Patterned progression of gut microbiota associated with necrotizing enterocolitis and late onset sepsis in preterm infants: a prospective study in a Chinese neonatal intensive care unit

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Necrotizing enterocolitis (NEC) and late-onset sepsis (LOS) are two common premature birth complications with high morbidity and mortality. Recent studies in Europe and America have linked gut microbiota dysbiosis to their etiology. However, similar studies in Asian populations remain scant. In this pilot study, we profiled gut microbiota of 24 Chinese preterm infants from birth till death or discharge from NICU. Four of them developed NEC and three developed LOS. Unexpectedly, we detected highly-diversified microbiota with similar compositions in all patients shortly after birth. However, as patients aged, the microbial diversities in case groups differed significantly from that of the control group. These differences emerged after the third day of life and persisted throughout the course of both NEC and LOS. Using a Zero-Inflated Beta Regression Model with Random Effects (ZIBR), we detected higher *Bacillus* (p = 0.032) and *Solibacillus* (p = 0.047) before the onset of NEC and LOS. During NEC progression, *Enterococcus*, Streptococcus and *Peptoclostridium* were the dominant genera while during LOS progression; *Klebsiella* was the only dominant genus that was also detected by the diagnostic hemoculture. These results warrant further studies to identify causative microbial patterns and underlying mechanisms.

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ABSTRACT

Necrotizing enterocolitis (NEC) and late-onset sepsis(LOS) are two common premature birth complications with high morbidity and mortality. Recent studies in Europe and America have linked gut microbiota dysbiosis to their etiology. However, similar studies in Asian populations remain scant. In this pilot study, we profiled gut microbiota of 24 Chinese preterm infants from birth till death or discharge from NICU. Four of them developed NEC and three developed LOS. Unexpectedly, we detected highly-diversified microbiota with similar compositions in all patients shortly after birth. However, as patients aged, the microbial diversities in case groups differed significantly from that of the control group. These differences emerged after the third day of life and persisted throughout the course of both NEC and LOS. Using a Zero-Inflated Beta Regression Model with Random Effects (ZIBR), we detected higher *Bacillus* (p = 0.032) and *Solibacillus* (p = 0.047) before the onset of NEC and LOS. During NEC progression, *Enterococcus*, *Streptococcus* and *Peptoclostridium* were the dominant genera while during LOS progression, *Klebsiella* was the only dominant genus that was also detected by the diagnostic hemoculture. These results warrant further studies to identify causative microbial patterns and underlying mechanisms.

INTRODUCTION

The gut microbiota is a crucial 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;

43 Jie et al., 2017; Jonsson and Bäckhed, 2017) and cancers (Gagliani et al., 2014; Irrazábal et al., 2014;
44 Sears and Garrett, 2014).

45 The microbiota in newborn infants undergoes dynamic changes in composition, abundance and
46 diversity before reaching homeostasis at around three years of age (Yatsunenko et al., 2012; Bäckhed
47 et al., 2015; Stewart et al., 2018). Temporal colonization pattern of the intestinal microbiota during early
48 stages of life may have an important contribution to the long-term health of an individual. Early life
49 microbiota disruption had been associated with the development of metabolic and immunological diseases
50 such as Type I diabetes (Giongo et al., 2011; Vatanen et al., 2018), asthma (Stokholm et al., 2018) and
51 allergies (Madan et al., 2012a; Savage et al., 2018).

52 In preterm infants, common medical practices including Cesarean delivery, formula feeding, sterile
53 incubator nursing and extensive use of broad-spectrum antibiotics may limit the normal microbiota
54 acquisition and development (La Rosa et al., 2014; Shin et al., 2015; Deweerdt, 2018). Resultant abnormal
55 microbiota colonization in the gut may then contribute complications such as necrotizing enterocolitis
56 (NEC) and late onset sepsis (LOS) (Sharon et al., 2015; Cernada et al., 2016).

57 Necrotizing enterocolitis is characterized by rapid ischemic necrosis of intestinal mucosa, resulting
58 in high morbidity (2% - 7%) and mortality (15% - 30%) (Neu and Walker, 2011; Stoll et al., 2015). Its
59 etiology remains largely unknown and likely to be multi-factorial. Previous studies in European and
60 American countries have associated microbial dysbiosis to NEC onset. Reduction in microbiota diversity
61 and unusual species colonization were observed in NEC patients (Jacquot et al., 2011; Warner et al.,
62 2016). No causative species have been identified so far. However, an increase in Proteobacteria phyla
63 and a decrease in Firmicutes were observed before NEC onset (Mai et al., 2011; Zhou et al., 2015).
64 Besides, blooming of *Gammaproteobacteria* and under-representation of *Negativicutes* were associated
65 with disease progression (Warner et al., 2016).

66 Late onset sepsis (LOS) is another common life-threatening disease for preterm infants. It is commonly
67 defined as a systemic infection with the isolation of pathogenic bacteria from the bloodstream after 72
68 hours of life (Rao et al., 2016; Pickering et al., 2012). Preterm infants have immature gastrointestinal and
69 immune systems. Therefore, it is easier for pathogenic bacteria or bacterial toxins that can cause systemic
70 inflammation to enter the bloodstream (Schwiertz et al., 2003; Bezirtzoglou et al., 2011; Cernada et al.,
71 2016; Sharon et al., 2015; Korpela et al., 2018), thus making the intestine a potential source of infections
72 and inflammation. Previous studies showed that the LOS patients' gut microbiota was less diversified, and
73 dominated by *Staphylococci* and *Enterobacter* but underrepresented by probiotic *Bifidobacteria* (Madan
74 et al., 2012b; Tarr and Warner, 2016; Stewart et al., 2017; Korpela et al., 2018; Ficara et al., 2018).

75 NEC and LOS are two major causes of morbidity and mortality in preterm infants worldwide and
76 have been exerting economic burdens on healthcare costs (Johnson et al., 2013, 2014; Mowitz et al.,
77 2018). Although early recognition and treatment regimen has improved clinical outcomes, both diseases
78 still account for morbidities in NICU survivors (Hintz et al., 2005; Zonnenberg et al., 2019; Shah et al.,
79 2015). In China, the rate of preterm birth is as high as 7.1% (Blencowe et al., 2012) and continuous
80 improvements in neonatal health care have greatly improved the survival of preterm infants. However,
81 the risk of developing NEC and LOS increases as well. Elucidating their pathogenesis and developing
82 preventive strategies would greatly benefit the health of preterm infants. Motivated by this, we carried out
83 this longitudinal pilot study to profile the microbiota of Chinese preterm NEC and LOS patients, with
84 the aim to examine if similar alterations in microbiota correlate with the onset and progression among
85 Chinese patients. Consistent with previous studies in Western countries, we observed lower bacterial
86 diversity among Chinese NEC and LOS patients. In contrast, we found that the Chinese patients in our
87 cohort showed different bacterial compositions.

METHODS

Ethics
90 This study was approved by the joint committee of ethics of Shanghai Children's Medical Center, Shanghai
91 Jiao Tong University School of Medicine (SCMCIRB-K2013022). Detailed written informed consent was
92 obtained from parents before enrollment.

Patients
94 Newly born preterm infants with a gestational age less than 33 weeks and birth weight over 950g were
95 enrolled from Neonatal Intensive Care Unit (NICU) at Shanghai Children's Medical Center from July 2013

96 to December 2014. The exclusion criteria were 1) diagnosed with early-onset sepsis, 2) hepatic diseases,
97 3) renal impairment (Cr >88 μM), 4) diagnosed with intestinal obstruction, 5) in foreseeable need of
98 major cardiovascular or abdominal surgeries (except for male circumcision or PDA ligation), 6) estimated
99 parenteral support to supply over 50% of daily caloric intake for more than four days, 7) given intravenous
100 antibiotics administration (except prophylactic regimen of cefotaxime, piperacillin-tazobactam and/or
101 metronidazole), 8) history of oral antibiotics administration, 9) grossly bloody stools at admission, and
102 10) over five days old.
103   NEC cases were defined as infants who met the criteria for Stage II and Stage III NEC diagnosis (Bell
104 et al., 1978), including radiographic intestinal dilation, ileus, pneumatosis intestinalis, and/or absent bowel
105 sounds with or without abdominal tenderness, and/or mild metabolic acidosis and thrombocytopenia. An
106 LOS case was defined if an infant 1) had a positive hemoculture or other suspicious loci of infection after
107 72 hours of life, or 2) presented with septic signs/symptoms reviewed and diagnosed independently by at
108 least two neonatologists, and had been responding well with advanced antibiotics (e.g., Meropenem) after
109 diagnosis. Infants with no infectious complications were regarded as controls.

Sample collection and handling

111 Fecal sample collection started from neonatal meconium until death or discharge, whichever came first.
112 Although we intended to collect fecal samples every day, due to working shifts and flexible clinical
113 scheduling, we set seven days as the maximum interval between two collections from every infant. Every
114 sample was collected from infants' diaper with a sterile spatula into cryogenic vials within 30 minutes of
115 defecation. Then the sample was immediately placed on dry ice and stored at - 80°C within 30 minutes
116 without additives. All samples were collected and stored before knowing the diagnosis of respective
117 patients.

