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Prolonged premature rupture of membranes with increased risk of infection is associated with gut accumulation of *Pseudomonas* from the environment

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ABSTRACT

Background: Preterm premature rupture of membranes (PPROM) contributes to over one-third of preterm births, and PPROM infants are more susceptible to infections. However, the risk factors remain poorly understood. We here aim to investigate the association of duration of premature rupture of membranes (PROM) and environmental microbiota with the gut microbiota and infection in PPROM infants.
Methods: Forty-six premature infants were recruited from two hospitals, and infant fecal and environmental samples were collected. 16 s rRNA sequencing was performed to analyze the fecal and environmental microbiome. Human inflammatory cytokines in cord vein plasma were measured.
Results: The gut microbiota composition of PPROM infants was different from that of non-PPROM infants, and the microbiome phenotypes were predicted to be associated with a higher risk of infection, further evidenced by the significantly increased levels of IL-6 and IL-8 in cord vein plasma of PPROM infants. The diversity of the gut microbiota in PPROM infants increased significantly as the duration of PROM excessed 12 h, and *Pseudomonas* contributed significantly to the dynamic changes. The *Pseudomonas* species in the gut of PPROM infants were highly homologous to those detected in the ward environment, suggesting that prolonged PROM is associated with horizontal transmission of environmental pathogens, leading to a higher risk of infection.
Conclusions: This study highlights that the duration of PROM is associated with the accumulation of environmental pathogens in the gut of PPROM infants, which is a risk factor for nosocomial infections. Improving environmental hygiene could be effective in optimizing the clinical care of PPROM infants.

1. Introduction

Over 15 million infants are born prematurely globally each year, accounting for 10 % of all births and showing an alarming upward trend [1]. Premature infants refer to live-born newborns with a gestational age of less than 37 weeks, and preterm birth complications are a major cause of under-five child mortality, resulting in approximately 1 million deaths a year. [2]. It is imperative to urgently develop and implement effective strategies to reduce the mortality rate associated with preterm births. Causes of preterm labour mainly include: maternal demographic characteristics, nutritional status, pregnancy history, present pregnancy characteristics, psychological characteristics, adverse behaviours, infection, uterine contractions and cervical length, and biological and genetic markers [3]. Accordingly, preterm birth can be classified into three distinct categories based on obstetric indicators (I) preterm premature rupture of the membranes (PPROM), (II) spontaneous onset of labor with intact membranes, and (III) induction of labor or cesarean delivery due to maternal or fetal indications [3]. PPROM, the rupture of

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the fetal membranes before 37 weeks of gestation, triggers preterm labor and is responsible for over 30 % of all preterm births. It is a significant risk factor for early-onset neonatal sepsis, neonatal aspiration pneumonia, severe septicemia, intracranial infections, and poses a grave threat to neonatal life [4]. It has been widely recognized that premature infants are at a higher risk of adverse outcomes due to their underdeveloped immune systems and the premature exposure to microbes. Premature rupture of membranes (PROM) compromises the protective barrier between the fetus and the external environment, leading to early microbial exposure and an increased risk of neonatal infection. The likelihood of adverse outcomes escalates with the duration of PROM [5–7].

Maternal and environmental microorganisms constitute the primary sources of early microbial exposure for neonates [8,9]. However, in cases where pregnant women experience PROM, they often encounter vaginal microbiota infections necessitating hospitalization. This circumstance significantly disrupts the natural source of early microbial exposure for PPROM infants, ultimately resulting in a disruption of their gut microbiota. Accumulating evidence suggests that a disrupted gut microbiota in premature infants is a significant contributor to neonatal infections [10–12]. In PPROM infants, the introduction of harmful microorganisms, originating from both maternal and environmental sources, plays a pivotal role in the development of infections. For instance, vaginal *Facklamia* spp. and *Winkia neuii* in pregnant women with PROM have been highly associated with sepsis in PPROM infants [13], which is of great significance in predicting the occurrence of neonatal sepsis in clinical practice. Additionally, hospital-acquired microorganisms, such as *Staphylococcus*, *Enterococcus*, *Pseudomonas*, and *Klebsiella*, have been linked to gut microbiota colonization and further nosocomial infections in preterm infants [14]. This indicates that environmental microorganisms may exert a considerable influence on the gut microbiota of hospitalized patients [15].

The main objective of this study is to dissect the characteristics of the gut microbiota in infants with PROM, and also aim to establish a potential link between the composition of the gut microbiota in PPROM infants, the hospital environment microbiota, and hospital-acquired infections in neonates.

2. Materials and methods

2.1. Study design and infants' fecal sample collection

A cohort consisting of 46 premature infants, gestated between 32 and 37 weeks, was enrolled in this study. These infants, who did not suffer from congenital abnormalities, early-onset sepsis, late-onset sepsis (LOS), necrotizing enterocolitis (NEC), focal intestinal perforation, or any other intestinal diseases, were recruited from Shenzhen People's Hospital (n = 34) and Shenzhen Baoan Women's and Children's Hospital (n = 12). Between September 2021 and September 2022, fecal samples were collected from these premature infants during their first week of life. The study cohort was divided into two distinct preterm groups I) PPROM group who was born less than 37 weeks, and had clinical symptoms of premature rupture of membranes before delivery (n = 15), and II) non-PPROM group infants who had premature delivery that was not caused by PROM (n = 31). To minimize pre-analytical variables, fecal samples were promptly frozen using liquid nitrogen or dry ice upon collection and stored at − 80 ℃ until further analysis. Pertinent information such as birth weight, birth date, gender, delivery method, gestational age, as well as the mother's medical history and medication use, were carefully documented. The Apgar score is used to assess the newborn's vitality at 5 and 10 min of life. Additionally, blood samples were collected from the newborns for cytokine detection.

2.2. Collection of environmental samples

Samples of environmental microbiota were gathered from Shenzhen People's Hospital. These samples were collected from standard wards, each consisting of four beds, at five different locations including the sink trap, toilet seat, bed rail, bed table and door handle. The swabbing process lasted for 2 min and the swabs were stored in their respective solutions. Swabs immersed in PBS were immediately placed on ice and sent for culturing while the remaining swabs were transported to a laboratory at room temperature and stored at − 80 °C. A total of 25 swabs were collected across four hospital wards.

2.3. DNA extraction and 16 s rRNA gene sequencing of preterm stool samples

The total DNA was extracted by fastDNA Spin Kit for Soil (MP) from preterm feces and environmental swabs. DNA concentration and quality were quantified using a Qubit 2.0 fluorometer (Invitrogen). Isolated bacterial genomic DNA was used as the template for PCR amplification of the V3 −V4 regions of the bacterial 16 s rRNA gene. The 16 s rRNA genes from the microbiota were amplified using bacterial primer set 341 F (5′-CCTACGGGNGGCWGCAG-3′) and 805 R (5′-GACTACHVGGGTATCTAATCC-3′). The amplification of a single 16 s rRNA gene sequencing library is performed according to the methodology outlined in our previous study [16]. Following that, the 16 s rRNA gene libraries were sequenced using the Illumina MiSeq platform with 300 bp paired-end reads at Bioyi Biotechnology Co., Ltd. in Wuhan, China.

