Analysis of endometrial microbiota in intrauterine adhesion by high-throughput sequencing

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Background: Intrauterine adhesions (IUA) arise from scar tissue formation between the endometrial surfaces in response to mechanical or infectious injuries. However, the potential role of endometrial microbiota in IUA remains unclear. We aimed to explore the composition of endometrial microbiota and its potential role in IUA.

Methods: We retrospectively enrolled 46 patients diagnosed with IUA and 21 infertility patients without intrauterine lesions, as control subjects. All cases were diagnosed with hysteroscopy and endometrial tissues were taken from the intrauterine cavity using a hysteroscopic cutting ring without electricity study. After endometrial samples were collected, DNA was extracted and amplified for barcoded Illumina high-throughput next-generation sequencing targeted to the 16S rRNA V4 region for microbiota. Microbiota data were compared between two groups using α-diversity, β-diversity and Nonmetric Multidimensional Scaling based on Weighted Unifrac distance.

Results: At the phyla level, the dominant bacteria included Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria. Proteobacteria accounted for more than 64.48%. At the genus level, the proportions of Klebsiella, Shewanella, and Lactobacillus were higher in patients with IUA than in non- IUA participants (20.67% and 8.77%, P=0.006, 13.37% and 4.53%, P=0.175, 12.74% and 6.95%, P=0.882; respectively). The proportion of Acinetobacter was significantly lower in patients with IUA than in non- IUA participants (P=0.005).

Conclusions: Endometrial microbiota differ between patients with IUA and infertility patients without intrauterine lesions, and the potential variation of endometrial microbiota might cause IUA.

Keywords: Intrauterine adhesions (IUA); endometrial microbiota; 16S rRNA

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Introduction

Intrauterine adhesions (IUA), also known as Asherman's syndrome, is an intrauterine condition characterized by the physiological endometrium being replaced by fibrotic tissue or scar after the mechanical or infectious injury of the endometrium (1,2). IUA could progressively cause hypomenorrhea, amenorrhea, infertility, spontaneous abortions, and even placenta implantation. Recently, the rate of IUA recurrence, especially in severe cases, remains high even after hysteroscopic adhesiolysis combined with various adjuvant therapies. Therefore, IUA therapy is still challenging (3). Endometrial damage and infection are recognized as the two major risk factors of IUA.

The balance of micro-ecology in the female reproductive tract plays a key role in health. An increasing body of evidence suggests that the change of composition and distribution of endometrial microbiota is related to endometrial diseases such as endometrial polyps, endometrial cancer, and infertility (4-6). Patients with IUA have micro-ecological imbalance in the lower genital tract, and the V4 region or the V3 and V4 region of the 16S rDNA in each sample was amplified by PCR method (7,8). However, there are few reports on endometrial microbiota in patients with IUA.

Moreover, a growing body of evidence shows that organ fibrosis is associated with microbiota. The specific microbial signatures can be used to distinguish the severity of liver diseases, such as mild disease and advanced fibrosis (9). Recent 454 pyrosequencing data have revealed that the progression of idiopathic pulmonary fibrosis is related to the presence of specific members in *Staphylococcus* and *Streptococcus* genera, suggestive of the role of microbiome in fibrotic processes (10).

Given the inflammatory profile and fibrosis essence of IUA, in this study we used high throughput sequencing techniques to characterize endometrial microbial communities in patients with IUA and the intrauterine microbial diversity difference compared to the females without intrauterine lesions. The authors present the study in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/atm-20-2813).