DNA extraction and quality control amplification and 16s rRNA gene sequencing

119 Microbial genomic DNA was isolated from each fecal specimen using the E.Z.N.A.® Soil DNA Kit
120 (Omega Bio-Tek, Norcross, GA, U.S.) according to the manufacturer's protocols. The concentration and
121 purity of the DNA were determined by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific,
122 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

124 The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified by PCR from each
125 sample using bacterialarchaeal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-
126 GGACTACHVGG GTWTCTAAT-3') using a thermocycler PCR system (GeneAmp 9700, ABI, USA).
127 The PCR reactions were as follows: 3 min of denaturation at 95 °C, 27 cycles of 30 s at 95 °C, 30 s
128 annealing at 55 °C and 45 s elongation at 72 °C, and a final extension at 72 °C for 10 min. The PCR
129 reactions were performed in triplicate, with each 20 μL mixture containing 4 μL 5X FastPfu Buffer, 2 μL
130 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL FastPfu Polymerase (TransGen Biotech, Beijing,
131 China) and 10 ng template DNA. PCR products were separated from impurities and genomic DNA by
132 running in 2% agarose gels. The PCR bands were further purified using the AxyPrep DNA Gel Extraction
133 Kit (Axygen Biosciences, Union City, CA, USA), and quantified using QuantiFluor™-ST (Promega, USA)
134 according to the manufacturer's protocols. Equimolar amounts of purified amplicons were pooled and
135 paired ended sequenced (2 x 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according
136 to the standard protocols of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The reads were
137 de-multiplexed using the Illumina software and separate FASTQ files were generated for each specimen
138 and deposited to the Sequence Read Archive NCBI under the BioProject accession PRJNA470548.
139 Another public archive repository is available at figshare doi: 10.6084/m9.figshare.7205102

Raw Data Processing

141 Raw data were processed according to the standard protocols provided by Majorbio Bio-Pharm Technology
142 Co. Ltd. (Shanghai, China) as previously described (Liu et al., 2018; Wang et al., 2018). In short, raw
143 sequencing data was first de-multiplexed. Sequence reads were then subjected to quality filtering utilizing
144 Trimmomatic software (Bolger et al., 2014) and were truncated at any site with a Phred score <20 over a
145 50bp-sized window. Barcode matching with the primer mismatch from 0 to 2 nucleotides was adopted and
146 reads containing ambiguous characters were removed. After trimming, FLASh (Fast Length Adjustment
147 of Short Read) (Magoč and Salzberg, 2011), a read pre-processing software, assembled and merged the

148 paired-end reads from fragments and generated >10 bp overlapped, with the dead match ratio of 0.2.
149 Unassembled reads were discarded. From the 192 fecal samples sequenced, a total of 7,472,400 optimized
150 V3-V4 tags of 16s rRNA gene sequences were generated(Table S1).
151   To unbiasedly compare all the samples at the same sequencing depth, the "sub.sample" command of
152 mothur program (version1.30.1) (Schloss et al., 2009) was used for normalization to the smallest sample
153 size (27994 sequences per sample). Chimera was detected and removed by UCHIME Algorithm. The
154 effective reads were then sorted by cluster size and processed using Operational Taxonomic Units (OTUs)
155 with 97% similarity cutoff UPARSE-OTU algorithm (implementing "cluster_otus" command) (Edgar,
156 2013) in USEARCH(v10)(UPARSE version 7.1). The taxonomy of each 16S rRNA gene sequence was
157 analyzed by RDP Classifier algorithm (Wang et al., 2007) against the Silva (SSU128) 16S rRNA database
158 (Quast et al., 2012) using confidence threshold of 70%. Each sequence was assigned the taxonomy by
159 QIIME (Caporaso et al., 2010). The representative sequences were allocated phylogenetically down to
160 the domain, phylum, class, order, family, and genus levels (Table S2). The relative abundance of a given
161 taxonomic group was calculated as the percentage of assigned sequences over total sequences.
162   Within-sample diversity(alpha diversity), including Shannon index and observed species richness
163 (Sobs), was obtained using the "summary.single" command of mothur program (version1.30.1) (Schloss
164 et al., 2009). Between-sample diversity(beta diversity) was obtained by calculating weighted UniFrac
165 distances between samples.

Statistical and Bioinformatics Analyses
Demographics and Clinical Sample comparisons
168 Kruskal-Wallis test and Wilcoxon rank-sum test were used to identify statistically significant differences in
169 continuous variables, including gestational age, birth weight, age at diagnosis and length of hospitalization.
170 The χ^2, or Fisher's exact test was used to identify differences in gender composition. α level was
171 considered 0.05 for all statistical tests. Other statistical analyses not involving microbiome 16s rRNA
172 sequencing data were performed using the *"stats"* package in R (v.3.5.1).

Microbiota and Bioinformatics Analyses
Disease-related Time Interval Definition   Considering that the sampling and disease onset time for
175 each patient were not identical, to illustrate the continuous longitudinal and repeated nature of the sampling
176 and its relationship with onset and progression of diseases, we divided the sampling span into seven time
177 intervals:

1. early post-partum(EPP): within 3 days after birth
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 disease onset; for control group patients, the equivalent onset time is set at the 16th day of life, as is the average diagnosis age of NEC and LOS groups.
4. early disease(ED): the first third interval of the whole disease span; for the control group, the equivalent ED interval is from day 16 to discharge.
5. middle disease(MD): the middle third interval of disease span
6. late disease(LD): the last third interval of disease span
7. post disease(PD): from the end of disease to discharge time-point

Modeling Strategies for Comparisons   To compare the dynamics of microbiota diversity and relative
189 taxonomic abundance preceding the disease, we applied the EPP, EPO, LPO and ED interval among all
190 patients into our model or comparisons.

Diversity Analyses   Kruskal Wallis tests were used to compare the differences in overall alpha diversity.
192 The MannWhitney U test was then applied to compare two adjacent time intervals. Differences in alpha
193 diversity over time were analyzed by a two-way repeated measures ANOVA, with the time interval
194 (EPP, EPO, LPO, ED, MD, LD, PD) as a within-subject factor and the group (NEC, LOS, control) as a
195 between-subject factor. If more than one sample of a patient were collected within a time interval, the
196 average of the α diversity indices was used as one data point. Beta diversity was obtained using weighted
197 Unifrac distance matrix and visualized through Principal Component Analysis (PCoA). The analysis of
198 similarity (ANOSIM) (Clarke, 1993) were used to determine the dissimilarity among the clusters revealed
199 by the PCoA plots.

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200 **Taxonomy Comparisons** Zero-Inflated Beta Regression Model with Random Effects (ZIBR) and
201 Linear Mixed-effects Model (LME) were used to test the association between OTU relative abundance
202 and clinical covariates (diseases-related time intervals) for longitudinal microbiome data (Chen and Li,
203 2016). *ZIBR* and *nlme* (Pinheiro et al., 2018) R packages were utilized for each model. If more than one
204 sample of a patients were collected within a time interval, the average of relative abundance of each genus
205 was used.

206 *Scripts and Figures Archiving*
207 Figures were generated with the *"ggpubr"* (Kassambara, 2017), *"ggplot2"* (Wickham, 2016) and *"ggsci"*
208 (Xiao, 2018) packages using R(v.3.5.1). RScripts for analyses as well as input and output files are available
209 at our GitHub repository.

RESULTS

Patients characteristics
212 From July 2013 to December 2014, a total of 130 preterm infants admitted to the neonatal intensive
213 care unit (NICU) of Shanghai Children's Medical Center met the criteria of our study and a total of
214 1698 samples were collected. 192 fecal samples from 24 well-sampled preterm infants were sequenced.
215 Four subsequently developed NEC (2 in stage IIA and 2 in stage IIB) and three developed LOS (2 with
216 positive hemoculture of Klebsiella pneumoniae; the other one was diagnosed upon sepsis-related signs
217 and symptoms, lab test of white blood cells >20 cells/microL and her effective reaction to vancomycin).
218 The remaining 17 served as matched controls (Figure1, Table S3). Fecal samples were collected between
219 days 1 and 69 of life. Numbers of samples collected and interval of sampling varied among patients but
220 met our preset criteria of less than 7 days between sampling. The average number of sample collected for
221 NEC, LOS and control patients was 11, 14 and 6 respectively. The number of samples per patient was
222 higher in the NEC and LOS groups because the severity of the disease required longer hospitalization (p
223 = 0.046).
224   All 24 infants profiled were delivered by Cesarean section, fed on infant formula and prescribed with
225 prophylactic antibiotics regimen (cefotaxime, piperacillin-tazobactam and/or metronidazole) right after
226 they were admitted to our NICU. No infant was prescribed probiotics during the study. There was no
227 significant difference in gestational age (p = 0.074), birth weight (p = 0.111) or gender proportions (p =
228 0.822) among the three groups. The average age at diagnosis for both disease groups was 16 days and
229 there was no statistical difference between the groups (p = 0.629) (Table 1). Therefore, we assigned day
230 16 to discharge as early disease interval, day 4-8 as early pre-onset interval and day 9-15 as late pre-onset
231 interval for the control group (Table S4).

