The association between vaginal microbiota disorders and early missed abortion: A prospective study

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Abstract

Introduction: The objective of this study was to explore the association between disordered vaginal microbiota and missed abortion to ascertain potential causes of missed abortion related to vaginal microbiology.

Material and methods: This study was a prospective cohort study with a comparison group (reference group). Vaginal secretions from the posterior fornix of women in the early stages of pregnancy were collected in the Xiangya Third Hospital of Central South University in Changsha, Hunan, China, from November 2018 to November 2019. A total of 54 cases of missed abortion (case group) and 50 cases of normal pregnancy requiring induced abortion (reference group) were analyzed. Bacterial DNA was extracted, hybridized with gene-specific primers, and then detected using a bacterial chip. The composition and relative abundance of vaginal microbiota in the two groups were compared using α-diversity analysis, β-diversity analysis, and the linear discriminant analysis effect size method.

Results: The α-diversity analysis showed that the Simpson index of the case group was lower than that of the reference group, whereas the Shannon index in the case group was higher. The relative abundance of Firmicutes in the case group (42.52%) was lower than in the reference group (51.03%, p < 0.05), as was the relative abundance of Lactobacillus (case group 16.51%, reference group 23.00%; p < 0.05). Interestingly, levels of Mycoplasma genitalium and Ureaplasma were lower in the case group (p < 0.05). The relative abundance of Lactobacillus crispatus, Lactobacillus jensenii, and Lactobacillus gasseri was also significantly lower in the case group than in the reference group (p < 0.05). The pathways enriched in the case group were predominantly related to metabolism, whereas the genetics-related pathways were predominantly enriched in the reference group.

Abbreviations: ACE, abundance-based coverage estimator; ANOVA, nonparametric multivariate analysis of variance; CE, chronic endometritis; KEGG, Kyoto Encyclopedia of Genes and Genomes; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; NMDS, nonmetric multidimensional scaling; OTU, operational taxonomic unit; Sobs, species information index.

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1 | INTRODUCTION

Early missed abortion usually occurs before the 12th week of pregnancy, though it is sometimes defined as occurring before the 14th week. A missed abortion occurs when embryonic or fetal development stagnates and cardiac activity ceases, but there is no natural expulsion from the uterine cavity for several days or weeks. It is defined as a pregnancy that fails to develop yet the cervix remains closed. It can have serious effects on the reproductive health of women of childbearing age. The known causes include genetic factors, endocrine factors, anatomical abnormalities of the reproductive tract, infection, and environmental, mental, male, and drug-related factors. The limitations of clinical detection methods mean that the cause of approximately 15% of early missed abortions remains unclear.

The vaginal tract provides an important habitat for the development of microbial communities and is broadly colonized by microorganisms known collectively as the vaginal microbiota. Researchers are giving increasing attention to the relation between changes in vaginal microbiota and pregnancy outcomes and are performing in-depth studies of vaginal microbiology. Vaginal flora is the first line of defense against foreign pathogenic bacteria and helps the body maintain a dynamic balance of interdependence and mutual restriction. A woman’s vaginal microbiota changes throughout her life and may be affected by changes in physiology, lifestyle, and hormone levels. The microbiology of healthy vaginal flora is dominated by Lactobacillus such as Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus iners, and Lactobacillus jensenii.

Although these microbial communities are dominated by Lactobacillus and are relatively simple compared with the gut microbiome, recent studies have shown that vaginal dysbacteriosis might be associated with gynecological cancer, gestational diabetes, adverse pregnancy outcomes, and preterm delivery.

To date, studies have shown that the vaginal microbiota is closely related to preterm delivery, but few studies have investigated the association between vaginal microbiota and early missed abortion. This study hypothesized that changes in vaginal microbial communities might be associated with syndromes and microenvironments of the host. Patients with early missed abortion (case group) and normal pregnancy requiring induced abortion (reference group) were enrolled to compare the differences in vaginal microbiota between the two groups.

Key message
Disordered vaginal microbiota may increase the risk of reproductive tract infection in early pregnancy and impair the energy intake of embryos, resulting in missed abortion.

