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The constitution and functional prediction of the microbiota in necrotizing enterocolitis with a gestational age of over 28 weeks

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Abstract

Background: To explore the features and function of gut microbiota in necrotizing enterocolitis patients over 28 gestational age weeks through a case-control study.

Methods: Fecal samples from patients with NEC over 28 gestational week age and matched control cases were collected. DNA of the fecal samples was extracted for 16s rRNA sequencing to estimate the composition of the microbiota. Functional inference analyses were conducted through PICRUSt based on the sequencing raw data.

Results: There was no significant difference in the total diversity of microbiota between the fecal samples from the patients with NEC and the controls ($P = .40$). *Propionibacterium* was more abundant in the NEC cases than in the controls. Conversely, *Lactobacillus, Phascolarctobacterium,* and *Streptococcus_salivarius* were found to be more plentiful in the controls through LEfSe analysis. Functional inference analysis revealed that the xenobiotic biodegradation and metabolic activity was lower in the NEC cases than in the controls ($P < .05$).

Conclusion: The NEC cohort with a gestational age of over 28 weeks has a different pattern of microbiota compared with the controls. Functional inference analysis indicated that the potential function of the microbiota may also differ between these groups.

Abbreviations: IL = interleukin, IQR = interquartile ranges, KEGG = Kyoto Encyclopedia of Genes and Genomes, KO = KEGG orthologs, LEfSe = Linear discriminant analysis effect size, NEC = necrotizing enterocolitis, OTUs = operational taxonomic units, PCoA = principal coordinate analysis, PICRUSt = Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, PPAR = peroxisome proliferator-activated receptor, *salivarius* = *Streptococcus salivarius*, TLR = toll-like receptor.

Keywords: functional inference, microbiota, necrotizing enterocolitis

1. Introduction

Necrotizing enterocolitis (NEC) is among the most common life threatening diseases in neonates and occurs mostly in preterm babies.[1] NEC has become one of the most difficult diseases to eradicate with a high mortality and thus has been a priority for research.[2] Although the exact cause of NEC still remains unknown, it is widely accepted that the colonization of abnormal microbiota plays a dominant role in the pathogenesis of NEC. Early studies have been limited by the use of a conventional culture based technique which is unable to thoroughly describe the human gastrointestinal microbiota, as more than 80% of bacteria cannot be cultivated.[3] In the last decade, advances in the 16S small subunit bacterial ribosomal RNA (rRNA) gene based sequencing method has provided a more detailed picture of the composition of the human intestinal microbiota.[4] Preliminary studies compared the bacterial populations found in patients with NEC to those found in healthy individuals. However, there was variability in these results, and the association between an increase in Proteobacteria, Clostridia, Staphylococci and a decrease in Firmicutes have been reported in different studies.[5–11] Some studies have also emphasized the correlation between a decrease in the diversity of the microbiota and the development of NEC.[10,12] However, most of the references above focused on extremely preterm babies. NEC is still one of the most common diseases which cause deaths in late preterm babies or even in term babies. The relationship between microbiota and NEC in preterm babies of over 28 gestational weeks remains unclear.[13]

Moreover, most previous studies have only described the constitution of gut microbes in patients with NEC and little is known about the molecular mechanisms through which these microbes contribute to NEC pathogenesis and the function of these microbes. Metagenomics is the best way to describe the function of

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microbiota. However, the amount of DNA required for this technique limits its use in neonates as the volume of fecal sample is limited. Advances in the 16S analysis technology has allowed us to apply functional inference analysis, a computational approach to predict the functional composition of a metagenome using marker gene data and a database of reference genomes, as an alternative choice in the identification of the functional factors that differ between patients with NEC and healthy individuals.

In this study, we compared the intestinal microbiota in 16 premature infants with NEC to that of 16 matched and unaffected control infants over 28 weeks through the 16S rRNA sequencing method and conducted a functional inference analysis to determine the potential functional shift that is present in the intestinal microbiota between patients with NEC and unaffected infants.

