MEDICAL UNIVERSITY - PLEVEN

Faculty of Medicine

Department of Obstetrics and Gynecology

Dr. Tatiana Trendafilova Bodurska-Petkova

**Composition and characterization of endometrial
microbiome in recurrent reproductive failure**

ABSTRACT

of a dissertation

to acquire an educational and scientific degree "Doctor"

Doctoral program "Obstetrics and Gynecology"

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Scientific supervisors:

Assoc. Prof. Tihomir Pankov Totev, MD, PhD

Assoc. Prof. Emiliana Ilieva Konova, MD, PhD

Official reviewers:

Prof. Elena Dimitrova Dimitrakova, MD, PhD

Assoc. Prof. Maria Angelova Angelova, MD, PhD

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The dissertation is written on 181 standard pages and illustrated with 45 figures and 6 tables.

The literary sources used include 267 titles in Latin.

The numbers of the figures and tables in the abstract do not correspond to the numbers of the figures and tables in the dissertation.

The author is a doctoral student in independent study at the Department of Obstetrics and Gynecology, Faculty of Medicine, Medical University – Pleven and is enrolled by order No. 3681/05.12.2022. She works as an obstetrician-gynecologist at the CIRM Medical Center – Pleven.

Three full-text publications have been published in connection with the dissertation work.

The dissertation was discussed, accepted and proposed for public defense at a meeting of the Departmental Council of the Department of Obstetrics and Gynecology at the MU - Pleven, held on 30.04.2024.

Scientific jury composed of:

Internal members for MU – Pleven:

1. Assoc. Prof. Petya Petrova Chaveeva, MD, PhD

2. Assoc. Prof. Nikola Kalinov Popovski, MD, PhD

Reserve internal member for MU - Pleven:

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External members for MU – Pleven:

1. Prof. Elena Dimitrova Dimitrakova, MD, PhD, Medical University - Plovdiv

2. Assoc. Prof. Maria Angelova Angelova, MD, PhD, Trakia University – Stara Zagora
3. Assoc. Prof. Vesela Ivanova Karamisheva, MD, PhD, Medical University - Sofia

Reserve external member for MU – Pleven:

Assoc. Prof. Kremen Tsvetanov Tsvetkov, MD, PhD, Medical University - Varna

The public defense of the dissertation work will take place on 8th of August 2024.

The defense materials are published on the website of the Medical University – Pleven.

CONTENTS

I. Introduction

Infertility affects between 8 and 12% of couples of reproductive age.

Although 40 years of assisted reproductive technology, implantation remains the last and most difficult barrier to successful pregnancy. Its success rate does not exceed 40% for 5-day-old embryos and 25% for 2- and 3-day-old embryos.

Recurrent miscarriages are one of the most challenging problems in reproductive medicine because their causes often remain unknown and effective treatment is rarely possible. The pathophysiology of miscarriages is multifactorial and often persists as a continuum causing new miscarriages in subsequent pregnancies. RPL affects about 5% of women in reproductive age. Standard tests leave about 50% of cases without a clear answer for pregnancy loss.

The development of new molecular genetic technologies made possible to detect microorganisms in organs considered sterile, such as uterus, placenta, testicles. As a result, a series of questions were raised - what are the normal microorganisms for the endometrium, how do they interact with the physiological processes in it, what leads to damage to the healthy microbiome, what are the consequences of its damage, what changes are most often found in the microbiome in repeated reproductive failures, how to investigate and treat them, what sources of error exist - from sampling (technique and menstrual cycle phase), contamination, interpretation of results, practical application, biomarker for ART outcome.

Reproductive failures may be fundamentally related to changes in the microbiome of the reproductive tract. Data in recent years have focused on the microbiome of the lower reproductive tract, but the microbiome of the upper reproductive tract has been relatively poorly studied.

II. Objective and tasks

Reproductive failures may be fundamentally related to changes in the microbiome of the reproductive tract. Data in recent years have focused on the microbiome of the lower reproductive tract, but the microbiome of the upper reproductive tract has been relatively poorly studied. In this regard, the aim of this dissertation is:

Objective: To implement a comprehensive approach to initially assess the composition and characterization of the endometrial microbiome, to correlate it with its role in patients with recurrent implantation failure and recurrent miscarriage, and to construct a gene-based protocol for its investigation technologies, with application in clinical practice.

Tasks:

Task 1: To determine the presence and amount of lactobacilli in the endometrium in patients with RIF (group 1) and RPL (group 2).

Task 2: To analyze the influence of age on the amount of lactobacilli in the endometrium in patients with RIF (group 1) and RPL (group 2).

Task 3: To investigate the composition and frequency of eubiotic and dysbiotic endometrial microbiome species in RIF (group 1) and RPL (group 2) patients.

Task 4: To characterize the bacterial composition by type and frequency of the isolated pathogenic microorganisms in a dysbiotic endometrial microbiome.

Task 5: To analyze the influence of the isolated dysbiotic microorganisms on the degree of damage to the microbiome and to compare it in the two studied groups.

Task 6: To explore approaches to the study of the endometrial microbiome and to propose the creation of an optimized protocol for its assessment using genome-based technologies.

III. Materials and methods
III.1. Study design and patient selection
III.1.2. Selection of patients
The studied clinical contingent includes 199 women with RIF and RPL, diagnosed, treated and followed up at the CIRM Pleven Medical Center for the period from October 2019 to November 2022, with an average age of 35.69±5.16 years. An inductive approach was used to assess interim status without looking for an association with subsequent episodes of RIF, RPL or live birth.

Inclusion criteria:

- patients with RIF and RPL;

- written informed consent to participate in the study.

Exclusion criteria:

- lack of written informed consent;

- untreated inflammatory processes of the vagina and cervix;

- present and untreated pathology of the adnexa and uterus;

- the presence of diseases or medical conditions that threaten the patient's health;

- technically difficult performance of endometrial biopsy due to anatomical features.

Patients are instructed to protect themselves from pregnancy by abstaining from sexual contact or using barrier methods of contraception. All patients provided written informed consent for genetic testing as part of approved standard operating procedures by all participating institutions. The studies strictly followed the principles of the

Declaration of Helsinki. Analyzes were performed under conditions of blinding of the person who performed all the bioinformative analyzes described below.

The cases of patients with reproductive failures included in the study met the current definitions of RIF and RPL. The average age of the patients was 35.69+-5.16 years in the range 24-54. Cases were retrospectively and prospectively studied.

III.1.2. Preparation of patients
In all patients, the endometrial biopsy was scheduled and performed on the $21^{st} - 22^{nd}$ day of a spontaneous menstrual cycle without use of hormonal preparations. Preparation of patients includes microbiological examination of vaginal contents within a maximum of 30 days before biopsy and PCR for Mycoplasma/Ureaplasma spp. from the cervix, carried out within the latest up to 3 months before the biopsy, Pap smear within the previous 1 year. In cases of pathological findings from the above tests, etiological treatment was carried out with subsequent control tests until they were remedied. Patients with cytological abnormalities were not included in the study until an accurate diagnosis and appropriate treatment were established.

III.2. Methods

III.2.1 Surgical method
The endometrial biopsy is performed in the mid-luteal phase - 21st - 22nd day of a spontaneous menstrual cycle. All patients underwent a transvaginal ultrasound examination to determine the phase of the menstrual cycle. The patients are asymptomatic, with no evidence of current colpitis or endometritis. Endometrial samples were obtained from 103 patients (51.8%) with RIF (group 1) and 96 patients (48.2%) with RPL (group 2).

III.2.1.1. Technique for obtaining endometrial sample

An obstetrician-gynecologist and a midwife actively participate in the process of obtaining an endometrial sample. All manipulations were performed by two obstetrician-gynecologists from the CIRM "St. Elisaveta" MC. No ultrasound assistance is used in the process. All participants, incl. the patient, are wearing face masks. Staff use powder-free sterile gloves. All instruments used were unpacked from sterile packaging immediately before manipulation and placed on a sterile field. In the patient's lithotomy position, after placing a sterile speculum, the vagina and cervix are thoroughly cleaned with NaCl 0.9%, removing as much vaginal contents and cervical

mucus as possible. Treatment of speculum and vagina with disinfectants is not applied. The patient must not have carried out special hygiene procedures, vaginal douches or applied topical medications or probiotic preparations. A flexible double-lumen catheter "Intra-uterine Insemination Catheter 180 mm" (Wallace®, Cooper Surgical, Inc., Shelton, CT, USA) is introduced into the uterine cavity. By applying negative pressure from a syringe with a volume of 1 ml mounted on the other end of the catheter and abrasive movements of the catheter in the uterine cavity, uterine mucosa is abraded and aspirated. Before aspiration of endometrial contents, the uterine cavity is not washed. Aspiration is terminated when the catheter is passed back at the level of the internal opening of the cervical canal. In order to prevent contamination with vaginal contents, any contact of the catheter with the vaginal walls is avoided. For the most correct interpretation of the obtained results, it is necessary that the sample contains a large amount of endometrial mucosa. The assessment of the amount of the sample was made subjectively by the physician performing it, and in case of suspicion of insufficiently obtained material, a repeat biopsy was performed. The sample should contain a minimum of blood and mucus. This is followed by careful and tight closing of the test tube containing the obtained material, its labeling and its transportation to the relevant laboratory. The sample is processed within one hour in order to preserve its integrity.

III.2.1.2 Sample storage protocol

The aspirated endometrial mucosa was stored in NaCl 0.9% and transported immediately after receipt to the DNA laboratory. The sample was homogenized with a tissue homogenizer DAKO Medimachine (Becton Dickenson, Heidelberg, Germany). The homogenate was then aspirated into a sterile syringe and stored in saline. Stored in physiological solution, the sample can be kept at a temperature between 2°C and 8°C for no more than 24 hours before analysis. In cases where the sample cannot be analyzed within 24 hours, the material is frozen, which can be stored at a temperature between -18^0 C and -22^0 C for 1 month.

III.2.2. Molecular diagnostic method

III.2.2.1. DNA extraction
DNA extraction was performed with the PREP-NA-PLUS reagent kit (DNA Technology LLC; Moscow, Russia) following a prescribed protocol provided by the manufacturer.

III.2.2.2. Sample analysis

All samples were processed with the Femoflor® 16 REAL-TIME PCR Detection Kit developed by "DNA-Technology Research & Production" in Moscow, Russia. The test uses polymerase chain reaction to amplify nucleic acids for the detection of opportunistic and normal flora in urogenital specimens. The sensitivity of the method is 99%, the specificity is 93%. The test makes a quantitative assessment of the total bacterial mass, of the endometrial normal flora - lactobacilli, of a complex of aerobic and anaerobic microorganisms, of urogenital mycoplasmas, fungi of the genus Candida, which can participate in the development of a dysbiotic process in the microbiome composition. The method is applicable to different populations and demographic groups, it can be used in clinical and diagnostic laboratories of medical institutions or research practices. The processing of all samples, the DNA-sequencing and the analysis of the obtained results were carried out under the direct control of a microbiologist at the CIRM "Sveta Elisaveta" - Pleven.

III.2.2.3. Method

It is based on PCR amplification of target DNA sequences. The Femoflor 16 Real Time PCR Detection Kit is based on a fluorescent modification of the PCR method. Fluorescence intensity was measured at each cycle of the reaction and analyzed with appropriate software.

Femoflor 16 Real Time PCR Detection Kit includes the following components: for multiplying the total bacterial mass, for multiplying Lactobacillus spp. and for the DNA of opportunistic flora.

In addition, there is a PCR mix for human DNA (sample intake control (SIC)), which excludes pre-analytical error. In each analysis, the amount of material obtained from the uterine cavity is taken into account. If the amount of collected material is not sufficient to carry out the analysis, it is necessary to repeat the sampling procedure.

The method also has an internal control (IC) whose task is to assess the quality of the polymerase chain reaction.

Upon completion of the process, the software performs a relative quantitative analysis of total bacterial DNA, species-specific lactobacilli DNA, and species-specific DNA of each opportunistic pathogen or flora. To exclude false-negative results, the amount of human DNA (SIC) is taken into account.

III.2.2.4. Data analysis

Registration and interpretation of PCR results is done in automatic mode. The sample ID, test name, and result of each test (quantity and chart that allows relative

comparison of normal flora and opportunistic pathogens in each sample) are displayed on the right side of the window. Qualitative pathogen analysis is performed. The resulting graph is plotted against fluorescence intensity over the number of cycles for each tube.