Conclusions: Bacteria are more diverse and unevenly distributed in patients with missed abortion. Decreases in the proportion of vaginal Lactobacillus and changes in Lactobacillus species in these patients may increase the chance of genital tract pathogenic bacterial infection. To our knowledge, our study was the first to observe that a decrease of Firmicutes levels in the vaginal microbiota might impair energy metabolism and have an association with missed abortion.

KEYWORDS
Firmicutes, Lactobacillus, missed abortion, Mycoplasma, vaginal microbiota

2 | MATERIAL AND METHODS

2.1 | Study participants

This study was a prospective cohort study with a comparison group (reference group). A total of 104 pregnant women were enrolled in the study from November 2018 to November 2019 in the Department of Gynecology of the Xiangya Third Hospital of Central South University in Changsha, Hunan, China. They were grouped into a case group comprising women with early missed abortions and a reference group of women with normally progressing pregnancies requiring induced abortion. All patients with missed abortion were diagnosed in the first trimester (pregnancy within 12 weeks); the gestational age of the reference group was similar to that of the case group. Participants in the two groups were aged between 21 and 40 years, and there was no significant difference in the mean ages between the case and reference groups (30.23±3.52 and 30.98±3.31, respectively; p = 0.91). All fetuses underwent diagnosis, and no chromosomal abnormalities were detected. Regular obstetric appointments, examinations, and treatments were undertaken for women in both groups according to the usual practices of the Third Xiangya Hospital.

The inclusion criteria for participants in this study were as follows: (1) women aged 21–40 years with a history of normal menstrual cycles; (2) no use of antibiotics and probiotics in the 3 months prior to enrolment; (3) no vaginal medication or lavage in the previous month; (4) no transvaginal operation in the previous 3 months; (5) no use of hormones during pregnancy; (6) no history of missed abortion, spontaneous abortion, recurrent abortion, or infertility; (7) no history of chronic disease such as diabetes, enteritis, hepatitis, or tuberculosis; (8) no history of smoking, drinking, or toxic exposure; and (9) able to understand and sign the informed consent. The exclusion criteria were as follows: (1)
known genetic or immunity-related spontaneous abortion, such as chromosomal abnormalities in either parent; (2) reproductive tract malformations or uterine space-occupying lesions, such as uterine leiomyoma, adenomyosis, endometrial polyps, uterine adhesions, or other diseases; (3) complications from severe medical diseases such as heart, liver, or kidney disease; (4) obvious abnormal endocrine functions, such as polycystic ovary syndrome, hyperprolactinemia, hypothyroidism, or hyperthyroidism; (5) abnormal blood coagulation with thrombosis; (6) fever or acute infection; (7) mental disorders; (8) unwillingness to cooperate with obstetric and gynecologic examinations and follow-up; (9) complications from cervical precancerous lesions or cervical cancer; or (10) absence of informed consent.

2.2 | Vaginal swab collection

The patients in the case group were sampled immediately after the diagnosis of missed abortion. The patients in the reference group completed the sampling when they decided to terminate the pregnancy. Vaginal discharge examination and gynecological examination were undertaken in preparation for subsequent termination of pregnancy. A gynecologist obtained samples from patients following basic medical practices. Each participant entered the gynecological examination room after urination and was placed in a lithotomy position. To obtain a sufficient quantity of vaginal secretion, two aseptic vaginal swabs were rotated three times in the same part of the posterior vaginal fornix. Immediately after collection, the specimens were placed in sterile test tubes filled with preservation solution (Sangon Biotech, Co. Ltd., Shanghai, China), vertically positioned for 10 minutes at room temperature, and then frozen at −80°C before testing.

