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

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, OTUs that matched to Bacillus ($p = 0.032$) and Solibacillus ($p = 0.047$) were higher in relative abundance in case groups before disease 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. [*Inconsistent 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(Yatsunenko et al., 2012; Bäckhed et al., 2015; 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. In line 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 calculating 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, for the control group, the equivalent ED interval is from the day 16 to discharge.
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 of each patient was calculated, if more than two samples were available within one analysis interval. 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 and fit the data into the model (supplementary 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.

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. Image credit: Icons made by Freepik from www.flaticon.com

All 24 infants sampled were delivered by cesarean section, fed on infant formula and prescribed with prophylactic antibiotics regimen of cefotaxime, piperacillin-tazobactam and/or metronidazole right after they were admitted to our NICU. 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 interval, day 4-8 as early pre-onset interval, day 9-15 as late pre-onset interval for the control group.

Longitudinal Microbiome Diversity of NEC and LOS patients

Microbiome Richness Analysis

To get an overview of gut microbiota in patients, we analysed the gut microbiome richness of the NEC and LOS patients over time. Similar to the normal group, the case groupsNEC and LOS disesae groups showed

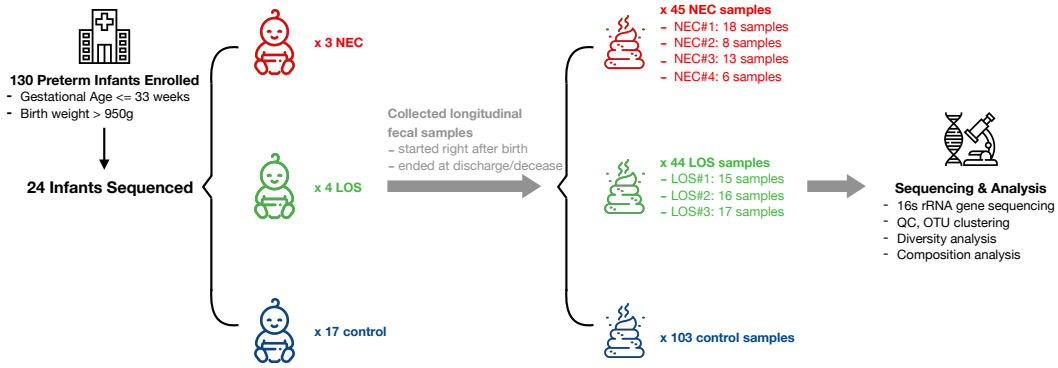

Figure 1. Schematic of Study Design

similar microbiota richness trend from early post-partum (EPP) to early disesse (ED), with the observed species (Sobs) decreased significantly from early post-partum stage (EPP) to early disease (ED) stage (Fig2 a. 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 onward with no significant difference between adjacent time intervals.

Fig2 Trend in microbiome richness (Sobs) over time.
Microbial richness trend in case 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 sample is depicted as one dot.

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,

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	—	—

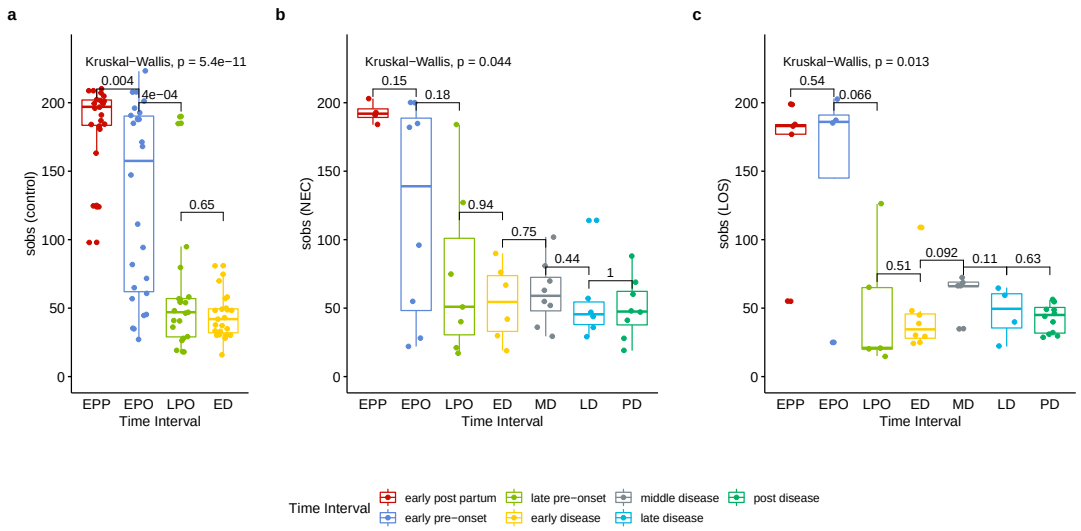

Figure 2. Post-partum microbiome richness (Sobs) trend in each group

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).

