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Patterned progression of gut microbiota predisposes preterm infants to necrotizing enterocolitis and late-onset sepsis

Jiayi Liu [1], **Jianhua Sun** [2], **Yuqing Li** [1], **Yi Feng** [1], **Liya Pan** [1], **Zhoulonglong Xie** [3], **Zhilong Yan** [3], **Jianhua Zhao** [4], **Li Hong** [Corresp. 1]

[1] Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiaotong University, Shanghai, China
[2] Department of Neonatology, Shanghai Children's Medical Center, School of Medicine Shanghai Jiaotong University, Shanghai, China
[3] Department of Surgery, Shanghai Children's Medical Center, School of Medicine Shanghai Jiaotong University, Shanghai, China
[4] Shanghai Majorbio Bio-pharm Technology Co., Ltd, Shanghai, China

Corresponding Author: Li Hong
Email address: hongli@scmc.com.cn

Background and objectives. Gut microbiota dysbiotis is frequently observed during the course of preterm complications including necrotizing enterocolitis (NEC) and late-onset sepsis (LOS). However, specific after-birth bacterial pattern of preterm newborns with these complications hasn't been well established. Here, we firstly profiled postpartum pattern progression of intestinal microbiome in these two diseases, with the aim of understanding their etiologic microbiota profiles from a dynamic perspective.

Methods. 24 preterm newborns were enrolled, among whom four subsequently developed NEC, three LOS, ???17 were healthy controls. Starting from the first stool after birth and continuing till discharge, 192 longitudinal fecal samples were prospectively collected from all patients. Bacterial V3~V4 region of 16s rDNA from each stool sample were amplified and sequenced.

Results. The postpartum gut microbiota colonization started to diverge among NEC, LOS and their matched control groups, from the second week after birth. Late-onset sepsis infants held the least diversified gut microbiota (Shannon index=1.66), with the control group held the most diversified one (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. After-birth colonization pattern of gut microbiome might predispose preterm newborns to necrotizing enterocolitis or late-onset sepsis, 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.

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[1] Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiaotong University, Shanghai,China
[2] Department of Neonatology, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiaotong University, Shanghai,China
[3] Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiaotong University, Shanghai,China
[4] Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiaotong University, Shanghai,China
[5] Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiaotong University, Shanghai,China
[6] Department of Surgery, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiaotong University, Shanghai,China
[7] Department of Surgery, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiaotong University, Shanghai,China
[8] Shanghai Majorbio Bio-pharm Technology Co., Ltd, Shanghai,China
[9] Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiaotong University, Shanghai,China

Corresponding Author:
Li Hong
No. 1678 Dongfang Rd, Shanghai Pudong, 200127,
Email address: hongli@scmc.com.cn

ABSTRACT

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INTRODUCTION

Gut microbe colonization starts right after a fetus is born out of the womb (Sommer & Backhed, 2013). Until the age of 1 to 2, the consortium of gut microbes developed into a relative stable microenvironment(Matamoros, Gras-Leguen, Le Vacon, Potel, & de La Cochetiere, 2013). During its colonization, its patterns are unstable and hyper variable (Rodriguez et al., 2015). For preterm infants, who are prone to disrupted microbiota exposure, are vulnerable to multiple preterm short-term complications, including necrotizing enterocolitis (NEC) and late-onset sepsis (LOS).

Necrotizing enterocolitis (NEC), as one of the most devastating gastrointestinal conditions in newborn infants, has been studied long for its etiology. The fact that preterm infants account for a large portion of NEC patients(Rees, Eaton, & Pierro, 2010) lead to the hypothesis that relatively immature intestinal epithelial integrity, disrupted barrier function and intestinal microbiota dysbiosis are causative for NEC onset(Halpern & Denning, 2015). Moreover, the fact that germ-free animals do not develop necrotizing enterocolitis also implicates the role of intestinal dysbiosis during the course of the disease (Morowitz et al., 2010; Musemeche, Kosloske, Bartow, & Umland, 1986).

Due to its multifactorial pathogenesis, it remains elusive for physicians to perform primary prevention in time before recognizing the very early signs and symptoms(Chen, Chung, Chang, & Lin, 2014). Although previous clinical studies have implicated dysbiotic status within the intestine of preterm infants (Hosny, Cassir, & La Scola, 2017; Rozé et al., 2017; Sim et al., 2015; Underwood et al., 2014), progressive effects of the microbiota on disease onset are still unknown. So far, three studies have explored the fecal microbiota in premature infants preceding NEC onset, especially in VLBW infants (Mai et al., 2011; Warner et al., 2016; Zhou et al., 2015). Mai et al. detected an increase in the *Proteobacteria* and a decrease in the *Firmicutes* phyla during three to seven days before 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).

