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MICROBIOMA UTERINO EM MULHERES FÉRTEIS SAUDÁVEIS E NO CASO DE PATOLOGIA DO ENDOMÉTRIO EM FALHAS MÚLTIPLAS DOS PROGRAMAS DE FERTILIZAÇÃO IN-VITRO

UTERINE MICROBIOME IN HEALTHY FERTILE WOMEN AND IN CASE OF ENDOMETRIUM PATHOLOGY IN MULTIPLE FAILURES OF IN-VITRO-FERTILIZATION PROGRAMS

МИКРОБИОМ ПОЛОСТИ МАТКИ У ЗДОРОВЫХ ФЕРТИЛЬНЫХ ЖЕНЩИН И ПРИ ПАТОЛОГИИ ЭНДОМЕТРИЯ ПРИ МНОГОКРАТНЫХ НЕУДАЧАХ ПРОГРАММ ВРТ

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RESUMO

Introdução: Graças às novas tecnologias de diagnóstico, não há dúvida de que o útero não é mais considerado estéril. Um microbioma comprometido do endométrio pode ser uma razão significativa para parto prematuro, infertilidade, recorrência de perda de gravidez e falhas repetidas de implantação em programas de fertilização in-vitro. **Objetivo:** estudar as características do microbioma endometrial em mulheres saudáveis férteis e em mulheres com múltiplas falhas na fertilização in-vitro. **Materiais e métodos:** Para avaliar as diferenças na microbiota endometrial de 20 mulheres com infertilidade e experimentando várias tentativas malsucedidas de fertilização in vitro e 15 mulheres férteis saudáveis, o microbioma endometrial foi estudado usando um NGS de 16S rRNA. **Resultados e Discussão:** *Lactobacillus* (29,4%), *Comamonas* (16,8%) e *Mesorhizobium* (6,0%) foram os gêneros mais abundantes no grupo de pacientes férteis saudáveis, sendo *Lactobacillus* (33,3%), *Ralstonia* (7,9%) e *Pediococcus* (4,8%) eram abundantes principalmente no grupo de pacientes inférteis. A abundância relativa média de *Lactobacillus* não diferiu significativamente entre os grupos e compreendeu 33,3% no 1º grupo e 29,4% no 2º grupo. Abundância relativa média significativamente mais elevada de bactérias do gênero *Brevundimonas* e *Ralstonia* foi registrada no primeiro grupo. As mulheres férteis do 2º grupo tiveram uma abundância relativa média estatisticamente significativamente maior de *Acidovorax*, *Brevibacillus*, *Caulobacter*, *Comamonas*, *Delftia*, *Distigma*, *Pseudomonas*, *Schlegelella*, *Thermus*. **Conclusões:** Os dados apresentados confirmam que o endométrio não é um tecido estéril, apesar dos conceitos antigos sobre sua esterilidade. *Lactobacillus* são gêneros dominantes; no entanto, não há dominância absoluta de *Lactobacillus* acima de 90%. A abundância relativa média de *Lactobacillus* no microbioma uterino em pacientes férteis e pacientes com múltiplas falhas de fertilização in-vitro não diferiu.

Palavras-chave: *microbioma endometrial, infertilidade, fertilização in-vitro, fertilidade.*

ABSTRACT

Background: Thanks to new diagnostic technologies, it is of no doubt that the uterus is no longer considered to be sterile. A disturbed microbiome of endometrium can be a significant reason for preterm birth, infertility, the recurrence of pregnancy loss, and repeated implantation failures in in-vitro-fertilization programs. **Aim:** Study the endometrial microbiome features in healthy fertile women and in women with multiple in-vitro-fertilization failures. **Methods:** To assess the differences in the endometrial microbiota of 20 women with infertility and experiencing multiple unsuccessful attempts of in-vitro-fertilization and 15 fertile, healthy women, endometrial microbiome was studied using an NGS of 16S rRNA. **Results and Discussion:** *Lactobacillus* (29.4%), *Comamonas* (16.8%), and *Mesorhizobium* (6.0%) were the most abundant genera in group of healthy fertile patients, and *Lactobacillus* (33.3%), *Ralstonia* (7.9%) and *Pediococcus* (4.8%) were most abundant in

the group of infertile patients. The mean relative abundance of *Lactobacillus* did not significantly differ between groups and comprised 33.3% in the group of infertile women and 29.4% in healthy fertile women. A considerably higher mean relative abundance of bacteria of the genus *Brevundimonas* and *Ralstonia* was recorded in the group of infertile women. The fertile women had a statistically significantly higher mean relative abundance of *Acidovorax, Brevibacillus, Caulobacter, Comamonas, Delftia, Distigma, Pseudomonas, Schlegelella,* and *Thermus*. **Conclusions:** The presented data confirm that endometrium is not a sterile tissue despite long-standing concepts concerning its sterility. *Lactobacillus* are dominant genera; however, there is no absolute dominance of *Lactobacillus* over 90%. The mean relative abundance of *Lactobacillus* in the uterine microbiome in fertile patients and patients with multiple in-vitro-fertilization failures did not differ.

Keywords: *endometrial microbiome, infertility, in-vitro-fertilization, fertility*

АННОТАЦИЯ

Введение: Благодаря новым диагностическим методикам, в настоящий момент не возникает сомнения, что полость матки не является стерильной. Нарушенный микробиом эндометрия может являться значимой причиной преждевременных родов, привычного невынашивания беременности, бесплодия и повторных неудач имплантации в программах ВРТ. **Цель:** изучить особенности микробиома эндометрия у здоровых фертильных пациенток и у пациенток с многократными неудачами ВРТ. **Материалы и методы:** С целью оценки различий микробиома эндометрия у 20 женщин с бесплодием и многократными неудачными попытками ВРТ и у 15 фертильных здоровых женщин был изучен микробиом эндометрия путем секвенирования нового поколения гена 16S рРНК. **Результаты:** Самыми представленными в группе здоровых фертильных пациенток были роды *Lactobacillus* 29,4%, *Comamonas* 16,8% и *Mesorhizobium* 6,0%, а в группе бесплодных пациенток с многократными неудачами ЭКО – *Lactobacillus* 33,3%, *Ralstonia* 7,9% и *Pediococcus* 4,8%. При этом доля лактобактерий у пациенток первой группы значимо не отличалась от пациенток 2ой группы (33,3% и 29,4% соответственно). В группе женщин с бесплодием и многократными неудачными попытками ЭКО регистрировалась достоверно более высокая относительная представленность бактерий рода *Brevundimonas* и *Ralstonia*. При этом у фертильных женщин 2ой группы регистрировалась статистически значимо более высокая представленность *Acidovorax*, *Brevibacillus*, *Caulobacter*, *Comamonas*, *Delftia*, *Distigma*, *Pseudomonas*, *Schlegelella*, *Thermus*. **Заключение:** Представленные данные подтверждают концепцию нестерильности эндометрия и существования маточного микробиома. Лактобактерии являются доминирующим родом в составе маточного микробиома, однако не абсолютно доминирующим, т.е. не более 90%. При этом доля лактобактерий у фертильных пациенток и у пациенток с многократными неудачами ВРТ значимо не отличалась.

