

Comparison of Endometrial Microbiota Results Between Patients with Unexplained Infertility and Fertile Patients through 16s rRNA Sequencing

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ABSTRACT

OBJECTIVE: The primary objective of our study was to compare the endometrial microbiota profiles of women with unexplained infertility and fertile women.

STUDY DESIGN: In this case control study a total of 15 patients with unexplained infertility and 15 patients who had a live birth in the last two years with proven fertility were recruited. Endometrial samples were collected and analyzed through next-generation sequencing of the bacteria-specific 16S ribosome gene.

RESULTS: *Lactobacillus* species represented the majority of the microbiome profile in both groups. The median percentage of the endometrial *Lactobacillus* between infertile patients and fertile patients was not statistically significant ($p=0.9$). Groups were divided into two categories: Lactobasillus dominant (*Lactobacillus* spp. > 90%, LD) and Non-*Lactobacillus* dominant (NLD). *Lactobacillus* dominance was detected in six of nine patients (66.7%) in the study group and four of nine patients (44.5%) in the control group, and no significant difference was observed between them ($p=0.64$).

CONCLUSION: According to our study results, no significant difference was observed between the endometrial microbiota profile of infertile and fertile patients. Further studies with larger sample sizes are needed to characterize the endometrial microbiota and its impact on reproduction.

Keywords: Endometrial microbiota, Microbiota, Unexplained infertility

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Introduction

Microbiota refers to the whole community of microorganisms inhabiting a particular environment. National Institutes of Health (NIS) “Human Microbiome Project” (HMP) showed the existence of a microbial environment in the human body and its impact on human health (1). Dysbiosis is defined as the presence of altered microbiota. Recent studies have shown that bacteria might affect immunity. The changes in the microbiota profiles of various body parts as a result of dysbiosis can lead to various diseases. (2,3).

One of the seven body regions evaluated by the Human Microbiome Project (HMP) was the reproductive tract (4). Only 9% of the human microbiota was detected in urogenital tracts (5). Several studies evaluated the possible effects of the microbiome profile on the reproductive system (6). It has been noted that the vaginal microbiota profile differs with various factors such as patient’s age, body mass index (BMI), and ethnicity and it might be related to reproductive outcomes. Although endometrium was formerly considered sterile, it is difficult to think that long-term exposure to microorganisms in the lower genital tract and a mucosa frequently invaded by sperm would not contain bacteria according to recent data (7). Nevertheless, during the last decade, available studies suggest

that the endometrium has its microbiome (8). Particularly, in patients with recurrent implantation failure (RIF) and recurrent pregnancy loss, the endometrial microbiota profile was shown to be altered when compared with otherwise healthy women (9,10). However, there is a paucity of data on whether alteration in microbiota itself might be solely associated with unexplained infertility.

The current study aimed to investigate whether endometrial microbiota profiles among patients with unexplained infertility and fertile patients present any diversity.

Material and Method

Participants: Fifteen patients diagnosed with unexplained infertility were recruited as a study group from the Assisted Reproductive Technology Unit, Department of Obstetrics and Gynaecology, Hacettepe University Hospital. Eligible patients who applied to the family planning department for intrauterine device insertion were included as the control group.

The inclusion criteria for the study group were age between 25 and 35 years, having a regular menstrual cycle (25–35 days), a normal uterus and ovaries as depicted by ultrasonography, bilateral tubal patency, and a BMI <30 kg/m². Tubal patency was assessed by hysterosalpingography (HSG) (11). Criteria for inclusion in the control group were age between 25 and 35 years, BMI <30 kg/m², having regular and ovulatory cycles, and having had a live birth within the last 2 years. Exclusion criteria for both groups were having a chronic disease, hormonal treatment for the last 3 months, or antibiotic treatment due to any reason (oral, systemic, or vaginal) in the last 3 months.

Written informed consent was obtained from all participants. The study protocol was maintained by the Declaration of Helsinki. Ethics approval was obtained from the institutional review board (Hacettepe University Clinical Research Ethics Committee, reference No: 2018/22-35).