Besides, increasing evidence is showing that altered intestinal flora influence enteric infections(Sekirov & Finlay, 2009), and are associated with higher prevalence of septic complications including bacteremic enteritis and higher risk of mortality in patients with systemic inflammatory response syndrome (Shimizu et al., 2011). In preterm infants, an overgrowth of certain bacterial genera that overlap with the causative genera of the condition has been reported in the intestine prior to late-onset sepsis (LOS) (Stewart et al., 2017). Dysregulation of the immune system could interact with the local gut microbiome (Sekirov & Finlay, 2009), so that we hypothesize that late-onset sepsis in preterm infants could correlate with gut microbiota dysbiosis. The human microbiome project, with the help of advanced sequencing (Fraher, O'Toole, & Quigley, 2012; Gevers, Pop, Schloss, & Huttenhower, 2012), and machine learning in genetics and genomics (Libbrecht & Noble, 2015) have enabled quantitative and comprehensive analyses of uncultivable intestinal bacteria (Saxena & Sharma, 2016).

Although early recognition and aggressive treatment of these two prematurity related short-term complications have improved over decades, it still accounts largely for long-term morbidity and mortality in newborn infants. The aim of our study was to describe, analyze and compare after-birth gut microbiome pattern of preterm infants who subsequently developed NEC or LOS, in order to determine the pathogenesis and etiology of these conditions.

MATERIALS & 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

Preterm infants with gestational age less than 33 weeks were enrolled in the study shortly after birth at the Neonatal Intensive Care Unit (NICU) in Shanghai Children's Medical Center from July 2013 to December 2014. The exclusion criteria were 1) early-onset sepsis, 2) hepatic diseases, 3) renal impairment (Cr> 88μM), 4) intestinal obstruction, 5) in foreseeable need of large 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) oral antibiotics administration, 9) grossly bloody stools at admission, and 10) over five days old.

Every infant was prospectively inspected and assessed for systemic, abdominal and radiographic signs, and diagnosed and classified based on the modified Bell staging criteria (Bell et al., 1978). NEC was diagnosed as Stage II, with radiographic intestinal dilation, ileus, pneumatosis intestinalis, and/or absent bowel sounds with or without abdominal tenderness, and/or mild metabolic acidosis and thrombocytopenia. LOS was diagnosed if the infant had a positive hemoculture or other suspicious loci of infection after 72 h 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 were collected from the infants beginning with their 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 2 h of defecation, either from the diaper or directly from around the perianal skin surface 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 before extraction.

DNA extraction and 16s rRNA sequencing

Microbial 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. 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'-GGACTACHVGGGTWTCTAAT-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 paired-end sequenced (2 × 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 No. PRJNA470548 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA470548).

Data processing

Raw FASTQ files were de-multiplexed, quality-filtered by Trimmomatic and merged by FLASH using the following criteria: 1) truncated reads at any site receiving an average quality score <20 over a 50 bp sliding window, 2) maximum 2 nucleotide mismatching of primers, with removal of reads containing ambiguous bases, and 3) sequences with longer than 10 bp overlap. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rRNA database using confidence threshold of 70%.

RESULTS

Patient and sample characteristics

A total of 1148 pre-term infants were admitted from July 2013 to December 2014, of which five developed NEC during that period; the incidence rate of 4.4% is in accordance with the overall rate (Llanos et al., 2002). One hundred and thirty infants met the criteria of our study, and 1698 samples were collected from them. Finally, 24 well-sampled infants, including four with NEC (2 in stage IIA and 2 in stage IIB), three with LOS, and 17 matched controls (Table S1) were selected, and a total of 192 samples were characterized using 16S rRNA sequencing. The mean gestational age of the infants was 30.5 weeks (28 to 33 weeks) and mean birth weight was 1440 g (945g to 1950g). There were three females and one male in the NEC group, two females and one male in the LOS group, and nine females and eight males in the Control group. All infants were delivered by cesarean section and fed on infant formula. The average NEC onset time was 16 days after birth (11 to 19 days) and the average day of life when diagnosed with LOS was 12 days. A demographic comparison of the three groups (Table 1) showed no significant difference in terms of gestational age, birth weight and gender.