2.4. Bioinformatics analyses

The QIIME2 (2019.4) feature-classifier plugin and a pre-trained Naïvees classifier were employed to assign taxonomic labels, using the Greengenes 13_8 99 % operational taxonomic units (OTUs) as training data. α-diversity metrics such as the Chao index and Shannon index-diversity measurements, were calculated. Linear discriminant analysis (LDA) of effect size (LEfSe) was conducted to identify the taxonomy that most likely contributes to group differences. Bugbase was utilized for functional profiling prediction of a microbial community based on RNA sequence data. Multiple testing correction was applied using the Benjamini-Hochberg false discovery rate (FDR). Source Tracker was used to analyze the proportion of PPROM infant gut microbiota derived from environmental microbiota.

2.5. Determination of cytokines in plasma

The levels of human inflammatory cytokines (including IL-12p70, TNF-α, IL-10, IL-6, IL-1β, and IL-8) in cord vein plasma of preterm infants were measured using the Human Inflammatory CK BD CBA Kit (No. 551811; Becton, Dickinson and Company, Franklin Lakes, NJ, USA), according to the manufacturer's instructions.

2.6. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 7 version 7.0 (GraphPad Software, USA). Multiple group comparisons were performed using one-way analysis of variance (ANOVA) with Tukey's post hoc test for multiple comparisons. Variables such as delivery method, sex, and antibiotic exposure were assessed using two-tailed χ^2 tests or Fisher's exact test (2 by 2 tables). The Wilcoxon test was employed to determine the statistical significance of differences between groups. Data are presented as mean ± standard deviation (SD). A p-value below 0.05 was considered statistically significant.

3. Results

3.1. Cohort characteristics

Forty-six premature infants, with gestational ages between 32 and 37 weeks, were enrolled at two tertiary hospitals between September 2021

and September 2022. Of these infants, six were born vaginally, and the rest were delivered by cesarean section. These premature infants were divided into two groups according to their obstetric history: PPROM (n = 15) and non-PPROM (n = 31). No significant differences in gestational age, birth weight, or Apgar scores recorded at one and five minutes were detected between the two groups. Additionally, maternal age, gravidity, and parity exhibited comparable patterns in both groups. Detailed characteristics of each group are listed in Table 1.

3.2. The composition of gut microbiota and the risk of neonatal infection are associated with PROM

3.2.1. PROM is associated with α-diversity changes of gut microbiota in PPROM infants

It is known that the development of gut microbiota in premature infants is delayed compared to term infants, resulting in a higher risk of dysbiosis [9,17]. However, the association between PROM and gut microbiota of PPROM infants remains largely unknown. Here, no significant differences were detected in the α-diversity of the gut microbiota between the non-PPROM and PPROM infants (Fig. 1A-D). However, observed species ($P = 0.0713$) and Chao1 index ($P = 0.0706$) showed an increasing trend in the PPROM group (Fig. 1A and B), suggesting that the α-diversity of the gut microbiota in PPROM infants shows trend of correlation with PROM. Considering that the fecal samples were collected from premature infants with different delivery methods in two hospitals, we evaluated the impact of these two factors on the α-diversity of the samples. No significant differences were detected in the α-diversity of samples collected from different hospitals or delivery modes (Fig. S1A and B), suggesting that the delivery methods and hospital factors had no significant influence on the α-diversity of gut microbiota in premature infants.

3.2.2. PROM is associated with a high infection risk in PPROM infants

To closely inspect the impact of PROM on the structure of intestinal microbiota in premature infants, a NMDS plot based on the Bray–Curtis dissimilarity matrix was performed to measure the overall microbiota composition between the PPROM and non-PPROM group. NMDS plots showed that the gut microbiome of the PPROM infants was significantly different from that of the non-PPROM infants (PERMANOVA; $P = 0.006$) (Fig. 1E), suggesting that the two groups shared a distinct microbial community structure. We additionally analyzed the effect of different delivery methods and hospitals on the microbiota structure, and no significant differences were found in Bray–Curtis dissimilarity of the gut microbiota between hospitals, or between delivery modes (Fig. S2A and B), further supporting that the delivery methods and hospitals had no significant influence on the structure of gut microbiota in premature infants. Redundancy analysis (RDA) was performed to identify the microbial factors that were probably involved in diversifying the microbial community structure between the two groups. Phylum level results showed that the clustering of the PPROM group was mainly driven by the phylum Proteobacteria and Actinobacteria, while that of the non-PPROM group was driven by Firmicutes. The genus level analysis showed that the clustering of the PPROM group was mainly driven by the genus *Pseudomonas*, while that of the non-PPROM group was driven by *Staphylococcus, Enterococcus, Clostridium*, and *Streptococcus* (Fig. 1F.and G). These results suggest that PROM is associated with alterative structures of intestinal microbiota in premature infants.

To further understand the compositional characteristics of the gut microbiota in PPROM premature infants, we performed Lefse analysis to identify differentially abundant bacterial taxa between the PPROM infants and non-PPROM infants. The organisms with significant differences in the two groups are shown by cladogram (Fig. 1H). There were no significant differences in the four dominant phyla (Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes) between the two groups. Significant variations were observed at the genus level between the two groups. Compared to the non-PPROM group, *Pseudomonas*, *Azospirillum*, *Silene*, *Phenylobacterium*, *Sporanaerobacter*, *Steroidobacter*, and *Roseateles* were relatively more abundant, whereas *Helicobacter* and *Clostridium* were relatively less abundant in the PPROM group (Fig. 1I).

To investigate whether changes in microbial diversity are associated with functional changes, BugBase analysis was performed to predict the phenotype of microbiome, including the proportions of aerobic, anaerobic, facultatively anaerobic, mobile element containing, forms biofilms, and potentially pathogenic microorganisms. In the PPROM group, the relative abundance of aerobics ($P = 0.0105$), form biofilms ($P = 0.0297$), and potentially pathogenic ($P = 0.0236$) was significantly higher than in the non-PPROM group (Fig. 3A, E, and F), indicating that PPROM infants are associated with higher risk of infections.

In order to verify the association between PROM and the risk of infections in PPROM infants, we further determined the concentration of inflammatory cytokines (IL-12p70, TNF-α, IL-10, IL-6, IL-1β, and IL-8) in umbilical vein blood (Fig. 1J-O). The concentration of IL-6 and IL-8 in PPROM infants was significantly higher than that of non-PPROM infants (Fig. 1M and O). In addition, the ratio of infections in premature infants from birth to discharge was calculated based on blood and radiologic diagnosis of neonatal lung disorders (Fig. S3 and Fig. S4), and the results showed that the incidence of infection in PPROM group was 53.33 %, significantly higher than that in non-PPROM group (22.58 %; $P = 0.0370$) (Fig. 1P). These results support that the PPROM infants have a higher risk of infection than the non-PPROM infants.

Table 1
Neonatal and maternal characteristics.

