Sex-specific gut microbiome profiles among preterm infants during the neonatal intensive care hospitalization

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Abstract

**Objectives:** The gut microbiota among preterm infants is shaped by sex and feeding types. However, sex-specific weekly patterns of gut microbiome profiles among preterm infants during their neonatal intensive care unit (NICU) hospitalization remain unclear. This study aimed to investigate the effect of sex on the weekly development of preterm neonatal gut microbiota in the first 4 weeks of NICU hospitalization.

**Methods:** This secondary data analysis included 28 preterm neonates with 261 stool samples collected from January 2014 to February 2015 in the Northeastern United States. The 16S rRNA V4 gene regions of the stool samples were sequenced and aligned against the SILVA 132 database by using Mothur 1.42.3. The sex-specific weekly diversity indexes and relative abundance of bacterial taxonomic composition were generated by Mothur and analyzed by R packages. Sex-specific weekly compositional patterns of the gut microbiome and predicted metabolic functions of gut microbiome profiles were compared, respectively.

**Results:** In each week of the NICU hospitalization, preterm females and males had significantly distinguished β-diversity indices and compositions of gut microbiota. Both females and males had significantly enriched \textit{Bifidobacterium}, a protection feature, in stool samples collected in the third week compared with those in the second week. The predicted metabolic pathways were significantly different between females and males in the second, third, and fourth week of the NICU hospitalization. Both females and males had significantly abundant pathways. Males consistently had more abundance of "lipopolysaccharide biosynthesis" than females in the second, third, and fourth week. Males also had a significant abundance of "membrane and intracellular structural molecules" and "glycan biosynthesis and metabolism" in the second and third week.

**Conclusions:** Sex shaped the weekly patterns of preterm neonatal gut microbiome profiles during the first 4 weeks of the NICU hospitalization. Further clinical interventions should consider the distinct gut microbiota compositions and predicted functional profiles between female and male preterm neonates.

**Keywords:** 16S rRNA, Composition, Gut microbiota, Preterm neonates, Sex

Introduction

Gut microbiota conducts crucial functions to maintain host health including mediation of immune response, regulation of energy metabolism, and protection against pathogens\textsuperscript{1-2}. The brain-gut axis, the signaling system between the gastrointestinal (GI) tract and central nervous system is connected by the gut microbiota\textsuperscript{3,4}. The gut microbiota has been found to play essential roles in a series of human disease conditions ranging from neonates\textsuperscript{5,6} to older adults\textsuperscript{7-9}. Research on the disease pathways of the brain-gut axis has found that variation of gut microbiota is involved in behavioral changes such as cognition decline, depression, and pain\textsuperscript{10-12}.

As one of the most vulnerable populations, preterm neonates’ health and neurobehavioral development is also mediated by gut microbiota\textsuperscript{4}. Researchers have demonstrated that neonates with necrotizing enterocolitis (NEC) and sepsis have an increased abundance of \textit{Enterobacteriaceae, Enterococcus,} and
Staphylococcus in the intestines compared with normal healthy subjects, suggesting that gut microbial-associated molecular patterns drive NEC and sepsis pathogenesis among preterm infants. Meanwhile, alteration of the gut microbiota composition is thought to result in impaired intestinal barrier function and splanchic circulation congestion which can lead to bacterial translocation and aggravating inflammatory states, that have been linked to pathologies of disease.

Understanding the factors that contribute to gut microbiota composition may have the potential to reveal the mechanisms of disease pathology, thus leading to disease prediction, treatment, and prevention. A study of mixed-sex twins reported that male neonates had a higher risk of neonatal mortality and morbidity than their female pair including a higher risk of congenital abnormalities and respiratory distress. The underlying mechanisms are still under-explored. Immune response and inflammatory driven by gut microbiota could be the potential pathology that has been confirmed in mice studies and preterm infants. Studies investigating adults gut microbiota also found that women and men had significantly different patterns of gut microbiota diversity, for example, different gut microbiota richness between women and men at different ages.