Ключевые слова: микробиом эндометрия, бесплодие, ЭКО, фертильность.

1. INTRODUCTION:

The endometrium has always been considered sterile in nonpregnant, pregnant women and postpartum women, as proven by cultural method diagnostics (Butler, 1958). Indeed, premature deliveries, recurrent miscarriage, and repeated implant failures in artificial reproductive technologies are known to be associated with the presence of highly concentrated microbial products, endotoxins, or infection process within the endometrium (Fox, *et al*., 2015; Drbohlav *et al*., 1998; Hernandes, *et al*., 2020; Cicinelli *et al*., 2014).

It is difficult to imagine that the endometrium can remain a colonization-free zone at the same time as being near various microorganisms of the lower reproductive system, as well as with sperm – which may carry bacteria.

The ascending path through the uterine cervix is considered the main source of bacterial existence within the endometrium (Mitchell *et al*., 2015). As an example, experimental models have shown that the labeled particles, identical to sperm cells (microaggregates of the human-derived protein albumin), may pass through the cervical canal to the womb within several minutes in cases of nonpregnant women (Zervomanolakis *et al*., 2007; Kunz, *et al.,* 1997). In this manner, the uterine peristaltic contractions, which facilitate the entry of sperm into the uterine cavity, may perform bacterial insemination within the endometrium. Simultaneously, the anatomical constitution of cervix and the cervical mucus protect the womb from vaginal bacteria. Due to this fact, data has shown a rapidly declining relative and absolute *lactobacillus* abundance in the lower third, middle third and upper third parts of the endometrium. Therefore, the ‘higher’ the definite part the womb

is, the lower the relative abundance of *lactobacillus* there (Mitchell *et al*., 2015), i.e. concentration of *lactobacillus* is less in the bottom of the uterus than near the cervical canal. Thus, it is obvious to conclude that the endometrium cannot be considered as sterile tissue.

Recent research in this field, with the implementation of molecular technology, has proven the existence of residential microflora within the human endometrium and its potential influence on the reproduction of women (Benner *et al.,* 2018). In particular, with the processes of ovum implantation and the outcomes of pregnancies (Marchesi and Ravel, 2015; Fang *et al*., 2016; Franasiak *et al*., 2016; Yan *et al.,* 2016).

The most commonly discussed hypothesis is the 'lactobacillary' and 'non-lactobacillary' microbiome in the endometrium (Moreno *et al*., 2016). This means that the dominance of *lactobacillus* (relative abundance is more than 90%) within the microbiota contents of the endometrium was generally associated with a successful implantation and a high frequency of live-borns in patients having undergone in-vitro-fertilization (Moreno *et al*., 2016; Kyono *et al*., 2018; Walther-Antonio *et al*., 2016). Consequently, it can be concluded that potentially increasing the relative abundance of *lactobacillus* by up to 90% or more in women with a non-*lactobacillus* microflora within the endometrium could lead to frequent successful implantations in infertile patients and can later be associated with a 'healthy' uterine microbiome.

Unfortunately, numerous scientific publications and the accumulated scientific material do not allow forming a solid understanding of what normal and a dysbiotic uterine microbiome is by now or explain how to correct in case of abnormality (Wee *et al*., 2018; Verstraelen *et al*., 2016; Tao *et al*., 2017; Miles *et al*., 2017; Pelzer *et al*., 2018; Chen *et al*., 2017).

This study aimed to assess the uterine microbiome in women with infertility who have experienced numerous failed attempts of in-vitro-fertilization and healthy fertile women without an obstetric or gynecological history who had at least one successful vaginal delivery.

2. MATERIALS AND METHODS:

2.1. Study Design

The study included 35 women divided into two groups (Table 1). All patients were examined at the Department of Obstetrics and Gynecology № 1 of the Federal State Budgetary Educational Institution of Higher Education, Rostov State Medical University, and Ministry of Health of Russia (Rostov-on-Don, Russia). Patients of both groups were comparable in body mass index, age of menarche, duration of the menstrual cycle and duration of menstruation itself, and age.

Group I included 20 women with infertility due to various origins and multiple unsuccessful in-vitro-fertilization attempts (two or more attempts), aged 20 to 42 years. All of these women underwent frozen embryo transfer protocols after preparation of endometrium with estrogens and progesterone. Only one embryo was transferred to the uterus, and preimplantation genetic testing was performed on all embryos before transfer. The exclusion criteria included any uterine pathology, along with congenital malformations, uterine fibroids, polyps, uterine synechia, and uterine manipulations within the last six months, as well as the presence of an IUD within the last six months, any systemic inflammatory disease or severe extragenital pathology, or the use of systemic antibiotic therapy in the last three months.

Group II consisted of 15 healthy women from 20 to 42 years old without any complaints, without burdened obstetric or gynecological history, who'd had at least one vaginal birth in the anamnesis. In addition to the criteria for group I, the exclusion criteria also included surgical abortions, pregnancy losses, non-developing pregnancies, and uterine cavity curettages in the postpartum period, together with a history of cesarean section.

2.2. Ethics

Each study participant completed an written informed consent. The local ethics committee of the Rostov State Medical University of the Russian Ministry of Health approved this study.

