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

Jiayi Liu[1], Jianhua Sun[2], Yuqing Li[3], Yi Feng[4], Liya Pan[5], Zhoulonglong Xie[6], Zhilong Yan[7], Jianhua Zhao[8], and Li Hong[9]

[1]Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University, Shanghai, China
[2]Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University, Shanghai, China
[3]Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University, Shanghai, China
[4]Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University, Shanghai, China
[5]Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University, Shanghai, China
[6]Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong University, Shanghai, China
[7]Department of Clinical Nutrition, Shanghai Children's Medical Center, School of Medicine Shanghai Jiao Tong 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 Jiao Tong University, Shanghai, China

Corresponding author:
Li Hong[9]

Email address: hongli@scmc.com.cn

ABSTRACT

Recent studies have associated necrotizing enterocolitis (NEC) and late-onset sepsis(LOS), two common complications of preterm births, to gut microbiota dysbiosis. Similar studies in Asian population remain scant. In this pilot study, we profiled gut microbiota of four NEC and three LOS patients among 24 preterm Chinese infants starting from birth until decease or discharge. The microbial community diversities in case stools differed significantly from those in control ones. These differences emerged only after the third day of life and persisted throughout the courses of both NEC and LOS. In ZIBR models, higer Bacillus (p = 0.032) and lower Solibacillus (p = 0.047) were associated with both NEC and LOS onset. *Enterococcus*, *Streptococcus* and *Peptoclostridium* were prominent in NEC progression and *Klebsiella* in LOS cases. Consistent (not really...) with studies in European and American countries, these changes in diversity and composition preceded the onset of diseases and might have played an essential role in disease progression. [*Inconsistant with studies in European an American countries, these results should be a starting point for further studying of microbial factors involved in preterm-associated complications specifically within Chinese population...???*] These results warrant further studies to identify correspondent microbial patterns, etiological strains and underlying mechanisms.

INTRODUCTION

The gut microbiota is a key contributor to human health. Imbalance of the microbial community, termed dysbiosis, is associated with various diseases, such as obesity and diabetes(Bouter et al., 2017; Rosenbaum et al., 2015; Winer et al., 2016; Cani, 2019; Zmora et al., 2019), immunity related diseases(Vogelzang et al., 2018; Pronovost and Hsiao, 2019; Vatanen et al., 2016), neurodevelopmental disorders(Sampson and Mazmanian, 2015; Pronovost and Hsiao, 2019), cardiovascular diseases(Tang et al., 2017; Jie et al., 2017; Jonsson and Bäckhed, 2017) and cancers(Gagliani et al., 2014; Irrazábal et al., 2014; Sears and Garrett, 2014).

The microbiota assembly in newborn infants undergoes dynamic changes in composition, abundance and diversity before reaching a homeostasis status at around three years old(Stewart et al., 2018). Temporal colonization pattern of the intestinal microbiota during early stages of life also provided evidence of its association with early life health events, including Type I diabetes(Giongo et al., 2011; Vatanen et al., 2018), asthma(Stokholm et al., 2018) and allergy(Madan et al., 2012a; Savage et al., 2018).

In preterm infants, common practices including cesarean sections, formula feeding, sterile incubator nursing and extensive use of broad-spectrum antibiotics may limit normal micrbiota acquisition and development(Shin et al., 2015; Deweerdt, 2018). Thus, microbioal dysbiosis, characterized by fewer species, less diversity, higher variability and more abundant potential pathogens such as *Escherichia coli*, *Enterococcus* sp., and *Klebsiella pneumoniae* (Schwiertz et al., 2003; Bezirtzoglou et al., 2011) is prevalent among preterm infants. It may then initiate a cascade of mucosal barrier integrity impairment leading to microbiota translocation or microbial toxins leakage(Cernada et al., 2016; Sharon et al., 2015). The normal gut microbiota breakdown or microbes harboring anomalies, as as result, has been implicated as key factors to the vulnerabilities to preterm-associated health consequences, such as necrotizing enterocolitis(NEC), sepsis and systemic inflammatory response syndrome(Sharon et al., 2015).

Necrotizing enterocolitis is characterized by unpredictive, rapid ischemic necrosis of intestinal mucosa. The morbidity(2% - 7%) and mortality(15% -30%)(Stoll et al., 2015) are exceptionally high among preterm newborns(Neu and Walker, 2011). Its etiologies remain largely unknown and likely to be multifactorial. Previous studies in European and American countries have suggested the contributing role of microbial dysbiosis in NEC onset. Microbial community diversity reduction and unusual species colonization are observed during NEC(Jacquot et al., 2011; Warner et al., 2016). No causative species have been identified so far. However, increase in Proteobacteria phyla and decrease in Firmicutes was observed before its onset(Mai et al., 2011; Zhou et al., 2015). In addition, blooming of *Gammaproteobacteria* and under-representing in *Negativicutes* was in chronological association with the condition(Warner et al., 2016).

Late onset sepsis (LOS) is another common life threatening disease of preterm infants. It is commonly defined as systemic infection with isolation of a bacterial pathogen from the bloodstream after 72 hours of life(Rao et al., 2016; Pickering et al., 2012). Preterm infants have an immature intestine with underdeveloped peristalsis, barrier function and immunity, allowing opportunistic and potential pathogens and toxins to enter the bloodstream(Korpela et al., 2018), thus making the intestine a potential source of infections and inflammation. Previous studies showed that the microbiota in LOS patients was less diversified, and dominated by *Staphylococci* and *Enterobacter* but underrepresented by probiotic *Bifidobacteria*(Madan et al., 2012b; Tarr and Warner, 2016; Stewart et al., 2017; Korpela et al., 2018; Ficara et al., 2018).

Here, we carried out a longitudinal pilot study to profile the microbiota of Chinese preterm NEC and LOS patients, with the aim to examine if similar alternations of microbiota correlate with disease onset and progression among Chinese patients. Consistent with previous studies in western countries, we observed lower bacterial diversity but higher variability among Chinese NEC and LOS patients(?). However, we found that Chinese patients showed different bacterial compositions compared to western patients.

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 patients' parents before enrollment.

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 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 were regarded as controls.

Sample collection and handling

Fecal samples collection began from neonatal meconium till decease or discharge, whichever comes first. 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-throughput 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

Raw data was processed according to the standard protocols provided by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai China) as previously described(Liu et al., 2018; Wang et al., 2018). In short, the protocols are as the followings: 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. From the 192 fecal samples sequenced, a total of 7,472,400 optimized V3-V4 tags of 16s rRNA gene sequences were generated. (Table S1)

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. Chimera was detected and removed by UCHIME Algorithm. The effective reads were then sorted by cluster size and processed using Operational Taxonomic Units (OTUs) with 97% similarity cutoff UPARSE-OTU algorithm (implementing "cluster_otus" command)(Edgar, 2013) in USEARCH(v10)(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 diversity) 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 by estimating weighted UniFrac distances between samples.

