



# Study on the correlation among dysbacteriosis, imbalance of cytokine and the formation of intrauterine adhesion

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**Background:** Intrauterine adhesion (IUA) is one of the most important causes of female infertility, while iatrogenic endometrial injury is the main, but not the entire, cause of IUA. The microorganisms of the female reproductive tract play an important role in its health and disease. The imbalance of immune regulation caused by the imbalance of reproductive tract dysbacteriosis may be an important link in the formation mechanism of uterine cavity adhesion.

**Methods:** We prospectively enrolled 30 patients diagnosed with IUA and 30 women with a history of intrauterine surgery, but without IUA, as control subjects. All participants were diagnosed with hysteroscopy while two swabs—one being leucorrhea drawn from the middle of the vagina and the other being cervical mucus drawn from the cervical canal—were taken. The bacterial load and community were identified by 16S rDNA quantitative polymerase chain reaction and pyrosequencing. Immunocytokines in serum were quantitatively detected by human T-helper cytokine kit. The correlation between Th cytokines and microorganisms in IUA and non-IUA groups was analyzed.

**Results:** Compared with non-IUA participants at the phylum level, patients with IUA had a significantly higher percentage of firmicutes in most samples, while the diversity of bacteria was significantly decreased. Some species that were members of vaginal and cervical canal bacterial phyla, including Euryarchaeota, Acidobacteria, Chlamydiae, Chlorobi, Planctomycetes and TM6 (Dependentiae), almost disappeared. The quantity in serum of IUA patients of classical proinflammatory cytokines IL-6 and TNF- $\gamma$ , released from immune cells, also known as profibrotic cytokines, were significantly higher than that of the non-IUA women in our study ( $P < 0.05$ ).

**Conclusions:** IUA is characterized by an increased bacterial burden, decreased diversity of bacterial communities in the vagina/cervical canal, and increased immune cytokines of pro-fibrosis, which may predict new and more effective therapeutic schemes for the treatment of IUA.

**Keywords:** Intrauterine adhesion (IUA); vaginal microbe; cytokines

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## Introduction

Intrauterine adhesion (IUA) was first reported by Joseph Asherman, and is thus also known as Asherman syndrome (1,2). Most patients with IUAs have fibrous band adhesions formed in the anterior and posterior walls of the uterus, and these vascular fibrous tissue adhesion bands are accompanied by different amounts of white blood cell infiltration (3). Adhesions begin to form when fibers and inflammatory exudate appear on the surface of the damaged endometrium, and then gradually develop into denser fibrous bands, eventually forming mass adhesions. Adhesion can lead to partial or total uterine cavity obliteration which reduces the implantation area of embryos. IUA hinders the endometrial blood supply and reduces the endometrial receptivity which is not conducive to the growth of the embryos (4,5). The common clinical manifestations of IUA include hypomenorrhea, amenorrhea, recurrent abortion, and secondary infertility. There are various causes of IUAs (6), and its prevalence rate increases yearly, which is mainly due to the increased incidence of iatrogenic endometrial trauma such as artificial abortion and postpartum curettage. Other causes include genital tuberculosis, pelvic irradiation, and uterine surgery (including myomectomy and surgery for uterine malformation, etc.) (7). Hooker *et al.* (8) reported that curettage was the most important reason for the formation of IUA during pregnancy. Iatrogenic endometrial trauma is the main cause of IUA, but this alone cannot fully explain the mechanism of adhesion formation. It is necessary to explore the potential pathogenic factors of IUA, so as to formulate specific preventive measures for IUA based on the study of these factors.

Here, we investigated the role of the uterine microenvironment: the microbiome in the formation mechanism of IUA. It has been known for some time that the microbiome of the female reproductive tract plays an important role both in maintaining a healthy state and in the development of diseases in the female reproductive system. It is also widely acknowledged that lactic acid-producing microorganisms dominate the vaginal microbial community (9), maintaining a relatively healthy reproductive tract microenvironment by lowering the vaginal PH value. Previous studies have shown that vaginal dysbacteriosis will lead to a variety of diseases, such as postpartum endometritis, preterm delivery, pelvic inflammatory disease, spontaneous abortion, delivery of low-birth-weight babies, etc. (10-12).

The etiology and progression mechanism of IUA caused by microorganisms is similar to that of other damaged tissues and organs with fibrosis (13). Tissue damage and bacterial imbalance not only promote inflammation, but also release a large number of proinflammatory and profibrotic cytokines by recruiting and activating innate and adaptive immune systems. For example, when infected by bacteria, Th1 response results in a release of large amounts of proinflammatory cytokines for the clearance of pathogens. The damaged tissues need an immune response, mainly of a profibrotic nature that is conducive to tissue repair. Meanwhile, a set of cytokines, including IL-25, IL-33, TSLP, IL-25, IL-33, and TSLP are released by damaged epithelial cells, while other cells can directly or indirectly promote the development of Th2 immune response that promotes fibrosis (14). In previous studies, many researchers have discussed the specific immune pathways and immune cytokines that lead to fibrosis. However, few studies have directly investigated the relationship between the microbes in the uterine environment and the immune response induced by these microbes in the formation of IUAs. As far as we know, the pathological changes of IUA also affect the physiology and metabolites of the uterus, further affecting the diversity of vaginal microorganisms. Therefore, we investigated the association and interaction among IUA and microbes in the vagina. In this study, the data of 30 IUA patients and 30 healthy women were collected to analyze the correlation among vaginal microorganisms, immune cytokines, and the formation of IUAs.