Methods

Patients

This study was conducted in accordance with the Declaration of Helsinki (as was revised in 2013). The study was approved by the Medical Ethics Committees at the Zhujiang Hospital of Southern Medical University (NO. 2019-KY-077-01) and all the patients provided informed consent. Demographic data of 67 participants from March 2016 to March 2019 were collected retrospectively. Among them, 46 patients with IUA were enrolled as the observation group (Group IUA), and 21 infertile females without intrauterine lesion were enrolled as the control group (Group C). Subjects eligible for Group IUA met the following criterion: diagnostic hysteroscopy confirmed the presence of adhesions in the intrauterine cavity. The inclusion criteria in Group C were the following: hysteroscopy and subsequent endometrial pathology excluded the lesions in the intrauterine cavity. The exclusion criteria of all participants were: women who had taken antibiotics within three weeks pre-operation, other intrauterine lesions such as endometrial polyps, submucosal myoma, endometrial cancer and endometrial hyperplasia, coagulopathy, vaginitis and acute pelvic inflammatory disease. Group IUA was further divided into three subgroups based on American Fertility Society Rating System: 20 patients scored from 1 to 4 were classified as Stage I (Group IUA-Mild), and 12 patients scored from 5 to 8 as Stage II (Group IUA-Moderate), and the remaining scored from 9 to 12 as Stage III (Group IUA-Severe). As shown in *Table 1*, clinical characteristics of these groups were comparable except for the number of intrauterine operations.

Samples

All samples were collected in the early stage of endometrial proliferation, or any day for the patients with secondary amenorrhea. After vaginal and cervical canal disinfection, endometrial tissues were taken gently from the intrauterine cavity using a hysteroscopic cutting ring without electricity. During the collection of endometrial samples, hysteroscope sheath was used to avoid contamination by vaginal microbiota. Each sample was placed in a sterile tube and immediately stored in liquid nitrogen for DNA extraction.

Genomic DNA extraction, bacterial 16S rRNA amplification and high-throughput sequencing

Genomic DNA was extracted from endometrial tissues using HiPure Bacterial DNA Kit (Magen, USA). The concentration and quality of purified DNA were determined by a spectrophotometer at 230 and 260

Table 1 Clinical characteristics of women enrolled in the study

Items	Group IUA-H	Group IUA-M	Group IUA-L	Group C	P value
Age (years)	29.79±1.01	30.58±1.25	31.85±1.45	33.14±1.02	0.251
Gravidity	2.64±0.39	2.17±0.32	2.90±0.35	1.95±0.15	0.176
Parity	0.71±0.83	0.25±0.13	0.85±0.18	0.85±0.25	0.254
No. of abortions/curettage	2.00±1.24	2.00±0.28	2.25±0.33	1.01±0.25	0.025

Group IUA-H, intrauterine adhesion patients with high-grade. Group IUA-M, intrauterine adhesion patients with middle-grade. Group IUA-L, intrauterine adhesion patients with low-grade. Group C, the control group. Mean ± SE are shown.

nm (NanoDrop-One, Thermo Fisher Scientific, MA, USA). The V4 region of the 16S rDNA was amplified using Premix-Taq (Takara Biotechnology, Dalian, China), and 515F/806R primers with 12 bp barcode (515F, 5'-GTGCCAGCMGCCGCGGTAA-3'; 806R, 5'-GGACTACHVGGGTWTCTAAT-3'). The cycling conditions for PCR were as follows: 94 ℃ for 5 min, followed by 30 cycles of 94 ℃ for 30 sec, 52 ℃ for 30 sec, 72 ℃ for 30 sec and another 10 min at 72 ℃. According to Gene Tools Analysis software (version 4.03.05.0, Syn-Gene), PCR products were mixed into one tube in equal proportions, and the amplicon mixture was purified with E.Z.N.A. Gel Extraction Kit (Omega, USA). The 16S rDNA sequencing was performed with a high-throughput next-generation Illumina Hiseq2500 platform (11).

DNA sequence data analysis and taxonomy

The quality filtering on the Paired-end reads from the original DNA fragments was processed with Trimmomatic software (12) (version 0.33, http://www.usadellab.org/cms/?page=trimmomatic) to remove paired-end reads with one or more ambiguous bases. The retained paired-end reads were trimmed at the 3' end to eliminate the continuous bases with a quality score <20 and reads with sequence length <100 bp to obtain high-quality reads. Paired-end clean reads were merged using FLASH (version 1.2.11, https://ccb.jhu.edu/software/FLASH/) according to the relationship of the overlap between the paired-end reads. The sequences were assigned to each sample based on their unique barcode and primer using Mothur software (13) (version 1.35.1, http://www.mothur.org), after which the barcodes and primers were removed to get effective Clean Tags.