Longitudinal Microbiome Diversity of NEC and LOS patients
233 To get an overview of gut microbiota in patients, we analyzed the microbial richness of the NEC and LOS
234 patients over time. Similar to the control group, the case groups showed a decreasing trend in observed
235 species (Sobs) from early post-partum stage (EPP) to early disease (ED) stage (Fig2 (A) control group, p
236 <0.01; (B) NEC group, p = 0.044; (C) LOS group, p = 0.013; Dataset S1, Sheet "Sobs" two way RM
237 ANOVA, p <0.0001). The greatest decline in sobs was from early pre-onset (EPO) to late pre-onset
238 (LPO). However, the decrease in the disease groups was less significant than the control group (control
239 group p = 0.0004, NEC group p = 0.18, LOS group p = 0.066). The Sobs then stabilized from LPO
240 onward with no significant difference between adjacent time intervals.
241   Next, we analyzed gut microbiome evenness over time. Similar to Sobs, the Shannon indices decreased
242 significantly from the early post-partum (EPP) to early disease (ED) stage (Fig3(A) control group 2.768
243 to 1.004 , p = 0.04; (B) NEC group, 3.141 to 0.578, p = 0.01; (C) LOS group, 2.641 to 0.470, p = 0.01).
244   Two way RM ANOVA showed significant Shannon index divergent among three groups before disease
245 onset (Dataset S1, sheet "Shannon", EPP to ED, p = 0.0017). Moreover, during early disease stage, the
246 Shannon indices were different among three groups (Fig4, facet "early disease", p = 0.0037), suggesting
247 that microbiota distortion may precede NEC and LOS onset. As diseases progressed, the NEC group
248 differed significantly with the LOS group during middle disease interval but insignificantly during late
249 disease interval (Fig4 facet "middle disease", p = 0.034; facet "late disease", p = 0.750). Upon alleviation
250 of both diseases, the Shannon indices rose back to the early pre-onset levels(Fig3 (B) NEC group. early

pre-onset at 1.925 vs. post disease at 1.320, p = 0.79; (C) LOS group, early pre-onset at 2.473 vs. post disease at 1.463, p = 0.16).

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 and used the analysis of similarity (ANOSIM) (Clarke, 1993) to determine the dissimilarity of the microbial composition among the groups. Bacterial composition of three groups showed non-significant difference during the early post-partum interval (ANOSIM R = 0.007, p = 0.385), with the first principal coordinates accounted for 33.01%(Fig5 (A)). Then the cluster of each group comtinued to separate from one another. The first principal coordinate one (PC1) increased from 33.01% at the early post-partum to 35.23% at the early pre-onset stage, 38.36% at the late pre-onset stage and eventually reaching 42.32% at the early disease stage (Fig5 (B) ANOSIM R = 0.151, p = 0.087, (C) ANOSIM R = 0.255, p = 0.015, to (D) R = 0.193, p = 0.077). 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 diseases progressed, the phylogenetic similarity between the NEC and the LOS disease groups diverged further and peaked at 59.53% in middle disease stage (ANOSIM R = 0.317, p = 0.028) then came down gradually to 42.8% at post disease stage (Fig5 (E)ANOSIM R = 0.317, p = 0.028, to (G) ANOSIM R = 0.203, p = 0.045). 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. Also, the further separation between the NEC and the LOS groups could be a result of different treatment strategies.

Colonization Trend at The Genus Level

In the analyses of intestinal microbiome alpha(Fig2, Fig3, Fig4) and beta diversity(Fig5), detectable differences were observed among the three groups, especially during the transition from the LPO to ED stage. This indicated that the microbiota assembly differences between the case groups and control group. To further investigate which microbiota composition was correlated with the onset and/or progression of NEC and LOS, we tracked the longitudinal compositional changes in the abundance of each genera. We filtered the genus of over 10% relative abundance among all samples and plotted relative abundance over time(Fig6).

At the 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% compared to 6.02% of control, p = 0.032) and *Solibacillus* (8.88% in NEC and 9.61% in LOS compared to 3.65% of control, p = 0.047) from the case groups (Dataset S2). Moreover, *Enterococcus* proportion (Fig6(B), purple area) was much higher in LOS patients (20.72%) than the normal controls (6.66%, p = 0.048, Fig6(A), purple area) but almost absent in NEC patients (0.51%) (Fig6(B)). While all three groups showed increases in *Klebsiella* and *Escherichia-Shigella* and decrease 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 a rapidly increase of *Klebsiella* (from 4.71% to 58.90%, p = 0.017), *Escherichia-Shigella* (from 2.02% to 18.16%, p = 0.034) and *Streptococcus* (from 1.22% to 12.68%, p = 0.029)(Fig6(C)). Together, these three genera accounted for almost 100% of all bacteria (Fig6(C)). In addition, *Lactococcus* decreased more rapidly than the other groups, from 24.54% at EPP to 0.94% before LPO (Fig6(C) magenta area, p = 0.037).

Besides, the increase of *Klebsiella* was the most minimal in NEC patients (Fig6(B) grey area, from 7.17% at EPP to 35.63% at ED). Moreover, a rapid surge of *Enterococcus*, *Staphylococcus* and *Streptococcus* from EPO to ED was only observed in NEC patients (Fig6(B), purple, dark and light blue area).

As NEC and LOS progressed with medical intervention, the genus in case groups underwent another round of drastic changes. Most notably, the fluctuation of *Enterococcus*, *Klebsiella*, *Staphylococcus* and *Peptoclostridium* during the disease stages (Fig6(B) and (C), stage ED to LD), which might be resultant from different healthcare strategies applied in two groups. Interestingly, as patients approached remission, the composition became more balanced and resembled more to that of the normal control, except for a higher level of *Clostridium*. In summary, relative to patients in the control group, we observed different patterns of temporal alterations 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 and were the most notable in LOS patients.

DISCUSSION

In this pilot study, we intend to investigate the etiopathology of NEC and LOS in Chinese preterm infants from the perspective of intestinal microbiota. We profiled the gut microbiome of NEC and LOS preterm infants from birth to death or discharge. Some of our findings are similar to previous larger-scale studies. Mainly, infants who developed NEC or LOS exhibit a different gut microbiota colonization pattern relative to the controls. Case groups showed a decline in diversity, although to a different extent. Moreover, NEC and LOS infants' intestines were prone to harbor potential pathogens prior to and after disease onsets, such as *Enterococcus*, *Staphylococcus*, *Peptoclostridium* and *Streptococcus*. There were also findings unique to this study will be discussed in the following paragraphs.

To our knowledge, few studies have analyzed stool bacterial alpha diversity in preterm infants as early as three days after birth. Unexpectedly, within three days after birth (i.e., early post-partum interval), the bacterial diversity of all three groups was the highest compared to the following stages. At this point, we do not know if this high bacterial richness and evenness within three days of life are universal. More data, especially from other countries, are needed to support this finding. After three days, the microbial alpha diversity exhibited a declining trend in both disease groups and the control group. The number of colonized species (sobs index) during this interval, in line with previous works (Mai et al., 2011, 2013), remained similar before disease onset in both case and control groups, suggesting a minor role of bacterial richness in the disease onset. Besides, a rapid decline in alpha diversity during the pre-onset stages was observed. This could be resultant from the standardized antibiotic regimen right after admission into our NICU. However, previous studies showed that the pervasive effect of antibiotics in reducing richness and evenness arose only after 1 week to 2 months of administration (DiGiulio et al., 2008; Dethlefsen and Relman, 2011; Fouhy et al., 2012; Greenwood et al., 2014; Tanaka et al., 2009). Thus, more research is needed to identify if additional factor(s) is involved in this rapid decline.

The role of empiric prophylactic antibiotics in NEC or LOS is 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 medications 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 can further induce microbiome dysbiosis that may increase the risk of developing these diseases and exacerbate the severity (Gibson et al., 2015; Kuppala et al., 2011; Martinez et al., 2017; Cantey et al., 2018). Our results showed limited differences in bacterial diversity and composition between two case groups and the control group despite continuously antibiotics administration. Although our results are in line with the dysbiotic effect of antibiotics, there was not enough evidence to support whether antibiotics per se induced or prevented NEC and LOS. Further studies are needed to confirm the causative relationships.

Furthermore, microbiota beta-diversity, which measures the phylogenetic similarity, drifted away continuously among three groups before the onset of both diseases. These findings were inconsistent with a previous study where the microbiota of NEC patients were shown to be similar to that of the healthy controls at three days before onset (Mai et al., 2011). With regards to the LOS patients, it is also inconsistent with the previous study where similar microbiota diversity was observed in LOS patients during the disease and 72 hours before onset (Mai et al., 2013). These discrepancies could be a result of differences in collection time points or differences in patients' demographics. Further studies are necessary to address these issues. As the diseases progressed, the beta-diversity of the NEC group and the LOS group separated further but converged again when diseases were alleviated. The exact cause of this divergence was not clear. It could be related to different treatment strategies or some intrinsic pathophysiology differences between the two diseases. Further studies should provide more insight.

In addition to bacterial diversity, we also tracked longitudinal changes in composition at the genus level by plotting the relative abundance over time. Overall, the control group exhibited more stable microbiota assembly, without drastic fluctuation in genus abundance and with less dominance of facultative anaerobes such as *Enterococcus* and *Staphylococcus* (Gibson et al., 2015; La Rosa et al., 2014; Grier et al., 2017). Based on our ZIBR model, an over-represented Bacillus and Solibacillus were detected during the pre-onset stages in case groups. However, both genera diminished after disease onset suggesting that the initial microbiota composition in preterms might contribute to their future health outcomes. Previous studies also observed a surge in Proteobacteria phyla (Mai et al., 2013, 2011) preceding LOS and NEC onset. In

line with this, LOS patients in our cohort were also characterized by a higher abundance of *Klebsiella* in their intestinal communities. On the contrary, NEC infants presented overgrowth of *Streptococcus* and *Staphylococcus* (both belong to phyla *Firmicutes*) before disease onset. Further work is warranted to identify specific genera and trends in association with the onset of NEC and/or LOS.