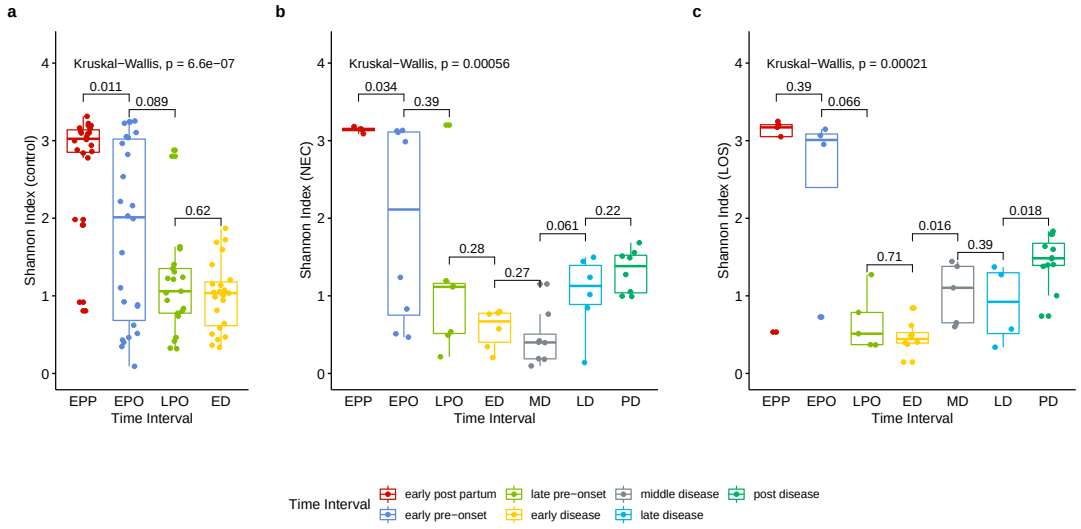

Figure 3. Post-partum microbiome evenness(shannon diversity index) trend in each group

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 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 (Fig3 a. NEC, early pre-onset 1.925 vs. post disease 1.320, p = 0.79; b. LOS, early pre-onset 2.473 vs. post disease 1.463, 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 compare the beta-diversity of the three groups over time,,we applied Principal Component Analysis (PCoA) to weighted UniFrac distance matrix. During early post-partum interval, beta-diversity among three groups was the lowest with the first principal coordinates accounted for 33.01%. Then beta diversity continued to drift away from one another. The first principal coordinate one (PC1) increased from 33.01% at early post-partum to 35.23% at early pre-onset stage, 38.36% at late-onset stage and eventually reaching 42.32% at early disease stage (Fig5 b to d). This continuous increase in beta-diversity suggested that the phylogenetic composition of the patients' microbiome started to deviate from the control group before the onset of diseases. As disease progress, the phylogenetic similarity between the NEC and the LOS disease groups separated further and peaked at 59.53% at middle disease stage then came down gradually to 42.8% at post disease stage (Fig5 e to g) This trend in phylogenetic dissimilarity suggested that the microbiome composition of the NEC and LOS patients might have deviated from normal even before the onset of diseases. While the further separation between the NEC and the LOS groups could be a result of different treatment strategies.

Fig 5. Beta diversity of the NEC, LOS and the control groups over time. Beta diversity of samples is depicted by principal coordinates analysis 'PCoA' plot showing unweighted UniFrac distance between samples. Each dot represents the microbiota of a single sample. Samples from the same group is represented by the same colors. Scatter plot shows principal coordinate 1 (PC1) versus principal coordinate 2 (PC2). Percentages shown are percentages of variation explained by the components. Samples that clustered closer together are considered to share a higher proportion of the phylogenetic tree and represented by a lower percentage in PC1.

Colonization Trend at 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 indicated that the microbiota assembly 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/or 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 relative abundance over time(Fig6).