Ultimately, an average of 158,737.64±112,78.96 reads per sample with a total of 10,635,422 sequence reads (47,952–421,485 reads per sample) (Table S1) were obtained after quality control. Using the usearch software (14) (version 10, http://www.drive5.com/usearch/), the sequences with 97% or higher similarity were grouped into the same operational taxonomic units (OTU).

Further processing was performed using QIIME (15). The taxonomic assignment of representative sequences was performed using the Ribosomal Database Project (RDP) Classifier (16) and a minimum confidence threshold to default was adjusted to 50%. Dominance and the Simpson indexes were used to measure α-diversity for species richness and evenness within different bacterial populations. The weighted UniFrac (17) was used to measure β-diversity for the diversity between bacterial communities in terms of ecological distance between samples. The difference in bacterial community composition was analyzed by Nonmetric Multidimensional Scaling (NMDS) based on Weighted Unifrac distance. Moreover, the difference in β-diversity among groups was analyzed by ADONIS, a multivariate analysis of variance based on distance matrices and permutation.

Statistical analysis

Statistical analysis was performed with Statistical Product and Service Solutions (SPSS) (version 20.0). Data normality was tested with the Kolmogorov-Smirnov test. Homogeneity of variance was detected with the Levene test. Data were presented as means ± standard deviations. Data were compared by the Mann-Whitney test or the Kruskal-Wallis analysis of variance on ranks, followed by Dunn's test. The statistical significance was set at two-side P<0.05.

Results

Sequencing coverage

To characterize the different bacteria population in endometrial tissues, we performed deep sequencing of the

