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Original Article

Correlation analysis between changes in oral and vaginal/intestinal microbiota during pregnancy and threatened preterm birth in pregnant women

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Abstract: Objectives: To explore the correlation between changes in oral and vaginal/intestinal microbiota during pregnancy and threatened preterm birth, providing a theoretical basis for further mechanism research. Methods: The study retrospectively analyzed pregnant women who gave birth in the gynecological outpatient department of a medical university affiliated hospital in East China from December 2021 to September 2022. The case group included pregnant women who experienced regular or irregular uterine contractions (with the cervical canal gradually shortening but the cervical opening not exceeding 1 cm) within 24 to 28 weeks of pregnancy and were at risk of preterm birth; The control group included individuals without such symptoms. Two sets of deoxyribonucleic acids (DNA) were extracted from oral and vaginal/intestinal microbiota, and bioinformatics (BI) methods were used to sequence and analyze these three sets. Results: Oral microbiota analysis showed significant differentiation between two groups, with significant clustering within each group. Vaginal microbiota analysis revealed enrichment of specific bacteria in Group D compared to Group C. Gut microbiota analysis showed varying proportions of different genera in Group E and F. Significant differences were observed in species composition between the groups. Conclusions: (1) Compared to normal and healthy pregnant women, there was a significant imbalance in the diversity of oral microbiota in patients with threatened premature delivery. (2) The overall composition and structure of vaginal microbiota in patients with threatened premature delivery have changed. The relative abundance of probiotic lactobacilli in the vaginal microbiota decreased in the case group. (3) There was no significant difference in the overall community structure of gut microbiota in patients with threatened premature delivery compared to normal pregnant women.

Keywords: Pregnant women, oral microbiota, vaginal/intestinal microbiota, threatened preterm birth, relativity

Introduction

Preterm birth refers to the occurrence of irregular or regular uterine contractions, accompanied by symptoms such as vaginal bleeding or bloody secretions, before 37 weeks of pregnancy. Afterwards, there will be regular uterine contractions, progressive shortening of the cervical canal, and enlargement of the uterine opening, ultimately leading to the entire process of delivering the fetus [1-3]. If preterm birth occurs, due to the young age of the fetus and the incomplete development of various organs and systems, it is likely to cause a series of near and far complications [4, 5]. The implementaion of China's two-child policy has led to an expansion of the overall fertility population, leading to an increasing trend in the incidence of preterm birth year by year. Numerous results have shown that an increase in gestational age leads to a decrease in the mortality rate of preterm birth, but the incidence of serious complications gradually increases [6, 7]. At present, the principle of managing preterm birth is still to extend the gestational week as much as possible while the fetal membrane is intact to reduce the prognosis of preterm birth. At the same time, it is also necessary to rest appropriately, promote fetal lung maturation, suppress uterine contractions, and, if necessary, undergo anti-infection treatment [8, 9]. However, a considerable number of preterm birth patients exhibit atypical prodromal symptoms and poor disease control, which accelerates the progression of the disease and ultimately results in preterm birth. Due to the unclear pathogenesis,

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there is currently no effective prevention and treatment method. It is worth noting that many scholars currently believe that infection and inflammatory reactions are important factors leading to preterm birth [10, 11].

Microbiomics is a new research field that has emerged in recent years and is closely related to various chronic diseases affecting the intestine, metabolism, reproduction, and nerves. It conducts sequence analysis, classification, and statistical analysis of the microbiome through methods such as tag gene analysis, metagenomic analysis, and metatranscriptome sequencing, revealing a high degree of diversity in microbial community structure and function [12, 13]. Multiple studies have shown that the abundance and diversity of vaginal microbiota in preterm and full-term pregnant women are higher than those in full-term pregnant women during the 11-16 weeks of pregnancy. The bacterial population of the Prevotella genus also showed obvious discrepancies. The abundance of vaginal soft membrane fungi in preterm pregnant women increases and is closely related to the gestational age of preterm birth [14]. However, there is currently limited research on the correlation between extremely early preterm birth in the Chinese population and the oral, vaginal, and intestinal microbiota of pregnant women. Based on this, this study investigates the correlation between changes in oral and vaginal/intestinal microbiota during pregnancy and threatened preterm birth in pregnant women. The purpose is to explore the etiology of threatened preterm birth from a microbial perspective, providing a theoretical basis for further mechanism research. Additionally, there are currently few studies analyzing the correlation between changes in oral and vaginal/intestinal microbiota during pregnancy and threatened preterm birth, and the study on the causes of threatened preterm birth from a microbial perspective has not been refined. Therefore, this study is innovative.

Material and method

Research object

According to the guidelines issued by the American College of Obstetricians and Gynecologists in 2016, preterm birth is defined as an actual pregnancy of 20 weeks but not more than 37 weeks. The study explores the extremely early preterm delivery of pregnant women who have reached 24 weeks but not exceeded 28 weeks.

(1) Oral analysis grouping criteria: This study retrospectively analyzed pregnant women who gave birth in the gynecological outpatient department of a medical university affiliated hospital in East China from December 2021 to September 2022. Pregnant women who were 24 weeks pregnant but less than 28 weeks old and had symptoms of threatened preterm birth (manifested as regular or irregular uterine contractions, while the cervical canal gradually became shorter, but the opening of the cervix did not exceed 1 cm) were used as the basis for grouping. Among these patients, 71 patients with symptoms were taken in the case group (CG). 83 asymptomatic patients were assigned to the health group (HG). According to the inclusion and exclusion criteria, a total of 109 oral samples were collected according to strict operating standards, including 57 samples in Group A (19 cases of premature delivery until full term delivery; 38 cases of premature delivery after fetal protection were considered as failed) and 48 samples in Group B.

(2) Vaginal analysis grouping criteria: Based on inclusion and exclusion criteria, a total of 93 vaginal samples were collected after strict operating procedures. They were also segmented into case group (Group C) and health group (Group D) according to the actual criteria for oral grouping. Among them, a total of 46 cases were collected for the former (15 cases were saved until full term delivery; 31 cases were still premature after the protection, indicating failure to protect the fetus), while a total of 47 cases were collected for the latter.

(3) Intestinal analysis grouping criteria: Based on inclusion and exclusion criteria, a total of 37 fecal samples were collected after strict operating procedures. They were case group (Group E) and health group (Group F) according to the actual criteria for oral grouping. Among them, there were a total of 15 cases of the former (6 cases of premature delivery until full term delivery; 9 cases of premature delivery after fetal protection), and a total of 21 cases of the latter.

