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ASSISTED REPRODUCTION TECHNOLOGIES

Effect of the vaginal microbiome on the pregnancy rate in women receiving assisted reproductive treatment

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Abstract

Purpose To investigate if the vaginal microbiome influences the IVF outcome.

Methods Thirty-one patients undergoing assisted reproductive treatment (ART) with own or donated gametes and with cryotransfer of a single euploid blastocyst were recruited for this cohort study. Two vaginal samples were taken during the embryo transfer procedure, just before transferring the embryo. The V3 V4 region of 16S rRNA was used to analyze the vaginal microbiome, and the bioinformatic analysis was performed using QIIME2, Bioconductor Phyloseq, and MicrobiomeAnalyst packages. Alpha diversity was compared between groups according to the result of the pregnancy test.

Results Fourteen (45.2%) patients did not and seventeen (54.8 %) did achieve pregnancy under ART. A greater index of alpha diversity was found in patients who did not achieve pregnancy comparing to those who did, although this difference was not significant ($p = 0.088$). In the analysis of beta diversity, no statistically significant differences were observed between groups established as per the pregnancy status. Samples from women who achieved pregnancy showed a greater presence of *Lactobacillus* spp. The cluster analysis identified two main clusters: the first encompassed the genera *Lactobacillus*, *Gardnerella*, *Clostridium*, *Staphylococcus*, and *Dialister*, and the second included all other genera. Women who achieved pregnancy were mainly detected microorganisms from the first cluster.

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Conclusions The vaginal microbiome can influence the results of ART. The profiles dominated by *Lactobacillus* were associated with the achievement of pregnancy, and there was a relationship between the stability of the vaginal microbiome and the achievement of pregnancy.

Keywords Infertility · Assisted reproductive techniques, Embryo transfer · pregnancy · Microbiota · Microbiome

Introduction

Any disruption in the balance of the human microbiome can severely alter its function, causing infections or other diseases [1]. The vaginal microbiome plays an important role in maintaining women's overall health. The main bacteria are in the *Lactobacillus* genus, producers of lactic acid that helps preserving vaginal acidic pH, which acts as a defense against pathogens. In pregnant women, the vaginal microbiome differs from that of women in other life stages, both in the childbearing years and after menopause. However, while the relationship between the vaginal microbiome patterns and the evolution of pregnancy are well studied, research has only just begun on vaginal microbiota in relation to other aspects of women's fertility [2].

About 10 to 15% of couples have trouble conceiving spontaneously [3], in part, due to delay in motherhood and current behavioral trends. Correct treatment for infertility depends on the cause. The association between vaginal flora and fertility has been well established throughout the past years; for example, bacterial vaginosis (*Gardnerella vaginalis*) is the alteration of vaginal flora associated with higher risk of miscarriages [4–6]. Other pathogenic microorganisms like *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Mycoplasma tuberculosis* are related to a lower gestation rate, causing subclinical changes that are risk factors for subfertility [7].

The first longitudinal study that compared the microbiomes of healthy, full-term pregnant women versus healthy women who were not pregnant showed differences between the two groups, with the pregnant group showing a more stable microbiome [8]. Graspeuntner et al. [9] concluded that the microbiome demonstrates a specific pattern, associated not only with reproductive problems but also with a possible etiology. Thus, current evidence suggests that the microbiome could have an influence on infertility. A further inquiry along this research line would be to assess whether the microbiome—in addition to affecting fertility—has an impact on the outcome of in vitro fertilization treatment (IVF). Other studies aim to reveal differences in the vaginal microbiome as per pregnancy rates, including studies by Kyono et al. [10], Singer et al. [11], Bracewell-Milnes et al. [12], and Kroon et al. [13]. In Van Oostrum [7] et al. study, it was shown that the incidence of bacterial vaginosis is significantly higher in patients with tubal infertility compared with patients with non-tubal infertility. Bacterial vaginosis does not affect implantation rates, but it is significantly associated with pregnancy loss, although not with early miscarriages during the first trimester of pregnancy. Thus, the primary aim of this study is to assess the influence of the diversity, composition, and distribution of the vaginal microbiome on the IVF outcome.