Statistical and Bioinformatics Analyses

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 hospitalization. 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. Other statistical tests not involving microbiome 16s rRNA sequencing data were performed using *"stats"* package using R(v.3.5.1).

Microbiota and Bioinformatics Analyses

Disease-related Time Interval Definition Considering 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 splitted 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 before disease onset
3. late pre-onset(LPO): from the end of EPO to the start of onset; for control group patients, the equivalent onset time is set at 16 days of life, as is the average diagnosis age of NEC and LOS groups.
4. early disease(ED): first third interval of whole disease span; for the control group, the equivalent ED interval is from the 16th day of life until discharge (EDe).
5. middle disease(MD): second third interval of whole disease span
6. late disease(LD): last third interval of whole disease span
7. post disease(PD): from the end of disease to discharge time-point

Diversity Analyses The average of α diversity, if more than two were available within one analysis interval, of each patient was calculated. Kruskal Wallis tests were used to test shannon diversity differences either among groups within a time interval or among time intervals within a certain group. Repeated measures two-way ANOVA, with time intervals(EPP, EPO, LPO, ED, MD, LD, PD) as a within-subject factor and groups (NEC, LOS, control) as a between-subject factor, along with Sidak's multiple comparisons test was used to test time-with-disease alpha diversity changes.

Modeling Strategies for Taxonomy Comparisons To compare the dynamics of microbiota diversity and relative taxonomic abundance preceding the disease, we took into account the EPP, EPO, LPO and

ED interval among all patients into the ZIBR modeland fit (what is fit, fitness? Or fit data into group?) (Supplemantary 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 dataset) and fit the data into the model. The average taxonomy 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 (Chen and Li, 2016). *ZIBR* and *nlme*(Pinheiro et al., 2018) R packages were utilized for each model.

Scripts and Figures Archiving
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.

RESULTS

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 (2 with positive hemoculture of Klebsiella pneumoniae; the other 1 was diagnosed upon sepsis-related signs and symptoms, lab test of white blood cells ¿20 cells/microL and her effective reaction to vancomycin), and 17 matched controls (Figure1, Supplemantary Table S2). Fecal samples were collected between days 1 and 69 of life. Numbers of samples collected and interval of sampling varied among patients but met our preset criteria of less than 7 days between sampling. The average number of sample collected for NEC, LOS and normal control patients was 15, 10 and 6 respectively. Number of sample per patients was higher for the NEC and LOS group than the control group because of longer period of hospitalization
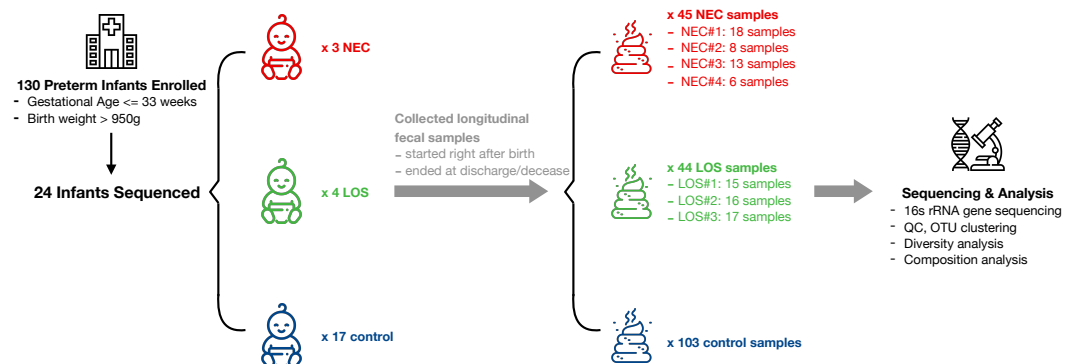

Figure 1. Schematic of Study Design

Fig 1 legend: Schematic of Study Design. Longitudinal fecal samples were collected over from birth to decease or discharge from preterm infants in the NICU. Bacterial diversity and compositions were then characterized.

All 24 infants sampled were delivered by cesarean section and fed on infant formula. No infant was prescribed probiotics during hospitalization. There was no significant difference in terms of gestational age(p = 0.074), birth weight(p = 0.11) or gender proportions(p = 0.820) among three groups. The average age at diagnosis for both disease group was 16 days and there was no statistical difference between the groups (p = 0.629) (Table 1). Therefore, we assigned day 16 until discharge as early disease, day 4-8 as early pre-onset, day 9-15 as late pre-onset interval for the control group.

Dynamics of Microbiome Diversity in diseases onset and progression
Microbiome Richness Analysis
Furthermore, pairwise comparions between any adjacent two time intervals within the same group(supplementary sobs-time-group!!!!!) didn't show significant alterations and differences in sobs, indicating the minor effect of microbiota richness on the diseases acquisition.

Overally, all three groups showed similar microbiota richness trend with the observed species (Sobs) decreased significantly from early post-partum period (EPP) to early disease (ED) stage (Fig2a. NEC group, $p = 0.044$; b. LOS group, $p = 0.013$; c. control group, $p < 0.01$; supplemantary!!! rm-matrix1-sobs, two way RM ANOVA, $p < 0.0001$). The biggest decrease in richness was between early pre-onset (EPO) to late pre-onset (LPO). However, the decrease in the disease groups was less significant than the normal group (NEC group $p=0.18$, LOS group $p=0.066$, normal $p=0.0004$). The Sobs then stabilized from LPO onword with no significant difference between adjacent time intervals.

Fig2. Post-partum microbiome richness (Sobs) trend in each group.
Shows microbial richness trend in stools from cases and controls (a. NEC group, b. LOS group, c. control group). Horizontal line shows median, box boundaries show 25th and 75th percentiles. Sobs index value of each stool is depicted as one dot. Sobs decreased significantly among three groups from early post-partum to early disease interval(two way RM ANOVA, p ¡0.01). Timewise comparisons however, shows non-significant trends in bacterial diversity associated with NEC or LOS onset in stools in each group.