Inclusion and exclusion criteria, physical examination and laboratory indicators

Inclusion criteria: (1) Pregnancy reached 24 weeks but did not exceed 28 weeks. (2) There was no history of acute or chronic gastrointestinal inflammation or surgical procedures in the

past. (3) No antibiotics (oral, intravenous or vaginal) were used within one month prior to actual sampling, and no probiotic preparations were used within two weeks. (4) There was no sexual activity and no use of vaginal suppositories within the three days prior to sampling. (5) Pregnant women gave informed consent and were willing to join this study.

Exclusion criteria: (1) Pregnant women with iatrogenic preterm birth who were forced to terminate their pregnancy due to the mother or fetus. (2) Cervical insufficiency, premature rupture of membranes, enlarged uterine opening > Length of Cervical Measurement (LCM), suspected chorioamnionitis, and physiological uterine contractions. (3) Patients with gestational diabetes, gestational hypertension, and other pregnancy complications. (4) Patients with complete placenta previa, partial placenta previa, blood vessel previa, and placental abruption.

Physical examination indicators: It was measured by professionally trained outpatient or ward nursing staff according to unified standards. Height and weight were measured using electronic measuring instruments, and the Body Mass Index (BMI) was calculated using relevant formulas.

Laboratory indicators: Clinical nursing staff and ward nursing staff who have received professional training were responsible for collecting blood samples and conducting blood routine tests.

Instruments and reagents

(1) Reagent: Contains sterile cotton swabs, physiological saline, sterile curved discs, and fecal sample bottles prepared by Guangzhou Peiyu Biological Products Co., Ltd. Disposable sterile speculum produced by Nanchang Aibo Medical Equipment Co., Ltd. 1.5 ml Eppendorf (EP) micro centrifuge tube, 96 well plate, and heat sealing film produced by American Aisijin Science Co., Ltd. 50 ml centrifuge tube produced by Corning Corporation in the United States. Phosphate buffer produced by Thermo Fisher Scientific (China) Co., Ltd. MinkaGene Bacterial Deoxyribonucleic Acid (DNA) test kit produced by Guangzhou Microchip Biotechnology Co., Ltd. Anhydrous ethanol, sodium dodecyl sulfate, Tween 20, chloroform, and sodium acetate produced by Guangdong Guanghua Technology Co., Ltd. Phenol produced by Shanghai McLean Biochemical Technology Co., Ltd. DNA extraction solution produced by Solebao Technology Co., Ltd. Primers produced by Shanghai Shenggong Biotechnology Service Co., Ltd. Real-time quantitative Polymerase Chain Reaction Detection System (qPCR) reagent kit produced by Dongyang Textile (Shanghai) Biotechnology Co., Ltd. These preparations were expressed as 1-20.

(2) Instruments: Micro pipettes and high-speed centrifuges (produced by Ebund AG in Germany). Mini centrifuge and vortex shaker (produced by Qilin Bell Instrument Manufacturing Co., Ltd. in Haimen City). Ultra pure water preparation system (produced by Sedoris Company). High-pressure sterilization pot [produced by Zhiwei (Xiamen) Instrument Co., Ltd.]. A commercial ice maker [produced by Scotsman Ice System (Shanghai) Co., Ltd.]. Electronic analytical balance (produced by Changshu Dual State Testing Instrument Factory). 4°C/-20°C ultra-low temperature refrigerator [produced by Hisense Rongsheng (Guangdong) Refrigerator Co., Ltd.]. -80°C ultra-low temperature refrigerator, trace nucleic acid protein concentration analyzer, and fluorescence quantitative PCR instrument [produced by Thermo Fisher Scientific (China) Co., Ltd.]. Electronic constant temperature stainless steel water bath pot (produced by Shanghai Yulong Instrument Equipment Co., Ltd.). These instruments were represented by 21-33. **Table 1** shows the specific content.

Experimental methods

Experimental method for changes in oral microbiota of pregnant women

(1) Oral swab collection standard: Pregnant women should not eat, smoke, or drink within 1 hour before sampling. After rinsing with 15 milliliters of sterile physiological saline, a trained doctor immediately scrapes and rotates 8-10 times on the tonsils of the pregnant woman's jaw and the lateral pharyngeal walls on both sides using a sterile cotton swab. The collected samples need to be immediately placed in the collection tube, sealed, labeled with the sample number and collection date, and temporarily stored in a -20°C refrigerator. They should be sent to a -80°C refrigerator as soon as possible for refrigeration.

Table 1. Instruments and reagents used in this research

Reagent				
1	2	3	4	5
Sterile cotton swab	Normal saline	Sterile curved disc	Fecal sample bottle	Disposable sterile speculum
6	7	8	9	10
1.5 ml EP	96-well plates	Heat Sealable Film	50 ml centrifuge tube	Phosphate buffer
11	12	13	14	15
MinkaGene Bacterial DNA Kit	Anhydrous ethanol	SDS	Tween 20	Chloroform
16	17	18	19	20
Sodium acetate	Phenol	DNA extraction solution	Primer	qPCR
Instrument				
21	22	23	24	25
Micropipette	High speed centrifuge	Mini Centrifuge	Vortex oscillator	Milli-Q Advantage
26	27	28	29	30
Autoclave	Commercial ice maker	Analytical balance	4°C/-20°C ultra-low temperature refrigerator	-80°C ultra-low temperature refrigerator
31		32	33	
Micro nucleic acid protein concentration analyzer		Real time PCR	Electronic constant temperature stainless steel water bath pot	

(2) Oral Deoxyribonucleic Acid (DNA) extraction: The sample is taken out of the -80°C refrigerator and placed on ice cubes to wait for melting. According to the instructions on the bacterial DNA extraction kit, the DNA of oral microorganisms is extracted. All assignments are completed on a super clean workbench and are carried out on ice.

(3) To achieve better amplification of the V4 region of the 16-Ribosomal Ribonucleic Acid (16RsRNA) gene, this study does not dilute the obtained DNA samples, but instead uses quantitative Polymerase Chain Reaction (qPCR) templates. Among them, the upstream primer of V4 is 514F-5 'GTGCCAGCMGCGGTAA3', and the downstream primer of V6 is 1063R-5 'ACAGCCATGCANCACCT3', amplifying the conserved region of the V4 region of the 16RsRNA gene in the sample. Add a 12 μL amplification reaction system to a 96 well plate, then cover it with a heat sealed film and put it on the machine. In the PCR reaction system, 5.0 μL of DNA template, 0.5 μL of upstream and downstream primers with a concentration of 10 μM, and 0.6 μL of fluorescent dye are selected. In the qPCR parameters, the pre-deformation temperature and time are set at 94°C and 2 min, the denaturation is set at 94°C and 30 s, the annealing is set at 59°C and 30 s, the elongation is set at 72°C and 30 s, and the final elongation is set at 72°C and 5 min, all with 28 cycles.

