Patterned progression of gut microbiota predisposes preterm infants to necrotizing enterocolitis and late onset sepsis: prospective pilot data from a non-Western population

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ABSTRACT

Background and Objectives
Intestinal microbiota dysbiosis might predispose preterm infants to necrotizing enterocolitis(NEC) and late onset sepsis(LOS). In this observational prospective study, we aimed to profile and compare postpartum microbiota progression patterns in non-Western preterm patients with either condition.
Methods
We enrolled preterm infants with gestational age less than 33 weeks and birth weight more than 950g, from July 2013 to December 2014. We began fecal sample collection from the the first stool after birth and prospectively collected until discharge. Bacterial V3 V4 region of 16s rRNA genes from each stool sample were amplified and sequenced. With the use of RM two-way ANOVA and Zero-Inflated Beta Random Effect models to account for repeated measures, we found out the development of NEC or LOS associated with gut bacterial communities.
Results
A total of 192 fecal samples from 24 patiens were studied, of whom four developed NEC, three LOS; the remaining 17 were used as controls. [The post-partum gut microbiota colonization started to diverge among NEC, LOS and their matched control groups, from the second week after birth. Microbiota of the LOS infants was the least diversified (Shannon index=1.66), while that of the control group was the most diversified(Shannon index=0.88, p=0.01). Potentially pathogenic genus Enterococcus (20.86%) and Staphylococcus (8.67%) were prominent in NEC patients and Klebsiella (42.15%) in LOS group. Both two groups addressed lower proportion of Lactococcus (7.98% and 13.76% in NEC and LOS group, respectively) than the control group (3.66%)].

Conclusions
Postpartum colonization pattern of gut microbiome might predispose preterm newborns to NEC or LOS, in which reduced diversity of the whole microbiota community and potentially pathogenic genus could have played an essential role in disease progression. Still, more studies are needed to identify etiological strains, underlying mechanisms and correspondent microbial patterns.

INTRODUCTION

Gut microbiota is a key contributor to human health and the dysbiosis of which are proven to be associated with multiple diseases, such as atherosclerosis(Tang et al., 2017), obesity(Bouter et al., 2017), neuropathy(Sarkar et al., 2016), liver diseases(Tilg et al., 2016), etc. Temporal colonization pattern of the intestinal microbiota during early stages of life also provided evidence of its association with early life events, including Type 1 diabetes(Giongo et al., 2011; Vatanen et al., 2018), asthma(Stokholm et al., 2018) and allergy(Madan et al., 2012; Savage et al., 2018). In light of less gut maturity, innate immunity and more C-setions birth modes, microbiome assembly in pretem infants often differs from that of term infants, especially presenting with lower *Bifidobacterium* spp. abundance and higher *Escherichia coli*, *Enterococcus* sp., and *Klebsiella pneumoniae*(Schwiertz et al., 2003; Bezirtzoglou et al., 2011). As as result, perturbation of postpartum microbiota haboring contributes to the vulnerablilty in preterm-associated health consequencese, such as necrotizing enterocolitis and late-onset sepsis.

Necrotizing enterocolitis, characterized by rapid progression, high morbidity and mortality, is one of the most devastating gestrointestinal neonatal emergencies, especially in preterm newborns; the etiologies of which remains elusive. Previous studies have suggested how intestinal microbiota pattern is implicated in the condition. Mai et al. reported an increase in the Proteobacteria and a decrease in the Firmicutes phyla during three to seven days prior to NEC onset (Mai et al., 2011). Zhou et al. reported a relatively higher abundance of Clostridium and Gamma-Proteobacteria in the proximity of NEC during early and late onset, respectively(Zhou et al., 2015). Dominance of (**?**) and/or Klebsiella pneumoniae was found to correlate with NEC risk in preterm infants

Among non-Western population, however, microbiota chronological dysbiosis preceding necrotizing enterocolitis or late onset sepsis remain scant so far. Hence, we conducted this prospective study with the aims to profile and compare post-postpartum pattern of intestinal microbiota in Chinese preterm infants who subsequently developed necrotizing enterocolitis and late onset sepsis, which may be critical in the etiopathogenesis of disease.