Material and methods

Prospective pilot case-control study in a private-assisted reproduction clinic in Spain in 2017 and 2018. This study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and was approved by the institutional review board (Reference:16/318; 29 November 2016). The study population comprised patients attending the clinic from April 2017 to January 2018. Inclusion criteria were as follows: 18 to 50 years of age; diagnosis of infertility; indication for assisted reproductive treatment (ART) with the woman's own or donated gametes and with cryotransfer of a single euploid blastocyst; at least 3 months since last antibiotic treatment; and signed informed consent form. Exclusion criteria were as follows: uterine malformations; untreated hydrosalpinx; known factors for implantation failure, defined as implantation failure following transfer of at least three good-quality embryos over at least two cycles.

Participants followed the usual ART protocol with controlled ovarian stimulation and intracytoplasmic sperm injection (ICSI). The embryos generated underwent to preimplantation genetic diagnosis techniques at the blastocyst stage to identify and select euploid embryos. Whole-genome amplification on each biopsy was performed using the Sureplex method and followed by NGS using Veriseq protocol (Illumina ®) with the MiSeq Sequencer (Illumina ®). Analyses for aneuploidy testing were performed using Bluefuse Multi Software (Illumina). Aneuploid embryos were discarded, while euploid embryos were frozen for a subsequent embryo transfer. In a cycle following the ovarian stimulation of the woman receiving the oocytes, endometrial preparation under the estrogen–progesterone replacement therapy was performed, and a single euploid embryo was transferred. A pregnancy test was performed 8 to 10 days after the embryo transfer by means of a human chorionic gonadotropin (hCG) blood test.

Vaginal samples

Two vaginal samples were taken during the embryo transfer procedure, just before transferring the embryo. The vaginal fluid was taken with a dry swab from the bottom of the rectouterine pouch, in the posterior fornix, visualized directly with the aid of a vaginal speculum and the patient being in the lithotomy position. After collecting the sample, we proceeded with the ultrasound-guided embryo transfer according to established protocols. All samples were preserved at − 80 °C for later analysis.

DNA extraction

DNA was extracted using the PureLink microbiome DNA purification kit (ThermoFisher) according to the manufacturer's instructions. DNA was quantified with the Qubit 2.0 fluorometer (ThermoFisher). Extracted DNA was preserved at −20 °C for later use.

Amplification of the V3 V4 region of 16S rRNA

The 16S rRNA has nine less conserved hypervariable regions (V1 to V9), which provide the most useful information for phylogenetic and taxonomic studies. There is no consensus about which region is the most representative for each microorganism. Regarding the hypervariable regions of 16S rRNA used for analyzing the vaginal microbiome, the main ones are V1 V2, V3 V4, V4, and V4 V5. In the present study, we chose the hypervariable region V3 V4.

The oligonucleotides used for amplifying the V3 V4 region are detailed in Supplementary file 1. The amplification of the region by polymerase chain reaction (PCR) was performed with 1.5 units of Taq DNA polymerase (KAPA HiFi HotStart, Roche) in presence of deoxynucleotide triphosphates (dNTPs), the oligonucleotides 357F and 806R at a final concentration of 1 μM and a mean 100 ng of DNA, and at a final reaction volume of 25 μL. PCR was carried out in the thermocycler (Verity, Applied Biosystems) with the following time and temperature program: initial denaturation at 95 °C for 3 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a final step of elongation at 72 °C for 5 min. To validate the PCR technique, all the amplification reactions included positive and negative controls without a DNA mold. The PCR products were visualized via agarose electrophoresis, verifying that the amplified DNA band was of the correct size (449 pb). All the amplification products were stored at − 20 °C for later sequencing.

Sequencing of the V3 V4 region of rRNA 16S

Once we obtained the amplicon for sequencing, a library was generated with the identifying indexes of each sample as well as the sequences employed. We used the Nextera XT sequencing kit (Illumina), the MiSeq sequencer (Ilumina), and the metagenomics workflow.