(4) Illumina Hiseq: Mix the PCR products from each well before sequencing the sample. The mixed samples are frozen with dry ice and sent to the Illumina Hiseq 2500 platform (Guangzhou Meige Gene) for 250-bp double ended sequencing. To reduce the error in sequencing depth, PCR amplification is performed on different samples. Then, a band scan and an Invitrogen fluorescence quantitative analyzer are used to perform grayscale scanning on each PCR product, and relative quantitative analysis is performed on the addition amount of each sample using the same 500 bp MarkerDL2000 band as a reference.

(5) Bioinformatics analysis: The 16RsRNAV4 region of bacteria belongs to a variable region, which is relatively conserved within species and exhibits inter-specific differentiation. It can also be used to indicate phylogenetic relationships between species. On this basis, this study aims to obtain the taxonomic characteristics of this bacterium by amplifying the 16RsRNAV4 region based on previous work. The first step is to obtain sample classification operation units and perform species dilution. The next is to perform Alpha (α) diversity analysis and continue with Beta (β) diversity analysis. Finally, the significance of inter-group differences is analyzed. In α diversity analysis, Shannon index, Chao 1 index, Observed Operational Taxonomic Units (Observed_OTUs) and PD_whole_tree are selected. In β diversity analysis, sample similarity distance (Bray Curtis), Binary_jaccard distance, Weighted, and Unweighted Unifrac distance are selected.

In the significance analysis of inter-group differences, molecular markers are used to identify

the characteristic species between each group. After a series of statistical calculations, significant inter-species differences are obtained, and then significant inter-group differences are analyzed to obtain species with significant differences at each level. Various methods are used to display Leukocyte Differentiation Antigen (LDA) numerical distribution histograms, species evolution branching maps, and feature tables. In addition, Linear Discriminant Analysis Effect Size (LEfSe) is a method that combines non-parametric testing with linear discriminant analysis. It can detect the most statistically significant biological characteristics between groups. Therefore, this study uses LDA values greater than 2 under oral microbiota LEfSe as cutoff values, only showing significant differences in species where the absolute value of LDA is greater than the actual preset value.

(6) Statistical analysis: The SPSS 20.0 statistical software is used to analyze and process all data. The number of use cases and the percentage of count data are represented as "(n)%". Chi square test or Fisher's precision test is used to compare the differences in data. The econometric data are first tested for normality using the Kolmogorovsimonov test. When the data are distributed normally, the mean ± standard deviation (x±sd) is used to express it and compared using the student *t*-test. If the data are not normally distributed, then they are represented by the median and compared using the Mann Whitney test. $P<0.05$ indicates a significant difference.

Experimental method for changes in vaginal microbiota in pregnant women

(1) Vaginal swab collection standard: No sexual history or vaginal medication history within 3 days before sampling. Pregnant women take bladder incisions and are sampled by professionally trained doctors. Under sterile endoscopy, vaginal secretions are extracted from both sides of the vagina and the posterior fornix area, and rotated 5-6 times without trying to touch the vulva and vaginal opening. The collected samples are immediately placed in the collection tube and then sealed. After pasting the sample number and collection date on it, it is temporarily stored in a refrigerator at -20°C and then moved to a refrigerator at -80°C as soon as possible.

(2) Vaginal DNA extraction: The sample is removed from the -80°C refrigerator, placed on ice, and waited for melting. Vaginal microbiota DNA is extracted in accordance with the bacterial DNA extraction kit instructions. The entire process is completed on an ultra clean workbench and is carried out at extremely low temperatures.

(3) Due to the high DNA content of actual vaginal specimens, it is necessary to uniformly dilute the concentration of vaginal DNA extraction products to 10 μL before amplification and use it as the template for the next PCR amplification step. Among them, the upstream and downstream primers are the same as the experimental method for changes in oral microbiota in pregnant women. The amplification reaction conditions are only set at 72°C and 6 minutes for final extension. The rest are the same as the experimental method for changes in oral microbiota. The actual PCR products are stored in a -20°C refrigerator.

(4) Illumina Hiseq sequencing, bioinformatics, and statistical analysis are the same as the experimental methods for changes in oral microbiota in pregnant women.

Experimental method for changes in gut microbiota of pregnant women

(1) Standard for collecting fecal samples: Trained doctors distribute sterile sample bottles to pregnant women and inform them how to collect fecal samples. After all urine is discharged, the feces are discharged into the disinfection tray provided to prevent contact with pollutants such as urine, blood, and vaginal discharge. Using a sterile sampler in a sample bottle, 5-6 g of samples are taken from the inner side of the middle of the feces. After the collection is completed, it is immediately covered, sealed and placed in a refrigerator at -20°C. It is transferred to a refrigerator at -80°C as soon as possible for frozen storage.

(2) DNA extraction from fecal samples: Samples are taken out of a refrigerator at -80°C and placed on ice. Use a sterile spoon to remove 2-3 grams of personal feces and place them in a 1.5 ml EP test tube. The rest of samples are stored at -80°C for freezing. All assignments are completed on a super clean workbench.

Table 2. Comparative results of clinical data of research subjects

Grouping and inspection	Age	Parity		Sampling period	White blood cell count	Hemoglobin
-		Primiparous woman	Menopausal women	-		
CG	30.00 years (20.00-43.00)	37.00 (52.10)	34.00 (47.88)	180.5±8.89 days	11.07±3.81 ×10⁹/L	111.49±11.48 g/L
HG	28.00 years (21.00-41.00)	50.00 (60.25)	33.00 (39.75)	182.29±8.93 days	9.24±1.97 ×10⁹/L	118.19±11.62 g/L
X^2/t	1.03			1.25	3.61	3.43
P	0.11	0.30		0.21	0.00	0.00

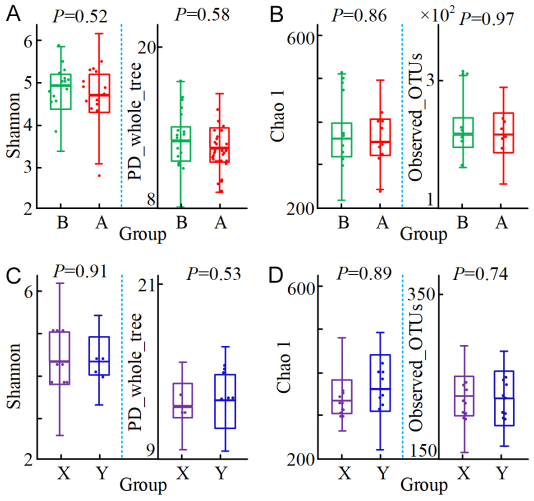

Figure 1. Oral microbiota of pregnant women α diversity analysis results. A. The results of Shannon index and PD_whole_tree index; B. The results of Chao 1 index and Observed_OTUs index; C. Comparison results of the first two indices between the two subgroups in Group A; D. Comparison results of two indices after two subgroups in Group A. Note: X and Y represent the subgroups of failed and successful abortion in Group A, respectively.

