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Original Article

Association between vaginal microbiomes and neonatal septicemia in pregnant women with preterm premature rupture of membranes based on metagenome sequencing

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Abstract: Background: Preterm premature rupture of membranes (PPROM) is closely associated with pathogenic microbiomes in the female reproductive tract, and can lead to neonatal septicemia. The current study aimed to investigate potential pathogenic microbiomes associated with neonatal septicemia based on DNA metagenome sequencing. Methods: In this study, a total of 7 pregnant women with PPROM presenting neonatal septicemia (experimental group) and 3 pregnant women with normal newborns (control group) were enrolled. Vaginal secretions at admission and before parturition as well as placental tissues after parturition were collected for DNA metagenome sequencing using whole genome shotgun method on the Illumina NovaSeq/HiSeq platform. Raw data were processed by BioBakery workflow, and MetaPhlAn4 was implemented for qualitative and quantitative analyses of microbiome. Lactobacillus crispatus, Gardneralla vaginalis, Fannyhessea vaginae and Streptococcus suis were specifically detected from the experimental group. The two groups were compared using Student's t-tests. Results: The indexes of Chao1 (P=0.00028/P=0.00072), abundance-based coverage estimator (ACE, P=0.00059/P=0.00026), Shannon (P=0.036/P=0.0065) and Simpson (P=0.007/P=0.041) in the experimental group were increased at admission and before parturition as compared with the control group. Several microbiomes, such as Lactobacillus crispatus, Gardneralla vaginalis, Fannyhessea vaginae and Streptococcus suis, were specifically detected in the experimental group. Notably, Gardnerella vaginalis and Streptococcus gallolyticus were identified from the vaginal secretions and placenta tissues of women with neonatal septicemia. Moreover, nucleic acid synthesis and carbohydrate metabolism-related pathways were enriched in the experimental group. Conclusion: This study enhanced the current understanding of the mechanisms underlying pathogenic microbiomes in PPROM-induced neonatal septicemia. The trial registry number is ChiCTR2300070666 (URL: https://www.chictr.org.cn/showproj.html?proj=195648).

Keywords: Preterm premature rupture of membranes, neonatal septicemia, microbiomes, whole genome shotgun, metabolic pathways

Introduction

Preterm premature rupture of membranes (PPROM), is the rupture of fetal membranes within 37 weeks of gestation, accounting for about 2%-4% of all pregnancies and 40% of spontaneous preterm births [1]. Rupture at a younger gestational age is associated with a greater possibility of infection. Moreover, infection develops in 15% of PPROM at above 34 weeks of gestation and 90% of PPROM at 28 weeks of gestation [2], which suggests that PPROM directly affects premature delivery of fetus and intrauterine infection.

Neonatal sepsis caused by PPROM is a challenge for gynecologists and neonatologists [3]. Early identification of pregnant women at risk of developing neonatal infection and implementing effective treatment and preventive programs for them pose significant challenges as well. Since 2009, we have successfully treated 665 extremely premature infants (gestational age less than 32 weeks), with a minimum ges-

tational week of 24 weeks and minimum weight of 550 g. Statistics showed that the success rate of treating premature infants above 28 weeks has reached 99.5%. However, the prognosis of PPROM patients with intrauterine infection remains relatively poor, especially for those with early-onset neonatal sepsis. In recent years, researchers have used Interleukin (IL)-6, IL-10, IL-33, matrix metalloproteinases (MMPs), fetal fibronectin (fFN), ultrasonic fetal thymus measurement, amniotic white blood cell count and other indicators for the early diagnosis of intrauterine infection [4-7], but due to limitations in laboratory conditions, patient compliance, potential risk of invasive procedures etc., these indicators have not been widely applied in clinical practice. The current study was the first prospective study combining retrospective exclusion for exploring vaginal microbiological characteristics of neonatal sepsis in pregnant women with PPROM using the latest Acer assay technology.

PPROM patients with intrauterine infection, especially those with early-onset neonatal sepsis, are associated with longer length of hospital stay, longer period in NICU, higher cost of treatment, poorer long-term prognosis than those of general newborns at the same gestational age [8]. Previous study indicated that infection plays a more substantial role than gestational age in affecting neonatal prognosis during PPROM pregnancies. In recent years, researchers have used inflammatory factors, MMPs, fFN, white blood cell count in amniotic fluid, etc. for the early diagnosis of intrauterine infection [9], but they have not been widely applied in clinical practice due to limitations in laboratory conditions, and potential risks of invasive procedures. Histological evidence of placental membranes is helpful for prognostic assessment of neonatal infection but not for early targeted prevention [10]. It is possible to analyze bacteria and reproductive tract microenvironment that potentially cause insidious and severe intrauterine infection by identifying the source of pathogens. This can then guide targeted antibiotic prevention and treatment strategies, tailored to the specific reproductive tract microecology and pathogens present in patients with PPROM for improving maternal and infant prognosis.

In China, Escherichia coli and Staphylococcus are the main pathogens of intrauterine infections of fetuses [11], and group B Streptococcus is a common pathogen [12]. According to the antimicrobial profiles of drugs in current guidelines, prophylactic regimens often cover several common pathogens, but the practical application effect is not satisfactory. Therefore, it is considered that variations in virulence, adhesion ability, glucose metabolism, drug resistance and other characteristics among different bacterial groups could contribute to differences in the affected tissues and organs. However, it is challenging to study these issues in-depth only by traditional experimental detection.

With the development of metagenomic high-throughput sequencing technology, a large number of microbial community species, abundance, and related biological information can be examined by total microbial DNA extraction and library construction from specific environments. As a result, a great amount of information about unculturable microbial flora can now be obtained without relying on traditional isolation and culture methods commonly used in microbial research. At present, metagenomic sequencing technology is employed as an important tool to study intestinal, reproductive and respiratory microorganisms. This study aimed to investigate the relationship between the neonatal infection outcomes and reproductive tract microecological characteristics, as well as disease-related functional genes. A prospective design was applied to retrospectively select representative cases, and metagenomic technology was utilized to analyze the distribution of reproductive tract microbial populations in patients with PPROM who exhibited different maternal and infant infection outcomes.