	PPROM (n = 15)	No-PPROM (n = 31)	P-value
Infant Characteristics			
Gestational Age at Birth (weeks)	34.9 ± 3.56	34.6 ± 2.54	0.7275
Birth Weight (grams)	2512.0 ± 809.20	2093.4 ± 649.60	0.0696
Male Sex	11 (73.3 %)	15 (51.7 %)	0.1283
Delivery (Vaginal/ Cesarean)	6/9	0/31	0.0005
Apgar (1 min)	9.3 ± 0.98	9.6 ± 0.79	0.3236
Apgar (5 min)	9.9 ± 0.29	9.9 ± 0.42	0.8378
Maternal Characteristics			
Age, years	30.93 ± 4.11	32.20 ± 4.00	0.4075
Gravidity count	2.2 ± 1.01	2.1 ± 1.30	0.9187
Parity count	1.4 ± 0.51	1.5 ± 0.51	0.6788
Antibiotics Exposure	2/13	4/27	0.9999
Pre-pregnancy BMI, kg/m^2	21.52 ± 1.68	21.50 ± 2.35	0.9753
BMI before delivery, kg/m^2	25.53 ± 1.99	27.50 ± 2.91	0.0670

Data are presented as mean ± SD. PPROM: Preterm premature rupture of membranes; No-PPROM: premature infants without premature rupture of membranes; BMI: Body mass index; *P*-value: two-dependent t test or *Chi*-squared test were as appropriate

3.3. PROM duration is a key factor associated with the enrichment of pathogenic bacteria in PPROM infants

3.3.1. 1 The diversity and similarity of gut microbiota in PPROM infants is associated with PROM duration

As aforementioned, PROM is associated with the gut microbiota disorders, resulting in a high risk of infection in PPROM infants. It is also known that the duration of PPROM, defined as the time interval between the rupture of the amniotic sac and the onset of labor, is associated with the risk of infection [5–7]. We therefore speculate that PROM duration may be one of the important factors affecting early bacterial exposure in premature infants. To estimate the association of PROM duration with the developmental dynamics of gut microbiota in premature infants, we analyzed the time dependence microbiome diversity in the cohort. Currently, a few studies have investigated the association of PROM duration with newborn gut microbiota, and various criteria have been used for classifying the PROM durations [6,7,18]. Considering that

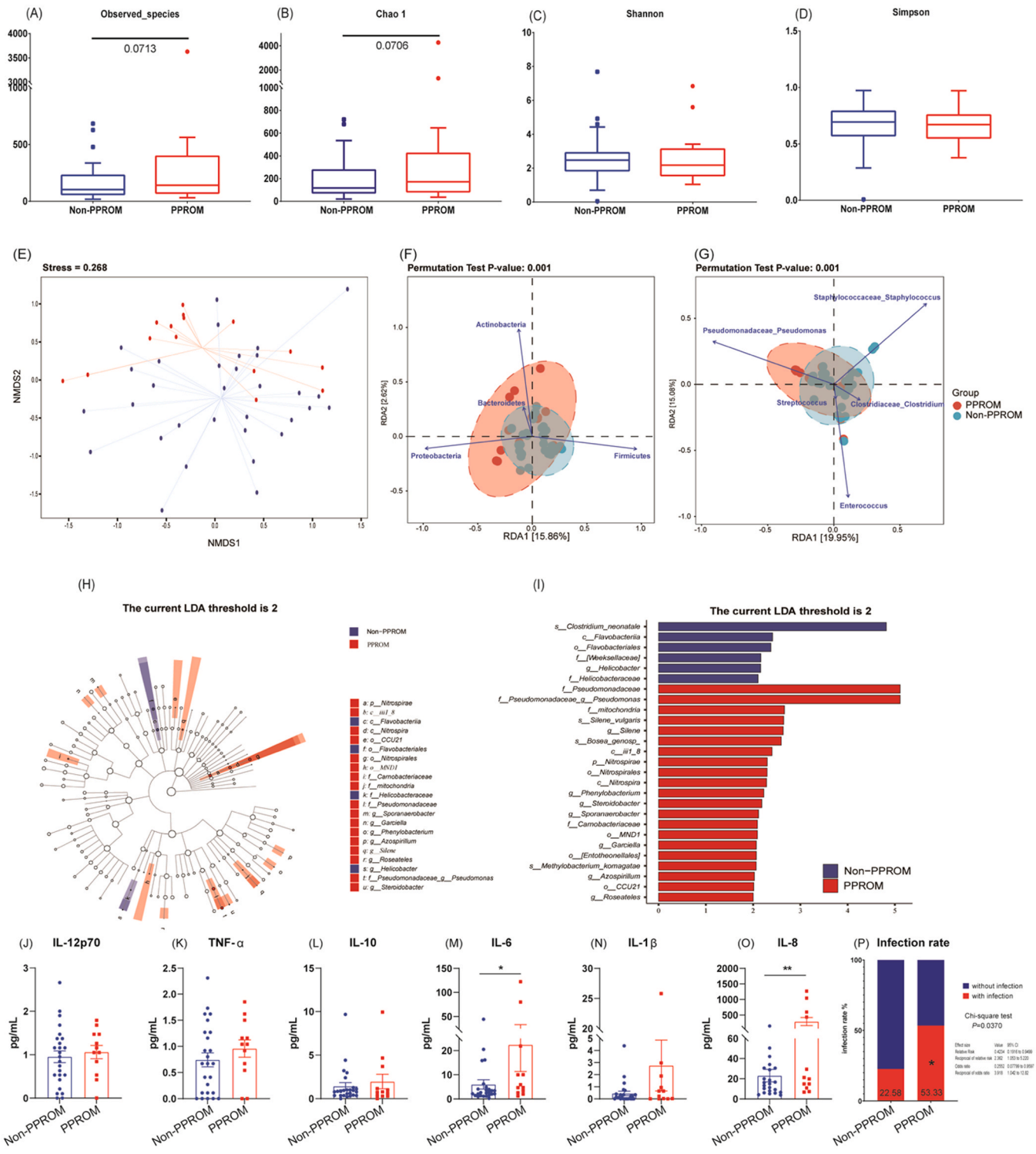

Fig. 1. PROM alters the structure of gut microbiota in PPROM infants and is associated with high infection risk. (A-D) observed_species, Chao 1 estimator, Shannon, and Simpson index between PPROM and non-PPROM group. (E) Two-dimensional non-metric multidimensional scaling (NMDS) plot of the microbial community composition in the PPROM and non-PPROM samples. (F-G) Redundancy analysis (RDA) at phylum and genus level in PPROM and non-PPROM groups. (H) LEfSe analysis depicting nodes within the bacterial taxonomic hierarchy that are enriched in fecal microbiota from PPROM versus non-PPROM. (I) Histogram of the LDA scored for differentially abundant genera between PPROM and non-PPROM groups. (J-O) Concentration of inflammatory cytokines in umbilical cord blood. (P) The incidence of infectious diseases in premature infants. * $P < 0.05$, * * $P < 0.01$.

infants and mothers who give birth after 12 h of PROM have a higher risk of infection, we therefore grouped the PPROM infants using the duration of 12 h as the cut-off: < 12 h (n = 7) and ≥ 12 h (n = 8). Both of the observed species ($P < 0.01$) and Chao1 ($P < 0.01$) showed a significant increasing trend with increasing duration of PROM, while Shannon and Simpson index showed no significant differences (Fig. 2A-D), indicating that the longer the duration of PROM, the higher loads and diversity of gut microbiota were detected in premature infants. These results suggest that the species richness of gut microbiota in PPROM infants may be positively correlated with the duration of PROM.

