells (36). Early establishment of normal intestinal flora is needed for every infant, but preterm infants, who have an immature self-adjustment ability, are more easily affected by external factors. These infants have a higher chance of developing a flora that reflects the NICU, due to the immaturity of their GIs and prolonged exposure to the NICU (37). Infants who have undergone surgical enterostomy placement often have multiple predisposing factors that can perturb the intestinal microbiome, including premature birth, history of bowel injury or perforation, antibiotic exposure, prolonged withholding of enteral feeds, and intestinal surgery (38). Infants requiring intensive care are usually nursed in high-sanitary incubators and have restricted breastmilk intake and limited contact with their mother's skin (3). In addition, infants undergoing surgery of the GI tract commonly require some period of fasting, and often the use of gastric acid suppressants (39). These factors can cause early life dysbiosis, involving a delayed and suboptimal colonization of the intestine, which has been associated with long-term health conditions in adulthood (40). Research on the effects of intestinal surgery in neonates on longer-term gut microbiome changes is rare. However, Younge et al. (27) reported an overall decline over time in bacterial diversity during their 9 weeks' study of premature infants with enterostomy. In pathological conditions such as NEC, the diversity of the intestinal flora is impaired, resulting in an obvious trend toward simplification of the flora (41). A study (5) in our series of studies on FICare demonstrated that FICare can improve the richness and diversity of the intestinal microbiome in premature infants who didn't undergo surgery, with the establishment of intestinal flora that was close to that of term infants in the NICU who were breastfed and did not undergo surgery. In our study, the types of intestinal flora in the two groups were significantly different from those of normal children (25). Further study is needed to determine whether there is a difference in the established intestinal flora between children who underwent surgery and normal children.

The diversity of the intestinal flora can be an indicator of health status. However, we also found that multiple samples from the same individual exhibited great differences in microbial components and community structure. Therefore, it is crucial to be aware that the microbiota composition in the period after birth can be highly variable within an individual, and there are also enormous inter-individual differences in the colonization dynamics regarding the infant intestine (2, 42). There are no standardized definitions of the composition of a "healthy" GI microbiota at different developmental stages, and little is known about the main factors that contribute to the establishment of the GI microbiota in early life (25). GI microbial composition and species abundance in infants are affected by many variables

that can directly or indirectly perturb the microbial community throughout the growth periods. The geographical region, host genetic factors, hygiene level of healthcare providers, mix-feeding (breastmilk and formula milk), brands and content of formula milk consumed, and other inter-individual variables are likely to contribute to GI microbiota development (43). One study (44) found that both the preterm infant microbiome and metabolome were highly specific to each individual and not associated with the infants' health conditions, and that the gut microbiome of preterm infants, who tend to be frequently treated with antibiotics, is enriched in microbes that commonly dominate after antibiotic exposure. Therefore, an individualized approach will be important to disentangle the health consequences of preterm infants' microbiomes.

It is important to note that the changes that we observed in the microbiome occurred in the context of the premature infant gut. Premature infants are still undergoing rapid intestinal growth and development, as well as maturation of the immune system. In addition, the infants were recovering from major intestinal injury and undergoing functional adaptation after loss of bowel length. It is possible that FICare may have differential effects on the microbiome and host at different stages of development. Given that the microbiome can vary significantly between individuals despite being in similar environments, and that some effects may take longer to occur than the length of this study, a larger, multicenter, longer-term study is necessary to explore the differences between FICare and non-FICare infants and to further examine the influence of confounding variables on the microbiome.

Limitations of this study were: (1) The sample size was small. It is very difficult to make a conclusion from a trial with such a small sample size. However, this was a pilot study for a group of specific preterm infants, who had enterostomy due to NEC with or without FICare during their recovering stage. Unfortunately, FICare is so far not a routine practice in NICUs in China yet, making the sample size even smaller. (2) The exposure time of our study might be too short to have detectable changes at later age (3–4 weeks postnatal) when stool microbiome is slower to change. Further studies are planned to follow these infants for their gut microbiome changes till their 1year corrected age. (3) Non-random allocation of the patients. Due to the ethical considerations, we have no way to randomize the eligible patients into FICare or non-FICare group, instead, we had to allocated the patients into 2 groups mainly based on their parents' willingness and choices. (4) The negative results of this study. As the first research to explore the feasibility and meaning of FICare for gut microbiome of NEC infants with enterostomy, we consider our study be a good try with fairly good novelty.

