Introduction

For nearly a century, the concept of Tissier (1) on the absolute sterility of the uterus was generally accepted in the scientific community. However, uterine peristalsis, which contributes to the capture of the sperm from the vagina, can accordingly contribute to the entry of vaginal microflora into the uterine cavity.

On the other hand, some studies proved the hematogenous pathway of bacteria entering various parts of the genital tract (2), either from the oral cavity (3) or the intestine (4). Other routes for the entry of microorganisms into the upper genital tract may include invasive procedures, embryo transfer procedures in in vitro fertilization (IVF) cycles, and the installation of intrauterine devices. With the advent of a new generation of sequencing technology, it has become possible to determine a wide range of microorganisms.

Considering the above-mentioned explanations, the purpose of this study was to reveal the difference between the uterine microbiomes of women with infertility and repeated IVF failures and those of normal healthy women with no burdened obstetrical or gynecological history.

Materials and Methods

Study Cohort

The study population included 42 women who were divided into two groups. All patients were recruited from the Department of Obstetrics and Gynecology №1, Rostov-on-Don State Medical University (Rostov-on-Don, Russia). The first group included 22 asymptomatic women with infertility and repeated IVF failures (2 or more attempts), who were in the age range of 20-46 years. The inclusion criteria were unexplained infertility, two and more unsuccessful attempts of IVF, the age range of 20-46 years, regular menstrual cycle (25-35 days), and normal anatomy of the uterus (proven by an ultrasound examination or hysteroscopy). On the other hand, the exclusion criteria included any pathology of the uterus, including leiomyomas, polyps, congenital malformations, and Asherman syndrome (intrauterine adhesions), as well as the usage of a contraceptive intrauterine device in the previous 6 months, any systematic inflammatory disease or severe extragenital pathology, and the recent use of systemic antibiotic therapy.

In addition, the second group included 20 healthy asymptomatic women aged 20-44 years. The inclusion criterion was the absence of burdened obstetrical or gynecological history, and the exclusion criteria were the same as those of the first group.

Sample Collection

The samples of uterine microbiomes were collected 7 days after the luteinizing hormone elevation, which was determined by the urine test. All measures were taken to avoid sample contamination. Finally, the massively parallel sequencing of the 16S ribosomal RNA gene was done in every uterine sample.
Due to development of technologies of new generation sequencing, it is no doubt that uterine microbiome exists. The predominance of Lactobacillus in uterine microbiome can be considered as a predictor of favorable reproductive outcomes. High microbial diversity of uterine microbiome in women with repeated IVF failures is a predictor of failed implantation.

The total DNA was extracted from the tissue samples using the "Rhibo Prep" kit (Public Entity Central Research Institute of Epidemiology, Russia) following the manufacturer’s recommendations.

Library Preparation and Sequencing
In general, 16S DNA libraries were prepared according to the Illumina protocol “16S Metagenomic Sequencing Library Preparation” (Part № 15044223 Rev. B). Moreover, 5 ng of the total DNA was used to amplify the target 16S rRNA gene fragment and Illumina adapter sequences were added using the recommended primers for V3 and V4 regions. The primer sequences included 16S Amplicon polymerase chain reaction (PCR) forward primer = 5’TCC TGG CCA CGG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3’, 16S Amplicon PCR reverse primer = 5’-GTC TGG CCT GCT CGG AGA TGT GAA GAG ACA GGA GTA CHV GGG TAT CTA ATGCC-3’. Accordingly, 25 cycles of PCR were conducted using the KAPA HiFi HotStart ReadyMix (2X, Roche Diagnostics, Switzerland).

After the solid phase reversible immobilization bead purification of PCR products, 5 ng of the resulting amplicons were indexed using KAPA HiFi HotStart ReadyMix (2X, Roche Diagnostics, Switzerland) and the Nextera XT Index Kit (Illumina, USA).

Finally, 8 cycles of index PCR were conducted following the Illumina protocol, and libraries were sequenced on the Illumina MiSeq System.

Sequencing
DNA Extraction
The total DNA was extracted from the tissue samples using the “Rhibo Prep” kit (Public Entity Central Research Institute of Epidemiology, Russia) following the manufacturer’s recommendations.

The age of the patients ranged from 20 to 46 years in both clinical groups. In the clinical group I (asymptomatic women with infertility and repeated IVF failures), the average age of the patients was 31.8 ± 4.9 years. In clinical group II, patients’ average age was 31.4 ± 4.9 years.

In addition, the body mass index (BMI) of all patients of both clinical groups ranged from 17 to 26, and that of the first and second groups was 21.9 ± 2.01 and 22.15 ± 2.2, respectively.