Material and methods

Sample collection and processing

We collected the vaginal secretions of 7 PPROM pregnant women (experimental group) presenting neonatal septicemia at admission and before parturition as well as the placental tissues after parturition from Chongqing Health Center for Women and Children. Another 3 pregnant women with normal newborns served as the control group. The clinical data are presented in Table S1. All the patients who participated in this study signed an informed consent

form, and this study was approved by Institutional Ethics Board of Chongqing Health Center for Women and Children (No. 2019-008). The trial registry number is ChiCTR-2300070666, and the trial URL is https://www.chictr.org.cn/showproj.html?proj=195648. Whole genome shotgun method [13] was employed for DNA metagenome sequencing on the Illumina NovaSeq/HiSeq platform [14]. The total DNA extracted from the microbial metagenome or the cDNA double strand synthesized by the metatranscriptomics, using mRNA served as template, was randomly fragmented into short fragments. Next, an inserted fragment library with appropriate length was developed and paired-end sequencing was performed using these libraries. During the sequencing, a library was built for each sample. The vaginal secretions were sequenced in the pregnant women, as shown in Tables S2 and S3. At admission, the total number of Reads in the control group and the experimental group was 71152412-72664200 and 67832966-73173554, respectively, and the total number of bases was 1089963000-10672861800 and 10461977400-10976033100, respectively. The Q20 (%) of the control group and experimental group was 96.48-96.75% and 97.55-97.86%, respectively, while the Q30 (%) was 91.20-91.73% and 93.36-94.15%, respectively. Additionally, the GC content (%) of the control group and experimental group was 40.95-41.17% and 41.17-41.62%, respectively. Negligible fuzzy base content was found in both groups. Before parturition, the total number of Reads was 68816778-71375810 and 66827234-78503436, and the total number of bases was 10322516700-10706371500 and 10024085100-11775515400 in the control group and experimental group, respectively. The Q20 (%) of the control group and experimental group was 96.16-96.53% and 97.58-97.79%, while the Q30 (%) was 90.56-91.42% and 93.48-94.00%, respectively. Meanwhile, the GC content (%) of the control group and experimental group was 40.85-41.43% and 41.35-42.83%, respectively. Negligible fuzzy base content was also found in both groups.

Analysis in microbiota and metabolic pathway

BioBakery workflow (https://huttenhower.sph.harvard.edu/biobakery_workflows/) [15] was used to process raw sequencing data. Briefly, Trimmatic software was applied to remove sequencing connectors. KneedData was employed to remove contaminated human sequences and low-quality readings. MetaPhlAn4 with default parameters was implemented for qualitative and quantitative analysis of the microbiome. The identified species were subjected to StrainPhlAn to characterize the bacterial strain diversity based on relaxed_parameters3. For each species with a minimum reading depth ≥ 5 times, the genome variation within the species-specific marker gene was used to develop single nucleotide polymorphism haplotype to represent the dominant strain in the metagenome samples. Using UniRef90 gene family and MetaCyc pathway database, HUMAnN2 was employed to characterize the gene content of species stratification and metabolic potentials. For each sample, the default abundance of the UniRef90 gene family was expressed as the reads per kilobase, which was then normalized by the total number of reading counts and multiplied by one million to obtain copy number per million. The data were processed in R software (version 3.6.3, https://www.r-project.org/ver. 3.6.3) [16]. Diversity values were measured using vegan package (version 2.5-6) [17]. Shannon index [18] was employed to evaluate species diversity in individual metagenome sample (α diversity). Bray-Curtis dissimilarity index [19] served to estimate the diversity among samples based on the generic relative abundance profiles (β diversity). The genes were annotated to obtain the abundance of each metabolic pathway using single-sample gene set enrichment analysis in GSVA package.

Results

Differences of microbial diversity between control and neonatal septicemia at admission

The workflow is shown in Figure S1. Pregnant women with PPROM at a gestational age fewer than 37 weeks were included. We evaluated microbial diversity between the control and experimental groups. As shown in **Figure 1**, the indexes of Chao1, ACE, and Shannon were higher in the experimental group than those in the control group (**Figure 2A**). In detail, a high abundance of Lactobacillus iners was detected in both groups. However, Lactobacillus crispus, Gardnella vaginalis, Fannyhessa vaginalis and

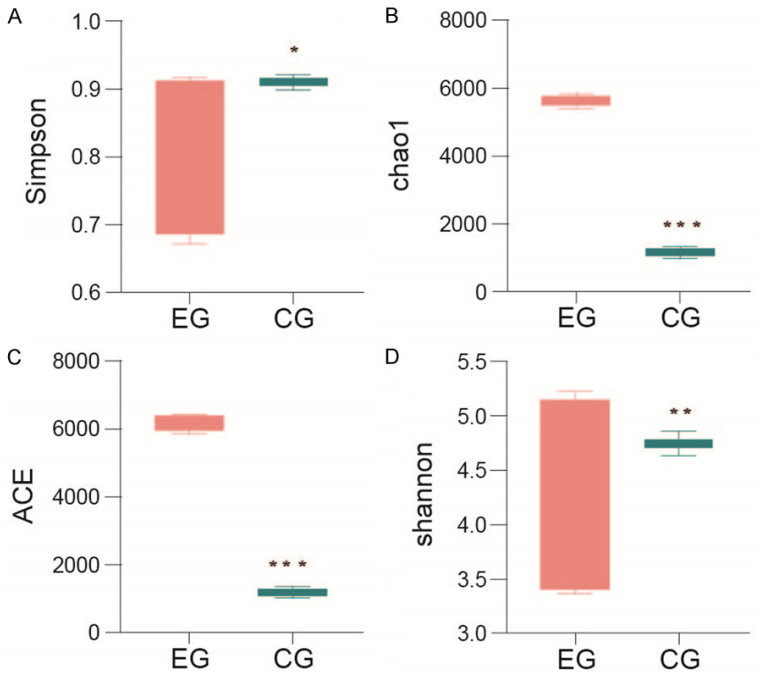

Figure 1. Differences of microbial diversity in vaginal secretions between the experimental group and control group at admission. A: The difference of Simpson between EG and CG group. B: The difference of chao1 between EG and CG group. C: The difference of ACE between EG and CG group. D: The difference of Shannon between EG and CG group. EG, experimental group; CG, control group; abundance-based coverage estimator (ACE). *P<0.05, **P<0.01, ***P<0.001.

Streptococcus suis were specifically detected in the experimental group (**Figure 2B**).

Differences of microbial diversity between control and neonatal septicemia before parturition

We further compared the differences of microbial diversity at a species level between the experimental group and the control group before parturition. Before parturition, the Chao1, ACE, Shannon and Simpson indexes in the experimental group were higher than those in the control group at a species level (**Figure 3**). The results from PCoA demonstrated that the genera abundance was remarkably altered between the two groups (**Figure 4A**). Meanwhile, most vaginal secretion samples exhibited a high abundance of Lactobacillus iners in both groups, while Lactobacillus crispus, Gardnella vaginalis, and Streptococcus suis were specifically detected in the experimental group (**Figure 4B**). These results are similar to those at admission. To further verify the results, we compared the species level in the experimental group between admission and before parturition. It was difficult to distinguish vaginal secretion samples at admission and before parturition using PCoA (**Figure 4C**). Notably, the abundance of Streptococcus suis increased over time (**Figure 4D**) and was significantly elevated in the experimental group before parturition in comparison with that in the control group (**Figure 4E**).