2.3. Sample collection

All samples of endometrium were collected between 22 to 24 days of the regular menstrual cycle. To avoid contamination with microorganisms from the vagina or cervical canal, and to obtain exclusive data on the species composition of the uterine cavity, a

catheter for transferring the embryo into the uterine cavity was used to collect the material, consisting of an 'outer hard part' and an 'inner soft part' (Figure 1). After visualization of the cervix, using a sterile gauze swab moistened with chlorhexidine solution, mucus was removed from the surface of the cervix, then the cervix was processed. The catheter for transferring embryos into the uterine cavity was carefully inserted into the cervical canal without touching the walls of the vagina. In contrast, the inner soft part of the catheter was completely inserted into the outer one. In this way, bacterial contamination of the cervical canal was avoided.

After entering the uterine cavity, the inner catheter was carefully pushed out from the outer one into the uterine fundus area. The material was taken from the fundus to study the microbiome of the uterus, then the inner catheter was again placed in the outer part, and the entire system was removed from the cervical canal and vagina. A sample of endometrial flora was placed in an Eppendorf tube with a special transport medium 'transport medium with mucolytic' (Federal Budget Institution of Central Epidemiological Research, Rospotrebnadzor, Moscow, Russia), the medium was kept at +4°C until DNA isolation.

The total DNA was isolated using the Ribo-prep kit (FBSI Central Research Institute of Epidemiology, Rospotrebnadzor, Russia) according to the manufacturer's protocol. This proprietary method is based on cell lysis with a guanidine thiocyanate (GuSCN) and a Triton X-100-based lysis buffer, followed by DNA precipitation and washing with isopropanol-based buffers. DNA is finally diluted in 10 mM Tris-HCl (pH = 8.0).

Libraries of 16S rRNA gene fragments were prepared according to '16S Metagenomic Sequencing Library Preparation' (Part No. 15044223 Rev. B) of Illumina. 5 ng of total DNA was amplified for 25 cycles using the primers recommended in the protocol for the V3 and V4 regions of the 16S rRNA bacteria gene and the ready-made mixture for PCR KAPA HiFi HotStart ReadyMix (2X) (Roche Diagnostics, Switzerland). The resulting DNA fragments were purified using AMPure XP beads paramagnetic particles (Beckman Coulter, USA). To carry out indexing PCR, 5 ng of DNA after the first round of amplification was subjected to eight amplification cycles using tag-coded primers from the Nextera XT Index Kit (Illumina, USA) and the KAPA HiFi HotStart ReadyMix (2X) PCR mixture (Roche Diagnostics, Switzerland). The obtained libraries of gene fragments were purified using paramagnetic particles, combined with an equimolar ratio, and sequenced on a MiSeq system (Illumina, United States) in a 2 * 151 paired end-sequencing mode.

2.4. Data analysis

The data analysis obtained during the sequencing of gene fragment libraries was carried out using a pipeline implemented in the R v.3.6 programming languages (R Core Team, 2014) and Python. At the first stage of the pipeline, the primer sequences were removed from the beginning of reads. Reads that did not contain primers were also removed. Then, the last 25 nucleotides of the reads were removed as low quality, and the obtained data were processed using the DADA2 protocol to detect exact sequence variants. After determining the exact variants of the sequences, forward and reverse readings were combined by concatenation, and the resulting sequences were used for Bayesian taxonomic classification (Wang *et al*., 2007) using the SILVA v132 reference database (Quast *et al*., 2013). Species identification was performed using the DADA2 full match algorithm using the SILVA v132 database sequences pre-processed with custom scripts to match the analysis.

2.5. Statistical analysis

The Shapiro–Wilk test was used to check the clinical and demographic characteristics of patients in the studied groups for normal distribution (Table 1). The concentrations of the studied microorganisms are not of a normal distribution and are significantly rarefied; therefore, they are presented as medians (Table 2). The nonparametric Mann–Whitney test was used to compare quantitative indicators in groups. The differences were considered statistically significant at the $p < 0.05$ level. The calculations were performed in R (version 3.2, R Foundation for Statistical Computing, Vienna, Austria).

3. RESULTS AND DISCUSSION:

The clinical and demographic characteristics of both patient groups are presented in Table 1. A study of the endometrial microbiome by sequencing made it possible to identify microorganisms belonging to 19 types, 26 classes, 89 families, 257 genera, and 366

different species by taxonomic classification. A comparison between groups was carried out at the taxonomic level of genera.

According to the genera level between the two groups, statistically, significant differences were revealed in the relative abundance of the following genera of microorganisms: *Acidovorax, Brevibacillus, Brevundimonas, Caulobacter, Comamonas, Delftia, Distigma, Pseudomonas, Ralstonia, Schlegelella,* and *Thermus*. In the group of women with infertility and repeated unsuccessful in-vitro-fertilization attempts, a significantly higher abundance of bacteria of the genus *Brevundimonas* (0.065 – 0; 0.38 in the Group I and 0 [0; 0] in the Group II) and *Ralstonia* (0.21 [0; 1.2] in the Group I and 0 [0; 0.12] in the Group II) was identified.

At the same time, the fertile women of Group II had a statistically significantly higher abundance of *Acidovorax* (0 [0; 1.33] in the Group I and 2.1 [0.75; 3.44] in the Group II), *Brevibacillus* (0 [0; 0] in Group I and 0.11 [0; 0.3] in Group II), *Caulobacter* (0 [0; 0.28] in Group I and 0.54 [0.15; 1.07] in Group II, respectively), *Comamonas* (0.01 [0; 6.37] in Group I and 7.44 [4.15; 18.2] in Group II), *Delftia* (0.025 [0; 1.26] in Group I and 2.4 [0.81; 4.11] in Group II), *Distigma* (0 [0; 0.22] in Group I and 0.64 [0.11; 1.22] in Group II, respectively), *Pseudomonas* (0 [0; 0.12] in the Group I and 0.48 [0.01; 2.26] in the Group II), *Schlegelella* (0 [0; 0.72] in the Group I and 1.18 [0.29; 2.14] in the Group II), *Thermus* (0 [0; 2.63] in the Group I and 2.57 [1.05; 5.62] in the Group II) (Table 2).