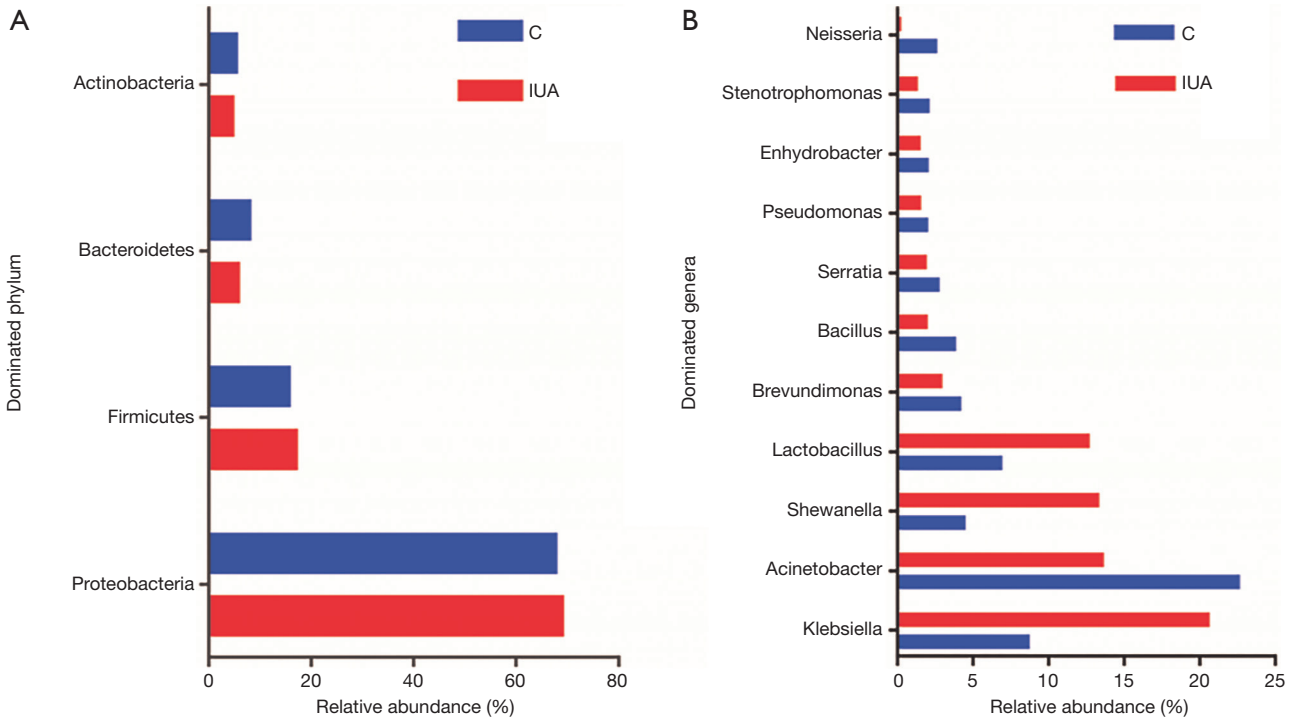

Figure 1 16S rRNA gene analysis revealed taxonomic variations and high diversity of endometrial microbiota between patients with IUA and infertile females without intrauterine lesion. (A) Bars plots showed the relative abundance of the 4 most abundant bacterial phyla in two groups. (B) Bars plots showed the relative abundance of the 10 most abundant bacterial groups at the genus level in two groups.

16S rRNA V4 region of all 67 samples. In total, 10,635,422 filtered clean reads (158,737.64 reads/sample) and 10,089,655 filtered clean tags (150,591.87 reads/sample) were obtained from all samples (Table S1). A total of 1,163 OTUs were identified from all samples.

Differences in bacterial population between Group IUA and Group C

The most abundant phylum in endometrium of Group C and Group IUA was *Proteobacteria*, which accounted for 68.11% and 69.36%, respectively, followed by *Firmicutes* (16.01% and 17.47%), *Bacteroidetes* (8.38% and 6.15%) and *Actinobacteria* (5.73% and 5.00%). There was no difference in the proportion between the two groups (P>0.05, *Figure 1A*). At the genus level (*Figure 1B*), bacterial communities in Group C were dominated by *Acinetobacter* (22.68%), followed by *Klebsiella* (8.77%), *Lactobacillus* (6.95%) and *Shewanella* (4.53%). However, bacterial communities in Group IUA were dominated by *Klebsiella* (20.67%), *Acinetobacter* (13.67%), *Shewanella* (13.37%) and *Lactobacillus* (12.74%). The proportion of *Acinetobacter* was significantly lower in Group IUA than in Group C (P=0.005), while the proportion of *Klebsiella* was significantly higher in Group IUA than in Group C (P=0.006). The rarefaction curves of Simpson index showed that α-diversity in Group IUA was significantly lower than that in Group C (P=0.01) (Figure S1A), while Group IUA had more OTUs than Group C (P=0.257) (Figure S1B). In addition, β-diversity analysis revealed the difference in microbiota structure. Endometrial microbiota showed significant difference in Group IUA and Group C (P=0.048, *Figure 2A*).

Overall microbiota structure difference among four groups

There was no difference in the observed species between the groups (P=0.491, Figure S2). As shown in the Figure S2, rarefaction curves of Simpson index indicated significant difference in α-diversity of endometrial bacteria between the different stage of IUA and Group C (P=0.011). Group C had the highest α-diversity of endometrial bacteria. Both endometrial α-diversity in Group IUA-Mild and