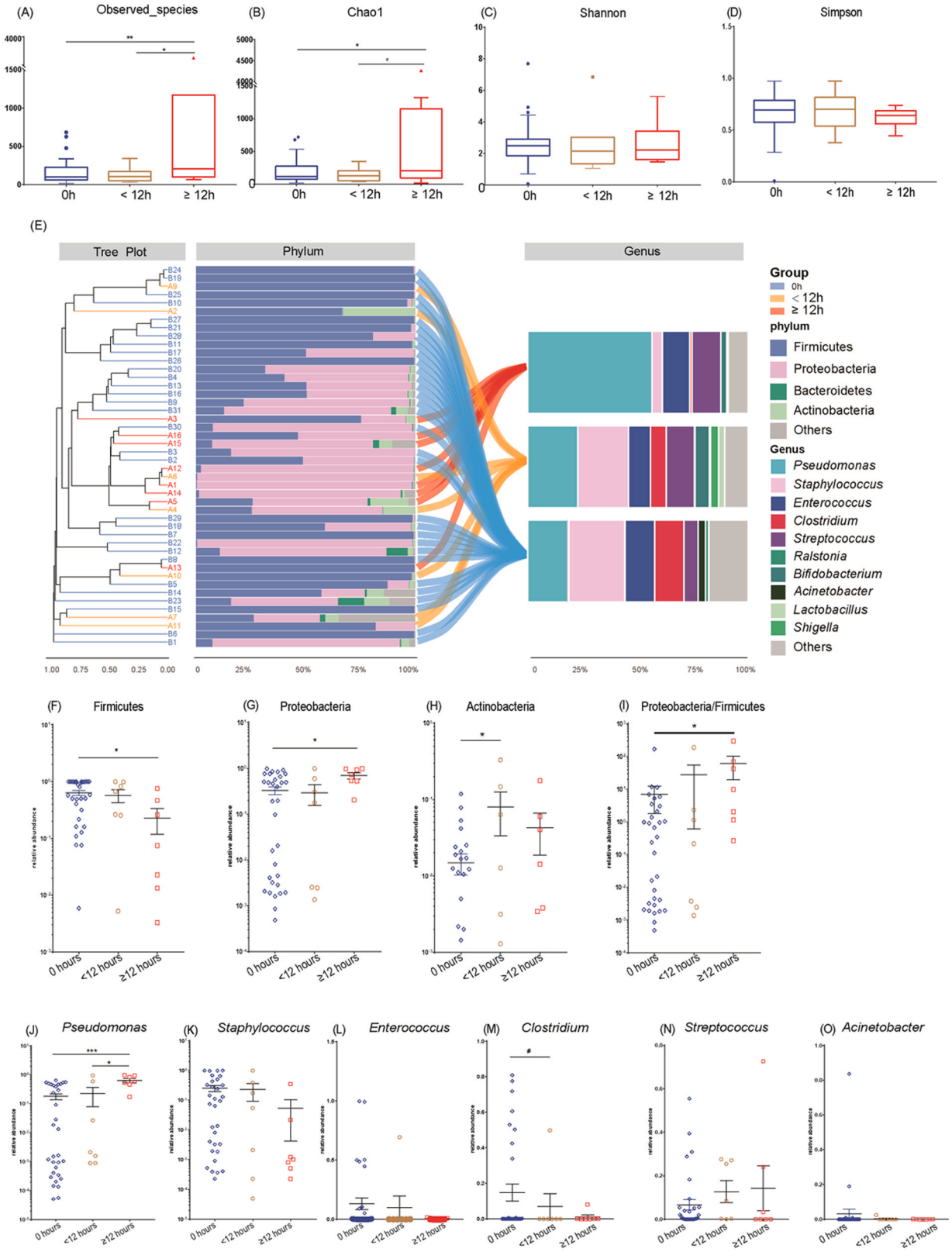

Fig. 2. The gut microbiota of premature infants changes significantly with the duration of PROM. (A-D) The number of observed species, the Chao 1 estimator, the Shannon index, and the Simpson index were measured for PPROM infants with different durations of PROM. (E) Microbiota profiles and abundances of bacterial taxa in different groups of infants were analyzed using niche-based hierarchical clustering. The average relative abundances of bacterial taxa at phylum and genus levels were determined for each group. Changes in the relative abundance of the top three phyla (F-I) and the top six genera (J-O) were assessed based on the duration of PROM. * $P < 0.05$, * * $P < 0.01$.

To understand the developmental characterizations of gut microbiota in PPROM infants after PROM, hierarchical clustering analysis was performed to evaluate the similarity of gut microbiota in PPROM infants. The results showed that the aggregation of individuals in the $\geq$ 12 h group was higher and significantly separated from the non-PPROM group (Fig. 2E; Bray_curtis, permanova; $P = 0.002$), indicating that with the increase of PROM duration, the gut microbiota of PPROM infants changed more significantly, and was significantly different from that of non-PPROM infants. Moreover, phylum-level analysis showed that Firmicutes and Proteobacteria were the top two phyla in the three subgroups. At the genus level, *Pseudomonas, Staphylococcus, Enterococcus, Clostridium*, and *Streptococcus* were the top five in all groups. Of note, the relative abundance of *Pseudomonas* increased with the duration increase of PPROM, with the $\geq$ 12 h group having the highest relative abundance compared to the 0 h and < 12 h groups (Fig. 2E). Given that *Pseudomonas* is composed of multiple important pathogens causing infections, the duration of PROM may be associated with the risk of infection in premature infants.

3.3.2. *Duration of PROM is associated with the relative abundance of Pseudomonas*

To further understand the relationship between gut microbiota composition and PROM duration in PPROM infants, we analyzed the characteristics of the changes in the dominant intestinal bacterial community with the duration of PPROM based on the results of the differences between PPROM and non-PPROM groups. With increasing