2. Materials and methods

2.1. Patients and sample collection

This study was a part of microbiota research of preterm babies and was approved by the Institutional Review Board for Human Studies of the Shenzhen Children's Hospital. The study was carried out from May 2016 to February 2018. All infants with definite or advanced NEC, corresponding to the Bell stages II and III, and with gestational age over 28 weeks were included in the NEC cohort. Infants with congenital diseases were excluded from the study. Healthy infants who matched the infants with NEC in gestational age, birth weight, date of birth (+/−2 months), mode of delivery, and feeding patterns were enrolled as controls.

Fecal samples from the patients with NEC at 28 gestational weeks or over were collected by the medical staff once a diagnosis of NEC was confirmed, with an average collection time of 10 hours after the diagnosis of NEC. The samples of the control infants were collected at the same postnatal day. The freshly evacuated faeces were gathered from diapers into sterile tubes and were then transported to the laboratory immediately. All samples were stored at −80°C for further processing.

2.2. DNA extraction

DNA was extracted from 250 mg of the frozen faecal samples using the QIAamp FAST DNA Stool Mini-Kit (Qiagen, Germany) according to the manufacturer's instructions. The extracted DNA was eluted using the 50 μL ATE buffer from the kit.

2.3. PCR amplification and illumina MiSeq sequencing

DNA was amplified according to the V3-V4 region of bacterial 16S rRNA gene with universal bacteria primers: 338F (5'-ACTCCTACGG-GAGGCAGCA-3') and 806R (5'-GGAC-TACHVGGGTWTCTAAT-3'), which contain an 8-base barcode sequence unique to each sample. The cleansed PCR products were pooled in equimolar amounts and submitted for paired-end sequencing (2 × 250) on an Illumina MiSeq platform according to the standard protocols.

2.4. Bioinformatic processing

Raw FASTq files were quality-filtered by QIIME according to the index sequence. Reads with over one nucleotide mismatch in the primer sequence, ambiguous characters, a length shortened by more than 50bp or those which could not be assembled were discarded. The sequences were binned into Operational Taxonomic Units (OTUs) using a similarity level threshold of 97%. The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed by Ribosomal Database Project.

Next, we applied Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), a technique that is based on the correlation between phylogeny and functions, to analyze the phylogenetic proximity between the public genomes and the 16S rRNA OTUs.[14,15] Additionally, the popular Kyoto Encyclopedia of Genes and Genomes (KEGG) was employed for annotation.[15] Functional predictions were exported as KEGG orthologs (KO). We obtained all KO annotations from the v3.5 of IMG25 to produce a table of 6909 abundances for all of the genomes that had identifiers in the Greengenes reference tree. Four levels of KO were used to compare the bacterial functional profiles.

2.5. Statistical analysis

Data were analyzed using SAS 9.0 (SAS Institute Inc, Cary, NC). Qualitative or categorical variables are expressed as frequencies and proportions while data with non-normal distribution are expressed as median and interquartile ranges (IQR). The differences between the two groups were tested using the Fisher Exact test for categorical variables and the nonparametric Wilcoxon paired test for continuous variables. The linear discriminant analysis effect size (LEfSe) was used to identify the phylogenetic features that differed significantly between all of the NEC cases and the controls[16] with an LDA score threshold of >3.0, based on the Kruskal-Wallis test. For all statistical analysis, a probability (P) value of <.05 was set as statistical significance.

3. Results

3.1. General information

During the study period a total of 21 patients were diagnosed with NEC. One case was excluded because of insufficient fecal sample collection, 2 because of congenital heart disease, and 2 patients died before sample collection could be completed. Finally, a total of 16 infants with NEC (NEC group) and 16 normal infants (control group) were enrolled in this study in the Shenzhen Children's Hospital, Shenzhen, China. The general demographic information is displayed in Table 1. Briefly, no

Table 1

Basic information of the enrolled infants in the NEC and control groups.