The relationship between vaginal microbial abundance and intrauterine infection before parturition

Secondary intrauterine infection caused by dysbacteriosis may result in bacterial infection of placenta tissue. Herein, we analyzed the alterations of microbiome between the placenta of the experimental group and the control group. It was found that PCoA displayed a separation of microbiome between the two groups (**Figure 5A**). The Streptococcus suis exhibited a high abundance in the placenta tissue of the experimental group (**Figure 5B**).

Identification of potential pathogen for PPROM complicated with neonatal septicemia

To identify the potential pathogenic microbiomes, we analyzed the microbiome alterations in vaginal secretions and placenta tissue at admission, before parturition, and after parturition. We found Gardnerella vaginalis and Streptococcus gallolyticus in vaginal secretions and placenta tissues of neonatal septicemia, which might be the potential pathogenic microbiomes leading to neonatal septicemia (**Figure 6A-D**).

Perturbation of metabolic pathways

Metabolic pathways in vaginal secretions and placenta tissues were studied. At admission, lactose and galactose degradation I, PWY-922: mevalonate pathway I (eukaryotes and bacteria), PWY-5667: CDP-diacylglycerol biosynthesis I, and PWY0-131: CDP-diacylglycerol biosyn-

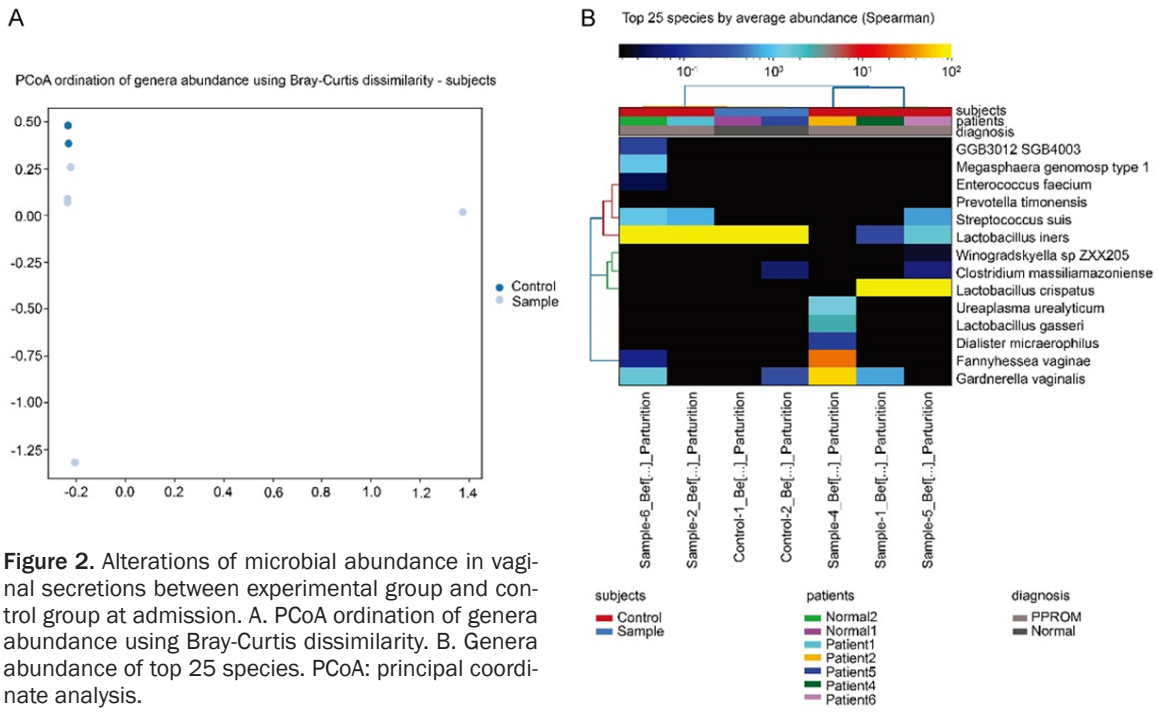

Figure 2. Alterations of microbial abundance in vaginal secretions between experimental group and control group at admission. A. PCoA ordination of genera abundance using Bray-Curtis dissimilarity. B. Genera abundance of top 25 species. PCoA: principal coordinate analysis.

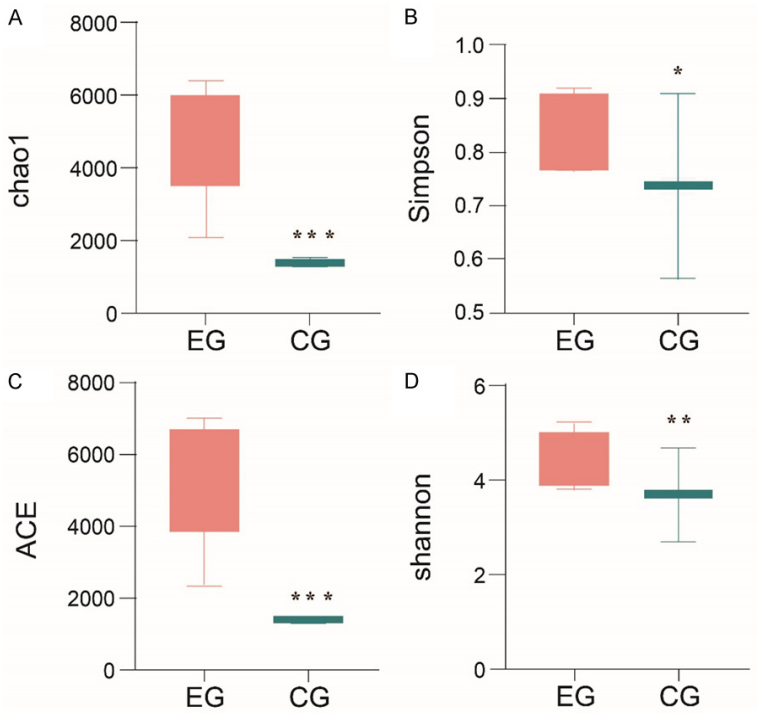

Figure 3. Differences of microbial diversity in vaginal secretions between experimental group and control group before parturition. A: The difference of Simpson between EG and CG group. B: The difference of chao1 between EG and CG group. C: The difference of ACE between EG and CG group. D: The difference of Shannon between EG and CG group. EG, experimental group; CG, control group; abundance-based coverage estimator (ACE). *P<0.05, **P<0.01, ***P<0.001.

thesis II were enriched (**Figure 7A**; **Table 1**). PWY-1042: glycolysis IV, TRNA-CHARGING-PWY: tRNA charging, and PWY-6609: adenine and adenosine salvage III were mainly enriched in vaginal secretions before parturition (**Figure 7B**; **Table 2**). We also observed the perturbed metabolic pathways at admission in comparison with that before parturition (**Figure 7C**; **Table 3**). Nucleic acid synthesis and carbohydrate metabolism-related pathways were found to be significantly enriched in the placenta of PPROM women (**Figure 7D**; **Table 4**).