Studies have also reported the impact of sex and feeding on the dynamic compositions of preterm infants’ gut microbiota, primarily explored the different impacts of formula and breast milk on gut microbiome development, while the other 2 reported sex-specific and feeding-specific gut microbiome compositions within 10 day intervals in the first 30 days of neonatal intensive care unit (NICU) hospitalization. However, weekly patterns and functions of preterm neonatal gut microbiota remain unclear, which may impede our understanding of the differentiation of mortality among female and male neonates. Given that the gestational age of preterm infants is calculated by weeks due to their development, this study aimed to investigate the effect of sex on the weekly development of preterm neonatal gut microbiota profiles, including compositions and functions, in the first 4 weeks of NICU hospitalization.

### Methods

#### Study design

The data used in this secondary data analysis were from a previous longitudinal study. The original study protocol received institutional review board approval from the University of Connecticut and Connecticut Children’s Medical Center. Informed consent was signed by the parents of all the participating preterm neonates. The deidentified dataset was used in the study.

#### Study samples

The original study enrolled 29 preterm neonates from May 2015 to April 2016 by convenience sampling. Preterm neonates were included if: (1) their gestational age of neonate born was between 28 and 33 weeks (28 0/7 to 32 6/7); (2) they were admitted to the NICU in the first week after birth; (3) their mothers were able (aged 18 y or older) to provide informed consent. Neonates were excluded if they had: (1) positive drug exposure (illicit drug use); (2) severe intraventricular hemorrhage (≥ Grade III); (3) undergone surgical procedures, (4) congenital anomalies.

Three hundred seventy-eight stool samples were collected by trained nurses during diaper changing from these 29 preterm neonates in the first 30 days of life during their NICU hospitalization. Each stool sample was labeled uniquely and immediately placed in a ~80 °C freezer until processing. Aseptic technique was employed throughout to ensure that the specimens were not contaminated. In this study, 261 stool samples from 28 preterm neonates were included based on the following inclusion criteria: (1) samples collected in the first 28 days (4 wk) of NICU hospitalization and (2) samples yielded more than 10,000 reads. Fifty-nine samples were excluded as they were collected after 28 days of NICU stay and of the remaining samples 58 were excluded due to reads that were <10,000.

### DNA extraction, sequencing, and sequence data processing

DNA extraction and sequencing were reported in previous studies. In this study, the Mothur pipeline of Miseq SOP (http://www.mothur.org/wiki/MiSeq_SOP) was followed to process the 16S rRNA gene V4 regions raw sequencing data. The SILVA 132 V4 alignment database was selected as 16S rRNA gene reference. The diversity indexes and operational taxonomic units (OTUs) were also generated by the Mothur pipeline following our previous protocol.

### Statistical Analysis

Demographic and clinical information of the neonates were described by mean and percentage. The OTU tables, α-diversity, and β-diversity indexes were analyzed by using R 3.6.0. Exploratory data analyses including taxonomy graph techniques were conducted to display the composition of the organisms in the preterm neonates’ gut-microbiota community. The α-diversity of the gut microbiome was measured using sobs, coverage, Chao, Shannon, Shannon-even, and Invsimpson diversity index with significance determined by a nonparametric Kruskal-Wallis test. β-diversity index analysis included Bray-Curtis, Jaccard, and Theta YC counts. The significance was determined by using a permutational multivariate analysis of variance test. Phylseq v1.16.2 of R package was used to visualize the abundance of bacterial taxonomic composition.

Linear discriminant analysis effect size (LEfSe) was used to identify the significantly enriched abundant taxa between microbiota composition of female and male fecal samples. The sex-specific weekly differences in gut microbiota composition were also compared by LEfSe. A taxonomic cladogram was used to visualize the discriminative features based on the settings including the α value (0.05), and the logarithmic linear discriminant score (2.0) of the LEfSe following our previous analysis.