Further, the mean age of patients’ menarche and the mean duration of the menstrual cycle were 13.27 ± 1.28 and 12.85 ± 1.50 years, as well as 28.18 ± 2.99 and 28 ± 2.71 days in the first and the second groups, respectively. Furthermore, mean menstrual duration comprised 5.59 ± 1.26 and 5.5 ± 1.39 days in the first and the second groups, respectively. Patients in both clinical groups were comparable in terms of age, BMI, age of menarche, duration of the menstrual cycle, and menstrual duration.

In general, 233 different microorganisms found in both groups were analyzed in this study. The comparison
of the relative abundance of microorganisms in the groups revealed significant differences in 14 of these microorganisms (Table 1).

Based on the results, higher relative abundance of *Lactobacillus iners* (37.2 [27.1; 44.6]), *Lactobacillus acidophilus* (7.24 [1.92; 12.6]), *Lactobacillus jensenii* (5.1 [3.59, 7.95]), *Lactobacillus crispatus* (7.38 [5.69, 10.2]), *Prevotella melaninogenica* (0 [0, 0.05]), *Bacteroides vulgatus* (0 [0, 0.05]), *Corynebacterium bouchardurhonense* (0 [0, 0.05]), *Bacteroides caccae* (0 [0, 0.005]), and *Bifidobacterium adolescentis* (0 [0, 0.005]) was detected in the group of healthy asymptomatic women of reproductive age with no burdened obstetric or gynecological history. Women in the first and second groups had a higher relative abundance of *Gardnerella vaginalis* and *Bifidobacterium gallinarum*, respectively (Figure 1).

Moreover, higher relative abundance of *Methylobacterium aerolatum* (0 [0, 0.21]) and *Comamonas testosteroni* (0 [0, 1.16]) was revealed in the group of women with infertility and repeated IVF failures. These microorganisms are not considered to be part of the normal human microbiome although evidence exists about their involvement in appendicitis (8). Therefore, they are regarded as a sign of the possible contamination of the sample medium or the air in the operation room.

The analysis of the mean relative abundance of various microorganisms in the uterine microbiome showed that women with infertility (first group) had higher microbiological diversity and variability as compared to healthy women in the second group (Figure 2). Based on the obtained data, the mean relative abundance of the *Lactobacillus* genus comprised 34.4% and 63.0% in the first and second groups, respectively. Eventually, the presence of *Streptococcus* spp. and *Gardnerella vaginalis* in small concentrations (3% and 3%, respectively) was also a hallmark of infertility and repeated IVF failures.

### Discussion

Our study had several limitations, one of which was the sample size. In general, 22 and 20 patients were included in infertile and fertile woman groups, respectively. Accordingly, the results cannot be extrapolated on the whole population and future studies on larger cohorts of women are of necessity.

Moreover, all women in our study were ethnically Caucasian, and thus the results cannot be generalized to women of other ethnicities. For example, Anahtar et al. found differences between the vaginal microbiomes of asymptomatic young South African women and Caucasian women (9). Considering the mechanism of peristaltic uterine contractions, which contribute to the capture of different bacteria from the vagina into the uterus, it could be argued that the uterine microbiomes of women of different ethnic groups can differ extensively.

The absence of negative controls was another limitation of the study (10). Some of the detected microbes in our study are commonly found in the air and the soil and thus can be the causative agents of postoperative infections or infections of the airways (8).

The findings of this study could suggest that higher microbial diversity in women with infertility and burdened obstetric and gynecological anamnesis may be the reason for failed IVF attempts. In healthy women with no history of intrauterine manipulations or miscarriages, having a predominance of *Lactobacillus* of various types in the uterine microbiome can be a predisposing factor for favorable reproductive outcomes. It is well-established that *Lactobacillus* species are capable of inhibiting other bacteria by producing lactic acid and hydrogen peroxide (11). It is also known that the epithelium thickens and