(3) Due to the high DNA content in gut microbiota samples, it is necessary to uniformly dilute the samples before amplification to 10 μL. The other operations are the same as the experimental method for vaginal microbiota changes.

(4) Illumina Hiseq sequencing, bioinformatics, and statistical analysis are the same as the experimental methods for changes in oral microbiota in pregnant women.

Results

Comparison of clinical data of research subjects

A total of 154 cases were collected, including 71 cases in the CG and 83 cases in the HG. According to relevant inclusion, exclusion criteria, and total medical history, 57 patients in Group A and 48 in Group B were selected for oral analysis. In vaginal analysis, there were 46 cases in Group C and 47 cases in Group D. In the intestinal analysis, there were 15 in Group E and 21 in Group F. As shown in **Table 2**, overall, in the comparison of general data between the CG and the HG, the differences in age, parity, and sampling period between the two groups were not statistically significant (*P*>0.05).

The α diversity analysis of oral and vaginal/intestinal microbiota in pregnant women

In the α diversity analysis of oral microbiota in pregnant women, the Shannon index, Chao 1 index, PD_whole_tree index, and Observed_OTUs index values of oral microbiota in Group B were slightly higher than those in Group A. The α diversity of oral microbiota in Group B was greater than that in Group A. Therefore, the richness and diversity of oral microbiota in Group A decreased, but the difference between the two groups was not statistically significant (*P*>0.05). The *P* values of the four indicators were 0.52, 0.58, 0.86, and 0.97, respectively. There was statistically no discrepancy in the α diversity of oral microbiota between pregnant women in Group A who has successfully and failed to conceive (*P*>0.05). The *P* values of the four indicators were 0.91, 0.53, 0.89, and 0.74, respectively. The specific content is shown in **Figure 1**.

In the analysis of vaginal microbiota diversity α, the values of Shannon index, Chao 1 index, PD_whole_tree index, and Observed_OTUs index in Group C were higher than those in Group D. The richness and diversity of its vaginal microbiota have increased compared to Group D. However, the difference did not have statistical significance (*P*>0.05). The *P* values of the

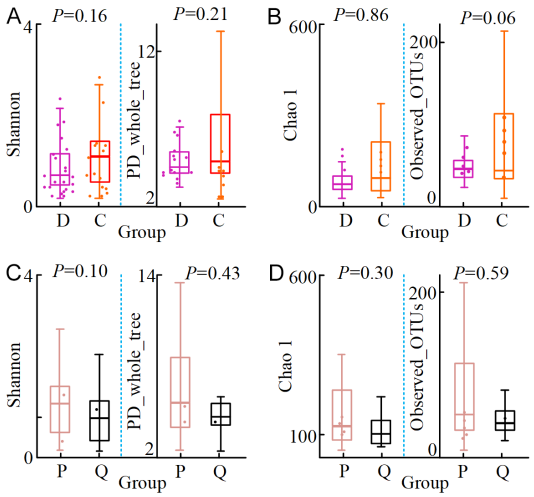

Figure 2. Two groups of vaginal microbiota α diversity analysis results. A. The results of Shannon index and PD_whole_tree index; B. The results of Chao 1 index and Observed_OTUs index; C. Comparison results of the first two indices between the two subgroups in Group C; D. Comparison results of two indices after two subgroups in Group C. Note: P and Q represent the subgroups of failed and successful abortion in Group C.

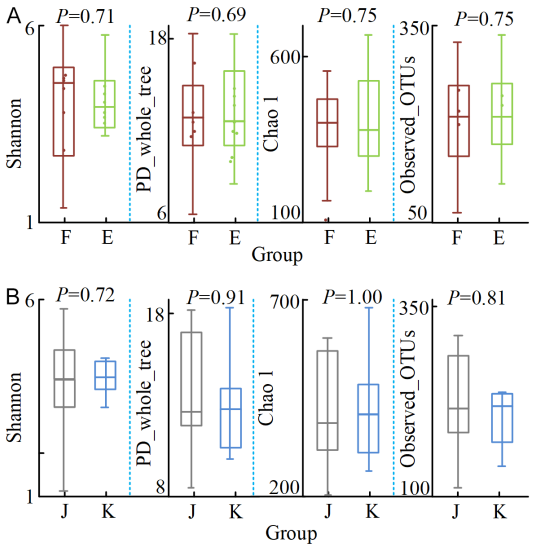

Figure 3. Two groups of gut microbiota α diversity analysis results. A. Analysis results of four indices; B. Analysis results of two subgroups and four indices within Group E. Note: K and J represent the subgroups of successful and failed abortion in Group E.

four indicators were 0.16, 0.06, 0.68, and 0.21, respectively. There was no significant difference in vaginal microbiota diversity α between the two subgroups in Group C ($P>0.05$), with P values of 0.10, 0.30, 0.43, and 0.59, respectively, as shown in **Figure 2**.

The four indicators of actual gut microbiota α diversity in Group F were higher than those in Group E, indicating a decrease in the richness and diversity of gut microbiota in Group E compared to Group F. The distinction was not significant ($P>0.05$). At this point, the P-values of the Shannon index, Chao 1 index, PD_whole_tree index, and Observed_OTUs index were 0.71, 0.75, 0.69, and 0.75, respectively. Additionally, there was no significant difference ($P>0.05$) in gut microbiota α diversity between the two subgroups of successful and failed abortion in Group E. At this point, the four P-values were 0.72, 1.00, 0.91, and 0.81, respectively, as shown in **Figure 3**.