Diarrhea is one of the typical symptoms in NEC patients and *Peptoclostridium* is conventionally regarded as a causative pathogen of hospital-acquired infectious diarrhea (Rodriguez et al., 2016; Pereira et al., 2016). In our study, we identified a transient bloom of *Peptoclostridium* in late NEC stage that coincided with the diarrhea symptom, possibly explaining the mechanism of common diarrhea symptom in NEC patients. Moreover, mucosal-adhering bacteria such as *Enterococcus* and *Streptococcus* were highly represented in pediatric enterocolitis (Normann et al., 2013; Zhou et al., 2016). Consistent with this, NEC patients from our cohort exhibited a higher abundance of *Enterococcus* during disease stage.

In contrast, the composition of our LOS patient samples was very different from previous studies where *Enterobacteria* and *Staphylococcus* were identified as the most prevalent genera (Stewart et al., 2017; Mai et al., 2013). In our cohort of LOS patients, *Klebsiella* was the most dominated genus. LOS is frequently caused by organisms, mostly bacteria, that translocate from the intestinal tract to the bloodstream. Consistently, *Klebsiella* was detected in hemoculture in two out of two of our LOS patients (hemoculture was not performed for the third patient). In addition, *Klebsiella pneumoniae* is one of the most common causes of sepsis in preterm patients of our hospital (JL and LH personal observation), suggesting that the most dominant and eventually infectious bacteria may be more specific to the environment.

Another notable point in our cohort was almost absent *Bifidobacteria*, an anaerobe that can ferment milk oligosaccharides (Gomez-Gallego et al., 2016) and thus commonly detected among breastfed infants (Murphy et al., 2017). We speculate that this extremely low level in our cohort was due to the lack of breastfeeding in the sterile hospital environment, being nurtured in the sterile NICU environment, continuous administration of antibiotics or the combinations of the above. Although *Bifidobacteria* has been generally considered a probiotic that serves to protect neonates against necrotizing enterocolitis and systemic infection (Nakayama et al., 2003; Khodayar-Pardo et al., 2014; Hermansson et al., 2019), recent randomized controlled trials are showing paradoxical results (Hays et al., 2016; Singh et al., 2019). Further studies on the role of probiotics in optimizing preterm infants' microbiota should address their effectiveness in preventing NEC and LOS.

This study was limited to only one hospital in one specific region (Shanghai) in China so how far these findings can be extrapolated remains to be determined. In addition, our sample size was relatively small since both diseases are rare (Neu and Walker, 2011; Cohen-Wolkowiez et al., 2009). Among the 1148 preterm infants admitted from July 2013 to December 2014, only five developed NEC and seven developed LOS. Nevertheless, this pilot study has provided essential information about NEC and LOS preterm patients within the Chinese population and serves as a starting point for future investigations into the etiology and pathogenesis of both diseases in the nation.

CONCLUSIONS

In this longitudinal study, we used next generation-sequencing to profile the 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 gut microbiota in NEC and LOS patients among the Asian population. Reduction in intestinal microbiota diversity and divergence of phylogenetic similarity from the control infants over time associated with both NEC and LOS onset. Overgrowth of potentially pathogenic genus *Enterococcus*, *Streptococcus* and *Peptoclostridium* were observed in NEC cases while *Klebsiella* was recognized as the dominant genus in LOS cases. In summary, our findings suggest that both NEC and LOS are dynamic processes involving abnormal microbiota assembly. This study is a starting point for further studying of microbial factors involved in preterm-associated complications in China. Accumulation of more data within China and perhaps from neighboring countries will allow us to build microbial signatures that can assist early diagnosis and development of novel treatment.

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Table 1(on next page)

Demographics Comparison among NEC, LOS and Control groups.

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

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Table 1. Demographics of NEC, LOS and Control groups.
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	NEC (n=4)	LOS (n=3)	Control (n=17)	Statistical test	p-value
GAge (weeks)[1]	29.25 (29-30)	30.00 (29-31)	30.94 (28-33)	Kruskal-Wallis test	0.074
BW (grams)[2]	1416.3 (773.4-2149.1)	1141.7 (633.4-1649.9)	1527.4 (1391.6-1663.1)	Kruskal-Wallis test	0.111
Gender[3]				Fisher's exact test	0.822
Female	3 (75%)	2(67%)	9 (53%)		
Male	1 (25%)	1 (33%)	8 (47%)		
Age when Diagnosed (days)[4]	16 (11-19)	12 (10-22)	/	Wilcoxon rank-sum test	0.629
Length of Stay[5] (days)	54.3 (13.5-95.0)	60.0 (24.8-95.2)	32.9 (26.3-39.5)	Kruskal-Wallis test	0.046
Total Number of Samples	45	44	103	/	/

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

Figure 1(on next page)

Schematic of Study Design.

Longitudinal fecal samples were collected from birth to death or discharge from the preterm infants in the NICU. Bacterial diversity and compositions were then characterized. Image credit: All the icons are made by Freepik from www.flaticon.com
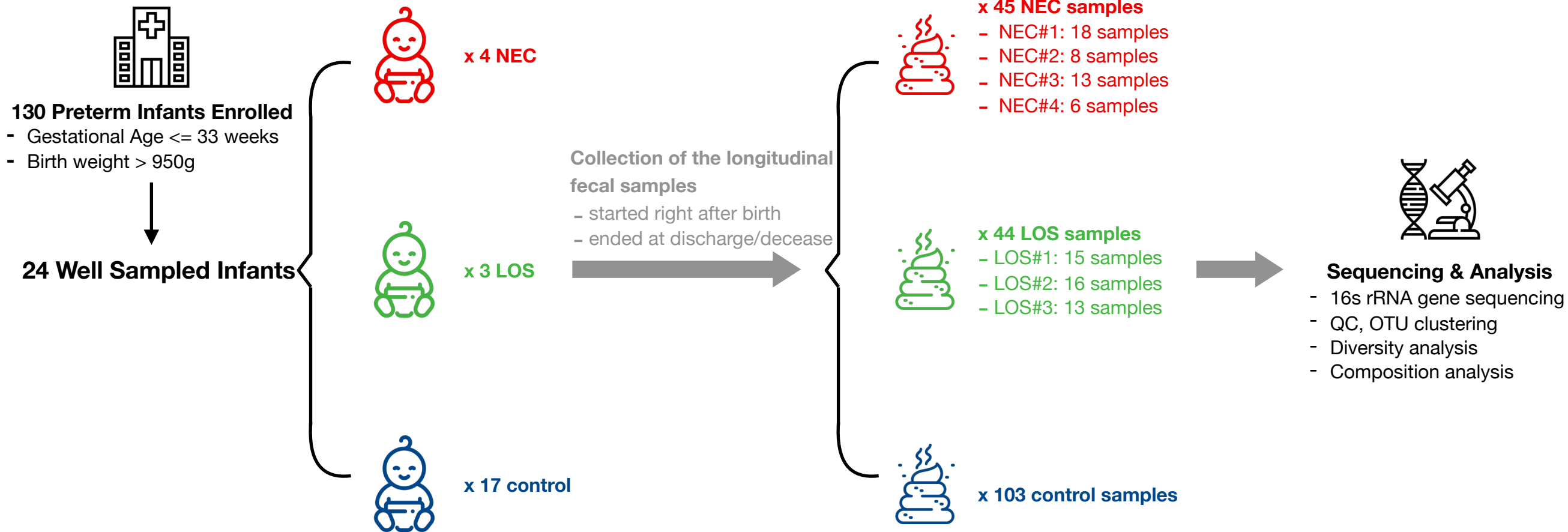
Figure 2(on next page)

Trend in microbiome richness (Sobs) over time.