## Methods

The trial enrolled 30 women aged between 20 and 40 years who were newly diagnosed with IUA during hysteroscopy examination at the Third Xiangya Hospital of Central South University between January 1<sup>st</sup>, 2018, and May 31<sup>st</sup>, 2018, IUAs were scored by one surgeon applying the American Fertility Society (AFS) classification system (15) (IUA group). IUAs were scored as follows: 1–4 (mild), 5–8 (moderate), and 9–12 (severe). Thirty women aged between 20 and 40 years who took routine gynecological health examination including hysteroscopy in the same hospital during the same period were recruited as the control group (non-IUA group). None of the participants had used vaginal medications, received cervical treatment, or performed douching within the previous 7 days, nor had they engaged in sexual activity within the previous 2 days. All participants had a history of uterine cavity operation, and none were

**Table 1** Demographic and clinical characteristics of the study groups

Basic clinical characteristics	VD (n=30)	CD (n=30)	P value
Age (year), mean ± SD	30.90±4.31	31.6±4.42	0.561
Height (m), mean ± SD	1.59±0.04	1.58±0.06	0.526
Weight (kg), mean ± SD	54.78±7.12	53.37±6.29	0.418
BMI (kg/m <sup>2</sup> ), mean ± SD	21.60±2.46	21.30±2.19	0.639
IUD, n (%)	0 (0)	0 (0)	NE
Other gynecological disease, n (%)	0 (0)	0 (0)	NE
Endocrine disorders, n (%)	0 (0)	0 (0)	NE

SD, standard deviation; BMI, body mass index; NE, not estimable (due to nullity of category in both groups); IUD, intrauterine device.

diagnosed with endocrine or autoimmune disorders, cancer, severe pelvic adhesion, hystero myoma, endometriosis, adenomyosis, or acute inflammation. All participants were subjected to two swabs by a doctor: one of leucorrhoea drawn from the middle of the vagina (marked zhong) and one of cervical mucus drawn from the cervical canal (marked kou) in the follicular phase of the menstrual cycle. All swabs were used to collect vaginal specimens which were immediately stored at -80 °C for DNA extraction, while anticoagulant blood vessels were centrifuged to separate the serum for cytokine detection.

DNA was extracted from 120 samples by using Fast DNA<sup>®</sup> SPIN Kit for Soil (MP Biomedicals) according to manufacturer's protocols, and the concentration and quality of purified DNA was determined via a spectrophotometer at 230 nm (A230) and 260 nm (A260; NanoDrop). Subsequently, the V3 and V4 region of the bacterial 16S rDNA gene was amplified by PCR using the following primers: 341FCCTACGGGNBGCASCAG/805R GACTA CNVGGGTATCTAATCC. PCR reactions were performed in a 25 µL mixture containing 5 µL of 5× GC Buffer, 0.5 µL of KAPA dNTP Mix, 0.5 µL of KAPA HiFi HotStart DNA Polymerase, 0.5 µL of each primer (10 pM), and 50–100 ng of template DNA. PCR reaction cycling included 95 °C for 3 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCR clean up used AMPure XP beads to purify the 16S V3 and V4 amplicon away from the free primers and primer dimer species. Purified product was amplified by PCR using the following

primers: 341F CCTACGGGNBGCASCAG/805R GACTACNVGGGTATCTAATCC, where the barcode was an eight-base sequence unique to each sample. PCR reactions were performed in 25 µL mixture containing 5 µL of 5× GC Buffer, 0.75 µL of KAPA dNTP Mix, 0.5 µL of KA-PA HiFi HotStart DNA Polymerase, 1.5 µL of each primer (10 pM), and 5 µL of purified product. PCR reaction cycling included 95 °C for 3 min, followed by 8 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The amplicons were subsequently purified by AMPure XP beads to clean up the final library before quantification. Last, purified amplicons were pooled in equimolar and paired-end sequenced (2×250) on an Illumina MiSeq platform according to the standard protocols.

Fast Length Adjustment of SHort reads (FLASH) was used to merge paired-end reads from next-generation sequencing (1). Low-quality reads were filtered by fastq\_quality\_filter (-p 90 -q 25 -Q33) in FASTX Toolkit 0.0.14, and chimera reads were removed by USEARCH 64 bit v8.0.1517. The number of reads for each sample was normalized based on the smallest size of samples by random subtraction. OTUs were aligned by the UCLUST algorithm with a 97% identity, and taxonomically classified using the SILVA 16S rRNA database v128. Alpha and beta diversities were generated in the Quantitative Insights Into Microbial Ecology (QIIME) and calculated based on weighted and unweighted Unifrac distance matrices.

Th1/Th2 cytokines in serum samples were quantified by Legendplex Assay (Human T helper Cytokine Panel, BioLegend). The correlation between Th cytokines and microorganisms was analyzed.