Bioinformatic analysis

Once sequencing was finalized, the primary analysis of the obtained sequences was performed, consisting of their demultiplexation with the MiSeqReporter software (Illumina). The paired-end sequences of each sample were exported from the MiSeq system for analysis in the FASTA format. The bioinformatic analysis of the sequences was performed in the VirtualBox of the Ubuntu Linux operating system, with different commands in the QIIME2 package [14, 15]. For the subsequent data analysis, we used R software (version 3.4.2) along with the Bioconductor Phyloseq [16] and MicrobiomeAnalyst [17] packages.

The demultiplexed paired-end sequencing protocol was used for importing the sequences and the dada2 denoise-paired command to delete the low-quality ones. The sequences were grouped in operational taxonomic units (OTUs) with 97% similarity. To estimate microbial diversity, a rarefaction analysis was performed on 1000 sequences per sample for different alpha diversity indexes (phylogenetic distance, observed OTUs, and Shannon index).

Alpha diversity measures the richness of species in a given community. In our particular case, it is focused on the number of different species present and identified in a sample. Beta diversity, on the other hand, analyzes different composition, in terms of abundance of different taxa, between different samples. Beta diversity can be measured qualitatively or quantitatively, in the first case considering the abundance of microorganisms observed, while in the second one taking into account their presence or absence. The Chao1 analysis of alpha diversity was performed.

The beta diversity analysis was done by calculating the weighted UniFrac distance between each pair of samples. The taxonomic assignment used a classification based on a filtrate of the 99_otus sequence, from the Greengenes database to the V3 V4 region. The sequences obtained were filtered and assigned to at least one genus. Finally, a specific analysis for each taxon or group was performed as per to the results obtained. Clustering and ordination methods were employed to search for patterns associated with pregnancy.

Study variables

On the day of the enrolment in the study, the following variables were collected for each participant: age, weight, height, tobacco use (yes/no), number of previous pregnancies, previous miscarriage (yes/no), and their number as well as prior fertility treatments (yes/no). On the day of sample collection, we recorded the date and the endometrial thickness. Two

weeks after the embryo transfer, we registered the result of the pregnancy test (positive/negative) according to the beta-hCG value.

Statistical analysis

Descriptive analysis of the data consisted of calculating the means for quantitative variables and the relative frequencies (%) for categorical variables. To determine the distribution of our variables, we performed a Shaphiro–Wilk test. The non-parametric test used was the Mann–Whitney U test and the parametric Student t test. We compared the mean values of quantitative variables by groups according to the result of the pregnancy. In regards to the variable distribution, parametric or non-parametric test was used. Alpha diversity between groups was compared by applying the Student t test using the Shannon and Chao1 indexes as both have normal distribution. We calculated the Pearson correlation coefficient between *Lactobacillus* and other types. A bivariable (chi-squared) analysis was performed to analyze the relative abundance of the different genera between samples from both groups. P values of less than 0.05 were considered significant.

Results

There were 31 included patients with a mean age of 40.0 years (standard deviation 4.3). Fourteen subjects (45.2%) did not and seventeen (54.8%) did achieve pregnancy under the ART. Table 1 shows participants' characteristics. There were 7,089,699 total sequences for the 31 samples (mean 228,699; range 2285 to 1,142,892).

Figure 1 shows the rarefaction curves for the Shannon diversity index, both for all the samples and separating the groups by whether the patients achieved the pregnancy or not. The box-plot for the Shannon alpha diversity index showed a greater index of diversity in patients who did not achieve pregnancy, although the difference was not significant ($p = 0.088$). Finally, a comparative

analysis was performed for the Chao1 diversity index (Fig. 2), yielding significant differences ($p = 0.039$).

In the analysis of beta diversity, using different indexes, no statistically significant differences were observed between groups according to pregnancy status. However, using the unweighted UniFrac index, a certain (non-significant) tendency was apparent ($p = 0.088$).