2.3 | Bacterial DNA extraction and labeling

Bacterial DNA was extracted from the vaginal specimens using a microorganism DNA extraction kit (Halgen Ltd., Guangzhou, China) in accordance with the manufacturer’s instructions. The primers F44 (GGTTGATYMTGGCTCAG) and R1543 (GGNTACCTKTACAGCTT) were used to amplify the variable regions 1–9 (V1–9) of the 16S rRNA. Approximately 5 ng of the extracted DNA was used in a 50 μl polymerase chain reaction (PCR). An initial denaturing step at 94°C for 3 minutes was followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, and a final extension step at 72°C for 3 minutes. Agarose gel electrophoresis was performed to confirm the success of the PCR amplification. The DNA products amplified by PCR were directly labeled with a DNA labeling kit (Halgen Co. Ltd., Guangzhou, China) without purification. Then, 5 μl of PCR-qualified DNA was loaded onto the slide of the Halgen array and mixed with hybridization buffer for hybridization according to the manufacturer’s instructions.

2.4 | Sequence processing

Based on species-specific information sequences, the operational taxonomic unit (OTU) sequence and corresponding relative abundances were simulated according to the off-chip results. The simulated OTU information was then used for OTU clustering, and species classification was completed by annotating each OTU. OTUs that did not affect the results and non-bacterial species were removed. The representative sequence after clustering was annotated and clustered using the Ribosomal Database Project (RDP classifier; v2.2, http://rdp.cme.msu.edu/). It was then compared with the Silva database v132 (https://www.arb-silva.de/; SSU128/16’s bacteria) with an 80% comparison threshold. Quantitative Insights Into Microbial Ecology (QIIME) v1.9.1 software was then used to cluster the sequences with 97% similarity. OTU tables were generated describing the relative abundance of bacterial phylotypes within each sample.

2.5 | Bioinformatics analysis

In the gene expression data of DNA microarray, the ratio of green fluorescein (Cy3) to red fluorescein (Cy5) reflects the relative expression level of genes in the sample. The relative abundance of each intestinal microbiota was proportional to the average value of its specific Cy5/Cy3 ratio. Therefore, the type of intestinal microbiota was determined by the ratios of each Cy5/Cy3 probe. A sequencing program (Halgen Co. Ltd., Guangzhou, China) was used to detect the species and relative abundance of microorganisms in vaginal swab samples. Both α- and β-diversity calculations and the QIIME software default original parameters were used to analyze the general characteristics of the intestinal microbiota. A Wilcoxon rank-sum test was used to calculate the α-diversity within each group and the differences in β-diversity among groups. Principal coordinate analyses and nonmetric multidimensional scaling analyses were performed using the QIIME module and visualized in R (version 3.5.2: https://www.r-project.org/).

The α-diversity showed the diversity of the microbial communities in the vaginal swab samples of the case and reference groups. The species information index (Sobs), Chao, abundance-based coverage estimator (ACE), Shannon, and Simpson indices were used for analysis. The Chao and ACE indices showed the abundance of the communities in each sample, indicating the number of microbial communities in a single sample without noting the relative abundance of each bacterial community; Shannon and Simpson indices were co-affected by sample richness and uniformity; sample diversity was positively correlated with the Shannon index and negatively correlated with the Simpson index. We used β-diversity analysis to compare the differences in species diversity between pairs of samples.

Linear discriminant analysis and linear discriminant analysis effect size (LEfSe) software was used to screen the significant differential microorganisms between the case and reference groups. Enrichment analysis was then performed to determine the Kyoto Encyclopedia of Genes and Genomes (KEGG; version 37) metabolic pathways related to the differentially expressed genes (p<0.05),
together with the metabolic pathways that were significantly altered under experimental conditions, including biochemical pathways and signal transduction pathways.

### 2.6 Statistical analysis

Statistical analyses were performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). Nonparametric tests were performed using Kruskal-Wallis and Wilcoxon tests. Relative abundance was compared between groups, and differential species selection was performed with the LEfSe software using linear discriminant analysis. A corrected $p$-value < 0.05 was considered statistically significant. A leave-one-out test was used for cross-validation in the random clustering algorithm to reduce the influence of over-fitting. Non-normally distributed data were analyzed using the Mann-Whitney U test.

### 2.7 Ethics statement

This study was approved by the Ethics Committee of the Third Xiangya Hospital of Central South University, Hunan, China on November 24, 2021 (IRB no. I 21146). All participants provided written informed consent.