To create a Specific report for Biocenose press the "Form the answer button". 🖼️
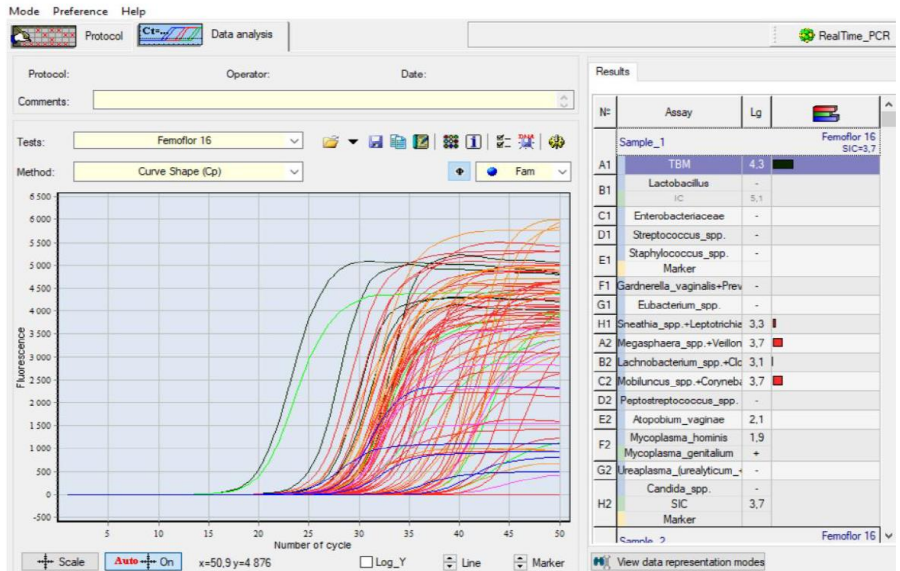
Figure 1. Detection channels of the amplified products

Urogenital microbiome composition test
Femoflor 16

Date
Number of tube
Patient name
Sex
Age
Organization
Clinician name
Comments

Information about laboratory

Sample ID: C+

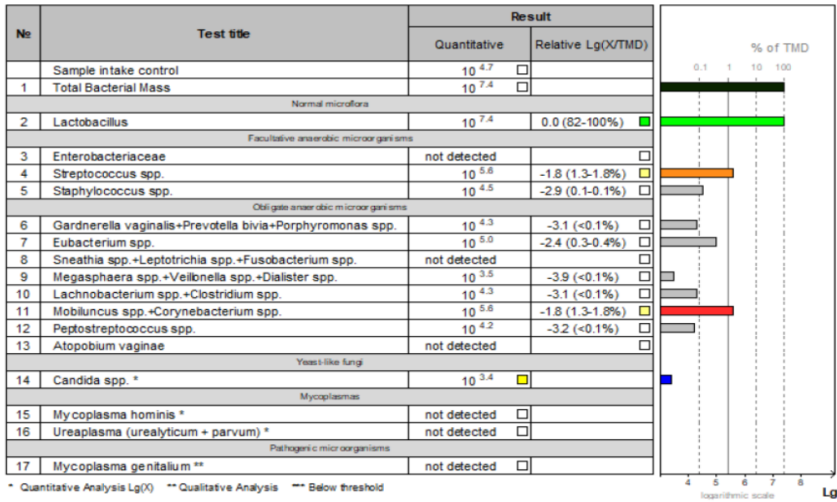| №	Test title	Result		% of TMD
		Quantitative	Relative Lg(X/TMD)	
	Sample intake control	$10^{4.7}$ ☐		
1	Total Bacterial Mass	$10^{7.4}$ ☐		
	Normal microflora			
2	Lactobacillus	$10^{7.4}$	0.0 (82-100%) ■	
	Facultative anaerobic microorganisms			
3	Enterobacteriaceae	not detected	☐	
4	Streptococcus spp.	$10^{5.6}$	-1.8 (1.3-1.8%) ☐	
5	Staphylococcus spp.	$10^{4.5}$	-2.9 (0.1-0.1%) ☐	
	Obligate anaerobic microorganisms			
6	Gardnerella vaginalis+Prevotella bivia+Porphyromonas spp.	$10^{4.3}$	-3.1 (<0.1%) ☐	
7	Eubacterium spp.	$10^{5.0}$	-2.4 (0.3-0.4%) ☐	
8	Sneathia spp.+Leptotrichia spp.+Fusobacterium spp.	not detected	☐	
9	Megasphaera spp.+Veillonella spp.+Dialister spp.	$10^{3.5}$	-3.9 (<0.1%) ☐	
10	Lachnobacterium spp.+Clostridium spp.	$10^{4.3}$	-3.1 (<0.1%) ☐	
11	Mobiluncus spp.+Corynebacterium spp.	$10^{5.6}$	-1.8 (1.3-1.8%) ☐	
12	Peptostreptococcus spp.	$10^{4.2}$	-3.2 (<0.1%) ☐	
13	Atopobium vaginae	not detected	☐	
	Yeast-like fungi			
14	Candida spp. *	$10^{3.4}$ ☐		
	Mycoplasmas			
15	Mycoplasma hominis *	not detected ☐		
16	Ureaplasma (urealyticum + parvum) *	not detected ☐		
	Pathogenic microorganisms			
17	Mycoplasma genitalium **	not detected ☐		

* Quantitative Analysis Lg(X)   ** Qualitative Analysis   *** Below threshold

Conclusion:
Absolute normocenosis

Figure 2. Exemplary test results with Femoflor 16

III.2.2.5. Specifications

The specificity of the Femoflor 16 REAL-TIME PCR Detection Kit has been assessed by bioinformatic analysis using available online databases with current and comprehensive genetic information. The specific oligonucleotides used in the assay were verified by sequences from the GenBank database. None of the sequences showed sufficient similarity for non-specific detection.

III.2.3. Statistical method

Data were entered and processed with the statistical package IBM SPSS Statistics 25.0. and Excel on Office 2021. A significance level at which the null hypothesis is rejected was $p < 0.05$.
The following methods were applied:
Descriptive analysis – the frequency distribution of the considered signs is presented in tabular form.
Graphical analysis – for visualization of the obtained results.
*Analysis of Variance* – calculating estimates of central tendency and statistical variance.
Fisher Freeman Haltonexact test, Fisher's exact test and χ2 test - for testing hypotheses about the presence of dependence between categorical variables.

IV. Results, own studies and discussion

IV.1. Main characteristics of the studied contingent
An ambispective clinical-epidemiological study covering 199 patients of the CIRM "St. Elisaveta" - Pleven for the period October 2019 - November 2022 was carried out. Two main groups were studied, designated RIF (recurrent implantational failure) n=103 (51.8%) and RPL (recurrent pregnancy loss) n=96 (48.2%) (Fig. 3).
The clinical contingent had an average age of 35.69±5.16 years in the range of 24 – 54.

RPL; 96; 48,2%   RIF; 103; 51,8%
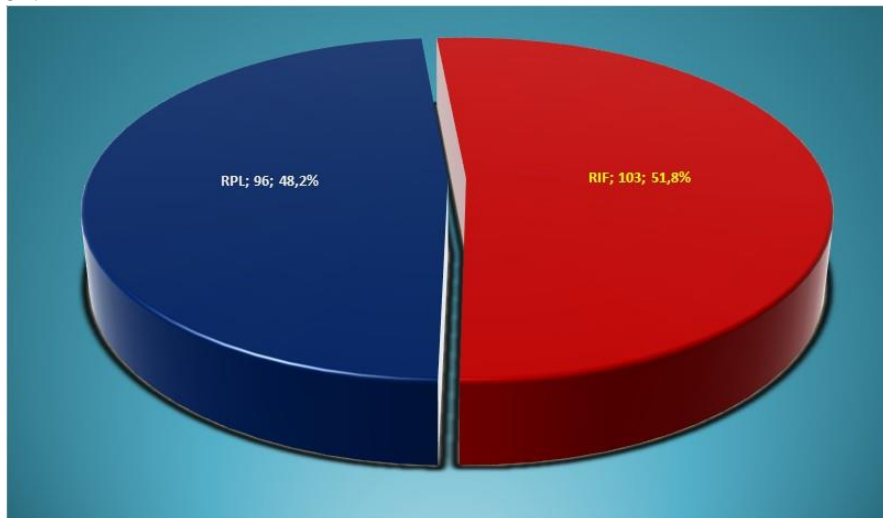
Figure 3. Frequency distribution of the clinical contingent by main study groups

The age group with the largest number (38) in RIF patients is 35-39 years, followed by 30-34 and 40-44 years with 23 each, and the smallest (0) – 20-24 years. Among women with RPL, the largest number (35) of the age group 35-39 years, followed by 30-34 years with 32 and the smallest (0) – 50-54 years (Fig. 4).
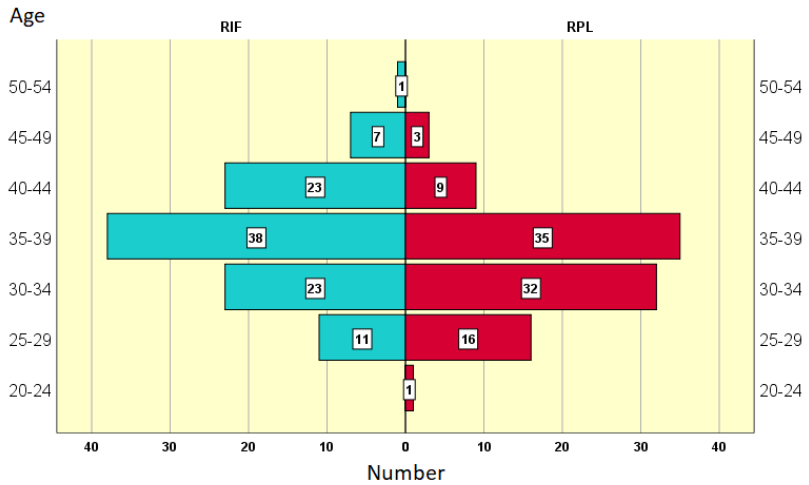
Figure 4. Distribution of the participants in the study by main and age groups
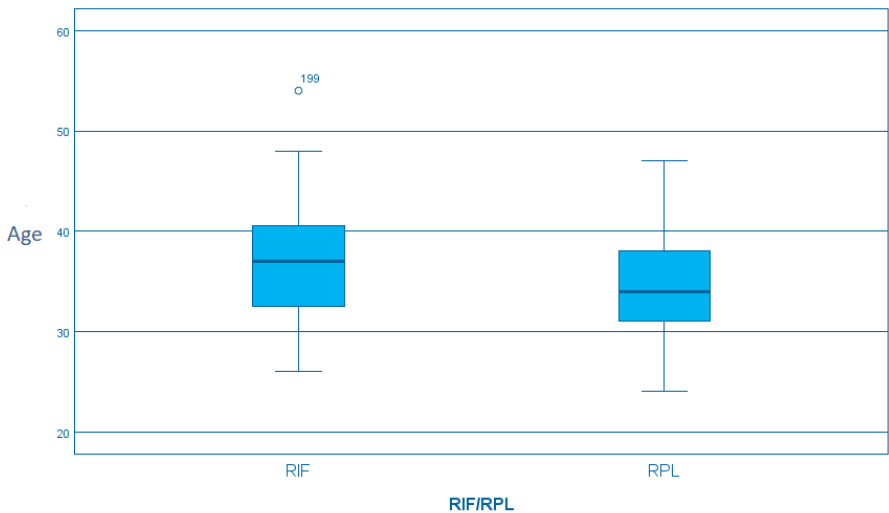
Figure 5. Boxplot by age in the two study groups

The mean age of patients in RIF is 36.91+/-5.35 years and in RPL is 34.39+/-4.63 years (p=0.0005) – the group of women with RIF is on average 2.5 years older.

IV.2. Composition of the endometrial microbiome in the two studied groups

Implantation remains one of the most unresolved issues of assisted reproduction today. It has been described as the final barrier to assisted reproductive technologies (Edwards, 2006). RIF continues to be a problem in 15% of patients and currently there are few and limited options to influence it (Busnelli et al., 2020).

Lactobacilli are the most represented microorganisms in the female genital tract. Their ability to inhibit infection without inducing inflammation may improve reproductive success (Witkin & Linhares, 2017). So far, research has shown that vaginal dysbiosis has a negative correlation with reproductive outcomes in IVF (Haahr et al., 2016).

Therefore, we set ourselves the task of investigating the presence and amount of lactobacilli in the endometrium in recurrent reproductive failures. In accordance with current literature data, we demonstrated a high percentage of deviations from the accepted normal concentrations of lactobacilli. A normal concentration of lactobacilli was found in only a quarter of the cases. In the remaining 76.4%, there is a significant disturbance in the normal composition of the microbiome.

In fig. 6 shows the amount of lactobacilli:
- With the largest relative share (62.8%) are the patients with missing ones, followed by those with levels above 85% (47 or 23.6%);
- Those with lactobacilli in the interval 51-85% are the least – 2.5%.
- The percentage of patients with a reduced amount of lactobacilli was 13.5 in both groups.
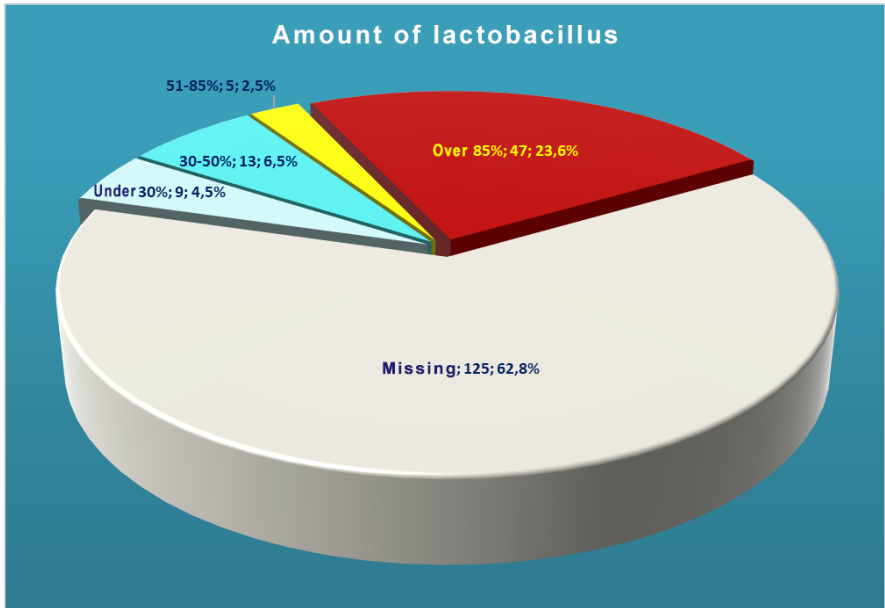
Figure 6. Frequency distribution of female patients by amount of lactobacilli

We additionally compared the violations in the amounts of lactobacilli in the two groups as the results of Table 1 and Fig. 7 show that there is no significant relationship between the amount of lactobacilli and belonging to the main groups - RIF and RPL. These data support a common pathogenetic mechanism of damage to the endometrial environment in relation to implantation and early pregnancy development.