METHODS

Ethics

This study was approved by the joint committee of ethics of Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University (SCMCIRB-K2013022). Detailed written informed consent was obtained from the parents prior to fecal sample collection.

Patients

Newly born preterm infants with gestational age less than 33 weeks, birth weight over 950g were enrolled from Neonatal Intensive Care Unit at Shanghai Children's Medical Center from July 2013 to December 2014. The exclusion criteria were 1) diagnosed with early-onset sepsis, 2) hepatic diseases, 3) renal impairment (Cr >88 μM), 4) diagnosed with intestinal obstruction, 5) in foreseeable need of cardiovascular or abdominal surgeries (except for male circumcision or PDA ligation), 6) estimated parenteral support to supply over 50% of daily caloric intake for more than four days, 7) given intravenous antibiotics administration (except prophylactic regimen of cefotaxime, piperacillin-tazobactam and/or metronidazole), 8) history of oral antibiotics administration, 9) grossly bloody stools at admission, and 10) over five days old.

NEC cases were defined as infants who met the criteria for Stage II and Stage III NEC disgnosis(Bell et al., 1978), including radiographic intestinal dilation, ileus, pneumatosis intestinalis, and/or absent bowel sounds with or without abdominal tenderness, and/or mild metabolic acidosis and thrombocytopenia. LOS cases was diagnosed if 1)an infant had a positive hemoculture or other suspicious loci of infection after 72 hours of life, with septic signs/symptoms reviewed independently by at least two neonatologists, and had been treated with advanced antibiotics (e.g., Meropenem) after diagnosis. Infants with no infectious complications or sepsis were regarded as controls.

Sample collection and handling

Fecal samples collection began from neonatal meconium till discharge. Although we intended to collect fecal samples on a daily basis, due to working shifts and flexible clinical scheduling, we set seven days as the maximum interval between two collections from every infant. Every sample was collected within 30 minutes of defecation from infants' diaper with a sterile spatula. The samples were immediately placed in a cryogenic vial on dry ice and stored at 80°C within 30 minutes without additives. All samples were collected and stored before knowing the diagnosis of respective patients.

DNA extraction and quality control amplification and 16s rRNA gene sequencing

Microbial genomic DNA was isolated from each fecal specimen using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.) according to manufacturer's protocols. The concentration and purity of the DNA were determined by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and the DNA quality was checked by 1% agarose gel electrophoresis.

Broad-range PCR and High-throuput Sequencing of 16s rRNA gene amplicons

The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified from each sample using bacterialarchaeal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGG GTWTCTAAT-3') using thermocycler PCR system (GeneAmp 9700, ABI, USA). The PCR reactions were as follows: 3 min of denaturation at 95 °C, 27 cycles of 30 s at 95 °C, 30 s annealing at 55 °C and 45 s elongation at 72 °C, and a final extension at 72 °C for 10 min. The PCR reactions were performed in triplicate, with each 20 μL mixture containing 4 μL 5X FastPfu Buffer, 2 μL 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL FastPfu Polymerase and 10 ng template DNA. The PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocols.

Equimolar amounts of purified amplicons were pooled and pairedend sequenced (2 x 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The reads were de-multiplexed using the Illumina software and separate FASTQ files were generated for each specimen and deposited to the Sequence Read Archive NCBI under the BioProject accession PRJNA470548. Another public archive repository is available at figshare doi: 10.6084/m9.figshare.7205102

Raw Data Processing

After pyrosequencing, de-multiplexed sequence reads were subjected to quality filtering utilizing Trimmomatic software(version????)(Bolger et al., 2014), and were truncated at any site with an Phred score $<$20 over a 50bp-sized window; barcode matching with the primer mismatch from 0 to 2 nucleotides was adopted and reads containing ambiguous characters were removed. After trimming, FLASh(Fast Length Adjustment of Short Read)(Magoč and Salzberg, 2011), a read pre-processing software, assembled and merged the paired-end reads from fragments and generated $>$10 bp overlapped, with the dead match ratio 0.2. Unassembled reads were discarded.