	NEC group (n = 16)	Control group (n = 16)	*P* value
Sex (males/females)	8/8	8/8	1
GA (week), median (IQR)	34.8 (33.4–36.1)	35.1 (33.1–36.5)	.83
Birth weight (grams)	2325 (2063–2575)	2345 (2025–2675)	.83
Caesarean section, n (%)	8 (50)	8 (50)	1
Feeding (breast milk/formula feeding or mixed feeding)	3/13	5/11	.69
Ages for stool analyzed (d), median (IQR)	9 (5–16)	14 (5–15)	.38
NEC treatment (surgical)	3/16 (18.755%)	NA	NA
NEC (II/III)	11/5	NA	NA
Mortality associated with NEC	2/16 (12.5%)	NA	NA

d = day, GA = gestational age, IQR = interquartile range, NEC = necrotizing enterocolitis.

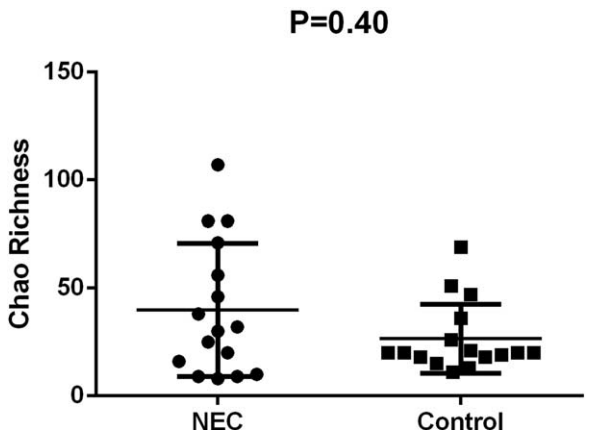

Figure 1. Comparison of the Chao index between the NEC and control groups. The diversity of the microbiota in the patients with NEC (closed circle) and the normal infants (controls, closed square) as determined by Chao-based estimate of the total numbers of OTUs present (the 25th and 75th percentiles).

differences were found between the NEC group and the control group for sex, gestational age, birth weight, delivery patterns, or feeding patterns. Among the samples, 5 cases progressed to stage III, 3 cases received surgery, and 2 infants died.

3.2. Microbiota analysis

A total of 32 fecal samples were analyzed through high-throughput sequencing. A total of 451,987 rRNA sequences with an average of 14,125 sequences per sample were obtained. These sequences were clustered at the 97.5% similarity level to obtain OTUs containing similar sequences for further microbiota analysis. The mean number of OTUs in each sample was 22.

As shown in Figure 1, the species richness, a diversity index that was determined by a Chao-based estimate of the total numbers of OTUs present, did not differ between the NEC and control cases ($P = .60$). Furthermore, we examined the samples using principal coordinate analysis (PCoA), which is based on UniFrac metric, to determine the microbiota structure. This analysis indicated that no clustering was detected according to infant status (Fig. 2). In the analysis of the overall microbiota structure for each group at the phylum and genus level, Proteobacteria and Firmicutes were the most abundant flora in both groups and these floras occupied over 90% of the total bacteria species composing the samples. However, no differences were found in the amount of Proteobacteria ($P = .81$) or Firmicutes ($P = .99$) between the 2 groups (Fig. 3). We, therefore, employed LEfSe analysis to investigate any difference in bacteria between the 2 groups. Interestingly, Propionibacteriales was more abundant in the NEC group than in the control group, while the abundance of Lactobacillus, Phascolarctobacterium and Streptococcus_salivarius was higher in the control group than in the NEC group (Fig. 4).

3.3. Functional inference analysis

Next, we performed functional inference analysis based on PICRUSt. By collapsing the data at KEEG level 2, we found that xenobiotic biodegradation and metabolism was significantly different between the groups ($P < .05$). Other methods of analysis, including environmental information processing, genetic information processing, and cellular processing did not show any differences between the NEC and control groups (Table 2).

4. Discussion

NEC has been identified as one of the most common causes of death in preterm babies at 26 to 28 gestational weeks.[17] As a