Sample collection: All samples were collected in the luteal phase of the menstrual cycle. Precautions were applied to avoid contamination and bacterial growth in all procedures. The sampling of the endometrium was performed via a pipelle cannula. After the insertion of a sterilized speculum, excessive vaginal secretions were removed by saline solution in a downward direction to avoid contamination. The Pipelle cannula was carefully placed into directly uterine cavity ensuring the tip of the catheter did not interact with the vaginal fornix. The samples were stored at -20 °C until DNA extraction. Samples were coded under “SG” for the study group and “CG” for the control group. All samples were given a number.

In the data collection form, patients' age, gravida, parity, BMI, HSG result, antral follicle count, partner sperm analysis result, menstrual pattern, history of hormone uses in the last 3 months, history of antibiotic use, duration of infertility, his-

tory of past infertility treatments, and chronic diseases were recorded.

Body mass index was calculated with the formula BMI = Body weight (kg) / height (m²).

DNA Extraction of Endometrium Samples: After all endometrial samples were collected, they were simultaneously thawed at room temperature and a tissue extraction kit (QIAamp DNA Mini Kit, Qiagene, Hilden, Germany) was used for DNA extraction. After DNA extraction, DNA quantification was performed with a Qubit fluorometer. After the process, agarose gel was prepared at 1.5% concentration and 5 µl of each sample was loaded. After the electrophoresis was completed, the next step was not continued for samples that did not form banding.

Metagenome Determination by 16s rRNA Sequencing: The Ion Torrent 16s Metagenomics kit (Thermo Fisher, Germany) was used for analysis. The kit contained two primer sets that selectively amplified hypervariable regions corresponding to the 16S region of bacteria:

- Primer set V2- 4-8
- Primer set V3-6, 7-9

The processing steps were performed according to the protocol of the Ion Torrent 16s Metagenomics Kit manual (12).

There were 2 separate primer sets in the metagenomics kit for real-time polymerase chain reaction (PCR). Therefore, two separate mixtures were prepared. *Escherichia coli* was used as a positive control and diluted at 1:20 according to the instructions. 30 µL of amplification mixture was prepared for each sample containing 15 µL master mix, 2 µL template, 3 µL primer, and 10 µL DNA. For each sample, amplicons 1 and 2 were combined in a volume of 40 µL.

Purification of amplification products: For purification, 72 µL ampure and 40 µL amplicons were combined and kept at room temperature for 5 minutes. Then, the tubes were kept in the Dynamag-2 magnet for 3 minutes and the resulting supernatant was discarded. 300 µL of 70% ethanol was added to each tube, left for 30 seconds, and then the supernatant was discarded. The tubes were incubated for 4 minutes at room temperature. After the tubes were removed from the magnet, 15 µL of nuclease-free water was added. The tubes were placed back into the Dynamag Magnet. After waiting for 1 minute, the supernatant was transferred to a new Eppendorf container. DNA quantification was performed with a Qubit fluorometer.

The available Ion Plus Library Kit was used for the library preparation phase. The Ion Xpress Barcode Adapters Kit was used for DNA barcoding. The prepared mixture components are shown in Table I. A separate barcode was used for each sample. The qPCR program of the mixture created in 0.2 mL tubes is shown in Table II.

Table 1: Blend components used for barcoding.

Component	Library volume
DNA	~25 µL
10X Ligase Buffer	2 µL
Ion P1 Adaptor	2 µL
Ion Xpress X Barkod	2 µL
DNTP Mixture	2 µL
Nüklease free water	49 µL
DNA Ligase	2 µL
Nick polymerase	8 µL
Total	100 µL

Table 2: qPCR program after barcoding

Stage	Temperature	Time
Hold	25°C	10 minutes
Hold	72°C	5 minutes
Hold	4°C	Hold

After the process, the entire mixture was transferred back into 1.5 ml tubes. Then, 140 µL of AMPure X was added for purification and kept at room temperature for 5 minutes. The tubes were placed on Dynamag-2 Magnet and waited for 3 minutes, then the resulting supernatant was discarded. 500 µL of 70% ethanol was added and the supernatant formed after a 30-second waiting period was discarded again. The tubes were kept at room temperature for 4 minutes. Then, 20 µL TE was added to the tubes taken from the magnet, and after waiting on the magnet again, the resulting supernatant was taken into 1.5 ml Eppendorf.