### TABLE 1 Characteristics of the case and reference groups

| General information          | Reference group | Case group | $p$-value |
|------------------------------|-----------------|------------|-----------|
| Age (years)                  | 30.98 ± 3.31    | 30.23 ± 3.52 | 0.910     |
| Gravidity (times)            |                 |            | 0.115     |
| 0                            | 15 (30.00)      | 12 (22.22)  |           |
| 1                            | 20 (40.00)      | 15 (27.78)  |           |
| ≥2                           | 15 (30.00)      | 27 (50.00)  |           |
| Parity (times)               |                 |            | 0.988     |
| 0                            | 24 (48.00)      | 26 (48.15)  |           |
| ≥1                           | 26 (42.00)      | 28 (51.85)  |           |
| Induced abortion (times)     |                 |            | 0.902     |
| 0                            | 20 (40.00)      | 20 (37.04)  |           |
| 1                            | 18 (36.00)      | 19 (35.19)  |           |
| ≥2                           | 12 (24.00)      | 15 (27.77)  |           |
| Gestational age (weeks)      | 9.67 ± 1.73     | 10.16 ± 1.78 | 0.159     |
| Educational level            |                 |            | 0.958     |
| Junior high school or lower  | 6 (12.00)       | 8 (14.81)   |           |
| Senior high school           | 9 (18.00)       | 10 (18.51)  |           |
| Undergraduate                | 23 (46.00)      | 25 (46.29)  |           |
| Graduate or higher           | 12 (24)         | 11 (20.37)  |           |
| Occupation                   |                 |            | 0.919     |
| Unemployed                   | 10 (20.00)      | 12 (22.22)  |           |
| Employed                     | 10 (20.00)      | 12 (22.22)  |           |
| White-collar workers         | 18 (36.00)      | 20 (37.03)  |           |
| Civil servants               | 12 (24.00)      | 10 (18.51)  |           |

### 3 RESULTS

#### 3.1 Basic demographic and vaginal microbiome characteristics

The basic demographic characteristics of the case and reference groups are shown in Table 1. A total of 104 women were analyzed in this study, including a case group of 54 women with missed abortion and a reference group of 50 women with voluntarily induced abortion. The mean ages in the case and reference groups were $30.23 ± 3.52$ years and $30.98 ± 3.31$ years, respectively ($p > 0.05$).

There was no significant difference between the groups in patients with a history of induced abortion (case group 62.96%, reference group 62.5%; $p > 0.05$), gestational age (case group $10.16 ± 1.78$ weeks, reference group $9.67 ± 1.73$; $p > 0.05$), gravidity, and parity ($p > 0.05$).

There was no significant difference in education and occupation between the two groups ($p > 0.05$). Therefore, the two groups were considered comparable (Figure 1).

A total of 2002 OTUs were obtained from the two groups. The number of overlapping OTUs in the two groups was 975. There were 52 OTUs in the case group. When the sequencing depth of random sampling was between 1000 and 2500, the dilution curve tended to be flat, indicating that this sequencing depth was enough to reflect the information of most species in the sample (Figure 2A).

![Figure 1](image1.png)

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3.2 | The α-diversity and β-diversity between the case and reference groups

The Shannon index for the case group was higher than that for the reference group (median 5.48 vs 5.18, respectively; \( p = 0.020 \), Figure 1D), whereas the Simpson index for the case group was lower than that for the reference group (median 0.01 vs 0.02, respectively; \( p = 0.001 \), Figure 1E). The Sobs, Chao, and ACE indices for the case group were higher than those for the reference group, with no statistical difference (Sobs: median 574.78 vs 538.80, respectively, \( p = 0.23 \); Chao: median 574.78 vs 538.80, respectively, \( p = 0.23 \); ACE: median 574.78 vs 538.80, respectively, \( p = 0.23 \)).