When analyzing the average relative abundance of bacterial genera, the most represented in the group of healthy fertile patients were *Lactobacillus* 29.4%, Comamonas 16.8% and Mesorhizobium 6.0%, and in the group of infertile patients with multiple in-vitro-fertilization failures – *Lactobacillus* 33.3%, Ralstonia 7.9%, and Pediococcus 4.8% (Figures 2, 3 and 4).

Meanwhile, the abundance of *Lactobacillus* in Group I patients did not differ significantly from the abundance of Lactobacilli in patients of Group II (33.3% and 29.4%, respectively). Also, it is not possible to make confident conclusions about the dominance of *Lactobacillus* over 90% in the composition of the uterine microbiome in healthy women, as Moreno claims from his observations (Moreno *et al*., 2016; Kyono *et al*., 2018). The category 'other microorganisms' included various microorganisms whose relative abundance in the microbiome was less than 1%. In patients of group I, 'other' microorganisms accounted for 18.7%, and 14.4% in Group II – 14.4%, respectively. Also, unclassified microorganisms – that is, those that could not be identified or whose genome is absent in the known databases – accounted for 10.5% in Group I and 7.5% in Group II. In general, it can be argued that the endometrial microbiome is quite diverse both in healthy fertile patients and in patients with multiple in-vitro-fertilization failures, which may be directly related to the place of collection of material for the microbiome study – namely, the uterine fundus. After all, the 'higher' the locus of material sampling in the female reproductive system, the lower the abundance of *Lactobacillus*. However, despite the diversity of microorganisms, attention was drawn to the almost complete absence (less than 1%) in fertile patients and the presence of the following genera of microorganisms in infertile women with in-vitro-fertilization failures: *Streptococcus* (3.2% in Group I), *Ralstonia* (7,9% in Group I), *Pediococcus* (4.8% in Group I), *Clostridium* (4.0% in Group I). At the same time, bacteria registered in fertile patients but not detected in significant proportions (more than 1%) in patients with infertility were *Acinetobacter* (1.7% in Group II), *Anaerobacillus* (1.3% in Group II), *Cupriavidus* (1.1% in Group II), *Curvibacter* (2.0% in Group II), *Delftia* (2.8% in Group II), *Paucibacter* (1.3% in Group II), *Prevotella* (1.7% in Group II), *Shlegella* (1.3% in Group II).

This research had many limitations. First, the sample size – 20 patients from Group I and 15 from Group II – may not represent population abundance as a whole but may indicate some trends for further study. Therefore, the results obtained require other studies carried out using larger cohorts of patients.

Another limitation of this study was the lack of negative control samples. In scientific papers devoted to studying the microbiome of biotopes of complex localizations, increasing attention is devoted both to the presence of control samples and their negative values. If the endometrial microbiota exists, then it is present in very low concentrations. Therefore, its molecular characteristics are influenced by background DNA contamination from extraction kits and reagents for PCR sequencing (Salter *et al*., 2014; Weyrich *et al.,* 2019). For this reason, contaminating DNA can account for a significant part, if not all, of the recorded molecular microbial signatures within the endometrium. It is,

therefore, imperative that endometrial microbiota studies include technical controls for potential sources of background DNA contamination (Salter *et al*., 2014), as well as a detailed presentation of the microbial profiles of these controls (Eisenhofer *et al.*, 2019). Most of the studies available to date do not include control samples, or the data on their microbial profile is insufficiently presented. When sequencing the control samples of nutrients or transport medium or air from the operating room, the number of reads should be hundreds of times less than the number of reads of material from the endometrium. Potential microbial contamination is possible from extraction kits, culture medium, and air, which can greatly affect the interpretation of the identified microorganisms' clinical significance. The role of negative control samples, therefore, is of paramount importance. This, however, increases research costs by almost three times, which further complicates work planning.

The third limitation was the method of material sampling for research – which was the transcervical approach. In most endometrial microbiome studies currently available, the sampling was carried out transcervically, possibly associated with the contamination of endometrial samples by microbes or microbial molecular signals from the vagina and cervical canal. In most studies to date, in which material for sequencing has been collected via transcervical access, the lactobacillary microflora characteristic of the vaginal microbiome was noted. At the same time, in studies where the material was collected in a transabdominal way, the variety of microorganisms was higher. Thus, Chen *et al.* used 16S rRNA sequencing to assess the microbiome of both the lower and upper sections of the female reproductive tract. This showed that, for the upper sections, the average proportion of *Lactobacillus* is only 1.7% (fallopian tubes). In the endometrium, the relative abundance of *Lactobacillus* was 30.6%, but *Acinetobacter* (9.1%), *Pseudomonas* (9.1%), *Vagococcus* (7.3%), *Sphingobium* (5.0%) and *Comamonadaceae* (4.9%) were widely abundant (Chen *et al*., 2017). Walther-Antonio *et al*. examined ten women after hysterectomy associated with benign neoplasms. In the myometrium and endometrium, *Lactobacillus* was found in extremely low proportions. The most represented in the endometrium were *Shigella* and *Barnesiella* (Walther-Antonio *et al*., 2016). In contrast, in a study that obtained material from the middle third of the endometrium after hysterectomy, there was also no dominance of *Lactobacillus* (Winters *et al*., 2019), and the endometrial bacterial profiles were mainly abundant with *Acinetobacter, Pseudomonas, Comamonadaceae* and *Cloacibacterium*. It turns out, therefore, that in all studies in which the method of sampling was transcervical, the predominance of *Lactobacillus* in the composition of the uterine microbiome was reported and, as with other sample obtaining methods, the microbiome was more variable.

The significance of the presented research lies in several positions. First, the presented data confirms the concept of endometrial non-sterility and the existence of the uterine microbiome as a whole (Altmäe *et al.*, 2018; Baker *et al.,* 2018). Unambiguously, sequencing makes it possible to detect the presence of bacteria in the endometrium, but the results of the analysis itself do not allow for quantifying this presence. That is, it is impossible to judge the level of bacteria found in a particular sample.

Statistically significant differences between groups were obtained in the following genera of microorganisms: *Acidovorax, Brevibacillus, Brevundimonas, Caulobacter, Comamonas, Delftia, Distigma, Pseudomonas, Ralstonia, Schlegelella,* and *Thermus*. However, their average relative representation in the uterine microbiome composition was less than 1%; therefore, the question of their influence on the implantation processes of a fertilized egg is debatable.