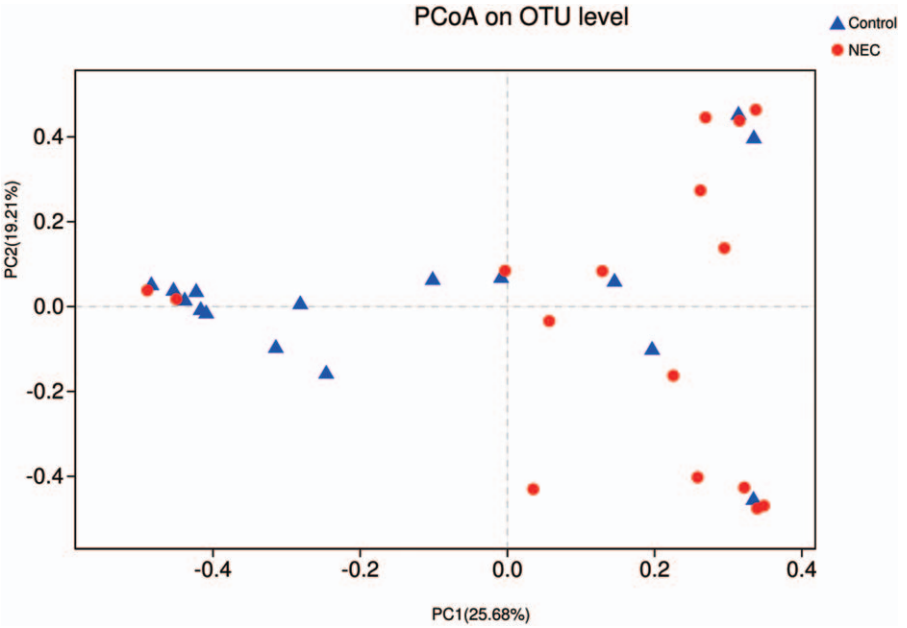

Figure 2. Principal coordinate analysis (PCoA) of the microbial communities of the groups. The microbiota cluster in the patients with NEC (red circle) and the normal infants (controls, blue triangle) were examined by principal coordinate analysis (PCoA).

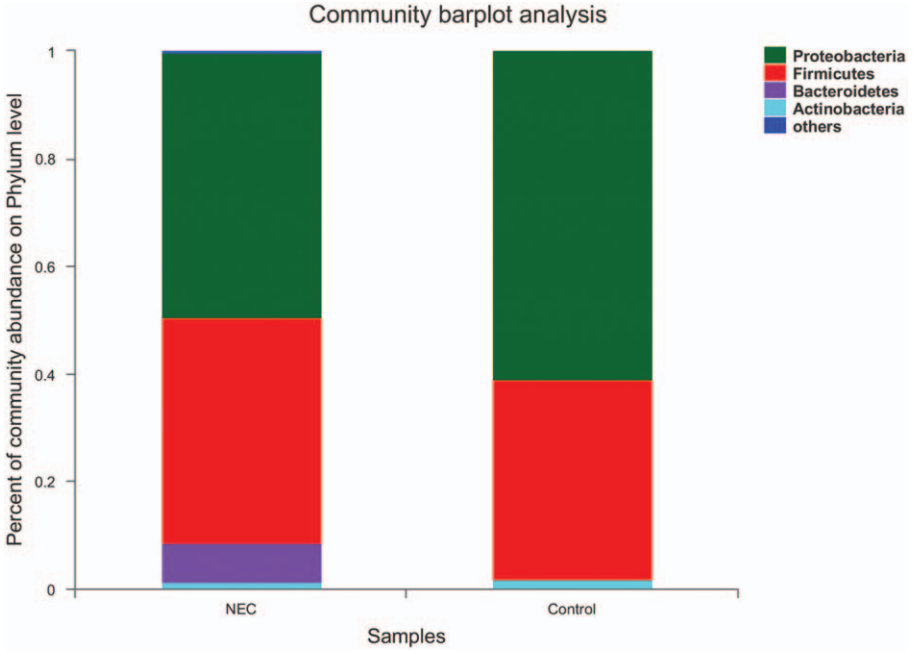

Figure 3. Overall microbiota structure of the 2 groups at the Phylumn level. The mean proportions of the major microbiota phyla [including Proteobacteria (green), Firmicutes (red), Bacteroidetes (purple), Actinobacteria (light blule), and others (dark blue)] structure of the patients with NEC (left) and the normal controls (right).

result, most of the studies that have focused on the pathogenesis of NEC have studied extremely preterm babies. In contrast with the findings in developed countries, in developing countries, such as China, only 10% of babies that are born before 28 weeks survive. However, the survival rate of infants that are born at this stage is more than 90% in developed countries. Thus, the survival gap between countries is 10: 90.[18] Most patients with NEC in developing countries are born over 28 weeks. This study explored the constitution and function of the microbiota in patients with NEC over 28 weeks in China to provide the possible pathogenic factors.