Discussion

Based on the Chinese National Free Preconception Screening Program database, a previous prospective study showed that a poor vaginal microenvironment was associated with longer time to pregnancy for nor-

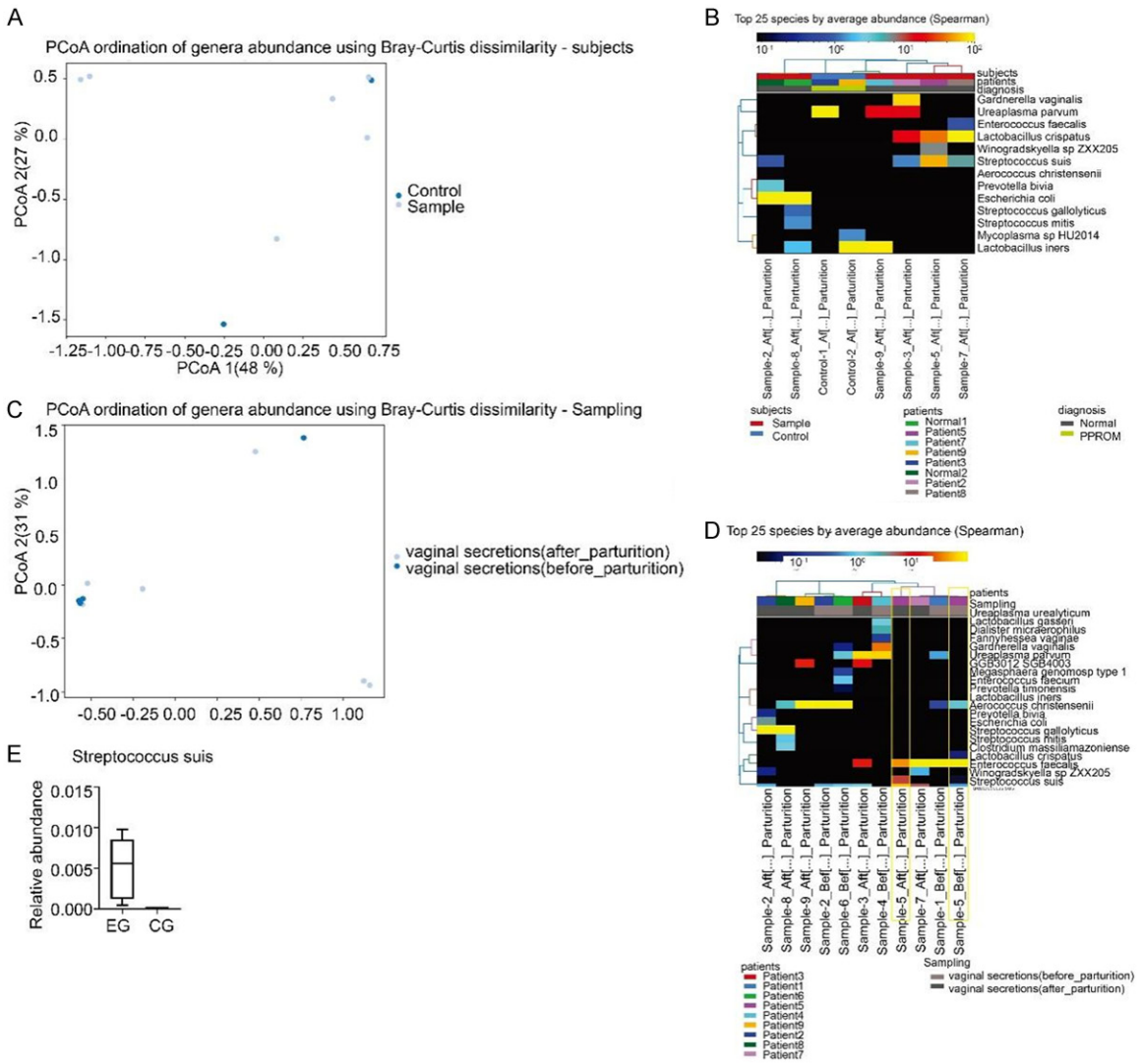

Figure 4. Alterations of microbial abundance in vaginal secretions before parturition. A. PCoA ordination of genera abundance using Bray-Curtis dissimilarity to distinguish the experimental group and the control group. B. Genera abundance of top 25 species in the experimental group and the control group. C. PCoA ordination of genera abundance shows a high consistency in genera abundance of vaginal secretions at admission and before parturition. D. Genera abundance of top 25 species at admission and before parturition. E. The abundance of Streptococcus suis in the experimental group and the control group before parturition. EG, experimental group; CG, control group. PCoA: principal coordinate analysis.

mal healthy women [20]. Using a Kenyan family planning cohort, another study found that women with bacterial vaginosis exhibited increased risk of low fertility [21]. Fannyhessea vaginae and Gardneralla vaginalis are associated with bacterial vaginitis [22]. The presence of Fannyhessea vaginae can cause biofilms to form in the vagina and become resistant to certain antibacterial substances. It has long been considered that Lactic acid bacteria are biomarkers for a healthy vaginal microenvironment for producing lactic acid to maintain a locally acidic environment so as to prevent pathogen colonization [23]. Local inflammation resulted from vaginal microbiome disturbance could decrease fertility, and elevate levels of IL-1B, IL-6, and IL-8 cytokines in the cervix were reported to be associated with infertility [24]. As shown by Li et al., vaginal probiotic Lactobacillus crispatus can greatly affect sperm motility and pregnancy through its adhesion properties [25]. Based on these results, it

