Microbiota in Uterine Cavity of Unexplained Recurrent Spontaneous Abortion (URSA) Patients in Early Pregnancy

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Research article

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Brief title:

Microbiota in early pregnant uterine cavity of URSA

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Microbiota in uterine cavity of unexplained recurrent spontaneous abortion (URSA) patients in early pregnancy

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Capsule：

Changes of *Lactobacillus* and *Curvibacter* dominated will lead to bad pregnancy outcome.

Abstract

Background: The majority of unexplained recurrent spontaneous abortion (URSA) was related to immune abnormalities. Inappropriate changes of microbiota could cause immune disorders. However, the role of uterine cavity microbiota in URSA has not be elucidated and few related studies are available for reference.

Methods: Using the double-lumen embryo transfer tubes to collect uterine cavity fluid samples from pregnant women in their first trimester. 16S rRNA sequencing was conducted to analysis the composition and abundance of the microbiota in samples.

Results: We enrolled 10 URSA cases and 28 induced miscarriage cases in their early pregnancy. Microbial communities were detected in all samples of URSA group (100%, n = 10) vs. none of control group (0%, n=28). Two most dominant microbes are *Lactobacillus* and *Curvibacter*.

Conclusions: This study showed *Lactobacillus* and *Curvibacter* dominated colonizing in uterine cavity of URSA patients during early pregnancy and associated with URSA. Changes of dominant microbiota will lead to bad pregnancy outcome.

Keywords: unexplained recurrent spontaneous abortion, microbiota, 16S rRNA sequencing

Background

The human microbiota named the "second human genome" play a prominent role in human health and become the focus of scientific research with the assistance of gradual development of genome sequencing techniques(1). The human body and the human microbiota have become a symbiotic relationship and complex interaction occurs in almost every part of the body. The uterine cavity has always been considered a classic sterile cavity(2), contrary to this notion, a lot of studies showed that there were special microbial communities colonization in there(3-7). At the same time, the impact of the microbiota on reproductive system made us stand on the higher level to understand them.

Data have showed that the alterations of *Lactobacilli* dominance in uterus were linked to inflammation which result in spontaneous preterm birth or other adverse obstetric outcomes(1). A recent study suggested that the microbiota composition in endometrial fluid of patients receiving in vitro fertilization and embryo transfer (IVF-ET) may link to implantation and pregnancy rates(5, 8). However, the role of uterine cavity microbiota in URSA has not be elucidated and few related studies are available for reference. Therefore, the intention of our study was to determine the uterine cavity microbial composition in URSA patients and to understand the correlation between URSA and uterine cavity microbiota.

Methods

Participants and sample collection

A total of 38 pregnant women in their early pregnancy who came to Sun Yat-sen Memorial Hospital of Sun Yat-sen University were enrolled in our study. Then we divided them into two groups according to entry requirements (Figure 1). Those that meet the following conditions served as control group: needed an induced abortion for personal reasons this time and had no abortion history, no history of uterine cavity operation or vaginal infection on the day of surgery (n=28). B-ultrasound indicated that embryo was alive and there was no bleeding in uterine cavity or in vagina (Figure 1A). Others (n=10) were enrolled in URSA group: who were diagnosed as URSA, defined as the loss of ≥3 consecutive pregnancies before 24 weeks' gestation(9) and the

embryo had stopped development this time but had no bleeding in uterine cavity or in vagina through B-ultrasound. The chromosomal karyotype of the aborted embryos in the URSA group were normal. They all needed uterine curettage and had no vaginal infection on the day of surgery. After disinfection the vagina and cervix, excess liquid was cleansed by insertion cotton buds. Then a double-lumen embryo transfer tube (Cook Medical) was used to collect uterine cavity fluid carefully(9). When the outer sheath of the tube reached the junction between the endocervical canal and the uterine cavity, the inner tube was advanced into the uterine cavity for sample collection (Figure 1B). 8-10 μl uterine cavity fluid was transferred to a cryopreservation tube containing 1 ml of RNase- and DNase-free water (Thermo Fisher Scientific) and snap-frozen in liquid nitrogen and then transferred to -80°C for future analysis.