In this study, our data were to some extent consistent with previous studies that identified no difference in the overall richness between patients with NEC (at 26–28 gestational weeks) and control infants.[7,9] However, at the phylum level, no changes were found in the Proteobacteria, Bacteroidetes, or Firmicutes between patients with NEC over 28 gestational weeks and the control group. This finding is different to those of previous studies.[7,9,11,19] This may be because the gut is well colonized in infants approaching 33 to 36 gestational weeks (the median gestational age was 34.8 for infants with NEC) in our study. Thus, the regular pattern for bacteria colonization in extremely premature babies may not apply to those babies at risk of NEC with a gestational age of over 28 weeks.[20]

Through IEfSe analysis, we noted that the level of *Streptococcus (S.)_salivarius* differed most significantly between the 2 groups. S. salivarius is one of the first colonizers of the gut after birth and, therefore, may contribute to the establishment of gut immune homeostasis and the modulation of host inflammatory responses. It has been reported that live *S salivarius* strains regulates innate intestinal immune inflammation through the prohibition of the NF-κB pathway and modulates peroxisome proliferator-activated receptor γ (PPARγ) transcriptional activity on intestinal epithelial cells.[21,22] The NF-κB pathway is a traditional signal involved in the promotion of transcription of proinflammatory factors and PPARγ has been shown to facilitate an anti-inflammatory antioxidant response that interacts with different enzymatic pathways, such as cyclooxygenase 2. Both of these factors have been reported to take part in the pathogenesis of NEC.[23–25] The other function of *S._salivarius* indicate that it may limit the expansion of other pathogens.[26] The above evidence could explain why we observed that *S._salivarius* was more abundant in the control group.

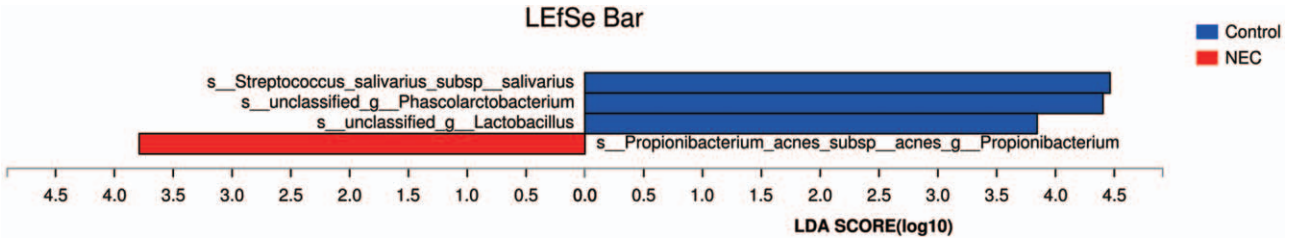

Figure 4. Comparison of the linear discriminant analysis effect size (LEfSe) between the groups. The phylogenetic features were tested by LEfSe to reveal the bacteria which significantly differed between the infants with NEC (left site, red) and the normal controls (right site, blue).

Table 2

Relative frequencies (Median with IQR) of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (level 2) between NEC and control infants.