CONCLUSION

In conclusion, short-term FICare had no significant positive effect on the establishment of intestinal flora diversity in premature NEC infants with enterostomy. Future studies will be conducted to assess the potential of FICare to modify infants' growth and development and intestinal adaptation, which may increase our ability to improve infants' development by improving their gut flora. We speculate that FICare may have broad practical implications, and the intervention will be improved by continuously assessing feedback and conducting follow-up studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: National Genomics Data Center, CRA004032.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Beijing Children's Hospital (Registration No. 2017-106). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MY, JD, MH, and MJ made substantial contributions to the conception of the study. MY, JD, QY, and WD helped the acquisition and analysis or interpretation of data for the work. MY drafted the work. MH and MJ revised it critically. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This draft was commented on by MH and MJ. MY and JD edited and approved the final draft. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Nie P, Li Z, Wang Y, Zhang Y, Zhao M, Luo J, et al. Gut microbiome interventions in human health and diseases. *Med Res Rev.* (2019) 39:2286–313. doi: 10.1002/med.21584
2. Milani C, Duranti S, Bottacini F, Casey E, Turroni F, Mahony J, et al. The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. *Microbiol Mol Biol Rev.* (2017) 81:e00036–17. doi: 10.1128/MMBR.00036-17
3. Cilieborg MS, Boye M, Sangild PT. Bacterial colonization and gut development in preterm neonates. *Early Hum Dev.* (2012) 88:S41–9. doi: 10.1016/j.earlhumdev.2011.12.027
4. Hei M, Gao X, Li Y, Gao X, Li Z, Xia S, et al. Family integrated care for preterm infants in China: a cluster randomized controlled trial. *J Pediatr.* (2021) 228:36–43. doi: 10.1016/j.jpeds.2020.09.006
5. Ouyang ZC, Gao XY, Du J, Li Y, Zhu X, Hei MY. Influence of family integrated care on the intestinal microbiome of preterm infants in the neonatal intensive care unit. *Zhonghua Er Ke Za Zhi.* (2019) 57:265–71. doi: 10.3760/cma.j.issn.0578-1310.2019.04.007