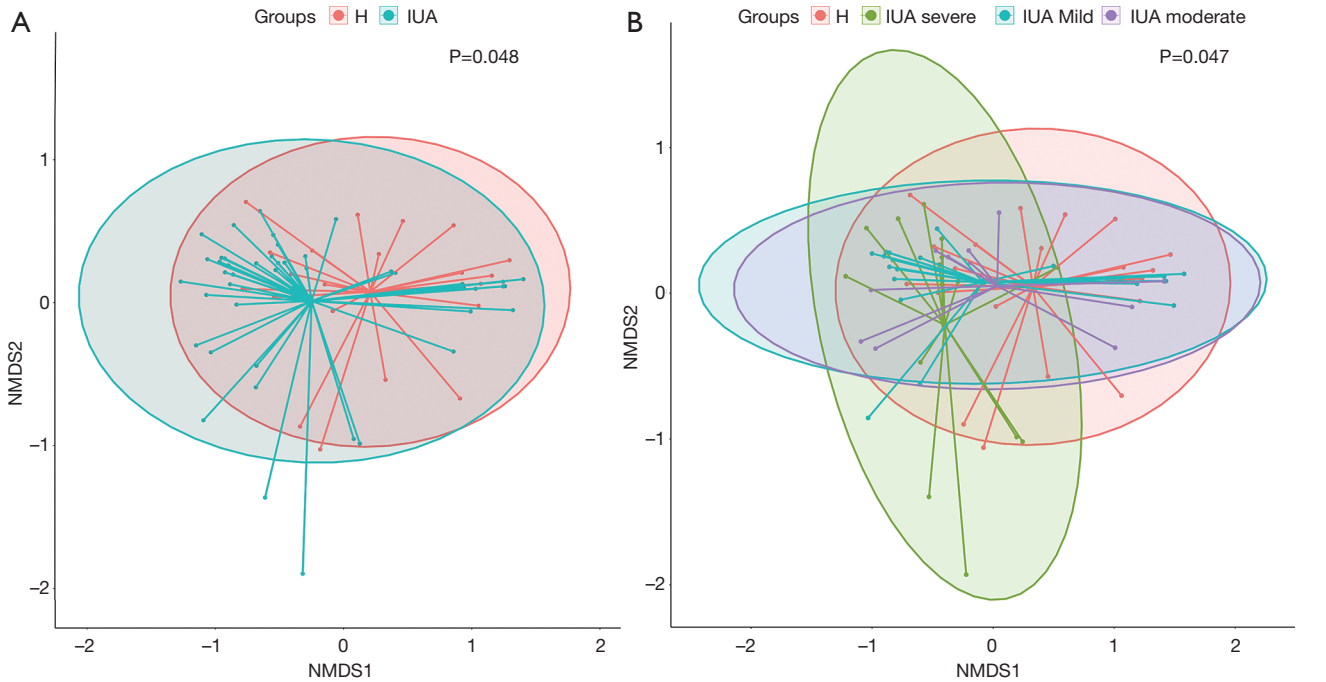

Figure 2 Ordination plot based on weighted UniFrac distance showed the relationship between different disease states. Each point represented a sample. The red color represented infertile females without intrauterine lesion, the green color represented patients with mild IUA, the purple color represented patients with moderate IUA, and the yellow color represented patients with moderate IUA. (A) The endometrial microbiota was significantly different between patients with IUA and infertile females without intrauterine lesion (P=0.048). (B) The endometrial microbiota showed difference between patients with mild IUA and infertile females without intrauterine lesion (P=0.022).

Group IUA-Severe were significantly different from that in Group C (P=0.012, P=0.002). No difference in α-diversity was found between Group IUA-Mild and group IUA-Moderate or Group IUA-Severe, respectively (P=0.090, P=0.512). There was significant difference between Group IUA-Moderate and Group IUA-Severe (P=0.046), but not between Group IUA-Moderate and Group C (P=0.811). The UniFrac-based nonmetric Multidimensional Scaling was carried out to explore the difference in bacterial community composition between groups. As shown in *Figure 2B*, consistent with the α-diversity analysis, there was significant difference in endometrial β-diversity among four cohorts (P=0.047). In addition, endometrial microbiome of Group IUA-Mild was different from that of Group C (P=0.022), but endometrial microbiome of Group IUA-Moderate and Group IUA-Severe were not different from that of Group C, and there was no difference in β-diversity among the three IUA subgroups. These results indicated a relationship between IUA and the variation of endometrial microbiota.

Bacterial composition and community structure in four groups

As shown in *Figure 3A*, at the phylum level, all groups were dominated by *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*. With the increase of the severity of adhesions, the proportion of *Proteobacteria* decreased (73.30%, 70.29% and 64.48%), while the proportion of *Firmicutes* increased (12.79%, 14.02% and 25.58%), although there was no difference. As shown in *Figure 3B*, compared with Group C, the abundance of *Klebsiella* sequences was significantly higher in endometrial communities of Groups IUA-mild, IUA-moderate and IUA-severe (31.44%, 12.51% and 18.06%, P=0.046), while *Acinetobacter* sequences was significantly lower (12.74%, 16.18% and 12.10%, P=0.020). In addition, Group IUA-Mild had the highest proportion of *Lactobacillus*.