After the process, the amount of DNA was determined with a Qubit fluorometer to decide the amount of the library. Dilution was performed according to this amount using the dilution formula (library volume in pM/26 pM). The created library was brought to a final volume of 25 µl. Then, the transfer of the library to the chip was completed by loading it into the Ion Chef device and the metagenome program was started. After this process, 16s rRNA sequencing was performed with the chip Ion Torrent 5S device.

Statistical analysis

Ion Reporter Software 5.12 was used for operational taxonomic unit (OTU), alpha diversity, and beta diversity analyses, and SPSS 21.0 programs were used to compare demographic data and *Lactobacillus* percentages. A comparison of two numerical values was made with the Mann-Whitney U test, one of the non-parametric tests. Spearman's rho test was used for correlation testing.

Results

The mean age of the patients in the study group was 28.5±3.2 (25-35) and the mean age of the control group was 29.9±3.5 (26-35). There was no significant difference between the mean ages of the two groups (p>0.05).

Considering the mean BMI of the groups, the study group

was 24.1±4.4 kg/m² and the control group was 24.4±2.4 kg/m². There was no statistical difference between the two groups (p>0.05).

The mean infertility duration of the patients in the study group was 29.4±9.7 months. Ten out of 15 patients (66.7%) in the study group had previously received intrauterine insemination treatment (n=10).

The average antral follicle count of the patients in the study group was calculated as 16.2±3.5.

In 12 out of 30 patients, due to the low DNA density, bands could not be formed during the agarose gel electrophoresis. OTU distribution of bacterial genera and species detected in the endometrial samples of the remaining 18 patients were represented in Figures 1 and 2. Since the most observed bacterial genus according to OTU distribution was *Lactobacillus* the groups were divided into two categories: *Lactobacillus* dominant (*Lactobacillus* spp>90%, LD) and Non-*Lactobacillus* dominant (NLD). *Lactobacillus* dominance was detected in six of nine patients (66.7%) in the study group and four of nine patients (44.5%) in the control group, and no significant difference was observed between them