Presented data confirm the current concept of the dominance of *Lactobacillus* in the uterine microbiome. Considering the anatomical proximity of the uterine cavity and endometrium with the main source of *Lactobacillus* in the reproductive system – with the vagina – and the existence of peristaltic uterine contractions, contributing to the capture of *Lactobacillus* from the vagina and the ascending pathway of their entry into the uterine cavity, the dominance of *Lactobacillus* becomes an understandable and logical fact. Various data on the percentage of *Lactobacillus* in the microbiome can be explained by the 'area' of sampling of the material for study: the closer to the area of the cervical canal, the higher the percentage of *Lactobacillus*; the closer to the area of the fundus, the less its percentage (Winters *et al.,* 2019).

Besides, the presented data refutes the theory of lactobacillary and non-lactobacillary microbiome in the genesis of fertilized egg implantation failures. According to the presented

data, the proportion of *Lactobacillus* in fertile patients and in patients with multiple in-vitro-fertilization failures did not differ significantly and amounted to 33.3% and 29.4%, respectively. This may be due to the area of sampling – in this study, this is the bottom of the uterus, e.g. the most distant from the source of *Lactobacillus* – the vagina – the locus of the uterus. Therefore, the dominance of *Lactobacillus* cannot be considered as a factor determining the absolute success of implantation. Besides, the relative abundance of *Lactobacillus* – over 50% in the microbiome – was recorded in only 5 out of 15 women from the group of fertile patients. In 7 out of 20 women from the group of women with in-vitro-fertilization failures (Figure 4). The absolute dominance of *Lactobacillus* – more than 90% – was noted in a total of three women out of the entire sample (35 patients), which does not correlate with Moreno's data (Moreno *et al*., 2016).

In all likelihood, it is not only the relative abundance of *Lactobacillus* that affects implantation success, as it is necessary to search for those pathogenic microorganisms that trigger a cascade of inflammatory reactions in the endometrium, thereby determining the implantation impairment. It is important to study the microbiome of the endometrium, together with markers of chronic endometritis, via a histological and immunochistochemical examination of the endometrium during biopsy (Agostinis *et al.,* 2018; Zhang *et al.,* 2019). The mechanisms providing a positive result during implantation of a fertilized egg are more subtle and include not only the influence of certain pathogenic microorganisms and their endotoxins but also the local immune response of the endometrium. In this regard, it is more expedient to study the composition of the endometrial microbiome in close connection with immunohistochemistry markers of chronic endometritis. Moreover, it is important to include in diagnostic algorithms an assessment of the functional state of the endometrium (dopplerometry of the uterine spiral arteries) and to assess tissue morphology during biopsy (; Liu Y *et al., 2018;*).

According to the presented data, in the group of women with infertility and repeated unsuccessful in-vitro-fertilization attempts, a significantly higher relative abundance of bacteria of the genus *Brevundimonas* and *Ralstonia* were recorded, and in fertile women of the second group, a statistically significant higher abundance of *Acidovorax, Brevibacillus, Caulobacter, Comamonas, Delftia, Distigma, Pseudomonas, Schlegelella,* and *Thermus* were recorded. Whether this statistically significant difference is of clinical importance or not remains unclear as, firstly, the average relative abundance of these bacterial genera was less than 1%. Secondly, all these microorganisms are opportunistic and lead to the development of the disease only under concomitant circumstances.

The most abundant in the group of healthy fertile patients were *Lactobacillus, Comamonas* and *Mesorhizobium*. While in the group of infertile patients with multiple in-vitro-fertilization failures *Lactobacillus, Ralstonia* and *Pediococcus* were most abundant. Regarding *Lactobacillus*, their biological role in the microbiota composition is more or less clear. As for *Comamonas*, this genus belongs to conditionally pathogenic microorganisms stable in the external environment and are characteristic of the gastrointestinal tract (Khalki *et al*., 2016). *Mesorhizobium* are also conditionally pathogenic microorganisms that occur in nature as a symbiont of plants, but there are several reports of their involvement in the development of peritonitis (Krishnan *et al*., 2016). *Ralstonia* are generally characteristic of such an econiche as water and are able to survive in conditions of low nutrient supply. In humans, they are involved in the occurrence of osteomyelitis and meningitis (Ryan and Adley, 2014). *Pediococcus* is a lactic acid-producing bacteria used as probiotics. The role of all these genera of microorganisms in the uterine microbiome composition remains insufficiently studied to date (Porto *et al*., 2017).

There are many interesting perspectives to potentially improve endometrial dysbiosis: among them is fecal transplantation, vaginal microbiota transplantation, and the administration of pro-and prebiotics (DeLong *et al.*, 2019; Lev-Sagie *et al.,* 2019; Quaranta *et al.,* 2019; Stallmach *et al*., 2019). All these methods have been discussed but are worth further investigation.

4. CONCLUSIONS:

The present study confirms that even such hard-to-reach locus as the endometrium are not sterile. The endometrium is not such a 'densely populated' biotope as, for example, the intestine, and the concentration of bacteria here is negligible. Still, one cannot ignore the role of detected microorganisms in the genesis of recurrent implantation failures. The most abundant genera, seen both in fertile and infertile

women with repeated implantation failures, was *Lactobacillus*. There was no significant difference in the mean relative abundance of *Lactobacillus* between two groups.

Infertile women with repeated in-vitro-fertilization failures and repeated impaired implantation had significantly higher mean relative abundance of bacteria of the genus *Brevundimonas* and *Ralstonia*. The fertile women had a statistically significantly higher mean relative abundance of *Acidovorax*, *Brevibacillus*, *Caulobacter*, *Comamonas*, *Delftia*, *Distigma*, *Pseudomonas*, *Schlegelella*, and *Thermus*. But the abundance of all these genera was very low. Thus, it is unclear whether such low concentrations can have clinical effects and influence implantation process.

Knowledge of the composition of the endometrial microbiome supports the need to develop algorithms for correcting dysbiosis. In this case, it is crucial to consider the pathways of microorganisms entering the uterine – ascending from the vagina, hematogenous from the intestine, and oral cavity. All this provides broad prospects for further research.