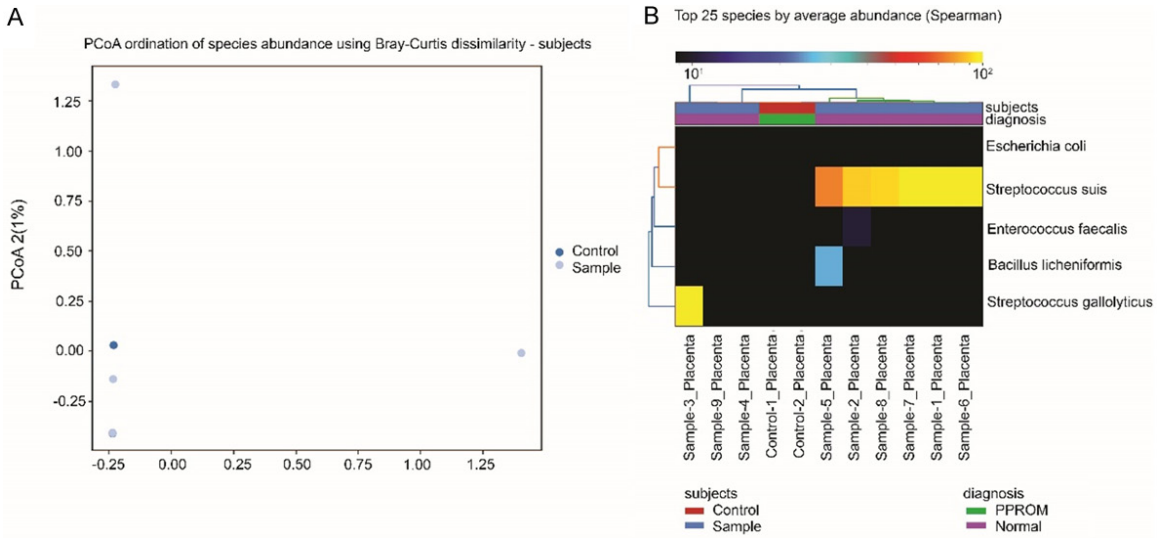

Figure 5. Relationship of vaginal microbial abundance and intrauterine infection before parturition. A. PCoA ordination of genera abundance portrays a separation of microbiome between the experimental group and the control group. B. Genera abundance of top 25 species of placenta tissue in the experimental and control groups. PCoA: principal coordinate analysis.

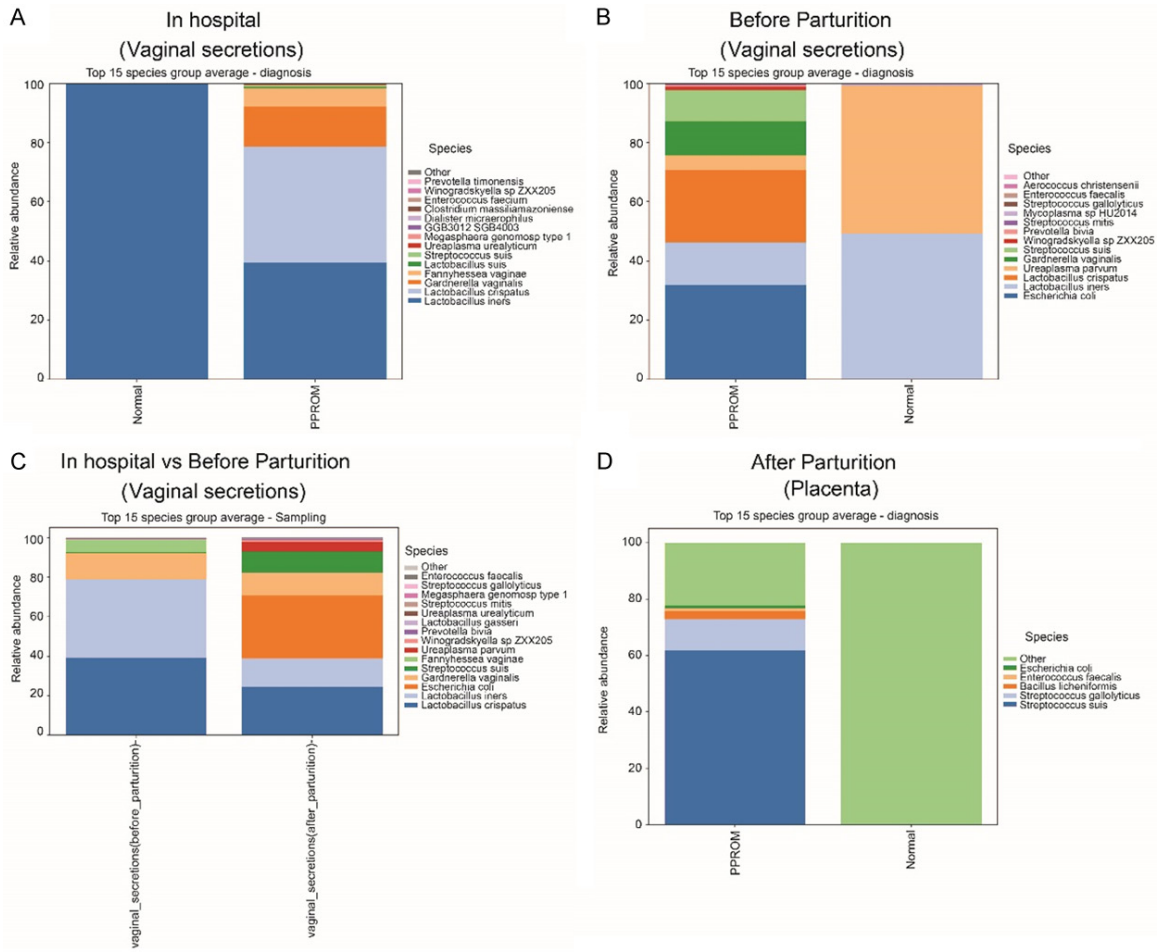

Figure 6. Identification of potential pathogen for neonatal septicemia in PPROM women. A. Potential pathogenic microbiomes in vaginal secretions at admission. B. Potential pathogenic microbiomes in vaginal secretions before

parturition. C. Differences of pathogenic microbiomes in vaginal secretions at admission vs. before parturition. D. Potential pathogenic microbiomes in placenta tissue after parturition. PPROM: Preterm premature rupture of membranes.