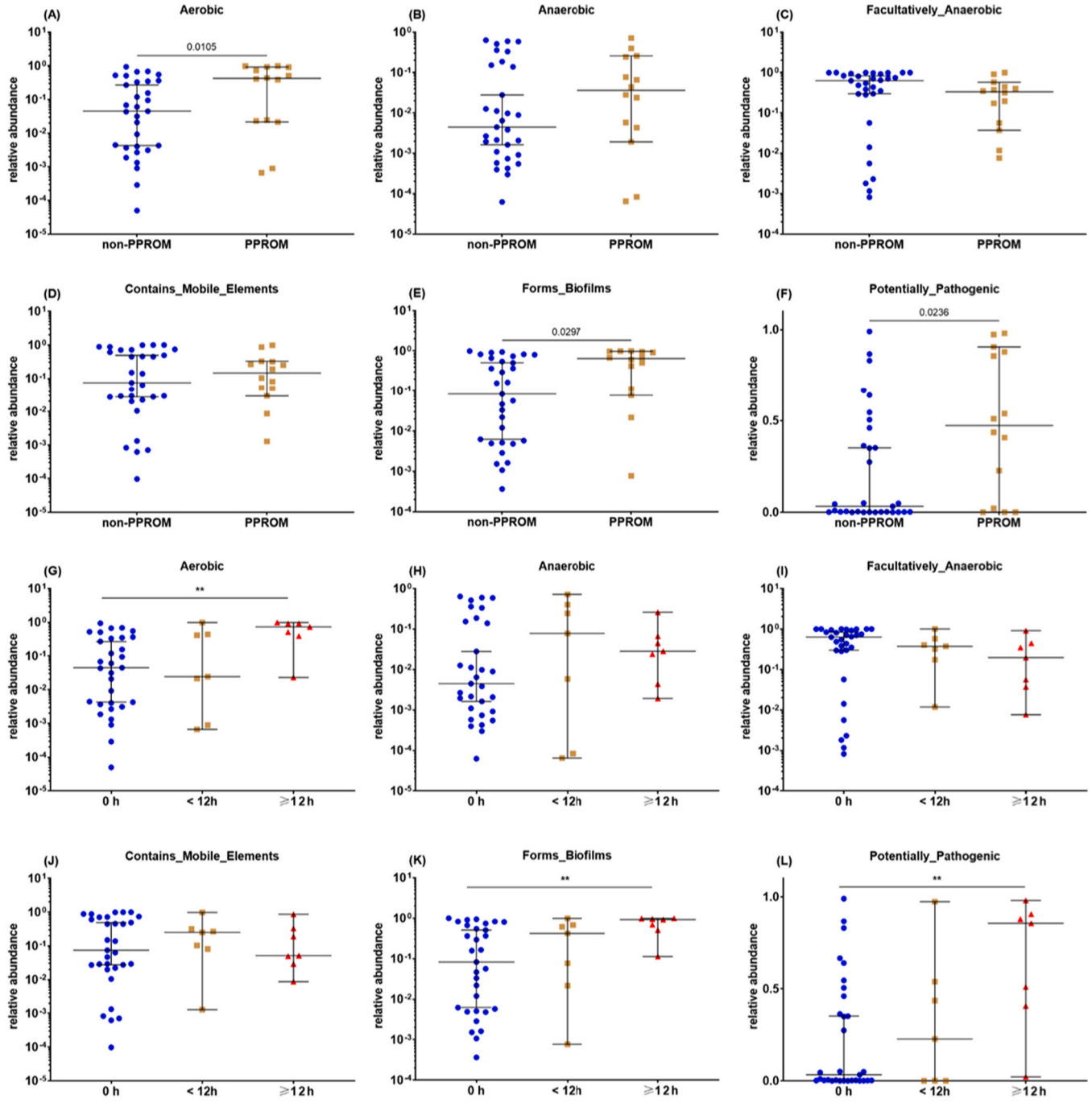

Fig. 3. BugBase analysis of gut microbiota in premature infants. The outcome is grouped according to the x-axis and the relative abundance is presented on the y-axis. Pairwise Mann–Whitney–Wilcoxon tests and FDR-corrected pairwise tests were performed for data analysis. * $P < 0.05$, * * $P < 0.01$.

duration of PROM, the abundance of Firmicutes significantly decreased (Fig. 2F), and that of Proteobacteria significantly increased (Fig. 2G). It is known that the gut microbiota in preterm infants with NEC has previously been characterized by increased relative abundances of Proteobacteria and decreased relative abundances of Firmicutes and Bacteroidetes [19]. The increased rate of Proteobacteria/Firmicutes (Fig. 2I) suggests that the premature infants may have a higher risk of NEC onset.

We additionally analyzed the association of the top 6 genera with the duration of PROM (Fig. 2J-O), and the results showed that the relative abundance of *Pseudomonas* significantly increased with the increase of PROM duration ($P < 0.05$, Fig. 2J), and *Clostridium* showed a downward trend ($0.05 < P < 0.1$, Fig. 2M). In addition, Bugbase analysis was performed for the changes of predicted phenotypes with PROM duration in PPROM infants. Compared to those of the non-PPROM group and the < 12 h group, the relative abundance of aerobic ($P = 0.0149$; Fig. 3G), biofilm-forming ($P = 0.0201$; Fig. 3K), and potentially pathogenic ($P = 0.0264$; Fig. 3L) microorganisms significantly increased in the ≥ 12 h group. Our results suggest that the duration of PROM is associated with the relative abundance of *Pseudomonas*, which may result in a high risk of intestinal microbiota disruption and further infection in premature infants.