At early post-partum stage, all three groups showed high proportion of Lactococcus, Bacillus and Pseudomonas. However, ZIBR model the disease groups showed significantly higher OTUs that matched to Bacillus (NEC 15.05% and LOS 15.97% compare to 6.02% of controlnormal, p = 0.032) and Solibacillus and higher Solibacillus (8.88% in NEC and 9.61% in LOS compare to 3.65% of controlnormal, p = 0.047) from the case groups (ZIBR table cited). Moreover, Enterococcus proportion (Fig6a, purple area) was much higher in LOS patients (20.72%) than the normal controls (6.66%, Fig. 6a, purple area) but almost absent in NEC patients (0.51%) (Fig6b). While all three groups showed increases in Klebsiella and Escherichia-Shigella, and decreases in Lactococcus from EPP to ED, the rates of change were different among the three groups. The LOS group exhibited the most drastic changes, with rapidly increased of Klebsiella (from 4.71% to 58,90%), Escherichia-Shigella (from 2.02% to 18.16%) and Streptococcus or Staphylococcus? (from 0.19% to 16.49%)(Fig6c). Together, these three genera accounted for almost 100% of all bacteria (Fig 6c). In addition, Lactococcus decreased more rapidly than the other groups, from 24.5416.0% at EPP to 0.94002% before LPO (Fig6c. magenta area).

On the other hand, increase of Klebsiella was the smallest in NEC patients (Fig6b grey area, from 7.17% at EPP to 35.63% to ED). Moreover, a rapid surge of Enterococcus, Staphylococcus and Streptococcus from EPO to ED was only observed in NEC patients (Fig. 6b, purple, dark and light blue area).

As disease progress with medical intervention, the composition of the disease group underwent another round of drastic changes. Most notably, the fluctuation of Enterococcus, Klebsiella, Staphylococcus and Peptoclostridium during the disease stages (Fig6b and c, stage ED to LD). This might be the result of difference regimen of antibiotics used to treat NEC versus LOS. Interestingly, as patients approached remission, the composition became more balanced, more resembled that of the normal control, except a higher level of Clostridium. In summary, compare to normal preterm infants, we observed different patterns of temporal changes in bacterial composition among NEC and LOS patients. Rapid changes in relative abundance of certain genera were revealed as early as early pre-onset of stages. These changes were especially obvious in LOS patience.

Fig 6. Stacked plots showing temporal development of microbiome in three goups. Genus of relative abundance over 10% were depicted among NEC, LOS and control infants.

DISCUSSION

NEC and LOS are major causes of morbidity and mortality in preterm infants worldwide and have been exerting economic burdens on healthcare costs(Johnson et al., 2013, 2014; Mowitz et al., 2018). Although early recognition and treatment regimen has improved clinical outcomes, both diseases still account for morbidites in NICU survivors(Hintz et al., 2005; Zonnenberg et al., 2019; Shah et al., 2015). China has a high rate of preterm birth(Blencowe et al., 2012) with ongoing improvements in neonatal healthcare which allow greater survival rates in preterm infants leading to increased numbers at risk of developing NEC and sepsis. Thus, elucidating their pathogenesis and developing preventive strategies would be invaluable to provide health benefits for preterm infants.

In this pilot study, we intended to investigate the etiopathology of NEC and LOS in Chinese preterm infants. We profiled the gut microbiome of NEC and LOS patients from birth to discharge. The sample size was relatively small. However, some of our findings is similar to that conducted in western countries. Mainly, microbiota of NEC and LOS patient showed very different early colonization patterns when compared to normal control. Moreover, case groups showed lower diversity, although to different degree. from a intestinal-microbial perspective: to profile 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*.

To our knowledge, few studies has analyzed stool bacterial diversity in preterm infants as early as three days after birth. Strikingly, the bacterial richness and evenness among all three groups were the highest within three days after birth (i.e. early post-partum interval). However, a generally declining trend of microbial alpha diversity was observed afterwards in both case groups and the control group. The number of colonized species (sobs index) over time, in line with previous studies(Mai et al., 2011, 2013), remained similar before disease onset in all three groups (might be largely due to the similar nursing environment within the NICU from which the infants acquired mostly their gut microbiota?), suggesting the minor role of bacterial richness in disease acquisition. Furthermore, the pervasive effect of antibiotics in reducing microbial richness has been reported in preterm infants: decrease in bacterial richness and diversity was reported 1 week to 2 months after early-life antibiotics(DiGiulio et al., 2008; Dethlefsen and Relman, 2011; Fouhy et al., 2012; Greenwood et al., 2014; Tanaka et al., 2009). In our cohort, the drastic decrease in sobs from early to late pre-onset interval (3 to 16 days of life), however, showed an earlier and more rapid effect of antibiotics.