6. O'Brien K, Bracht M, Macdonell K, McBride T, Robson K, O'Leary L, et al. A pilot cohort analytic study of family integrated care in a Canadian neonatal intensive care unit. *BMC Pregnancy Childbirth.* (2013) 13:S12. doi: 10.1186/1471-2393-13-S1-S12
7. Guyton K, Alverdy JC. The gut microbiota and gastrointestinal surgery. *Nat Rev Gastroenterol Hepatol.* (2017) 14:43–54. doi: 10.1038/nrgastro.2016.139
8. Brower-Sinning R, Zhong D, Good M, Firek B, Baker R, Sodhi CP, et al. Mucosa-associated bacterial diversity in necrotizing enterocolitis. *PLoS ONE.* (2014) 9:e105046. doi: 10.1371/journal.pone.0105046
9. Barreiros MI, Marques C, Faria A, Neto MT, Cordeiro-Ferreira G, Virella D, et al. Colonisation of the proximal intestinal remnant in newborn infants with enterostomy: a longitudinal study protocol. *BMJ Open.* (2019) 9:e28916.
10. Hei M, Gao X, Gao X, Nong S, Zhang A, Zhang Q, et al. Is family integrated care in neonatal intensive care units feasible and good for preterm infants in China: study protocol for a cluster randomized controlled trial. *Trials.* (2016) 17:22. doi: 10.1186/s13063-015-1152-9
11. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R, UCHIME. improves sensitivity and speed of chimera detection. *Bioinformatics.* (2011) 27:2194–200. doi: 10.1093/bioinformatics/btr381
12. Edgar RC, UPARSE. highly accurate OTU sequences from microbial amplicon reads. *Nat Methods.* (2013) 10:996–8. doi: 10.1038/nmeth.2604
13. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* (2013) 41:D590–6. doi: 10.1093/nar/gks1219
14. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol.* (2009) 75:7537–41. doi: 10.1128/AEM.01541-09
15. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. *Genome Biol.* (2011) 12:R60. doi: 10.1186/gb-2011-12-6-r60
16. Hout MC, Papesh MH, Goldinger SD. Multidimensional scaling. *Wiley Interdiscip Rev Cogn Sci.* (2013) 4:93–103. doi: 10.1002/wcs.1203
17. O'Brien K, Robson K, Bracht M, Cruz M, Lui K, Alvaro R, et al. Effectiveness of family integrated care in neonatal intensive care units on infant and parent outcomes: a multicentre, multinational, cluster-randomised controlled trial. *Lancet Child Adolesc Health.* (2018) 2:245–54. doi: 10.1016/S2352-4642(18)30039-7
18. Broom M, Parsons G, Carlisle H, Kecskes Z, Thibeau S. Exploring parental and staff perceptions of the family-integrated care model: a qualitative focus group study. *Adv Neonatal Care.* (2017) 17:E12–9. doi: 10.1097/ANC.0000000000000443
19. Cheng C, Franck LS, Ye XY, Hutchinson SA, Lee SK, O'Brien K. Evaluating the effect of family integrated care on maternal stress and anxiety in neonatal intensive care units. *J Reprod Infant Psychol.* (2019) 39:166–79. doi: 10.1080/02646838.2019.1659940
20. He SW, Xiong YE, Zhu LH, Lv B, Gao XR, Xiong H, et al. Impact of family integrated care on infants' clinical outcomes in two children's hospitals in China: a pre-post intervention study. *Ital J Pediatr.* (2018) 44:65. doi: 10.1186/s13052-018-0506-9
21. Benzies KM, Shah V, Aziz K, Isaranuwatchai W, Palacio-Derflingher L, Scotland J, et al. Family integrated care (FICare) in level II neonatal intensive care units: study protocol for a cluster randomized controlled trial. *Trials.* (2017) 18:467. doi: 10.1186/s13063-017-2181-3
22. Franck LS, Kriz RM, Bisgaard R, Cormier DM, Joe P, Miller PS, et al. Comparison of family centered care with family integrated care and mobile technology (mFICare) on preterm infant and family outcomes: a multi-site quasi-experimental clinical trial protocol. *BMC Pediatr.* (2019) 19:469. doi: 10.1186/s12887-019-1838-3
23. Franck LS, Waddington C, O'Brien K. Family integrated care for preterm infants. *Crit Care Nurs Clin North Am.* (2020) 32:149–65. doi: 10.1016/j.cnc.2020.01.001
24. Iizumi T, Battaglia T, Ruiz V, Perez PG. Gut microbiome and antibiotics. *Arch Med Res.* (2017) 48:727–34. doi: 10.1016/j.arcmed.2017.11.004
25. Backhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, et al. Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe.* (2015) 17:690–703. doi: 10.1016/j.chom.2015.04.004
26. Sun B, Hu C, Fang H, Zhu L, Gao N, Zhu J. The effects of *Lactobacillus acidophilus* on the intestinal smooth muscle contraction through PKC/MLCK/MLC signaling pathway in TBI mouse model. *PLoS ONE.* (2015) 10:e128214. doi: 10.1371/journal.pone.0128214
27. Younge N, Yang Q, Seed PC. Enteral high fat-polyunsaturated fatty acid blend alters the pathogen composition of the intestinal microbiome in premature infants with an enterostomy. *J Pediatr.* (2017) 181:93–101. doi: 10.1016/j.jpeds.2016.10.053
28. Smith A, Saiman L, Zhou J, Della-Latta P, Jia H, Graham PR. Concordance of gastrointestinal tract colonization and subsequent bloodstream infections with Gram-negative bacilli in very low birth weight infants in the neonatal intensive care unit. *Pediatr Infect Dis J.* (2010) 29:831–5. doi: 10.1097/INF.0b013e3181e7884f
29. Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, et al. Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network. *Pediatrics.* (2002) 110:285–91. doi: 10.1542/peds.110.2.285
30. Cole CR, Frem JC, Schmotzer B, Gewirtz AT, Meddings JB, Gold BD, et al. The rate of bloodstream infection is high in infants with short bowel syndrome: relationship with small bowel bacterial overgrowth, enteral feeding, and inflammatory and immune responses. *J Pediatr.* (2010) 156:941–7. doi: 10.1016/j.jpeds.2009.12.008
31. Arrieta MC, Stiemsma LT, Amenyogbe N, Brown EM, Finlay B. The intestinal microbiome in early life: health and disease. *Front Immunol.* (2014) 5:427. doi: 10.3389/fimmu.2014.00427
32. Schwiertz A, Gruhl B, Lobnitz M, Michel P, Radke M, Blaut M. Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full-term infants. *Pediatr Res.* (2003) 54:393–9. doi: 10.1203/01.PDR.0000078274.74607.7A
33. Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT, et al. Structure, function and diversity of the healthy human microbiome. *Nature.* (2012) 486:207–14. doi: 10.1038/nature11234
34. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol.* (2009) 9:313–23. doi: 10.1038/nri2515
35. Caporaso JG, Lauber CL, Costello EK, Berg-Lyons D, Gonzalez A, Stombaugh J, et al. Moving pictures of the human microbiome. *Genome Biol.* (2011) 12:R50. doi: 10.1186/gb-2011-12-5-r50
36. Beura LK, Hamilton SE, Bi K, Schenkel JM, Odumade OA, Casey KA, et al. Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature.* (2016) 532:512–6. doi: 10.1038/nature17655
37. Groer MW, Luciano AA, Dishaw LJ, Ashmeade TL, Miller E, Gilbert JA. Development of the preterm infant gut microbiome: a research priority. *Microbiome.* (2014) 2:38. doi: 10.1186/2049-2618-2-38
38. Moss RL, Dimmitt RA, Barnhart DC, Sylvester KG, Brown RL, Powell DM, et al. Laparotomy versus peritoneal drainage for necrotizing enterocolitis and perforation. *N Engl J Med.* (2006) 354:2225–34. doi: 10.1056/NEJMoa054605
39. Amin SC, Pappas C, Iyengar H, Maheshwari A. Short bowel syndrome in the NICU. *Clin Perinatol.* (2013) 40:53–68. doi: 10.1016/j.clp.2012.12.003
40. Xu Z, Harvey KA, Pavlina T, Dutot G, Hise M, Zaloga GP, et al. Steroidal compounds in commercial parenteral lipid emulsions. *Nutrients.* (2012) 4:904–21. doi: 10.3390/nu4080904
41. Lindberg TP, Caimano MJ, Hagadorn JI, Bennett EM, Maas K, Brownell EA, et al. Preterm infant gut microbial patterns related to the development of necrotizing enterocolitis. *J Matern Fetal Neonatal Med.* (2020) 33:349–58. doi: 10.1080/14767058.2018.1490719
42. Chong C, Bloomfield FH, O'Sullivan JM. Factors affecting gastrointestinal microbiome development in neonates. *Nutrients.* (2018) 10:274. doi: 10.3390/nu10030274
43. Nagpal R, Tsuji H, Takahashi T, Nomoto K, Kawashima K, Nagata S, et al. Ontogenesis of the gut microbiota composition in healthy, full-term, vaginally born and breast-fed infants over the first 3 years of life: a quantitative bird's-eye view. *Front Microbiol.* (2017) 8:1388. doi: 10.3389/fmicb.2017.01388
44. Wandro S, Osborne S, Enriquez C, Bixby C, Arrieta A, Whiteson K. The microbiome and metabolome of preterm infant stool are personalized and not driven by health outcomes, including necrotizing enterocolitis