Table 1. Frequency distribution of female patients by amount of lactobacilli and main groups (p=0.926)

Amount of lactobacilli	Frequency	Total	RIF	RPL
Missing	n	125	65	60
	%	62,8	63,1	62,5
Under 30%	n	9	4	5
	%	4,5	3,9	5,2
30 – 50%	n	13	6	7
	%	6,5	5,8	7,3
51 – 85%	n	5	2	3

		%	2,5	1,9	3,1
Over 85%		n	47	26	21
		%	23,6	25,2	21,9
Total		n	199	103	96
		%	100,0	100,0	100,0
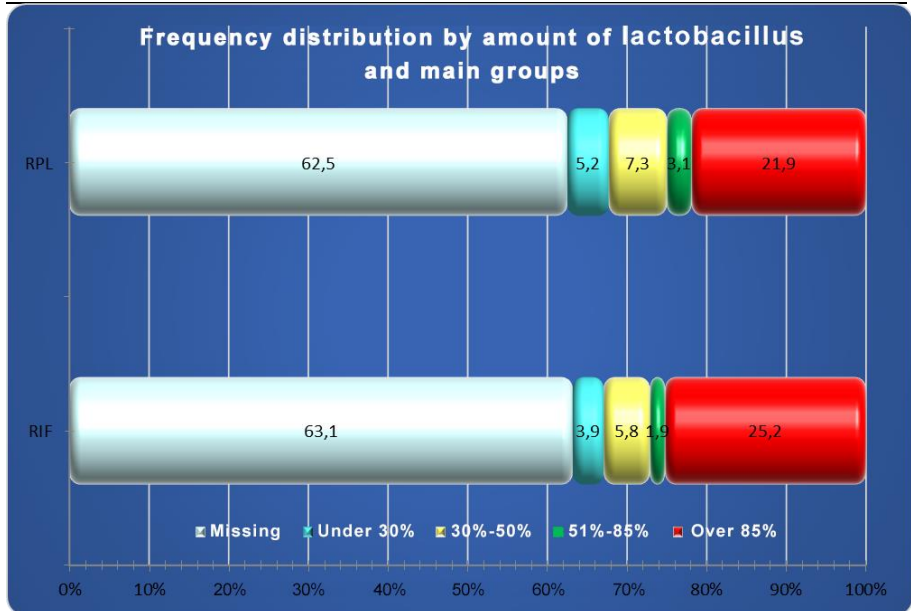
Figure 7. Frequency distribution of female patients by amount of lactobacilli and main groups

Initial research on the endometrial microbiome is a major breakthrough in breaking the stereotype of uterine sterility. Viewed chronologically, they range from strong proponents of lactobacilli dominance creating uterine eubiosis to opposing lactobacilli as a condition for successful pregnancy. All, however, are adamant about her non-sterility. However, there is still no clear definition and unified opinion on endometrial eubiosis.

Fang *et al.* (Fang et al., 2016) studied the endometrial microbiome in healthy women and, surprisingly, at this time found that the uterus was not sterile. This team found lactobacilli in the endometrium in only 6%. These data contradict what was later accepted as normal high levels of lactobacilli for adequate and healthy reproductive function (implantation, early pregnancy development and live birth). These initial

works are the starting point for all subsequent research, breaking the dogma accepted until then about the sterility of the uterine cavity.

Franasiak *et al*. (Franasiak et al., 2016) found a dominance of lactobacilli in all 35 studied patients who underwent an in vitro procedure, regardless of the achieved result - live birth or negative pregnancy test.

In a study of endometrial samples from 13 fertile women, Moreno *et al.* (Moreno et al., 2016) the dominance of lactobacilli species was established again. This leads to the emergence of the hypothesis of two possible types of monometric bacterial composition: lactobacillus - dominated microbiome with the presence of more than 90% lactobacilli of all isolated microorganisms and non-lactobacillus - dominated microbiome with the presence of less than 90% lactobacilli. This hypothesis is based on the implantation results depending on the amount of lactobacilli. In a microbiome with reduced lactobacilli less than 90% and a prevalence of opportunistic microorganisms greater than 10%, compared with a microbiome greater than 90%, implantation rates were 23.1% vs. 60.7% (p=0.02), per pregnancy 33.3 % vs. 70.6% (p=0.03), on developing pregnancy 13.3% vs. 58.8% (p=0.02), on live birth 6.7% vs. 58.8% (p=0.02).

Moreno *et al.* (Moreno et al., 2016) reported that half of women with reproductive failure have non-Lactobacillus-dominated endometrium. According to this team, reproductive outcomes differed by 30% between the two types of microbiome—Lactobacillus-dominated and non-Lactobacillus-dominated.

Our results differ from those of Moreno *et al*. in the frequency of non-Lactobacillus dominated endometrium - we found it in 76.4%, only 23.6% of our women had a normal according to the criteria of Moreno *et al*. endometrium. Moreover, these results are similar in the two studied groups – RIF and RPL. These significant differences in the results can be explained by the strict criteria of selection and preparation of the patients for the study. Another possible influencing factor that should not be overlooked is the homogenous ethnic background of the female patients – all of them are from the Bulgarian ethnic group. This high frequency of non-Lactobacillus dominated endometrium in our group means also a high frequency of reproductive failures in case of missing diagnosis and not carried out etiological treatment.

Another collective (Kitaya et al., 2019) compared the richness and diversity of microbial species in the endometrial microbiome in patients with RIF and infertile patients without evidence of RIF. They concluded that among the infertile population, especially IVF patients, a Lactobacillus-dominated microbiome was less common, as well as a statistically significant lower percentage of Lactobacilli.

Our results remain again with a high frequency of non-Lactobacillus dominated endometrium, even if the cut-off of 80% proposed by Kyono *et al*. (Hashimoto & Kyono, 2019; Kyono, Hashimoto, Kikuchi, et al., 2018). According to this collective, pregnancy occurs in 61.3% with lactobacilli above 80% and in 40% with lactobacilli below 80%.

Regarding group 1 (RIF), our results are consistent with Ichiyama *et al.* (Ichiyama et al., 2021), who in 145 RIF patients found lactobacilli absent in $51.2 \pm 37.5\%$. In their study, a control group of healthy women was also included, in which lactobacilli were present in only 28.6%, and lactobacilli were absent in $51.6 \pm 38.3\%$, i.e. there is no statistically significant difference between the two groups. For this reason, the above authors do not accept the concept of a Lactobacillus-dominated microbiome as a biomarker for implantation success. A similar opinion was expressed by the team of Kyono (Kyono, Hashimoto, Kikuchi, et al., 2018) in their study. According to their data, there is no difference in the outcome of the IVF procedure in patients with Lactobacillus-dominated and non-Lactobacillus-dominated microbiome. Therefore, they believe that it is necessary to revise the reference limits for lactobacilli in fertile women in cases where the presence and amount of lactobacilli is used as a biomarker for implantation failure. Diaz-Martinez *et al.* (Diaz-Martínez et al., 2021) are of the same opinion. They who found no difference in reproductive outcome in patients with and without a Lactobacillus-dominated microbiome. For these reasons, many researchers are beginning to focus on pathogenetically relevant interactions between the endometrial microbiota and endometrial immunity, rather than simply confirming the presence and abundance or absence of microorganisms in the uterus.

Again, the team of Moreno (Moreno et al., 2016) in the same study obtained conflicting results. In cases of endometrial polyps there is a high abundance of lactobacilli. This suggests that a delicate balance in their quantity is necessary to create a eubiotic microbiota sufficiently stable and protective of dysbiosis with subsequent association with pathological conditions.

Such a difference in the results of such large studies leaves the question of the relationship between the amount of lactobacilli in the endometrium and the successful outcome of a pregnancy highly debatable. In these cases, when interpreting the results and discussing behavior for further research and treatment, the hypothesis of the Ravel group, which suggests that in the absence of pathological signs, a non-Lactobacillus-dominated microbiota can be considered normal, should be taken into account (Ravel et al., 2011). These considerations are the basis of subsequent developments and the consideration of this type of microbiome as a variant of the norm.

Our results are consistent with those of Vomstein *et al.* (Vomstein et al., 2022), who also found a decrease in lactobacilli in female patients with RIF and RPL. This team conducted a dynamic study of the endometrial microbiome at three time points within the menstrual cycle in patients with RIF, RPL, and a control group of healthy women. Only in the group of healthy women was there a change in the composition of the endometrial microbiome in the form of stabilization as the phases of the menstrual cycle progressed. Similar plasticity of the endometrial microbiome is absent in RIF and RPL patients, which supports the hypothesis of a favorable endometrial

microenvironment created by lactobacilli for implantation and early embryonic development.

Cela *et al.* (Cela et al., 2022) in female patients with RIF found very different results from ours – using a cut-off of 90%, eubiosis was present in 50% of the examined women, in the other half a different pronounced dysbiotic condition was present, and in 20% of the group with dysbiosis lactobacilli are not detected at all.

The team of Keburiya (Keburiya et al., 2022) in patients with RIF found the presence of microflora in 89.2% of samples with dominance of lactobacilli. According to these authors, there is no clear evidence that the presence of Lactobacillus-dominated endometrium has a beneficial effect on the occurrence and outcome of pregnancy. However, according to them, the restoration of lactobacillus dominance has a positive effect on implantation. They found no effect of opportunistic bacteria on pregnancy rates. Most likely, all this is due to the fact that the uterine microbiota is a set of functionally associated microorganisms (Verstraelen et al., 2016). Certain microorganisms in the uterine cavity are involved in maintaining its homeostasis in a healthy state. The formed biofilms, which are bacterial communities, play an important role, and the bacteria in them have certain physiological properties. Normal biofilms in the human body are represented by microbial communities that form the physiological microflora of the skin, oral cavity, vagina, and intestines. The formation of pathological biofilms is associated with chronic inflammatory processes. These authors found fairly close values of lactobacilli in pregnant and non-pregnant patients. In the group with the first IVF procedure, lactobacilli were 80.0% in women who became pregnant and 73.7% in non-pregnant women. In the RIF group, they were 90.2% and 79.0%, respectively. Their results do not support the hypothesis of a role for lactobacilli in the process of implantation and its outcome.

All these research data, some of which are completely contradictory, the lack of statistical evidence to confirm the influence of certain microorganisms on the reproductive outcome is a strong enough motive for deepening and expanding future studies. Not only the presence or absence of a particular microorganism should be evaluated, but the individual state of the organism and the interactions of microorganisms in the host organism should be taken into account. Understanding the essence of dysbiotic processes in the uterus and the onset of a chronic inflammatory process, as well as timely setting of a correct diagnosis with appropriate treatment, are important for adequate behavior towards patients with reproductive failures.

In the results of the team Lozano (Lozano et al., 2023) in female patients with RIF there was a lower abundance of lactobacilli. The absence or reduction of lactobacilli in these cases most likely creates favorable conditions for the ascension of microorganisms in the uterus with a subsequent negative effect on the occurrence and development of a normal pregnancy, which places the microbiome with low biomass

as a potential gateway for pathogens. Kyono *et al.* (Kyono, Hashimoto, Nagai, et al., 2018) reported similar data and found that a non-Lactobacillus dominaria microbiome was found more frequently in infertile Japanese women. Ichiyama *et al.* (Ichiyama et al., 2021) comparing the vaginal and endometrial microbiome in women with RIF found a 30% non-Lactobacillus dominated microbiome. An important result of this research is the combination between the two localizations – when dysbiosis develops in the vagina, it also occurs at the level of the endometrium. Increasing lactobacilli saturation above 90% in the endometrium could have a beneficial effect in these women. An endometrium dominated by lactobacilli is more receptive than one with a low saturation of lactobacilli and a high diversity of microorganisms. For this general reason, understanding how to diagnose and treat microbiome dysbiosis can improve reproductive outcomes (García-Velasco et al., 2020).

In a 2023 study of 141 women with RIF a lactobacillus-dominated microbiome was present in only 20 patients, in the remaining 121 lactobacilli were absent or greatly reduced (Zou et al., 2023).

The results of the Fujii 2023 team are a very serious cause for reflection and study (Fujii & Oguchi, 2023). They investigated the relationship of endometrial receptivity to the amount of lactobacilli in the endometrium. The conclusion they make is that the reduction of lactobacilli, especially their absence, is associated with a shift in the implantation window. They believe that lactobacilli are important in preparing the uterus for implantation and synchronizing with the preimplantation embryo.

Although sampling may affect the obtained result through possible contamination from the vagina and cervix, we found a high percentage of absence of lactobacilli in the endometrium – 62.8% of all examined. This result supports the credibility and reliability of the transcervical approach for studying the endometrial microbiome.

Studies on the endometrial microbiome in RPL are scarce and varied in design. However, they are very similar to the available data from patients with RIF, most likely due to the similar pathogenetic mechanism of disruption of early pregnancy - absence or reduction of lactobacilli and the presence of dysbiosis with pathogenic microbes and a wide variety of bacteria.

In our RPL results, lactobacilli were absent in 62.5%. In a normal amount they are in 21.9%.

The team of Churchill (Churchill et al., 2018) when examining endometrial fluid in the secretory phase of RPL patients found an abnormal microbiome with a wide variety of isolated pathogens in half of the cases. In these cases, there is a combination with a non-lactobacillus dominated microbiome - lactobacilli vary between 12 and 68%. Lactobacilli-dominated microbiome is present in half of the examined women, which strongly differs from our results in such patients – 21.9%.

Barinova *et al.* (Barinova et al., 2022) studied the endometrial microbiome in healthy fertile women and in women with RPL. In the RPL group, lactobacilli were the most abundant at 30.3%. In the group of healthy fertile women in 29.4%. There was no statistically significant difference between the two groups. Their results are in confirmation of the dominance of lactobacilli, although this abundance is below the accepted 90%, including in the group of healthy fertile women, but they cannot prove the negative effect of reduced lactobacilli on the reproductive outcome.

IV.3. Age-related changes in the amount of lactobacilli

To analyze the influence of age on the endometrial microbiome, we performed a study of the amount of lactobacilli by age group.

Age is a known risk factor for eubiosis damage in the genital tract. Hormonal changes, which are the result of depletion of the ovarian reserve, result in a hypoestrogenic environment, atrophy of the mucous membranes in the reproductive tract, a change in pH, creation of unfavorable conditions for the colonization and maintenance of lactobacilli and the corresponding development of other commensal or opportunistic bacteria.