To fairly compare all the samples at the same sequencing depth, the "sub.sample" command of mothur program(version1.30.1)(Schloss et al., 2009) was used for normalization to the smallest sample size. UCHIME Algorithm detected chimeric sequences, removed chimera to obtain effective reads, which were then sorted by cluster size and processed using Operational Taxonomic Units(OTUs) with 97% similarity cutoff using USEARCH v7(UPARSE version 7.1). The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm(Wang et al., 2007) against the Silva (SSU128)(Quast et al., 2012) 16S rRNA database using confidence threshold of 70%. Each sequence was assigned the taxonomy by QIIME(Caporaso et al., 2010). The representative sequences were allocated phylogenetically down to the domain, phylum, class, order, family, and genus levels. The relative abundance of a given taxonomic group was calculated as a percentage of the sequences number belonging to that group devided by the total number of obtained sequences.

Within-sample diversity(alpha diversty) analysis, including Shannon index and Observed species richness (sobs), were obtained using the "summary.single" command of mothur program(version1.30.1)(Schloss et al., 2009). Between-sample diversity(beta diversity) analysis was obtained estimating weighted UniFrac distances between samples.

Statistical and Bioinformatics Analysis

Demographics and Clinical Sample comparisons

Kruskal-Wallis test and Wilcoxon rank-sum test were used to identify statistically significant differences in continuous variables ,including gestational age, birth weight, age when the patients were diagnosed and length of hospitilisation. The χ^2, or Fisher's exact test were used to identify differences in gender composition. α level was considered 0.05 for all statistical tests . All statistical test not involving microbiome 16s rRNA sequencing data was performed using *"stats"* package using R(v.3.5.1).

Microbiota and Bioinformatics Analyses

Diversity Analyses α diversity

Disease-related Time Interval Determination Under the circumstance that the sampling and disease onset timepoints for each patient were not perfectly universal, to illustrated the continuous longitudinal and repeated nature of the sampling and its relationship with onset and progression of diseases, we seperated the whole sampling span into 7 time intervals:

1. early post-partum(EPP): within 3 days afterbirth
2. early pre-onset(EPO): from the end of EPP to at least four days befor disease onset
3. late pre-onset(LPO): from the end of EPO to the start of onset
4. early disease: first third interval of whole disease span
5. middle disease: second third interval of whole disease span
6. late disease: last third interval of whole disease span
7. post disease: from the end of disease to discharge timepoint

Modeling Strategies To compare the dynamics of microbiota diversity and relative taxonomic abundance preciding the disease, we took into account the EPP, EPO, LPO and ED interval among all patients and fit(Summlemantary matrix1). To compare the microbiome profile right after birth until disease alleviation, we selected EPP, EPO, LPO, ED, MD and LD interval of NEC and LOS patients(Supplementary matrix2). The average value of diversity indexes and relative abundances, if more than two were available within one analysis interval, of each patient was calculated. Zero-Inflated Beta Regression Model with Random Effects (ZIBR) and Linear Mixed-effects Model(LME) were used to test the association between OTU relative abundance and clinical covariates (diseases-related time intervals) for longitudinal microbiome data (**?**). *ZIBR* package were utilized for modeling.

ZIBR package were utilized for modeling. Figures were generated with the *"ggpubr"*(Kassambara, 2017) and *"ggplot2"*(Wickham, 2016) packages using R(v.3.5.1). Scripts for modeling and figures plotting, input and output files, figures are available at our github repository.