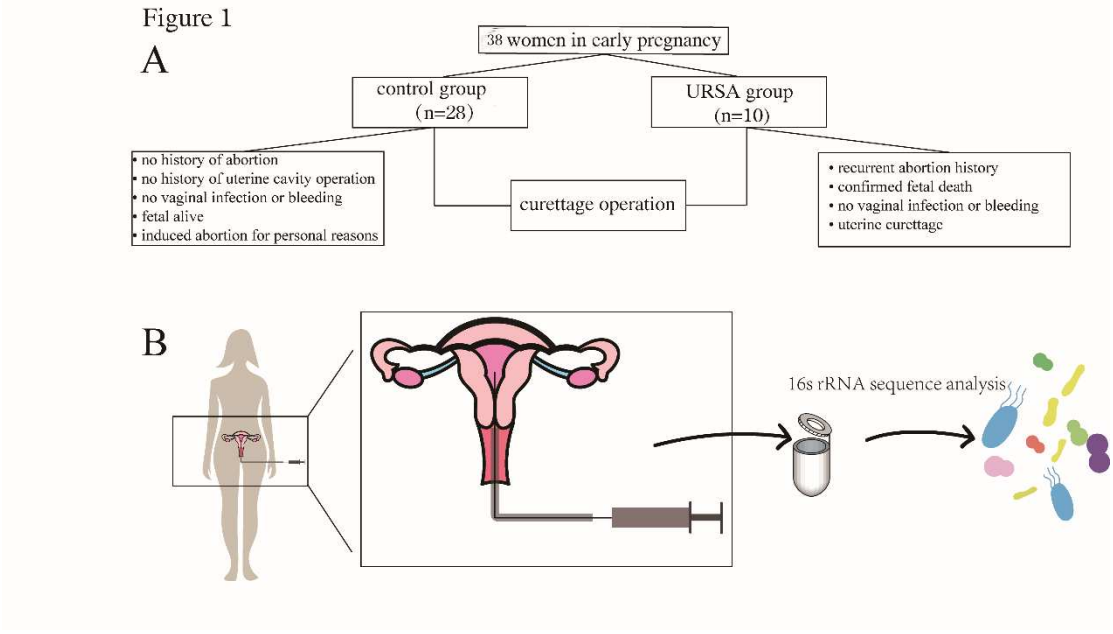

Figure 1. The enrolled criteria and sample collection method. (A) The workflow and enrolled criteria. (B) When the outer sheath of the tube reached the junction between the endocervical canal and the uterine cavity, the inner tube was advanced into the uterine cavity for collection 8-10 μl uterine cavity fluid. Then transferred the sample to a cryopreservation tube containing 1ml of RNase- and DNase-free water for future analysis.

Genomic DNA extraction and 16S rRNA sequencing

Isolation of genomic DNA from frozen uterine cavity fluid sample and the V3-V5 region of the 16S rRNA gene sequencing was completed at LC Sciences (Hangzhou, China) according to their standard service workflow. In brief, genomic DNA was extracted by QIAmp DNA mini-kit (Qiagen, USA). After 16S rRNA gene amplifcation by PCR following the Illumina protocols, the amplicons were sequenced using MiSeq® Reagent Kit v3 (Illumina) on a MiSeq-Illumina platform (Lifesequencing sequencing service, Valencia, Spain). PCR amplifcation and libraries controls were sequenced as negative controls to reduce sequencing error rate. We also used RNase- and DNase-free water as a blank control to remove background noise which may have impacted microbiota results.

Bioinformatics and data analysis

All the sequences were clustered into operational taxonomic units (OTU) based on their sequence similarity using QIIME, setting the sequence similarity threshold to 0.97. Singletons and

OTUs with a relative frequency below 0.01 were removed. Shannon was used to analysis Alpha diversity using QIIME. Beta diversity was done using Bray-Curtis distance and principal component analysis (PCA). Calypso software version 8.10.was used to perform the total statistical analysis on bacterial taxonomic identification.

Statistical analysis was performed using Graphpad Prism 8.0, and the measurement data were expressed as mean ± standard deviation (X ± SD). The mean comparison between groups was performed by t-test. $P<0.05$ was considered to be statistically significant.

Results

The general characteristics of the participants were listed in Table 1 including age, gestational week, history of uterine cavity operation and reproductive tract infection. The mean age of control group was 28.96 years old, which was much younger than URSA group (P=0.0272). Significant differences were not found in gestational week (5.89 ± 0.19, n=28 vs 6.46 ± 0.39, n=10, P=0.1422). None of the control group had the uterine operation history while all participants of the URSA group had history of uterine cavity operations such as uterine curettage, hysteroscopy, etc. None participant had bacterial vaginosis before surgery. 70% of URSA group ever had a live birth.

Table 1 General characteristics of participants

	control group(n=28)	URSA group(n=10)	*P* value
age(years), mean ± SD	28.96±1.10	33.08±1.13	0.0272
Gestational week(weeks), mean ± SD	5.89±0.19	6.46±0.39	0.1422
History of uterine cavity operation,no.(%)	0 (0%)	10 (100%)	-
Reproductive tract infection,no.(%)	1 (3.57%)	0 (0%)	-
Ever pregnant,no.(%)	14 (50%)	10 (100%)	0.005
Ever had a live birth,no.(%)	9 (32.14%)	7 (70%)	0.037
History of miscarriage,no.(%)	0 (0%)	10 (100%)	-

Surprisingly, all uterine cavity fluid samples of URSA group (n=10) showed positive detection of microbial communities by 16s rRNA sequencing analysis, while none sample showed positive detection from control group (Figure 2). After qualify negative sample, a total of 488,380 reads were obtained. The average of operational taxonomic units (OTUs) per uterine cavity fluid sample observed was 90.5. The valid reads were assigned to 84 OTUs in URSA group. The Alpha diversity measured by Shannon diversity, Chao 1 index and Simpson index were shown in Table 2.