The results obtained in the entire group confirm the negative effect of age on lactobacilli saturation and dominance. The highest percentage of missing lactobacilli was found in the age group of 41-45 years, 66.67%, and the lowest in the age group of 26-30 years – 48.57%. This group of young patients has the highest percentage of lactobacillus - dominated microbiome -34.29%, compared to the group 41-45 years old - 13.89%.
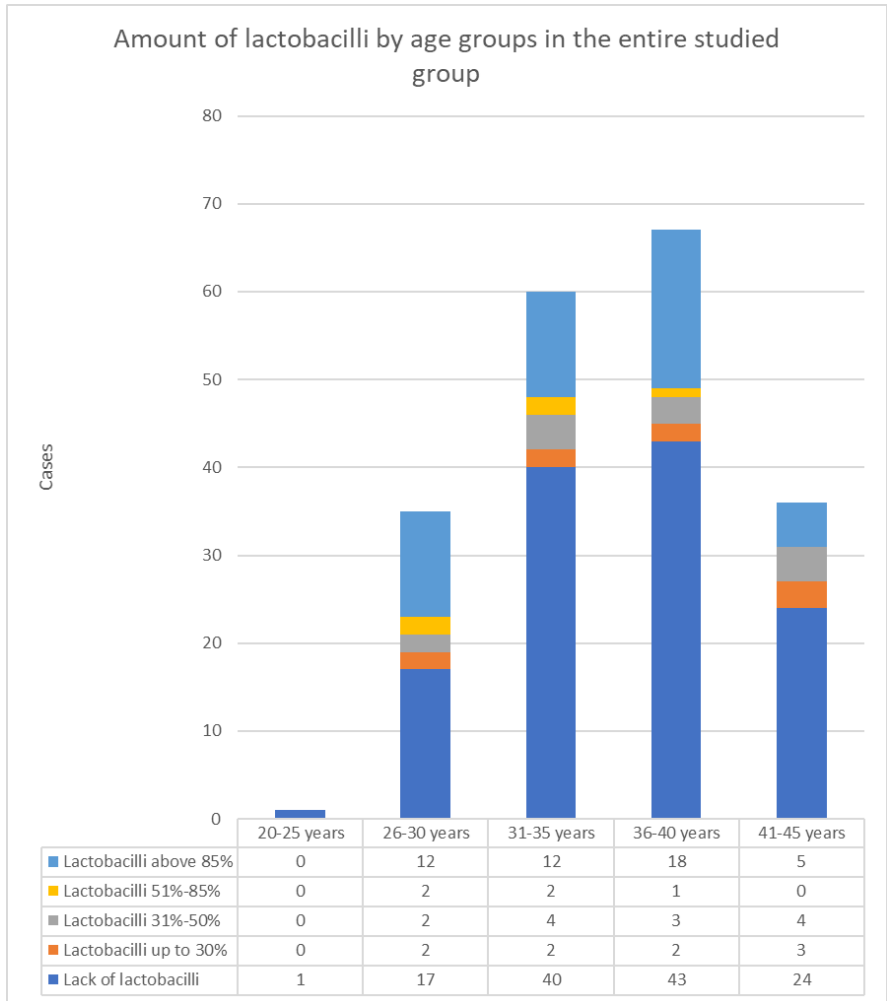
Figure 8. Lactobacillus-dominant and non-Lactobacillus-dominant endometrial microbiome incidence by age group
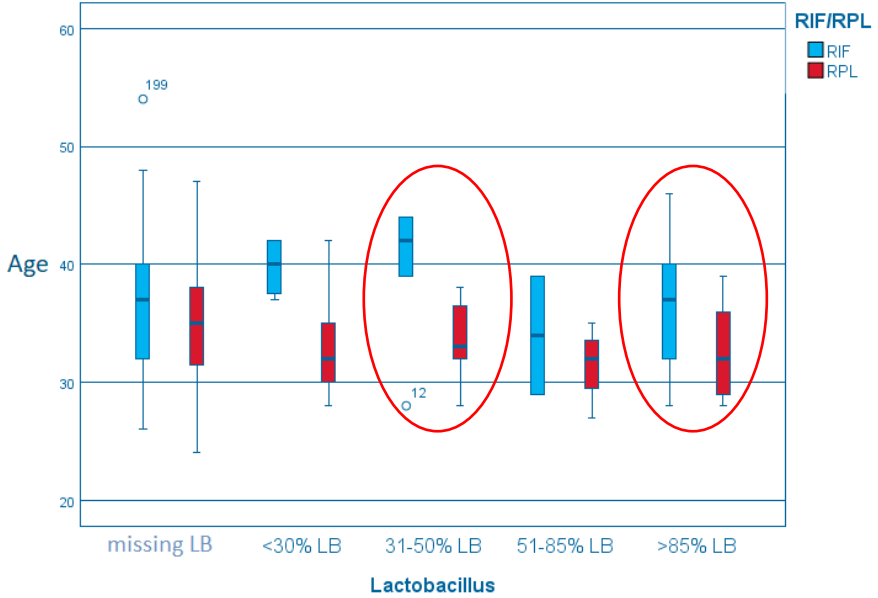
Figure 9. Boxplot of the amount of lactobacilli by age in the two studied groups

A statistically significant difference in mean age between the RIF and RPL groups was found in patients with 31-50% lactobacilli and in patients with lactobacilli greater than 85%, namely, patients with RIF and 31-50% lactobacilli had a mean age of 39.8+/- 6 years, and those with RPL and lactobacilli 31-50% had an average age of 33.7+/-3.5 years (p=0.046). For patients with RIF and lactobacilli greater than 85%, the mean age was 36.4+/-4.8 years, and for RPL the mean age was 32.5+/-3.5 years (p=0.004).
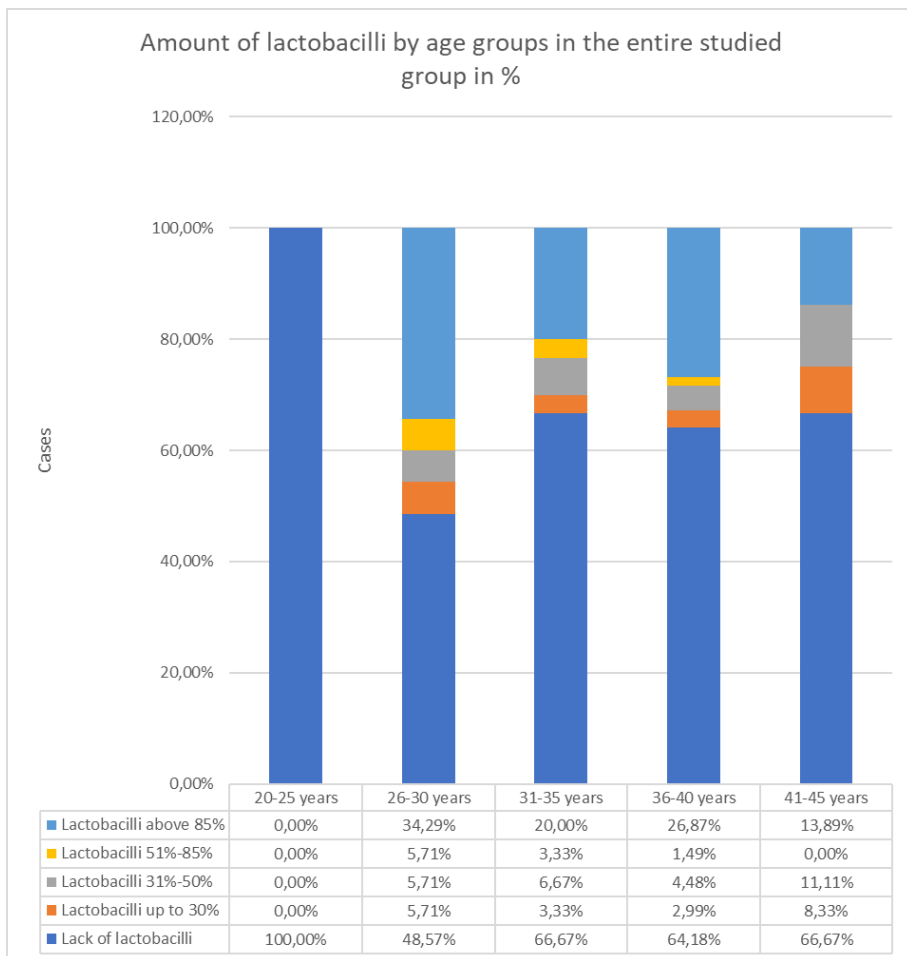
Figure 10. Cases of Lactobacillus-dominated and non-Lactobacillus-dominated endometrial microbiome by age groups presented in percentages

No statistical significance was found in the frequency distributions of RIF and RPL patients with respect to lactobacilli groups in the different age groups.

In the RIF group, the highest share of lactobacillus - dominant microbiome in the 31-35 year group - 29.17%, again in this age group the highest percentage of missing lactobacilli - 70.83%. In the other age groups, there is a different degree of lactobacilli reduction.
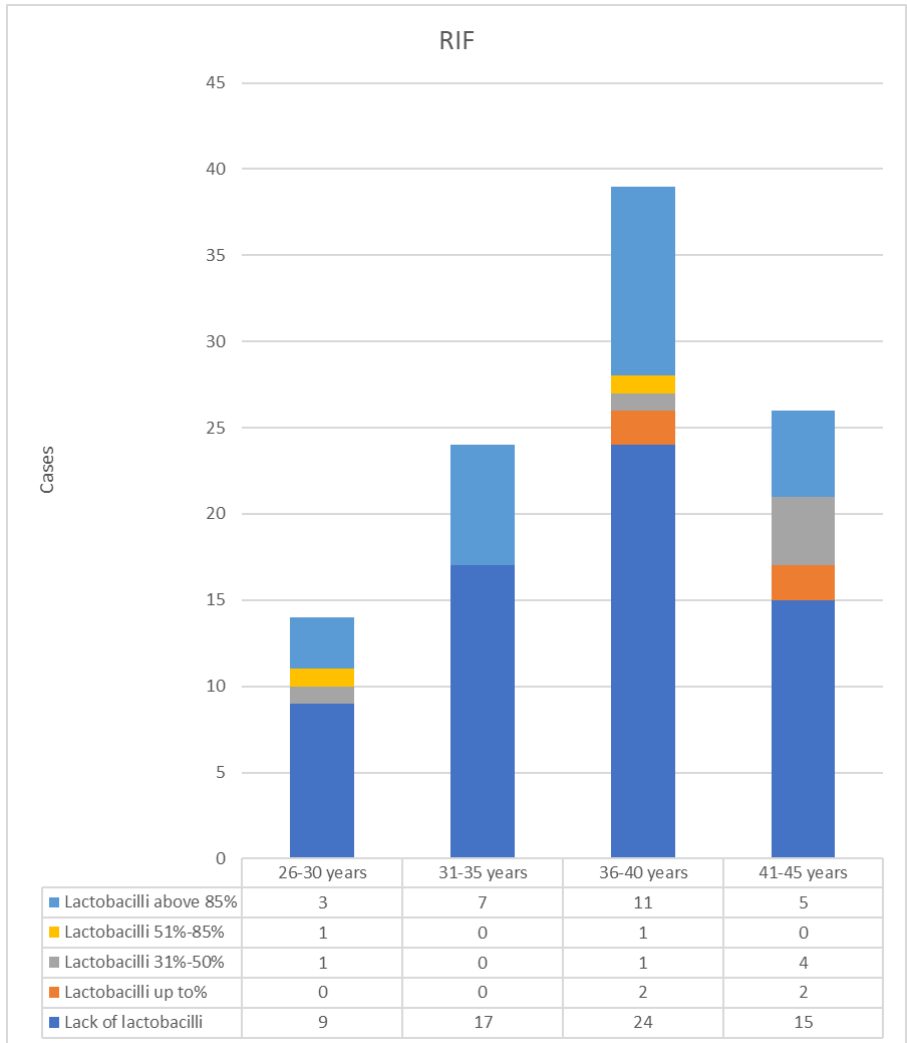
Figure 11. Cases of Lactobacillus-dominant and non-Lactobacillus-dominant endometrial microbiome in patients with RIF
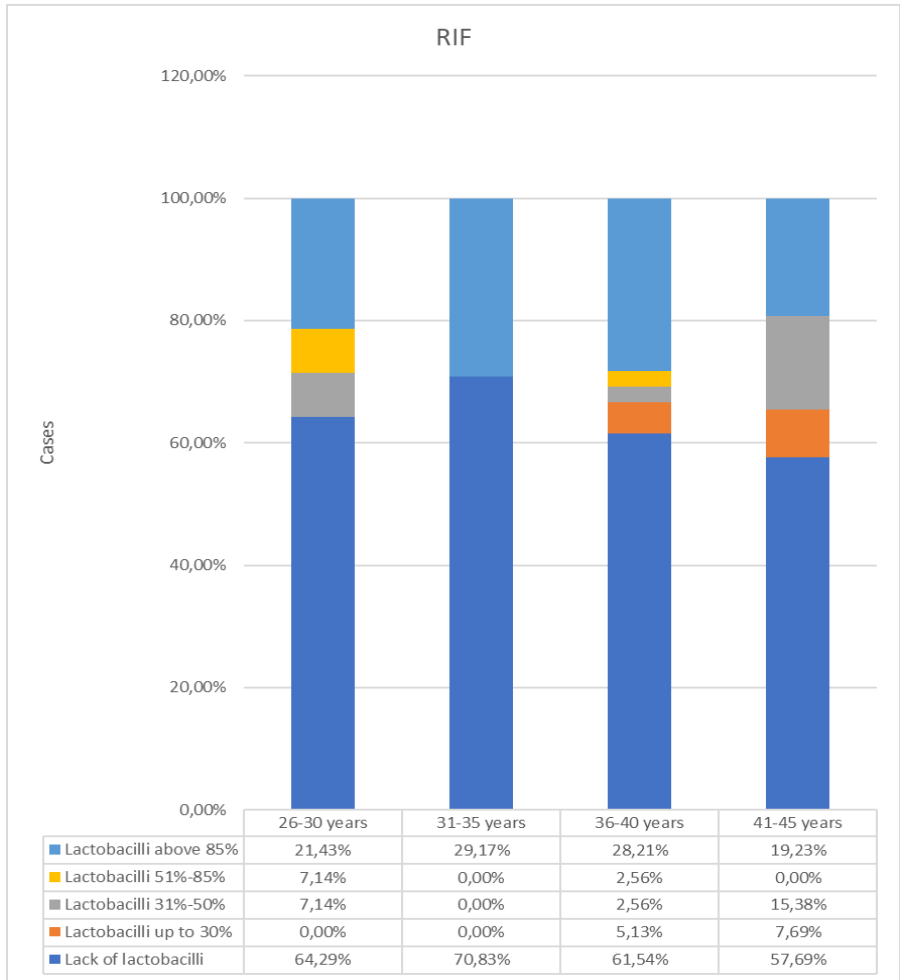
Figure 12. Lactobacillus-dominant and non-Lactobacillus-dominant endometrial microbiome incidence in RIF patients presented as percentages

In the RPL group Lactobacillus - dominated microbiome has the highest percentage in the age group 26-30 years - 42.86%, which is in great contrast with the age group 41-45 years - 0%. Accordingly, in this group the missing lactobacilli are in 90%, the lowest frequency of missing lactobacilli is in the 26-30 year old group – 38.1%.