In contrast, microbiota structure, which measures the phylogenetic similarity of stool microbiota (using a weighted UniFrac based PCO analysis), drifted away continuously after birth among three groups until alleviation of the disease. These findings were in accordance with a previous study revealing the heterogeneous microbiota one week before NEC onset(Mai et al., 2011); while inconsistant with previous findings that microbiota within 72 hours before LOS onset resembled the one during LOS(Mai et al., 2013).

Roles of empiric prophylactic antibiotics in NEC or LOS are controversial. In animal models, antibiotics

eliminating Gram-negative bacteria enhance gut function and diminish mucosal injury to the bowel thus preventing necrotizing enterocolitis or bacterial leakage into the bloodstream(Carlisle et al., 2011; Jensen et al., 2013; Birck et al., 2015). In clinical practices, broad-spectrum antibiotics (the most commonly prescribed medicine in the NICU) are recommended to empirically prevent and treat both NEC and LOS(Bury and Tudehope, 2001; Brook, 2008; Kimberlin et al., 2018). However, antibiotics could further result in microbiome dysbiosis therefore increase the risk of developing these conditions(Gibson et al., 2015; Kuppala et al., 2011; Martinez et al., 2017; Cantey et al., 2018). Our results showed limited differences between two case groups and the control group with respect to bacterial diversity and composition when antibiotics were continuously administered. Therefore, although antibiotic indeed cause dysbiosis in our cohort, we still hesitate to draw the conclusion that antibiotics per se induced or prevented the diseases.

Our results identified that over-represented Bacillus and Solibacillus as they initially colonized the intestine (although both diminished within days afterbirth) were implicated in the vulnerability of NEC and LOS. Furthermore, blooming and dominance of potentially pathogenic genus in both case groups are consistent with the hypothesis that dysbiosis precedes NEC or LOS.

Our study was limited to only one hospital in one specific geographical region in China and so could not be generalized to a larger population. Our results, therefore, 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

In this longitudinal studies, we used next generation sequencing to profile microbiota of 24 Chinese preterm infants from birth to discharge. Among them, four developed NEC and three developed LOS. To our knowledge, this is the first profiling of NEC and LOS patients among Asian population. Intestinal microbiota diversity reduction and phylogenetic similarity 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.

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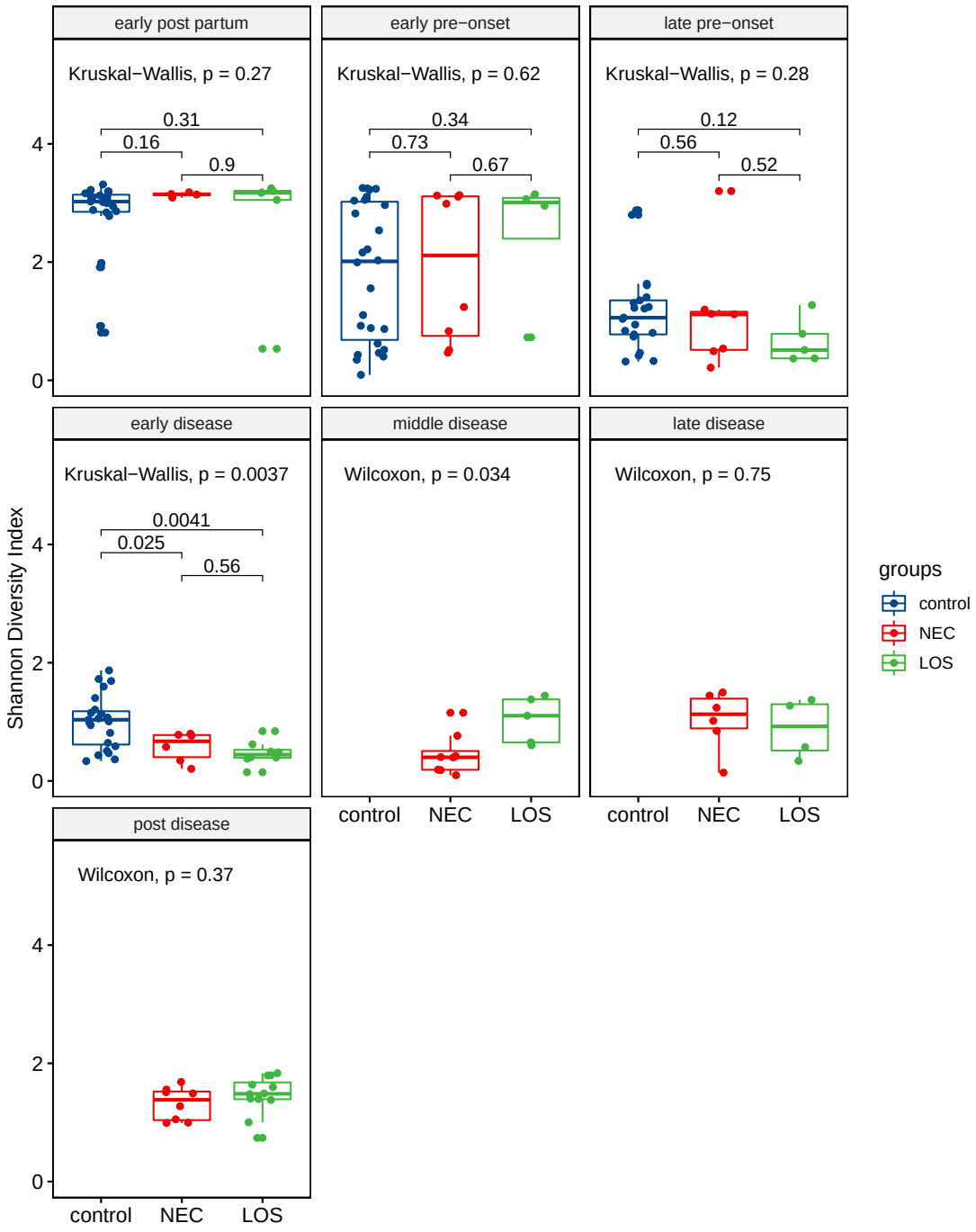