Microbiome Evenness Analysis
Similar to microbiota richness, the microbiome evenness represented by shannon diversity indices, decreased from early post-partum (EPP) to early disease (ED)(Fig3, supplemantary!!!! rm_matrix1_shannon, $p < ¡0.0001$), the shannon index of the NEC and LOS groups, from the early pre-onset interval to early disease decreased significantly (Fig3a.NEC group, early pre-onset = 1.92, early disease = 0.58, $p = 0.04$, b. LOS group, early pre-onset = 2.47, early disease = 0.47, $p = 0.01$), while the control group did not show the similar trend (Fig3c.control group, early pre-onset = 1.81, early disease =1.00, $p = 0.05$).

Fig3. Post-partum microbiome evenness (Shannon) trend in each group.
Shows microbial richness trend in stools from cases and controls (a. NEC group, b. LOS group, c. control group). Horizontal line shows median, box boundaries show 25th and 75th percentiles. Shannon index value of each stool is depicted as one dot. p=0·004 for NEC and p = 0.010 for LOS from early pre-onset to early disease indicating significantly discordant trends in bacterial diversity preceding disease onset.
The inter-time-interval comparison among three groups showed significant shannon index divergent during during early pre-onset interval (two way RM ANOVA, $p = 0.0017$ supplementary!!!) and the early disease stage(Fig4, $p = 0.0037$), implying the role of microbiota distortion in triggering NEC and

Table 1. Demographic characteristics of Preterm NEC, LOS and control groups.

|	NEC (N=3)	LOS (N=4)	Control (N=17)	Statistical Test	*p value*
Gestational Age (weeks)	29(29-30)	30(29-31)	31(28-33)	Kruskal-Wallis test	0.074
Birth Weight(g)	1416.3 (773.4-2149.1)	1141.7 (633.4-1649.9)	1527.4 (1391.6-1663.1)	Kruskal-Wallis test	0.111
Gender				Fisher's exact test	0.820
Female	3(75%)	2(67%)	9(53%)		
male	1(25%)	1(33%)	8(47%)		
Diagnosis Age(days)	16(11-19)	16(10-22)	—	Wilcoxon rank-sum test	0.629
Length of Stay(d)	54.3 (13.5-95.0)	60.0 (24.8-95.2)	32.9 (26.3-39.5)	Kruskal-Wallis test	0.046
Number of Samples	46	42	103	—	—
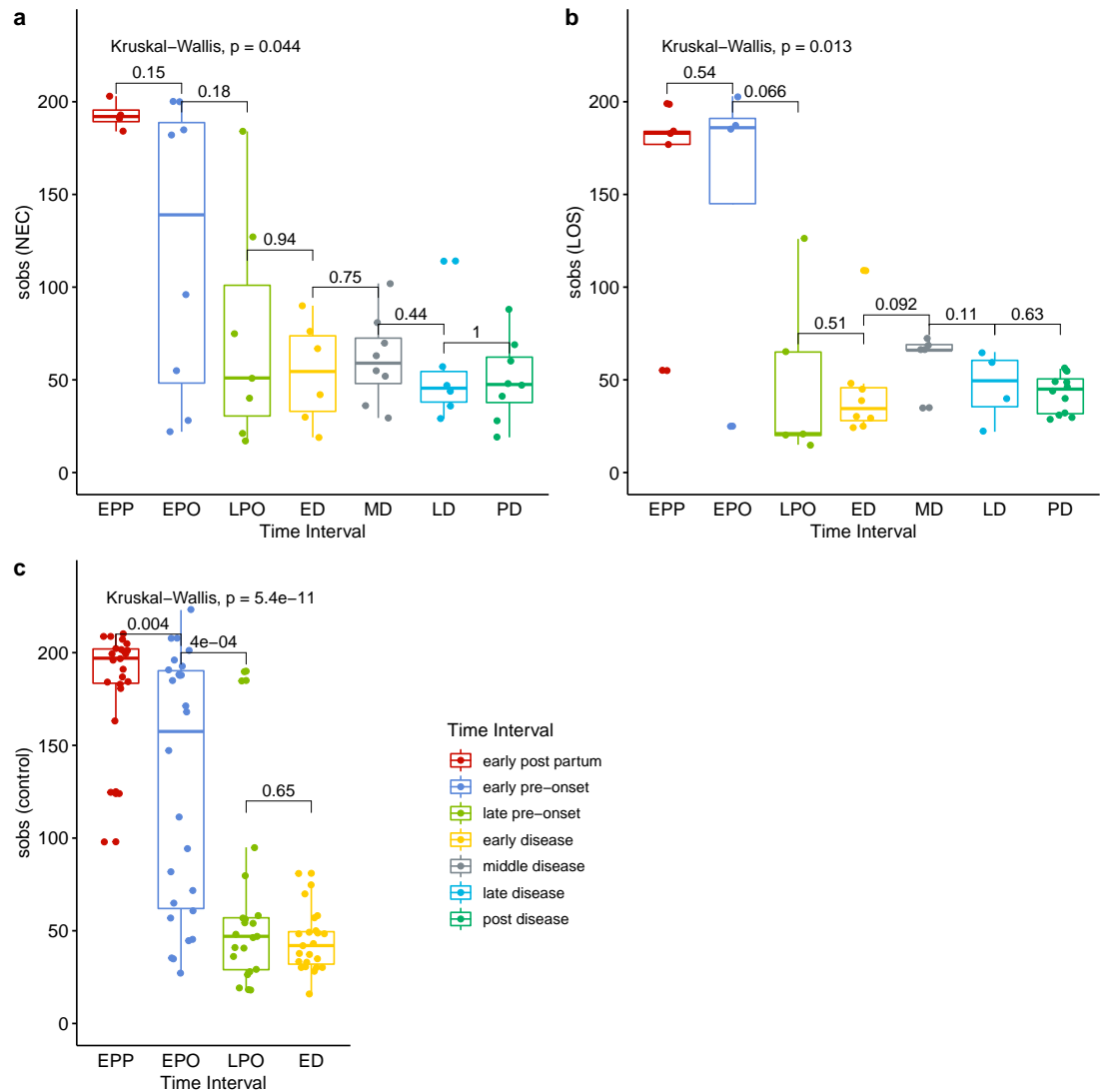
Figure 2. Post-partum microbiome richness (Sobs) trend in each group

LOS. As diseased progressed, two disease groups showed a non-significant difference in community evenness(Fig4 facet "middle disease", p = 0.034), indicating a similar community distribution pattern in both NEC and LOS development. Finally, alleviation of both diseases restored the microbiota evenness back to the early pre-onset level respectively (Fig3 a. NEC, early pre-onset vs. post disease, p = 0.79; b. LOS, early pre-onset vs. post disease, p = 0.16). *Fig4. Post-partum microbiome evenness (Shannon) in each time-interval.*

Shows microbial richness trend in stools from cases and controls (a. NEC group, b. LOS group, c. control group). Horizontal line shows median, box boundaries show 25th and 75th percentiles. Shannon index value of each stool is depicted as one dot. p=0·004 for NEC and p = 0.010 for LOS from early pre-onset to early disease indicating significantly discordant trends in bacterial diversity preceding disease onset.