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Table 1. *Clinical and demographic characteristics of patients in the surveyed groups*

Index	Group I (infertile) N=20	p-value (Shapiro–Wilk test) in the Group I	Group II (Fertile women) N=15	p-value (Shapiro–Wilk test) in the Group II	p-value (Mann–Whitney test)
Age	33.3 (3.8)	0.99	32 (6.26)	0.16	0.06
BMI	23.8 (4.51)	0.32	21.3 (1.99)	0.79	0.08
Menarche	12.9 (1.19)	0.14	13.5 (0.74)	0.25	0.3
Length of the menstrual cycle	27.2 (2.43)	0.37	30.9 (8.83)	0.19	0.3
Duration of menstruation	4.67 (1.18)	0.14	4.57 (0.84)	0.16	0.06
Number of in-vitro-fertilization procedures	4,72 (2,11)	-	-	-	-
Number of births in history	-	-	2,14 (0,75)	-	-

Note: The data in the table is given as mean and standard deviation. Differences were considered statistically significant at $p < 0.05$.

Table 2. *Comparison of the mean abundance of the most common genera of microorganisms in the endometrial microbiome of women in Group I (infertile patients with repeated IVF failures) and Group II (healthy fertile women without a burdened obstetric-gynecological history), only statistically significant differences*

Index	Median [Lower quartile; Upper quartile]		Mean p
	Group I (infertile) N=20	Group II (Fertile women) N=15	
Acidovorax	0 [0; 1.33]	2.1 [0.75; 3.44]	0,009
Brevibacillus	0 [0; 0]	0.11 [0; 0.3]	0,01
Brevundimonas	0.065 [0; 0.38]	0 [0; 0]	0,03
Caulobacter	0 [0; 0.28]	0.54 [0.15; 1.07]	0,03
Comamonas	0.01 [0; 6.37]	7.44 [4.15; 18.2]	0,02
Delftia	0.025 [0; 1.26]	2.4 [0.81; 4.11]	0,004
Distigma	0 [0; 0.22]	0.64 [0.11; 1.22]	0,02
Pseudomonas	0 [0; 0.12]	0.48 [0.01; 2.26]	0,03
Ralstonia	0.21 [0; 1.2]	0 [0; 0.12]	0,02
Schlegelella	0 [0; 0.72]	1.18 [0.29; 2.14]	0,02
Thermus	0 [0; 2.63]	2.57 [1.05; 5.62]	0,009

Note: in the table, mean values are presented as Median [Lower quartile; Upper quartile]; comparison was carried out using the Mann–Whitney test. Differences were considered statistically significant at $p < 0.05$.

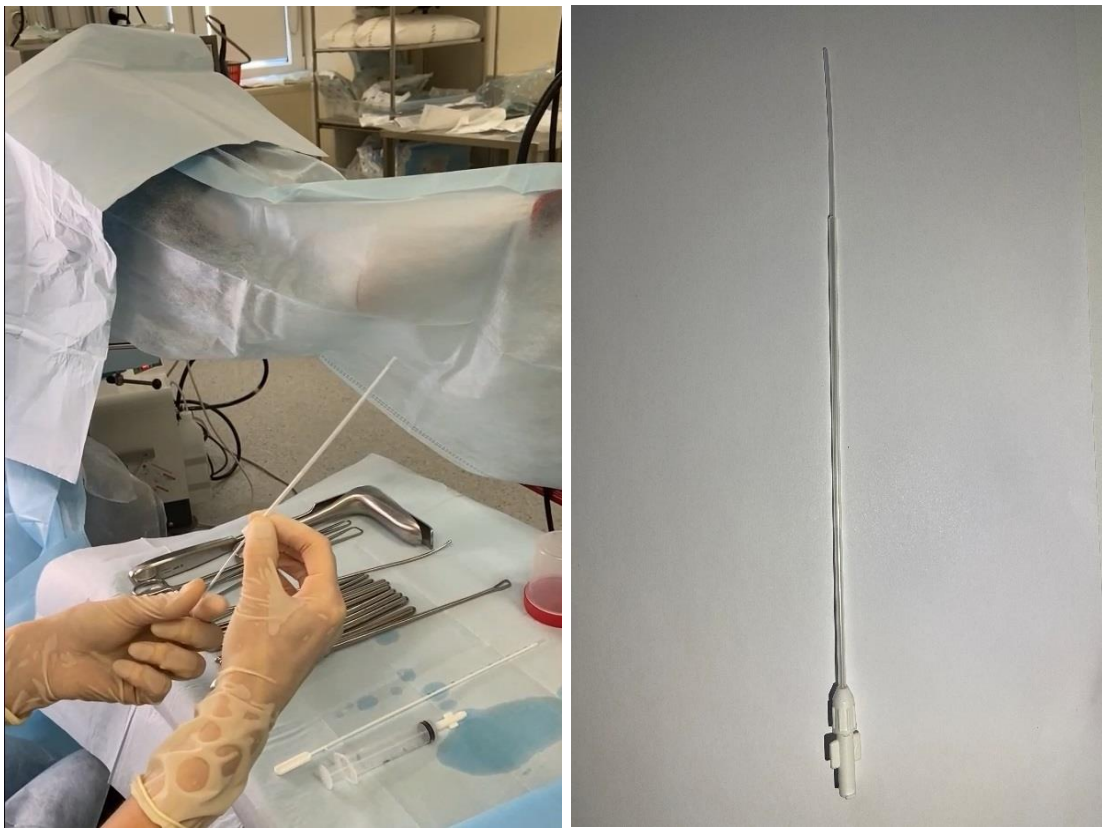


Figure 1. *A sampling of microbiome of endometrium using double-sheathed catheter for embryo transfer to avoid contamination with microorganisms from the vagina or cervical canal and to obtain data exclusively on the species composition of the uterine cavity.*