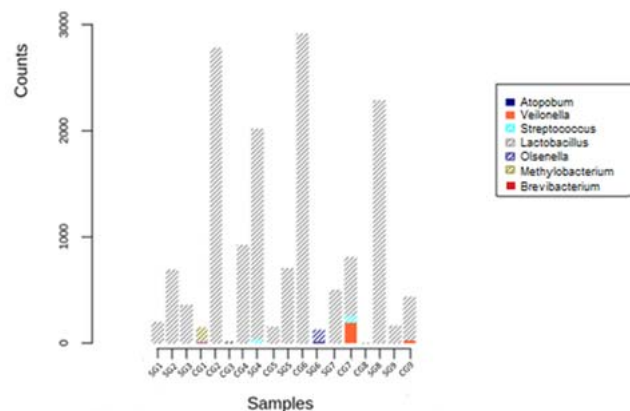


Figure 1: OTU distribution of bacterial genera detected in endometrial samples

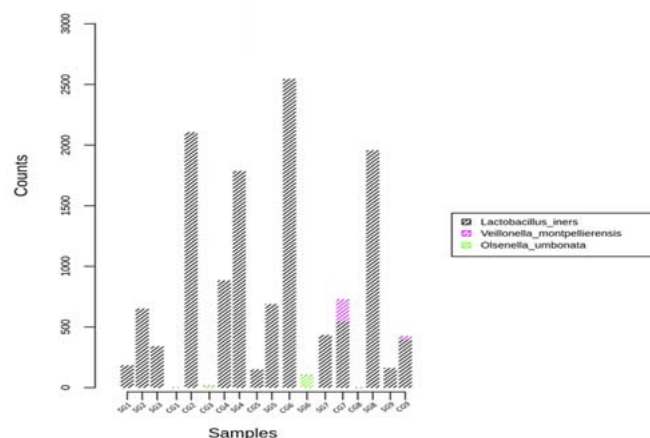


Figure 2: OTU distribution of bacterial species detected in endometrial samples.

($p=0.64$). In the unexplained infertility group, no correlation was found between infertility duration and *Lactobacillus* dominance ($p=0.272$).

When the distribution of bacterial species was evaluated, it was noted that the *Lactobacillus iners* species constituted the majority in the endometrial microbiota distribution. Whereas eight out of nine patients in the study group had *Lactobacillus iners* species, it was noticed in six out of nine patients in the control group.

In non-*Lactobacillus* dominant samples, *Olsenella umbonata* ($n=1$) for the study group, *Olsenella umbonata* ($n=1$) for

the control group, and bacteria belonging to the Caulobacteraceae family ($n=2$) were observed (Figure 3).

Beta diversity is used to compare bacterial species between two communities. For this purpose, coordinates analysis was performed and the Bray-Curtis index was used (Figures 4 and 5).

Two alpha diversity methods were used to evaluate the bacterial diversity and number of species contained in the samples. In this context, Shannon diversity analysis and Chao-1 indices are shown in Figures 6 and 7.