The metabolic functions (KEGG pathways) of gut microbiota compositions were predicted by using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt). The sex-specific differences of KEGG pathways at level 3 were detected by using Statistical Analysis of Metagenomic Profiles (STAMP). Benjamini-Hochberg procedure was used to decrease the false discovery rate for multiple test corrections. The P-value was set as 0.05.

### Results

#### Subject characteristics

The characteristics of these preterm neonates were summarized in Table 1. The average gestation age of female neonates was 30.98...
weeks (SD = 1.81) and the average gestation age of males was 31.66 (SD = 1.72) weeks. Approximately half of the neonates were delivered by cesarean section, and the majority were White. There was no significant difference in demographic characteristics between females and males.

Table 2 presents the weekly feeding regimen of the preterm neonates. According to our previous study, 6 groups including mother’s own milk (MOM), human donor milk (HDM), formula, MOM and HDM, MOM and formula, HDM and formula were determined based on > 70% of the total frequency of feeding in each week. Five females and 3 males did not receive any enteric feeding during the first week of NICU stay (nothing by mouth), and 1 female did not receive enteric feeding in the fourth week. Most of the neonates received their MOM. No significant difference was found regarding weekly feeding (all \( P > 0.05 \)).

Table 3 summarized the antibiotic use of these neonates; there was no significant difference between females and males (all \( P > 0.05 \)). Most of the neonates received antibiotics in the first week of NICU stay. Six female and 10 male neonates did not receive antibiotics after the third day of NICU stay. Four female and 2 male neonates did not receive any antibiotics during their NICU stay.

### Microbiome community and diversity analysis

The median number of stool collections from the neonates was 9 with an average number of 9 ± 4.8 collections per neonate. Among the 261 samples included (Supplementary File 1, Supplemental Digital Content 1, http://links.lww.com/NR9/A1), 133 were from females. During their first week of stay, 28 samples were analyzed and 14 were from females; while in the second week, 86 were analyzed and 45 were from females. In the third and fourth week, 92 and 55 samples were analyzed, respectively, and 44 and 30 were from females. Of the 261 fecal samples, 7674 OTUs were identified belonging to 26 phyla, 72 classes, and 176 orders. The compositions of the relative abundance of bacteria were calculated for each sample for both female and male neonates each week (Fig. 1). The most abundant phyla of the total reads were Firmicutes, Bacteroidetes, and Proteobacteria.

The sobs, Chao, Shannon, Shannon-even, and Invsimpson diversity indices of bacterial community among female samples and male samples collected in the third week of stay in the NICU were significantly different (Supplementary Figs. 1 a–e, Supplemental Digital Content 1, http://links.lww.com/NR9/A1). No other significant differences in \( \alpha \)-diversity were found between female of male samples collected in the first, second, and fourth weeks. For male samples, the bacterial community of gut samples in the fourth week showed relatively higher sobs, Chao, Shannon and Shannon-even, and Invsimpson diversity indices compared with the gut samples collected in the first and third week (Supplementary Figs. 1 a–e, Supplemental Digital Content 1, http://links.lww.com/NR9/A1). However, there were no significant weekly differences regarding the \( \alpha \)-diversity indices of females’ samples.

The Bray-Curtis, Jaccard, and Theta YC dissimilarities (Supplementary Figs. 2 a–c, Supplemental Digital Content 1, http://links.lww.com/NR9/A1) between female and male samples during the first 4 weeks of stay in NICU were determined by using the permutation multivariate analysis of variance test. At each week, female and male samples were found to present significantly different Bray-Curtis dissimilarities, so as the Jaccard and Theta YC dissimilarities (all \( P < 0.05 \)). Among the female samples, weekly distance metrics (the Bray-Curtis, Jaccard, and Theta YC dissimilarities) were significantly different from each other, so as those in males (all \( P < 0.05 \)).