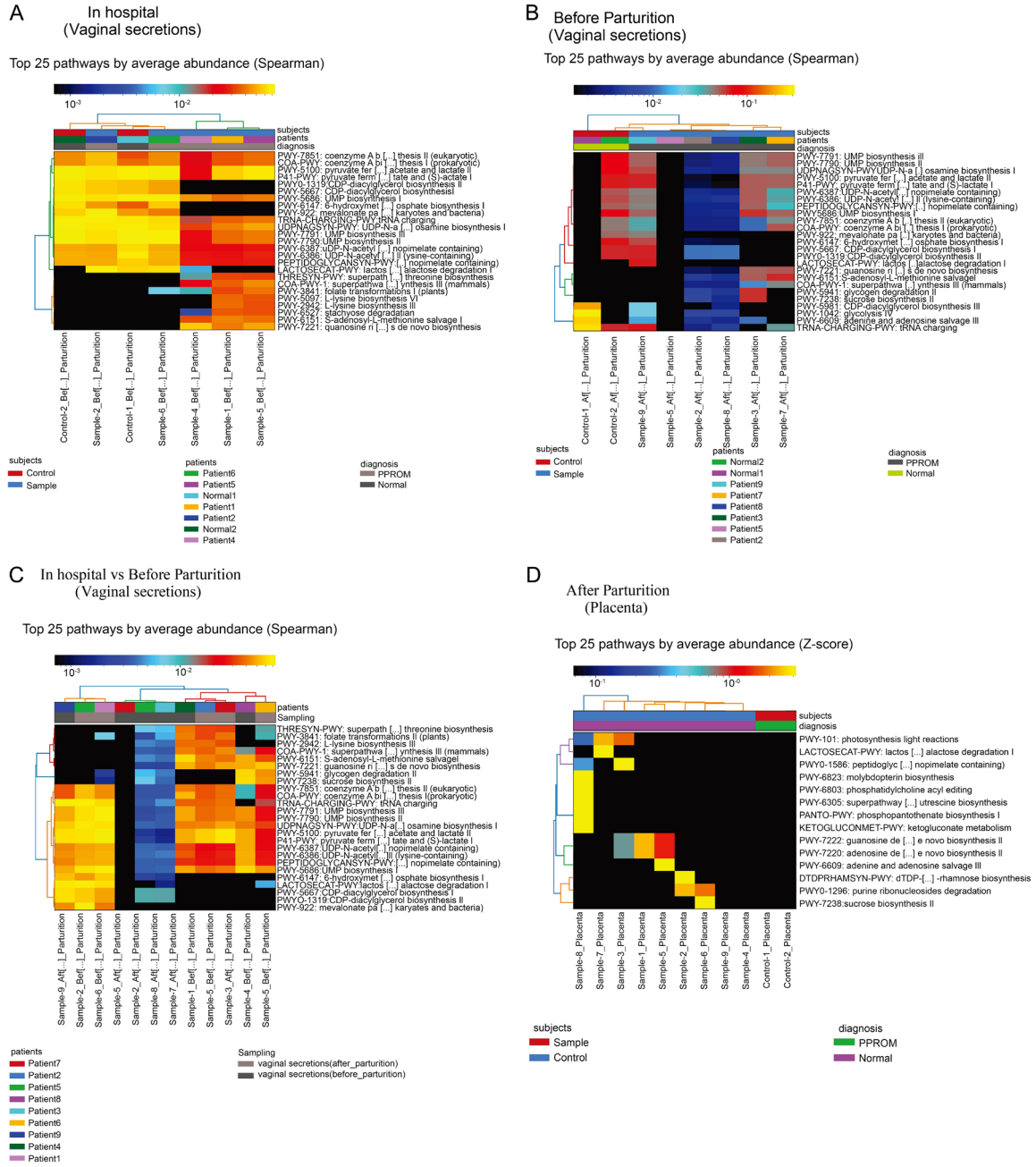

Figure 7. Perturbation of metabolic pathways. A. Perturbed metabolic pathways in vaginal secretions at admission. B. Perturbed metabolic pathways in vaginal secretions before parturition. C. Differential perturbed metabolic pathways in vaginal secretions at admission vs. before parturition. D. Perturbed metabolic pathways in placenta tissue after parturition.

could be reasonably concluded that vaginal microbial environment may have important effects on reproductive outcomes.

Metagenomic analysis involves the extraction of DNA from all microorganisms in environmental samples with metagenomic library construc-

Table 1. Differences in microflora pathways between vaginal secretions of pregnant women with PPROM and normal samples at admission

Pathways	Average abundance	Variance
P41-PWY: pyruvate fermentation to acetate and (S)-lactate I	0.0606	0.000259
PWY-5100: pyruvate fermentation to acetate and lactate II	0.0606	0.000259
PWY-5686: UMP biosynthesis I	0.0572	0.000208
UDPNAGSYN-PWY: UDP-N-acetyl-D-glucosamine biosynthesis I	0.0558	0.000199
PWY-7790: UMP biosynthesis II	0.0543	0.000324
PWY-7791: UMP biosynthesis III	0.0543	0.000324
TRNA-CHARGING-PWY: tRNA charging	0.051	0.000352
PWY-6386: UDP-N-acetylmuramoyl-pentapeptide biosynthesis II (lysine-containing)	0.0422	0.000209
PEPTIDOGLYCANSYN-PWY: peptidoglycan biosynthesis I (meso-diaminopimelate containing)	0.0411	0.000159
PWY-6387: UDP-N-acetylmuramoyl-pentapeptide biosynthesis I (meso-diaminopimelate containing)	0.0402	0.000202
COA-PWY: coenzyme A biosynthesis I (prokaryotic)	0.0389	8.92E-05
PWY-7851: coenzyme A biosynthesis II (eukaryotic)	0.0383	9.30E-05
PWY-5667: CDP-diacylglycerol biosynthesis I	0.0319	0.000768
PWY0-1319: CDP-diacylglycerol biosynthesis II	0.0319	0.000768
PWY-922: mevalonate pathway I (eukaryotes and bacteria)	0.0309	0.000791
PWY-6147: 6-hydroxymethyl-dihydropterin diphosphate biosynthesis I	0.0286	0.000679
LACTOSECAT-PWY: lactose and galactose degradation I	0.028	0.000962
PWY-7221: guanosine ribonucleotides de novo biosynthesis	0.0207	0.00059
PWY-6151: S-adenosyl-L-methionine salvage I	0.0164	0.000363
COA-PWY-1: superpathway of coenzyme A biosynthesis III (mammals)	0.0147	0.000319
PWY-3841: folate transformations II (plants)	0.0127	0.000233
THRESYN-PWY: superpathway of L-threonine biosynthesis	0.0107	0.000192
PWY-2942: L-lysine biosynthesis III	0.0103	0.000269
PWY-5097: L-lysine biosynthesis VI	0.0103	0.000268
PWY-6527: stachyose degradation	0.0102	0.000236

PPROM: Preterm premature rupture of membranes; PWY: Pathway.

Table 2. Differences in microflora pathways between vaginal secretions of pregnant women with PPROM and normal samples during parturition

Pathway	Average abundance	Variance
TRNA-CHARGING-PWY: tRNA charging	0.0542	0.00672
PWY-1042: glycolysis IV	0.0438	0.0108
PWY-6609: adenine and adenosine salvage III	0.0332	0.00577
PWY-5686: UMP biosynthesis I	0.0303	0.000874
P41-PWY: pyruvate fermentation to acetate and (S)-lactate I	0.029	0.000819
PWY-5100: pyruvate fermentation to acetate and lactate II	0.029	0.000822
PWY-7790: UMP biosynthesis II	0.0281	0.000786
PWY-7791: UMP biosynthesis III	0.0281	0.000787
UDPNAGSYN-PWY: UDP-N-acetyl-D-glucosamine biosynthesis I	0.0281	0.000813
PWY-5981: CDP-diacylglycerol biosynthesis III	0.0267	0.00376
PWY-6386: UDP-N-acetylmuramoyl-pentapeptide biosynthesis II (lysine-containing)	0.0241	0.000517
PWY-6387: UDP-N-acetylmuramoyl-pentapeptide biosynthesis I (meso-diaminopimelate containing)	0.0231	0.00048
PEPTIDOGLYCANSYN-PWY: peptidoglycan biosynthesis I (meso-diaminopimelate containing)	0.0223	0.000457
COA-PWY: coenzyme A biosynthesis I (prokaryotic)	0.0184	0.000406
PWY0-1319: CDP-diacylglycerol biosynthesis II	0.0183	0.000669
PWY-5667: CDP-diacylglycerol biosynthesis I	0.0183	0.000669
PWY-7851: coenzyme A biosynthesis II (eukaryotic)	0.0169	0.000322