Oral and vaginal/intestinal microbiota of pregnant women β diversity analysis

In the analysis of oral microbiota β diversity in pregnant women, the corresponding Principal Coordinate Analysis (PCoA) was performed using unweighted Unifrac distance, and the contribution rates of Principal Coordinate 1 (PC1) axis and PC2 axis were 8.77% and 5.73%, respectively. There was a significant distinction between the two groups of samples, and significant clustering occurred within the group. The difference in the species composition structure of the oral microbiota between the two groups in the analysis of inter-group differences had statistical significance ($P=0.002<0.05$). The contribution of PCoA using Binary_jaccard distance to the PC1 axis was 5.14%, and to the PC2 axis was 4.19%. The samples of Group B and Group A could be clearly distinguished, but there were independent clusters between each group, and the differences between the groups had statistical significance ($P=0.001<0.05$). In addition, in the PCoA of the oral microbiota for successful and failed abortion based on Unweighted Unifac and Binary_jaccard in Group A, there was no significant difference ($P>0.05$) in the composition of the microbiota between the two subgroups, as displayed in **Figure 4**.

In the analysis of vaginal microbiota β diversity, when using unweighted Unifrac distance for corresponding PCoA, the contribution rate of the PC1 axis was 11.47%, and the contribution rate of the PC2 axis was 9.03%. There was a significant clustering within the two groups,

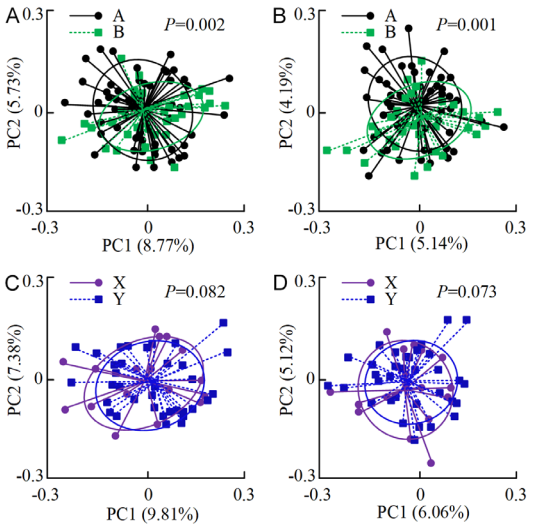

Figure 4. Oral microbiota of pregnant women β diversity analysis results. A. Results of using Unweighted Unifac distance between groups; B. Results of using Binary_jaccard distance between groups; C. Results of using Unweighted Unifac distance within Group A; D. Results of using Binary_jaccard distance within Group A. Note: * indicates $P<0.05$. ** indicates $P<0.01$.

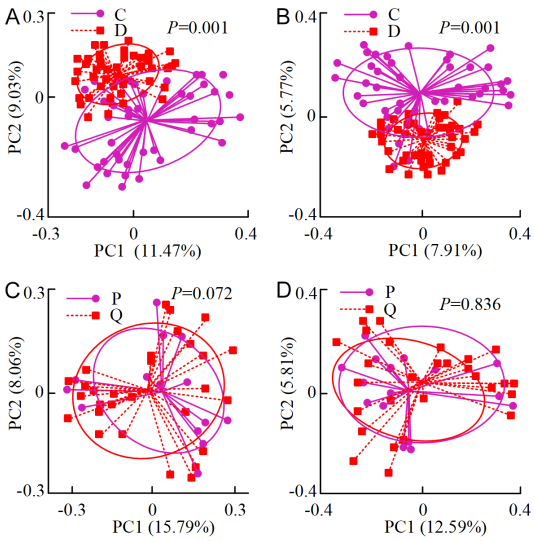

Figure 5. Two groups of vaginal microbiota β diversity analysis results. A. Results of using Unweighted Unifac distance between groups; B. Results of using Binary_jaccard distance between groups; C. Results of using Unweighted Unifac distance within Group C; D. Results of using Binary_jaccard distance within Group C. Note: * means $P<0.05$; ** means $P<0.01$.

and the samples between the groups were separated from each other. There was a significant difference in the composition of vaginal microbiota between Group C and Group D ($P=0.001<0.05$). In the PCoA using Binary_jaccard distance, the contribution of the PC1 axis was 7.91%, and the contribution of the PC2 axis was 5.77%. The samples of Group C and Group D were clearly separated, and the samples of the same group showed good clustering ($P=0.001<0.05$). In addition, there was no statistically significant difference in the β diversity of related vaginal microbiota between the two subgroups in Group C using unweighted Unifac distance and Binary_jaccard distance for PCoA ($P>0.05$). The specific content is shown in **Figure 5**.

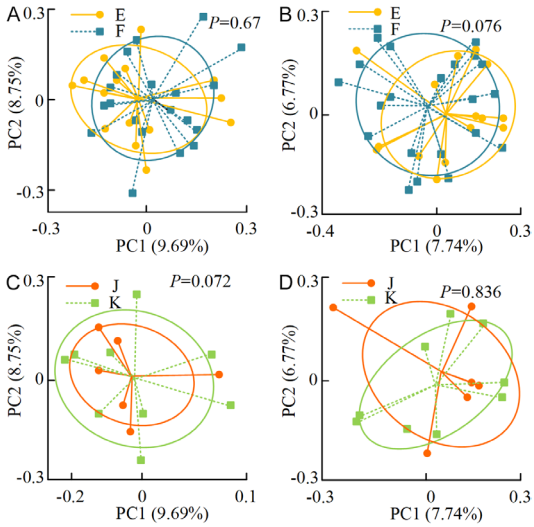

Figure 6. Two groups of gut microbiota β diversity analysis results. A. Results of using Unweighted Unifac distance between groups; B. Results of using Binary_jaccard distance between groups; C. Results of using Unweighted Unifac distance within Group E; D. Results of using Binary_jaccard distance within Group E.

In the analysis of gut microbiota β diversity, when using in the overall composition structureunweighted Unifrac distance and Binary_jaccard distance for PCoA, it was not possible to distinguish between the Group E and Group F. There was no significant difference ($P>0.05$) in the overall composition structure between the two sample groups. Among them, there were $P=0.67$ for unweighted Unifrac distance and $P=0.76$ for Binary_jaccard distance. In addition, there was no significant difference ($P>0.05$) in gut microbiota β diversity between the two subgroups when using unweighted Unifrac distance and Binary_jaccard distance for PCoA between the successful and failed abortion groups in the Group E. The specific content is shown in **Figure 6**.