### Weekly gut microbiota composition difference between/ among female and male neonates

**Differences between females and males during each week**

LEfSe analysis indicated that female and male preterm neonates had significantly different gut microbiota compositions each week. The differences were largely described at the family level. Enriched taxa in male samples included *Enterobacteriaceae*; while enriched taxa in females including *Archangiacae*, *Rhizobiacae*, and *Kunminococacae* in the first week (Figs. 1, 2 A). In the second week, females had a significantly higher abundance of...
Clostridiaceae, Enterococcaceae, Peptostreptococcaceae, and Staphylococcaceae. The male samples had significantly greater abundance of Bacteroidaceae, Muribaculaceae, Spirochaetaceae, and Tannerellaceae (Figs. 1, 2B). The significantly abundant taxa in female samples included Clostridiaceae, Lachnospiraceae, Muribaculaceae, Peptostreptococcaceae, and Rhodobacteraceae in the third week; with Enterobacteriaceae in male samples (Figs. 1, 2C). When compared the compositional difference in the fourth week, male samples had significantly higher abundance of Muribaculaceae, and Prevotellaceae; female samples had significantly higher abundance of Clostridiaceae, Enterococcaceae, Halomonadaceae, Pseudomonadaceae, and Staphylococcaceae (Figs. 1, 2D).

Weekly patterns among females

Results from the LEfSe analysis also showed that weekly patterns of gut microbiota compositions shifted among females. The significantly discriminative bacteria between samples collected in the first and second week among females are presented in Supplementary Figure 3a (Supplemental Digital Content 1, http://links.lww.com/NR9/A1). Samples collected in the first week had significantly enriched taxa including Blattabacteriaceae, Caulobacteraceae, and Enterobacteriaceae. Samples collected in the second and third week, the significantly enriched taxa in the second week included Gemmataceae and Staphylococcaceae; the significantly enriched taxa in the third week included Bifidobacteriaceae, Clostridiaceae, Endomicrobiaceae, and Lachnospiraceae (Supplementary Fig. 3b, Supplemental Digital Content 1, http://links.lww.com/NR9/A1). The compositional differences of gut microbiota between the third and the fourth week among females are shown in Supplementary Fig. 3c (Supplemental Digital Content 1, http://links.lww.com/NR9/A1). Samples in the third week had a significantly higher abundance of Bacteroidaceae, whereas samples in the fourth week had more enriched features including Lactobacillales (order).

Weekly patterns among males

Among the male neonates, the weekly patterns of gut microbiota compositions were identified by the LEfSe analysis. The significantly enriched taxa in the first and second week among males are presented in Supplementary Figure 4a (Supplemental Digital Content 1, http://links.lww.com/NR9/A1). Samples collected in the first week had significantly higher abundance of Staphylococcaceae, Spirochaetaceae, and Ruminococcaceae; and Enterococcaceae was significantly enriched in the second week. When comparing the difference between the second and third week, the significantly enriched taxa in the second week included Gemmataceae and Staphylococcaceae; the significantly enriched taxa in the third week included Bifidobacteriaceae, Clostridiaceae, Endomicrobiaceae, and Lachnospiraceae (Supplementary Fig. 4b, Supplemental Digital Content 1, http://links.lww.com/NR9/A1), including significantly higher

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*Figure 1.* Relative abundance of gut microbiota among preterm neonates. The relative abundance of the included neonates was presented on a weekly basis in the first 4 weeks of neonatal intensive care unit stay. Each column presents one infant. A blank column indicates no stool sample was obtained in that week. The different colors of the bar represent selected taxonomic levels.
abundance of Endomicrobiaceae and Lachnospiraceae in the second week, significantly higher abundance of Bacteroidaceae, Bifidobacteriaceae, and Enterobacteriaceae in the third week. 