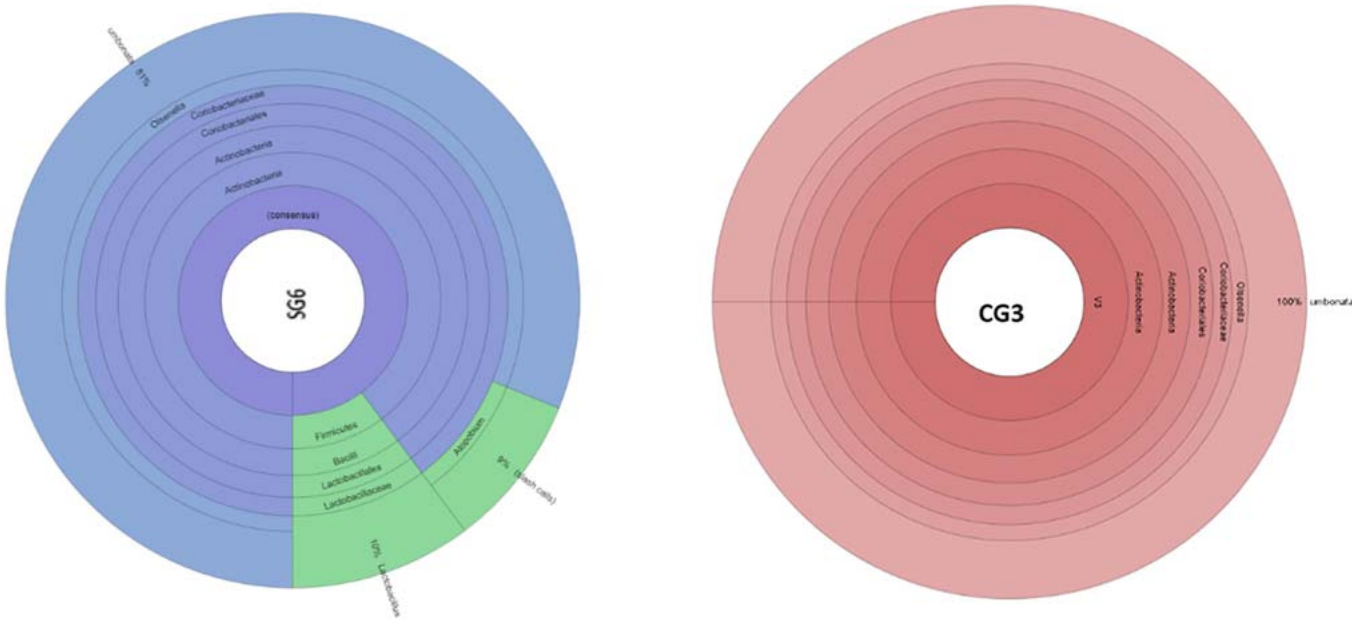


Figure 3. Bacterial distribution of endometrial samples of patients in the NLD group

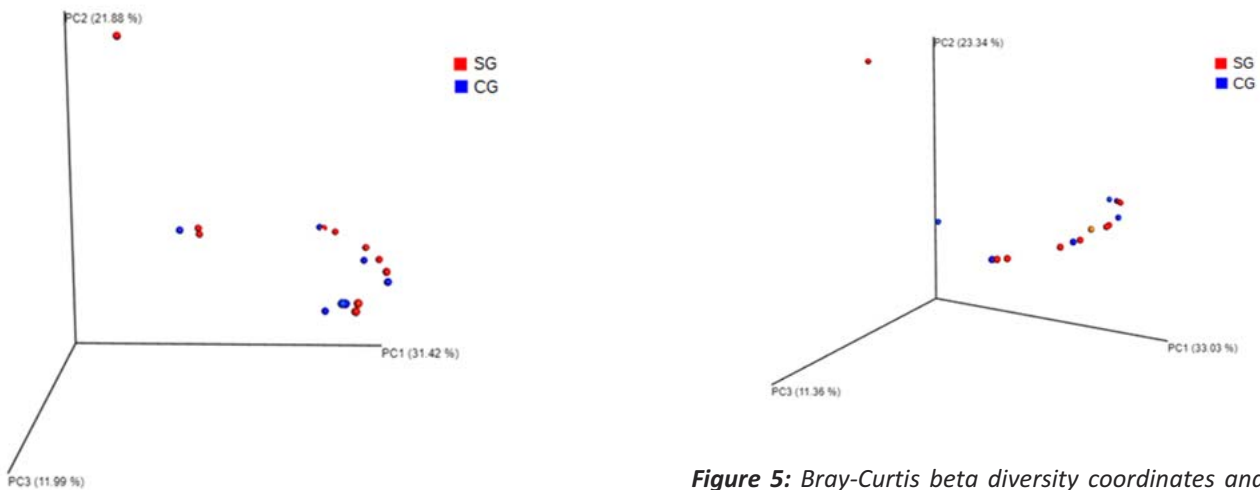


Figure 4. Bray-Curtis beta diversity coordinates analysis according to bacterial species (SG: Study group including unexplained infertility patients, CG: Control group).

Figure 5: Bray-Curtis beta diversity coordinates analysis according to bacterial species

(SG: Study group including unexplained infertility patients, CG: Control group).

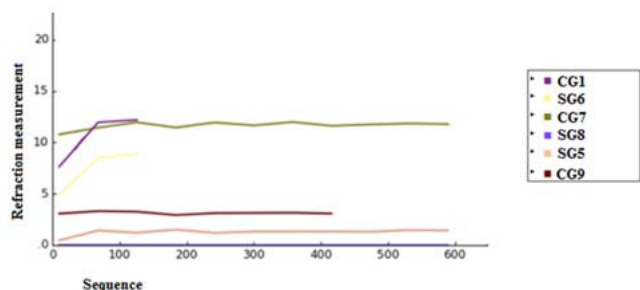


Figure 6: Shannon Diversity Analysis according to bacterial genera

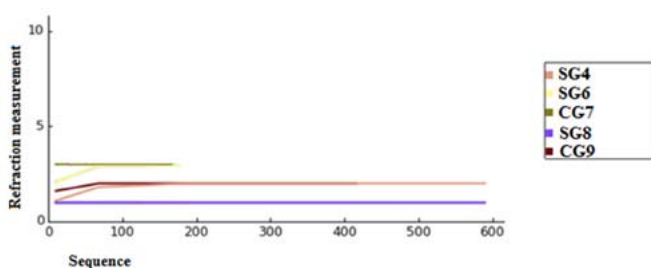


Figure 7: Chao1 index according to bacterial genera.

Discussion

Although there have been many studies about endometrial microbial composition and its effect on assisted reproduction treatments, there is a paucity of data on the validity of screening for microbiota particularly in cases with unexplained infertility. To our knowledge, this is one of the limited studies that has been conducted in this field.

Several studies showed that endometrial microbiome profiles might have a critical role in conception. Moreno et al. presented a multi-center study analyzing 342 infertile patients' endometrial microbial composition before embryo transfer and concluded that endometrial microbiome might be considered as a biomarker to predict a reproductive outcome. Patients with a *Lactobacillus* dominant microbiota were more likely to have a live birth (13). Chen et al also analyzed the endometrial microbiota of 111 patients by using 16 s rRNA sequencing technology. They found that the structure and composition of the endometrial microbiota community of patients with RIF were significantly distinct from the control group. The authors reported that the abnormality of microbial structure and composition might interfere with the implantation of embryos by affecting the immune adaptation of the endometrium (14).

In another study conducted by Moreno et al., (2006) endometrial fluid samples of 35 IVF patients were studied (9). The endometrial microbiota profile of these patients was divided into two groups: *Lactobacillus* dominant (LD>90%) and non-*Lactobacillus* dominant (NLD). A significant difference was found in the live birth rate per transfer between the two groups in favor of the LD group. In this context, even though the disrupted endometrial microbiome environment in

infertile couples could potentially be considered as a factor, the design and cohort of the studies differ widely.

Several studies did not detect any association between the microbiome and reproductive outcomes. Frasnjak et al. classified the endometrial microbiota of 33 patients who underwent single, euploid embryo transfer with the 16s rRNA sequencing method and did not find any significant difference between positive and negative pregnancy results (13). In the study conducted by Kyono et al. in 2018, the endometrial and vaginal microbiota of 109 patients (79 IVF, 23 non-IVF infertile patients, and 7 healthy volunteers) were compared. Patients were evaluated as LD (>90%) and NLD. LD endometrium in IVF patients was significantly lower than in healthy volunteers (38.0% vs. 85.7%). However, no significant difference was observed between the median *Lactobacillus* percentages of the 3 groups which is consistent with our study (15).

One of the limitations of profiling the endometrial microbiota is the low bacterial load. It is shown that bacterial content in the endometrium is 10,000 times less than in the vagina (16). In the study by Wee et al, samples were taken from three different reproductive regions (vagina, cervix, and endometrium) of 16 infertile and 15 fertile female patients who underwent hysteroscopy for various reasons and compared with the 16s rRNA sequencing method (17). It has been stated that the bacterial load in endometrial samples is significantly lower compared to other regions. It was also mentioned that the DNA extraction step of the studies could be challenging because of the low bacterial load. In the descriptive study conducted by Tao et al. in 2018, the embryo transfer catheters of 70 IVF patients were evaluated. It was mentioned that it is difficult to create a microbiota profile due to the low endometrial bacterial load (18). It is also consistent with our study.

As can be noticed from the studies summarized above, some of the endometrial sampling was done by aspiration of endometrial fluid and some by obtaining endometrial tissue itself. In a study evaluating both endometrial fluid and endometrial tissue samples from 25 patients with recurrent pregnancy loss, diversity and read sequence per OTU were detected to be more intense in endometrial tissue samples than in endometrial fluid samples (19). It can also be speculated that the sampling method also has a role in profiling endometrial microbiota. These results indicate that the ideal sampling method may be endometrial tissue itself rather than endometrial superficial fluid, and suggest that further studies are needed to determine the most appropriate method in this regard. Considering these findings in our study, we preferred to collect the endometrial tissue sample with a pipelle cannula.

Another challenge could be stated as the possibility of contamination. It could not be eliminated because the sample collection is usually performed transcervically. In the study conducted by Kitaya et al., the microbiota of endometrial fluid and vaginal secretions of 28 recurrent implantation failure pa-

tients and 18 patients who started their first IVF attempt were compared. It has been stated that the majority of endometrial microbiota samples consist of *Lactobacillus*. LD samples were detected in 64.3% of the patients in the recurrent implantation failure group and 38.9% of the patients with their first IVF attempt, no statistical difference was found between them (20). In this study, the species distributions of both endometrial fluid and vaginal secretions were found to be similar. This also raises the question about the correct sampling method to avoid cervicovaginal contamination when taking samples from tissue with low biomass such as endometrium (10,18). For example, *Lactobacillus* species were found to be dominant in the majority of studies using transcervical sampling. In a study by Verstraelen et al. Samples were collected with the help of an endometrial brush with a protective sheath to prevent cervicovaginal contamination (21).

In order to elucidate the shadowing effect of contamination on the evaluation of endometrial microbiota, studies were conducted from hysterectomy materials. In this method, in which vagino-cervical contamination is theoretically minimized, *Lactobacillus*, *Acinetobacter*, and *Corynebacterium* species were still detected in endometrial samples (22,23). Vanstokstraeten et al investigated to what extent the endometrial microbiome corresponds to that of the vagina, applying culturomics on paired vaginal and endometrial samples. An additional vaginal swab was taken from each participant right before hysteroscopy. Both endometrial biopsies and vaginal swabs were analyzed. In total, 101 bacterial and two fungal species were identified among patients. On average, 28% of species were found in both the endometrial biopsy and vaginal swab of a given patient. Of the 56 species found in the endometrial biopsies, 13 were not found in the vaginal swabs (24). The data suggest the potential existence of a unique endometrial microbiome that is not merely a presentation of cross-contamination derived from sampling.

The dominance of different bacterial species in endometrial microbiota studies suggests that there is no uniform microbiota profile in all healthy women. Although it has been stated in studies that differences in the intestinal microbiota vary with age, BMI, and ethnicity, it is not clear which factors affect the endometrial microbiota, and further studies are needed on this subject (25,26).

To minimize potential endometrial microbiota differences that could be associated with demographic reasons between the study group and the control group, care should be taken to include individuals with similar demographic characteristics.

To conclude, we might assume that there is no significant difference between infertile and fertile patients with regard to endometrial microbiota. A low bacterial load was found in endometrial tissue samples, consistent with other studies. More studies with a large sample size and a careful design are needed to investigate the reproductive effects of endometrial dysbiosis.

Conclusion

Further studies with large cohorts are required to establish the contribution of microbiota for clinical application. Results between different studies are barely comparable due to the varying and limited study designs. The question needs to be clarified in future studies with larger cohorts.

Declarations

Ethics approval and consent to participate: The study protocol was maintained by the Declaration of Helsinki. Ethics approval was obtained from the institutional review board (Hacettepe University Clinical Research Ethics Committee, reference No: 2018/22-35). All participants signed informed written consent before being enrolled in the study.

Availability of data and materials: The data supporting this study is available through the corresponding author upon reasonable request. / The datasets and code used and/or analyzed during the current study are available from the corresponding author upon reasonable request. *Competing interests:* The authors declare that they have no competing interests.

Conflict of interest statements: Authors declare no conflict of interest in this study. *Acknowledgments:* We thank all participants who voluntarily participated in this study. *Authors' contributions:* GOD: and CT are responsible for the data collection. GOD: and GB: are responsible for the application for ethical approval and the writing of the manuscript. GOD: and GB: are responsible for the statistical analysis of the study, graphic, and figure design. GÖD: and SM: are responsible for the study design. PZ: and GB: are responsible for data collection and supervision.

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