RPL

	20-25 years	26-30 years	31-35 years	36-40 years	41-45 years
Lactobacilli above 85%	0	9	5	7	0
Lactobacilli 51%-85%	0	1	2	0	0
Lactobacilli 31%-50%	0	1	4	2	0
Lactobacilli up to 30%	0	2	2	0	1
Lack of lactobacilli	1	8	23	19	9

Figure 13. Cases of Lactobacillus-dominant and non-Lactobacillus-dominant endometrial microbiome in patients with RPL
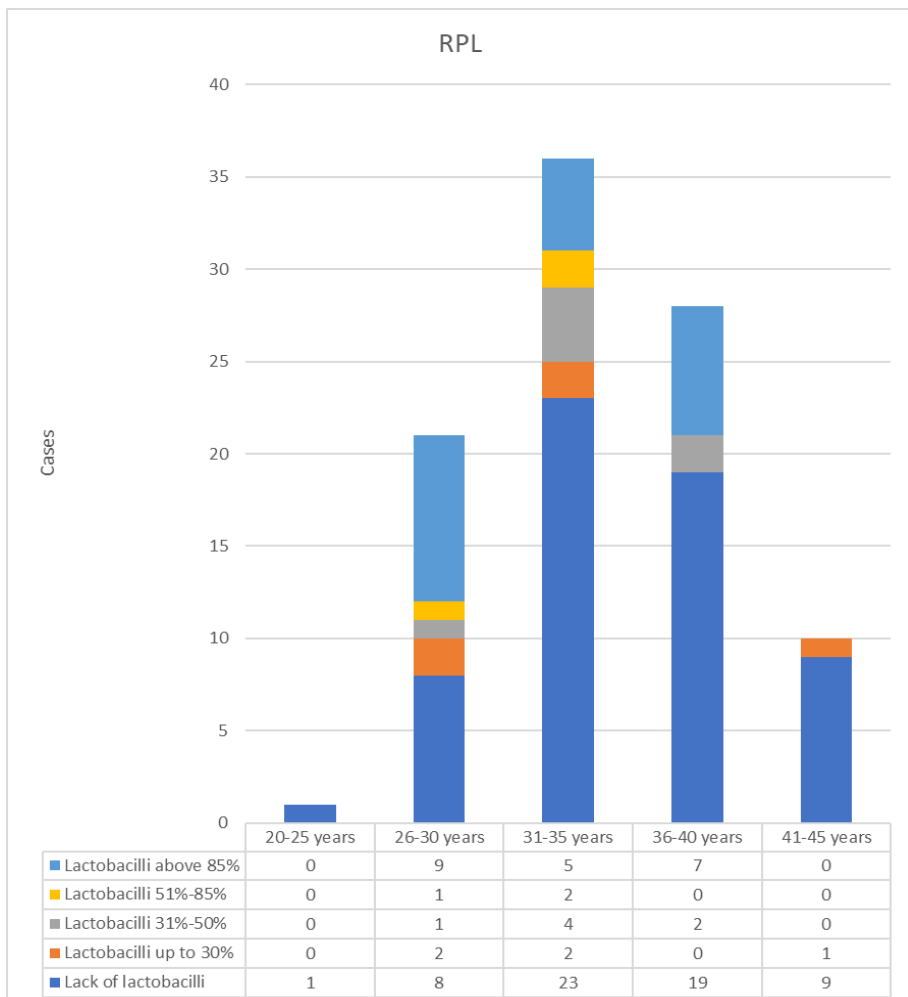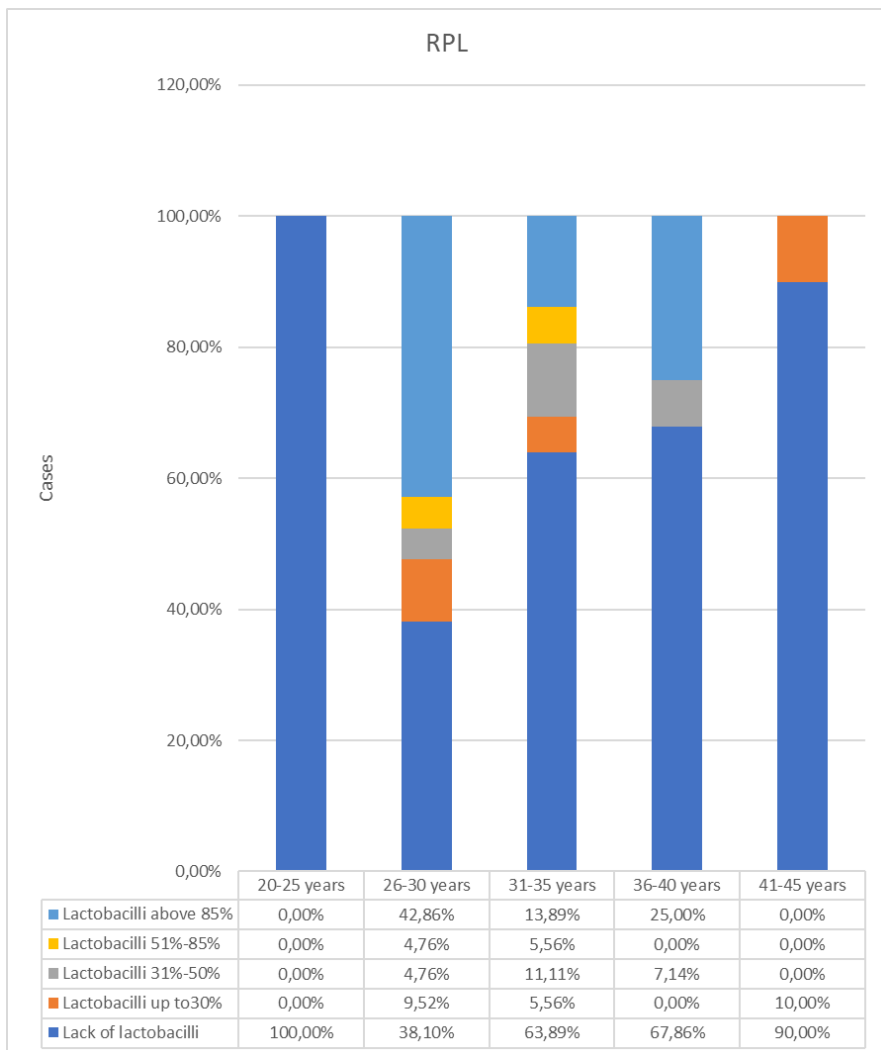
Figure 14. Lactobacillus-dominant and non-Lactobacillus-dominant endometrial microbiome incidence in RPL patients presented as percentages

Many studies discuss the influence of age on the amount of lactobacilli in the endometrium. Age is a known factor that is associated with a decrease in lactobacilli saturation and an increase in microbial diversity in the endometrium with the development of dysbiosis. Our results are similar to the available literature.

Fujii's group (Fujii & Oguchi, 2023) investigated the relationship of age with endometrial receptivity and the amount of lactobacilli. They found a shifted implantation window with advancing age. Since advanced age is accompanied by a decrease in lactobacilli, they believe that lactobacilli are responsible for preparing the uterus for implantation. In their study, the included patients were up to 41 years old, there is no information about lactobacilli saturation after this age that we have.

Wang's 2021 team (Wang et al., 2021) did a broader analysis by age. According to them, lactobacilli are stable until about 40 years of age, after which they begin to fluctuate, and in the period of 40+ to 60 years, they suffer significant damage. The study was conducted in the follicular phase of the menstrual cycle and included a control group of healthy women.

The Odawara team (Odawara et al., 2020) conducted a study among Japanese women on the factors that influence the intrauterine microbiota. They found a significant difference in terms of age and nativity. In women over 36 years of age, there was a significant decrease in intrauterine lactobacilli, especially in parous compared with nonparous. The most likely reasons for this are two - the broken integrity of the cervix at birth, which becomes an open door to the uterine cavity, and postpartum amenorrhea with low estrogen levels, which favors colonization with bacteria other than lactobacilli. In our research, we did not analyze the influence of previous births, abortions, curettages and other intrauterine manipulations, incl. embryo transfer and intrauterine insemination, which could be responsible for breaching the natural barrier for the ascension of microorganisms from the vagina.

IV.4. Composition and frequency of eubiotic and dysbiotic endometrial microbiome species in RIF (group 1) and RPL (group 2) patients

To analyze the obtained results, we defined four types of endometrial microbiome according to current attempts to classify it. For a normal microbiome we accept the presence of more than 90% lactobacilli without dysbiotic bacteria. Low biomass microbiome – lactobacilli are absent, dysbiotic bacteria are absent. Moderately disturbed microbiome - lactobacilli below 90% with presence of dysbiotic bacteria above 10%. Severely disturbed microbiome – lack of lactobacilli, presence of dysbiotic bacteria over 10%. Currently, the first two types of microbiome are considered normal.

From fig. 15 it becomes clear that in microbiome types:

- The most (74 or 37.2%) are those with a highly disturbed microbiome, followed by those with a low biomass microbiome (25.1%);
- The least (15.1%) are those with moderately impaired;
- A normal microbiome is present in 22.6% of patients.
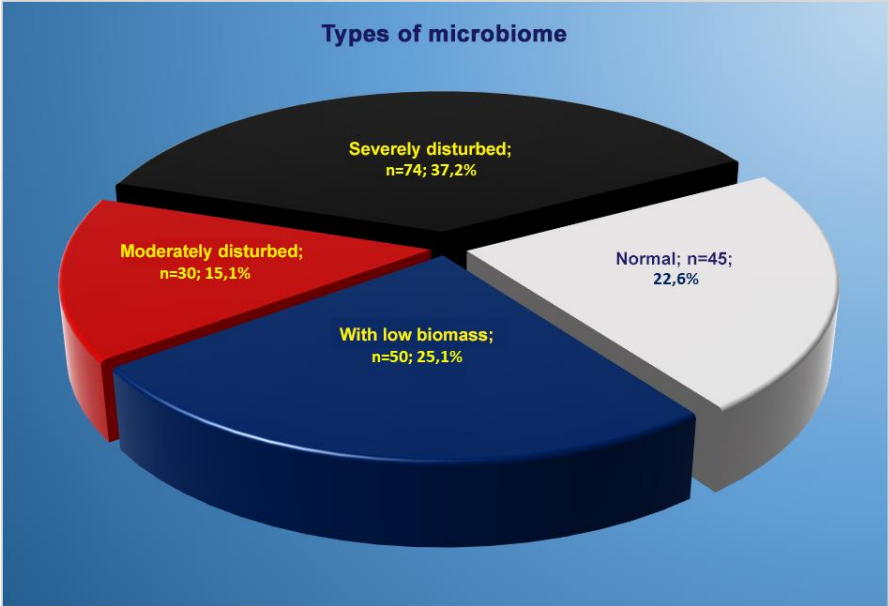
Figure 15. Frequency distribution of female patients by types of microbiome

Table 2. Frequency distribution of female patients by types of microbiome in the entire sample and by main groups (p=0.942)

Microbiome types	Frequency	Total	RIF	RPL
Normal	n	45	25	20
	%	22,6	24,3	20,8
Low bio-mass microbiome	n	50	26	24
	%	25,1	25,2	25,0
Moderately disturbed	n	30	15	15
	%	15,1	14,6	15,6
Severely disturbed	n	74	37	37
	%	37,2	35,9	38,5
Total	n	199	103	96
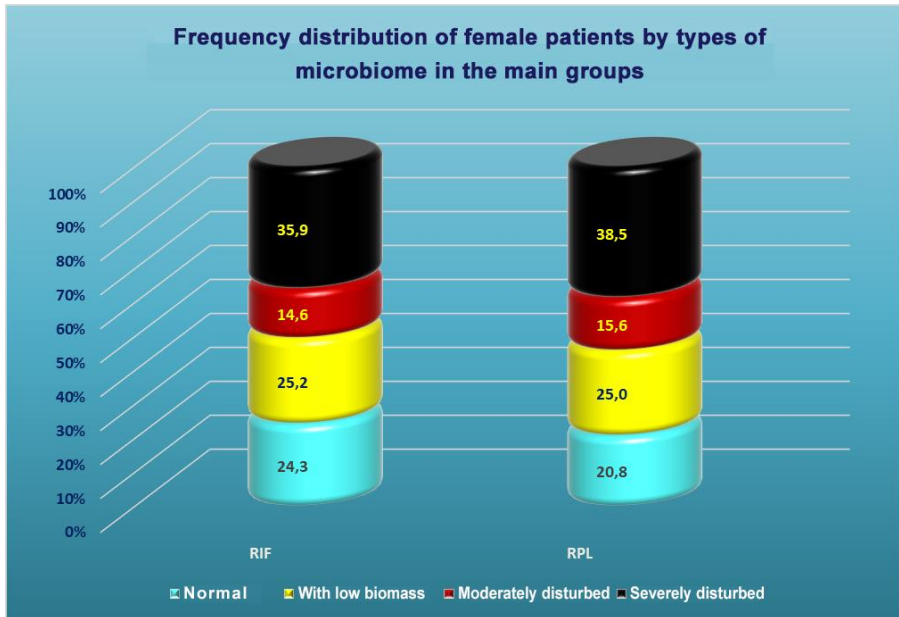
Figure 16. Frequency distribution of female patients by types of microbiome in the main groups

The comparative analysis of the two main groups by microbiome species (Table 2 and Fig. 16) showed that there was no significant relationship between them (p=0.942).
In group 1 (RIF) 50.9% have a disturbed microbiome - strongly and moderately disturbed, and in group 2 (RPL) such are present in 54.1%.
In their original 2016 study, Moreno's team (Moreno et al., 2016) proposed the division of the endometrial microbiome into Lactobacillus-dominated and non-Lactobacillus-dominated. They accept a limit of 90% to distinguish them. In the second microbiome group, there is a different concentration of lactobacilli with different combinations of pathogenic and opportunistic microbial species. Due to the small number of patients included in the study, they were unable to further classify these different variants. In this study, they link a non-lactobacillus-dominated endometrium with a negative pregnancy outcome and discuss the creation of an inflammatory environment in the uterus in the absence of lactobacilli as a possible cause. However, they make a very general analysis - they do not further consider the presence, quantity and types of other

types of bacteria - pathogens and commensals in non-Lactobacillus - dominated endometrium.