The principal coordinate analyses method was used to analyze differences in species diversity between the two groups. Percentages represent the contribution of principal coordinate components to sample composition differences. The closer the two sample points, the more similar the species composition of the two samples. The results of nonparametric multivariate analysis of variance tests for assessing the diversity of weighted UniFrac \( (p = 0.001 \), Figure 2B), unweighted UniFrac \( (p = 0.003 \), Figure 2C), and Bray–Curtis \( (p = 0.001 \), Figure 2D) showed significant differences in vaginal microbiota diversity between the two groups.

3.3 | Specific phylum comparisons between the case and reference groups

Database comparisons were performed to classify the OTUs by phylum, class, order, family, genus, and species. We used LEfSe analysis to determine the statistically significant differences between the vaginal microbiota of the two groups.

A total of 12 microbial phyla were detected, four of which had a relative abundance of more than 1%. Of these, Firmicutes accounted for 46.61% of the total abundance, followed, from most to least, by Proteobacteria, Actinobacteria, and Bacteroidetes (Figure 3A). Among the 12 microbial phyla, there were six with significant differences between the two groups: Firmicutes (reference group 51.03%, case group 42.52%), Proteobacteria (reference group 26.12%, case group 29.05%), Actinobacteria (reference group 14.76%, case group 18.07%), Chlamydiae (reference group 0.01%, case group 0.03%), Fusobacteria (reference group 0.36%, case group 0.75%), and Saccharibacteria (reference group 0.09%, case group 0.05%) (Figure 3B).

3.4 | The relative abundance of Mycoplasma in the vaginal microbiota did not increase in the case group

We further compared the relative abundance of the three most common Mycoplasmas in the female genital tract between the two groups. Interestingly, the relative abundance of Mycoplasma genitalium (reference group 13.09%, case group 10.38%) and Ureaplasma (reference group 9.18%, case group 6.59%) was lower in the case group than in the reference group (\( p < 0.05 \)), whereas the relative abundance of Mycoplasma hominis (reference group 3.46%, case group 3.89%) was not significantly different between the two groups (\( p > 0.05 \)) (Table 2). These results showed that Mycoplasma did not increase in patients with missed abortion, and some species of Mycoplasma decreased.

3.5 | The relative abundance of Lactobacillus in the vaginal microbiota decreased in the case group

In both groups, the relative abundance of Lactobacillus was the highest, accounting for 19.76% of the total richness. However, the relative abundance of Lactobacillus in the case group (16.51%) was lower than that in the reference group (23.00%, \( p < 0.05 \)). Since the most common Lactobacillus in vaginal microbiota are L. crispatus, L. gasseri, L. iners, and L. jensenii, we compared the proportion of these types of Lactobacillus between the two groups. The relative abundance of L. crispatus, L. jensenii, and L. gasseri in the case group was lower than in the reference group, and the differences for L. jensenii \( (p = 0.00078 \) and L. gasseri \( (p = 0.00069 \)) were significant (Table 2). The relative abundance of L. iners was not significantly different between groups \( (p > 0.05 \), Table 2). These results showed that not only was the relative abundance of Lactobacillus decreased in patients with missed abortion but also the composition of the Lactobacillus subtypes changed.

3.6 | Other kinds of pathogenic bacteria increased in the case group

A total of 982 microbial species were detected at the species level. L. iners, Arthrobacter, Gemella-sanguinis, Burkholderia, Prevotella, Pseudomonas, Salinicoccus-roseus, Staphylococcus-repidermidis, and Yokenella-regensburgei were dominant in the two groups (relative abundance >1%). There were 345 species with statistically significant differences between the two groups. Pathogenic bacteria in the case and reference groups predominantly included Streptococcus pneumoniae (0.01% vs 0.09%, respectively), treptococcus (0.04% vs 0.06%, respectively), hemolytic streptococcus (<0.01% vs 0.05%, respectively), Pseudomonas (0.01% vs 0.04%, respectively), Klebsiella pneumoniae (<0.01% vs 0.04%, respectively), salmonella (0.01% vs 0.04%, respectively), Helicobacter pylori (0.01% vs 0.03%, respectively), campylobacter (0.01% vs 0.03%, respectively), Chlamydia trachomatis (<0.01% vs 0.02%, respectively), and Streptococcus agalactiae (<0.01% vs 0.02%, respectively) (Figure 4).