## Results

### Basic clinical characteristics of participants

Between January 1<sup>st</sup>, 2018, and May 31<sup>st</sup>, 2018, 60 women were recruited to either the IUA group (30 patients with IUA) or the non-IUA group (30 healthy women). Baseline characteristics between the two groups including age, height, weight, BMI, IUD, and other gynecological diseases and endocrine disorders, showed no statistical differences (Table 1). Two groups of participants had a 1- or 2-time history of induced abortion, AFS scores were moderate to severe in the IUA group patients (11 moderate IUA, 19 severe IUA), and no adhesions were diagnosed in the non-IUA group. Each participant was subjected to a vaginal

swab (marked zhong) and a cervical canal swab (marked kou); a total of 120 samples were available for high-throughput sequencing.

### Data quality control and optimization

Samples were distinguished according to barcode sequences, and the extracted data were stored in the standard fastq files. Data quality evaluation of sequencing samples was conducted according to the fastq file. The number of paired reads in the sample, the base ratio with Q value greater than 20, the mass value in the sample higher than 30, and the GC content of the sample data are listed in *Table 2*.

### Operational taxonomic unit (OTU) clustering and species notes

As indicated by the 97% sequence similarity level, Uclust method in the QIIME software package was used for OTU clustering analysis. The classification results of all the samples at the levels of phylum, class, order, family, and genus were analyzed. Based on the cumulative histogram of species in the top 15 of abundance, the microorganism composition differences among samples were compared and presented in a list. The community structure analysis results of the whole sample at the phylum and genus level are shown in *Figure 1*. At the phylum level, Firmicutes, Actinobacteria, Proteobacteria Bacteroidetes, and Fusobacteria constituted five of the most predominant phyla in the following regions: the cervical canal of the IUA group (69.55%, 11.05%, 6.85%, 8.40%, and 1.53%, respectively), the middle of the vagina of the IUA group (69.20%, 11.16%, 6.84%, 8.38% and 1.53%, respectively), the cervical canal of the non-IUA group (41.67%, 14.03%, 14.10%, 11.90%, and 8.61%, respectively), and the middle of the vagina of the non-IUA group (41.68%, 14.41%, 14.03%, 11.77%, and 8.61%, respectively). In the IUA group, the proportion of Firmicutes in most samples was more than 60%, while the diversity of bacteria was significantly reduced, with, for example, the Euryarchaeota, Acidobacteria, Chlamydiae, Chlorobi, Planctomycetes, and TM 6 (Dependentiae) phyla almost all disappearing. Statistical analysis also showed that the proportion of Firmicutes in IUA group was significantly higher than that in non-IUA group, and there were significant differences in *Lactobacillus*, *Cetobacterium* and *Bifidobacterium* ( $P < 0.05$ ) (*Figure 2*).

**Table 2** Sequencing sample data output and quality evaluation statistics

Sample	PE_reads	Q20 (%)	Q30 (%)	GC (%)
Sample (VD)				
A101kou	7702	97.66	95.89	51.99
A101zhong	137219	97.73	96.03	52.48
A102kou	129313	97.88	96.25	54.06
A102zhong	158034	97.87	96.20	53.62
A103kou	147018	97.90	96.33	53.48
A103zhong	137450	97.78	96.12	53.55
A104kou	144249	97.33	95.71	53.24
A104zhong	164513	97.69	96.09	53.66
A105kou	165721	96.78	95.12	52.15
A105zhong	122235	97.71	96.09	51.85
A55kou	135442	97.56	95.90	51.66
A55zhong	118568	97.77	96.08	50.83
A56kou	159992	96.29	94.62	51.86
A56zhong	149826	97.66	96.00	52.42
A57kou	138006	97.81	96.25	51.85
A57zhong	139832	97.76	96.15	52.04
A59kou	147775	97.85	96.25	52.06
A59zhong	135591	97.71	96.09	51.97
A62kou	144575	97.75	96.15	52.25
A62zhong	124962	97.88	96.26	51.82
A63kou	148910	97.85	96.28	51.97
A63zhong	127930	96.90	95.26	51.82
A65kou	140301	97.43	95.86	51.85
A65zhong	136975	97.88	96.29	52.00
A67kou	190662	97.38	95.84	52.56
A67zhong	180826	93.56	91.86	51.88
A68kou	146383	97.73	96.17	52.39
A68zhong	137618	97.87	96.30	52.06
A74kou	162131	97.02	95.37	52.45
A74zhong	139848	97.48	95.84	52.19
A75kou	219948	90.84	88.99	51.31
A75zhong	151254	97.83	96.22	52.13
A77kou	163859	97.35	95.79	51.97
A77zhong	149796	97.64	96.09	52.25
A78kou	153324	97.76	96.15	52.23
A78zhong	146487	97.81	96.18	52.01
A82kou	145602	97.74	96.15	52.06
A82zhong	166547	97.01	95.36	52.14
A84kou	152770	97.56	95.94	52.31