Scripts and Figures Archiving

Figures were generated with the *"ggpubr"*(Kassambara, 2017) and *"ggplot2"*(Wickham, 2016) packages using R(v.3.5.1)

RESULTS

A total of 7,472,400 optimized V3-V4 tags of 16s rRNA gene sequences were produced from 192 fecal samples, with an average length of 448 bp.(Table S1)

Patients characteristics

Totally 130 infants met the criteria of our study, and 1698 samples were collected from them in the neonatal intensive care unit (NICU) at Shanghai Children's Medical Center from July 2013 to December 2014. Among whom, we studied 192 fecal samples from 24 well-sampled preterm infants, including four subsequently diagnosed with NEC (2 in stage IIA and 2 in stage IIB), three with LOS, and 17 matched controls (Figure1 ,Table S2). Fecal samples were collected between days 1 and 69 of life. Sampling timepoints and numbers of samples varied among each infant.

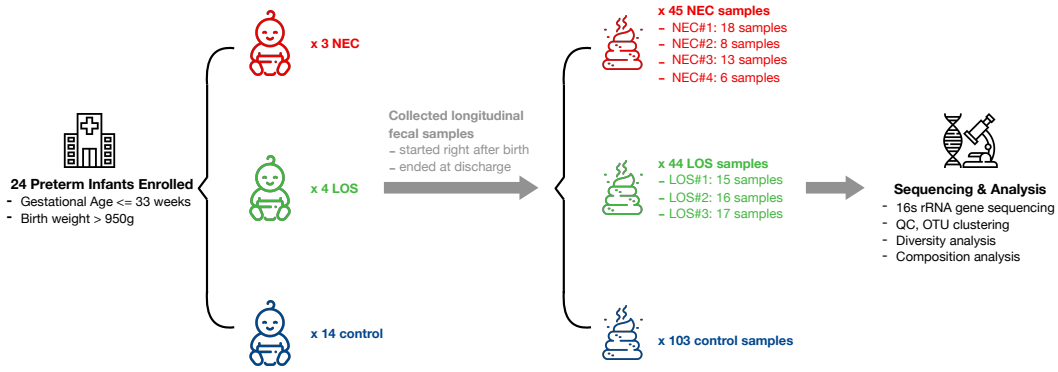

Figure 1. Flow of Study Design

All infants were delivered by cesarean section and fed on infant formula. No one was prescribed probiotics during hospitalization. Comparisons showed no significant difference in terms of gestational age, birth weight and gender proportions, diagnosed age among three groups (Table 1). Lenth of stay mong three groups was significantly different however rational since NEC and LOS patients usually require longer period of healthcare because of there worse conditions compared with the control group. All infants were delivered by cesarean section and fed on infant formula. No one was prescribed probiotics during hospitalization.

Microbiota Diversity

DISCUSSION

are consistent with the hypothesis that dysbiosis precedes this severe event. The

Our study has its limitations. We acknowledge that the sample size is limited since this study is single-center-based and the incidence of both diseases are relatively low: among the 1148 preterm infants admitted within July 2013 to December 2014, only five developed NEC. Furthermore, the resultant overfitting possibility inevitably rose up, which became the pitfall in understanding the true microbiota patterns preceding NEC and LOS.

CONCLUSIONS

Necrotizing enterocolitis, a worldwidely concern that threatern

ACKNOWLEDGMENTS

We appreciate the support from enrolled patients, their families, and all staffs at Shanghai Children's Medical Center.

SOME LATEX EXAMPLES

Use section and subsection commands to organize your document. LaTeX handles all the formatting and numbering automatically. Use ref and label commands for cross-references.

Figures and Tables

Use the table and tabular commands for basic tables — see Table 1, for example. You can upload a figure (JPEG, PNG or PDF) using the project menu. To include it in your document, use the includegraphics command as in the code for Figure 2 below.

Figure 2. An example image.

Item	Quantity
Widgets	42
Gadgets	13

Table 1. An example table.

Citations

LaTeX formats citations and references automatically using the bibliography records in your .bib file, which you can edit via the project menu. Use the cite command for an inline citation, like **?**, and the citep command for a citation in parentheses (**?**).

Mathematics

LaTeX is great at typesetting mathematics. Let $X_1, X_2, \ldots, X_n$ be a sequence of independent and identically distributed random variables with $\mathrm{E}[X_i] = \mu$ and $\mathrm{Var}[X_i] = \sigma^2 < \infty$, and let

$$S_n = \frac{X_1 + X_2 + \cdots + X_n}{n} = \frac{1}{n}\sum_{i}^{n} X_i$$

denote their mean. Then as n approaches infinity, the random variables $\sqrt{n}(S_n - \mu)$ converge in distribution to a normal $\mathcal{N}(0, \sigma^2)$.

Lists

You can make lists with automatic numbering ...

1. Like this,
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... or bullet points ...

- Like this,
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... or with words and descriptions ...

Word Definition

Concept Explanation

Idea Text

We hope you find writeLaTeX useful for your PeerJ submission, and please let us know if you have any feedback. Further examples with dummy text are included in the following pages.

METHODS

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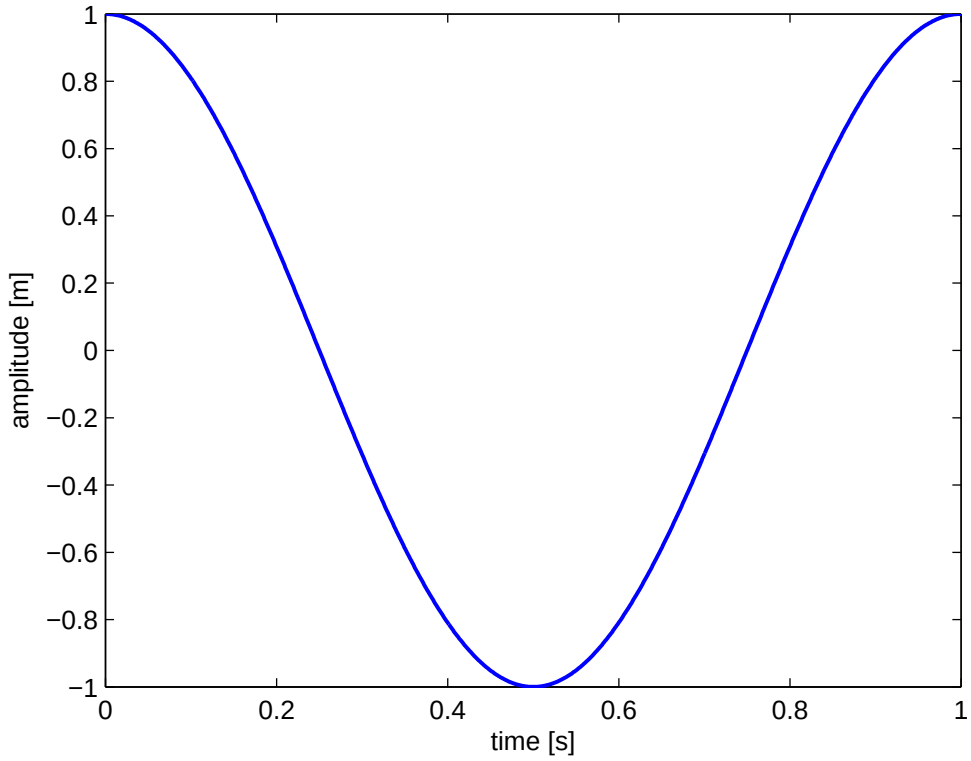

Figure 3. In-text Picture

Reference to Figure 3.

RESULTS AND DISCUSSION

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Nulla ac nisl. Nullam urna nulla, ullamcorper in, interdum sit amet, gravida ut, risus. Aenean ac enim. In luctus. Phasellus eu quam vitae turpis viverra pellentesque. Duis feugiat felis ut enim. Phasellus pharetra, sem id porttitor sodales, magna nunc aliquet nibh, nec blandit nisl mauris at pede. Suspendisse risus risus, lobortis eget, semper at, imperdiet sit amet, quam. Quisque scelerisque dapibus nibh. Nam enim. Lorem ipsum dolor sit amet, consectetuer adipiscing elit. Nunc ut metus. Ut metus justo, auctor at, ultrices eu, sagittis ut, purus. Aliquam aliquam.

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