Kinetics of Microbiome Composition

To evaluate time-wise differences in beta-diversity between the microbiomes, we applied Principal Component Analysis (PCoA) to weighted UniFrac distance matrix, considered by timer intervals. During early post-partum interval, beta-diversity among three groups was the lowest with the first principal coordinates accounted for 33.01%. Over time, beta diversity started to drift away from one another from early pre-onset to early disease, with the first principal coordinate accounted for 35.23% at early pre-onset phase, to 42.32% in early disease phase((Fig5)b to d), implicating that the phylogenetic compositions of
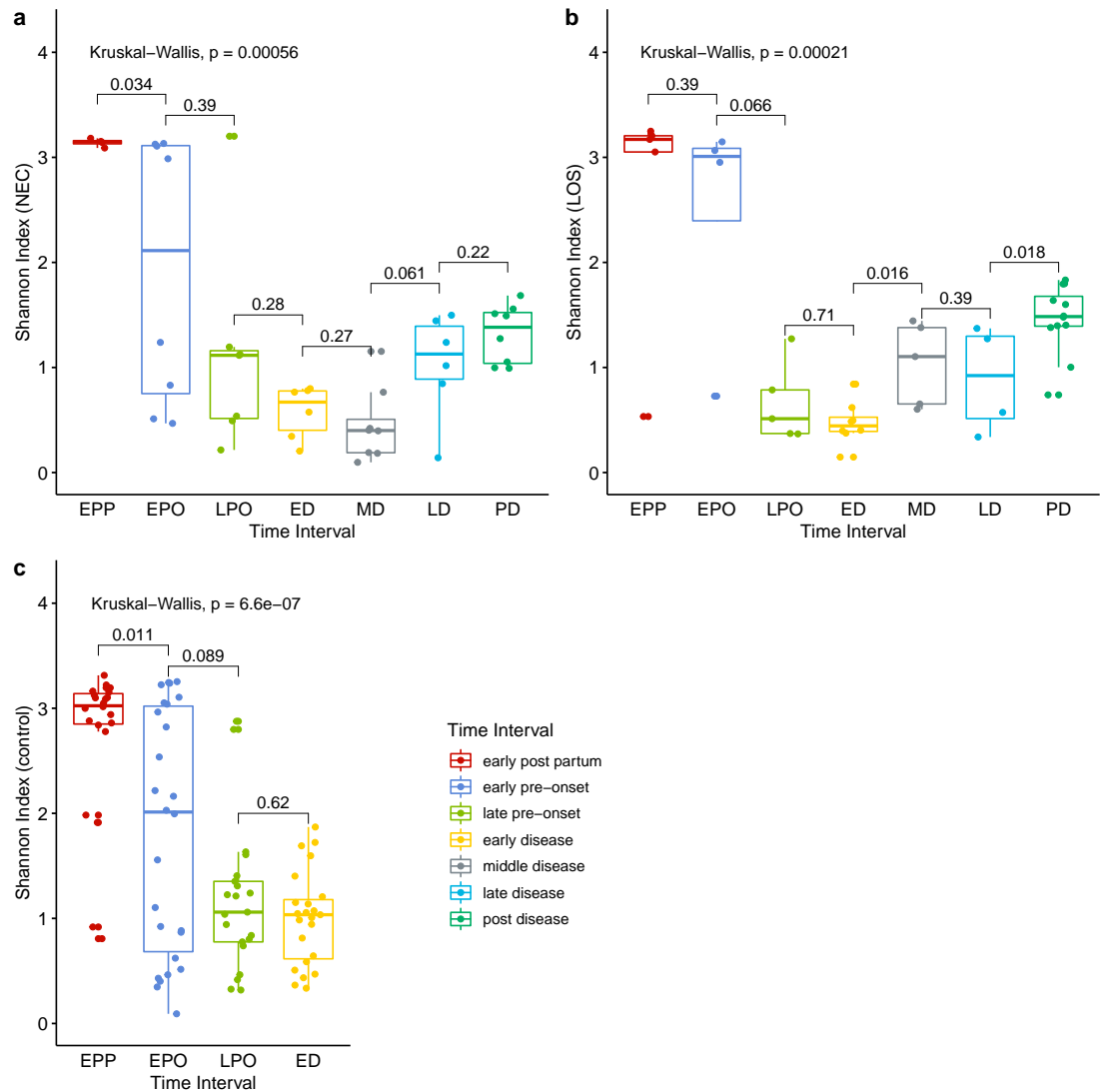
Figure 3. Post-partum microbiome evenness(shannon diversity index) trend in each group

the case stools already deviated from the control ones before the onset of disease. As disease progress, the phylogenetic dissimilarity between the disease groups further separated and peak at middle disease stage (59.53%) then came down gradually to 42.8% at post disease stage (Fig. 5 e to g) This phylogenetic dissimilarity may suggest difference in etiopathology at the pre-onset stage while the differences between the disease group may be a result of different antibiotic regimes for NEC and LOS.

Fig 5 Principal coordinates analysis 'PCoA' plot (beta diversity) demonstrating unweighted UniFrac distance between sample with group points colored for NEC, LOS and control infants. Scatter plot shows principal coordinate 1 (PC1) versus principal coordinate 2 (PC2). Percentages shown are percentages of variation explained by the components. Each dot represents the microbiota of a single sample, and colors reflect the grouping information for that sample. Samples that are clustered closely together are considered to share a larger proportion of the phylogenetic tree in comparison to samples that are more separated. During early post-partum interval, three groups have similar beta diversity. But as approaching disease status beta diversity drifted further away between case groups and the control group.

Colonization Trend on Genus Level

In the analyses of microbiome alpha(Fig2) and beta diversity(Fig**??**), detectable differences was observed among the three groups, especially during transition from LPO to ED stage. This suggested that the

microbiota compositions of the two disease groups and the normal group might be different. To further investigate if any change in microbiota composition correlate with the onset and progression of NEC and LOS, we tracked the changes in bacteria at genus level during hospitalization. We filtered the genus of over 10% relative abundance among all samples and plotted proportion over time.