3.7 | The pathways enriched in the case group were mainly related to metabolism

In the present study, vaginal bacterial metabolic functions were enriched and predicted based on KEGG level 3. There were 32 KEGG pathways with significant differences (Figure 5). Compared with the
reference group, there were 15 additional vaginal bacterial enrichment pathways in the case group, including amino acid, cofactors and vitamins, xenobiotics, porphyrin and chlorophyll II, arginine, and proline metabolisms ($p < 0.05$). Amino acid metabolism was more abundant in the case group than in the reference group. However, pathways including genetic information processing, membrane transport, replication, and repair were significantly enriched in the reference group.

**FIGURE 1** Comparative $\alpha$-diversity results for the two groups (x±s). The $\alpha$-diversity of the vaginal microbiota from Sobs (A), Chao (B), ACE (C), Shannon (D), and Simpson (E) indices for the case and reference groups. Data are summarized in Table (F). The five lines from the bottom to the top of the box chart show the minimum, first quartile, median, third median, and maximum values, respectively. ACE, abundance-based coverage estimator; Sobs, number of observed species.

| Groups           | Sobs        | Chao        | ACE        | Shannon    | Simpson     |
|------------------|-------------|-------------|------------|------------|-------------|
| Reference group  | 538.80±139.69 | 538.80±139.69 | 538.80±139.69 | 5.18±0.53  | 0.02±0.01   |
| Case group       | 574.78±163.44 | 574.78±163.44 | 574.78±163.44 | 5.48±0.66  | 0.01±0.01   |
| $P$ value        | 0.231       | 0.231       | 0.231       | 0.020      | 0.001       |

**DISCUSSION**

A decrease in *Lactobacillus* or an imbalance in the proportions of the different *Lactobacillus* strains may adversely affect pregnancy outcomes. Recent clinical studies have indicated that the vaginal bacterial composition in cases of first-trimester miscarriage is associated with a reduced abundance of *Lactobacillus*, leading to the
The relative abundance of vaginal Lactobacillus was significantly decreased in patients at risk of early pregnancy miscarriage compared with a reference group. Moreover, some studies have found that different subtypes of Lactobacillus have different effects on pregnancy outcomes. Vaginal microbiota with dominant L. iners appears to be associated with an increased risk of the development of specific pregnancy-related pathologies, such as preterm delivery. In contrast, vaginal microbiota dominated by L. crispatus, L. jensenii, and L. gasseri is very common in pregnancy and is associated with a lower risk of preterm delivery. To our knowledge, the present study was the first to indicate that the relative abundance of Lactobacillus, especially of L. crispatus, L. gasseri, and L. jensenii, was significantly lower in patients with missed abortion, though the abundance of L. iners was not. We observed that the decreased proportion of Lactobacillus in the case group weakened the protective effect of Lactobacillus on the normal physiological function of the reproductive tract. Our results were consistent with the discussed research on early pregnancy miscarriage and premature delivery.

Vaginal Lactobacillus species can create a barrier against pathogen invasion since the metabolic secretions from Lactobacillus in the cervicovaginal fluid can play an important role in the inhibition of bacterial and viral infections. Vaginal dysbiosis is associated with the activation of inflammatory pathways and premature delivery.
with increased levels of proinflammatory cytokines, and decreased levels of the Lactobacillus family might allow the richness and diversity of potential pathogens to increase. An increased risk of premature delivery and miscarriage has been detected in patients with low levels of Lactobacillus and increased bacterial diversity. Therefore, detecting the levels of Lactobacillus in the vaginal microbiome could be a way of evaluating the risk of miscarriage in clinical diagnosis.