Once NEC was suspected, the infant was immediately given supportive care and antibiotic therapy. Supportive care included bowel rest with cessation of enteral feeding, TPN, fluid replacement, correction of metabolic acidosis, etc. The empiric antibiotic combination used in NICU to prevent foreseeable concomitant bacteremia is piperacillin-tazobactam, cefotaxime, and metronidazole. Three infants recovered and restarted their enteral feeding after 28 and 15 days management, respectively. One infant died of NEC after her family consented to terminate medical treatment and declined surgical intervention (FIG. 1).

One infant from the LOS group was diagnosed with *Klebsiella pneumoniae* bacteremia based on the positive results of bacterial hemoculture and was given meropenem for 21 days. Another infant was diagnosed with *K. pneumoniae* and *Pseudomonas aeruginosa* bacteremia and given meropenem for 38 days. The third newborn given meropenem for 13 days since he did not react well to cefotaxime.

No patient was administered with probiotics before or during hospitalization.

Taxonomic Analysis

In order to determine the causative species of NEC and LOS, and compare the abundance of different bacteria in the three groups, we analyzed their microbiota composition at the OTU, phylum, class, order, family, and genus level. The control group had the maximum OTU count of 933, indicating greater bacterial diversity under relatively healthy conditions. The least diversified intestinal microbiota was in the LOS group (OTU counts = 567), suggesting a significant impact of advanced antibiotics on the diversity of gut intraluminal micro-environment (Table 2.)

Although *Firmicutes*, *Proteobacteria,* and *Actinobacteria* were the dominant phyla along the intestinal epithelium in all three groups, the mean relative abundance of *Firmicutes* was the highest in the NEC group (59.79%) compared to the other two groups (39.54% in the LOS group and 44.88% in the control group) (FIG. 2, Dataset S2.a). The *Actinobacteria* abundance in the NEC group (1.10%) was similar to that of the LOS group (0.63%), both of which were lower compared to that in the control group. In contrast, *Proteobacteria* abundance was lower in the NEC group compared to the other two groups.

Klebsiella, *Enterococcus,* and *Lactococcus* were the most prominent genera across the three groups (FIG. 3, Dataset S2.b). *Klebsiella* abundance was highest in the LOS group (42.5%), which is plausible since it is the causative pathogen (Table S1.) On the other hand, the NEC group had the highest abundance of *Enterococcus*, *Streptococcus* and *Staphylococcus,* representing 20.86%, 8.67% and 9.01% of all OTUs, respectively. Furthermore, the probiotic *Lactococcus,* along with *Staphylococcus* and *Pseudomonas,* were the least abundant in the LOS group (one way ANOVA, $p = 0.0006$), while the pathogenic *Escherichia-Shigella* was the most abundant group (12.99%), although the difference was weakly significant (one-way ANOVA, $p = 0.11$) (Dataset. S2.b).

Longitudinal Bacterial Colonization Variation within and among Three Groups

We analyzed the overall gut microbiome composition of the three groups on a weekly basis using the principal coordinate analysis (PCoA) of weighted UniFrac distances to compare their diversities. The intestinal microbiota significantly differed overall between the groups (FIG. S1a~c, a. Anosim, r=0.40, p=0.001; b. Anosim, r=0.36, p=0.001; c. Anosim, r=0.40, p=0.001), which was consistent with previous studies (Moles et al., 2013).

Cross-sectional comparison of the three groups, as well as between any two groups, showed that the gut microbiota did not differ significantly before the 14th and after the 21st day of life. From the 14th to 21st day postnatally, the first and second principal coordinates accounted for 71.54% and 8.61% of the inter-sample variance, respectively (FIG. 4a, b, d, and e, a. Anosim, r=0.02, p=0.797, b. Anosim, r=0.12, p=0.079, d. Anosim, r=0.21, p=0.087, e. Anosim, r=0.11, p=0.183). The phylogenetic composition of the intestinal microbiota also differed significantly among the NEC, LOS and control groups during the third week after birth (FIG. 4c, Anosim r=0.32 p=0.001), suggesting the likely association of microbial pattern and the onset of NEC. Furthermore, the comparison of the bacterial composition of the NEC and control groups showed significant differences from the 21st to 28th day of life (FIG. S2, ANOSIM r=0.27, p=0.030), indicating persistent dysbiosis after the disease onset, although the divergence after the 28th day of life was less significant (Anosim, r=0.22, p = 0.052). Considering the potential influence of individual differences, we also compared the microbiome composition of singular cases using PCoA on a bi-weekly basis and found a significant chronological pattern of intestinal microbiome after birth for three NEC infants (FIG. 5a~d; a. Anosim, r=0.36, p=0.01, b. Anosim, r=1.00, p=0.035, c. Anosim, r=0.610, p=0.014, d. Anosim, r=0.09, p=0.800).