*Table 2 (continued)*

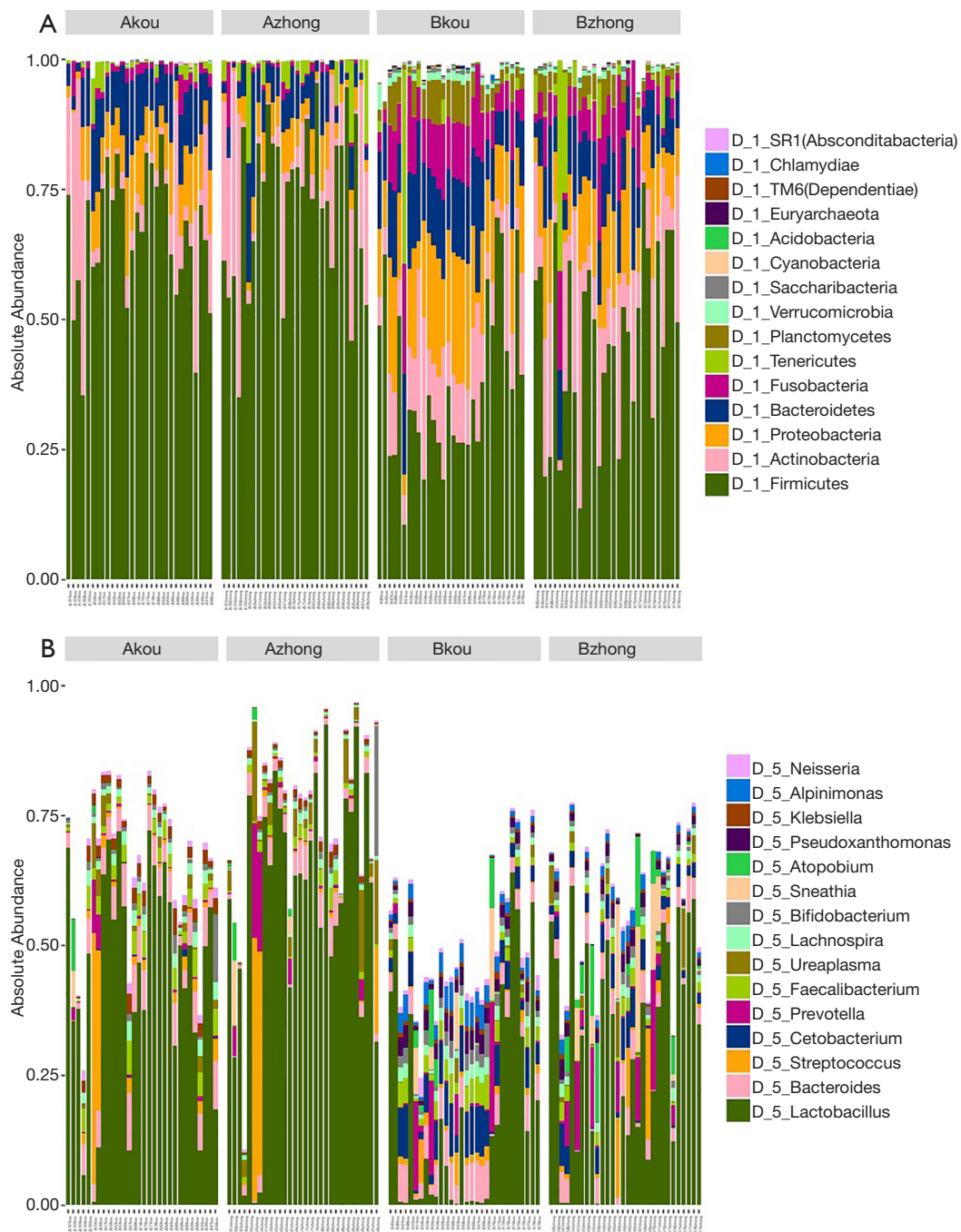
Table 2 (continued)

Sample	PE_reads	Q20 (%)	Q30 (%)	GC (%)
A84zhong	153188	98.01	96.46	52.19
A85kou	151276	97.99	96.48	52.16
A85zhong	150678	97.68	96.06	52.35
A86kou	168598	97.40	95.81	52.35
A86zhong	178303	97.71	96.09	51.81
A88kou	163560	97.82	96.21	53.23
A88zhong	142423	97.77	96.13	53.22
A89kou	142954	97.30	95.68	52.13
A89zhong	150183	97.80	96.15	51.65
A90kou	133372	97.63	95.97	52.20
A90zhong	179171	97.77	96.11	52.52
A92kou	170181	97.34	95.74	52.44
A92zhong	160550	97.84	96.19	52.04
A95kou	89529	97.33	95.71	53.15
A95zhong	137312	96.80	95.14	51.98
A96kou	170898	97.51	95.90	52.19
A96zhong	147055	97.93	96.36	51.92
A97kou	145110	97.80	96.15	52.36
A97zhong	134741	97.87	96.19	52.65
A98kou	176908	97.61	96.05	52.71
A98zhong	138250	97.86	96.23	52.57
Sample (CD)				
B45kou	164485	91.62	89.74	50.67
B45zhong	106021	97.15	95.44	52.14
B46kou	158701	95.22	93.53	51.15
B46zhong	152103	96.23	94.57	52.07
B47kou	196685	94.39	92.70	52.07
B47zhong	184698	91.86	90.05	51.79
B48kou	175716	91.76	89.95	51.50
B48zhong	137551	92.64	90.93	52.22
B50kou	142384	94.40	92.65	51.91
B50zhong	139164	93.14	91.36	51.38
B51kou	144403	96.19	94.63	49.57
B51zhong	121438	97.86	96.29	49.11
B53kou	174179	84.30	81.95	50.05
B53zhong	122428	93.59	91.86	51.52
B54kou	182883	94.45	92.76	51.91
B54zhong	158752	92.72	90.99	51.09
B55kou	165246	96.89	95.26	53.00
B55zhong	133782	97.77	96.20	53.35
B58kou	140464	94.06	92.32	52.29

Table 2 (continued)