Before disease onset, ZIBR model showed significant time-by-NEC and time-by-LOS interaction factors for the progression over time of Bacillus and Solibacillus. At early post-partum stage, all three groups were dominated by Lactococcus, Bacillus and Pseudomonas. However, a rapid decline in was observed in both disease groups showed significantly higher Bacillus($p = 0.032$) and lower Solibacillus($p = 0.047$) relative to control samples(ZIBR table cited). Furthermore, both case groups experienced a rapid *Bacillus* decline when approaching disease stages(NEC from 15.053% in early post-partum to 0.011% in early disease interval; LOS from 15.967% in in early post-partum to 0.002% in early disease interval).

For NEC samples, *Enterococcus* increased significantly from 0.009% in late pre-onset phase, to 28.801% in early disease and 58.959% in middle disease interval ($p = 0.032$). Meanwhile, after its transient boost in late pre-onset interval(27.231%), Staphylococcus had been constantly presented (12.571%) until late disease interval of NEC. Relative to control samples(6.010%), NEC samples showed higher Streptococcus(14.439%, $p = 0.023$) starting from late pre-onset stage to late disease stage. Peptoclostridium increased during late disease stage(#diarrhea!!)(Fig6a).

For LOS patients, a significant outburst of Klebsiella (EPP: 4.7%, EPO: 2.376%, LPO: 58.902%) with diminishment of other genus were characterized prior to the disease onset. Later on, Klebsiella aggressively dominated the intestine throughout the whole course of disease(ED: 73.529%, MD: 69.506%, LD: 45.333%). By contrast, Escherichia-Shigella, which might well be impacted by advanced antibiotics including Meropenem, was absent from late pre-onset to early disease interval(0.005%, $p = 0.026$ and 0.003%, $p = 0.045$, respectively), restored until middle disease interval(14.762%, $p = 0.042$) and became the second abundant genera dominating the intestine in late disease(48.336%, $p = 0.262$)(Fig6b)

DISCUSSION

With a prospective study design, we investigated the longitudinal microbiome diversity and composition, from the Chinese preterm infants who subsequently developed necrotizing enterocolitis or late-onset sepsis. The main findings were that among case stools, the overall microbiota diversity was reduced and specific compositional characteristics of the microbiota was associated with potentially pathogenic genus, such as *Enterococcus*, *Staphylococcus*, *Peptoclostridium* and *Streptococcus*.

Inter-sample diversity decrease postpartum,

compare to control, phylogenetic dissimilarity emerged preceding disease consistent with previous studies. continuous

oppotunistic initially

Both case groups and control group showed decrease in richness and evenness after birth. In previous studies Several studies suggested a reduction in microbiota community diversity Community richness

1. alpha 1.1 evenness minor rols 1.2 richness dicrease during (corresponding 4 7 dol) may precede diseases, less diversified ¡-¿ s/s? # consistent with the hypothesis that dysbiosis precedes this severe event. 2. beta 3. over/underrepresenting of certain genus 4. Microbiome optimization – a novel strategy

Our study was limited to 1 hospital in one specific geographical region in China and so could not be generalized to a larger population. Our results, however, showed the needs of an exceptionally larger, more heterogenous study population to confirm our findings 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. The resultant overfitting possibility inevitably rose up, which became the pitfall in understanding the true microbiota patterns of NEC and LOS. Our results, however, showed the needs of an exceptionally larger, more heterogenous study population and longer follow ups, to draw a more meaningful and solid conclusion within the non-western population.

CONCLUSIONS

To our knowledge, our study is the first to report intestinal microbiota of Chinese preterm infants using 16s rRNA gene NGS data. Intestinal microbiota diversity reduction and phylogenetic dissimilarity away from healthy infants over time is associated with both NEC and LOS onset. Over-growth of potentially

pathogenic genera were recognized, i.e. *Enterococcus*, *Streptococcus* and *Peptoclostridium* in NEC cases; *Klebsiella* in LOS cases. Our results is a starting point for further studying of microbial factors involved in preterm-associated complications within China range. A better understanding of microbial risk signatures in longitudinal microbial community assembly during early life, including its colonization mechanism and interaction with intestinal immunological responses, would assist in better understanding the etiology and assist in development of alternative biomarkers for early diagnosis and facilitate novel prevention and treatment strategies, to protect predisposed preterm infants in Chinese population.

ACKNOWLEDGMENTS

REFERENCES

Bell, M. J., Ternberg, J. L., Feigin, R. D., Keating, J. P., Marshall, R., Barton, L., and Brotherton, T. (1978). Neonatal necrotizing enterocolitis. therapeutic decisions based upon clinical staging. *Annals of surgery*, 187(1):1.

Bezirtzoglou, E., Tsiotsias, A., and Welling, G. W. (2011). Microbiota profile in feces of breast-and formula-fed newborns by using fluorescence in situ hybridization (fish). *Anaerobe*, 17(6):478–482.

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics*, 30(15):2114–2120.

Bouter, K. E., van Raalte, D. H., Groen, A. K., and Nieuwdorp, M. (2017). Role of the gut microbiome in the pathogenesis of obesity and obesity-related metabolic dysfunction. *Gastroenterology*, 152(7):1671–1678.

Cani, P. D. (2019). Severe obesity and gut microbiota: does bariatric surgery really reset the system? *Gut*, 68(1):5–6.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I., et al. (2010). Qiime allows analysis of high-throughput community sequencing data. *Nature methods*, 7(5):335.

Cernada, M., Bäuerl, C., Serna, E., Collado, M. C., Martínez, G. P., and Vento, M. (2016). Sepsis in preterm infants causes alterations in mucosal gene expression and microbiota profiles compared to non-septic twins. *Scientific Reports*, 6(1):25497.

Chen, E. Z. and Li, H. (2016). A two-part mixed-effects model for analyzing longitudinal microbiome compositional data. *Bioinformatics*, 32(17):2611–2617.

Deweerdt, S. (2018). How baby's first microbes could be crucial to future health. *Nature*, 555(7695):S18–S19.

Edgar, R. C. (2013). Uparse: highly accurate otu sequences from microbial amplicon reads. *Nature methods*, 10(10):996.

Ficara, M., Pietrella, E., Spada, C., Della Casa Muttini, E., Lucaccioni, L., Iughetti, L., and Berardi, A. (2018). Changes of intestinal microbiota in early life. *The Journal of Maternal-Fetal & Neonatal Medicine*, pages 1–8.

Gagliani, Nicola, Hu, Bo, Huber, Samuel, Elinav, and Richard (2014). The fire within: Microbes inflame tumors. *Cell*, 157(4):776–783.