According to the latest research on the subject, an opinion is beginning to prevail about the presence of pathogenic microbes in the uterus, which have an adverse effect, and not so much the presence of a certain bacterial taxon, which would have a positive effect on reproduction. This view is supported by the results of Moreno (Moreno et al., 2021), according to which the absence of bacteria, incl. of lactobacilli is associated with a positive result. They found 49% live births in the bacteria-free group, incl. lactobacilli compared to 40.5% in the group with bacteria present. This could mean that in the absence of pathogenic microbes, lactobacilli are not required for implantation and introduce the existence of a second microbiome group, a variant of the norm – a low-biomass microbiome. Another conclusion this team makes is that the composition of the microbiome at conception is associated with reproductive outcome. Lactobacilli dominance has a negative correlation with pathogenic microorganisms and a positive correlation with commensal microorganisms. This can be important for maintaining stability in the ecosystem. The authors believe that the role of lactobacilli in reproduction consists in preventing the colonization of the uterine cavity by pathogenic bacteria. These data on the association of lactobacilli and pathogenic microbes are also demonstrated in our study - in the RIF group we demonstrate a 35.9% highly disturbed microbiome with missing lactobacilli and a high percentage of pathogenic microbes, in the RPL group this percentage is 38.5.

Cariati *et al.* (Cariati et al., 2023) studied the endometrial microbiome in infertile women and compared the results between pregnant and non-pregnant women. In the group of pregnant women, 33.3% lack bacteria, incl. lactobacilli. In our study, however, without examining reproductive outcome, lactobacilli were absent in 25.1% - the low biomass microbiome group.

Iwami *et al.* (Iwami et al., 2022) in a study of endometrial microbiome in 195 RIF patients found dysbiosis in 48.9%, lactobacillus-dominated microbiome in 51.1%. Of these 48.9%, 22.9% had a severely disturbed microbiome, 17.6% had a moderately disturbed microbiome, and 8.4% had a low biomass microbiome. These results differ from ours, which are respectively: 37.2%, 15.1%, 25.1%, similarity only with respect to moderately disturbed microbiome. According to our results, a normal microbiome is present in 22.6%, i.e. two times less. In the study by Iwami et al. only RIF patients were included, while in our study there was also a group of RPL patients. However, we did not find a statistically significant difference in microbiome types between RIF and RPL, so we cannot attribute these discrepancies to the different profile of the patients included in the study.

In our study, we divide the results into 4 main groups – Lactobacillus-dominated microbiome and 3 groups of non-Lactobacillus-dominated endometrium: low biomass, moderately disturbed and highly disturbed microbiome. In the available literature, we were able to find results similar to ours only in the study of the Iwami group from 2023. This subdivision of microbiome types is convenient and effective

for clinical practice because it provides clarity about the microbiome composition of the endometrium, the degree of its damage, the need for treatment or follow-up study after treatment. In this sense, it is an effective biomarker for ART procedure prognosis. However, the judgment for treatment must always be strictly individual, discussed with the patient and taken into account with her reproductive and chronological age.

In about half of the patients with RIF and RPL, a varying degree of reduction to a complete absence of lactobacilli can be expected, and in the other half, the presence of pathogenic microbes with a different degree of dysbiosis. According to Iwami *et al*. (Iwami et al., 2022) in about half of the patients with RIF and RPL, a varying degree of reduction to a complete absence of lactobacilli can be expected, and in the other half, the presence of pathogenic microbes with a different degree of dysbiosis. According to Iwami only 23% would need antibiotic treatment to normalize the lactobacilli content and concentration in the endometrium. At the same time, courses of treatment longer than 8-10 days are not recommended due to the negative effect on lactobacilli from long courses. Customizing the need for antibiotic treatment according to the obtained RT-PCR result, as well as choosing an antibiotic only and when necessary, reduces the use of broad-spectrum antibiotics and the development of antibiotic resistance. The question of the correction of dysbiotic endometrium has remained open since the use of sequencing techniques. There are two main approaches to restoring eubiosis – antibiotics (systemic or local) and probiotics. The widespread use of antibiotics carries risks in addition to the development of antibiotic resistance and damage to the normal microbiome, not just the endometrial. Therefore, the blind application of antibiotics based only on the sequencing results cannot be a favorable prospect for the treatment of endometrial dysbiosis. The administration of probiotics may be a promising and motivated method of dysbiosis correction. Two main theories have been accepted for colonization of the endometrium with microbes - ascension from the vagina and hematogenous dissemination from the oral cavity and intestine. The similarity in the microbiomes of the reproductive and gastrointestinal tracts are grounds for the future development of the so-called microbial transplants.

Whether the microbiome is the new hope in reproductive medicine is currently in question. In the future, there will be more clarity to what extent its influence extends to reproductive outcome – implantation, initial pregnancy, parturition, and how the microenvironment can be modulated to a desired state.

IV.5. Characterization of the bacterial composition by type and frequency of the isolated pathogenic microorganisms in a dysbiotic endometrial microbiome

We analyzed the dysbiotic composition of the disordered microbiome, which showed the most common microorganisms and also their influence on the degree of disorder in the two studied groups.

According to the amount of microorganisms in the entire sample (Fig. 17, Table 3):

- Most (116 or 58.3%) are from *Fungi Candida spp*., followed by OAHM *Atopobium vaginae* with 30.7% and OAHM *Mobiluncus spp.* plus *Corynebacterium spp.* with 22.1%. The current paper does not address the impact of the mycobiome on reproductive failure, and therefore lacks further discussion of the established *Candida species*.

- They are least of the type Pathogenic microorganisms *Mycoplasma genitalium* – 0%. The comparative analysis of the main groups according to the presence of the studied microorganisms showed that (Table 3):

- A statistically significant difference was found in three of the microorganisms - FAHM *Staphylococcus spp*. (p=0.021), OAHM *Megasphaera spp*. plus *Veillonella spp*. plus *Dialister spp*. (p=0.029) and *Fungi Candida spp*. (in the case of the latter, the difference is borderline significant - $p<0.1$).

- OAHM *Megasphaera spp*. plus *Veillonella spp*. plus *Dialister spp*. has a statistically significantly greater relative share in the RIF group, and the other two in the RPL group.

- Regarding *Fungi*, a tendency for higher values in the group with RPL ($p<0.087$) was established, although not with high statistical significance.

- For the rest of the microorganisms, the difference between the main groups is statistically insignificant.
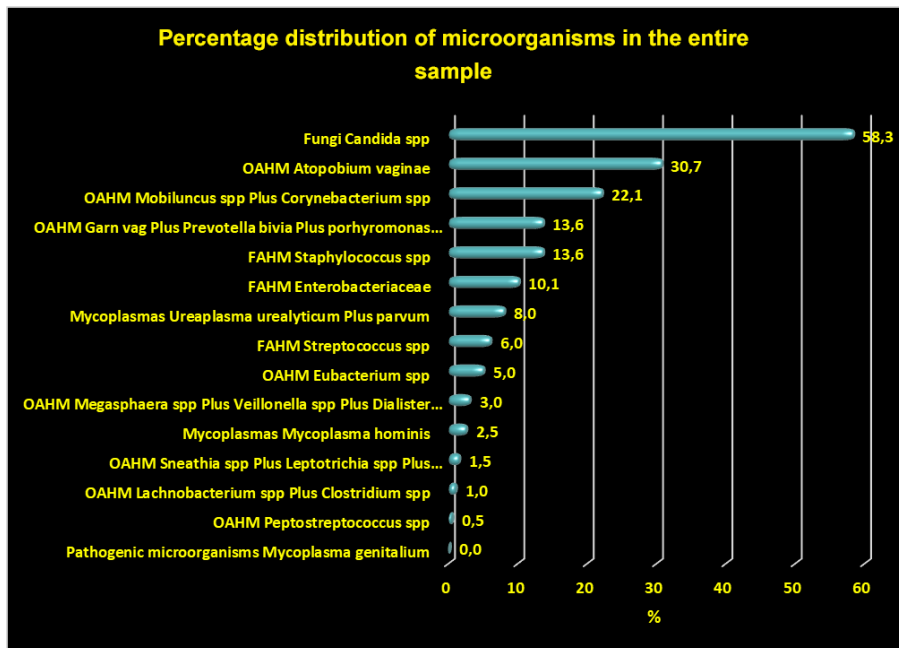
Figure 17. Frequency distribution of the investigated microorganisms in the entire sample

Table 3. Frequency distribution of the studied microorganisms in the whole sample and main groups

Microorganisms	Frequency	Total	RIF	RPL	P
FAHM *Enterobacteriaceae*	n	20	9	11	0,639
	%	10,1	8,7	11,5	
FAHM *Streptococcus spp.*	n	12	6	6	1,000
	%	6,0	5,8	6,3	
FAHM *Staphylococcus spp.*	n	27	8	19	**0,021**
	%	13,6	7,8	19,8	
OAHM *G.vaginalis* plus *Prevotella bivia*	n	27	13	14	0,836
	%	13,6	12,6	14,6	

plus *Porhyromonas spp.*					
OAHM *Eubacterium spp.*	n	10	6	4	0,749
	%	5,0	5,8	4,2	
OAHM *Sneathia spp.* plus *Leptotrichia spp.* plus *Fusobacterium spp.*	n	3	1	2	0,610
	%	1,5	1,0	2,1	
OAHM *Megasphaera spp.* plus *Veillonella spp.* plus *Dialister spp.*	n	6	6	0	**0,029**
	%	3,0	5,8	0,0	
OAHM *Lachnobacterium spp.* plus *Clostridium spp.*	n	2	2	0	0,498
	%	1,0	1,9	0,0	
OAHM *Mobiluncus spp.* plus *Corynebacterium spp.*	n	44	18	26	0,124
	%	22,1	17,5	27,1	
OAHM *Peptostreptococcus spp.*	n	1	1	0	1,000
	%	0,5	1,0	0,0	
OAHM *Atopobium vaginae*	n	61	32	29	1,000
	%	30,7	31,1	30,2	
Fungi Candida spp.	n	116	54	62	*0,087*
	%	58,3	52,4	64,6	
Mycoplasmas Mycoplasma hominis	n	5	3	2	1,000
	%	2,5	2,9	2,1	
Mycoplasmas Ureaplasma urealyticum plus *parvum*	n	16	8	8	1,000
	%	8,0	7,8	8,3	

Pathogenic microorganisms	n	0	0	0	
Mycoplasma genitalium	%	0,0	0,0	0,0	-

IV.5.1. Incidence of pathogens in RIF

The incidence of chronic endometritis in the general population has been estimated at 19% (Yoshii et al., 2013) and 45% in an infertile population (Kushnir et al., 2016). This high incidence is primarily associated with recurrent implantation failure (RIF) and recurrent pregnancy loss (RPL) rather than other causes of infertility (Johnston-MacAnanny et al., 2010), (Yang et al., 2014), (Cicinelli et al., 2015), (Cicinelli et al., 2014). Because of this high frequency and the silent, asymptomatic course of chronic endometritis, we expect the results to identify pathogenic microbes that are associated with it. Dysbiosis in the lower genital tract, which is a source of bacteria for ascending to the uterus, in combination with a decrease in endometrial lactobacilli, create suitable conditions for a high frequency of endometrial dysbiosis and chronic inflammation of the uterine lining.

In patients with chronic endometritis, Liu *et al.* found a higher frequency of *Dialister, Prevotella, Gardnerella* and *Anaerococcus*, which is also consistent with our results (Liu et al., 2019). These data support the hypothesis that microbial imbalance can trigger a pathological immune response with impairment of endometrial receptivity and subsequent RIF (P. Chen et al., 2021). Dysbiotic vaginal microbiome and associated pro-inflammatory response may damage the cervical epithelial barrier, thereby allowing translocation of pathogenic bacteria to the endometrium with subsequent local inflammatory process (Borgdorff et al., 2016). This result strongly correlates with our findings – all cases of *Dialister* presence were only in RIF patients. *Prevotella bivia* belongs to the group of microorganisms that have a negative impact on human reproduction (Campisciano et al., 2021).

Ichiyama's group in 2021 (Ichiyama et al., 2021) analyzed 145 RIF patients and 21 healthy control women. In the RIF group, 14 different genera of microbes were found: *Atopobium, Megasphaera, Gardnerella, Prevotella, Schlegelella, Delftia, Burkholderia, Sphingobacterium, Dietzia, Enterococcus, Micrococcus, Rlstonia, Leucobacter* and *Hydrogenophaga*. The difference with the control healthy group is significant. Of these 14 genera, *Atopobium, Gardnerella, Prevotella* and *Megasphaera* are the same that associate pathological vaginal microbiome with RIF, i.e. can ascend from the vagina and have a negative effect on the endometrium and association with RIF. Vaginal dysbiosis with pathogenic bacteria (*Atopobium, Gardnerella, Prevotella* and *Megasphaera*) suggests the presence of endometrial dysbiosis of vaginal origin. These same bacteria we find in high frequency in our RIF patients.

Since endometrial biopsy is invasive and carries the risk of ascending infection, and obtaining a vaginal sample is easy and reproducible, in some cases the isolation of these bacteria can be used as a biomarker for RIF. This approach can be used in certain cases – technical difficulties, financial constraints, individual preferences.

Diaz-Martinez (Diaz-Martínez et al., 2021) in 48 patients undergoing an IVF procedure observed a negative association of *Streptococcus* and *Prevotella* with reproductive outcome and a positive association of lactobacilli. In our sample, *Streptococcus* had a frequency of 6% in both studied groups, and *Prevotella* 13.6%.

Zou *et al.* (Zou et al., 2023)) in 141 women with RIF found pathogenic bacteria in 88.7%, the most common being *Streptococcus, Staphylococcus, Neisseria* and *Klebsiella*. These pathogens are known to damage the integrity of the endometrial epithelium, which may be the cause of implantation failure (Cicinelli et al., 2015). Zou *et al*. (Zou et al., 2023) confirmed this fact, there was a significant increase in implantation rates after antibiotic treatment. In our study, *Staphylococcus* and *Garnderella* have a frequency of 13.6% in the two studied groups, and another obligate anaerobe - *Atopobium*, is found in 30.7% of the entire sample.

Comparing the results of IVF procedures in "naïve" IVF patients and RIF patients with established microorganisms in the endometrial microbiome, Keburiya *et al*. (Keburiya et al., 2022) found no difference in the final outcome, although there was a higher incidence of *Gardnerella vaginalis* in the former group (12.8% vs. 1.6% in RIF). In the second group with RIF, these authors found obligate anaerobes (*Streptococci, Enterobacteriaceae*), but in low concentrations that were not significant.

Cela *et al.* (Cela et al., 2022) in a study of endometrial microbiome in patients with RIF found dysbiosis in 50% of cases, using a cut-off of 90%. The pathogenic microorganisms most often isolated from them are *Streptococci* and *Gardnerella vaginalis.*

Iwami *et al.* (Iwami et al., 2022) again demonstrated *Streptococci* and *Gardnerella vaginalis* as the most common pathogens in RIF patients. After antibiotic treatment, pregnancy rates were 64.5% versus 33.3% in the untreated control group.