Discussion

With the development of high-throughput sequencing

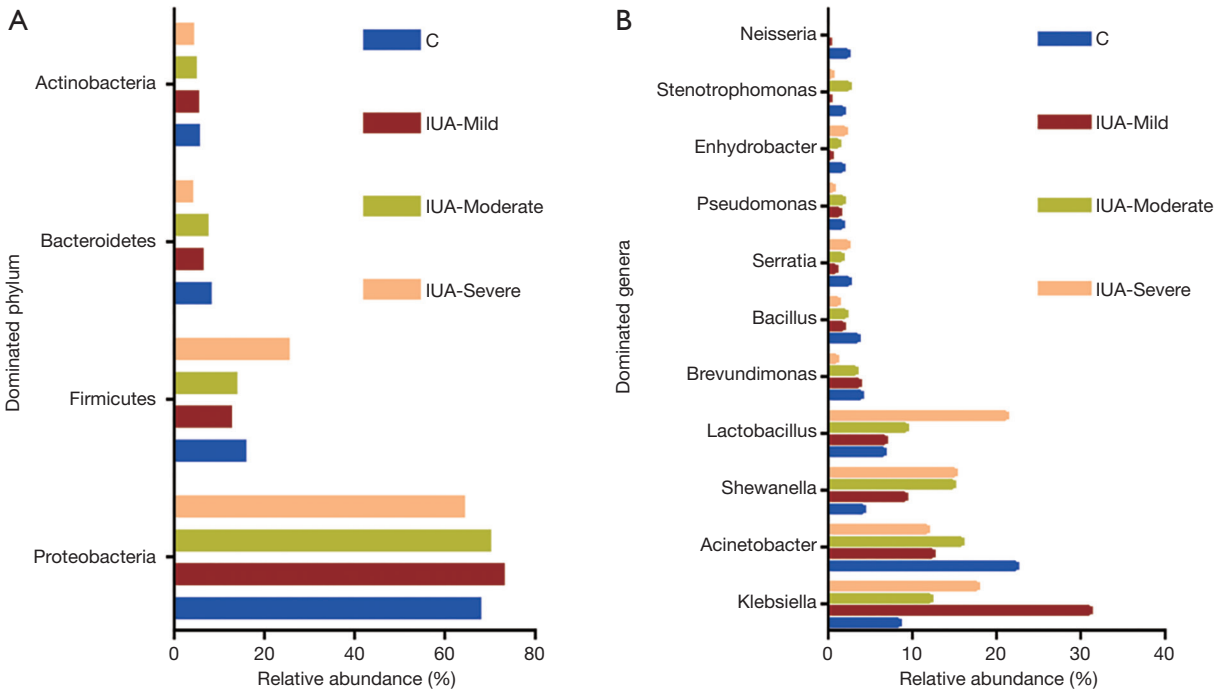

Figure 3 16S rRNA gene analysis showed taxonomic variations and high diversity of endometrial microbiota among patients with three IUA subgroups and infertile females without intrauterine lesion. (A) Bars plots showed the relative abundance of the 4 most abundant bacterial phyla in four groups. (B) Bars plots showed the relative abundance of the 10 most abundant bacterial groups at the genus level in four groups.

technology, some uncultivable, low abundant and unclassified microorganisms in the upper reproductive tract have been recognized. Recent evidence has revealed the association between the microbiota in the vagina or cervical canal and IUA (7,8). Up to now, there is few report on the structure and distribution of endometrial microbiota in patients with IUA. Here, we provided the first evidence that endometrial microbiota in patients with IUA were different from those in infertility patients without intrauterine lesions, and the potential variation of endometrial microbiota might be related with the occurrence of IUA.

In present study, for all participants, the genera with relatively high abundance of endometrial bacteria were *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Lactobacillus*. These results are consistent with previous reports on uterine microbiota in patients with endometrial polyps, endometrial cancer, and infertility (4-6). *Klebsiella*, *Acinetobacter*, *Shewanella*, *Brevundimonas*, *Bacillus*, *Serratia*, *Pseudomonas*, *Stenotrophomonas*, *Neisseria*, were also detected in all participants. However, our results are not consistent with some results reported previously (7,8). Liu *et al.* (7) showed that patients with IUA had significantly lower percentage of *Firmicutes* and higher percentage of *Actinobacteria* in the vagina, and half of the patients had overgrowth of *Gardnerella* and *Prevotella* accompanied with the reduction of *Lactobacillus* in the vagina. Zhao *et al.* (8) demonstrated that the proportion of *Firmicutes* was higher in vagina and cervical canal from most cases with IUA, but some species including *Acidobacteria*, *Euryarchaeota*, *Chlamydia*, *Chlorobi*, *Planctomycetes* and *TM6 (Dependentiae)* almost disappeared. We found that the proportion of *Actinobacteria* was lower than that of *Firmicutes*, while *Lactobacillus* increased among endometrial microbiota in patients with IUA. This discrepancy might be related to different severity of IUA, different sites of samples and different ecological niches in the reproductive tract.