	NEC group (n = 16)	Control group (n = 16)	*P* value
Cellular Process			
Cell Growth and Death	0.41 (0.35–0.47)	0.28 (0.24–0.45)	.14
Cell Motility	1.46 (0.80–3.21)	1.58 (1.10–2.03)	.72
Cellular Processes and Signaling	4.71 (3.15–5.27)	4.97 (4.55–5.08)	.83
Metabolism			
Amino Acid Metabolism	8.37 (7.89–8.92)	8.57 (8.42–8.80)	.38
Carbohydrate Metabolism	10.65 (8.85–11.52)	10.76 (10.62–11.42)	.46
Biosynthesis of Other Secondary Metabolites	0.69 (0.61–0.78)	0.76 (0.70–0.79)	.28
Energy Metabolism	4.93 (4.55–5.28)	4.67 (4.54–4.95)	.44
Metabolism of Cofactors and Vitamins	4.03 (3.49–4.46)	3.72 (3.56–4.12)	.56
Glycan Biosynthesis and Metabolism	2.45 (1.92–2.90)	2.16 (1.92–2.26)	.18
Lipid Metabolism	2.85 (2.77–2.94)	2.78 (2.74–2.83)	.17
Nucleotide Metabolism	3.86 (3.39–4.46)	3.05 (2.76–4.18)	.10
Metabolism of Terpenoids and Polyketides	1.54 (1.43–1.67)	1.48 (1.43–1.58)	.77
Enzyme family	2.01 (1.92–2.10)	2.00 (1.98–2.03)	.70
Xenobiotics Biodegradation and Metabolism	1.90 (1.66–2.21)	2.48 (2.10–2.57)	.01
Genetic Information Processing			
Folding, Sorting and Degradation	2.30 (2.01–2.65)	2.07 (1.98–2.26)	.12
Replication and Repair	8.04 (7.00–8.63)	6.30 (5.65–8.17)	.11
Transcription	2.89 (2.75–3.10)	3.21 (2.88–3.28)	.06
Translation	4.74 (4.24–5.39)	3.70 (3.28–5.15)	.16
Environmental Information Processing			
Membrane Transport	15.8 (13.6–17.8)	18.2 (16.1–19.9)	.21
Signal Transduction	2.12 (1.58–2.54)	2.41 (1.77–2.50)	.69
Signaling Molecules and Interaction	0.15 (0.11–0.26)	0.13 (0.12–0.21)	.69

Meanwhile, it is not a surprise that we identified an increase in the abundance of Lactobacillus in the control group. Lactobacillus has been widely accepted to be a kind of probiotic. Many randomized clinic trials have demonstrated that Lactobacillus has a preventative action in NEC.[27–29] The mechanism though which Lactobacillus prevents NEC appears to involved the attenuation of interleukin (IL)-1β-induced IL-8 and IL-6 expression, decreased toll-like receptor (TLR)2, TLR4, and TLR9 expression, increased levels of specific negative regulators of inflammation (such as Single Ig IL-1-related receptor and Toll interacting protein), and the restoration of the ratio of CD4(+) Foxp3(+) regulatory T cells, which can inhibit the inflammatory activity of TH17 cells.[30–33] In addition, we discovered that Propionibacteriales was more abundant in the NEC group than in the control group, while Phascolarctobacterium was more abundant in the control group than in the NEC group. Both of these the bacteria can produce propionic acid that has been reported to maintain the intestinal immune homeostasis.[34–36] However, both of these bacteria occupied less than 0.1 percent of the total bacteria in our analysis. Thus, the influence of these bacteria may be very small.

Through function inference analysis, we also identified that NEC cases showed impaired xenobiotic biodegradation and metabolism. Xenobiotics contain drugs environmental pollutants, dietary supplements, and food additives.[37] Dysregulation of xenobiotic metabolism may contribute to some intestinal diseases, such as ulcerative colitis.[38] Thus, this may be the reason for the increased rate of the dysregulation of xenobiotic metabolism in patients with NEC.

The most valuable result of this study is the difference in the patterns of microbiota that were observed between patients with NEC and controls at a gestational age of over 28 weeks, in contrast to those of extremely preterm babies. One limitation for this research is that we did not explore the differences in microbiota between different gestational groups in the NEC cases below 28 gestational weeks. However, we could compare the composition of the microbiota that we observed with those that have been reported previously. Another limitation is our research is a small scale single-center study. The local geographic area may also affect the constitution of micobiota. Moreover, the fact that the incidence rate for NEC over 28 weeks' gestation is low (previously reported as low as 0.2%) limits our sample size.[39] Thus, an independent multi-center validation cohort is necessary to confirm what we found in this study.

5. Conclusion

Lactobacillus and *Streptococcus_salivarius* are less abundant in NEC at over 28 gestational weeks than in controls. In addition, NEC cases presented with lower xenobiotic biodegradation and metabolism by functional prediction analysis.

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