Lozano *et al.* (Lozano et al., 2023) in RIF patients reconfirmed *Streptococci* and *Gardnerella vaginalis* as the most common pathogens in the endometrium. Statistically significant other pathogenic bacteria in their study were *Prevotella, Bifidobacterium* and *Dialister*. In addition to proving them, the authors found a negative relationship between them and lactobacilli. This result is in confirmation of other studies that associate the presence of pathogenic microbes with poor reproductive outcome.

Cariati *et al.* (Cariati et al., 2023) examined the endometrial microbiome before embryo transfer and compared the results with reproductive outcome. In the group of

pregnant women, there was a significant difference in the isolation of bacteria from the families *Staphylococcaceae* (8% vs. 35% in non-pregnant women) and *Enterobacteriaceae* (60% vs. 100% in non-pregnant women).

IV.5.2. Frequency of pathogens in RPL

Churcill *et al.* (Churchill et al., 2018) in endometrial fluid in the luteal phase of patients with RPL in half of the women detected *Streptococcus, Enterococcus*, *Staphylococcus, Gardnerella, Atopobium, Prevotella, Megasphaera, Clostridiales*. In all these cases, a combined pathogen presence and lactobacilli reduction of between 12 and 68% was observed.

Peuranpaa *et al.* (Peuranpää et al., 2022) in RPL observed a decrease in endometrial lactobacilli and an increase of *Gardnerella*. They did a detailed analysis of the species of lactobacilli in the endometrium in RIF. According to them, *L.crispatus* is reduced, a similar reduction is also found in chronic endometritis (Liu et al., 2019). *L. iners* was the most dominant microbe in their samples. Several studies have associated *L.iners* with dysbiosis (Petrova et al., 2017) and poor reproductive outcomes, incl. subfertility (Campisciano et al., 2021), miscarriages (Nasioudis et al., 2017), premature birth (Kindinger et al., 2017). In their results, there was an association between vaginal and endometrial colonization with *Gardnerella vaginalis.* A known fact is its connection with spontaneous abortions - early (Haahr et al., 2019), and especially late (Leitich & Kiss, 2007). Women with RPL and vaginal colonization with *Gardnerella vaginalis* have higher levels of peripheral NK cells, which may explain the association of dysbiotic reproductive tract microbiota with inflammation and miscarriage (Kuon et al., 2017).

In female patients with RPL, Shi *et al.* (Shi et al., 2022), in addition to reduction in lactobacilli in the endometrium, found that the presence of *Gardnerella* was associated with preterm birth in subsequent pregnancies, and the relative dominance of *Ureaplasma spp*. is an independent risk factor for miscarriage in chromosomally normal pregnancies and a risk factor for preterm birth.

Fen-Ting *et al.* (Fen-Ting et al., 2022) when examining the endometrium of patients with RPL found a significant reduction of lactobacilli and transformation of the uterine cavity into a multimicrobial environment. The phylum *Proteobacteria* dominates, which includes *Acinetobacter, Hydrogenophilus, Schlegelella, Serratia, Delftia*. Instead of Lactobacillus *Acinetobacter spp*. becomes the predominant species in the endometrium.

All these studies conducted in the last 5 years have very similar results - association of specific pathogens with poor reproductive outcome. Our results are largely

consistent with the conclusions drawn so far. They confirm the role of certain bacterial species in the development of dysbiosis, which can be a negative participant in the pathogenesis of reproductive failures.

IV.5.3. Frequency distribution of the types of microorganisms studied in moderately and severely disturbed microbiome

According to the analysis of the frequency distribution of the studied microorganisms in moderately and severely disturbed microbiome (table 4):

A statistically significant difference was found in 4 of the microorganisms: *Staphylococcus* (p 0.049), OANM *Gardnerella vaginalis* plus *Prevotella bivia* plus *Porphyromonas spp.* (p 0.002), OAHM *Mobiluncus spp.* plus *Corynebacterium spp.* (p 0.046), *Mycoplasmas*, Ureaplasma urealyticum plus *parvum* (0.036), and their proportion was higher in the group with moderately disturbed microbiome. In the highly disturbed microbiome group, there was a trend close to significant for an increased frequency of FAHM *Enterobacteriaceae* (p 0.056). From the obtained results, no dysbiotic microorganism could be identified to be significantly associated with a highly disturbed microbiome. There is no statistically significant difference between the two studied groups regarding the other isolated microorganisms.

In accordance with (Moreno et al., 2021), we also found a negative relationship between lactobacilli and facultative and obligate anaerobes – *Staphylococcus, Enterobacteriaceae, Gardnerella, Prevotella, Porphyromonas, Mobiluncus* and *Corynebacterium*, and this negative relationship was most pronounced in *Enterobacteriaceae*.

Table 4: Frequency distribution of the investigated microorganisms in moderately and severely disturbed microbiome

Microorganisms	Frequency	Moderately disturbed	Severely disturbed	P
FAHM *Enterobacteriaceae*	n	2	17	*0,056*
	%	6,7	23,0	
FAHM *Streptococcus spp.*	n	5	7	0,321
	%	16,7	9,5	
FAHM *Staphylococcus spp.*	n	12	15	**0,049**
	%	40,0	20,3	
	n	14	12	**0,002**

OAHM *G.vaginalis* plus *Prevotella bivia* plus *Porhyromonas spp.*	%	46,7	16,2	
OAHM *Eubacterium spp.*	n	4	6	0,469
	%	13,3	8,1	
OAHM *Sneathia spp.* plus *Leptotrichia spp.* plus *Fusobacterium spp.*	n	2	1	0,199
	%	6,7	1,4	
OAHM *Megasphaera spp.* plus *Veillonella spp.* plus *Dialister spp.*	n	3	3	0,352
	%	10,0	4,1	
OAHM *Lachnobacterium spp.* plus *Clostridium spp.*	n	1	1	0,496
	%	3,3	1,4	
OAHM *Mobiluncus spp.* plus *Corynebacterium spp.*	n	17	25	**0,046**
	%	56,7	33,8	
OAHM *Peptostreptococcus spp.*	n	1	0	0,288
	%	3,3	0,0	
OAHM *Atopobium vaginae*	n	15	38	1,000
	%	50,0	51,4	
Fungi Candida spp.	n	24	51	0,336
	%	80,0	68,9	
Mycoplasmas Mycoplasma hominis	n	2	3	0,625
	%	6,7	4,1	
Mycoplasmas Ureaplasma urealyticum plus *parvum*	n	7	5	**0,036**
	%	23,3	6,8	
Pathogenic microorganisms *Mycoplasma genitalium*	n	0	0	-
	%	0,0	0,0	

IV.6. Exploring the association of established pathogens with perturbed microbiome species

According to our results, in a moderately and severely disturbed microbiome, a statistically significant difference is found in 4 groups of microorganisms: *Staphylococcus spp.,* OAHM including *Gardnerella, Prevotella spp*., *Porphyromonas spp.,* OAHM including *Mobiluncus spp*. and *Corynebacterium spp., Mycoplasma* and *Ureaplasma.* The changes are more pronounced in a moderately disturbed microbiome. In a highly disturbed microbiome, there was a trend close to significant for an increased frequency of *Enterobacteriaceae*. From these results, no conclusion can be drawn for a dysbiotic microorganism to be significantly associated with a highly disturbed microbiome. These results are similar to those obtained by Moreno *et al.* (Moreno et al., 2021). Their data showed a negative correlation of lactobacilli with *Gardnerella*, *Bifidobacterium, Atopobium, Staphylococcus, Streptococcus* and a positive correlation with commensal microbes, thus maintaining the stability of the system. Their subsequent analysis of live births showed a negative correlation with *Streptococcus, Corynebacterium, Haemophilus, Staphylococcus, Atopobium*, *Gardnerella, Klebsiella* and *Escherichia*. Patients with live births have a greater abundance of lactobacilli, compared to the others - with reduced or dysbiotic microbes present. According to their hypothesis, the composition of the microbiota in patients with live births is a physiological scenario and does not affect the functional reproductive potential. In our research, we find an analogous correlation to the above: depletion and reduction of lactobacilli lead to the creation of an environment that favors the development of pathogenic microbes. In this case, the opposite scenario can also be discussed - the presence of pathogens does not favor the presence and dominance of lactobacilli.

Cela's team (Cela et al., 2022) found significantly increased proinflammatory interleukins in dysbiosis due to *Streptococci* and *Gardnerella vaginalis*. They associate the presence of these particular pathogens in the endometrium and the resulting dysbiosis with the development of immune dysregulation. According to them, the dominance of lactobacilli in the endometrial microbiota is of key importance for maintaining a physiological non-inflammatory microenvironment with proper activation of the local immune response, creating optimal conditions for implantation. When dysbiosis is established, the metabolomic and proteomic composition of the endometrium changes. There are changes in processes related to the immune response, inflammation and cell adhesion, as well as a change in naturally occurring antimicrobial peptides that inhibit the growth of various pathogens. Kyono *et al.* (Kyono, Hashimoto, Nagai, et al., 2018) and Moreno *et al.* (Moreno et al., 2016) defined two groups - of eubiosis and dysbiosis. For a state of eubiosis, they defined an 80% lactobacilli or bifidobacteria dominant microbiome. In the group with dysbiosis, the most represented genera were *Atopobium, Gardnerella* and *Streptococcus,* without

finding an effect of their proportions on the pregnancy rate (Hashimoto & Kyono, 2019).

More extensive future research will be able to establish more precisely which is the leading mechanism in the interactions between microorganisms and how these interactions affect the host organism.

IV.7. Exploring approaches to study the endometrial microbiome and creating an optimized protocol for its assessment using genome-based technologies

Despite initial encouraging data on the presence of an endometrial microbiome, some studies have cast doubt on this and yielded very conflicting conclusions. Concerns about the reliability of the results are diverse in nature, from reagents, small or heterogeneous cohort groups, demographic characteristics to methods of sample collection, sequencing methods with post-processing and interpretation of results.

One of the biggest concerns is regarding sample contamination. The currently available literature lacks a precise description of how to obtain material and avoid its contamination. Contamination can be of a different nature - from the vagina and cervix in transcervical access to staff gloves, patient skin, uterine manipulators, cervical dilators in transabdominal access (Baker et al., 2018).

Other difficulties regarding the adequate interpretation of the obtained results originate from the groups of patients that are included in the studies - uteri from hysterectomy for gynecological disease, benign or malignant, since naturally healthy uteri cannot be removed, as well as IVF patients who although they do not have severe disease, they are infertile and cannot be used as controls.

Specific primers for the 16S rRNA gene V region are a potential cause of discordance as they may underestimate or overestimate certain taxa (Tremblay et al., 2015). Another source of variation across studies is the DNA extraction methods used and the classification of taxonomic units (Sinha et al., 2015).

Despite various data, the composition of the endometrial microbiome is quite unclear and its role remains a field of debate (Baker et al., 2018), (Simon, 2018), (Peric et al., 2019). The topic is quite controversial, insufficiently and fully researched and opens up the possibility for in-depth and wider research. There is a lack of large cohort studies, limited by the invasiveness of the study, lack of comparability of the obtained results. The establishment of standard protocols for sampling and processing and analysis of the results is urgent. The small number of patients included in the available studies has a direct negative effect on the statistical power of the analysis. Due to its invasiveness, conducting the study in healthy controls remains a challenge.

One of the major limitations in processing the results is the selection of patients for the study. Patient characteristics—age, ethnicity, sexual behavior, hormonal, antibiotic, probiotic treatment—must be taken into account in cohort selection, as these factors can lead to large fluctuations in the results obtained with subsequent ambiguity.

In addition to the inter-individual characteristics, the individual characteristics of the included patients should also be taken into account - menstrual cycle, indication for the study, conducting an ART procedure. Depending on the phase of the menstrual cycle, changes in the composition of the endometrial microbiome are found. In ART, microbiome testing should be performed under conditions equivalent to the implantation period in order to avoid ambiguity in the interpretation of results due to hormonal fluctuations.

Regarding the obtained results, external factors must also be taken into account. In patients with RIF and RPL, intrauterine manipulations are more often performed - dilations, curettages, embryo transfers, hysteroscopies. These manipulations can lead to direct inoculation and colonization of the endometrium with vaginal and cervical flora, including pathogenic ones.

One of the topics that needs to be further explored and clarified is what constitutes a normal microbiome. In the studies cited above, the microbiome sequenced in healthy women varied greatly among authors. At present, with the available data, it is difficult to build a consensus on a healthy or core microbiome.

The uterine microenvironment is unique from other mucosae in that it is the site of embryonic implantation and placentation and is under the strict control of female sex hormones. Therefore, unlike the vagina, lactobacilli cannot be used as a criterion for uterine health. Furthermore, they can trigger a series of pathophysiological changes after ascending to the cavity (Baker et al., 2018).

The presence of the uterine microbiome can impair the genomic stability of the uterine epithelium by modulating transcription factors and other genomic and epigenetic damage with subsequent shutdown of autophagy. Suppression of the expression of cell junctions is a key factor in disruption of the epithelial barrier, which allows the movement of bacteria between epithelial cells. Additionally, the integrity of the epithelial barrier is damaged by the degradation of the extracellular matrix by matrix metalloproteinases. Products secreted by microorganisms, such as short-chain fatty acids, can promote the growth of certain species and suppress others. Free oxygen radicals and changes in the pH of the uterine microenvironment can also trigger a pathological process. Activation of inflammatory circuits can activate immune cells with subsequent secretion of antimicrobial peptides, reducing the bacterial load. Therefore, determining the healthy uterine microbiome that creates a state of eubiosis and is associated with a favorable reproductive outcome is a starting point for the diagnosis and treatment of patients with reproductive failure.

