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

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ABSTRACT

Background and Objectives
Recent studies associate intestinal microbiota dysbiosis with necrotizing enterocolitis(NEC) and late onset sepsis(LOS) preterm infants in western countries. To date, there is no report of similar studies performed among non-Western population. In this pilot study, we profiled gut microbiota of NEC and LOS patients from birth to deceased or discharged.
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
post-partum 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 various 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 post-partum microbiota haboring contributes to the vulnerablilty in preterm-associated health consequencese, such as necrotizing enterocolitis and late-onset sepsis.

#% early pattern a/w Nec

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 and collegues 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). Boost of *Gammaproteobacteria* and decline in *Negativicutes* was in chronological association with the condition(Warner et al., 2016).

#% early pattern a/w LOS

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 postpost-partum 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 both conditions.

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

Raw data was processesed 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.

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 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 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

Disease-related Time Interval Definition 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 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 befor disease onset
3. late pre-onset(LPO): from the end of EPO to the start of onset; for control group patients, the 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
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 timepoint

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) and Sidak's multiple comparisons test was used to test time-with-disease shannon diversity changes.

Modeling Strategies for Taxomony Comparisons 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(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). 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.
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); Scripts for data analysis are available at our github repository

RESULTS

Overall 16s Sequencing Report and Coverage
A total of 7,472,400 optimized V3-V4 tags of 16s rRNA gene sequences were produced from 192 fecal samples, with an average read 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 ,Supplemantary Table S2). Fecal samples were collected between days 1 and 69 of life. Sampling timepoints and numbers of samples varied among each infant.

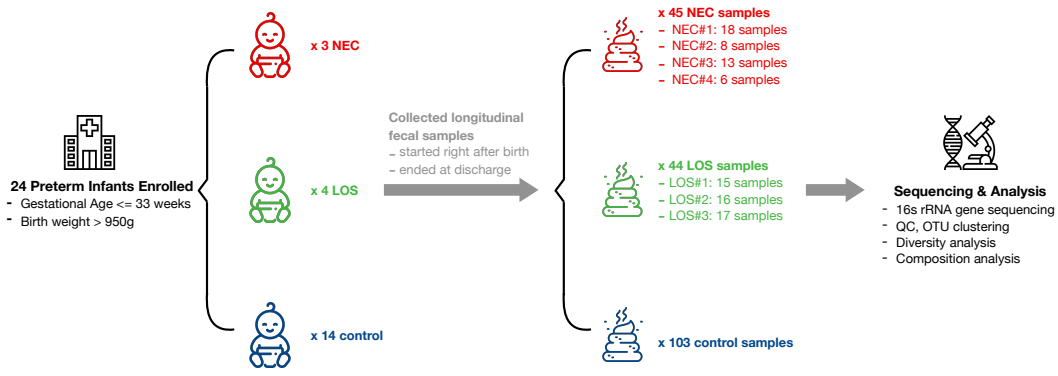

Figure 1. Flow of Study Design

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 their worse health status often required longer health care time than the control group. All infants were delivered by cesarean section and fed on infant formula. No one was prescribed probiotics during hospitalization.

Dynimics of Microbiome Diversity in diseases onset and progression

Mircobiome Richness Plays Minor Roles

Overally, all three groups shared similar microbiota richness trend after birth, that the observed richness (Sobs) decreased significantly from post-partum period until the late disease 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). Nevertheless, the inter-time-interval comparison among groups (supplementary sobs-time-group!!!!!) didn't show any significant alterations and differences in sobs, indicating the minor effect of microbiota richness on the diseases acquisition.

Recession of Mircobiome Evenness Preceded NEC and LOS

Although, microbiota communities evenness among all patients, reflected by shannon diversity indices, tended to decrease after birth(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).

The inter-time-interval comparison among three groups showed signifant 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 LOS. As infants aged, two disease groups showed a non-significant difference in community evenness(Fig4 facte "middle disease", p = 0.034), indicating a similar community distribution pattern in both NEC and LOS development. Furthermore, alleviation of both diseases restored the microbiota evenness back to the level during early pre-onset interval (Fig3 a. NEC, early pre-onset vs. post disease, p = 0.79; b. LOS, early pre-onset vs. post disease, p = 0.16).

Microbiome Composition Kinetics

To evaluate time-wise differences in beta-diversity between the microbiomes, we applied Principal Component Analysis (PCoA) to weighted UniFrac distance metric matrices, considered by timer intervals. During early post partum interval, there appears to be no clear difference in beta-diversity among three groups, with the first principal coordinates accounted for 33.01%. In the process of entering diseases status, beta diversity continued drifting away, with the first principal coordinates accounted for 35.23% in early pre-onset phase, 38.36% in late pre-onset phase and 42.32% in early disease phase(Fig5). During the middle, late and post disease interval, samples from NEC and LOS groups clustered together respectively(). This phylogenetic dissimilarity indicated the different etiopathology and antibiotic regimes between NEC and LOS.

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	0.82
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	—	—

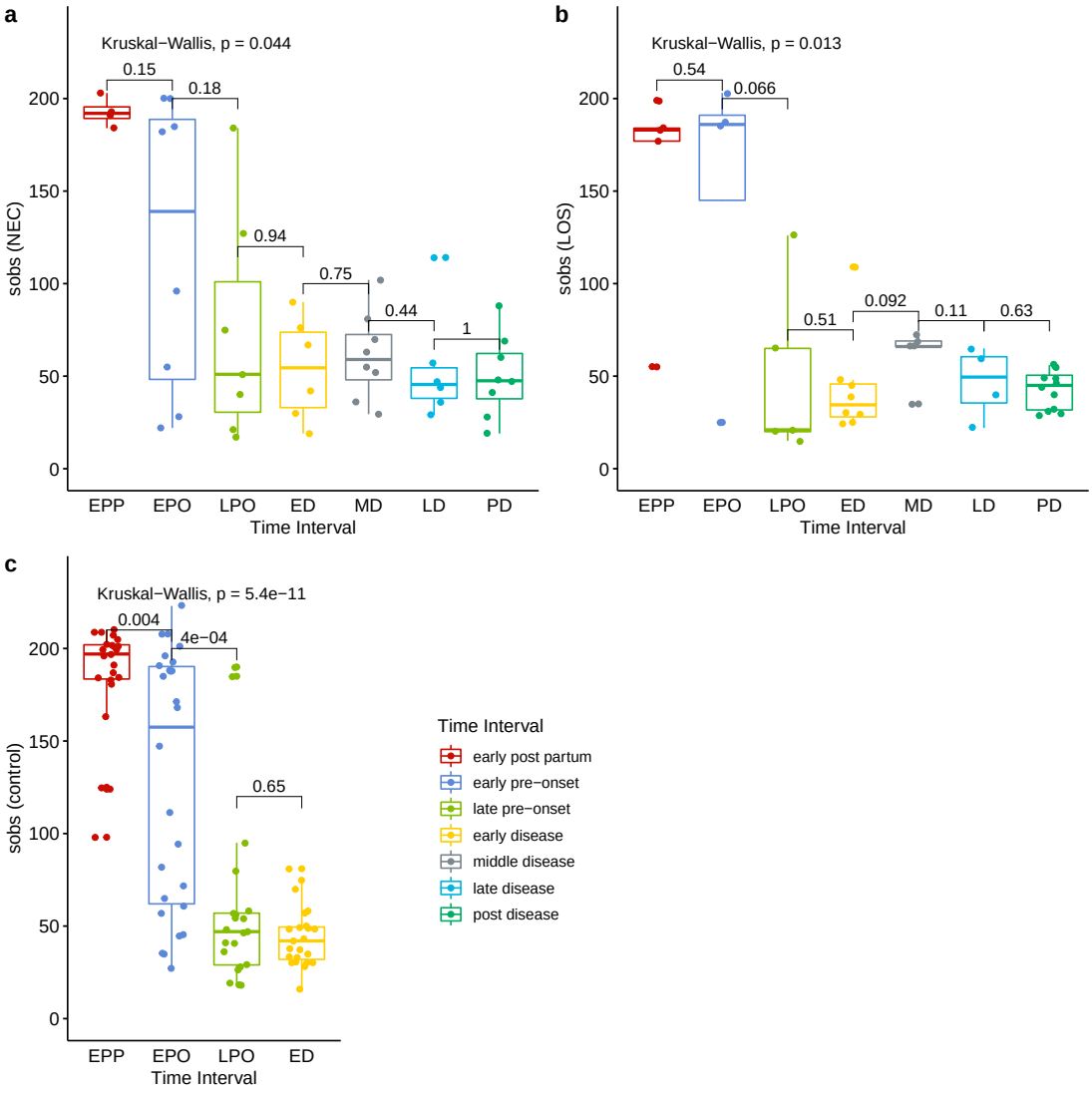

Figure 2. Post-partum microbiome richness (Sobs) trend in each group

Potentially Pathogenic Genus Represented Case Groups

To investigate specific genera that affect the course of diseases development, we filtered the genus of over 10% relative abundance among all samples and performed tests on a time-interval scale.

Before disease onset, ZIBR model showed significant time-by-necrotising-enterocolitis interaction factors for the progression over time of Bacillus and Solibacillus. Diseases samples showed significantly higer Bacillus($p = 0.032$) and lower Solibacillus($p = 0.047$) relative to control samples(ZIBR table cited). Furthermore, both case groups experienced a rapid decline in Bacillus 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 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!!). The development of LOS, however, was characterized by

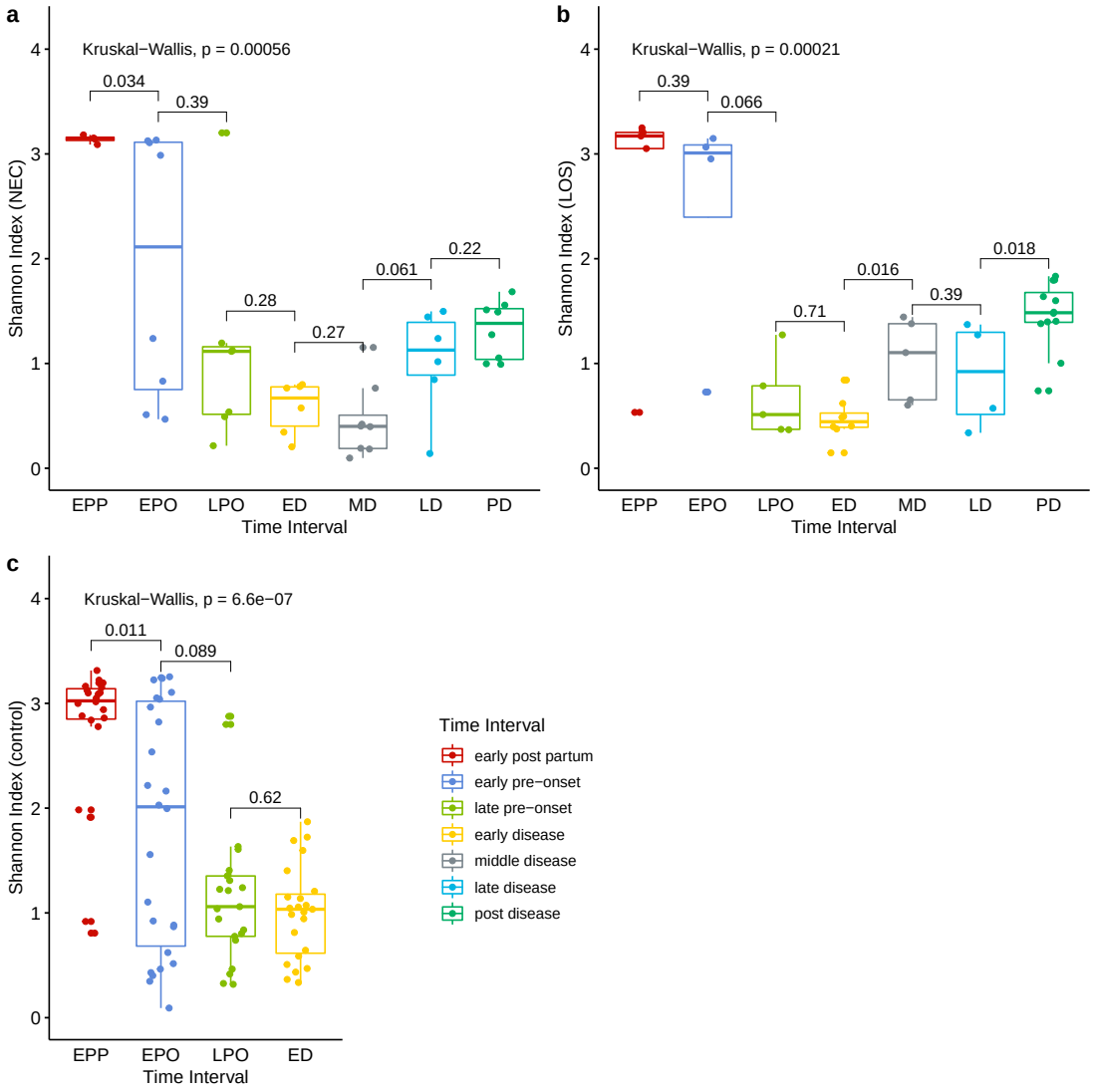

Figure 3. Post-partum microbiome evenness(shannon diversity index) trend in each group

DISCUSSION

During the first months of life, the microbiota undergoes hypervariable changes

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 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 preceding NEC and LOS.

Our results, however, showed the needs of larger study population and longer follow ups.

CONCLUSIONS

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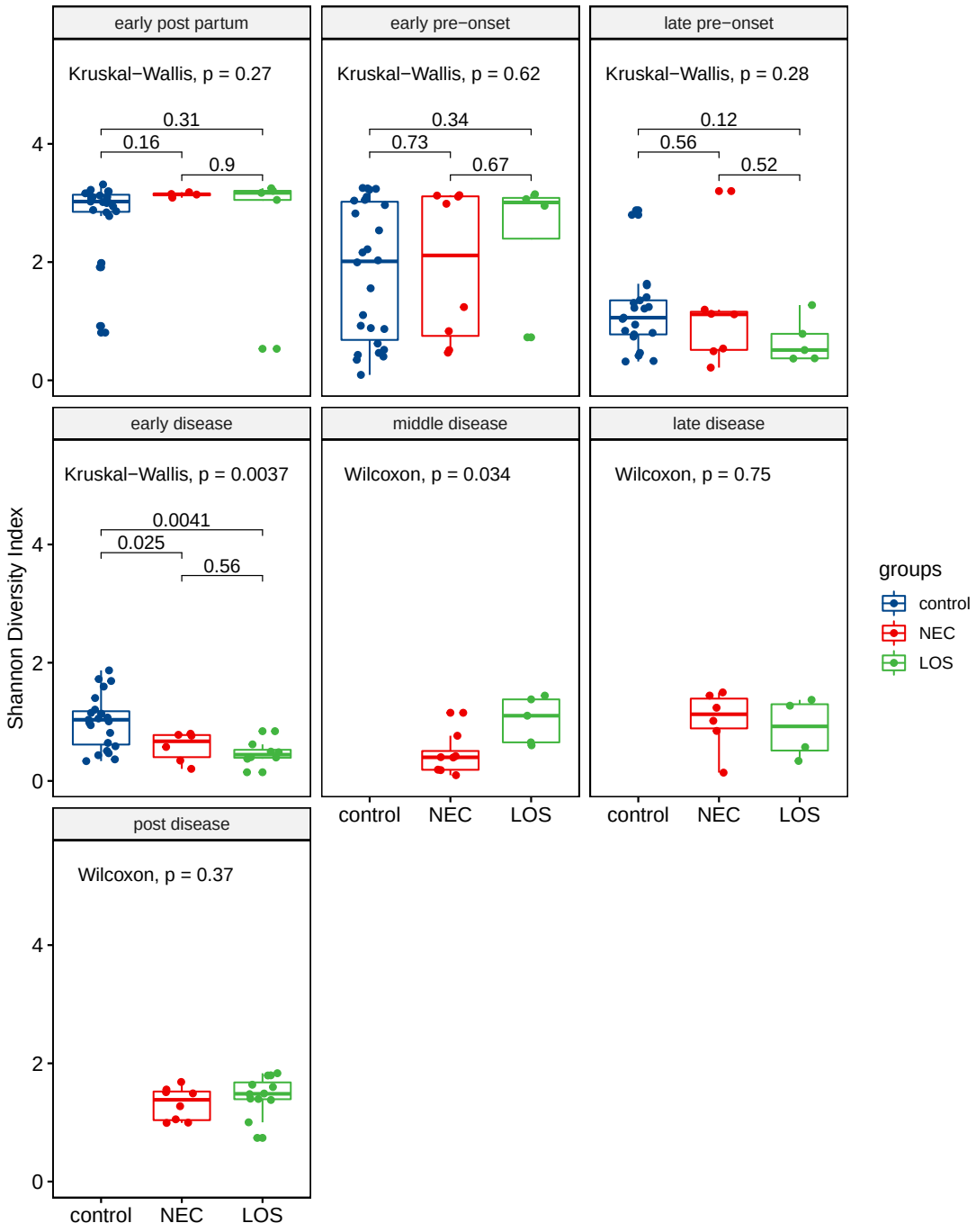

Figure 4. Post-partum microbiome evenness(shannon diversity index) trend in each time interval

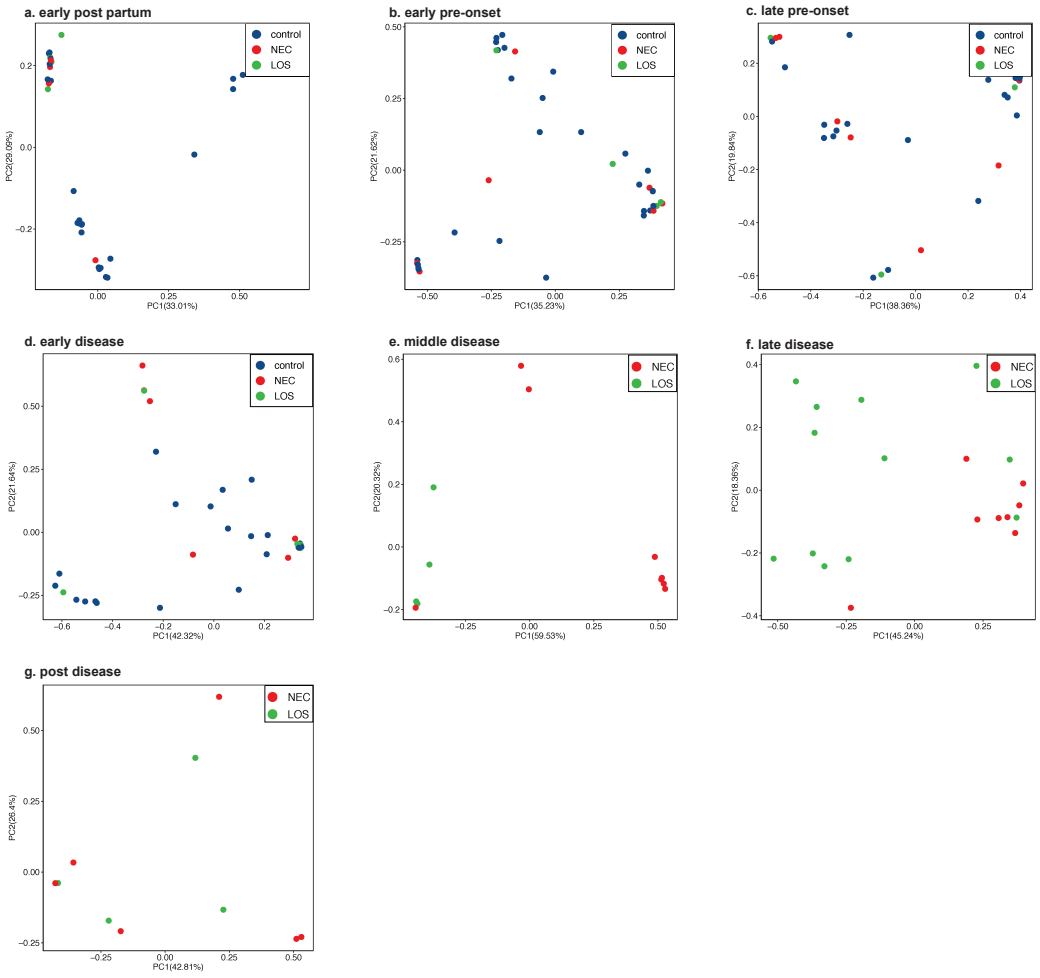

Figure 5. Beta Diversity

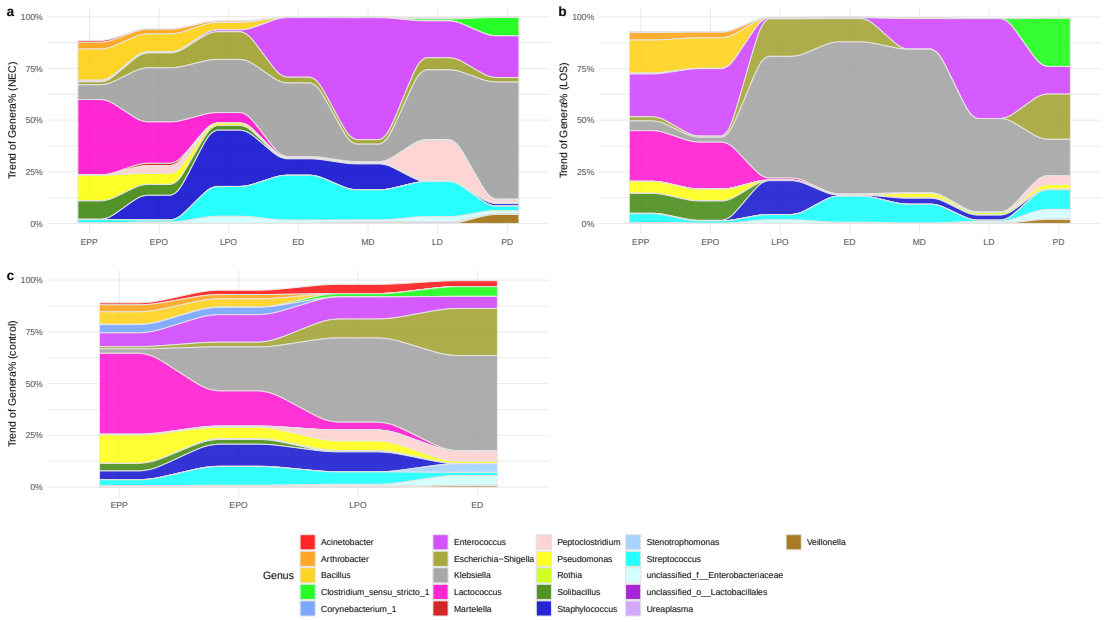

Figure 6. Kinetics of Genera Relative Abundance