Next, we analyzed the microbial communities. As seen in Figure 3, we found that *Firmicutes*was the most dominant phylum in samples of URSA 4, 7, 8 and 9 (74.02, 97.49, 55.28 and 81.09%; respectively); followed by *Proteobacteria* which was the most dominant phylum in samples of URSA 5, 10 (64.09, 81.22%). *Deinococcus-Thermus* was the most abundant in samples of URSA 3, 6 (96.92, 93.55%). *Actinobacteria* (67.92%) was dominant in samples of URSA 1. We further analyzed the relative abundance of genus in each sample. As shown in Figure 4, *Lactobacillus* were higher in URSA 4, 7, 8, 9 (72.50, 96.83, 52.68, 79.29%; respectively) and *Ureaplasma* were higher in URSA 3, 6 (96.92, 93.55%). Obviously, *Curvibacter* was the subdominant bacteria.

Table 2 Microbiota community α diversity with URSA samples

Samples	Number of raw reads	Number of valid reads	Observed OTUs	Chao 1 index	Shannon index	Simpson index
URSA1	87517	76526	78	78.20	3.53	0.80
URSA2	80588	77217	60	61.75	1.00	0.28
URSA3	83582	80623	105	65.80	1.28	0.52
URSA4	52897	46516	111	111.00	3.13	0.70
URSA5	82413	73825	113	116.00	5.70	0.95
URSA6	85788	82415	51	53.14	1.46	0.56
URSA7	83205	78248	105	110.08	1.11	0.28
URSA8	83032	70919	91	91.00	3.59	0.74
URSA9	86105	72305	96	96.00	1.87	0.38
URSA10	84602	77064	79	79.33	4.91	0.93

Table 3 the abundance Top 3 bacteria in genus level and the gestational week of surgery or embryo damage of all samples

Sample	Gestational week of surgery or embryo damage(weeks)	Top 1 bacteria	Top 2 bacteria	Top 3 bacteria
URSA1	5	Gardnerella	Curvibacter	Acinetobacter
URSA2	6	Sneathia	Anaerococcus	Ureaplasma
URSA3	6	Ureaplasma	Lactobacillus	Curvibacter
URSA4	6+	Lactobacillus	Curvibacter	Gardnerella
URSA5	6+	Curvibacter	Ralstonia	Prevotella
URSA6	7	Ureaplasma	Lactobacillus	Gardnerella
URSA7	7+	Lactobacillus	Curvibacter	Bradyrhizobium
URSA8	8+	Lactobacillus	Curvibacter	Acinetobacter
URSA9	8+	Lactobacillus	Curvibacter	Sphingobium
URSA10	9+5	Curvibacter	Bradyrhizobium	Rubellimicrobium

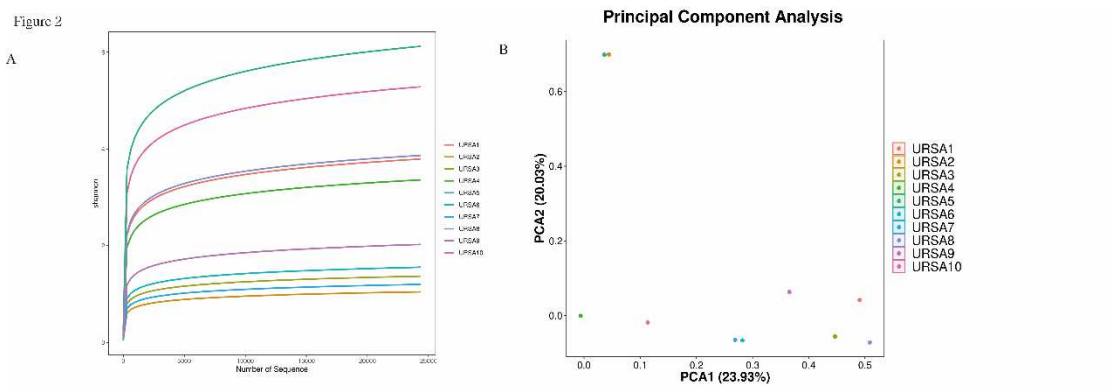

Figure 2. 16S rRNA sequencing analysis of microbial communities. (A) Alpha diversity was measured by Shannon index. (B)Beta diversity was showed by principal component analysis (PCA).

Figure 3

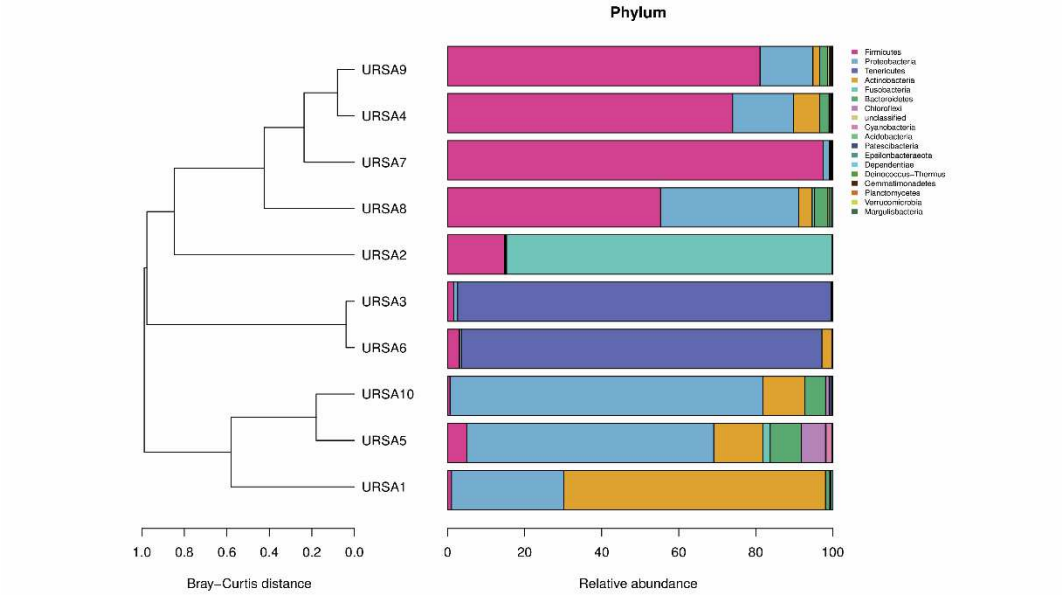

Figure 4

Figure 3. The composition of microbiome in Phylum level of all samples.

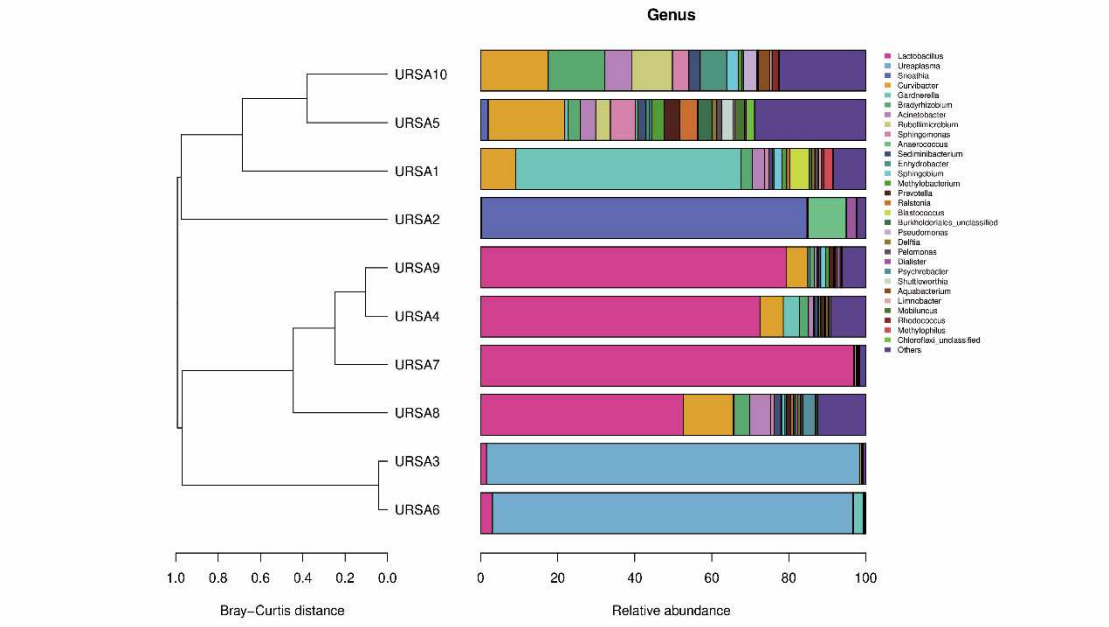

Figure 4. The composition of microbiome in Genus level of all samples.

Then, we listed the Top 3 abundant bacteria in genus level and the gestational week of embryo damage of all samples (Table 3). *Lactobacillus* or *Curvibacter* was the most dominant in most samples. The gestational week of embryo damage of samples which dominated by *Lactobacillus* was later than that dominated by other bacteria from URSA group (7.25±0.48 vs 6.50±0.56, P=0.375).

Discussion

To the best of our knowledge, this is the first study to detect the microbiota in uterine cavity in first-term pregnancy. Due to the limited availability of materials, most studies focused on the microbial communities in uterine cavity in the unpregnant state(3, 7, 8, 10, 11). And some other studies put their attention on the microbiome of amniotic fluid and placenta(10, 12, 13). Therefore, the status of the uterine cavity microbiota in pregnancy has yet to be elucidated.

Embryo chromosomal abnormalities were one of the most relevant causes of miscarriage, so our karyotype analysis of aborted embryos in the URSA group eliminated this important confounding factor. To further ensure the credibility of our findings, we attempted to rule out the possibility of sample contamination from cervix and vagina meticulously. After disinfection before surgery, we used a sterile and sleeved embryo transfer tube for sample collection. In order to avoid contamination of blood, the patient would be rejected once she had vaginal bleeding or bleeding in the uterine cavity through B-ultrasound. Therefore, the possibility of false positives in our results was very minimal.

In our study, we recognized that, in first-term pregnant uterine cavity of URSA women whose embryo stopped developing, there were microbiome such as *Firmicutes, Proteobacteria, Deinococcus-Thermus, Lactobacillus,* etc. It is in line with several previous studies which focused on microbiome colonization in female upper genital tract (6, 7). Altered microbiota in uterine cavity may be associate with URSA. A study showed that there were distinct differences in the relative abundance of microbial groups between URSA and control group on family and genus levels. URSA samples showed increased abundance of *Firmicutes*, which was consistent with our research results(9). *Lactobacillus* was a dominated resident microbiota in healthy endometrium and several studies detected it may be associated with infertility, endometriosis, chronic endometritis, endometrial polyps and dysfunctional menstrual bleeding(14, 15). It also was the most dominated microbiota in the vagina. And Brown et al. found that reduced relative abundance of *Lactobacillus* is associated with premature cervical dilation(16). We compared the cases and found that the gestational week of embryo damage of URSA patients whose uterine cavity dominated by the bacteria was later than others. This may indicate *Firmicutes*-*Lactobacillus* domination may be conducive to embryo development. We did not find *Lactobacillus* colonized in control group and we thought that they may only exist in unpregnant uterine cavity and disappear during early pregnancy in normal females.

Mounting studies showed that *Ureaplasma* was a common microbiota which contribute to bad pregnancy outcomes like preterm delivery and chronic inflammation of the productive tract(17-19). A recent review focused on the correlation between *Ureaplasma* exposure and the important morbidities of prematurity and got conclusion that *Ureaplasma* was attributed to neonatal morbidities of bronchopulmonary dysplasia, intraventricular hemorrhage, and necrotizing enterocolitis(20). Another pilot study showed that infertile women were more likely had *Ureaplasma* in the vagina(21). In our study, *Ureaplasma* was dominant in URSA 3 and 6, and the Top 3 highest abundance in URSA 2. Therefore, we speculate that increased abundance of

Ureaplasma may lead to worse early pregnancy outcomes. We guessed that different dominant microbiome will lead to different pregnancy outcome. In some samples of URSA group, we also found some other reproductive tract pathogens like *Gardnerella*, *Anaerococcus*, etc. The most likely reason was that these patients had multiple uterine cavity operations before, and these pathogens ascended through the lower reproductive tract and then colonized in the uterine cavity, which had a negative impact on embryo implantation and development.

Curvibacter being dominant or predominant microbe was detected in almost all samples of URSA group in our study. It was previously isolated from various aqueous environments and had identified in atherosclerotic plaques(22). *Curvibacter* was first reported existed in the amniotic fluid and vaginal fluid microbiota of healthy pregnant women in a recent study (23). But there was no *Curvibacter* existed in the control group in our study, so whether it be harmful to pregnancy still need to discuss in the future.

It has been suggested that fetus development needs a fertile environment(24). Interestingly, in our study, all samples in control group were sterile of microbiota. A study showed that 40% of the endometrial samples obtained by abdominal hysterectomy detected no microbiome(25). This exciting phenomenon raises doubts whether uterine microbiome colonization in all early pregnant women or just in URSA women, whether the detected microbiota is in the uterus temporarily or permanently, or whether they are just contamination(26). Therefore, in the near future, we will concentrate on a series of related work to verify our assumption that there may be a "microbiota-containing time window" in the uterine cavity in the early pregnancy. As the pregnancy progresses, the uterine cavity gradually changes from a microbiota-containing state to a sterile state. In the latter part of pregnancy, it becomes a bacterial fertile state again. Perhaps, lengthened or shortened "microbiota-containing time window" may lead to severe pregnancy outcomes.

Our research still has some limitations should be considered. Firstly, in the materials of 28 uterine cavity fluid samples of control group, none sample was microbiome positive that lead to statistical analysis of differences of diversity and abundance between two groups unavailable. A small sample of negative data may not represent the whole truth. We cannot rule out the influence of previous uterine cavity operation history on the uterine cavity flora as a confounding factor. Secondly, our data just showed the functions of whole microbiota instead of the dominant ones so that how they affect URSA is not clear. And the causal relationship between the uterine cavity flora and the abortion is difficult to determine.

Conclusion

In summary, our study showed an interesting finding of a phenomenon that microbiota could be found in the pregnant uterine cavity of URSA patients. We speculate that either the presence of microbiota leads to URSA or this could corroborate the theory of uterine fertile of microbiota. Nevertheless, studies are still needed to replicate these initial findings to either confirm the interesting phenomenon or to open a new field of the existence and function of microbiota in pregnant uterine which may be neglected. Questions of which microbiota is the real culprit of URSA and whether there is a "microbiota-containing time window" for microbiota in the uterine cavity remains to be elucidated in future studies.

Abbreviations

unexplained recurrent spontaneous abortion (URSA)

in vitro fertilization and embryo transfer (IVF-ET)

operational taxonomic units (OTU)

principal component analysis (PCA)

Declarations

Ethics approval and consent to participate

Approval for our study was obtained from the Ethics Committee of Sun Yat-sen Memorial Hospital of Sun Yat-sen University (SYSEC-KY-KS-2020-146) and written informed consent was obtained from all participants.

Consent for publication

All of the authors have agreed to publish this manuscript and signed the consent.

Availability of data and material

The datasets used and analysed during the current study available from the corresponding author on reasonable request.

Competing interests

No conflicts of interest in this study.

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Authors' contributions

Shiyu Bai performed research development and wrote the manuscript; Shuai Fu performed data collection and manuscript editing; Manqi Chen, Bingqian Huang and Lihao Hu performed project development and data collection; Hui Chen and Jianping Zhang performed the supervision, data interpretation and manuscript revision. All the authors reviewed the manuscripts.

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Figure legends

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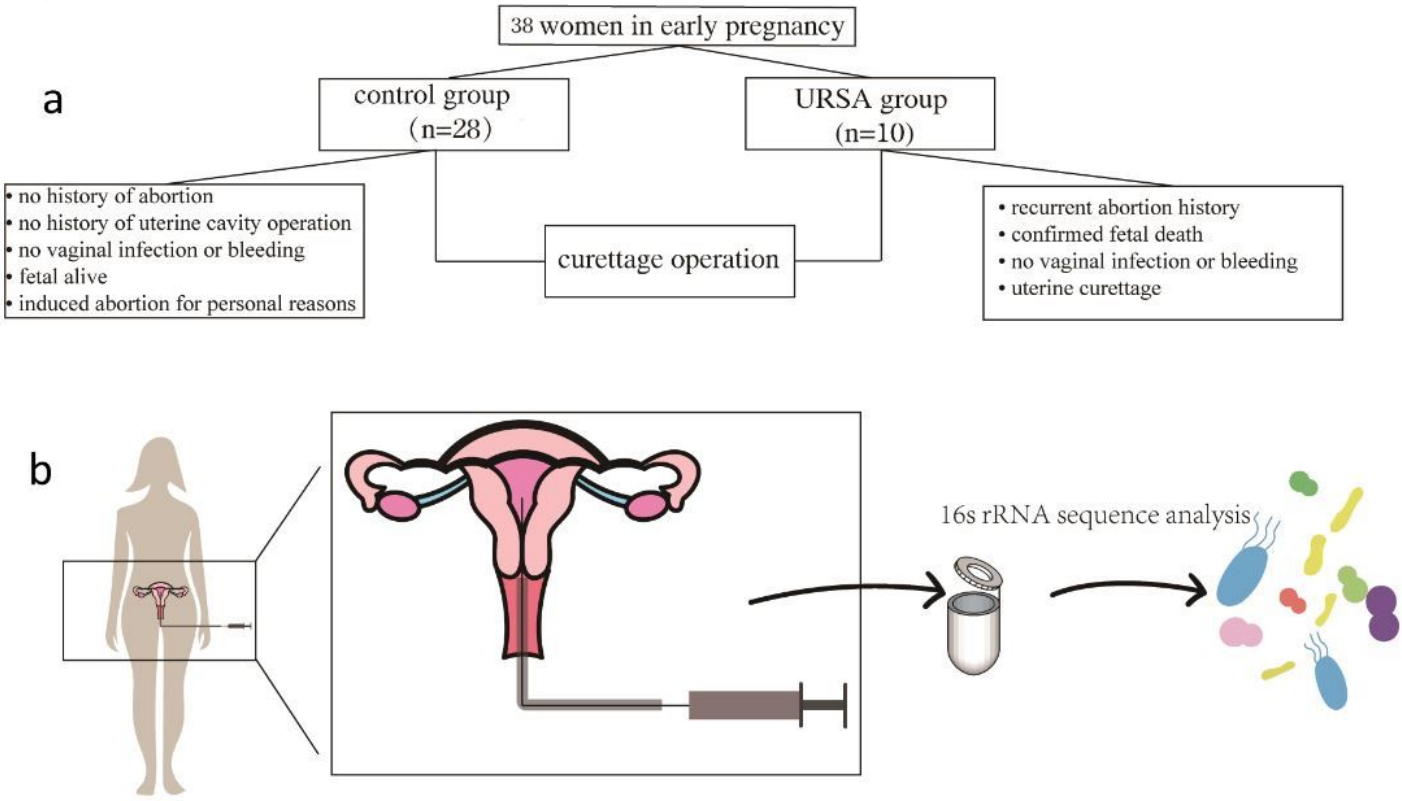
Figures



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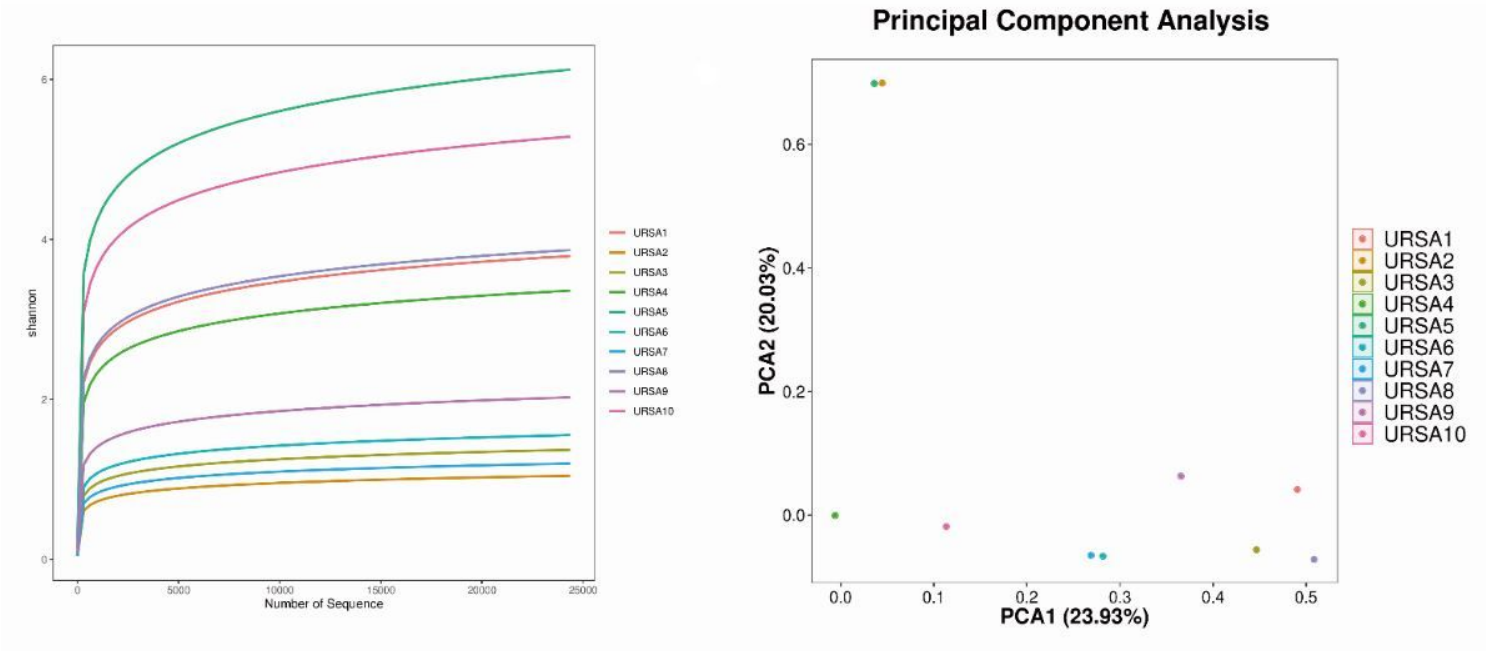

Figure 2

16S rRNA sequencing analysis of microbial communities. (A) Alpha diversity was measured by Shannon index. (B)Beta diversity was showed by principal component analysis (PCA).

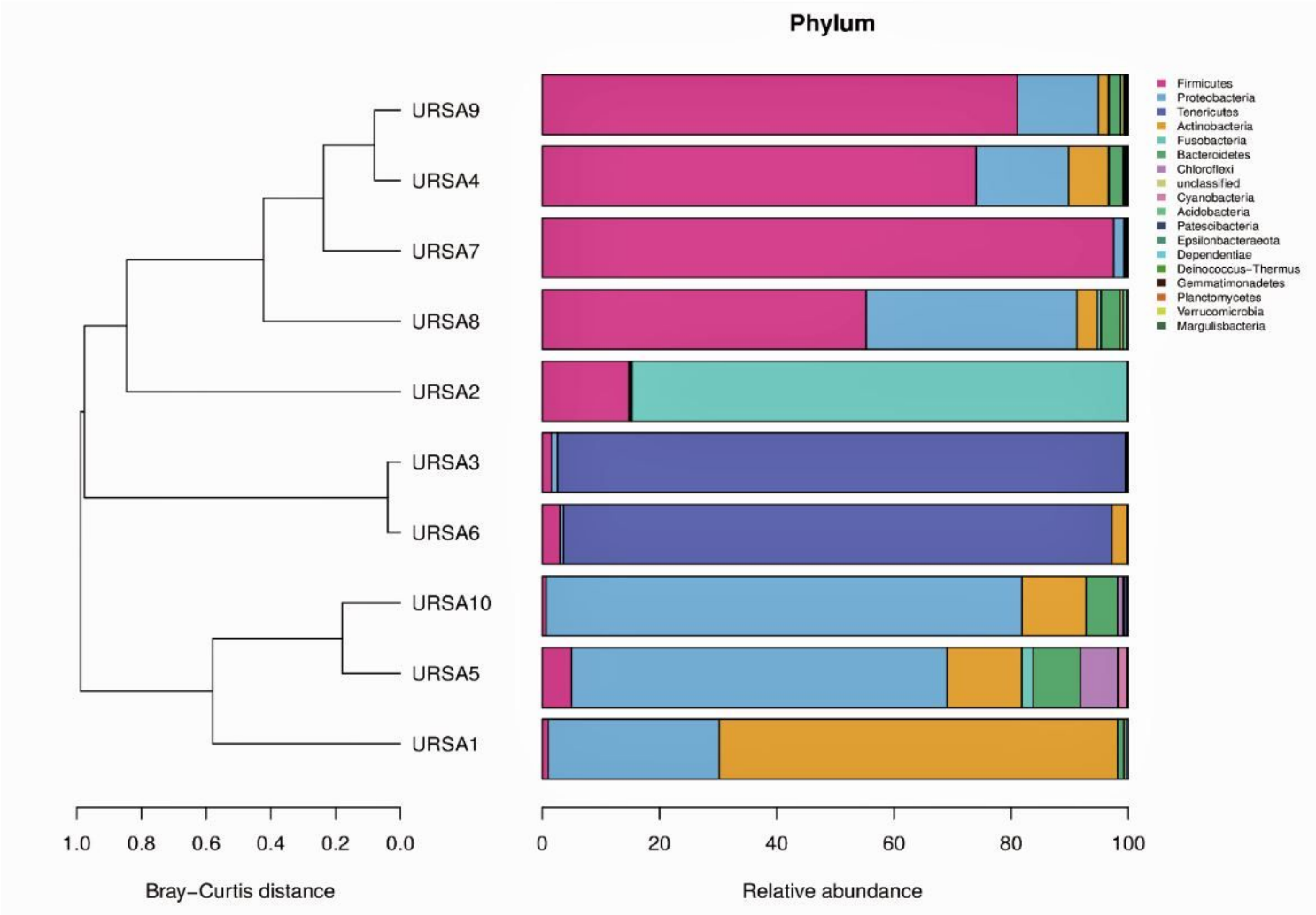

Figure 3

The composition of microbiome in Phylum level of all samples.

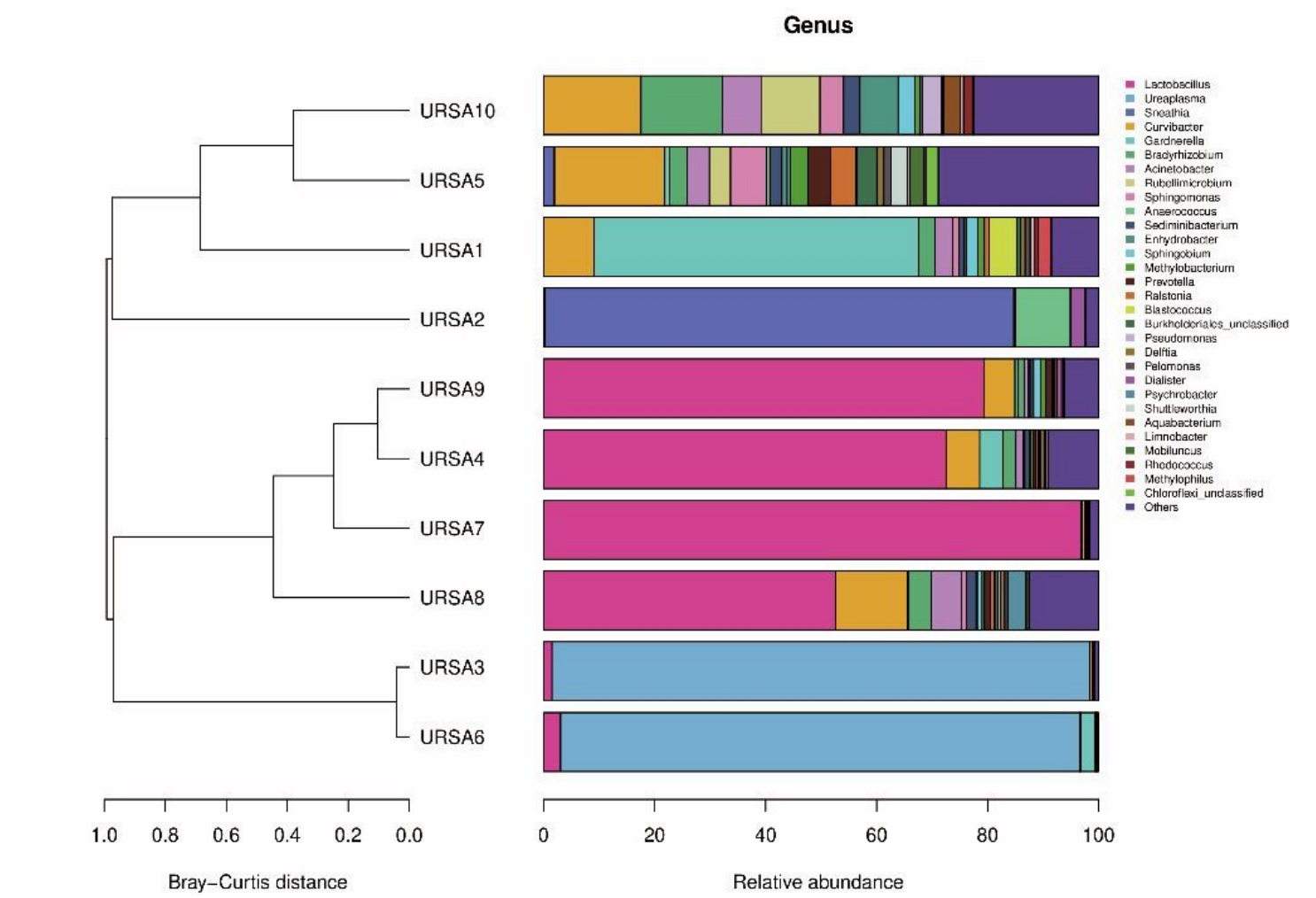

Figure 4

The composition of microbiome in Genus level of all samples.