and late-onset sepsis. *MSphere.* (2018) 3:e00104–18. doi: 10.1128/mSphere.00104-18

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Pediatrics

CASE REPORT
published: 02 December 2021
doi: 10.3389/fped.2021.771467

Case Report: *Clostridium neonatale* Bacteremia in a Preterm Neonate With Necrotizing Enterocolitis

Nadim Cassir[1,2]*, *Isabelle Grandvuillemin*[3], *Manon Boxberger*[1,2], *Priscilla Jardot*[1,2], *Farid Boubred*[3,4] *and Bernard La Scola*[1,2]

[1] Department of Clinical Microbiology, Aix Marseille Université, Institut de Recherche pour le Développement, Assistance Publique des Hôpitaux de Marseille, Microbes, Evolution, Phylogénie et Infection, Marseille, France, [2] Institut Hospitalo-Universitaire Méditerranée Infection, Marseillle, France, [3] Department of Neonatology, Hôpital La Conception, Assistance Publique des Hôpitaux de Marseille, AMU, Marseille, France, [4] Neonatal Unit, C2 VN, Hospital University La Conception, Assistance Publique des Hôpitaux de Marseille, AMU, Marseille, France

Necrotizing enterocolitis is a life-threatening acquired gastrointestinal disorder among preterm neonates and is associated with a high mortality rate and long-term neurodevelopmental morbidity. No etiologic agent has been definitively established; nonetheless, the most implicated bacteria include members of the *Clostridium* genus. We reported here on a case of *Clostridium neonatale* bacteremia in a preterm neonate with necrotizing enterocolitis, providing more information regarding the potential role of this bacterium in pathogenesis of necrotizing enterocolitis. We emphasized the sporulating form of *C. neonatale* that confers resistance to disinfectants usually applied for the hospital environmental cleaning. Further works are needed to establish the causal relationship between the occurrence of NEC and the isolation of *C. neonatale,* with promising perspectives in terms of diagnostic and therapeutic management.

Keywords: case report, necrotizing enterocolitis, preterm neonate, *Clostridium neonatale*, gut microbiota

OPEN ACCESS

Edited by:
Yuan Shi,
Children's Hospital of Chongqing Medical University, China

Reviewed by:
Jan B. F. Hulscher,
University Medical Center Groningen, Netherlands
Brenda Law,
University of Alberta, Canada

***Correspondence:**
Nadim Cassir
cassirnadim@gmail.com

Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: *06 September 2021*
Accepted: *28 October 2021*
Published: *02 December 2021*

Citation:
Cassir N, Grandvuillemin I, Boxberger M, Jardot P, Boubred F and La Scola B (2021) Case Report: Clostridium neonatale Bacteremia in a Preterm Neonate With Necrotizing Enterocolitis. Front. Pediatr. 9:771467. doi: 10.3389/fped.2021.771467

INTRODUCTION

Necrotizing enterocolitis is a life-threatening acquired gastrointestinal disorder among preterm neonates, especially those of very low birth weight (<1,500 g) for which the incidence reaches 7% (1). Necrotizing enterocolitis (NEC) is associated with a high mortality rate (15–30%) and long-term neurodevelopmental morbidity (1). Although NEC pathophysiology remains unclear, prematurity, enteral feeding strategies, gut bacterial colonization, and inappropriate proinflammatory response are major factors involved in NEC development (1). Gut dysbiosis is considered the cornerstone of NEC pathogenesis. One of the main factors leading to gut dysbiosis is pre- and post-natal antibiotic exposure (1).