Table 2 (continued)

Sample	PE_reads	Q20 (%)	Q30 (%)	GC (%)
B58zhong	159405	96.00	94.37	53.23
B59kou	172022	95.80	94.15	52.16
B59zhong	139824	95.67	94.03	51.87
B60kou	158457	93.91	92.25	51.89
B60zhong	136713	95.88	94.31	51.70
B62kou	143449	93.70	91.95	51.94
B62zhong	165476	94.18	92.43	51.86
B63kou	135867	95.00	93.34	51.52
B63zhong	158737	96.24	94.60	51.03
B64kou	174318	95.00	93.27	52.07
B64zhong	142343	95.13	93.43	52.01
B65kou	185074	90.67	88.80	51.14
B65zhong	146121	93.47	91.63	51.90
B66kou	174426	93.47	91.75	51.75
B66zhong	203522	92.99	91.22	51.39
B67kou	181635	92.17	90.38	51.42
B67zhong	122453	97.03	95.43	52.32
B68kou	141852	94.35	92.65	51.83
B68zhong	139997	94.76	93.07	51.80
B69kou	144565	87.20	85.12	51.02
B69zhong	232848	86.72	84.62	50.57
B70kou	101082	96.37	94.77	52.70
B70zhong	162161	97.73	96.12	52.92
B71kou	150398	91.81	90.01	51.31
B71zhong	123454	95.50	93.78	51.09
B72kou	161306	96.11	94.47	51.73
B72zhong	153212	96.97	95.37	51.75
B73kou	188604	91.29	89.43	51.17
B73zhong	165029	84.44	82.28	50.15
B74kou	179825	96.50	94.99	52.05
B74zhong	326549	83.51	81.04	49.50
B75kou	128424	96.36	94.75	52.17
B75zhong	168641	96.71	95.06	52.20
B76kou	188426	89.99	88.11	51.40
B76zhong	131517	97.40	95.77	52.65
B77kou	174054	88.58	86.67	50.70
B77zhong	108945	92.95	91.21	51.22
B78kou	164436	93.73	91.99	51.43
B78zhong	141726	95.54	93.62	51.84
B79kou	186324	90.85	88.99	51.55
B79zhong	166931	88.20	86.19	50.97