Screening for and treatment of Mycoplasma during pregnancy remains controversial. Mycoplasmas are the smallest free-living microorganisms and predominantly include M. genitalium, M. hominis, and Ureaplasma. Some research has indicated that testing high-risk

Table 2: Comparison of the average relative abundance of Mycoplasma and Lactobacillus between the two groups

|                | Reference group (n = 50) | Case group (n = 54) | Z     | p-value |
|----------------|-------------------------|---------------------|-------|---------|
|                | Median | Q1     | Q3     | Median | Q1     | Q3     |       |         |
| Mycoplasma genitalium | 0.1309 | 0.8775 | 0.1797 | 0.1038 | 0.0551 | 0.1402 | -2.311 | 0.021   |
| Mycoplasma hominis     | 0.0346 | 0.0000 | 0.0761 | 0.0389 | 0.0000 | 0.0726 | -0.365 | 0.715   |
| Ureaplasma            | 0.0918 | 0.0678 | 0.1147 | 0.0659 | 0.0401 | 0.1119 | -2.213 | 0.027   |
| Lactobacillus crispatus | 1.0759 | 0.8100 | 1.5611 | 0.8701 | 0.2134 | 1.6451 | -2.084 | 0.037   |
| Lactobacillus gasseri  | 0.4893 | 0.3764 | 0.6994 | 0.3636 | 0.1505 | 0.5138 | -3.39  | 0.001   |
| Lactobacillus jensenii | 0.1312 | 0.0690 | 0.2127 | 0.0671 | 0.0301 | 0.1374 | -3.359 | 0.001   |
| Lactobacillus iners    | 0.0691 | 0.0415 | 0.1018 | 0.0595 | 0.0303 | 0.1262 | -0.567 | 0.571   |

Note: Mann–Whitney U test was used. p > 0.05 = no significant difference; p < 0.05 = significant difference.

Figure 3: The relative abundance of species at the phylum level in the two groups. (A) Relative abundance of the species at the phylum level. (B) Summary of the relative abundance of different bacteria at the phylum level.
symptomatic women for *M. genitalium* may be warranted, as *M. genitalium* infection has been significantly associated with increased risk of preterm birth and spontaneous abortion. However, some research has reported that *Mycoplasmas* are ubiquitous in the vaginal milieu and do not appear to affect pregnancy outcomes, suggesting that treatment is only needed when symptoms occur. At present, literature on infection with *Mycoplasma* in the lower genital tract of Chinese patients with missed abortion is lacking. Our population-based research showed that the relative abundance of *M. genitalium* and *Ureaplasma* in the case group was lower than in the reference group, whereas the relative abundance of *M. hominis* was not significantly different between the two groups. We found no evidence of elevated *Mycoplasma* infection rates in the case group, so we cannot easily link *Mycoplasma* infections to this pregnancy outcome. We suspect that *Mycoplasma* does not need to be included in routine screening for genital secretions and that it should not be over-treated in the early stages of pregnancy. However, more evidence is needed to verify these suspicions.

This study found that the relative abundance of pathogens, including *S. pneumoniae*, *treptococcus*, *hemolytic Streptococcus*, *Pseudomonas*, *K. pneumoniae*, *Salmonella*, *H. pylori*, *Campylobacter*, *C. trachomatis*, and *S. agalactiae* was higher in the case group than in the reference group (p < 0.05). Chlamydia an important cause of spontaneous abortion. It can impact the endometrium of pregnant women, interfere with the normal immune system, and then induce the placenta accreta process, weakening the mother’s protective effect on the fetus and finally cause spontaneous abortion. *S. agalactiae*, also known as group B Streptococcus, is a conditional pathogen and can lead to premature rupture of membranes, endometritis, and neonatal infection. Cardaropoli et al. compared the serum-positive rate of *H. pylori* between women with spontaneous abortion and healthy control patients and found that *H. pylori* infection was related to primary spontaneous abortion but not to recurrent abortion. *K. pneumoniae* is a conditional pathogen that often causes lung infection, urinary tract infection, and vaginitis, resulting in recurrent redness and swelling of the vaginal wall, copious leukorrhea, and sticky, purulent, or bloody jelly-like discharge. We found significantly higher pathologic bacterial levels in the case group and speculated that an increase in the relative abundance of vaginal pathogenic bacteria might lead to adverse pregnancy outcomes by increasing the risk of reproductive tract infection.