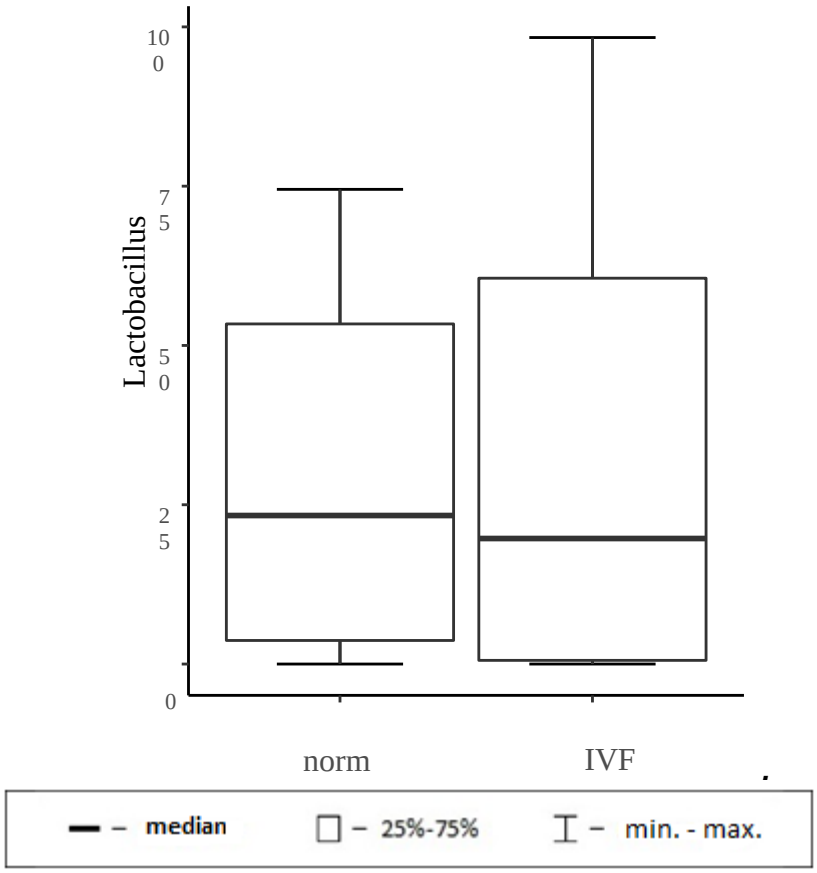


Figure 2. *Boxplot of relative abundance of Lactobacillus in groups I and II in the composition of the endometrial microbiota. The bold line within the box is drawn to the median of each group, the bottom and top of the box to the 25th and 75th percentiles, respectively. The whiskers are drawn to the 10th and 90th percentiles.*