The two main methods of studying the microbiome, culture and genomic sequencing, have their advantages and disadvantages. The cultural method is widely used, cheap and accessible, but it does not give information about all the microorganisms present in a given habitat. Reasons for this are believed to be low mass microbiomes and also the impossibility of creating an environment to cultivate all possible microorganisms Currently there are data that only 1% of microorganisms can be cultivated, the remaining 99% lack growth, which is due to strict growth requirements – optimal combination of nutrients, oxygen concentrations, temperature conditions, need for co-cultivation with other bacterial species (Cicinelli et al., 2009), (Cicinelli et al., 2015). A great advantage of cultural methods is the information about the viability of the isolated microbes and their antibiotic resistance. Genomic sequencing provides accurate results for the microorganisms present based on detection of their DNA. Enables taxon resolution, culture-free microbiome analysis, quantitative representation of the microbiome, short turnaround time, no need to work in aerobic and anaerobic conditions, no heavy workload. Disadvantages of the method are its high cost and the lack of information about the viability of microbes, their biological activity and antibiotic resistance. Despite these drawbacks, we chose and used the RT-PCR method to obtain comparable and reliable results.
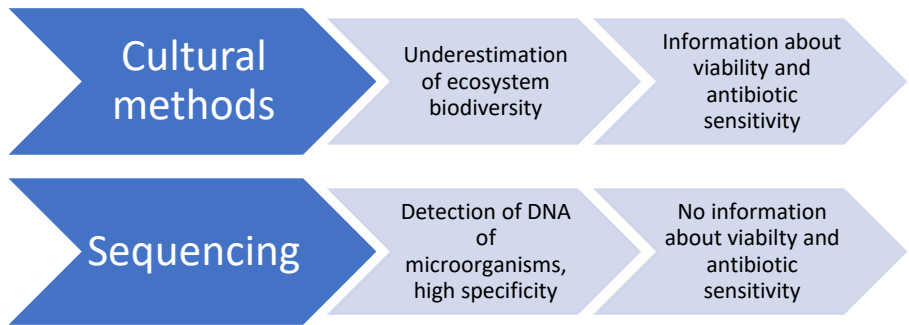
Figure 18. A comparison between the two main methods of microbiome research

In developing an optimized protocol for studying the endometrial microbiome, we explored the different ways to reach the uterine cavity. We chose the transcervical approach, despite the real risks of contamination of the sample from vaginal and cervical contents, because we accept the hypothesis of Chen's group (C. Chen et al., 2017) about the existence of a continuum of microbiota in the female reproductive tract. In making this decision, we considered the risk of the data of Moreno's group (Moreno et al., 2016) for the presence of a 20% difference in the composition of the microbiota between the lower and upper reproductive tract. We chose the transcervical

approach to the uterine cavity over the transfundal approach, for obvious reasons. Obtaining material for the study of the endometrial microbiome is an invasive procedure, which, however, does not require preoperative and preanesthetic preparation, with risks for the patient that do not exceed the average frequency of complications for intrauterine manipulations. The risk of hemorrhage, pain, ascension of infectious agents, rupture and perforation of the uterus is not increased.

When choosing the sampling technique, we also considered the ability of bacteria to invade mucous membranes. Some bacteria adhere only to the surface of the endometrial epithelium, while others colonize the deep layers of the endometrium. Due to this fact, we chose the technique of endometrial biopsy over aspiration, in order to have the possibility of obtaining material for examination also from the deep layers of the endometrium.

Regarding the phase of the menstrual cycle, after analyzing the scientific literature, we adopted a mean luteal phase for sampling. This is the period in which endometrial receptivity develops and there is the greatest stability of the uterine ecosystem with the dominance of lactobacilli.

When developing the protocol, we also unified the selection and preparation of the patients for research. The detailed information is described in the Materials and Methods chapter. We also unified the surgical method and the instruments used. Bioinformatic analysis was also identical in all cases studied.

The microbiome has its own physical structure. Research shows that microorganisms form their own three-dimensional biofilm with an outer and inner layer. This adds a complexity that is little explored at present. The existence of this biofilm from the vagina to the fallopian tubes allows for complex and dynamic relationships between gametes and the embryo and maternal tissue surfaces (Swidsinski et al., 2013).

Low-biomass microbiomes (endometrial, blood, urine) can play an important role in microbial homeostasis and physiology, but their study is often hampered by potential contamination from bacteria or bacterial DNA present in the air, laboratory equipment and reagents that are inadvertently involved in sample collection, processing and analysis. One of the most significant sources of bacterial DNA contamination comes from DNA extraction kits and laboratory reagents, even when they are sterile. Therefore, it is critical that researchers working with low-biomass samples follow strict protocols to avoid misleading conclusions about the sequenced output. These measures include prevention of contamination before sequencing by maximally protective equipment, during sequencing by including sufficient negative and blank controls, and after sequencing by developing bioinformatic pipelines to track and remove potential contaminants from the true microbiome present in the sample (Weiss et al., 2014).
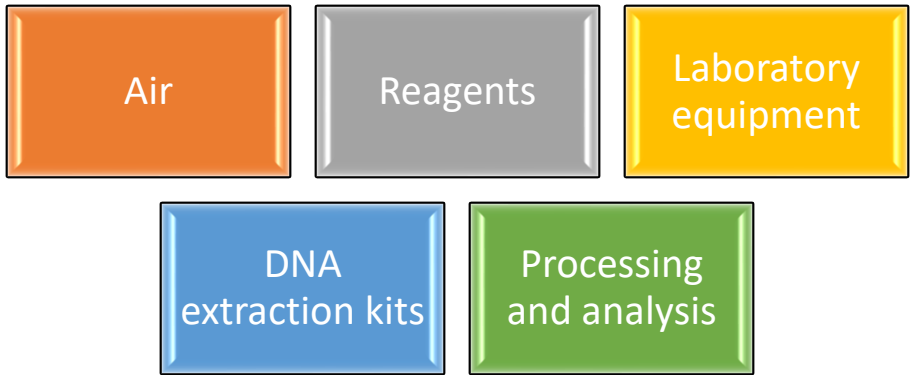
Figure 19. Potential sources of compromising genomic sequencing results

In our research, we have tried as much as possible to limit the factors influencing the obtaining and interpretation of the results. We researched and selected research methods and subsequent analysis. We built a protocol for the selection of patients, their preparation, technique of obtaining material from the uterine cavity, processing and analysis of the results. According to our studies, the problem has not been discussed and studied in a large homogeneous group among the Bulgarian population.

As a result of these studies, selection of methods and analysis, we believe that the results obtained by us have a high level of accuracy and reliability and enable the creation of logical conclusions with scientific and practical application.

We believe that our proposed protocol is standardized and reproducible with a high level of accuracy and reliability and with great potential for application in clinical and research work. In its construction, we have taken into account all influencing factors and have developed a methodology aimed at minimizing them and obtaining a non-misleading bacterial composition. As a result, the obtained results can serve as a basis for clinical behavior and to expand and build on research in the future. They provide an opportunity to create logical conclusions with practical and scientific application and expand our knowledge in this still unknown and wide field.

When analyzing the microbiome, its dynamics over time should always be taken into account, as it may not correspond to the current microbiome at the time of embryo transfer and implantation. Because of this fact, the results of his research should not be taken as absolute and limiting reproductive intentions.

V. Conclusion - main conclusions

V.1. Conclusions
Recurrent reproductive failures are characterized by a dysbiotic microbiota of the female reproductive tract, particularly in the uterus. According to available data from the literature, 74% of women with reproductive failure have one or more types of bacteria isolated. These data definitely open the discussion for endometrial research before embryo transfer. To this must be added the fact that in 20% of cases there is a discrepancy between the microbiome composition of the vagina and cervix with that of the endometrium. 46% of IVF patients with receptive endometrium have non-Lactobacillus-dominated endometrium and this microbiome profile is associated with poor reproductive outcome in terms of implantation, pregnancy and live birth.

A normal endometrial microbial environment is key to reproductive success. Its damage can activate an incorrect immune response at the time of implantation and placentation. Expanding research and increasing understanding of the influence of the microbiota will enable the assessment of future pregnancy risk based on the bacterial profile, the possibility of modulating the microbiome composition and enhancing reproductive outcomes in couples with RIF and RPL.

We found diversity in the types of pathogens in the endometrium. Most likely, this is related to the lack of a dominant type of microorganism (what is the current perception of a healthy microbiome - more than 90% relative presence of lactobacilli according to available scientific data). This can lead to colonization by other bacterial species with an increase in the diversity of the environment, the occurrence of dysbiosis and an association with infertility.

In the future, research will need to focus on defining and more clearly understanding the healthy microbiome, not just simply proving the presence or absence of lactobacilli. There is a need to study the mechanisms that guide the interaction between the microbiota and the host organism.

V.2. Main conclusions:

Having followed the aim of the present work and fulfilled all the set research tasks, the following conclusions can be drawn:
1.   The uterine cavity is not a sterile environment.
2.   In patients with recurrent reproductive failures, there is a high percentage of missing lactobacilli. This is found in more than half of the investigated cases. No difference was found between the two studied groups (RIF and RPL), which supports the main pathogenetic mechanism of damage to the endometrial microbiome profile.
3.   Recurrent reproductive failures are associated with endometrial dysbiosis in half of the studied cases.

4.   In a detailed analysis of microbiome types, a pronounced association of repeated reproductive failures with a severely disturbed microbiome is found.

5.   There is no statistically significant difference in the two studied groups (RIF and RPL) regarding endometrial dysbiosis, supporting the common pathogenetic mechanism of early pregnancy disruption.

6.   In a detailed analysis and comparison of the pathogenic microbes in the two studied groups (RIF and RPL), in the RIF group the obligate anaerobic Megasphaera spp., Veilonella spp., Dialister spp. are statistically reliable, in the RPL group the facultative anaerobic ones are statistically reliable Staphylococcus spp.

7.   In a moderately disturbed microbiome, there is statistical reliability for facultatively anaerobic Staphylococcus spp., obligately anaerobic Gardnerella spp., Prevotella spp., Porphyromonas spp., Mobiluncus spp., Corynebacterium spp., Mycoplasmas, Ureplasma urealyticum, Ureaplasma parvum.

8.   In a severely disturbed microbiome, there is no statistically reliable result for a specific pathogenic microbe. There was a trend close to significance for an increased frequency of facultatively anaerobic Enterobacteriaceae.

VI. Contributions

VI.1. Contributions of original character:

1.   An analysis was made of the temporal status of the endometrium in a large group of patients with reproductive failure without looking for a relationship with subsequent episodes of RIF and RPL.

2.   The frequency of the dysbiotic endometrial microbiome in a large group of Bulgarian patients with reproductive failure was investigated.

3.   We identify the pathogenic microorganisms most often associated with reproductive failures.

4.   We are developing a methodology for the classification of endometrial microbiome types.

5.   We validate RT-PCR as a reliable and precise method in the study of the endometrial microbiome.

6.   We introduce the study of the endometrial microbiome as a biomarker for RIF and RPL risk assessment.

7.   We are developing a protocol for research and subsequent personalized etiological treatment in cases of pathological results, which gives an additional opportunity to limit the use of antibiotics and the development of antibiotic resistance.

8.   The developed protocol gives results with a high level of accuracy and reliability, with the possibility of creating logical conclusions with scientific and practical application.

9.   We introduce a method in the complex evaluation of the uterine cavity, in particular the uterine mucosa, with the possibility of wide use in order to improve the reproductive outcome in RIF and RPL.
10.   The analysis will benefit the improvement of family planning and potentially improve the discussion between patient and treating physician regarding informed expectations and consideration of treatment options.

VI.2. Contributions of confirmatory character:

1.   We confirm that the endometrium is not a sterile environment.
2.   We confirm that there is a high incidence of dysbiotic endometrial environment in patients with reproductive failure.
3.   We confirm the reliability of the method in the set of studies in patients with RIF and RPL.

VII. Dissertation related publications and scientific announcements
VII.1. Publications related to the dissertation work:

Bodurska T., Konova E, Pachkova S, Yordanov A. *Endometrial microbiome and women's reproductive health-review of the problem endometrial microbiome and reproductive health*. Journal of pure and applied microbiology, 2021, 15(4):1727-1734; ISSN: 0973-7510; Web of Science, Scopus

Bodurska T., Konova E., Pachkova S., Yordanov A. *The role of uterine infection in pregnancy outcome: A case report*. Journal of biomedical and clinical research 2021; 14(2):178-181; ISSN:1313-6917; Web of Science (CABI)

Blazheva S., **Bodurska T.**, Ivanov P., Pachkova S., Konova E. *Endometrial immune cells and endometrial microbiome in women with recurrent implantation failure*. Bulgarian Journal of Clinical Immunology, 2022, 15(1): 3-12; ISSN:2738-7046

VII.2. Participation in research projects related to the dissertation work:

        Project 22/2022: „ A study of the endometrial immune profile in women with recurrent implantation failures". Lead researcher: Assoc. Dr. Emiliana Ilieva Konova, "Clinical Laboratory, Clinical Immunology and Allergology", Laboratory of Clinical Immunology" at UMHAT "Dr. Georgi Stranski".

VII.3. Participation in scientific forums in Bulgaria:

Bodurska T, Pachkova S, Yordanov A, Konova E. "Clinical case of chronic endometritis and recurrent miscarriages" 20th Anniversary National Congress on Clinical Microbiology and Infections of the Bulgarian Association of Microbiologists, Plovdiv, 16.09-18.09.2022;

Pachkova S, Blazheva S, Ivanov P, **Bodurska T**, Konova E. "Endometrial microbiome in women with reproductive problems" 20th Anniversary National Congress on Clinical Microbiology and Infections of the Bulgarian Association of Microbiologists, Plovdiv, 16.09-18.09.2022;

Blazheva S, Pachkova S, Ivanov P, **Bodurska T**, Konova E. Influence of the endometrial microbiome on endometrial immune cells in women with reproductive failure. Annual Scientific Conference on Immunology, Sofia, 16.12.2022;

Bodurska T, Blazheva S, Pachkova S, Konova E, Totev T. Study of endometrial microbiome in patients with recurrent reproductive failures. XXIV Congress on Sterility and Reproductive Health, 06-09.04.2023, Borovets;

Blazheva S, **Bodurska T**, Ivanov P, Pachkova S, Konova E. Study of endometrial immune cells in women with disturbed endometrial microbiome and reproductive failures. XXIV Congress on Sterility and Reproductive Health, 06-09.04.2023, Borovets;

S. Blazheva, **T. Bodurska,** S. Ivanova**,** P. Ivanov, E. Konova. Research of the endometrial immune cells in women with reproductive failure and impaired endometrial microbiota. 4th International World of Microbiome Conference, 26-28.10.2023, Sofia.

VII.4. Participation in scientific forums abroad:
M. Atanasova, E. Konova, S. Blazeva, **T. Bodurska**. Serum levels of anti-elastin and anti-fibrillin 1 autoantibodies in recurrent pregnancy loss (RPL) patients. 16th Dresden Symposium on Autoantibodies, 12-15.09.2023, Dresden, Germany.