### Table 1. Relative Abundance of Microorganisms in the Studied Groups, Including Only Statistically Significant Differences

| Microorganism                  | 1st Group (Infertile Women) | 2nd Group (Healthy Women) | P Value |
|--------------------------------|-----------------------------|---------------------------|---------|
| *Lactobacillus iners*          | 0.17 [0; 24.4]              | 37.2 [27.1; 44.6]         | 0.0005  |
| *Lactobacillus acidophilus*    | 0 [0; 1.42]                 | 7.24 [1.92; 12.6]         | 0.003   |
| *Lactobacillus jensenii*       | 0 [0; 0]                    | 5.1 [3.59; 7.95]          | <0.0001 |
| *Lactobacillus crispatus*      | 0 [0; 0]                    | 7.38 [5.69; 10.2]         | <0.0001 |
| *Methylobacterium aerolatum*   | 0 [0; 0.21]                 | 0 [0; 0]                  | 0.01    |
| *Comamonas testosteroni*       | 0 [0; 1.16]                 | 0 [0; 0]                  | 0.003   |
| *Gardnerella vaginalis*        | 0 [0; 0]                    | 0 [0; 0]                  | 0.03*   |
| *Bifidobacterium gallinarum*   | 0 [0; 0]                    | 0 [0; 0]                  | 0.03*   |
| *Prevotella melaninogenica*    | 0 [0; 0]                    | 0 [0; 0.05]               | 0.01    |
| *Bacteroides vulgatus*         | 0 [0; 0]                    | 0 [0; 0.05]               | 0.01    |
| *Corynebacterium bouchardurhonense* | 0 [0; 0]                   | 0 [0; 0.05]               | 0.02    |
| *Finegoldia magna*             | 0 [0; 0.02]                 | 0 [0; 0]                  | 0.007   |
| *Bacteroides caccae*           | 0 [0; 0]                    | 0 [0; 0.005]              | 0.01    |
| *Bifidobacterium adolescentis* | 0 [0; 0]                    | 0 [0; 0.005]              | 0.01    |

Note. Average values are presented as median (Lower quartile, Upper quartile). In addition, the comparison was carried out using the Mann-Whitney test.

* Symbol indicates the significance of differences at the zero median, and zero quartiles are explained by differences for the participants of the last quartile, which in particular, can be found by the different maximum concentrations presented in the following graphs (Figure 1).
releases glycogen under the influence of estrogens, thus favoring glucose-fermenting microorganisms such as *Lactobacillus* (12). Possibly, a thin endometrium, which is a rather common case in women with repeated IVF failures, is directly associated with the low abundance of *Lactobacillus* in the endometrial microbiome. Fang et al indirectly suggested this issue and reported that women with endometrial polyps had higher levels of *Lactobacillus* (13).

Moreover, this study included 3 women in the group with repeated IVF failures who had an abundance of *Lactobacillus* of more than 80% in the uterine microbiome. Therefore, further studies are needed regarding the relationship between the disturbed uterine microbiome and the markers of chronic endometritis (14).

**Conclusions**

The findings of our study confirm the current understanding of the “lactobacillary” and “non-lactobacillary” microbiota of the endometrium and their effect on the success of embryo implantation. The concept of “normal” endometrial microbiota, as well as the analysis of dysbiotic microflora shifts in a close correlation with the presence of the histochemical markers of endometritis require further evaluations.

The question of the correction of endometrial dysbiosis remains open since the sequencing method, unlike the classical cultural method, provides no data about sensitivity to antibiotics. Currently, two methods of correcting dysbiosis are generally used, including the administration of antibiotics, systemically or locally, and probiotics. Antibiotic resistance is generally a growing public health concern worldwide and represents a significant risk for the disruption of the normal microbiome. Therefore, the “blind” prescription of antibacterial drugs based only on sequencing results cannot be a favorable prospect for the treatment of endometrial dysbiosis.

Additionally, the administration of probiotics can be a promising and reasonable method for treating endometrial dysbiosis. Nowadays, two theories are generally accepted about the bacterial entry to the uterine cavity, including the ascending vaginal way and the hematogenous way from the oral cavity and intestines.
In experimental models on cows, Jeon et al showed that the same bacterial cultures were isolated from the blood and the uterine cavity (15). In another study, Fardini et al confirmed the hematogenous pathway by injecting human saliva into the tail veins of pregnant mice and later detecting microorganisms from the saliva in the placenta of the mice (3). Additionally, there are several publications confirming the presence of the same species of bacteria both in the human placenta and intestinal and oral cavities (16,17). The similarity of the intestinal, vaginal, and upper reproductive tract microbiomes gives a reason to hope for new strategies regarding treating genital dysbiosis with the transplantation of fecal microbiota as an alternative to aggressive antibiotic therapy (18).

Authors’ Contribution
BVV wrote the paper, conceived and designed the analysis, collected data. SKM collected the data, performed the analysis. KNB conceived and designed the analysis. PDE performed the analysis, contributed analysis tool. AMV performed the analysis, contributed analysis tool.

Conflict of Interests
Authors declare that they have no conflict of interests.

Ethical Issues
The study was approved by the Local Ethics Clinical Research Committee of Rostov-on-Don State Medical University. Further, informed consent was obtained from all participants.

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