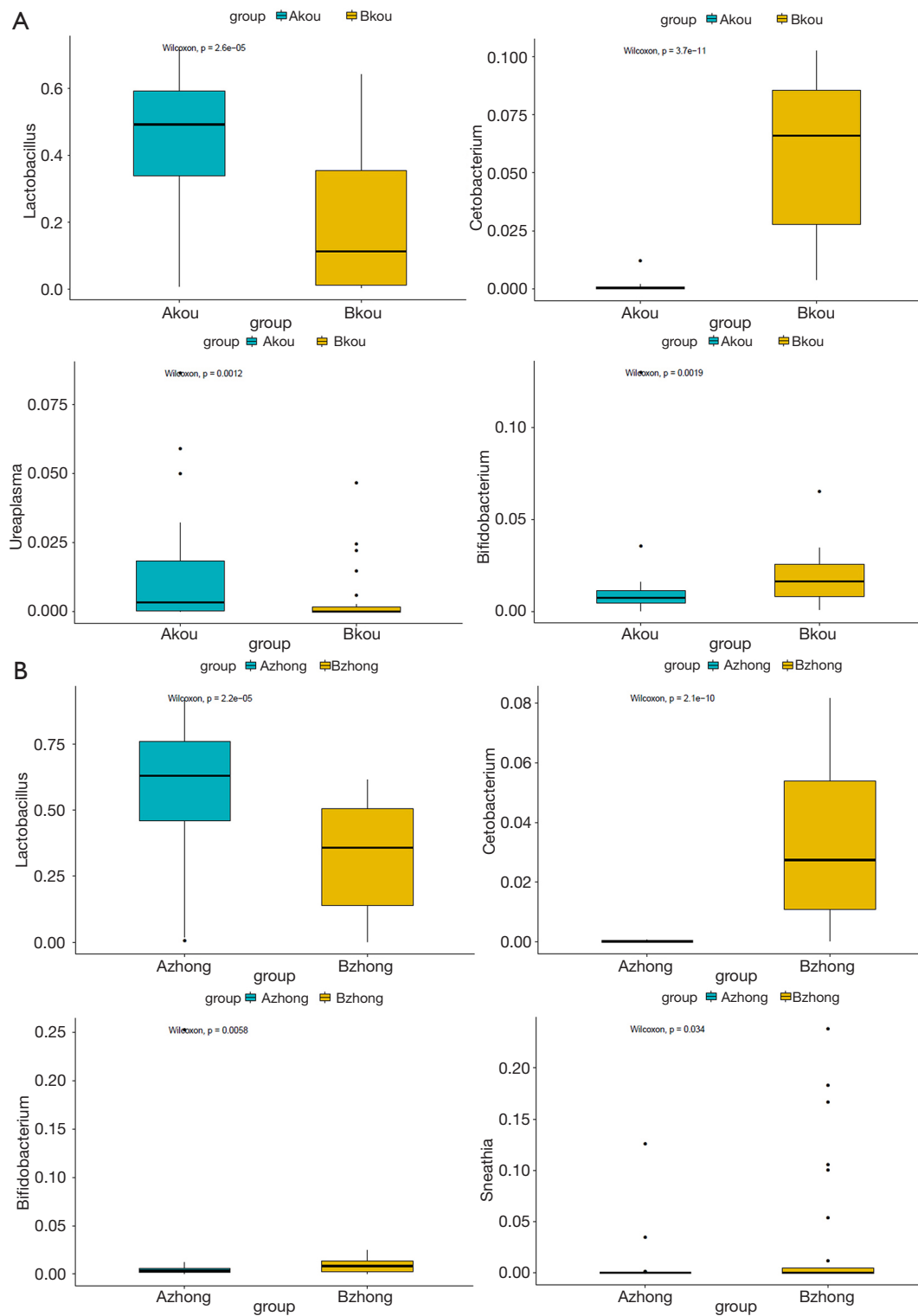


**Figure 1** The cumulative histogram of species in the top 15 of abundance at the phylum (A) and genus (B) level.

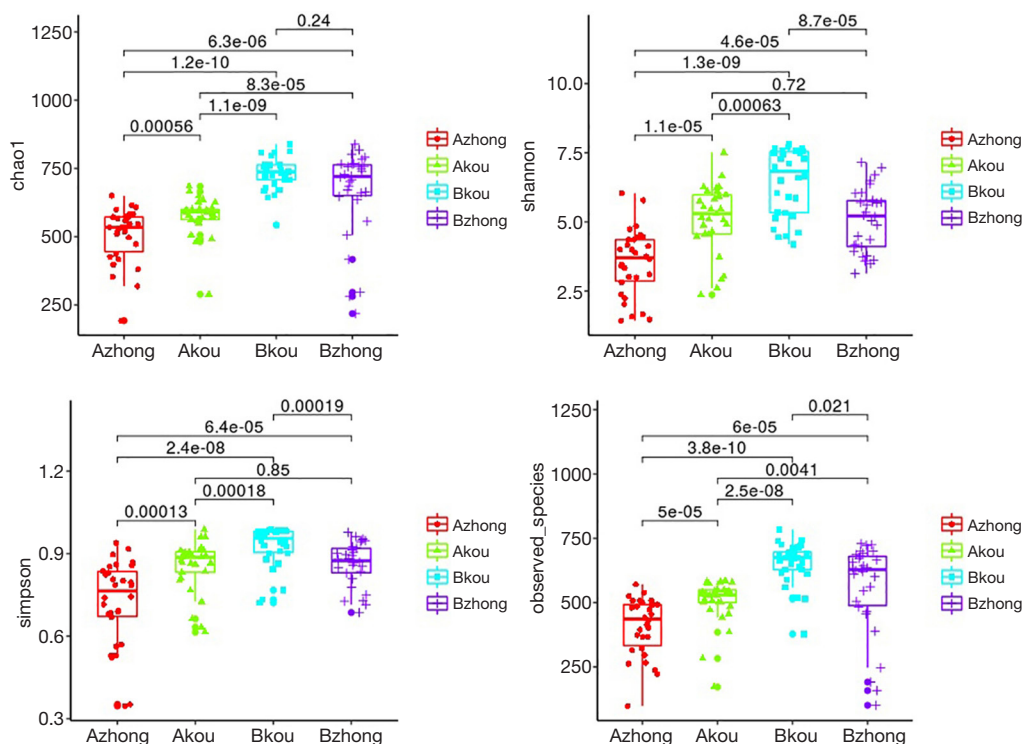
### *Alpha diversity analysis*

The Alpha diversity index includes the species abundance (chao1, ACE), the diversity index of integrating species abundance and uniformity (Shannon, Simpson), and the observed species, which reflects the low OTU coverage

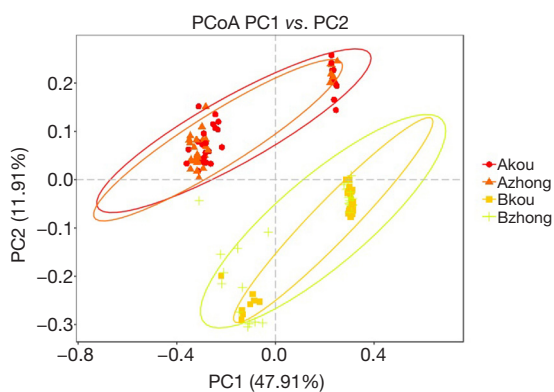
of samples in Good's coverage and OTU number. Among them, abundance measures species in a single sample by estimating the OTU number, while the diversity index measures the heterogeneity of communities. As shown in *Figure 3*, the species diversity in the middle vagina and cervix of the IUA group was significantly lower than that of



**Figure 2** Significant differences of species between the IUA and non-IUA groups in the vagina (A) and cervical canal (B) at the phylum level. IUA, intrauterine adhesion.



**Figure 3** Alpha diversity analysis in the middle vagina and cervix of the IUA group was significantly different from that of the non-IUA group. IUA, intrauterine adhesion.



**Figure 4** PCoA of Beta diversity analysis for the abundance and composition of species in the vagina and cervix between the groups.

the non-IUA group, and the species diversity in the middle vagina of the IUA group was the lowest ( $P < 0.001$ ).