There is evidence of the role of specific bacteria in the pathogenesis of NEC. Indeed, outbreaks of NEC have long been described, although the reported causal agents greatly differed (1). *Pneumatosis intestinalis* is one of the specific radiological signs of NEC that likely represents submucosal gas produced by bacterial fermentation (2). In addition, it has been shown that NEC could not be reproduced in germ-free animals and rarely occurs until at least 8–10 days postpartum in preterm neonates, after strict anaerobic bacterial populations establishment (2).

We report here on a case of *Clostridium neonatale* bacteremia in a preterm neonate with necrotizing enterocolitis providing more information regarding the potential role of this bacterium in the pathogenesis of NEC.

CASE

A 24^{+4}-week-old gestational age female infant, with a birth weight of 800 g (95th percentile), was diagnosed with severe necrotizing enterocolitis (NEC) at the day of life (DoL) 21. She was born after cesarean delivery for preterm premature rupture of membranes and after antenatally completing a course of betamethasone. She was immediately admitted to our neonatal intensive care unit (NICU) and required exogenous surfactant combined with nasal continuous positive airway pressure for respiratory distress syndrome. The initial intravenous antibiotics (ampicillin and gentamycin) administered for suspected neonatal infection were stopped on DoL 2. Own breastfeeding was started from DoL1 (colostrum) and was well tolerated. On DoL 21, clinical signs of feeding intolerance suddenly occurred with bilious gastric residuals, abdominal distention, and rectal bleeding. Abdominal X-ray showed *pneumatosis intestinalis* (**Figure 1A**). White blood cells count, and C-reactive protein (CRP) respectively peaked at 40×10^9 /L (normal value range: 4.5 to 11×10^9 /L) and 240 mg/L (normal value < 5 mg/L). Enteral feeding was stopped and broad-spectrum antibiotics with vancomycin, cefotaxime, amikacin, and metronidazole were initiated. The yield of blood cultures allowed for the isolation of *Clostridium neonatale*. More specifically, 24 h after blood sampling, one anaerobic bottle out of two blood culture sets tested positive. Colony growth was observed on Columbia agar medium (Oxoid, Dardilly, France) supplemented with 5% (vol/vol) sheep blood and incubated for 24 h at 37°C in an anaerobic chamber (80%N2, 10% CO2, and 10% H2) (AES Chemunex, Bruz, France). Colonies were cream-colored and opaque after 24 h of growth (**Figure 1B**). By electronic microscopy using Hitachi SU5000 scanning electron microscope (Hitachi High-Tech Corporation, Tokyo, Japan), colonies were bacilli (**Figure 1C**). Bacterial cells were Gram-positive (not shown). Sporulation was observed after 24 h under aerobic conditions (**Figure 1D**). Using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry Vitek MS (bioMérieux, France), the organism was identified as *Clostridium* spp., and this was confirmed as *C. neonatale* through 16S rRNA PCR and whole-genome sequencing. In brief, strain Marseille-Q4564 exhibited a 99.66% 16S rRNA sequence similarity with *C. neonatale*^{T} (Accession number AF275949.1), the phylogenetically closest bacterium standing in nomenclature. Furthermore, a digital DNA–DNA hybridization revealed a maximum identity similarity of only 91%, and an OrthoANI parameter provided values of 98.89 and 98.95%, respectively, between Marseille-Q4564 and *C. neonatale*^{T} (NZ_PDCJ01000001.1 and NZ_LN890312.1). Taken together, these results confirm the status of this strain belonging to the *C. neonatale* species (**Figure 2**).

Antimicrobial susceptibility testing was performed. Minimum Inhibitory Concentration (MIC) of tigecycline and vancomycin were determined using Etest (Biomérieux, France), while imipenem, metronidazole, and clindamycin MICs were obtained by the disk diffusion method using SIRscan (i2a diagnostics, France). Strain Marseille-Q4564 was susceptible to all the above-mentioned antibiotics tested.

While stool culture under anaerobic conditions after a heat shock was negative, specific RT-PCR of *C. neonatale* tested on stools was positive (3).

Because of a sub-occlusive digestive syndrome with systemic inflammation, surgery was performed 7 days after NEC (DoL28). A severe ischemic jejunum injury was found and was treated with a conservative ileostomy. Culture and RT-PCR specific for *C. neonatale* tested on the intestine fragment were negative. Cefotaxime and metronidazole were continued for 2 weeks post-surgery. Prolonged parenteral nutrition with minimal enteral feeding was required and preserved adequate growth and a good outcome at 6 months follow-up.