DISCUSSION

More than 10 trillion microbes reside in the human intestinal epithelia (Ley, Peterson, & Gordon, 2006), which shapes many aspects of human health (Sekirov, Russell, Antunes, & Finlay, 2010). The disruption of the intestinal microenvironment might hinder this homeostasis and result in various disease including obesity (Liu et al., 2017), IBD (Ley et al., 2006), cardiovascular diseases (Z. Wang et al., 2011), as well as mental disorders (Rogers et al., 2016), among others. Although evidence is still lacking regarding the long-term impact of early bacterial colonization on human health, it is still a valuable parameter in elucidating newborns' health, or alternatively their predisposition to diseases. So far, little studies have focused on either NEC or LOS in adults, and that on systemic inflammation or septic conditions are limited. The most recently identified causative genus of NEC is *Clostridium* (Hosny et al., 2017). However, no specific pathogen has been implicated in its etiology, and a 2013 report suggested that early dysbiosis during 4–9 days of life may be strongly involved in NEC pathophysiology (Morrow et al., 2013). Therefore, instead of searching for a single pathogen, it is more logical to look for bacterial patterns to provide new insights into their etiology.

In this study, we described and compared the chronological postnatal intestinal microbiota of subsequently developed NEC and LOS patients and their matched controls. We sequenced 16S rDNA from a total of 192 intestinal specimens: 46 from the NEC patients, 42 from the newborns with LOS, and 103 from the control group. A total of 7, 472, and 400 sequences were generated using the Illumina-MiSeq platform for all samples and helped detect rare OTUs that would have been missed with less extensive sequencing platforms.

In contrast to an early study, which indicated an abundance of *Proteobacteria* during NEC onset in the preterm infants, our data showed high abundance of *Firmicutes* (59.79%) in NEC patients while *Proteobacteria* in the control and LOS groups. In addition, NEC patients addressed the highest mean abundance of *Enterococcus* and *Staphylococcus*, and increasing abundance of *Klebsiella* and *Staphylococcus* compared to the control group within 1–3 and 3–7 days, respectively, prior to the onset (FIG. S3). These findings indicate a possible role of the potentially pathogenic *Staphylococcus* genus in the pathogenesis of NEC along with conventional pathogenic species such as *S. aureus* and *S. haemolyticus*. The trend of increasing abundance of *Klebsiella,* a gram-negative genus, indicated a role of LPS, which is a ligand for TRL–4 and facilitates bacterial transcytosis in Caco–2 cells in vitro (Panigrahi et al., 1996) and increases bacterial translocation (Deitch, Berg, & Specian, 1987).

In addition, prominent *Enterococcus* in the LOS group suggests a considerable dysbiogenesis impact of advanced antibiotic therapy. Although the exact microbiota composition in sepsis remains to be elucidated through animal studies (Hand et al., 2012; Sekirov & Finlay, 2009), the mechanisms underlying sepsis, immunological responses, and microbiota disruption and their consequences may facilitate our understanding toward sepsis progression and help developing strategic prophylactic solutions. In a clinical study on adults, a decrease in total obligate anaerobes and an increase in pathogenic bacteria in the gut were associated with higher septic complications and mortality in patients with SIRS (Shimizu et al., 2011). Although our relatively small sample size showed only limited evidence of the involvement of a specific strain in sepsis, it is noteworthy to illustrate the host-microbiota-pathogen interplay in pediatric patients with sepsis.

Earlier reports have shown postpartum colonization of intestinal microbiota in preterm infants (Dutta, Ganesh, Ray, & Narang, 2014) and associated abnormal early colonization with hospital-related events including mechanical ventilation and antibiotic therapy (Moles et al., 2013). Another study found that the rate of colonization and abrupt population changes in the gut microbiota of preterm infants might be a result of the birthing mode, type of feeding, and gestational age. A succession of bacterial classes starting from *Bacilli*, *Gammaproteobacteria*, to *Clostridia* has been observed in preterm infants (La Rosa et al., 2014), and was also seen in most of our study subjects including the LOS patients (FIG. S4). We corroborated the postnatal bacterial colonization pattern on a weekly basis and found significant differences between the intestinal microbiota of each group. Furthermore, our data showed that intestinal microbiota differed significantly between the NEC and control groups after the 14th day of life, despite similar colonization pattern during the first 14 days after birth (FIG. 4c, Anosim r=0.32

p=0.001), strongly indicating an association between dysbiosis and NEC onset. Finally, the difference in intestinal microbiota from the 21st to 28th day postnatal (<FIG. S2, ANOSIM r=0.27, p=0.030) indicated persistent dysbiosis during NEC progression, which is consistent with previous cross-sectional studies (Y. Wang et al., 2009).