### Beta diversity analysis

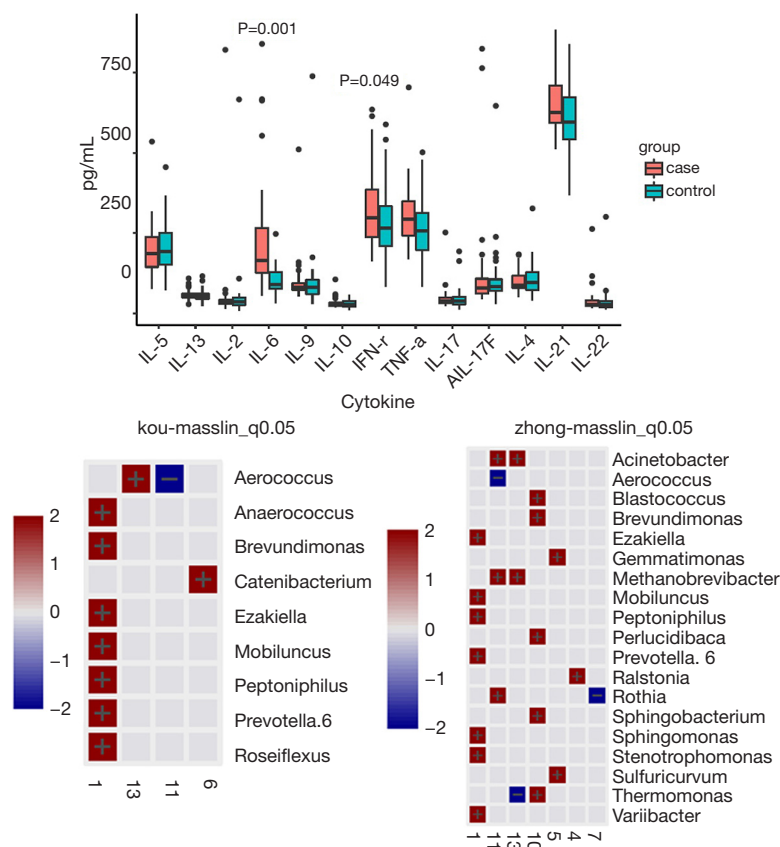
The principal coordinate analysis (PCoA) was based on the

distance matrix to find the principal coordinates. Through a series of eigenvalues and eigenvectors following sorting, the top several main characteristic values were selected to effectively find the “main” elements and structure between the two groups of sample data visualization of similarities or differences (Figure 4). The abundance and composition of species in the vagina and cervix were more similar in the same group, but the difference between the groups was significant.

### Differences of cytokines in the IUA and non-IUA groups

The cytokine kit quantitatively detected 13 human cytokines secreted by Th1/Th2 immune cells, including IL-2, 4, 5, 6, 9, 10, 13, 17A, 17E, 21, 22, along with IFN- $\gamma$ , and TNF- $\alpha$ . IL-6 and IFN- $\gamma$  were significantly different between the two groups, with the IUA group being significantly higher than the non-IUA group ( $P < 0.05$ ). Correlation analysis was conducted between immune cytokines and microbial genera, and the correlation heat map is shown in Figure 5. At the genus level, Aerophilus, Brevundimonas, Ezakiella, Mobiluncus, Peptoniphilus, and Prevotella 6 were highly





**Figure 5** Correlation heat map between immune cytokines and microbial genus level.

correlated with cytokines.

## Discussion

With the wide application of hysteroscopy in clinical practice, the incidence and diagnosis rate of IUA in females of childbearing age has sharply increased to 2.2–36.8% (16,17). Iatrogenic endometrial trauma is the primary condition of IUA, which is mainly caused by curettage during pregnancy. It has been reported that more than 90% of IUAs are caused by curettage (18,19). Other causes include removal of pregnancy residues, cesarean section, diagnostic curettage, myomectomy, and correction of uterine anomalies. Taskin *et al.* reported that IUA could be formed after hysteroscopic myomectomy, with a probability of 31.3% (20). If multiple myomectomy was performed, the incidence of IUA would rise to 45.5%, and 6.7% after hysteroscopic resection of a septum. Although hysteroscopic adhesiolysis (HA) has been widely used in clinic (21), the high recurrence rate of IUA and poor pregnancy outcomes

after HA present a great challenge for clinical treatment and prevention (22).

To date, researchers have studied the role of microorganisms in the lower reproductive tract in reproductive system tumors, vaginitis, adverse pregnancy, and other diseases (23–29); however, the interaction between vaginal microbiota, immune response, and IUA has not been investigated.

Microorganisms are the richest source of biodiversity on earth. The investigation of microbial diversity has important theoretical and practical significance for human diseases, environmental governance, and the exploitation and utilization of microbial resources. The human-coexisting microorganisms have important influences on human growth and development, physiology and pathology, immunity, nutrition metabolism, and other processes (30–35). Most of these microbiotas have a mutually beneficial relationship with their human hosts, and only a few opportunistic pathogens can cause chronic infections or other serious diseases under certain conditions. These

microbial communities are also considered the body's first line of defense against foreign pathogens, competing to eliminate other invasive pathogens. As is well known, the symbiotic microorganisms are very important to the human body, but incredibly, people know little about the community composition and function of the microorganisms. Furthermore, we barely understand how microorganisms interact with each other, or how they can form a balanceable ecoenvironment. At present, many investigators are focusing on the research of human microorganisms, in order to get a better idea of the real role of these microorganisms in human health and disease (36).