DISCUSSION

Regarding necrotizing enterocolitis (NEC), several causative microorganisms have been proposed. Nonetheless, the most implicated bacteria include members of the *Clostridium* genus, especially *Clostridium butyricum, C. perfringens*, *C. paraputrificum*, and *C. neonatale*. Indeed, *C. butyricum* has frequently been recovered from biological samples of premature neonates suffering from NEC (4). Additionally, in quail and chicken animal models of NEC, *C. butyricum, C. perfringens, and C. paraputrificum* were shown to be responsible for NEC-like lesions (5). *C. neonatale* and *C. butyricum* were reported to be significantly overrepresented among colonic mucosal samples from premature piglets with NEC (6). In 2002, an outbreak of NEC occurred in a Canadian neonatal intensive care unit (7). Blood cultures from three out of six premature neonates grew the same strain further described as *C. neonatale*, belonging to cluster I of the *Clostridium* genus sensu stricto (8). Another research team that performed microbiota analysis in 16 cases and 78 controls showed an association between the isolation of *Clostridium neonatale* from the stools of preterm neonates and the occurrence of NEC (9). A specific *rpoB*-based quantitative real-time PCR has been recently designed and developed to detect *C. neonatale* directly from the stool specimens of patients. In a case-control study, *C. neonatale* was significantly more prevalent in stools from preterm neonates with NEC than in controls (respectively 30/88 (34%) vs. 9/71 (13%); $p = .003$) (3). By whole-genome sequencing, genes encoding the secretion of bacterial toxins, especially hemolysins and *C. difficile* toxins A/B (TcdA/B) had been previously found (10). These latter are involved in the pathogenic mechanism of *C. difficile* colitis, which is the consequence of TcdA (enterotoxin) and TcdB (cytotoxin) production inducing colonic tissue damage (1).

The preterm neonate acquires its microbiota within the confines of the NICU where colonization is influenced by iatrogenic manipulations including the hospital environment. Recently, an outbreak of NEC was epidemiologically linked to sporulating *C. butyricum* contamination within the hospital (11). Knowing that spore-forming bacteria are highly resistant to usual hospital disinfectants, sporicidal agents should be used

Abbreviations: NEC, necrotizing enterocolitis; DoL, days of life; CRP, C-reactive protein.

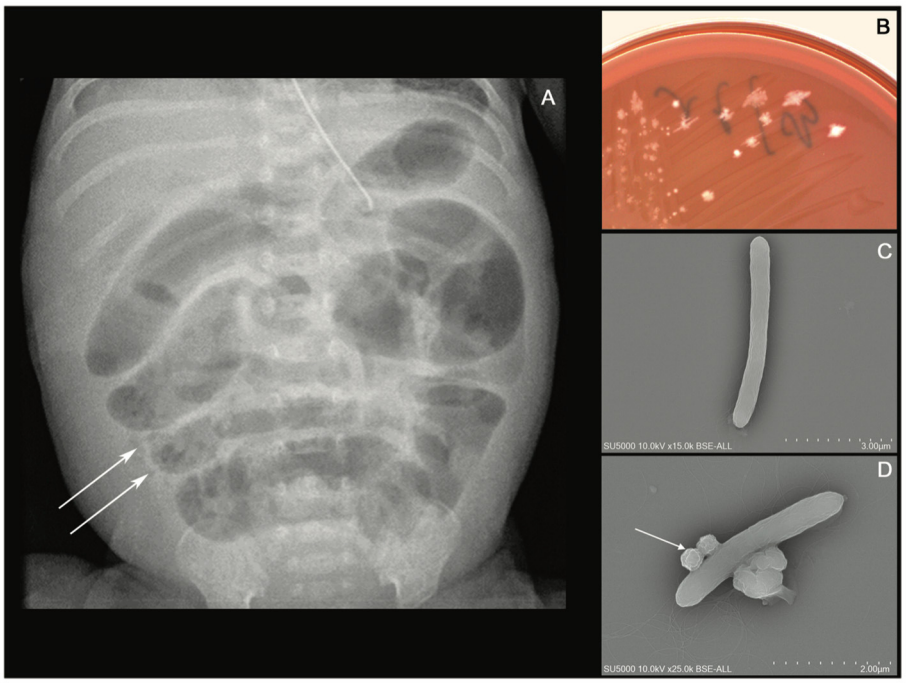

FIGURE 1 | (A) Abdominal X-ray showing intramural bowel gas also known as *pneumatosis intestinalis*, **(B)** culture growth of cream-colored colonies on COS after 24 h of incubation at 37°C in the anaerobic chamber, **(C)** *Clostridium neonatale* rod-shaped cells using Hitachi SU5000 scanning electron microscope. Scale bar and acquisition settings are shown on the original micrograph, and **(D)** sporulating *C. neonatale* cell.