For the structure of the microbial community, mild IUA cohort could be noticeably distinguished from the control cohort. This indicates that the microbiota may play a role in the early stage of endometrial fibrosis. However, the structural difference was not significant between the moderate or severe IUA cohort and the mild IUA

cohort, indicating differential microbial community of endometrium associated with the severity of IUA.

For the distribution of endometrial microbiota, the uniformity of mild IUA cohort was the lowest, and the overall uniformity of all IUA cohorts was still lower than that of the control cohort. There may be a period of transient microflora disturbance, and then the endometrial microbiota would be renormalized to a new equilibrium state.

In addition, the number of endometrial *Klebsiella* in patients with IUA was significantly higher than that of the control group, and this bacterium was mainly enriched in the mild IUA cohort. *Klebsiella* is a kind of encapsulated Gram-negative bacilli in *Enterobacteriaceae*, and it is a typical conditional pathogen (18). Some strains of *Klebsiella* can produce virulence factors such as lipopolysaccharide (LPS), which acts on the receptor Toll-like receptors 4 (TLR4) to induce fibrosis and inflammation (19). As we know, the expression of TLR4 is constant in female endometrium (20). Liu *et al.* showed that LPS-induced endometrial infection played an important part in the occurrence of IUA (2). Therefore, endometrial *Klebsiella* may be involved in the occurrence of IUA. Further studies are needed to define which *Klebsiella* species are present in endometrial samples and whether they are capable of inducing pathogenic state.

Furthermore, we found that the proportion of *Lactobacillus* increased with the severity of adhesion. Although *Lactobacillus* has long been recognized as the dominant flora of healthy female vagina, not all *Lactobacillus* is beneficial. It has been proved that most strains of *Lactobacillus* such as *Lactobacillus jensenii*, *Lactobacillus crispatus*, and *Lactobacillus gasseri* are probiotics (21), while *Lactobacillus iners* produces less hydrogen peroxide and has weaker ability to resist pathogens (22). Therefore, endometrial *Lactobacillus* could exist in the micro-ecological imbalance or balance state. In different state, more than 10% of gene expression of the strain is different, and the expression of related metabolic enzymes increases (23). All the above results suggest that high abundance of *Lactobacillus* in patients with IUA may contain *Lactobacillus iners*, which need subsequent isolated culture. Moreover, due to the different structure and microenvironment of uterine cavity and vagina, the distribution or structure of endometrial *Lactobacillus* may differ from that of vaginal *Lactobacillus*.

This study has some limitations. First, the sample size may be small, which might cause that we did not detect significant differences for some comparisons. Second, we did not choose healthy women as the control due to ethical restriction, so we were unable to evaluate whether infertile factors could affect endometrial microbiota. Nevertheless, endometrial microbiota should be confined to intrauterine cavity, and the patients with IUA might have other infertility factors. Moreover, this study lacked the inclusion of negative controls because of ethical restriction. With such low abundance microbiota, this type of control is essential.

In conclusion, the uterine cavity is not sterile and actually contains various bacteria. Uterine microbiota are different between patients with IUA and infertility patients without intrauterine lesions and all potential variation of uterine microbes might cause IUA. Maintaining the balance of reproductive tract microbiota could enhance the repair of endometrium after injuries.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at: http://dx.doi.org/10.21037/atm-20-2813). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related

to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as was revised in 2013). The study was approved by the Medical Ethics Committees at the Zhujiang Hospital of Southern Medical University (No. 2019-KY-077-01) and informed consent was taken from all the patients.

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