There are many kinds of microorganisms in the vagina, and 29 kinds of microorganisms have been isolated from vaginal secretions (37-39). The most important one is lactobacillus (40,41), and the other microflora present in the vagina are mainly composed of bacteria, fungi, protozoa, and virus. Under normal circumstances, the microbes in the vagina can reach an equilibrium state. Any factors that cause vaginal microecological imbalance can lead to inflammation, such as long-term application of antibiotics, frequent intercourse, vaginal flush, and a decline in estrogen (42). The balance of vaginal microecology can effectively resist the invasion of various foreign pathogens. The female reproductive tract is highly susceptible to bacterial infection. Once the immunity of the body is reduced, foreign bacteria or parasitic bacteria in the body may increase infection, causing cervicitis, endometritis, etc. (43). The main cause of IUA is injury to the endometrium associated with reproductive tract infection. Patients with IUA are usually infected with a variety of reproductive tract pathogens. Once these pathogens invade the vagina, they can enter the uterine cavity through the cervix, causing endometritis, salpingitis, or pelvic inflammatory disease. Abortion preoperative cervicitis or endometritis in the case of no cure pre-abortion can lead bacteria into the uterine cavity and then cause intrauterine infection or increase the chances of IUA formation. In this study, compared with the control group, patients with IUA had a higher bacterial load and density, but less diversity in the vagina/cervix, a condition which may cause persistent damage by pathogens in the impairment of the endometrium. Some of the bacterial communities in IUA patients that disappeared in our study may play an important role in maintaining the health of the reproductive tract, and their absence may lead to a compromised repair function of the endometrium. After the iatrogenic injury of the endometrium occurred, the load of the opportunistic bacteria increased and the

essential bacteria became absent, so that the normal repair function of endometrium tissue was reduced and replaced by fiber repair which lost normal function.

To our knowledge, tissue injury and inflammation are important causes of tissue fibrosis. This study focused on the pathways from tissue damage and inflammation to fibrosis. The vaginal immune function is a component of the systemic immune function, including humoral and cellular immunity. The functions of systemic and local immunity jointly form an immune response network. When the local mucosa is attacked by pathogens, the immune response will quickly be generated in the blood and other mucosal sites. Some immune mediators released by immune T cells have both profibrotic and antifibrotic properties. After iatrogenic damage to the endometrium, pathological biological bacteria of the vagina and cervical canal invade into the endometrium, causing local inflammation and triggering the immune response of the endometrium, which will eliminate some pathogenic microorganisms and further change the composition of bacteria. Endometrial injury destroys the function of the surface barrier and increases the entry of harmless symbiotic bacteria or their products into the endometrium, resulting in the aggravation of the inflammatory response and fibrosis repair of the endometrium. Immune Th cells can be divided into two subgroups according to different functions, TH1 and TH2 cells, which play an important role in Th response. For inflammatory diseases, most available literature shows that Th1 expression is weak, while Th2 expression is strong, leading to the imbalance of TH1 with TH2. Cytokines secreted by immune Th cells are key regulators of immune responses to health and disease. As mentioned above, damaged endometrial epithelial cells release a series of immune cytokines, which directly or indirectly promote the development of Th2 immune response that is thought to promote fibrosis (44). The innate immune cells can release classical proinflammatory cytokines IL-6, IL-1, and TNF- $\gamma$  which are also profibrotic. In our study, the IL-6 and IFN- $\gamma$  quantities in the serum of IUA patients were significantly higher than those of the non-IUA women ( $P < 0.05$ ). In repeated peritonitis models, inflammatory Th1 cells and cytokines IL-6 and IFN- $\gamma$  are essential factors in the occurrence and development of fibrosis (45). It was found that cytokine IL-6 increased the Th1 cell survival rate and up-regulated IFN- $\gamma$  production, while negative feedback decreased MMP activity. Therefore, the fibrogenic effect of IL-6 is mainly due to the reduced degradation of matrix protein. Other studies have explained the role of IL-6

in promoting fibrosis by regulating the TGF- $\beta$  pathway (46,47). Profibrotic effects of IL-6 were observed in fibrosis models of different organs, including the lung (48,49), heart (50,51), kidney (47), and eye (52).

Microorganisms play a role in mediating inflammation/immune response to trigger related pathways, a phenomenon which has become an area of intense research. IUAs seriously endanger the reproductive health of childbearing females, and its pathogenesis is complex. Therefore, it is important to study the pathogenesis of IUAs and to find effective treatment. We analyzed the differences of vaginal microbiological structure between patients with IUA and healthy women. Our study found that vaginal microbial imbalance can cause endometrial inflammation and inflammation factors or related cytokines released in great quantities. Proinflammatory cytokines IL-6 and IFN- $\gamma$  were massively increased in IUA patients and played a role in promoting fibrosis, which in turn caused fibrinogen accumulation, extracellular matrix deposition, and eventually induced IUAs. A comprehensive understanding of microorganisms and immune function will allow us to better clarify the role of microorganisms and immune function in the development of IUA. From a new perspective of vaginal dysbacteriosis mediating a Th1-Th2 imbalance, the mechanism of IUA was revealed. Moreover, it was found that fibrosis could be successfully prevented by inhibiting pathogens, inflammatory factors, and cytokines. These findings provide a new platform for the prevention and treatment of IUA targeted at microbial and cytokine intervention.

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### Footnote

**Conflicts of Interest:** 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

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