Regarding the taxonomic characterization, Fig. 3 shows the taxonomic diversity at the genus level in the analyzed samples, with *Lactobacillus* spp. standing out as the most prevalent genus. It was majorly represented by *L. crispatus* (47.05%), *L. helveticus* (22.85%), *L. iners* (21.95%), and *L. jenseii* (3.97%). Samples from women who achieved pregnancy showed a greater proportion of *Lactobacillus* spp. Although none of the results from bivariable analyses comparing the abundance of different genera between groups reached the level of significance, for *Gardnerella*, the p value was 0.11, and for *Lactobacillus*, it was 0.20 (Fig. 4).

Finally, the cluster analysis identified two main clusters: the first encompassed the genera *Lactobacillus*, *Gardnerella*, *Clostridium*, *Staphylococcus*, and *Dialister*, and the second included all other genera. In women who achieved pregnancy, the microorganisms mainly from the first cluster were detected. The ordination analysis did not show any clear pattern except for that already observed for *Lactobacillus* and *Gardnerella*. The correlation graph (Fig. 5) indicates that *Lactobacillus* is positively correlated with the *Propionibacterium*, *Bacillus*, *Clostridium*, and *Caloramator* genera. On the other hand, it is mainly negatively correlated with *Gardnerella*, *Dialister*, *Burkholderia*, and *Finegoldia*.

Discussion

The results of this study show that the vaginal microbiome could influence the results of ART, as the profiles dominated by *Lactobacillus* are associated with the achievement of pregnancy,

Table 1 Participant characteristics and comparison between groups according to achievement of pregnancy

Variable	Total ($N = 31$)	Pregnancy achieved ($N = 17$)	Pregnancy not achieved ($N = 14$)	P value
Age, median and range (years)	40 (32-47)	38.5 (32-47)	42 (38-47)	0.019*
BMI, mean (kg/m²)	24.28	25.22	23.50	0.23
% Smokers	12.90	15.4	11.1	0.73
Previous pregnancies, mean n	0.77	0.82	0.69	0.66
% with prior miscarriage	70.96	70.6	76.9	0.69
Prior miscarriages, mean n	1.53	1.41	1.69	0.69
Previous ART, %	80.00	92.3	70.6	0.14
Endometrial thickness, mean (mm)	8.25	8.46	7.96	0.37

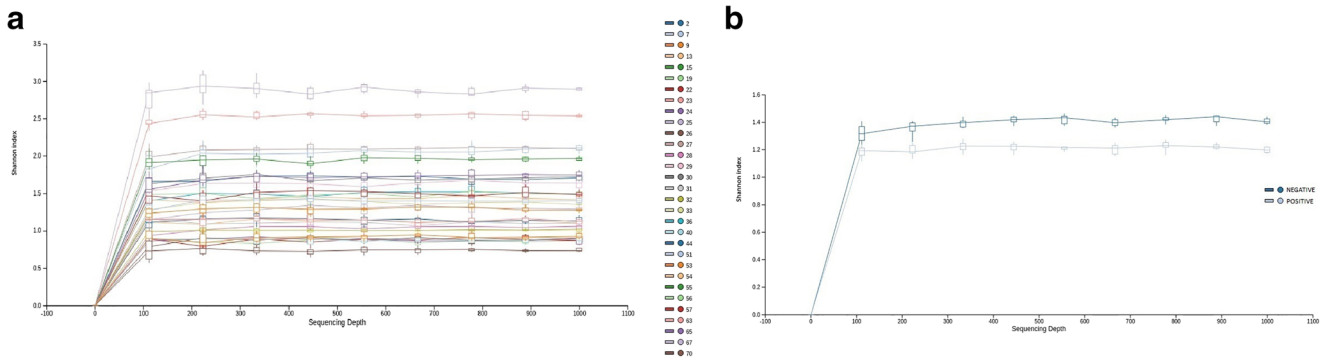*Statistical significance

ART assisted reproductive treatment

Fig. 1 Rarefaction curves for the Shannon index for **a** each sample and **b** according to result of pregnancy test (color)

and there is a relationship between the stability of the vaginal microbiome (Chao1 index) and the achievement of pregnancy.