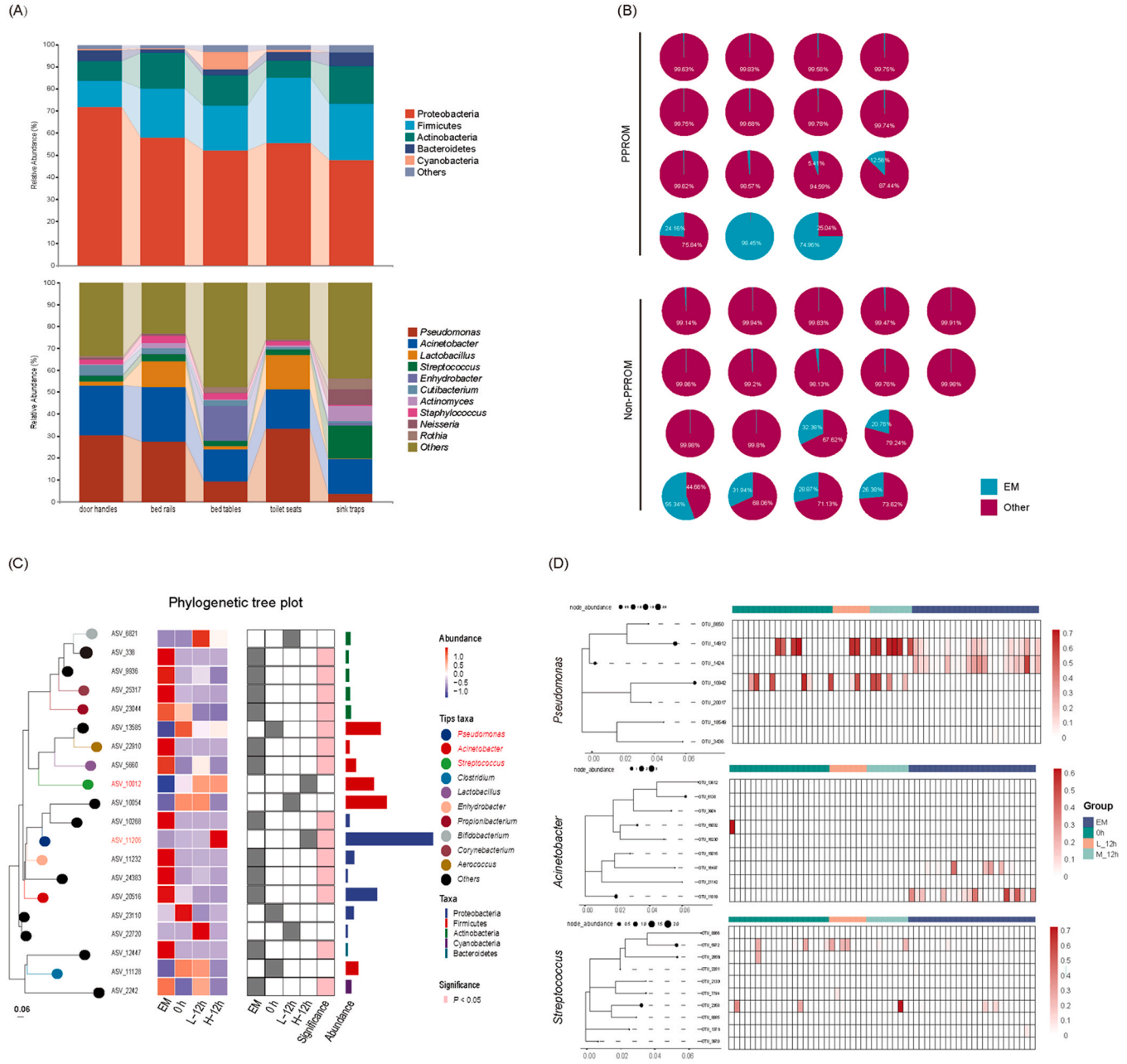

Fig. 4. Relationships between environmental microbiota and premature infants gut microbiota. (A) Taxonomics at the phylum and genus levels of environmental microbiota. (B) Source tracking analysis to reveal the contributions of environmental microbiota to the bacterial communities of premature infants. (C) 16 s rRNA gene-based phylogenetic tree of top 20 ASV between environmental microbiota and premature infants. (D) Phylogenetic trees based on the OTUs of *Pseudomonas*, *Acinetobacter*, and *Streptococcus*, and heatmap of their relative abundance across samples. EM: environmental microbiota; 0 h: non-PPROM; L_12 h: < 12 h group; and M_12 h: ≥ 12 h group; Red represents significant enrichment of OTU in the sample ($P < 0.05$).

3.4. Hospital-environment Pseudomonas strains shared a high homology with those of PPROM infants

Our results showed that the abundance of some potential pathogens, e.g. *Pseudomonas,* was significantly associated with the duration of PROM. We then tended to identify the source of *Pseudomonas*. It is known that the gut microbiota in early life of newborn is mainly obtained from the maternal and environmental microbiota. Given that the relative abundance of *Pseudomonas* is very low in the vagina [13,20], we thus hypothesized that these bacteria may have come from the nosocomial environment during the mothers' hospitalization. To test this hypothesis, we performed 16 s rRNA sequencing on the environmental samples collected from maternity wards, including the sink trap, toilet seat, bed rail, bed table, and door handle in one of the two hospitals. At the phylum level, the dominant bacteria of the ward environment were Proteobacteria, Firmicutes, and Actinobacteria, and those at the genus level were *Pseudomonas*, *Acinetobacter*, *Lactobacillus*, and *Streptococcus* (Fig. 4A), suggesting that the ward environment indeed carried some potential pathogens. Source Tracker was then used to predict the association of the environmental microbiota with the premature infants' microbiota by analyzing the samples collected at the same hospital. Compared to non-PPROM infants, the composition of the gut microbiome of PPROM infants showed greater similarity to that of the environmental microbiome. (42.86 % in the PPROM infants group, 30.00 % in the non-PPROM infants group, $\chi^2 = 0.5961$, $P = 0.4401$) (Fig. 4B).

To further verify the association of the microbiome between the environment and premature infants, we analyzed the enrichment of the top 20 ASVs in the environment and in the infants' gut. At the phylum level, all of the top 20 AVSs mainly belong to Proteobacteria, Firmicutes, Actinobacteria, Cyanobacteria, and Bacteroidetes. ASV_11206, a member of *Pseudomonas*, was significantly enriched in PPROM infants (Fig. 4C). To further understand the homology of *Pseudomonas* significantly enriched in premature infants and in the environment, we analyzed the enrichment OTUs with relative abundance ≥ 0.01 % in ≥ 5 environmental and preterm samples containing *Pseudomonas*. OTU_14912 was detected in both the environment and the intestine of premature infants (Fig. 4D), indicating that the intestine of premature infants shared certain population of *Pseudomonas* with the environment. Moreover, the abundance of OTU_14912 in PPROM infant increased significantly with the duration of PROM (0 h: 21.05 %; <12 h: 28.57 %; $\geq$ 12 h: 75 %; χ^2 (0 h vs $\geq$ 12 h) = 7.349; $P = 0.0254$) (Fig. 4D). In addition, we also analyzed the enrichment of two important healthcare-related organisms, i.e. *Acinetobacter* and *Streptococcus*, and the results showed that OTU_2358 of *Streptococcus* was detected both in the environment and in the intestines of premature infants, while no shared OTU of *Acinetobacter* was detected in the two niches (Fig. 4D). These results show a similarity of *Pseudomonas* and *Streptococcus* found in the hospital environment and in the gut of PPROM infants, indicating the potential horizontal transmission of *Pseudomonas* and *Streptococcus* between the environment and PPROM infants.

4. Discussion

The acquisition and subsequent colonization of the gut microbiota in the early-life period have crucial and long-term impact on the healthy development of newborns. Premature infants are a particularly vulnerable population whose gut microbiota develops late and is disordered, leading to delayed immune development and more severe and long-lasting adverse outcomes, e.g. gut inflammation, NEC, sepsis, and even death [12,17,21,22]. Hence, understanding the characteristics of the gut microbiota and fostering its balanced growth represents a viable approach to improve the quality of life for premature infants. Infants with PPROM account for more than 30 % of all preterm births and face an elevated infection risk, which are often linked to gut microbiota dysbiosis. However, the underlying mechanism remains poorly understood. In this investigation, we pinpoint the risk factors that contribute to gut microbiota imbalances and infections among PPROM infants.

Upon comparing the gut microbiota between PPROM and non-PPROM infants, we found that the diversity and composition of the gut microbiota in PPROM infants was significantly different between that in non-PPROM infants. Proteobacteria is the dominant phylum in the PPROM infant feces, while Firmicutes is predominant in the feces of non-PPROM infant, which is similar to previous studies [9,12,23]. It has been shown that the developmental process of neonatal gut microbiota is from Proteobacteria and Actinobacteria to Firmicutes and Bacteroidetes [24,25], which means that the development of the gut microbiota in PPROM infants is slower than in non-PPROM infants. Of concern, the higher relative abundance of Proteobacteria and the lower relative abundance of Firmicutes in the fecal microbiota of premature infants has been identified as a risk factor for necrotizing enterocolitis [19], suggesting that alternative gut microbiota is associated with disease in premature infants. To verify such an association, we further measured the levels of biomarker cytokines (IL-6 and IL-8) associated with neonatal infection in cord blood [26–31], and found that PPROM infants may have a higher risk of infection, as shown by the significantly increased levels of plasma cytokines and radiographic changes in lung X-rays.