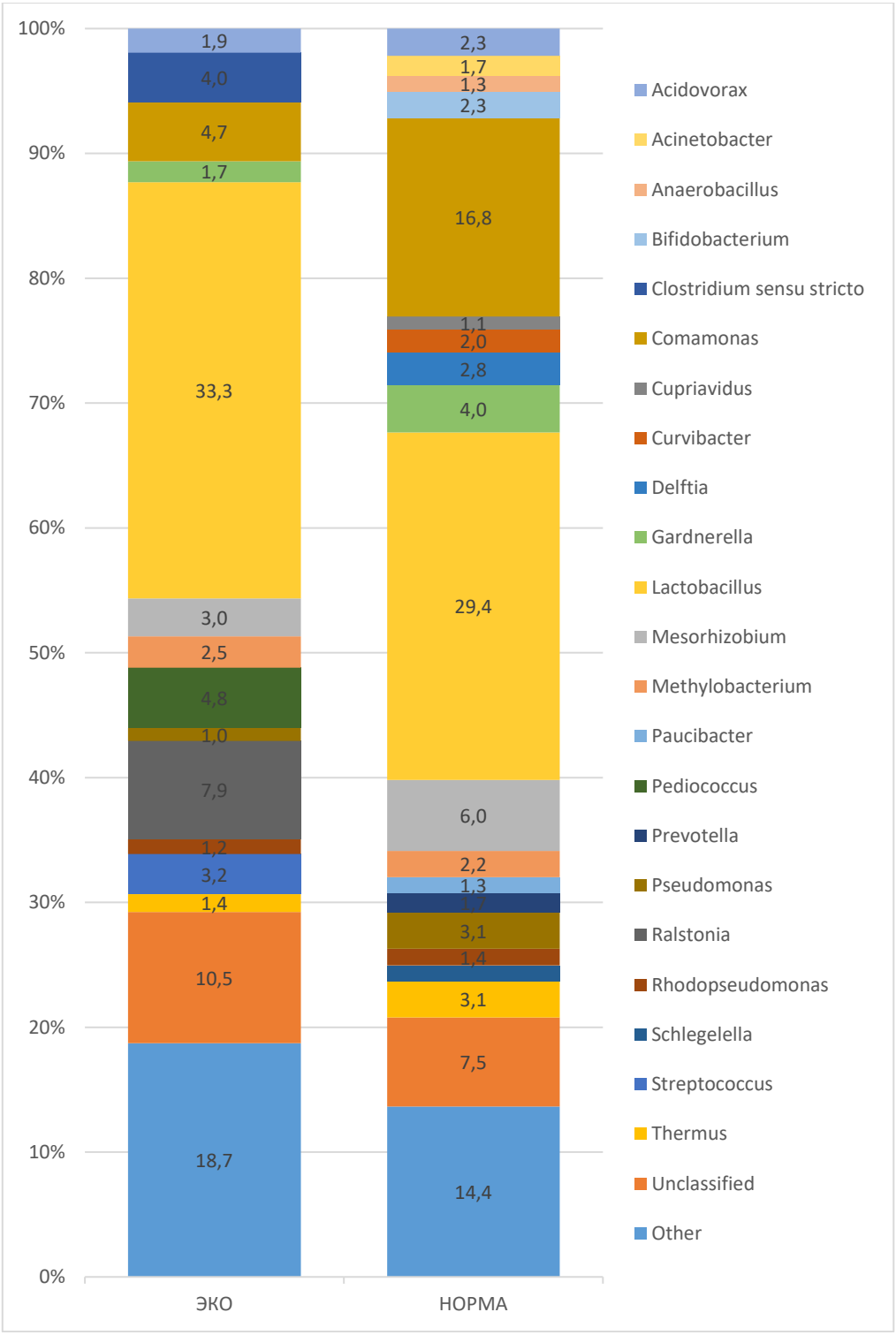
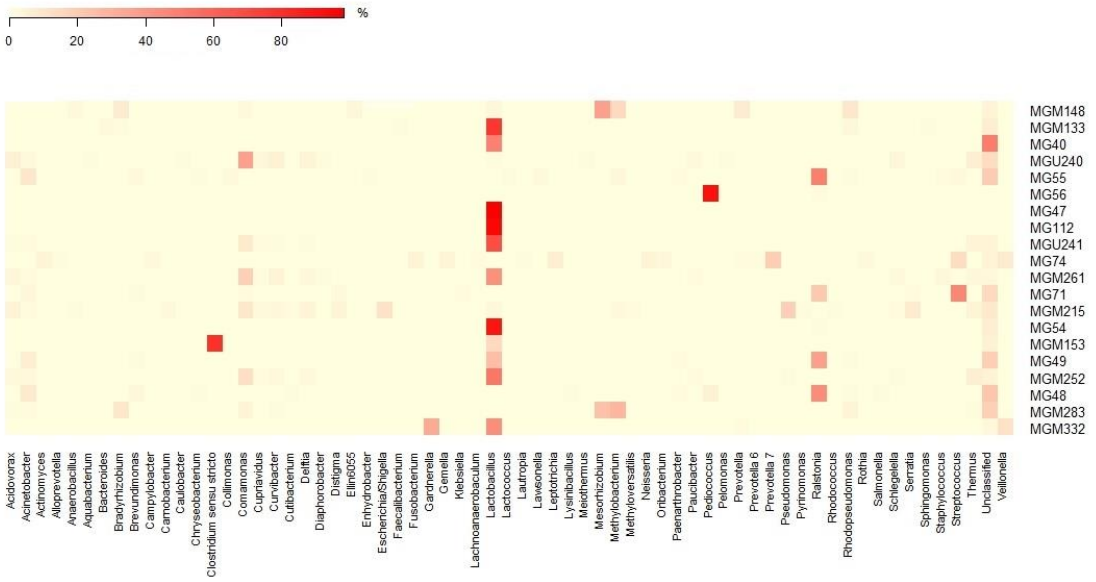
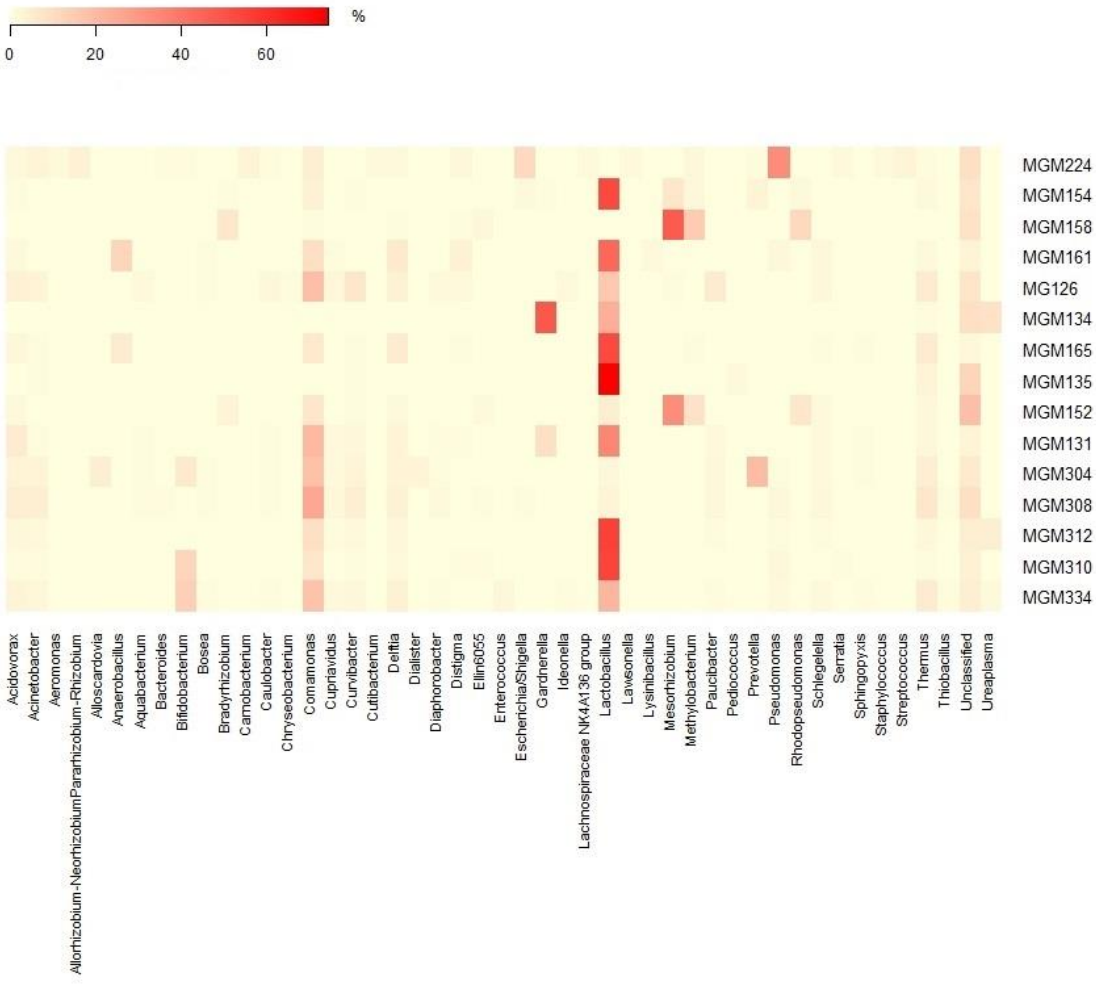


Figure 3. *The mean relative abundance of the most common genera of microorganisms in the endometrial microbiome of women in group I (infertile patients with repeated in-vitro-fertilization failures) and group II (healthy fertile women without a burdened obstetric-gynecological history) 'Other' bacteria include various bacteria whose average proportion was less than 1%.*



a



b

Figure 4. *Heat map of the mean relative abundance of bacteria in patients of group I (infertile patients with repeated in-vitro-fertilization failures) (a) and group II (healthy fertile women without burdened obstetric and gynecological history) (b). The figures show the genera of bacteria, whose relative abundance in the microbiome was 1% and higher.*