The sequencing results from other studies on vaginal and/or endometrial microbiome populations vary in terms of the
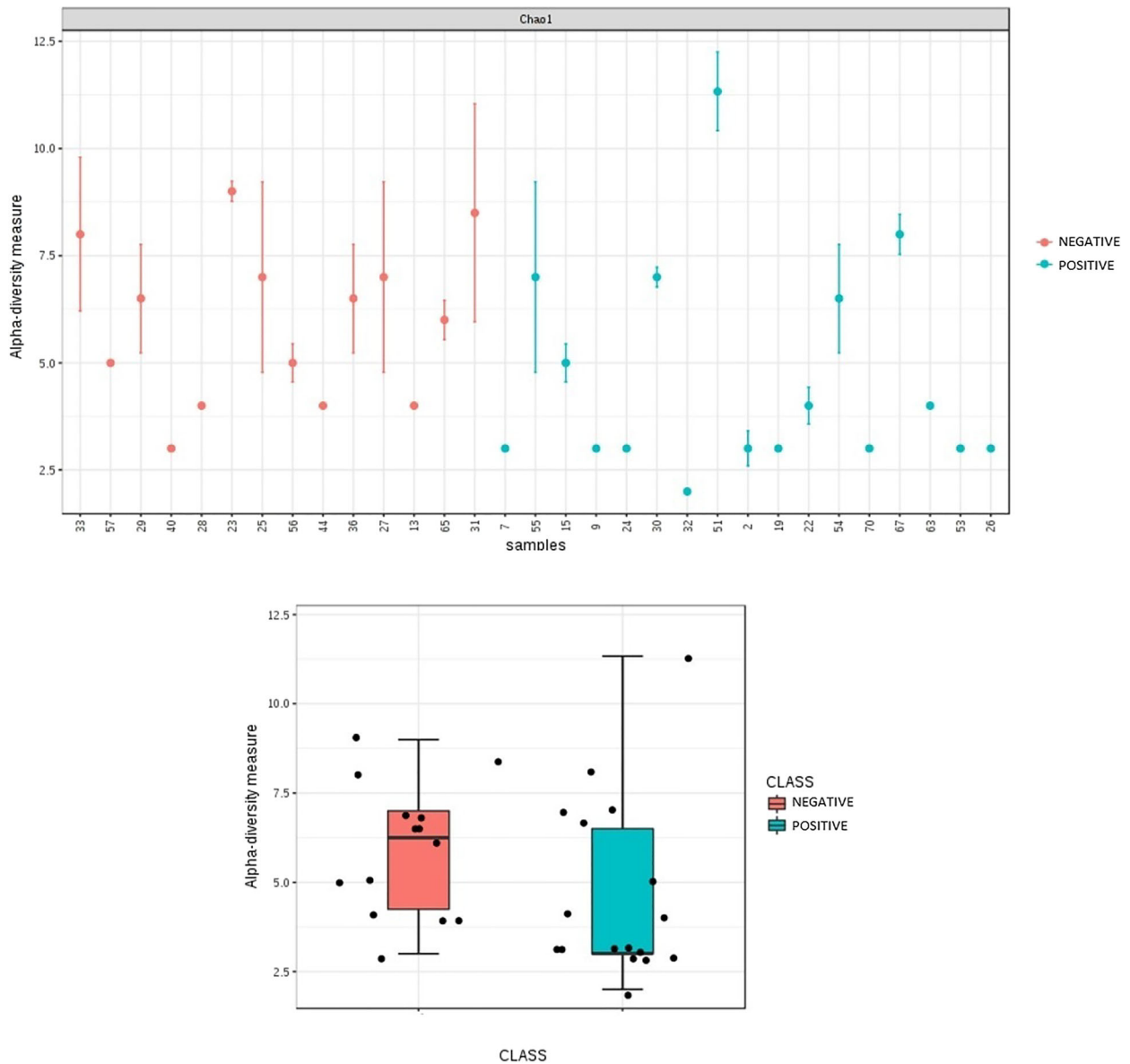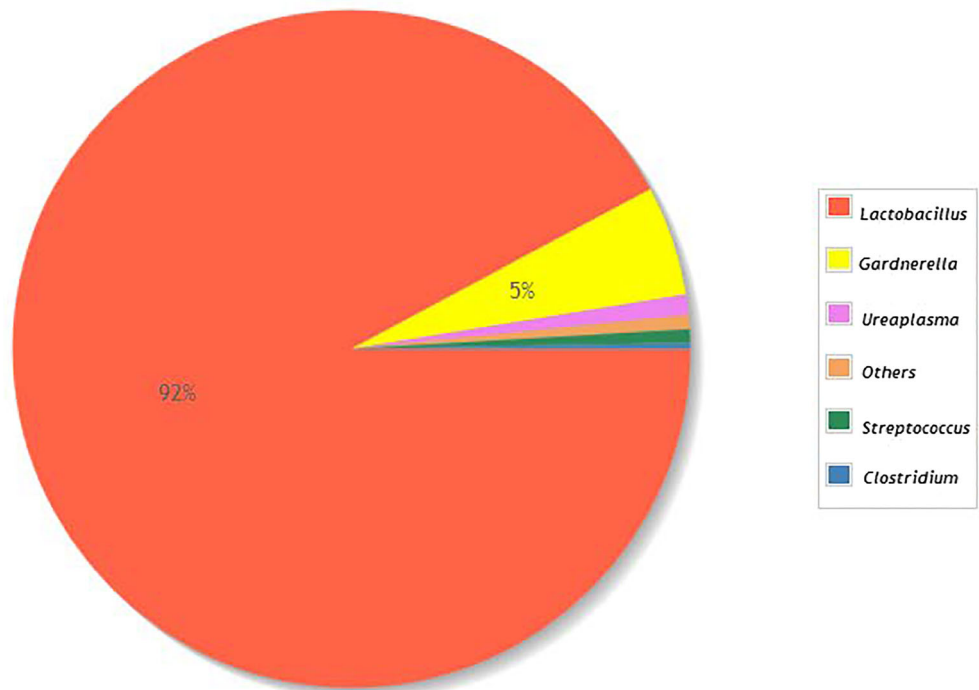