Supplementary Figure 4c (Supplemental Digital Content 1, http://links.lww.com/NR9/A1) shows the different gut microbiota compositions between the third and fourth week among male samples, samples in the third weeks had a significantly higher abundance of Enterobacteriaceae, samples in the fourth week had significantly higher abundance of Lachnospiraceae, Muribaculaceae, and Rikenellaceae.

Weekly function profile difference between female and male neonates

PICRUSs prediction revealed that female and male preterm neonates had significant differences in metabolic profile at level 3 of KEGG categories during their first four weeks of NICU stay except in the first week. After FDR correction, males had 19 abundant metabolic features including the bacterial secretion system (\(P < 0.05\)) and nicotinate and nicotinamide metabolism (\(P < 0.001\)), while the females had 10 abundant metabolic features in the second week (Supplementary Fig. 5a, Supplemental Digital Content 1, http://links.lww.com/NR9/A1). In the third week, females had a greater abundance of “methane metabolism” and “DNA repair and recombination proteins,” males had an abundance of “bacterial secretion system” and “lipopolysaccharide biosynthesis” (Supplementary Fig. 5b, Supplemental Digital Content 1, http://links.lww.com/NR9/A1). In the fourth week, “bacterial motility proteins” and “signal transduction mechanisms” were more abundant in female samples, “RNA degradation” and “lipopolysaccharide biosynthesis” were higher in the male samples (Supplementary Fig. 5c, Supplemental Digital Content 1, http://links.lww.com/NR9/A1). Males consistently had an abundance of “lipopolysaccharide biosynthesis” than the females in the second, third, and fourth weeks. The males also had a significant abundance of “membrane and intracellular structural molecules” and “glycan biosynthesis and metabolism” in the second and third weeks.

Discussion

The brain-gut axis has been shown to mediate the neonates’ neurodevelopment and immunity and influence the lifelong health of humans\(^3\). Since information about the dynamic changes of preterm neonates’ gut microbiota compositions and

Figure 2. Weekly compositional difference of gut microbiota between females and males. A–D. The compositional difference of gut microbiota between females and males during the first, second, third, and fourth week of neonatal intensive care unit stay.
related factors is limited, potential pathogens and pathogenesis of common diseases involving the gut microbiota are still not well understood. This study reported sex-specific gut microbiota composition after controlling demographic characteristics and feeding among preterm infants during their NICU hospitalization; both females and males demonstrated weekly distinguished gut microbiota patterns.

The most abundant phyla of the total reads were *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, which supported results in the original analysis and previous studies. Sexual dimorphism in gut microbiota composition was identified over time after the first week, while same-sex preterm infants had similar gut microbiota patterns. In the first 4 weeks of stay in NICU, the alpha-diversity of preterm neonates’ gut microbiota significantly increased in the fourth week among the male samples. Male neonates were likely to have a lower diversity than females, which is in line with the original study that reported male neonates tended to have a higher abundance of *Enterobacteriales* and a lower abundance of *Clostridiales* than females. Distinct abundance of features was also found that females had more abundance of gut protection features, for example, *Enterococcusaceae* and *Lachnospiraceae*, with the potential function of promoting hematopoiesis and attenuating GI damage, while males had more abundant features potentially related to inflammation, for example, *Enterobacteriales*. Because differentiation of clinical outcomes may be associated with these variations of gut microbiota composition, further multiple-center studies with larger sample sizes are needed to confirm these findings.

The host's cardiometabolic diseases and brain disease risk is associated with the alternation of bacterial community composition. In the present study, obvious shifting patterns in the gut microbiota composition were observed in preterm neonates’ stool samples collected in their first 4 weeks of stay in NICU. The number of significantly enriched species was much lower in the second week, which may result from the inflammatory burden or stress-response in preterm neonates while acclimating to the NICU environment. In addition, both females and males had significantly enriched *Bifidobacterium* in stool samples collected in the third week compared with those in the second week (Supplementary Figs. 3 b, Fig. 4 b, Supplemental Digital Content 1, http://links.lww.com/NR9/A1), which may also support a higher inflammatory burden or stress-response in the second week of NICU stay given the potential protective role of enriched *Bifidobacterium* in stool samples among preterm infants.