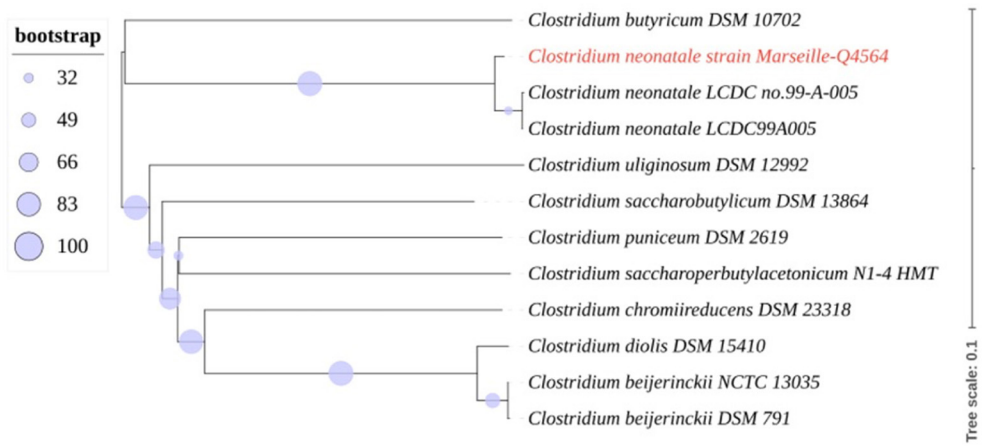

FIGURE 2 | Whole-genome (nucleotides) based phylogenetic tree highlighting the position of *C. neonatale* strain Marseille-Q4564 relative to other closely related bacterial taxa.

for environmental cleaning in case of sporulating *C. neonatale* infection. Therefore, we hypothesized that enteric isolation precautions like those applied for patients with *C. difficile* colitis may be effective in controlling NEC outbreaks.

Perinatal exposure to antibiotics has been identified as a risk factor for NEC, in particular when administered more than 5 days (12). The postnatal antibiotic exposure, in this case, may have increased the risk of NEC. Moreover, in this case, the use of large spectrum antibiotics during the episode of NEC may have participated in the secondary worsening 7 days after the initial diagnosis of NEC, by promoting gut dysbiosis. The length of treatment and choice of antimicrobial agents for presumed and proven episodes of NEC vary among centers due to a lack of supportive evidence and guidelines. The study of Cantey et al. showed recently that the implementation of antibiotic stewardship programs including appropriate dosages

and narrow-spectrum regimens was effective in reducing unnecessary antibiotic use in NICUs (13). In addition, probiotics have been extensively studied to mitigate gut dysbiosis in preterm neonates and prevent the occurrence of NEC (14). A low to moderate level of certainty about the effects of probiotic supplementation on the risk of NEC has been reported. To date, product safety and quality remain of concern especially in a vulnerable population such as preterm neonates. Therefore, probiotics are not routinely used in our NICU. In this case, we cannot rule out the possibility that the administration of a combination of different strains of probiotics may have prevented the occurrence of NEC. Further works are needed to address this issue.

Cultivation of strictly anaerobic species is challenging. Only a few studies analyzing the gut bacterial profile associated with NEC have used strategies to optimize the growth of strictly anaerobic bacteria such as heat shock for sporulating anaerobes, direct inoculation in anaerobic culture bottles, selective culture media, and the use of an anaerobic chamber (14). However, it is too early to state that routine microbiological exams in preterm neonates with NEC should include bacterial culture under anaerobic conditions and RT-PCR testing specific for *C. neonatale*, *C. butyricum,* and *C. perfringens* on a stool, blood, and surgical samples. This case of *Clostridium neonatale* bacteremia in a preterm neonate with necrotizing enterocolitis provides more information regarding the potential role of this bacterium in the pathogenesis of necrotizing enterocolitis. We hypothesize that the pathophysiological mechanism of *C. neonatale* may be like that of toxigenic *C. difficile* in adults with pseudomembranous colitis. Further works are needed to establish the causal relationship between the occurrence of NEC and the isolation of *C. neonatale* (15), with promising perspectives in terms of diagnostic and therapeutic management.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, NZ_PDCJ01000001.1.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participant's legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

NC: conceptualized and designed the study, drafted the initial manuscript, and reviewed and revised the manuscript. IG, MB, and PJ: designed the data collection instruments, collected data, carried out the initial analyses, reviewed, and revised the manuscript. BL and FB: conceptualized and designed the study, supervised data collection, performed analysis and interpretation of data, and critically reviewed the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

FUNDING

This research was supported by the French Government under the Investissements d'avenir (investments for the future) program managed by the Agence Nationale de la Recherche grant number [10-IAHU-03].