We then found that the risk of infection in PPROM infants is associated with the duration of PROM, which is similar to the findings in term infants [5,32]. To understand the underlying mechanism, the structural characterization of the gut microbiota in PPROM infants was further dissected dynamically with the duration using 12 h as a cutoff. The most important finding is that PROM duration is associated with the relative abundance of potential pathogens, e.g. *Pseudomonas*. *Pseudomonas* spp. is capable of colonizing both humans and animals, and some of species (e.g. *P. aeruginosa*) are also important opportunistic pathogens causing hospital acquired infections [33]. It has been suggested that *Pseudomonas* spp. can cause a variety of infectious diseases in newborns, including septicemia, meningitis, NEC, and intestinal injury [34–38]. Taken together, our results suggest that increasing abundance of *Pseudomonas* spp. with PROM duration may be an important factor in PPROM infants susceptible to infection.

Understanding the source of *Pseudomonas*, which increases with PROM duration, is imperative to prevent infection in PPROM infants and improve their outcomes. The gut microbiota in early life of newborn is mainly obtained from the maternal and environmental microbiota. The impact of vaginal microbiota on the gut microbiota of PPROM infants has been reported, and it has been found that vaginal *Facklamia* spp. and *Winkia neuii* in pregnant women with PROM are closely associated with premature infant infection [13]. However, *Pseudomonas* is a common environmental pathogen, and was found with the extremely low relative abundance in the vagina [13,20]. We therefore hypothesized that the source of *Pseudomonas* could be from the nosocomial environment. The Source Tracker analysis strongly supports our hypothesis, and found that the microbial community of PPROM infants was highly homologous with the environmental microbiota of the inpatient wards of pregnant women with PROM before delivery, and the OTU of *Pseudomonas* is highly homologous in premature infants and the environment. To our knowledge, this is the first report that the environmental bacteria, such as *Pseudomonas*, are preferentially exposed to the fetus and colonized in the intestine of PPROM infants, ultimately leading to a higher risk of infection in PPROM infants. Our findings are in line with the "founder's hypothesis" that *Pseudomonas* has the priority to contact premature infants after PROM resulting in colonization advantage [39–41], and hold significant implications for enhancing the prevention and control of PPROM infection in neonatal hospital settings.

In summary, our investigation suggests a potential link between prolonged PROM and the accumulation of environmental microbes in the gut of PPROM infants, highlighting the importance of stricter environmental measures for infants with PPROM in clinical settings.

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Ethical statement

According to Good Clinical Practice guidelines, the study (LL-KY-2024020–01) was approved by the medical ethics committees of the Shenzhen People's Hospital.

CRediT authorship contribution statement

Xing Shi: Validation, Methodology. **Yi'e Huang:** Resources, Data curation. **Lingfeng Li:** Methodology, Investigation. **Jiacheng Zhong:** Validation. **Kai Zhou:** Writing – review & editing, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Quanhang Xiang:** Writing – original draft, Validation, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Xudong Yan:** Investigation, Data curation. **Shaohui Tang:** Formal analysis, Conceptualization. **Wei Shi:** Resources, Methodology, Formal analysis, Data curation. **Tingting Xu:** Methodology.

Declaration of Competing Interest

The authors have declared no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2024.07.007.