Throughout pregnancy, to support the normal development of the fetus, the mother increases her energy intake and storage. At the phylum level, *Firmicutes* have been associated with increased energy storage. Studies in animals fed high-fat or high-fiber diets have shown more abundant *Firmicutes*. We found that *Firmicutes* constituted the highest abundance in both groups, although it was lower in the case group than in the reference group (42.52% vs 51.03%, respectively). Combined with the results of KEGG analysis, the pathway changes in the case group were mainly concentrated in the energy metabolism pathway. We speculated that a decrease in *Firmicutes* might impair energy metabolism and be associated with missed abortion. To our knowledge, our study is the first to propose and observe the role of *Firmicutes* in the vaginal microbiota of patients with missed abortion. However, more experiments are needed for further confirmation.

We also found that the proportion of vaginal *Lactobacilli* decreased, or the relative abundance of pathogenic bacteria increased, in the case group, which may have affected endometrial receptivity. In a study using culture-based methods, Cicinelli et al. reported that the most frequent infectious agents detected at the endometrial level are common bacteria, accounting for 69% of all infections. A decrease in the abundance of vaginal *Lactobacillus* activates the inflammatory pathways, thereby

![Figure 4](image-url) Relative abundance of species at the species level. YD19T100047 to YD19T100096 from left to right are the reference group samples. YD19T010001 to YD19T100046 from left to right belong to the case group.
Reducing endometrial receptivity and implantation. 29 L. crispatus has been reported as present in the endometrium before spontaneous abortion, whereas L. iners has been the most prevalent microbe found in the endometrium during early pregnancy. 29 Endometrial infection has been proposed as a possible etiologic factor of chronic endometritis (CE). Positive endometrial cultures have been reported in 75% of women with histologically confirmed CE, with common bacteria (Escherichia coli, Enterococcus faecalis, S. agalactiae) most frequently found (77.5%), followed by Mycoplasma, Ureaplasma (25%), and C. trachomatis (13%). 48-50 Moreover, the prevalence of CE ranges from 0.8% to 19% in the general population and from 30% to 45% in infertile patients, leading to recurrent implantation failure and recurrent pregnancy loss. 31-35 We found that the proportion of Lactobacillus decreased in the case group, which was consistent with previous research on the trends of Lactobacillus in the uterine cavity. In addition, the relative abundance of pathogenic bacteria increased. Such changes may increase the risk of ascending uterine infection, especially after operations such as assisted reproduction or hysteroscopy. However, several recent studies have drawn attention to uterine populations of microorganisms, which reached only a 30% concordance with those of the cervical-vaginal flora. Therefore, the composition of vaginal microbiota cannot accurately reflect the situation of uterine microbiota, and the direct relation between the change of vaginal microbiota and the change of endometrial receptivity needs to be further discussed.

This study had some limitations. More complete clinical parameters, health habits, and other data are needed; the causal relation between vaginal microbiota dysbiosis and missed abortion needs to be verified with further experiments; and other causes of missed abortion, such as genetic factors, endocrine abnormalities, and autoimmune diseases could not be eliminated completely.
We found a preliminary correlation between vaginal microbiota dysbiosis and missed abortion. The decrease in the proportion of vaginal *Lactobacillus* and the change of *Lactobacillus* species in patients with missed abortion may have increased the risk of genital tract pathogenic bacteria infection, but this study does not allow for causal interpretation; however, no increase in Mycoplasma infections was observed. A decrease of *Firmicutes* in the vaginal microbiota might impair energy metabolism, which leads to missed abortion. Comprehensive insights regarding vaginal microbiota may facilitate improved understanding and the development of novel diagnosis, therapeutics, and preventive strategies for early missed abortion.

**AUTHOR CONTRIBUTIONS**

CX Cheng and DB Xu conceived and designed the study, D Sun and XP Zhao drafted the manuscript and analyzed the data. Q Pan and BS Gao handled the picture and article format. F Li collected specimens. AQ Zhang and H Huang reviewed the data. All authors contributed to the article and approved the submitted version.

**CONFLICT OF INTEREST**

None.

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