Microbial richness trend in case and controls. (A) control group. (B) NEC group. (C) LOS group. Horizontal line shows median, box boundaries show 25th and 75th percentiles. Sobs index value of each sample is depicted as one dot. Indices are analyzed using the Kruskal-Wallis test followed by the Mann-Whitney U test in comparisons between two adjacent intervals.
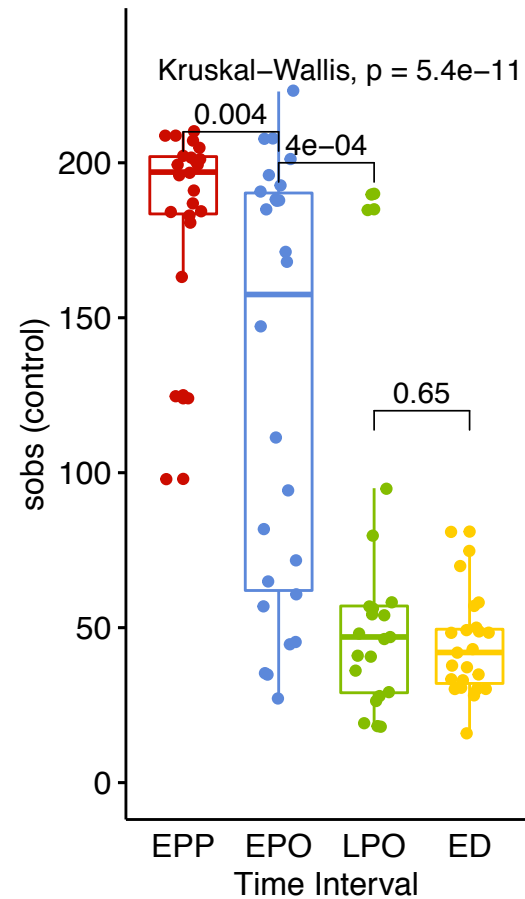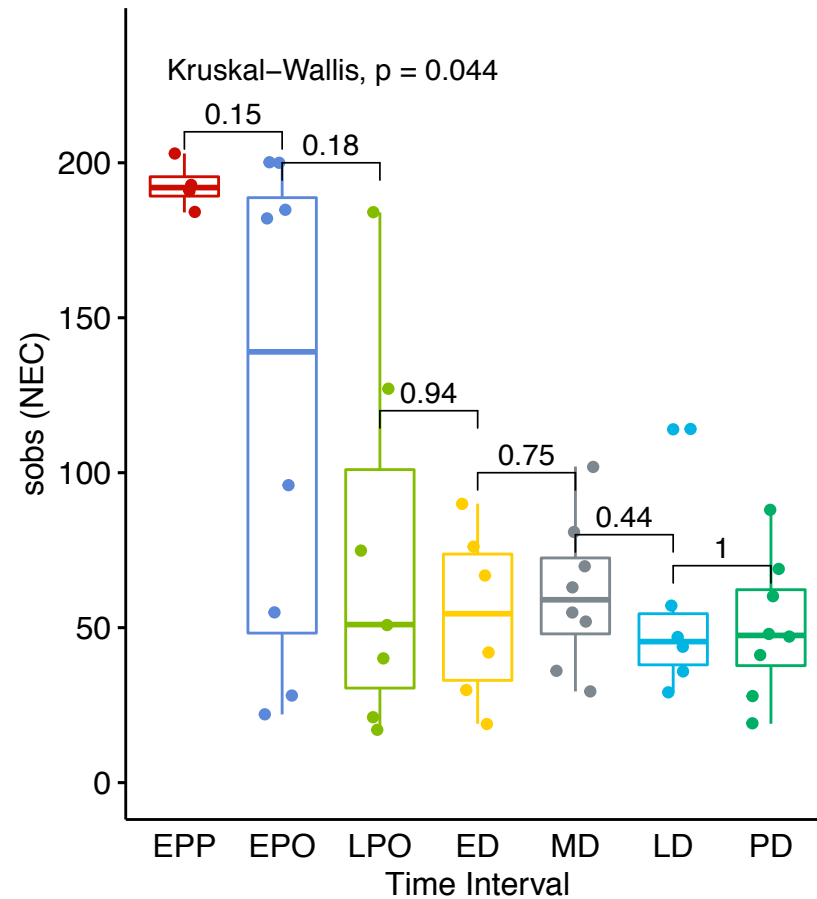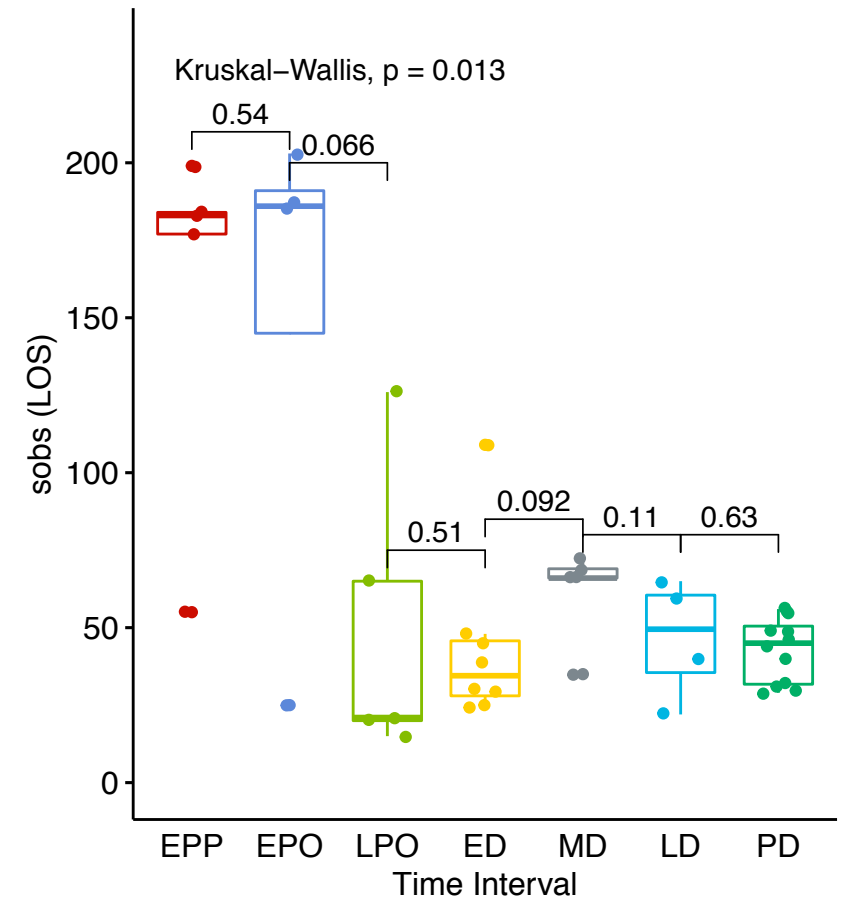
Figure 3(on next page)

Post-partum microbiome evenness (Shannon diversity) trend in each group.

Shows microbial richness trend in stools from cases and controls. (A) control group. (B) NEC group. (C) LOS 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 (two way RM ANOVA) indicating significantly discordant trends in bacterial diversity preceding disease onset. p values between two adjacent intervals were calculated by the Mann-Whitney U test.
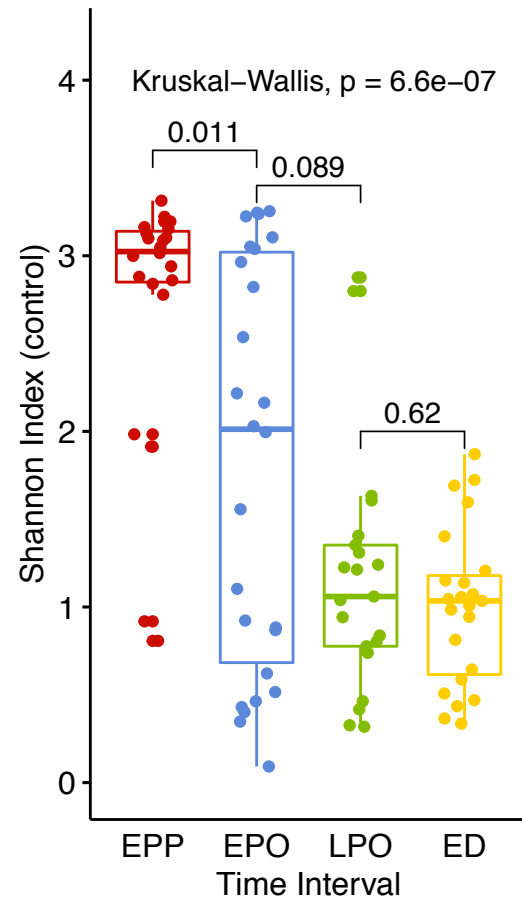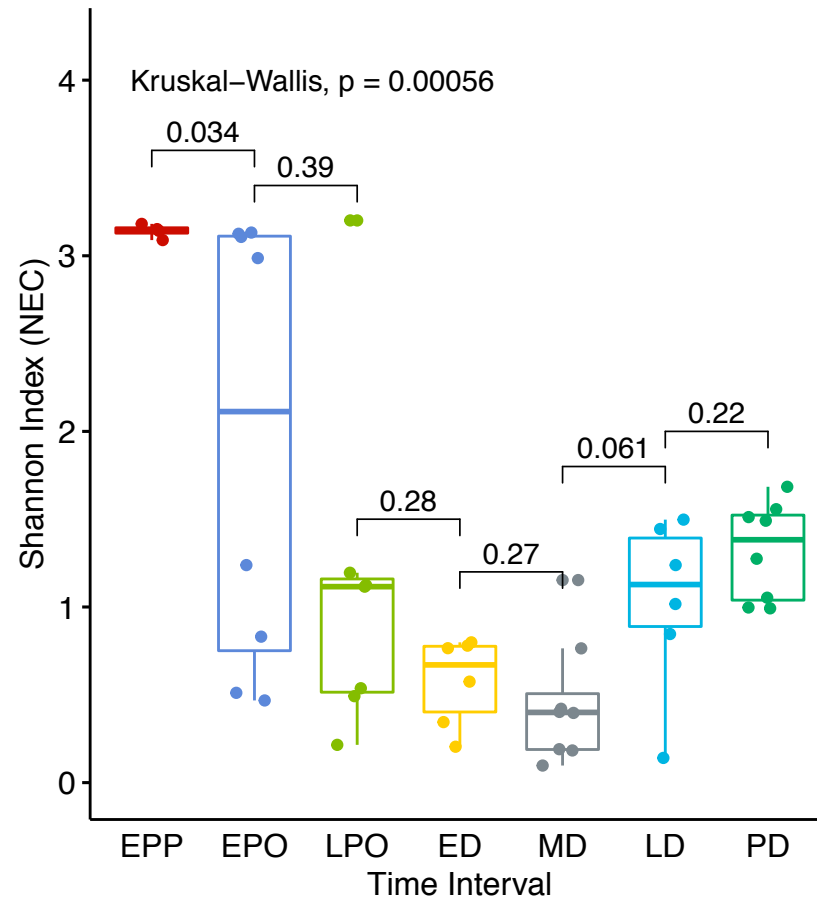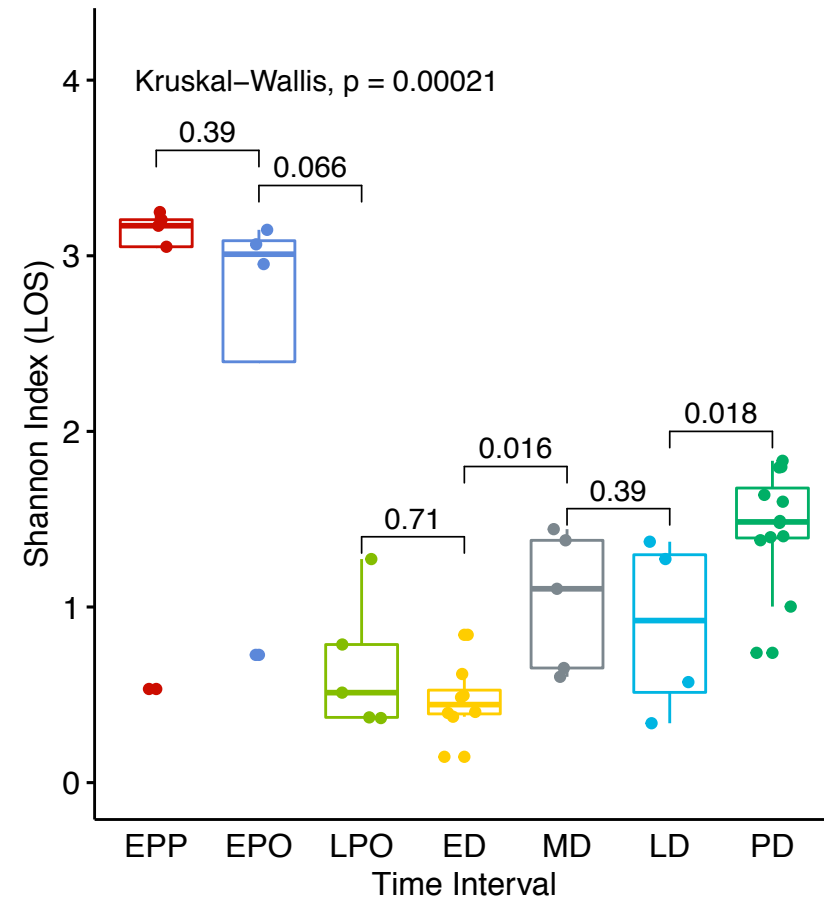
Figure 4(on next page)

Post-partum microbiome evenness (Shannon diversity) in each time-interval.

Shows microbial richness trend in stools from cases and controls ((A) control group. (B) NEC group. (C) LOS group. Horizontal line shows median, box boundaries show 25th and 75th percentiles. Shannon index value of each stool is depicted as one dot. p values among three groups were calculated by Kruskal-Wallis test. Comparisons between two groups were calculated by the Mann-Whitney test.
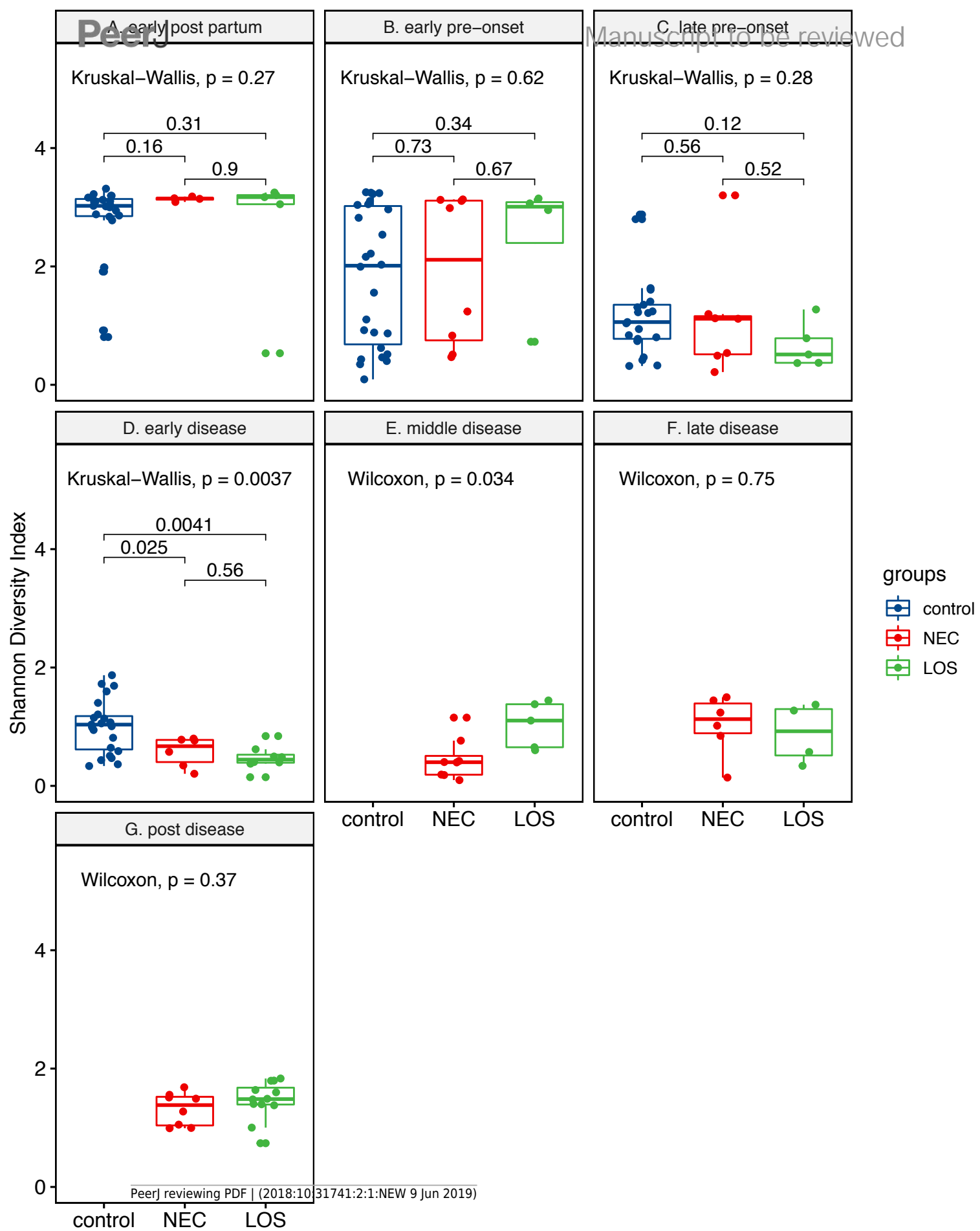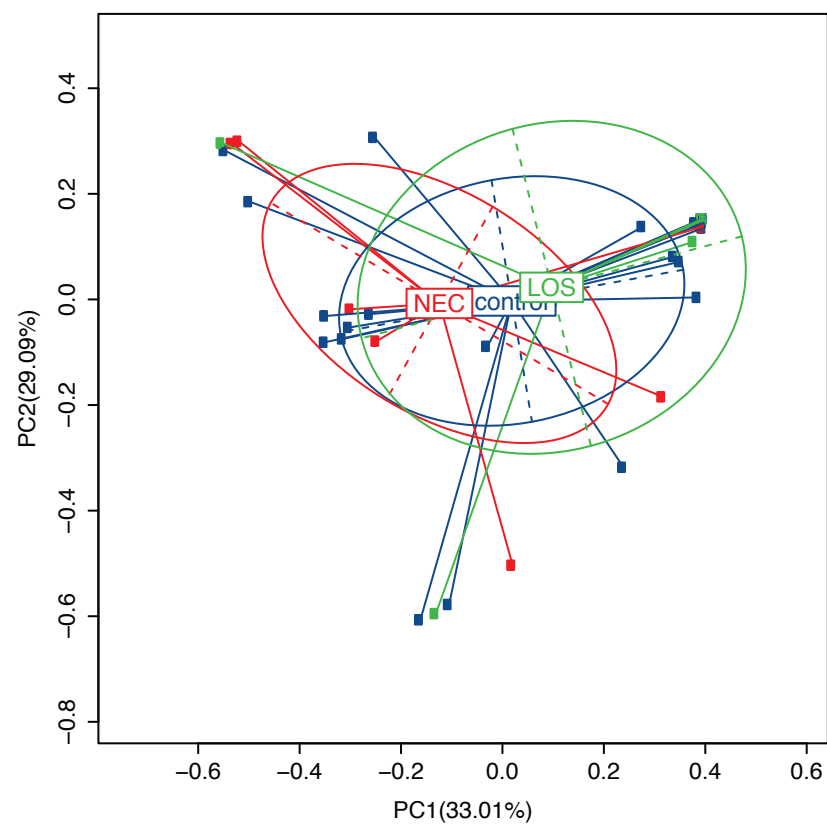
Figure 5(on next page)

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 higher percentage in PC1.
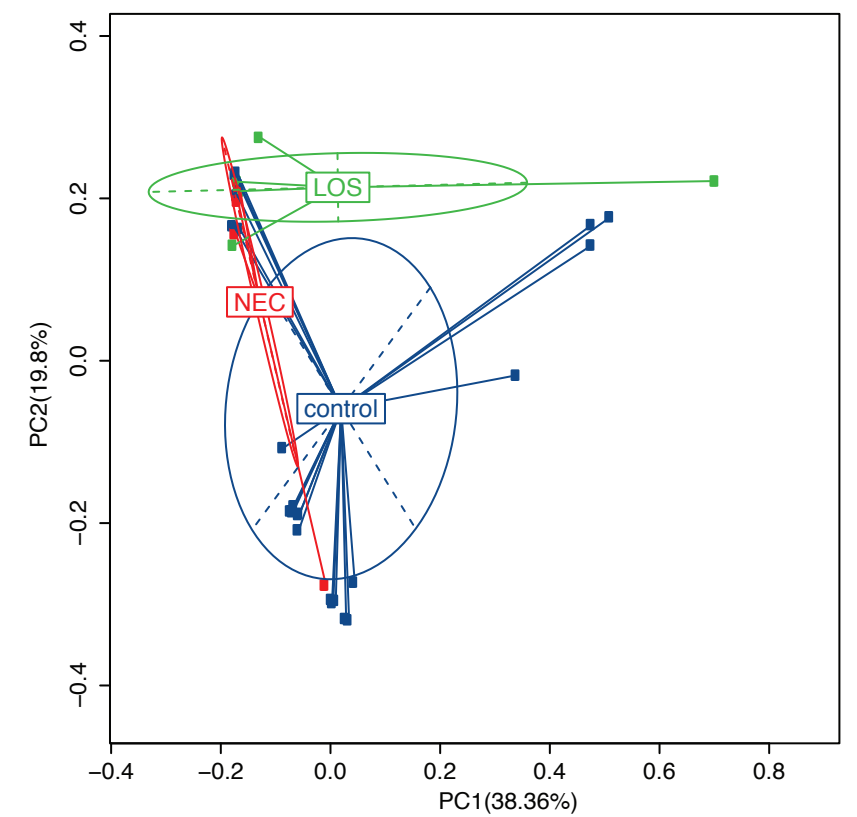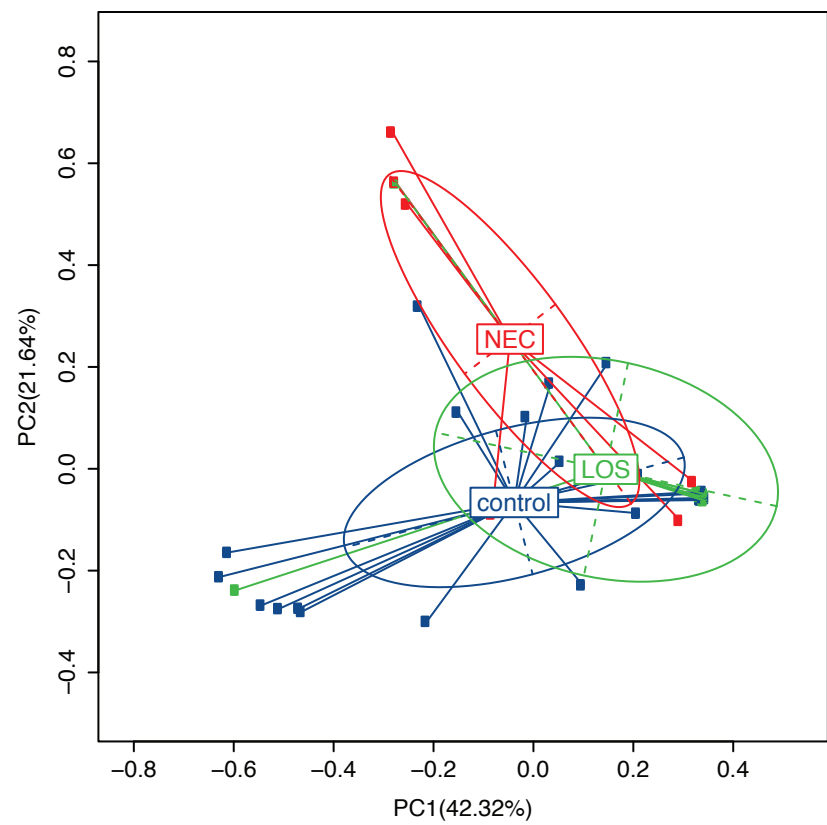
A. early post partum
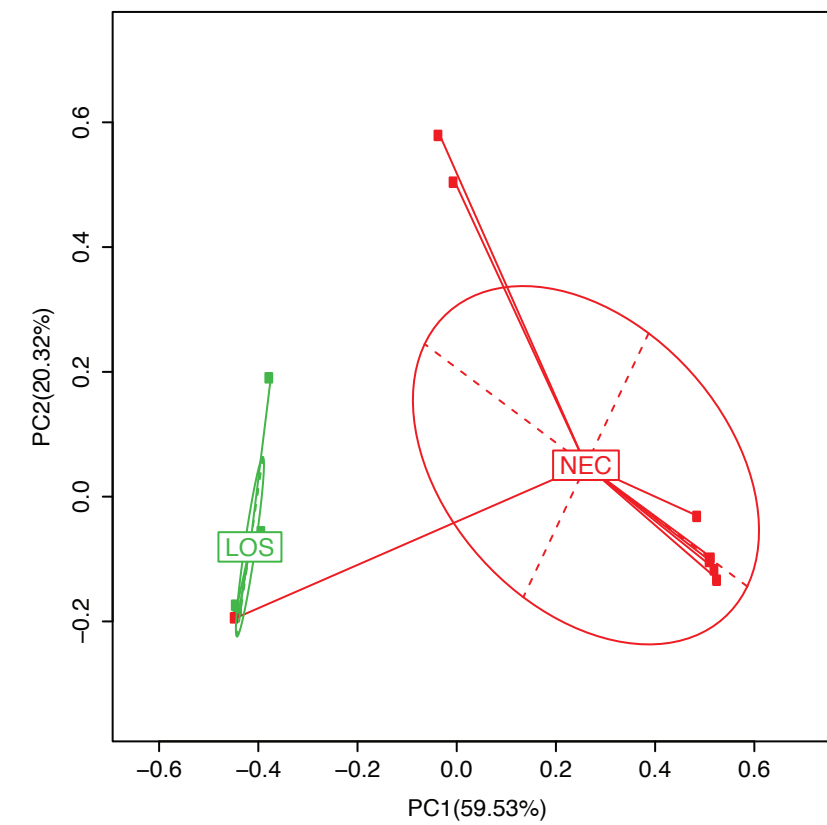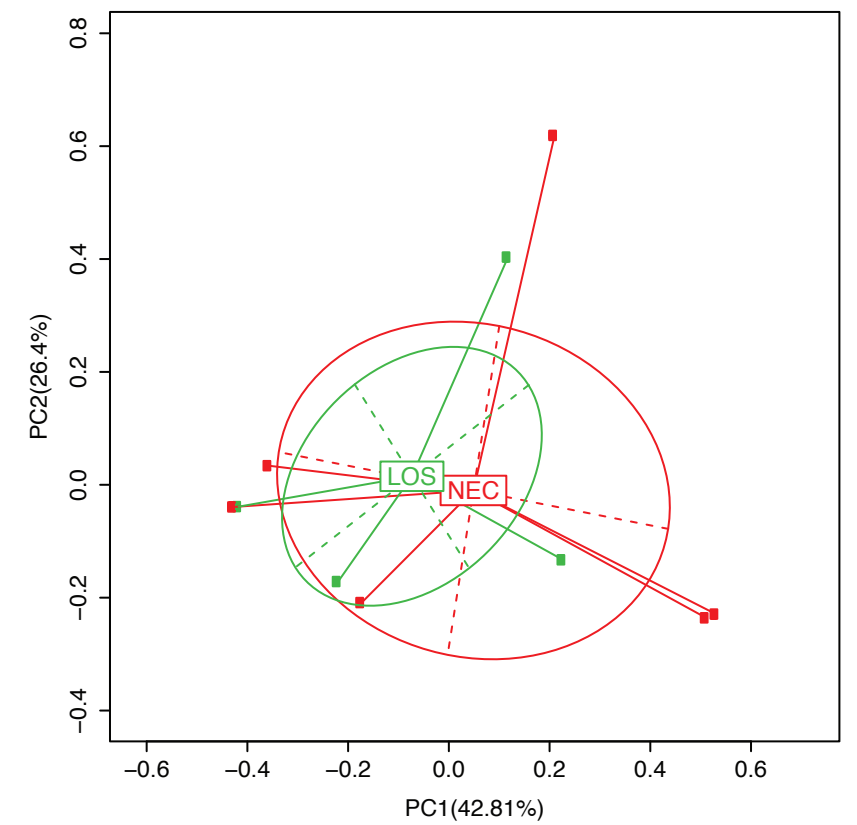

B. early pre-onset
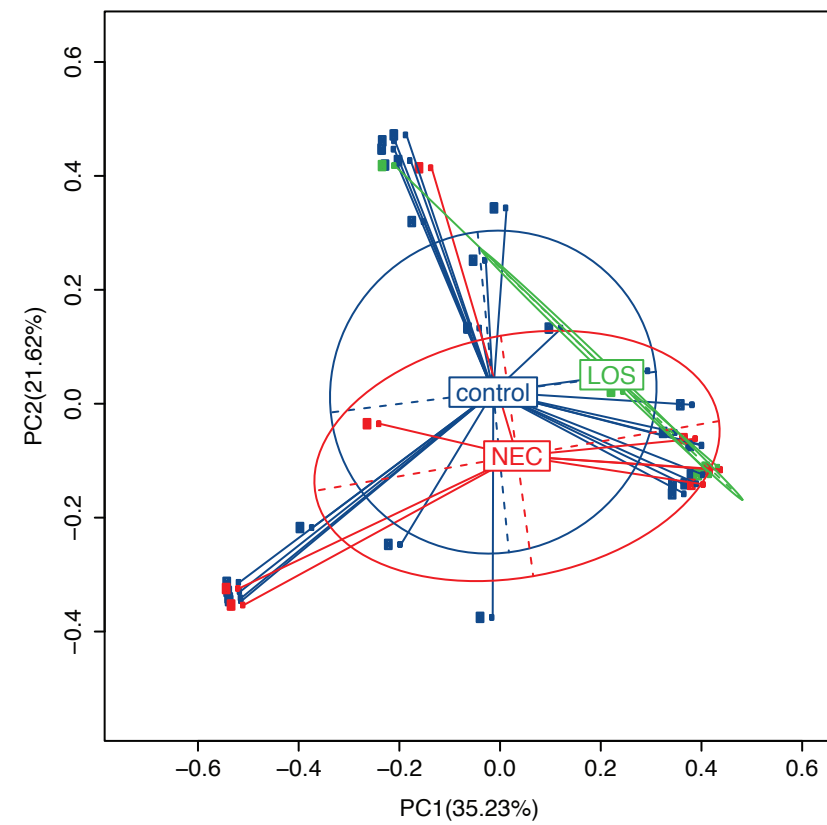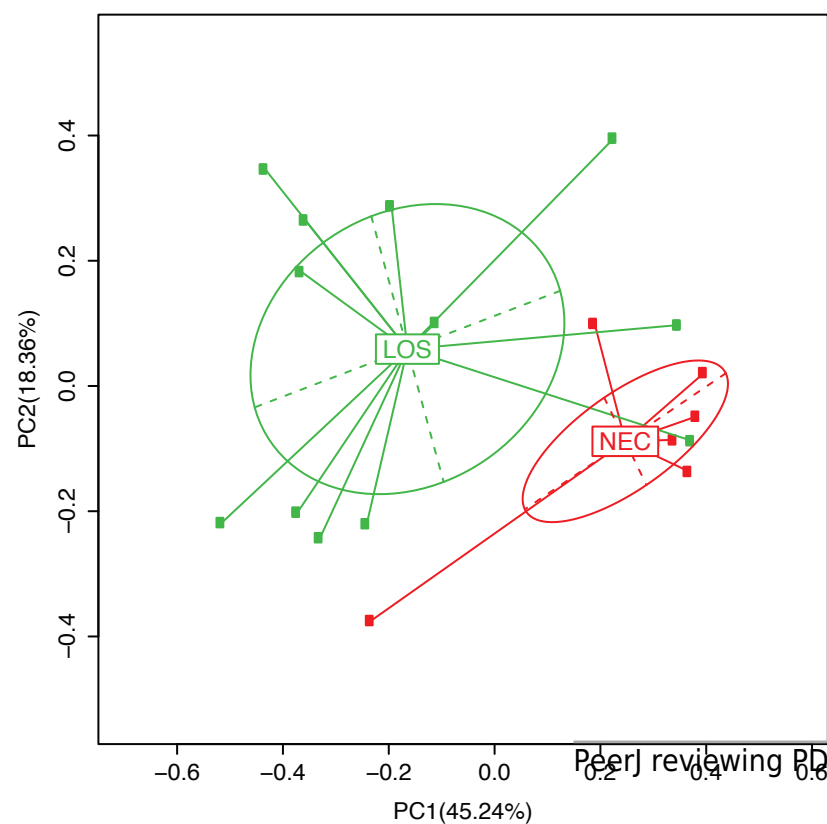

C. late pre-onset

D. early disease

E. middle disease
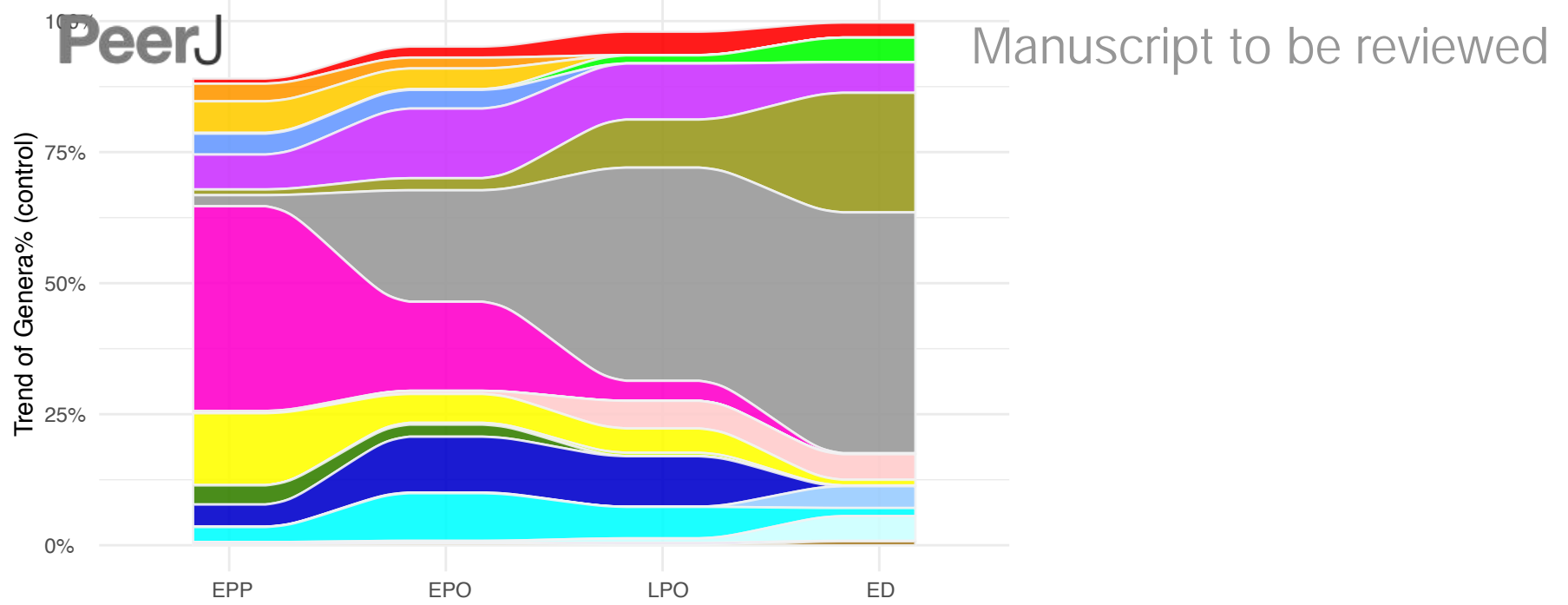

F. late disease

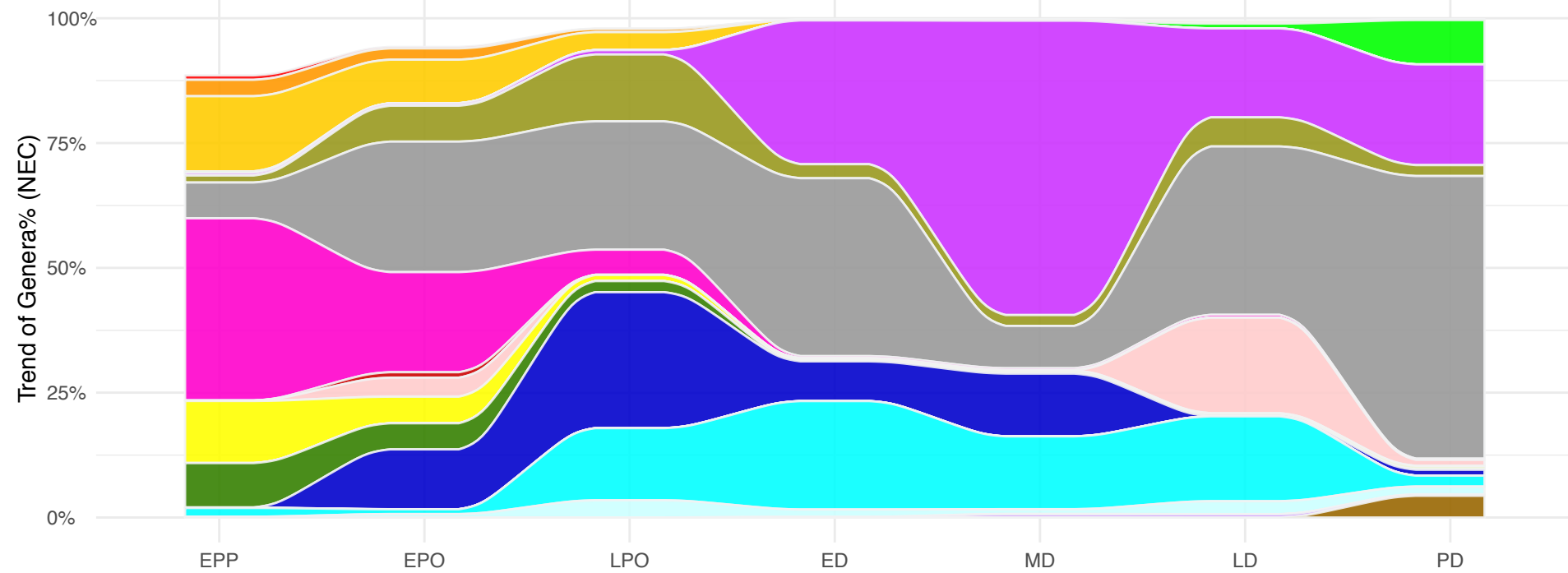**G. post disease**

Figure 6(on next page)

Alluvial diagrams showing temporal development of microbiome in NEC, LOS and the control groups.

Genus of relative abundance over 10% were depicted. (A) control group. (B) NEC group. (C) LOS group.

Genus

Acinetobacter	Enterococcus	Peptoclostridium	Stenotrophomonas	Veillonella
Arthrobacter	Escherichia−Shigella	Pseudomonas	Streptococcus	
Bacillus	Klebsiella	Rothia	unclassified_f__Enterobacteriaceae	
Clostridium_sensu_stricto_1	Lactococcus	Solibacillus	unclassified_o__Lactobacillales	
Corynebacterium_1	Martelella	Staphylococcus	Ureaplasma	