Figure 4. Post-partum microbiome evenness(shannon diversity index) trend in each time interval

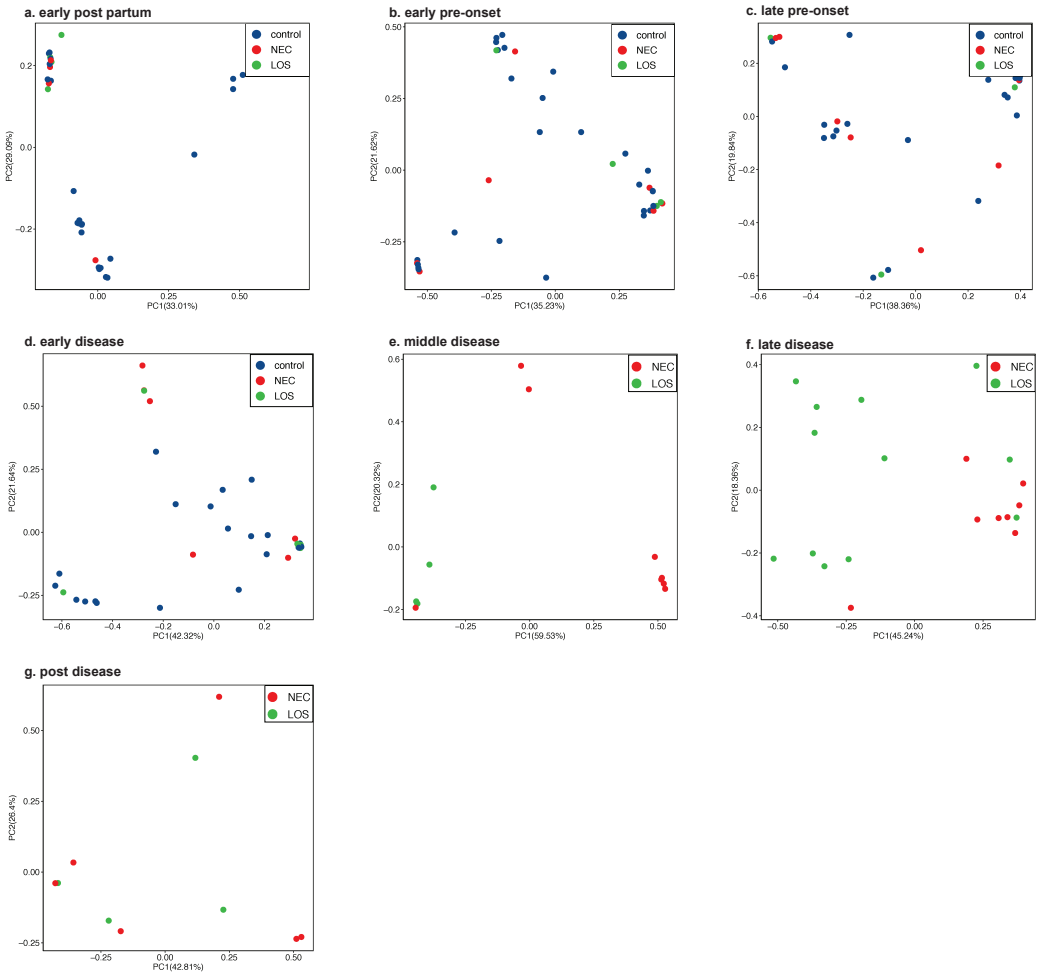