ACKNOWLEDGMENTS

We acknowledge Lina Barrassi, Anthony Fontanini, and Ludivine Brechard for their technical assistance.

REFERENCES

1. Cassir N, Simeoni U, La Scola B. Gut microbiota and the pathogenesis of necrotizing enterocolitis in preterm neonates. *Future Microbiol.* (2016) 11:273–92. doi: 10.2217/fmb.15.136
2. Neu J, Walker WA. Necrotizing enterocolitis. *N Engl J Med.* (2011) 364:255–64. doi: 10.1056/NEJMra1005408
3. Hosny M, Baptiste E, Levasseur A, La Scola B. Molecular epidemiology of *Clostridium neonatale* and its relationship with the occurrence of necrotizing enterocolitis in preterm neonates. *New Microbes New Infect.* (2019) 32:100612. doi: 10.1016/j.nmni.2019.100612
4. Cassir N, Benamar S, Khalil JB, Croce O, Saint-Faust M, Jacquot A, et al. *Clostridium butyricum* strains and dysbiosis linked to necrotizing enterocolitis in preterm neonates. *Clin Infect Dis.* (2015) 61:1107–15. doi: 10.1093/cid/civ468
5. Waligora-Dupriet A-J, Dugay A, Auzeil N, Huerre M, Butel M-J. Evidence for clostridial implication in necrotizing enterocolitis through bacterial fermentation in a gnotobiotic quail model. *Pediatr Res.* (2005) 58:629–35. doi: 10.1203/01.PDR.0000180538.13142.84
6. Azcarate-Peril MA, Foster DM, Cadenas MB, Stone MR, Jacobi SK, Stauffer SH, et al. Acute necrotizing enterocolitis of preterm piglets is characterized by dysbiosis of ileal mucosa-associated bacteria. *Gut Microbes.* (2011) 2:234–43. doi: 10.4161/gmic.2.4.16332
7. Alfa MJ, Robson D, Davi M, Bernard K, Van Caeseele P, Harding GKM. An outbreak of necrotizing enterocolitis associated with a novel clostridium species in a neonatal intensive care unit. *Clin Infect Dis.* (2002) 35:S101–105. doi: 10.1086/341929
8. Bouvet P, Ferraris L, Dauphin B, Popoff M-R, Butel MJ. Aires J. 16S rRNA gene sequencing, multilocus sequence analysis, and mass spectrometry identification of the proposed new species "Clostridium neonatale." *J Clin Microbiol.* (2014) 52:4129–36. doi: 10.1128/JCM.00477-14
9. Rozé JC, Ancel PY, Lepage P, Martin-Marchand L, Al Nabhani Z, Delannoy J, et al. Nutritional strategies and gut microbiota composition as risk factors for necrotizing enterocolitis in very-preterm infants. *Am J Clin Nutr.* (2017) 106:821–30. doi: 10.3945/ajcn.117.152967
10. Benamar S, Cassir N, La Scola B. Genome sequence of a *clostridium neonatale* strain isolated in a Canadian neonatal intensive care unit. *Genome Announc.* (2016) 4:e01431–15. doi: 10.1128/genomeA.01431-15

11. Dong Y, Li Y, Zhang D, Nguyen S, Maheshwari N, Hu Y, et al. Epidemiological and genetic characterization of *Clostridium butyricum* cultured from neonatal cases of necrotizing enterocolitis in China. *Infect Control Hosp Epidemiol.* (2020) 41:900–7. doi: 10.1017/ice.2019.289
12. Cotten CM, Taylor S, Stoll B, Goldberg RN, Hansen NI, Sánchez PJ, et al. Prolonged duration of initial empirical antibiotic treatment is associated with increased rates of necrotizing enterocolitis and death for extremely low birth weight infants. *Pediatrics.* (2009) 123:58–66. doi: 10.1542/peds.2007-3423
13. Cantey JB, Wozniak PS, Pruszynski JE, Sánchez PJ. Reducing unnecessary antibiotic use in the neonatal intensive care unit (SCOUT): a prospective interrupted time-series study. *Lancet Infect Dis.* (2016) 16:1178–84. doi: 10.1016/S1473-3099(16)30205-5
14. Murphy K, Ross RP, Ryan CA, Dempsey EM, Stanton C. Probiotics, prebiotics, and synbiotics for the prevention of necrotizing enterocolitis. *Front Nutr.* (2021) 8:667188. doi: 10.3389/fnut.2021.667188
15. Cassir N, Benamar S, La Scola B. Clostridium butyricum: from beneficial to a new emerging pathogen. *Clin Microbiol Infect.* (2016) 22:37–45. doi: 10.1016/j.cmi.2015.10.014

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

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership