

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

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27 **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, sequenced and analyzed.

## 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 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 interindividual variability in gut maturity, innate immunity, birth mode and enviromental exposures, preterm infants are predisposed to complications post-partum intestinal microbiota development

In preterm infants, necrotizing enterocolitis and late onset sepsis

Necrotizing enterocolitis, characterized by rapid progression, high morbidity and mortality, is one of the most devastating gastrointestinal neonatal emergencies, especially in preterm newborns. Previous studies have suggested well how intestinal microbiota pattern is implicated prior to, during and after the course of this 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. found a relatively higher abundance of *Clostridium* and Gamma-Proteobacteria in the proximity of NEC during early and late onset, respectively (Zhou et al., 2015).

late onset sepsis is

Among non-Western population, however, evidence (few studies on?) of microbiota chronological dysbiosis preceding necrotizing enterocolitis or late onset sepsis has been scant so far. Hence, conducting a prospective study among Chinese patients using high-throughput DNA sequencing, we aimed to profile and compare post-postpartum pattern of intestinal microbiota in 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 ( $\text{Cr} > 88 \mu\text{M}$ ), 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 diagnosis (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 patients' diaper with a sterile spatula. The samples were immediately placed in a cryogenic vial on dry ice and stored at  $80^\circ\text{C}$  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-throughput Sequencing of 16s rRNA gene amplicons

The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified from each sample using bacterial/archaeal 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  $95^\circ\text{C}$ , 27 cycles of 30 s at  $95^\circ\text{C}$ , 30 s annealing at  $55^\circ\text{C}$  and 45 s elongation at  $72^\circ\text{C}$ , and a final extension at  $72^\circ\text{C}$  for 10 min. The PCR reactions were performed in triplicate, with each 20  $\mu\text{L}$  mixture containing 4  $\mu\text{L}$  5X FastPfu Buffer, 2  $\mu\text{L}$  2.5 mM dNTPs, 0.8  $\mu\text{L}$  of each primer (5  $\mu\text{M}$ ), 0.4  $\mu\text{L}$  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 paired-end 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 archive repository is available at figshare doi: 10.6084/m9.figshare.7205102

## Bacterial Species Classification Reference Set Creation

### Statistical Analysis

#### 0.0.1 Demographics and Clinical Sample comparisons

Non-parametric tests, including Kruskal-Wallis test and Wilcoxon rank-sum test (SPSS Version ???(SPSS Inc. )) were used to compare gestational age, birth weight, age when the patients were diagnosed as NEC

131 or LOS and length of stay at  $\alpha$  level of 0.05 among three groups. !!!!SPSS version check!!!Fisher's exact  
132 test

### 133 *0.0.2 Microbiota Analyses*

## 134 RESULTS

### 135 Patients and samples characteristics

## 136 DISCUSSION

137 Previous studies have

## 138 CONCLUSIONS

139 Necrotizing enterocolitis, a worldwide concern that threatern

## 140 ACKNOWLEDGMENTS

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

## 143 SOME L<sup>A</sup>T<sub>E</sub>X EXAMPLES

144 Use section and subsection commands to organize your document. L<sup>A</sup>T<sub>E</sub>X handles all the formatting and  
145 numbering automatically. Use ref and label commands for cross-references.

### 146 Figures and Tables

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



**Figure 1.** An example image.

## 150 Citations

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

Item	Quantity
Widgets	42
Gadgets	13

**Table 1.** An example table.

## 154 Mathematics

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

$$S_n = \frac{X_1 + X_2 + \dots + X_n}{n} = \frac{1}{n} \sum_i^n X_i$$

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

## 157 Lists

158 You can make lists with automatic numbering ...

- 159 1. Like this,
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161 ... or bullet points ...

- 162 • Like this,
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164 ... or with words and descriptions ...

165 **Word** Definition

166 **Concept** Explanation

167 **Idea** Text

168 We hope you find write $\text{\LaTeX}$  useful for your PeerJ submission, and please let us know if you have any  
169 feedback. Further examples with dummy text are included in the following pages.

## 170 METHODS

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Reference to Figure 2.

## RESULTS AND DISCUSSION

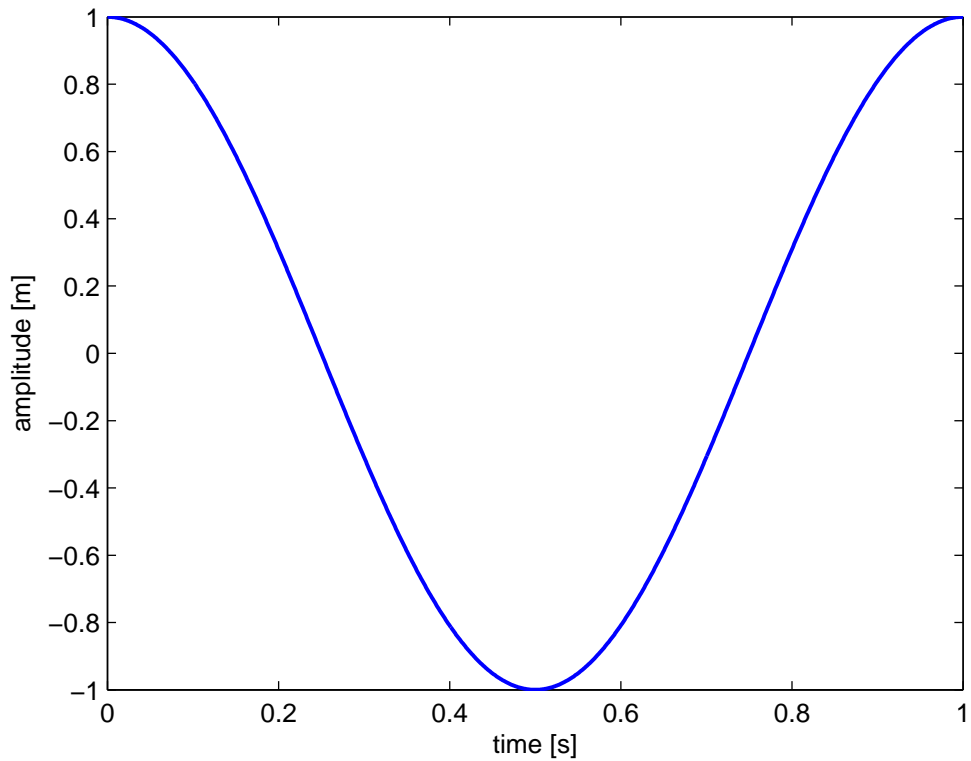
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**Figure 2.** In-text Picture

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279 ultrices eu, sagittis ut, purus. Aliquam aliquam.

## 280 ACKNOWLEDGMENTS

281 So long and thanks for all the fish.

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