Figure 5. Beta Diversity

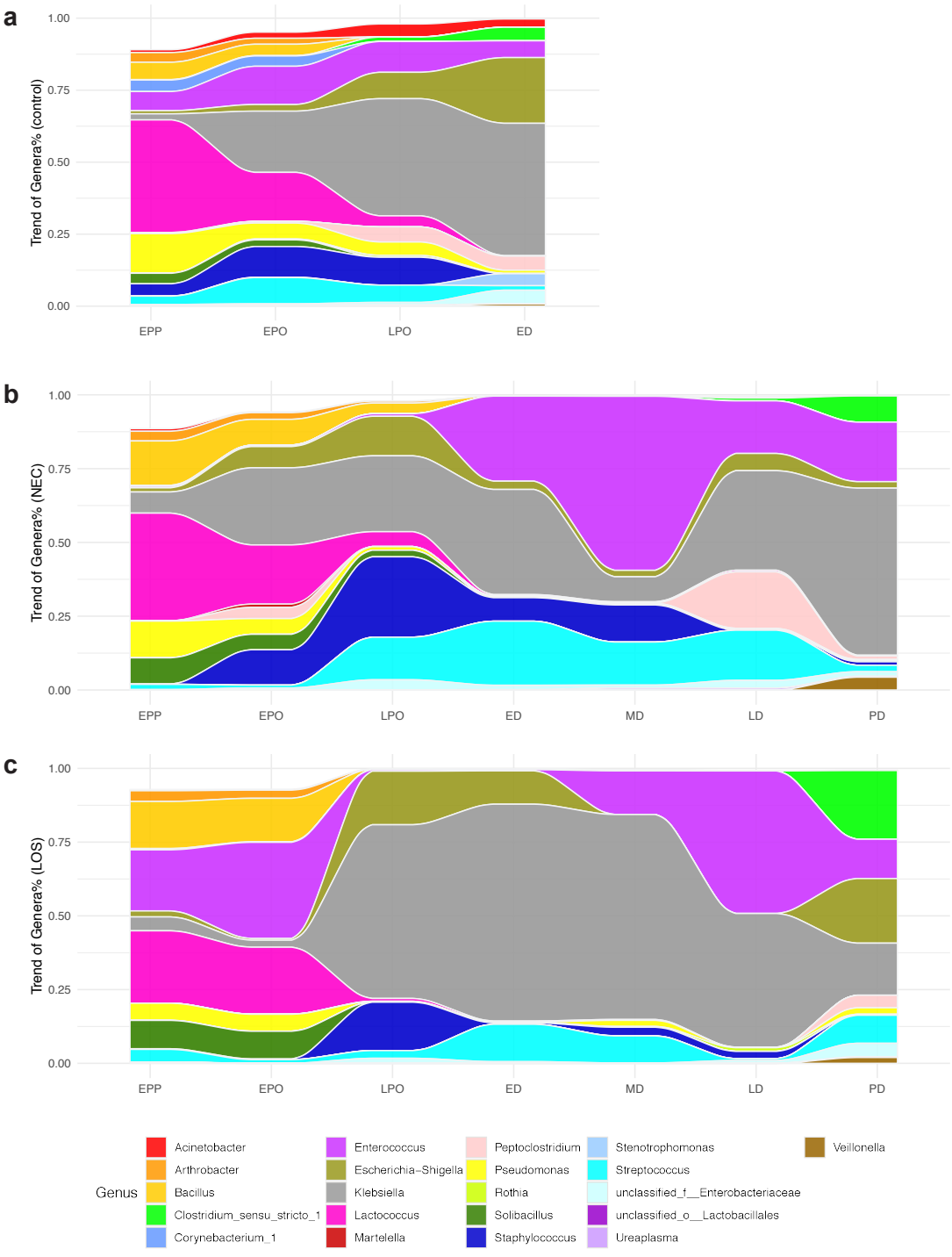

Figure 6. Kinetics of Genera Relative Abundance