Fig. 2 Comparative analysis of Chao1 diversity index

Fig. 3 Taxonomic diversity by
genus in included study samples

average number of OTUs filtered per sample, which range from 40 [18] to 250 [19]. Thus, factors such as the DNA extraction kit used, the hypervariable regions selected from the sequenced 16S rRNA gene, the pair of primers employed, and the kit or system for generating massive sequencing libraries could all strongly influence the sequencing of the 16S rRNA gene. In terms of the OTUs, we identified with the deblur protocol, the total of 116 within the range of values expected for this type of vaginal sample.

The vaginal microbiome has low levels of diversity according to studies performed in the framework of the human microbiome project [20, 21], with some variability existing in terms of the location in the vagina and the gestational week. Our analysis of alpha diversity showed generally low values; women who did not achieve pregnancy showed significantly more alpha diversity than those who did. Moreno et al. [22] performed this analysis in the endometrial microbiome and did

not observe the same correlation. In previous studies that analyzed vaginal microbiome by week of gestation, the authors reported lower diversity indexes in patients with ongoing pregnancies [21]. This suggests that the vaginal microbiome changes during pregnancy; however, since we sampled the microbiome on the same day as the embryo transfer, the correct interpretation may be that a microbiome with little diversity favors the achievement of an ongoing pregnancy.

With regard to the beta diversity, previous studies describe differences in the vaginal microbiome according to gestational week and location in the vagina [20]. Moreover, some authors have observed differences in the beta diversity between pregnant and non-pregnant women [23]. We also observed differences, but these were not statistically significant, perhaps due to the small sample size.