PWY-6147: 6-hydroxymethyl-dihydropterin diphosphate biosynthesis I	0.0157	0.000688
PWY-6151: S-adenosyl-L-methionine salvage I	0.015	0.000569
PWY-7221: guanosine ribonucleotides de novo biosynthesis	0.0132	0.000468
PWY-922: mevalonate pathway I (eukaryotes and bacteria)	0.0113	0.000384
PWY-7238: sucrose biosynthesis II	0.00894	0.000396
PWY-5941: glycogen degradation II	0.00882	0.000388
LACTOSECAT-PWY: lactose and galactose degradation I	0.00837	0.000467
COA-PWY-1: superpathway of coenzyme A biosynthesis III (mammals)	0.00752	0.000176

PPROM: Preterm premature rupture of membranes; PWY: Pathway.

Table 3. Differences of microflora pathway in vaginal secretions of pregnant women with PPROM at admission and parturition

Pathways	Average abundance	Variance
P41-PWY: pyruvate fermentation to acetate and (S)-lactate I	0.0409	0.00074
PWY-5100: pyruvate fermentation to acetate and lactate II	0.0408	0.000743
PWY-5686: UMP biosynthesis I	0.0383	0.000563
UDPNAGSYN-PWY: UDP-N-acetyl-D-glucosamine biosynthesis I	0.0359	0.00051
PWY-7790: UMP biosynthesis II	0.0349	0.000526
PWY-7791: UMP biosynthesis III	0.0349	0.000527
TRNA-CHARGING-PWY: tRNA charging	0.0305	0.000677
PWY-6386: UDP-N-acetylmuramoyl-pentapeptide biosynthesis II (lysine-containing)	0.0289	0.000336
PEPTIDOGLYCANSYN-PWY: peptidoglycan biosynthesis I (meso-diaminopimelate containing)	0.0281	0.000328
PWY-6387: UDP-N-acetylmuramoyl-pentapeptide biosynthesis I (meso-diaminopimelate containing)	0.0276	0.000323
COA-PWY: coenzyme A biosynthesis I (prokaryotic)	0.0267	0.000366
PWY-7851: coenzyme A biosynthesis II (eukaryotic)	0.0256	0.000324
PWY-7221: guanosine ribonucleotides de novo biosynthesis	0.0228	0.000596
PWY-6151: S-adenosyl-L-methionine salvage I	0.0213	0.000524
LACTOSECAT-PWY: lactose and galactose degradation I	0.0185	0.000838
PWY0-1319: CDP-diacylglycerol biosynthesis II	0.0172	0.00058
PWY-5667: CDP-diacylglycerol biosynthesis I	0.0172	0.00058
COA-PWY-1: superpathway of coenzyme A biosynthesis III (mammals)	0.0148	0.000289
PWY-6147: 6-hydroxymethyl-dihydropterin diphosphate biosynthesis I	0.0146	0.000539
PWY-922: mevalonate pathway I (eukaryotes and bacteria)	0.0141	0.000581
PWY-3841: folate transformations II (plants)	0.0125	0.000249
THRESYN-PWY: superpathway of L-threonine biosynthesis	0.0115	0.000212
PWY-2942: L-lysine biosynthesis III	0.0108	0.00026
PWY-7238: sucrose biosynthesis II	0.0108	0.00042
PWY-5941: glycogen degradation II	0.0107	0.000407

PPROM: Preterm premature rupture of membranes; PWY: Pathway.

tion and the use of high-throughput sequencing techniques to study the genetic composition and community function of these microorganisms [26]. The types and amounts of microbes vary significantly according to diet, species, sex, and age. The microbes in the vagina are relatively conservative, but pregnancy could disturb the balance [27]. Microbial infection has been confirmed as an important risk factor leading to adverse pregnancy outcomes, such as recurrent abortion, eclampsia, intrauterine growth retardation, preterm rupture of membranes, and preterm delivery [28, 29]. Due to the changes of hormones and complexity of microorganisms, the traditional methods cannot meet the demand of current data analysis. The development of metagenomics and high-throughput sequencing technology has greatly promoted the study of reproductive tract microorganisms [30]. Prior research used

Table 4. Differences of bacterial pathways in placenta between pregnant women with PPROM and normal pregnant women

Pathway	Average abundance	Variance
PWY-7220: adenosine deoxyribonucleotides de novo biosynthesis II	0.107	0.0274
PWY-7222: guanosine deoxyribonucleotides de novo biosynthesis II	0.107	0.0274
PWY0-1296: purine ribonucleosides degradation	0.103	0.0482
LACTOSECAT-PWY: lactose and galactose degradation I	0.0672	0.0451
PWY-101: photosynthesis light reactions	0.0495	0.00824
PWY-7238: sucrose biosynthesis II	0.0447	0.02
PWY0-1586: peptidoglycan maturation (meso-diaminopimelate containing)	0.0379	0.00992
DTDPRHAMSYN-PWY: dTDP-β -L-rhamnose biosynthesis	0.0342	0.0117
PWY-6609: adenine and adenosine salvage III	0.0294	0.00864
PWY-6803: phosphatidylcholine acyl editing	0.0203	0.00411
PWY-6823: molybdopterin biosynthesis	0.0119	0.00141
PWY-6305: superpathway of putrescine biosynthesis	0.0111	0.00123
PANTO-PWY: phosphopantothenate biosynthesis I	0.0071	0.000503
KETOGLUCONMET-PWY: ketogluconate metabolism	0.00587	0.000345

PPROM: Preterm premature rupture of membranes; PWY: Pathway.

multi-platform metabolomic analysis to show that the normal vaginal flora of pregnant women consists of Prevotella, Sneathia, Gardnerella, Atopobium, Lactobacillus crispatus, Lactobacillus iners, Dialister and Megasphaera species [31]. A high-throughput pyrosequencing study on 16S RNA genes in vaginal microbiota demonstrated stable microbiota during pregnancy in health pregnant women [32]. In this study, Q20 (%) was higher than 96% and Q30 (%) was higher than 90% in both the control and septicemia groups.