CONCLUSIONS

Scientists have been striving to explore the microbial etiology of NEC and sepsis for years. Although specific pathogens or microbial patterns have not been elucidated, the existing research has helped improve the clinical strategies and minimized the complications in preterm infants. This study, meanwhile, is the first one which described, compared and analyzed the role of afterbirth dynamic pattern of postpartum gut microbiota in preterm infants with NEC and LOS using high-throughput sequencing. Our findings support the dysbiosis hypothesis that an "aberrant colonization development" of intestinal microbiota acts as the trigger in the progression of NEC in preterm infants.

Further, this study is also consistent with previous reports that disrupted gut flora is crucial for LOS progression, and antibiotics, specifically glycopeptide ones, could reduce the abundance of the gut microflora in preterm infants. This study, however, has its limitations. For instance, all infants in this study were fed on a formula diet, and thus the protective impact of human breast milk could not be evaluated. In addition, the sample size of the NEC and LOS groups was very small. Since an empirical antibiotics regime was administered to the infants, it was difficult to explain the extent to which the antibiotics affected the intestinal microbiota. Therefore, our findings still need to be validated in further studies with larger sample sizes and more prudent methodologies. Based on the current findings, future studies should include more cases for the identification of a "highly risky" microbial pattern for NEC and LOS based on metagenomic sequencing. Our study, along with others, provide a rationale for discerning the human gut microbiome in the pathogenesis of NEC and LOS in preterm infants to develop more accentuated prophylactic interventions and diagnostic tools, and more effective treatment to reduce the morbidity and severity of these conditions.

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Table 1(on next page)

Demographics of NEC, LOS and Control groups

There was no statistical differences in gestational age, birthweight, gender and age when diagnosed among NEC, LOS and Control group. The mean length of stay differs among the three groups, which is within our expectation because it takes longer time for NEC or LOS patients to recover. Besides, no difference was observed when it was compared between LOS and NEC group.

1. GAge: Gestational Age = mean (range);

2. BW: Birth Weight = mean (95% CI);

3. Age when diagnosed = mean (range);

4. Gender = number (%);

5. Length of Stay = mean (95% CI).

Table 1. Demographics of NEC, LOS and Control groups.
There was no statistical differences in GA, BW, gender and diagnosing age among NEC, LOS and Control group. The mean length of stay differs among the three groups, however, no difference was observed when it was compared between LOS and NEC group.

	NEC (n=3)	LOS (n=4)	Control (n=17)	Statistical test	p-value
GAge (weeks)[1]	29.25 (29-30)	30.00 (29-31)	30.94 (28-33)	Kruskal-Wallis test	0.074
BW (grams)[2]	1416.3 (773.4-2149.1)	1141.7 (633.4-1649.9)	1527.4 (1391.6-1663.1)	Kruskal-Wallis test	0.111
Gender[3]				Fisher's exact test	0.822
Female	3 (75%)	2(67%)	9 (53%)		
Male	1 (25%)	1 (33%)	8 (47%)		
Age when Diagnosed (days)[4]	16 (11-19)	12 (10-22)	NA	Wilcoxon rank-sum test	0.629
Length of Stay[5] (days)	54.3 (13.5-95.0)	60.0 (24.8-95.2)	32.9 (26.3-39.5)	Kruskal-Wallis test	0.046
Total Number of Samples	46	42	103	/	/

[1] GAge: Gestational Age = mean (range);
[2] BW: Birth Weight = mean (95% CI);
[3] Age when diagnosed = mean (range)
[4] Gender = number (%);
[5] Length of Stay = mean (95% CI)

Table 2(on next page)

Total number of taxons in three groups

The control group addressed the most taxons from phylum to species level. Taxon numbers of the LOS group is the least among three groups, indicating the least diversified intestinal microbiota in severely infected patients.

Table 2. Total number of taxons in three groups.