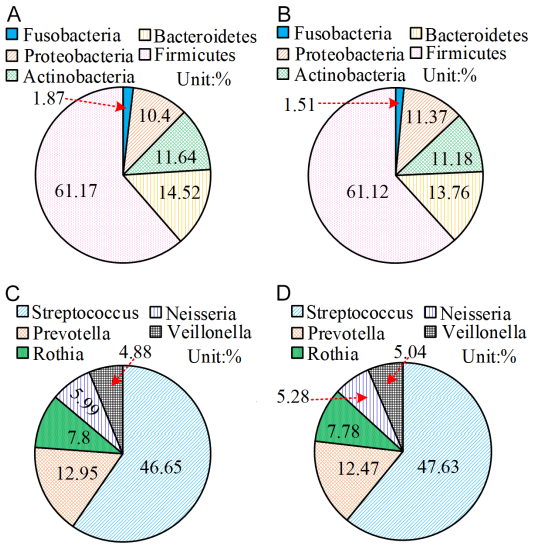

Figure 7. Analysis of the phylum level composition of the community structure of two groups of oral microbiota. A. Group A Gate Level Analysis Results; B. Group B Gate Level Analysis Results; C. Analysis results of genus level in Group A; D. Analysis results of genus level in Group B.

Phylum level analysis of the composition of oral and vaginal/intestinal microbiota community structure in pregnant women

In the horizontal analysis of the composition phyla of oral microbiota community structure in groups A and B, the oral microbiota genus of pregnant women was mainly composed of thick walled, rod-shaped, actinomycete, deformed, and shuttle rod-shaped bacteria, accounting for up to 99.4%. Other bacteria included cyanobacteria and spirochetes. The proportion of Firmicutes in Group A and Group B was 61.17% and 61.12%, respectively. Bacteroidetes accounted for 14.52% and 13.76%, Actinobacteria accounted for 11.64% and 11.18%, Proteobacteria accounted for 10.40% and 11.37%, and Fusobacteria accounted for 1.87% and 1.51%. In the genus level analysis, the two groups of oral microbiota were mainly composed of Streptococcus, accounting for 47.1%, followed by Prevotella, Rochella, Neisseria, and Vibrio. In addition, the proportion of Streptococcus in Group A and Group B was 46.65% and 47.63%, respectively. Prevotella accounted for 12.95% and 12.47%, Rochella accounted for 7.80% and 7.78%, Neisseria accounted for 5.99% and 5.28%, and Vibrio accounted for 4.88% and 5.04%. However, there was no significant difference in the actual composition and structure of the oral microbiota between the two groups at the phylum level ($P>0.05$), as shown in **Figure 7**.

In the phylum level analysis of the community structure of vaginal microbiota in groups C and D, the vaginal microbiota in groups C and D belonged to 6 phyla. Among them, Firmicutes accounted for 82.99% and 80.98%, Actinobacteria accounted for 12.18% and 18.27%, Bacteroidetes accounted for 2.27% and 0.48%, Firmicutes accounted for 1.65% and 0.01%, Proteobacteria accounted for 0.71% and 0.07%, and Fusobacteria accounted for 0.08% and 0.07%, respectively. In addition, the vaginal microbiota of Group C and Group D were mainly composed of Lactobacillus genus, followed by genus 3, genus 1, and genus 10 under the Streptococcidae family. The proportion of genus 13 in group C and group D was 43.53% and 46.00%, respectively. The proportion of genera (represented by v) under the Streptococcidae family was 33.29% and 33.31%, with genus 3 of 7.75% and 12.71%, genus 1 of 3.00% and 2.30%, and genus 10 of 1.03% and 3.04%. The specific results are shown in **Figure 8**.

In the phylum level analysis of the composition of gut microbiota community structure in groups E and F, the gut microbiota in groups E and F belonged to 5 phyla. The proportion of Firmicutes was 77.77% and 71.16%, Actinobacteria was 7.82% and 12.09%, Proteobacteria was 6.81% and 12.65%, Bacteroidetes was 2.02% and 2.88%, and Verrucomycetes was 4.80% and 0.37%, respectively. In addition, the gut microbiota of Group E and Group F were mainly composed of genera under the family G, genera under the family H, Bifidobacterium, Macromonas, and Streptococcus. The proportion of genera under the family G in Group E and Group F was 34.88% and 38.66%, under the family H was 4.32% and 11.05%, under the genus Bifidobacterium was 6.30% and 7.26%, under the genus Macromonas was 5.69% and 5.05%, and under the genus Streptococcus was 4.05% and 3.80%. **Table 3** shows the details.

Linear discrimination and cladistic analysis of species evolution of oral and vaginal/intestinal microbiota in pregnant women

In the linear discriminant analysis of oral microbiota in groups A and B, there were a total

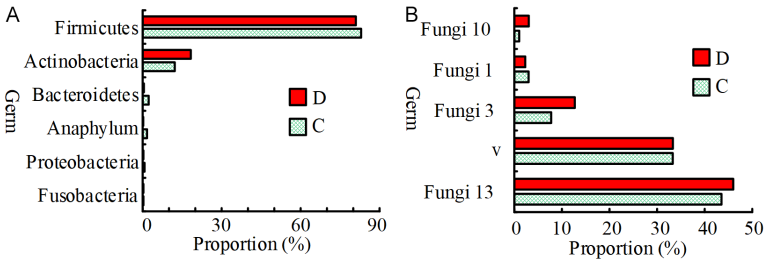

Figure 8. Analysis results of two groups of vaginal microbiota community structure composition at the phylum level. A. Gate level analysis results; B. Genus level analysis results.

of 32 common characteristic species between groups A and B in the oral microbiota, set at 1-32. 1-10: Bifidobacteria, Bifidobacteriaceae, Gardnerella, Burkholderia, Lauteropus, Coldbacteria, Gram vaginal bacteria, Actinobacteria, Red hoppers, and strange bacteria. 11-24: Red stink bug order, Lactobacillus family, Lactobacillus genus, Skadobia genus, Campylobacter genus, Campylobacter order ε-Proteobacteria, Campylobacter family, Vibrio order, Macroglobular genus, Vibrio family, Vibrio genus, red snail order, and acetobacter family. 25-32: Twins, Twins, Botanidae, Streptomyces, Clostridium, Bacteroidei, Bacteroideaceae, and δ-Proteobacteria. Among them, the first 24 species were enriched in Group B, and the last 8 species were enriched in Group A.

In addition, in the analysis of the cladistic diagram of species evolution in Group A and Group B, at the class level, Group B mainly included: Order Rhodobacter, ε-Proteobacteria, Related Bifidobacteria under Actinobacteria, Related Lactobacillus bacteria under the Bacillus class, α-Related red snail bacteria under the class Proteobacteria, and γ-Related Vibrio bacteria under the class Proteobacteria. At the level of group A, there were: δ-Proteobacteria, Related Bacillus bacteria under the Bacillus class, and Lactobacillus bacteria. The specific content is shown in **Figure 9**.

In the analysis of group A and group B at the genus level, the microorganisms in Group A's oral cavity were mainly enriched in the genera Clostridium, Bacteroides, and Streptococcus. The microorganisms in Group B's oral cavity were mainly enriched in the genera Gardnerella, Lactobacillus, Bifidobacteria, and Gram negative bacteria. **Figure 10** shows the specific results.

LDA values greater than 2.5 under the vaginal microbiota LEfSe were considered cutoff values. In the linear discriminant analysis and species evolution branch analysis of vaginal microbiota in groups C and D, there were a total of 15 species with significant differences between the actual vaginal microbiota in groups C and D, set at a~o. It included: genera Bifidobacteria, Aerococcus, Balloon Bacteriaceae, Clostridia, Clostridia, Gammaproteobacteria, Rumen bacteria, Enterobacteriaceae family, Enterobacterales order, Trichospira, Salmonella, Pseudomonadaceae, Pseudomonas, Corynebacterium, and Xanthomonas. Among them, a~c was enriched in Group D, while the rest was enriched in Group C. The results showed a $P<0.05$ in the species diversity of vaginal microbiota between Group C and Group D, as shown in **Figure 11**.

LDA values greater than 2 under the intestinal microbiota LEfSe were used as cutoff values. In linear discriminant analysis and cladistic analysis of species evolution of gut microbiota in groups E and F, the gut microbiota in group E was enriched in the genus Trichospira, Erysipelotrichia, Erysipelotrichales, and Erysipelothrix, designated as r~u. However, there was no significant enrichment of characteristic bacterial genera in Group F. The specific content is shown in **Figure 12**.

Discussion

The oral cavity is an open and microcirculation system, which is an organ in contact with the outside world. There are over 1.9×10^4 types of microorganisms in the oral cavity of normal individuals, including bacteria, fungi, and viruses. The symbiotic bacteria within the biofilm can promote the development of the immune system, compete, or inhibit foreign pathogens, thereby maintaining the homeostasis of the oral microbiota. However, if the environment in the oral cavity changes, they will become a pathogen and participate in the development of the disease [15-17]. The disorder of microorganisms in the oral cavity can lead to the occurrence of oral and maxillofacial diseases, which can lead to adverse pregnancy results such as miscarriage and preterm birth [18, 19]. In addi-

Table 3. Results of phylum level analysis on the composition of two groups of gut microbiota community structure

Analysis results of intestinal hilum level					
-	Firmicutes	Actinobacteria	Proteobacteria	Bacteroidetes	Verrucomicrobia
E	7.77%	7.82%	6.81%	2.02%	4.80%
F	71.16%	12.09%	12.65%	2.88%	0.37%
Analysis results of gut genus level					
-	Genus under Fungaceae g	Genus under Mycobacteriaceae h	Bifidobacterium	Macromonas	Streptococcus
E	34.88%	4.32%	6.30%	5.69%	4.05%
F	38.66%	11.05%	7.26%	5.05%	3.80%

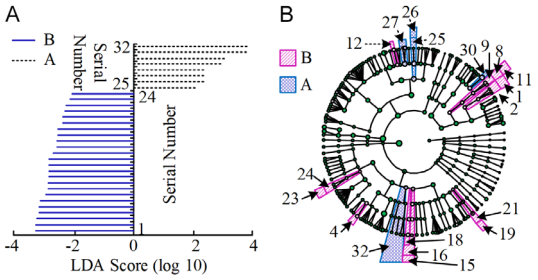

Figure 9. Linear discriminant analysis of two groups of oral microbiota and branch of species evolution. A. Linear discriminant analysis results of two groups of oral microbiota; B. Results of two groups of species evolutionary branches.

tion, the vaginal microbiota is the primary barrier to maintaining female reproductive health, and lactobacilli play an important role in it. When the number of lactobacilli decreases, it can cause a large number of pathogenic bacteria to colonize the vagina, leading to vaginal microbiota imbalance [20, 21]. Many potential pathogenic microorganisms can invade cells through adhesion, secrete toxic factors, obtain nutrients, evade host defense, and cause inflammation of the lower reproductive tract, such as bacterial vaginosis. Severe cases can lead to uterine infections, ultimately leading to diseases such as preterm birth, premature rupture of membranes, and pelvic inflammatory disease [22, 23].

At the same time, gut microbiota maintains the microecological balance of the body through a series of ways under long-term symbiotic conditions, thereby affecting a series of complex life processes such as nutritional metabolism, growth and development, and immune regulation of the body [24]. Among them, the intestinal mucosal barrier blocks symbiotic bacteria and intestinal epithelium. The symbiotic bacteria on one side of the lumen can strengthen the mucosal barrier. The immune cells on the mucosa can prevent the aggregation and translocation of symbiotic bacteria. The two interact and play an important role in maintaining the integrity of the body's immune barrier, preventing pathogen colonization, and regulating the body's immune balance. The imbalance of intestinal microecology during pregnancy is closely related to many pregnancy complications, such as pre-eclampsia, pregnancy diabetes, obesity, etc. Numerous studies have shown a correlation between changes in oral and vaginal/intestinal microbiota and threatened preterm birth. Therefore, this study conducts a detailed analysis of the three factors to determine their correlation with threatened preterm delivery during pregnancy in pregnant women and provides a theoretical basis for further mechanism research.

The experimental results showed that in the analysis of oral microbiota β diversity in pregnant women, the contribution rates of PC1 axis and PC2 axis were 8.77% and 5.73%, respectively, when using unweighted Unifrac distance for corresponding PCoA analysis. There was a significant distinction between the two groups of samples, and significant clustering occurred within the group. The difference in the species composition structure of the oral microbiota between the two groups in the analysis of intergroup differences had statistical significance (P=0.002<0.05). In the analysis of vaginal microbiota β diversity, when using unweighted Unifrac distance for corresponding PCoA, the contribution rate of the PC1 axis was 11.47%, and the contribution rate of the PC2 axis was 9.03%. There was a significant clustering within the two groups, and the samples between the groups were separated from each other. There was a significant difference in the composition of vaginal microbiota between Group C and

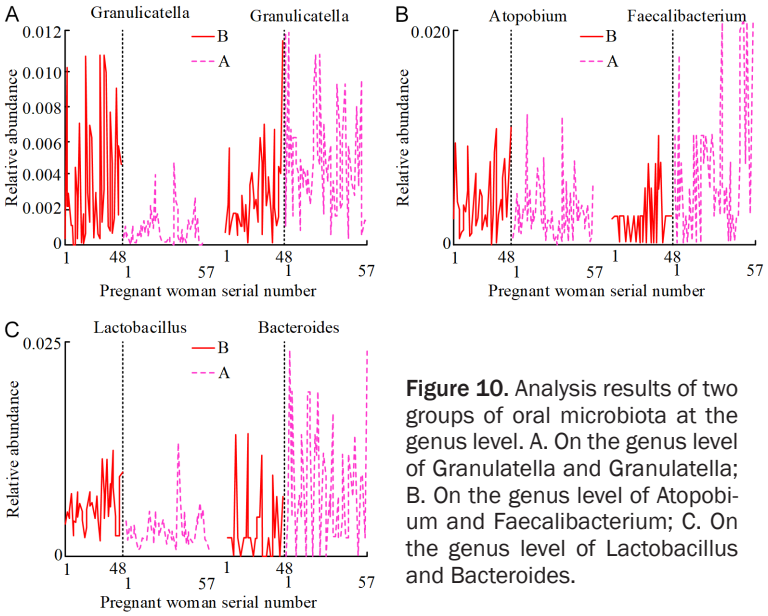

Figure 10. Analysis results of two groups of oral microbiota at the genus level. A. On the genus level of Granulatella and Granulatella; B. On the genus level of Atopobium and Faecalibacterium; C. On the genus level of Lactobacillus and Bacteroides.

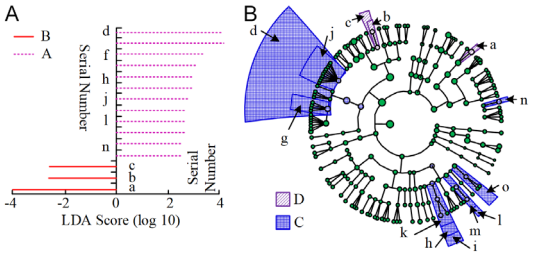

Figure 11. Results of linear discriminant analysis and branch analysis of species evolution for two groups of vaginal flora. A. Linear discriminant analysis results of two groups of vaginal microbiota; B. Species evolution analysis results of two groups of vaginal microbiota.

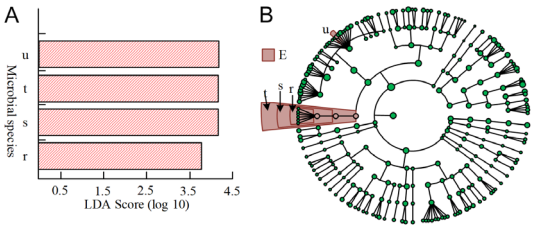

Figure 12. Results of linear discriminant analysis and branch analysis of species evolution for two groups of intestinal microflora. A. Linear discriminant analysis results of two groups of gut microbiota; B. Branch analysis results of species evolution of two groups of gut microbiota.

Group D (P=0.001<0.05). This result is basically correlated with the findings of Borkent et al. [25]. In the genus level analysis, the proportions of various bacterial groups in Group A and Group B were 46.65% and 47.63% for Streptococcus, 12.95% and 12.47% for Prevotella, 7.80% and 7.78% for Rochella, and 5.99% and 5.28% for Neisseria. The proportion of genus 13 in Group C and Group D was 43.53% and 46.00%, respectively, while the proportion of genus under the Streptococcidae was 33.29% and 33.31%, respectively. The proportion of genera under the bacteriaceae g in Group E and Group F was 34.88% and 38.66%, respectively, while under the bacteriaceae h it was 4.32% and 11.05%, with Bifidobacterium for 6.30% and 7.26%, and Macromonas for 5.69% and 5.05%. The richness of the analysis content is significantly better than the results of Fu et al. [26]. In the CG, the study found that the relative abundance of probiotic lactobacillus in Group E decreased. The reason may be the changes in hormone levels during pregnancy and the regulation of the immune system, which leads to changes in the ecological balance of the vaginal microbiota, thereby affecting the growth environment of lactic acid bacteria. The occurrence of preterm birth is usually associated with inflammation, infection, etc. These factors may inhibit the growth of lactobacillus, so that pathogenic bacteria or other harmful microorganisms can reproduce and further cause microbial imbalance. The findings show clear differences between the oral microbiota of patients with threatened preterm birth and that of healthy pregnant women, suggesting that characteristics of the oral microbiota may be associated with the risk of preterm birth. Consequently, further research and analysis, particularly longitudinal and large-scale clinical studies, are necessary to more accurately ascertain the validity and reliability of oral microbiota in predicting preterm birth. This will provide new directions for early intervention and clinical management.

The overall structure of the gut microbiota in patients with threatening preterm birth was not observed to be significantly different from that of healthy pregnant women in this study, which is different from the conclusions of some previ-

ous studies. Yin et al. reported that patients with preterm birth had significant changes in the gut microbiota and showed a higher proportion of common oral bacteria [27]. The differences in these results may result from a number of factors. First, geographical and ethnic differences in the study subjects may lead to changes in microbial composition, affecting the comparability of the results. Second, differences in study design and methods, such as sample collection time, sample size, and data analysis methods, may affect the detection results of microbiota. Finally, the dynamic properties of the microbiome and individual differences may lead to different microbial structures being observed in different studies at specific points in time. Therefore, future studies need larger samples and cross-regional comparisons to more fully reveal the association between gut microbiota and preterm birth.

Conclusion

Overall, the enrichment of Streptococcus granulosa in Group A led to a decrease in the immune system and mucosal immune function of pregnant women during pregnancy, leading to preterm birth. The enrichment of Lactobacillus and Bifidobacterium in Group B enhanced the stability of the cavity microbiota. In addition, when threatened with preterm birth, the overall composition of the vaginal microbiota might change. The number of beneficial bacteria and lactic acid bacteria in the vaginal microbiota of Group C in clinical samples significantly decreased, while pathogenic bacteria such as Enterobacterium and Gammaproteobacteria were abundant, leading to vaginal microbiota imbalance. Meanwhile, compared with healthy pregnant women, there was no significant change in the overall structure of gut microbiota in patients with threatened preterm delivery. However, in Group E, the abundance of rumen cocci decreased, the abundance of gut microbiota Firmicutes increased, while actinomycetes, Proteobacteria, and Bacteroides decreased. Overall, changes in oral and vaginal microbiota are strongly correlated with threatened preterm birth, while changes in intestinal microbiota are weakly correlated with threatened preterm birth. However, the actual sample size included in this study is still relatively limited, and it is necessary to increase the sample size to verify the current results.

Disclosure of conflict of interest

None.

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