Giongo, A., Gano, K. A., Crabb, D. B., Mukherjee, N., Novelo, L. L., Casella, G., Drew, J. C., Ilonen, J., Knip, M., Hyöty, H., et al. (2011). Toward defining the autoimmune microbiome for type 1 diabetes. *The ISME journal*, 5(1):82.

Irrazábal, T., Belcheva, A., Girardin, S. E., Martin, A., and Philpott, D. J. (2014). The multifaceted role of the intestinal microbiota in colon cancer. *Molecular Cell*, 54(2):309–320.

Jacquot, A., Neveu, D., Aujoulat, F., Mercier, G., Marchandin, H., Jumas-Bilak, E., and Picaud, J.-C. (2011). Dynamics and clinical evolution of bacterial gut microflora in extremely premature patients. *The Journal of pediatrics*, 158(3):390–396.

Jie, Z., Xia, H., Zhong, S. L., Feng, Q., Li, S., Liang, S., Zhong, H., Liu, Z., Gao, Y., and Zhao, H. (2017). The gut microbiome in atherosclerotic cardiovascular disease. *Nature Communications*, 8(1).

Jonsson, A. L. and Bäckhed, F. (2017). Role of gut microbiota in atherosclerosis. *Nature Reviews Cardiology*, 14(2):79.

Kassambara, A. (2017). ggpubr:"ggplot2" based publication ready plots. r package version 0.1. 6.

Korpela, K., Blakstad, E. W., Moltu, S. J., Strømmen, K., Nakstad, B., Rønnestad, A. E., Brække, K., Iversen, P. O., Drevon, C. A., and Vos, W. (2018). Intestinal microbiota development and gestational age in preterm neonates. *Scientific reports*, 8(1):2453.

Liu, Y., Li, J., Jin, Y., Zhao, L., Zhao, F., Feng, J., Li, A., and Wei, Y. (2018). Splenectomy leads to amelioration of altered gut microbiota and metabolome in liver cirrhosis patients. *Frontiers in Microbiology*, 9.

Madan, J. C., Farzan, S. F., Hibberd, P. L., and Karagas, M. R. (2012a). Normal neonatal microbiome variation in relation to environmental factors, infection and allergy. *Current opinion in pediatrics*, 24(6):753.

Madan, J. C., Salari, R. C., Saxena, D., Davidson, L., O'toole, G. A., Moore, J. H., Sogin, M. L., Foster, J. A., Edwards, W. H., Palumbo, P., et al. (2012b). Gut microbial colonisation in premature neonates predicts neonatal sepsis. *Archives of Disease in Childhood-Fetal and Neonatal Edition*, 97(6):F456–F462.

Magoč, T. and Salzberg, S. L. (2011). Flash: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, 27(21):2957–2963.

Mai, V., Young, C. M., Ukhanova, M., Wang, X., Sun, Y., Casella, G., Theriaque, D., Li, N., Sharma, R., Hudak, M., et al. (2011). Fecal microbiota in premature infants prior to necrotizing enterocolitis. *PloS one*, 6(6):e20647.

Neu, J. and Walker, W. A. (2011). Necrotizing enterocolitis. *New England Journal of Medicine*, 364(3):255–264.

Pickering, L. K., Baker, C. J., Kimberlin, D. W., et al. (2012). *Red Book, (2012): Report of the Committee on Infectious Diseases*. Am Acad Pediatrics.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., and R Core Team (2018). *nlme: Linear and Nonlinear Mixed Effects Models*. R package version 3.1-137.

Pronovost, G. N. and Hsiao, E. Y. (2019). Perinatal interactions between the microbiome, immunity, and neurodevelopment. *Immunity*, 50(1):18–36.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glöckner, F. O. (2012). The silva ribosomal rna gene database project: improved data processing and web-based tools. *Nucleic acids research*, 41(D1):D590–D596.

Rao, S. C., Srinivasjois, R., and Moon, K. (2016). One dose per day compared to multiple doses per day of gentamicin for treatment of suspected or proven sepsis in neonates. *Cochrane database of systematic reviews*, (12).

Rosenbaum, M., Knight, R., and Leibel, R. L. (2015). The gut microbiota in human energy homeostasis and obesity. *Trends in Endocrinology & Metabolism*, 26(9):493–501.

Sampson, T. R. and Mazmanian, S. K. (2015). Control of brain development, function, and behavior by the microbiome. *Cell Host & Microbe*, 17(5):565–576.

Savage, J. H., Lee-Sarwar, K. A., Sordillo, J., Bunyavanich, S., Zhou, Y., O'connor, G., Sandel, M., Bacharier, L. B., Zeiger, R., Sodergren, E., et al. (2018). A prospective microbiome-wide association study of food sensitization and food allergy in early childhood. *Allergy*, 73(1):145–152.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and environmental microbiology*, 75(23):7537–7541.

Schwiertz, A., Gruhl, B., Löbnitz, M., Michel, P., Radke, M., and Blaut, M. (2003). Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full-term infants. *Pediatric research*, 54(3):393.

Sears, C. L. and Garrett, W. S. (2014). Microbes, microbiota, and colon cancer. *Cell Host & Microbe*, 15(3):317–328.

Sharon, U., Alain, S., Prakeshkumar, S., David, M., and O'Connor, D. L. (2015). Gut microbiota of the very-low-birth-weight infant. *Pediatric Research*, 77(1-2):205.

Shin, H., Pei, Z., Martinez, K. A., Rivera-Vinas, J. I., Mendez, K., Cavallin, H., and Dominguez-Bello, M. G. (2015). The first microbial environment of infants born by c-section: the operating room

microbes. *Microbiome*, 3(1):59.

Stewart, C. J., Ajami, N. J., O'Brien, J. L., Hutchinson, D. S., Smith, D. P., Wong, M. C., Ross, M. C., Lloyd, R. E., Doddapaneni, H., Metcalf, G. A., et al. (2018). Temporal development of the gut microbiome in early childhood from the teddy study. *Nature*, 562(7728):583.

Stewart, C. J., Embleton, N. D., Marrs, E. C. L., Smith, D. P., Fofanova, T., Nelson, A., Skeath, T., Perry, J. D., Petrosino, J. F., and Berrington, J. E. (2017). Longitudinal development of the gut microbiome and metabolome in preterm neonates with late onset sepsis and healthy controls. *Microbiome*, 5(1):75.

Stokholm, J., Blaser, M. J., Thorsen, J., Rasmussen, M. A., Waage, J., Vinding, R. K., Schoos, A.-M. M., Kunøe, A., Fink, N. R., Chawes, B. L., et al. (2018). Maturation of the gut microbiome and risk of asthma in childhood. *Nature communications*, 9(1):141.

Stoll, B. J., Hansen, N. I., Bell, E. F., Walsh, M. C., Carlo, W. A., Shankaran, S., Laptook, A. R., Sánchez, P. J., Van Meurs, K. P., Wyckoff, M., et al. (2015). Trends in care practices, morbidity, and mortality of extremely preterm neonates, 1993-2012. *Jama*, 314(10):1039–1051.

Tang, W. W., Kitai, T., and Hazen, S. L. (2017). Gut microbiota in cardiovascular health and disease. *Circulation research*, 120(7):1183–1196.

Tarr, P. I. and Warner, B. B. (2016). Gut bacteria and late-onset neonatal bloodstream infections in preterm infants. In *Seminars in Fetal and Neonatal Medicine*, volume 21, pages 388–393. Elsevier.

Vatanen, T., Franzosa, E. A., Schwager, R., Tripathi, S., Arthur, T. D., Vehik, K., Lernmark, Å., Hagopian, W. A., Rewers, M. J., She, J.-X., et al. (2018). The human gut microbiome in early-onset type 1 diabetes from the teddy study. *Nature*, 562(7728):589.

Vatanen, T., Kostic, A., D'Hennezel, E., Siljander, H., Franzosa, E., Yassour, M., Kolde, R., Vlamakis, H., Arthur, T., and Hämäläinen, A. M. (2016). Variation in microbiome lps immunogenicity contributes to autoimmunity in humans. *Cell*, 165(4):842–853.

Vogelzang, A., Guerrini, M. M., Minato, N., and Fagarasan, S. (2018). Microbiota—an amplifier of autoimmunity. *Current opinion in immunology*, 55:15–21.

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive bayesian classifier for rapid assignment of rrna sequences into the new bacterial taxonomy. *Applied and environmental microbiology*, 73(16):5261–5267.

Wang, R., Xiao, Y., Lv, F., Hu, L., Wei, L., Yuan, Z., and Lin, H. (2018). Bacterial community structure and functional potential of rhizosphere soils as influenced by nitrogen addition and bacterial wilt disease under continuous sesame cropping. *Applied Soil Ecology*, 125:117–127.

Warner, B. B., Deych, E., Zhou, Y., Hall-Moore, C., Weinstock, G. M., Sodergren, E., Shaikh, N., Hoffmann, J. A., Linneman, L. A., Hamvas, A., Khanna, G., Rouggly-Nickless, L. C., Ndao, I. M., Shands, B. A., Escobedo, M., Sullivan, J. E., Radmacher, P. G., Shannon, W. D., and Tarr, P. I. (2016). Gut bacteria dysbiosis and necrotising enterocolitis in very low birthweight infants: A prospective case-control study. *The Lancet*, 387(10031):1928–1936.

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.

Winer, D. A., Luck, H., Tsai, S., and Winer, S. (2016). The intestinal immune system in obesity and insulin resistance. *Cell metabolism*, 23(3):413–426.

Zhou, Y., Shan, G., Sodergren, E., Weinstock, G., Walker, W. A., and Gregory, K. E. (2015). Longitudinal analysis of the premature infant intestinal microbiome prior to necrotizing enterocolitis: a case-control study. *PloS one*, 10(3):e0118632.

Zmora, N., Suez, J., and Elinav, E. (2019). You are what you eat: diet, health and the gut microbiota. *Nature Reviews Gastroenterology & Hepatology*, 16(1):35–56.
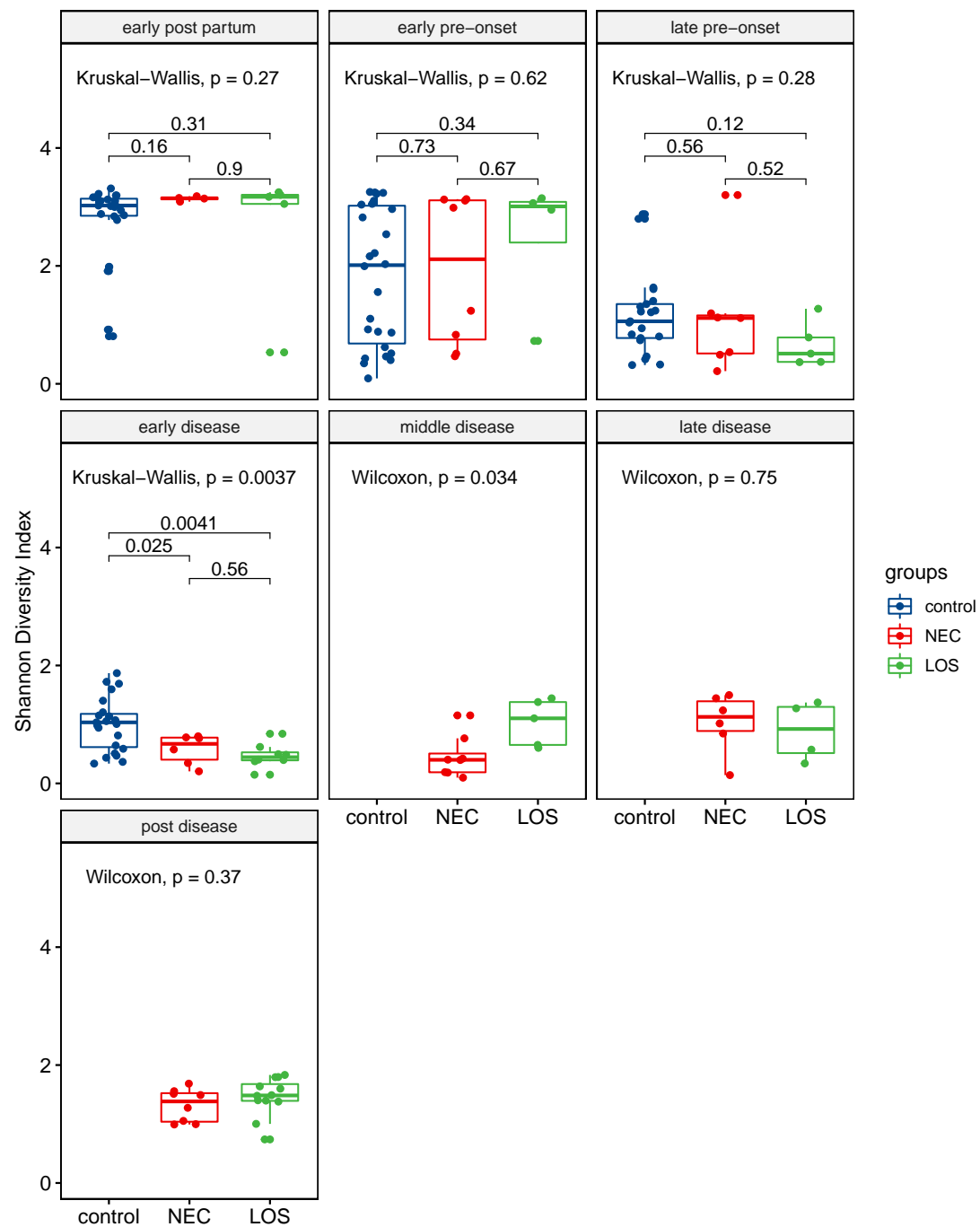
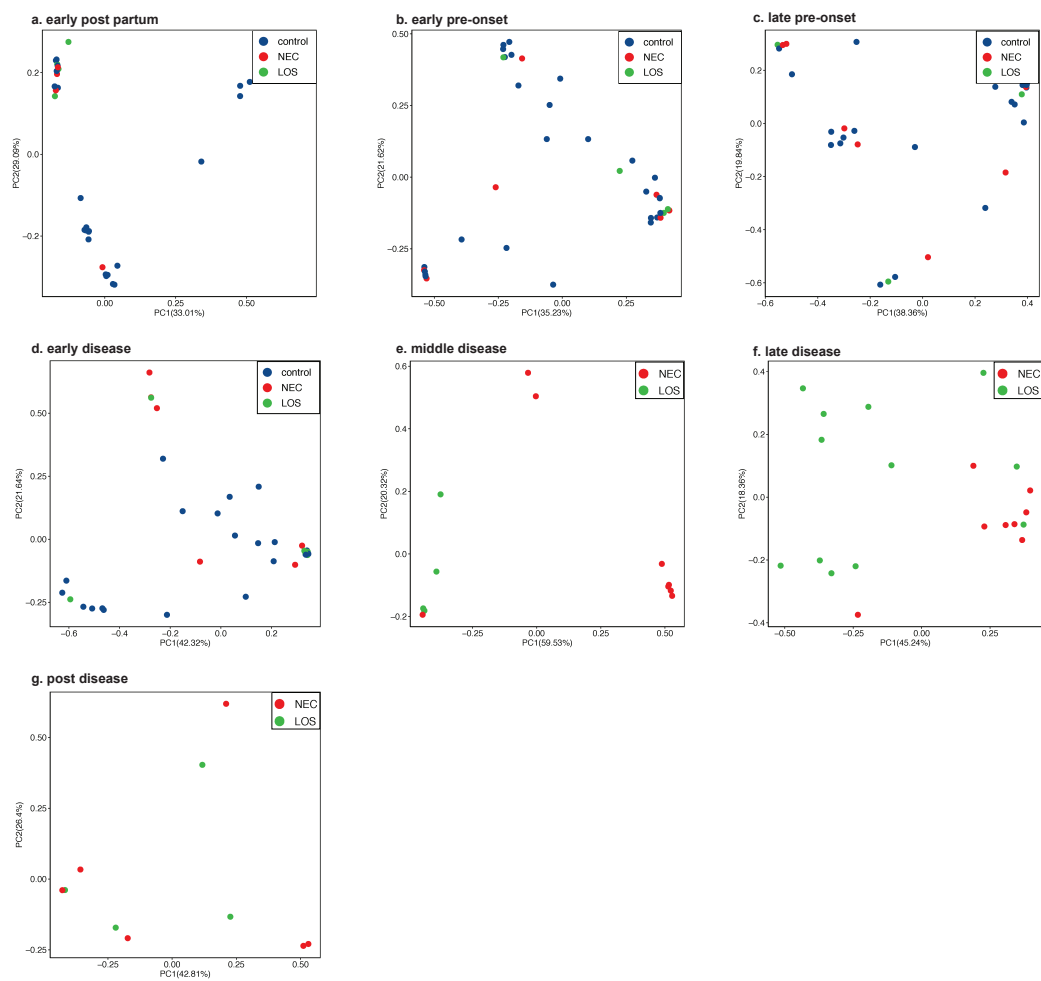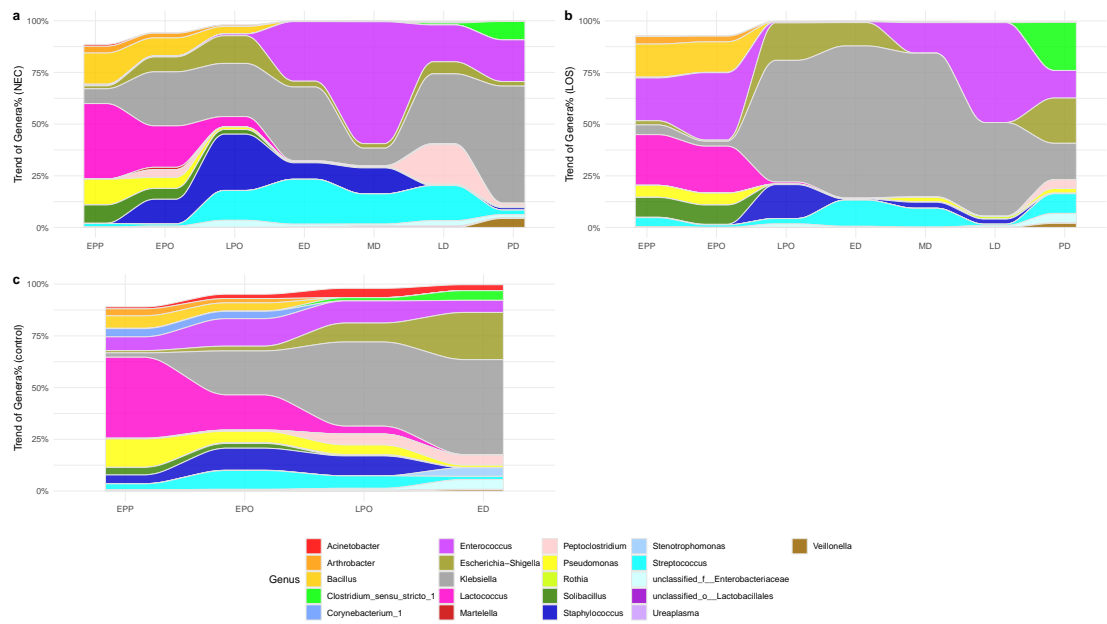**Figure 4.** Post-partum microbiome evenness(shannon diversity index) trend in each time interval

Figure 5. Beta Diversity

Figure 6. Kinetics of Genera Relative Abundance