The control group addressed the most taxons from phylum to species level. Taxon numbers of the LOS group is the least among three groups, indicating the least diversified intestinal microbiota in severely infected patients.

	Phylum	Class	Order	Family	Genus	Species	OTU
Total	24	43	93	166	350	537	1109
NEC	18	34	72	128	271	411	744
LOS	14	26	47	96	204	315	567
Control	21	39	85	153	318	477	933

Figure 1

Onset and Relieving Age in NEC and LOS Groups, in comparison with a mean length of stay of Control group.

The average onset age for NEC was 16th day of life and 12th day for LOS patients. For Control group, the mean length of stay was 24 days.

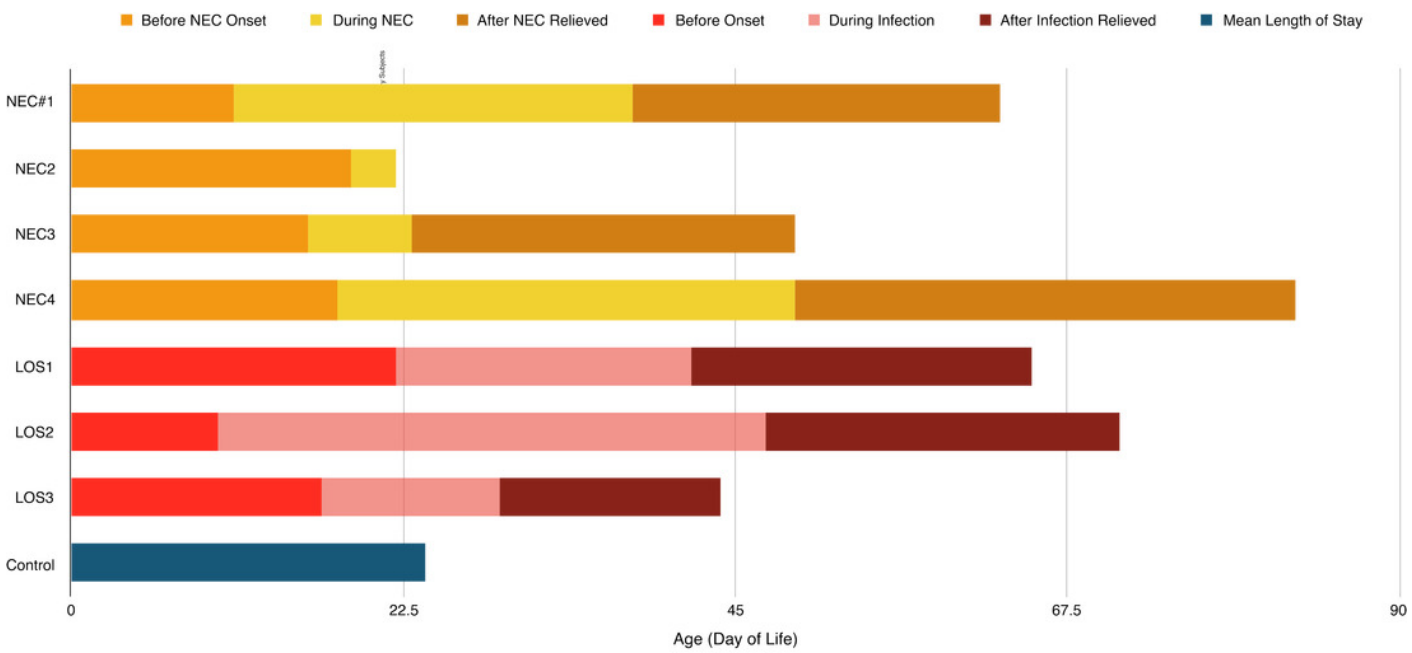

Figure 2

figure2

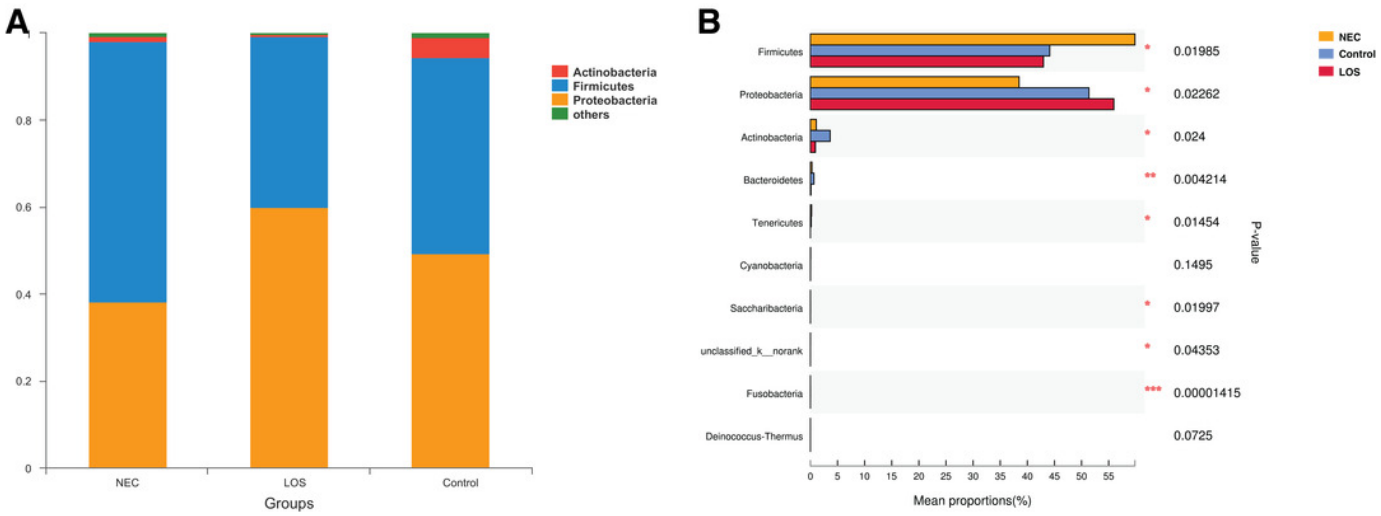

Figure 3

Relative abundance of genus in intestinal microbiota.

(A) Klebsiella was the most prominent genus in LOS patients (42.5%). NEC addressed the most abundant Enterococcus (20.86%), Streptococcus(8.67%) and Staphylococcus (9.01%) in the intestinal microbiota. **(B)** Abundance of Klebsiella, Lactococcus and Pseudomonas were significantly different among three groups

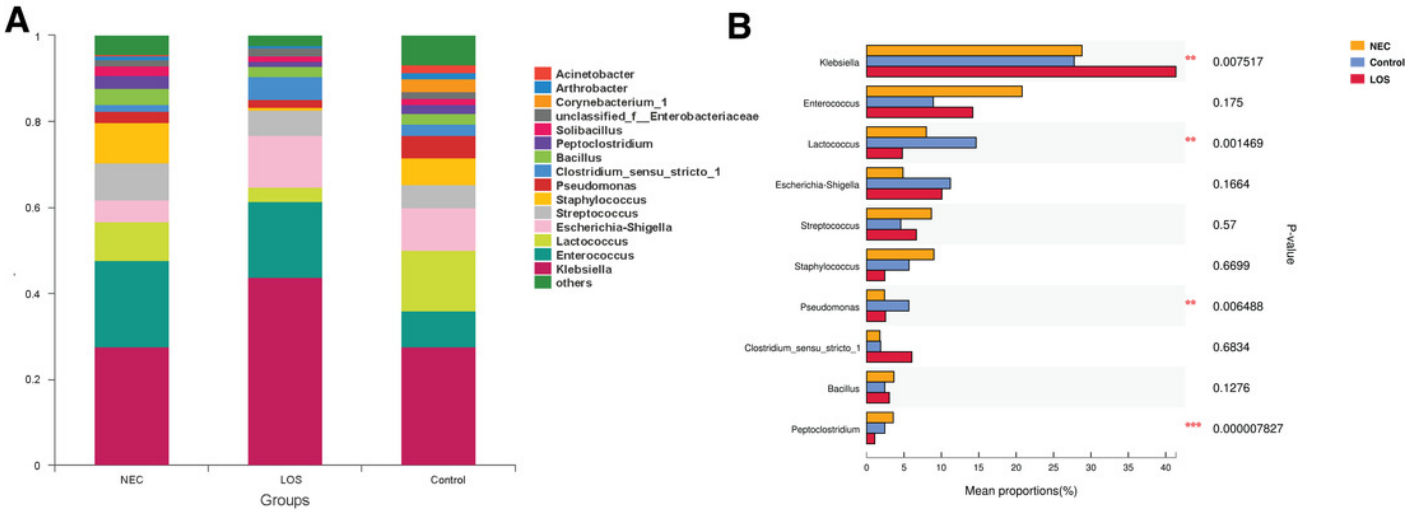

Figure 4

The PCoA of microbiota colonization among the three groups on a weekly basis after birth.