The taxonomic characterization showed the unequivocal dominance of the *Lactobacillus* genus in the vaginal
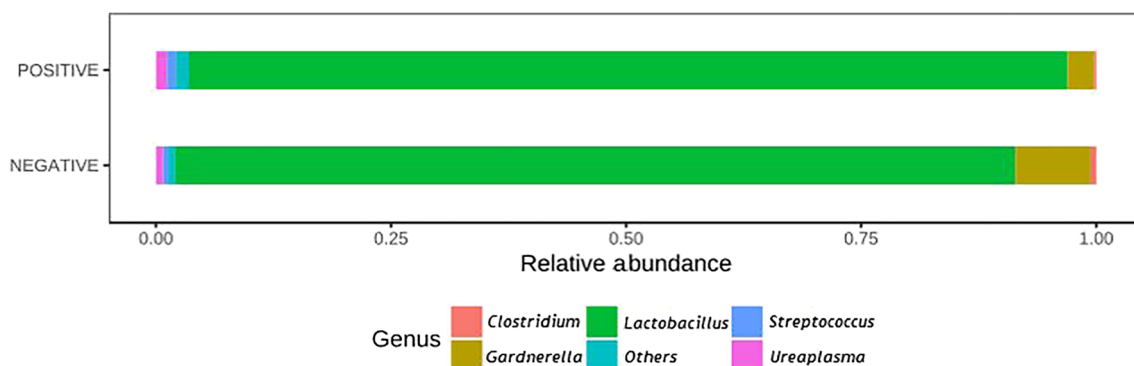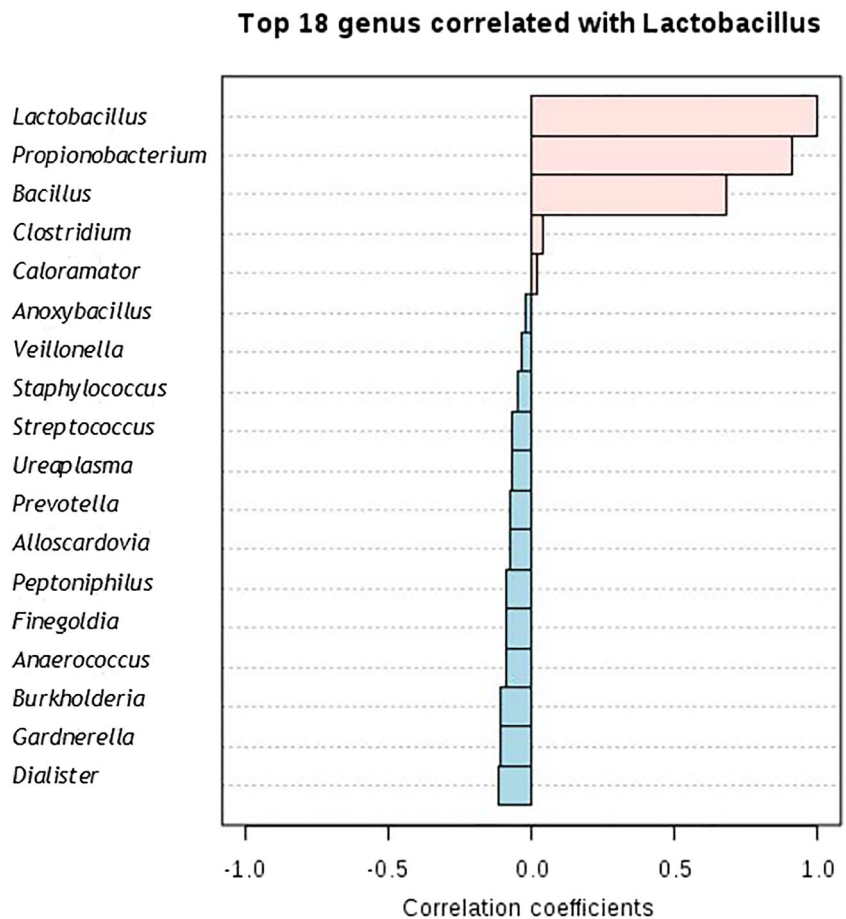

Fig. 4 Relative frequency of most abundant genera, according to the result of pregnancy test (positive/negative)

Fig. 5 Analysis of correlation
with *Lactobacillus*. Top 18 genera
correlated with *Lactobacillus*

Top 18 genus correlated with Lactobacillus

microbiome. The vaginal microbiome is mainly composed of microorganisms from the *Lactobacillus* genus [24], which creates an acidic environment that protects against sexually transmitted and other opportunistic infections. Moreno et al. [22] reported that an endometrial microbiota dominated by *Lactobacillus* spp. showed higher rates of embryo implantation, ongoing pregnancy, and lower rate miscarriage than this that was not. Nevertheless, the differences we found were not statistically significant.