In humans, up to 9% of the total bacterial load come from various microbiota in the female reproductive tract [33]. The stability of microecological environment plays an important role in health regulation [34, 35]. Using gene sequencing to examine the vaginal microbiome derived from a total of 1,958 pregnant women in the first and second trimesters of pregnancy, a previous study reported that preterm birth was a result of decreased lactic acid bacteria rather than an increase in other microbiota [36-38]. Gardnerella as the most common genus of bacteria is increased from 3.8% to 15.4% in women with a first conception and to 14.3% in those with a previous miscarriage or live birth [39]. The commensal role of Gardnerella in many healthy women could be observed by the frequent presence of Gardnerella vaginalis in women with lactobacilli-dominated vaginal microbiome [40]. This study observed that maternal microbial diversity was greater in the experimental group than the control group both at admission and before parturition. However, to maintain the stability of vaginal microecology, there is a tendency for decrease microbial diversity and increased abundance of microflora, particularly beneficial bacteria, in women of childbearing age during the transition from pre-pregnancy to early pregnancy and mid-to-late pregnancy [41, 42]. In our study, with the change of gestational age, the vaginal microbial species of pregnant women in the experimental group changed significantly and caused functional changes. In the control group, such a significant change in microflora did not cause functional abnormalities, suggesting that the function of microflora played an important role in the development of neonatal sepsis rather than the type of microflora.

There are some limitations in our study. Firstly, multicenter and prospective clinical studies are needed to validate our hypothesis. Secondly, this study was only based on bioinformatics analysis and lacked *in vivo* and *in vitro* experimental verification in cells and animal models. Thirdly, the specific mechanism of PPROM associated with microorganism remained to be further studied.

The current research detected several microbiomes such as Lactobacillus crispatus,

Gardneralla vaginalis, Fannyhessea vaginae and Streptococcus suis specifically in the neonatal septicemia group. Notably, Gardnerella vaginalis and Streptococcus gallolyticus were distinctly identified from the vaginal secretions and placenta tissues of neonatal septicemia. Moreover, nucleic acid synthesis and carbohydrate metabolism-related pathways were enriched in the neonatal septicemia group. This has contributed to enhancing our current understanding of the underlying mechanisms involving pathogenic microbiomes in PPROM-induced neonatal septicemia.

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Disclosure of conflict of interest

None.

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Table S1. The clinical information of samples

Cases	Weight (after pregnancy)	Weight (before pregnancy)	Ethnicity	Comorbid	Number of pregnancies
Sample 1	67	50.8	Han	Gestational hypertension	5
Sample 2	55	48	Han	/	3
Sample 3	70	55	Han	/	1
Sample 4	65	58	Han	/	0
Sample 5	58.5	42.5	Han	/	0
Sample 6	75	54.5	Han	/	0
Sample 7	75	50	Han	/	1
Control 1	53.5	41	Han	/	1
Control 2	52	39	Han	Gestational diabetes	1
Control 3	63.5	54	Han	/	2

Table S2. Sequencing data of vaginal microbiome of PPROM at admission

CAses	Weight (after pregnancy)	Weight (before pregnancy)	Ethnicity	Comorbid	Number of pregnancies
Sample 1	67	50.8	Han	Gestational hypertension	5
Sample 2	55	48	Han	/	3
Sample 3	70	55	Han	/	1
Sample 4	65	58	Han	/	0
Sample 5	58.5	42.5	Han	/	0
Sample 6	75	54.5	Han	/	0
Sample 7	75	50	Han	/	1
Control 1	53.5	41	Han	/	1
Control 2	52	39	Han	Gestational diabetes	1
Control 3	63.5	54	Han	/	2

Table S3. Sequencing data of vaginal microbiome of PPROM before birthing

Sample	Raw data	Reads	Q20 (%)	Q30 (%)	N (%)	GC (%)
GR2_2	10210096200	68067308	97.79	94.00	0.00018	41.65
GR5_2	10386697200	69244648	97.62	93.60	0.00018	41.65
GR7_2	10024085100	66827234	97.77	93.94	0.00018	41.54
GR8_2	11775515400	78503436	97.70	93.78	0.00018	42.83
GR9_2	10952760900	73018406	97.58	93.48	0.00018	41.35
CK10_2	10706371500	71375810	96.16	90.56	0.00134	40.85
CK11_2	10322516700	68816778	96.53	91.42	0.00044	41.43

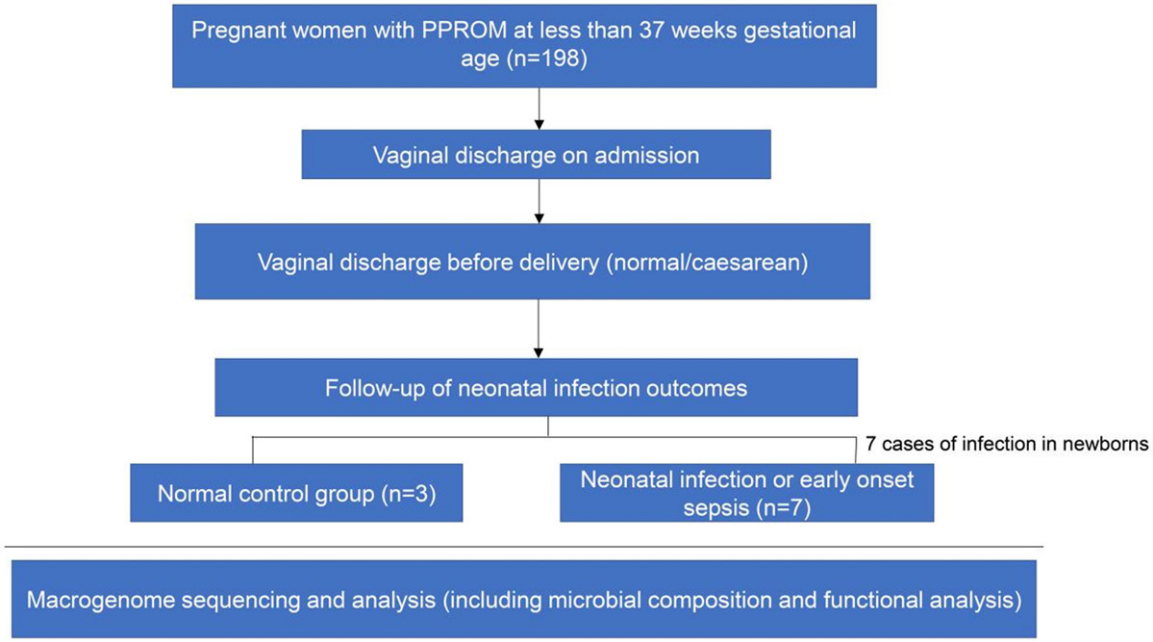

Figure S1. Workflow chart.