The colonization patterns during the first two weeks after birth (0 to the 14th day of life) were similar among the three groups. However, from the 21st-day after birth gut microbiota colonization of NEC, LOS and the Control group differed, which was the time when most NEC occurs.

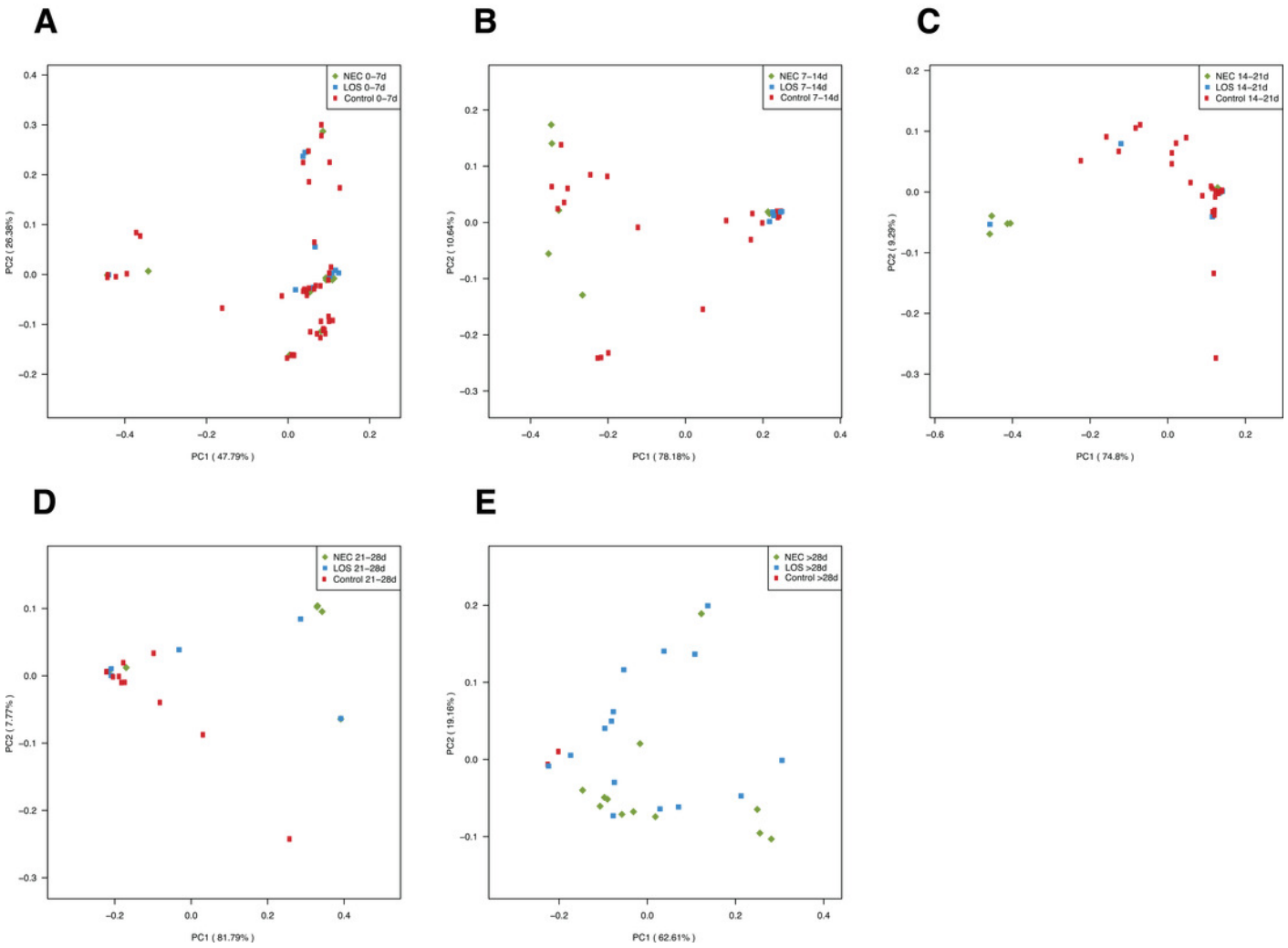

Figure 5

The PCoA of afterbirth colonization in each NEC patient.

(A) NEC patient No. 1, (B) NEC patient No. 2, (C) NEC patient No. 3, (D) NEC patient No. 4.