References

[1] Walani SR. Global burden of preterm birth. Int J Gynaecol Obstet 2020;150(1):31–3. https://doi.org/10.1002/ijgo.13195.
[2] Liu L, Oza S, Hogan D, Chu Y, Perin J, Zhu J, et al. Global, regional, and national causes of under-5 mortality in 2000-15: an updated systematic analysis with implications for the Sustainable Development Goals. Lancet 2016;388(10063):3027–35. https://doi.org/10.1016/S0140-6736(16)31593-8.
[3] Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. Lancet 2008;371(9606):75–84. https://doi.org/10.1016/S0140-6736(08)60074-4.
[4] Parry S, Strauss JF. Premature rupture of the fetal membranes. N Engl J Med 1998;338(10):663–70.
[5] Herbst A, Källén K. Time between membrane rupture and delivery and septicemia in term neonates. Obstet Gynecol 2007;110(3):612–8.
[6] Lorthe E, Ancel P-Y, Torchin H, Kaminski M, Langer B, Subtil D, et al. Impact of latency duration on the prognosis of preterm infants after preterm premature rupture of membranes at 24 to 32 weeks' gestation: a national population-based cohort study. J Pedia 2017;182. https://doi.org/10.1016/j.jpeds.2016.11.074.
[7] Namavar Jahromi B, Ardekany MS, Poorarian S. Relationship between duration of preterm premature rupture of membranes and pulmonary maturation. Int J Gynaecol Obstet 2000;68(2):119–22.
[8] Vatanen T, Jabbar KS, Ruohtula T, Honkanen J, Avila-Pacheco J, Siljander H, et al. Mobile genetic elements from the maternal microbiome shape infant gut microbial assembly and metabolism. Cell 2022;185(26). https://doi.org/10.1016/j.cell.2022.11.023.
[9] Healy DB, Ryan CA, Ross RP, Stanton C, Dempsey EM. Clinical implications of preterm infant gut microbiome development. Nat Microbiol 2022;7(1):22–33. https://doi.org/10.1038/s41564-021-01025-4.
[10] Grier A, Qiu X, Bandyopadhyay S, Holden-Wiltse J, Kessler HA, Gill AL, et al. Impact of prematurity and nutrition on the developing gut microbiome and preterm infant growth. Microbiome 2017;5(1):158. https://doi.org/10.1186/s40168-017-0377-0.
[11] Ho TTB, Groer MW, Kane B, Yee AL, Torres BA, Gilbert JA, et al. Dichotomous development of the gut microbiome in preterm infants. Microbiome 2018;6(1):157. https://doi.org/10.1186/s40168-018-0547-8.
[12] Lee C-C, Feng Y, Yeh Y-M, Lien R, Chen C-L, Zhou Y-L, et al. Gut dysbiosis, bacterial colonization and translocation, and neonatal sepsis in very-low-birth-weight preterm infants. Front Microbiol 2021;12:746111. https://doi.org/10.3389/fmicb.2021.746111.
[13] Dos Anjos Borges LG, Pastuschek J, Heimann Y, Dawczynski K, Schleußner E, Pieper DH, et al. Vaginal and neonatal microbiota in pregnant women with preterm premature rupture of membranes and consecutive early onset neonatal sepsis. BMC Med 2023;21(1):92. https://doi.org/10.1186/s12916-023-02805-x.
[14] Brooks B, Olm MR, Firek BA, Baker R, Thomas BC, Morowitz MJ, et al. Strain-resolved analysis of hospital rooms and infants reveals overlap between the human and room microbiome. Nat Commun 2017;8(1):1814. https://doi.org/10.1038/s41467-017-02018-w.
[15] Chng KR, Li C, Bertrand D, Ng AHQ, Kwah JS, Low HM, et al. Cartography of opportunistic pathogens and antibiotic resistance genes in a tertiary hospital environment. Nat Med 2020;26(6):941–51. https://doi.org/10.1038/s41591-020-0894-4.
[16] Xiang Q, Wu X, Pan Y, Wang L, Cui C, Guo Y, et al. Early-life intervention using fecal microbiota combined with probiotics promotes gut microbiota maturation, regulates immune system development, and alleviates weaning stress in piglets. Int J Mol Sci 2020;21(2). https://doi.org/10.3390/ijms21020503.
[17] Groer MW, Luciano AA, Dishaw LJ, Ashmeade TL, Miller E, Gilbert JA. Development of the preterm infant gut microbiome: a research priority. Microbiome 2014;2:38. https://doi.org/10.1186/2049-2618-2-38.
[18] Liu J, Feng Z-C, Wu J. The incidence rate of premature rupture of membranes and its influence on fetal-neonatal health: a report from mainland China. J Trop Pedia 2010;56(1):36–42. https://doi.org/10.1093/tropej/fmp051.
[19] Pammi M, Cope J, Tarr PI, Warner BB, Morrow AL, Mai V, et al. Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis. Microbiome 2017;5(1):31. https://doi.org/10.1186/s40168-017-0248-8.
[20] Pruski P, Correia GDS, Lewis HV, Capuccini K, Inglese P, Chan D, et al. Direct on-swab metabolic profiling of vaginal microbiome host interactions during pregnancy and preterm birth. Nat Commun 2021;12(1):5967. https://doi.org/10.1038/s41467-021-26215-w.
[21] Groer MW, Gregory KE, Louis-Jacques A, Thibeau S, Walker WA. The very low birth weight infant microbiome and childhood health. Birth Defects Res C Embryo Today 2015;105(4):252–64. https://doi.org/10.1002/bdrc.21115.
[22] Baranowski JR, Claud EC. Necrotizing enterocolitis and the preterm infant microbiome. Adv Exp Med Biol 2019;1125:25–36. https://doi.org/10.1007/5584_2018_313.
[23] Moles L, Gómez M, Heilig H, Bustos G, Fuentes S, de Vos W, et al. Bacterial diversity in meconium of preterm neonates and evolution of their fecal microbiota during the first month of life. PloS One 2013;8(6):e66986. https://doi.org/10.1371/journal.pone.0066986.
[24] Milani C, Duranti S, Bottacini F, Casey E, Turroni F, Mahony J, et al. The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. Microbiol Mol Biol Rev 2017;81(4). https://doi.org/10.1128/MMBR.00036-17.
[25] Roswall J, Olsson LM, Kovatcheva-Datchary P, Nilsson S, Tremaroli V, Simon M-C, et al. Developmental trajectory of the healthy human gut microbiota during the first 5 years of life. Cell Host Microbe 2021;29(5). https://doi.org/10.1016/j.chom.2021.02.021.
[26] Krueger M, Nauck MS, Sang S, Hentschel R, Wieland H, Berner R. Cord blood levels of interleukin-6 and interleukin-8 for the immediate diagnosis of early-onset infection in premature infants. Biol Neonate 2001;80(2):118–23.
[27] Büscher U, Chen FC, Pitzen A, Menon R, Vogel M, Obladen M, et al. Il-1 beta, Il-6, Il-8 and G-CSF in the diagnosis of early-onset neonatal infections. J Perinat Med 2000;28(5):383–8.
[28] Berner R, Tüxen B, Clad A, Forster J, Brandis M. Elevated gene expression of interleukin-8 in cord blood is a sensitive marker for neonatal infection. Eur J Pediatr 2000;159(3):205–10.
[29] Nakstad B, Sonerud T, Solevåg AL. Early detection of neonatal group B streptococcus sepsis and the possible diagnostic utility of IL-6, IL-8, and CD11b in a human umbilical cord blood in vitro model. Infect Drug Resist 2016;9:171–9. https://doi.org/10.2147/IDR.S106181.
[30] Fan Y, Yu J-L. Umbilical blood biomarkers for predicting early-onset neonatal sepsis. World J Pedia 2012;8(2):101–8. https://doi.org/10.1007/s12519-012-0347-3.
[31] Kurokawa CS, Hashimoto M, de Souza Rugolo LMS, Bentlin MR, Golin MdA, Peraçoli JC, et al. Cord blood cytokine levels in focal early-onset neonatal infection after preterm premature rupture of membranes. Turk J Pedia 2013;55(6):598–605.
[32] Zhuang L, Li Z-K, Zhu Y-F, Ju R, Hua S-D, Yu C-Z, et al. Latency period of PROM at term and the risk of neonatal infectious diseases. Sci Rep 2022;12(1):12275. https://doi.org/10.1038/s41598-022-16593-6.

[33] Lupo A, Haenni M, Madec J-Y. Antimicrobial resistance in Acinetobacter spp. and Pseudomonas spp. Microbiol Spectr 2018;6(3). https://doi.org/10.1128/microbiolspec.ARBA-0007-2017.
[34] Xu Y, Jin D, Ye H, Liang Y. A rare case of Pseudomonas aeruginosa bacteremia in a newborn with 58 perforations in the small intestine. BMC Pedia 2021;21(1):9. https://doi.org/10.1186/s12887-020-02466-2.
[35] Wareham DW. Sepsis in a newborn due to Pseudomonas aeruginosa from a contaminated tub bath. N Engl J Med 2001;345(22):1644–5.
[36] Smith RT. Septicemia and meningitis in a newborn infant; report of an unusual example due to simultaneous infection by Escherichia coli and Pseudomonas aeruginosa, with recovery. J Pediatr 1955;47(6):740–5.
[37] Gupta AK, Shashi S, Mohan M, Lamba IM, Gupta R. Epidemiology of Pseudomonas aeruginosa infections in a neonatal intensive care unit. J Trop Pedia 1993;39(1):32–6. https://doi.org/10.1093/tropej/39.1.32.
[38] Krasna IH, Kurgan A, Noy S. Pseudomonas septicemia; necrotizing bowel lesions (NEC) and skin lesions in a 5-mo-old child. J Pedia Surg 1979;14(4):481–2.
[39] Litvak Y, Baumler AJ. The founder hypothesis: A basis for microbiota resistance, diversity in taxa carriage, and colonization resistance against pathogens. PLoS Pathog 2019;15(2):e1007563. https://doi.org/10.1371/journal.ppat.1007563.
[40] Martinez I, Maldonado-Gomez MX, Gomes-Neto JC, Kittana H, Ding H, Schmaltz R, et al. Experimental evaluation of the importance of colonization history in early-life gut microbiota assembly. Elife 2018;7. https://doi.org/10.7554/eLife.36521.
[41] Sprockett D, Fukami T, Relman DA. Role of priority effects in the early-life assembly of the gut microbiota. Nat Rev Gastroenterol Hepatol 2018;15(4):197–205. https://doi.org/10.1038/nrgastro.2017.173.