Lactobacillus genus is the most abundant vaginal bacteria in women. They inhibit the binding of other bacteria to the epithelial cells and produce lactic acid that kills or inhibits the growth of many other bacteria. Lactic acid blocks histone deacetylases, improving gene transcription and DNA repair. Lactic acid induces autophagy in epithelial cells to degrade intracellular microorganisms and promote homeostasis. Lactobacilli are tolerated by vaginal epithelial cells and inhibit the induction of proinflammatory cytokines. Emotional stress can reduce the abundance of lactobacilli in the vaginal microbiota and increase inflammation. The ability of lactobacilli to inhibit infection without inducing inflammation can maximize fertility and the successful outcome of pregnancy in women [25].

A pilot study (Kyono et al. 2019) aimed to analyze the results of pregnancy in patients under IVF treatment who presented *Lactobacillus*-dominated microbiota (LDM) and non-*Lactobacillus*-domesticated microbiota (NLDM) in the endometrium and reports cases that were treated for NLDM simultaneously with antibiotics and prebiotic/probiotic supplements in a Japanese infertile population [25]. The study of Singer et al. (2019) concludes that women with an abnormal vaginal microbiota have approximately 1.4 times less chance of pregnancy after an IVF treatment compared with women with normal microbiota [11].

The comparison of relative abundance (Fig. 4) provided more data on the relationship between the two main types of vaginal microbiomes. On one hand, those dominated by *Lactobacillus* and showing less diversity were characterized by greater reproductive success. The other profile is not dominated by *Lactobacillus* and contains the *Gardnerella* genus; according to previous reports, the proportion of *Gardnerella* genus bacteria exceeds 10% in patients with infection-related infertility. There is also a negative correlation between the abundance of this genus and of *Lactobacillus* [9].

Another factor evaluated in other studies, which differs from the methodology applied in the present one, opens the possibility

of parallel analysis or complementary methods: it is bacterial contamination of the catheter tip [26]. The objective of that study was to evaluate the effect of bacterial colonization of the tip of the catheter used for embryo transfer on the clinical pregnancy rate in IVF treatments and concluded that, indeed, it is associated with the decrease of clinical pregnancy rate.

In another study of Selman et al. (2007), the presence of vaginal–cervical microbial contamination at the time of embryo transfer was analyzed. Samples were taken from the following different sources: the bottom of the vagina, the cervix, the culture medium of the embryo before and the after transfer procedure, the tip of the catheter, and the external sheet. This study also concluded that vaginal–cervical microbial contamination at the time of transfer can be associated with significantly lower pregnancy rates [27].

Limitations

The main limitation of this study is the small sample size. Future studies with more statistical power could confirm some of the tendencies that we observed. The cause of the infertility has not been analyzed either. Nevertheless, we have taken into account the patients diagnosed with recurrent implantation failure as they could alter the results in terms of embryo implantation and the maintenance of pregnancy. Another limitation of the study is the difficulty in the extrapolation of the results to other populations, since all patients analyzed are Caucasian. The fact that all subjects received the same endometrial preparation protocol helps us to normalize the population of the study and, therefore, also the validity of the results. At the same time, this uniformity makes it difficult to extrapolate the results to other endometrial preparation protocols.

Future studies with more statistical power are needed to confirm the tendencies that were observed in the present project.

Conclusions

The results of this study show that the vaginal microbiome has an influence on the results of assisted reproductive treatment. The microbiome profile that seemed to favor the achievement of pregnancy was dominated by *Lactobacillus*, while the notable presence of *Gardnerella* spp. was associated with the opposite outcome. The abundance of these genera shows a negative correlation. Moreover, there was a greater Chao1 alpha diversity in women who did not achieve pregnancy.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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