Our predicted functional profile analysis showed significant differences in bacterial functional pathways between females and males in their NICU stay except during the first week. These pathways are related to immune response and inflammation mediation such as nicotinate and nicotinamide metabolism, which may be related to disease pathologies since research also reported that male neonates had a higher risk of neonatal morbidity and mortality. However, the present findings should be confirmed by shotgun metagenomics analysis from animal studies, and then longitudinal studies observing the effect of different gut microbiota composition and function on neonatal morbidity and mortality.

The results of this study expanded upon the original analysis since only stool samples collected in the first 4 weeks (28 d) were included as opposed to later time points. From the results, we found that preterm neonates had distinct gut microbiota composition in each of the 4 weeks and different predicted function profiles of gut microbiota after adjusting the related factors such as feeding and antibiotic use. Two important methodological differences should be noted between the original study and the current study. First, SILVA 132 instead of Green Genes was used as a reference in this study. In addition, the Mothur 1.42.3 pipeline was used for sequencing data analysis such as Usearch and Chimera detection in the current study as opposed to the QIIME pipeline. These methodological modifications could have contributed to differences in the findings reported in the original study.

Some limitations should be addressed in this study. Although differentiation of gut microbiota composition and predicted functional profiles between female and male preterm neonates was found, we cannot speculate on the functional differences that may be involved in pathologies of disease or clinical outcomes such as mortality and morbidity. Further studies should explore the contribution of different gut microbiota compositions to clinical outcomes by using shotgun sequencing to capture metagenomic functions of gut microbiota in preterm neonates to validate the predicted function of 16S sequencing by PICRUSt. We did not record the detailed dosage of antibiotics use. In addition, only 261 samples from 28 preterm neonates collected in 2 clinic sites were analyzed in this study. Multicenter studies that include a large sample size are necessary to validate findings from this study.

Given the essential role of the gut microbiome on growth and development among preterm infants, for example, weight gain and neurological outcomes, several implications emerged. Further studies could investigate the effect of precise sex-specific nutrition support on preterm infants in their NICU hospitalization since the present study did not find significant feeding differences between females and males. Manipulating the gut microbiome by supplementing prebiotics and probiotics was effective to improve memory and cognitive functions. Sex-specific prebiotics and/or probiotics use could be another option to ameliorate the early gut microbiota dysbiosis and improve the deficit neurobehavioral development outcomes among preterm infants since the sex-specific gut microbiome compositions and predicted functions, as well as the impact of the gut microbiome on neurological outcome and cognitive function have been reported.

Conclusions

This study included preterm neonates’ stool samples collected during their first 4 weeks of NICU hospitalization. The most abundant phyla were *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, which is similar to the original analysis even though a different reference database and pipeline were used in this study. Female and male preterm neonates were shown to have distinct gut microbiota composition and predicted functional profiles, and both females and males demonstrated weekly shifted gut microbiota patterns in the NICU hospitalization. Gut microbiome-based interventions such as precise sex-specific feeding, sex-specific prebiotics, and/or probiotics use could be employed to ameliorate the early gut microbiota dysbiosis and improve the development outcomes among preterm infants.

Author contribution

J.C.: conceptualization, formal analysis, methodology, writing—original draft, writing—review and editing. H.L.: formal analysis, methodology, writing—review and editing. K.M. and M.-H.C.:
conceptualization, formal analysis, methodology, writing—review and editing. A.S.: conceptualization, methodology, writing—review and editing. X.C.: conceptualization, formal analysis, methodology, funding acquisition, project administration, writing—review and editing.

**Conflict of interest disclosures**

The authors declare that they have no financial conflict of interest with regard to the content of this report.

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