

The Impact Of The Vaginal And Endometrial Microbiome Pattern On Assisted Reproduction Outcomes

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Abstract

Background:

The vaginal microbiome plays an important role in maintaining health, and there is evidence that microbial colonization of the upper genital tract can also influence successful embryo transfer. The aim of this study is to determine whether the vaginal and endometrial microbiome in people undergoing assisted reproduction techniques could affect the pregnancy rate.

Results:

Regarding the microbiome dynamics during the cycle, we observed a decrease in alpha diversity from the follicular to luteal phase in the control group, in contrast to a stable pattern in the repetitive implantation failure group. As for endometrial and vaginal microbiome, alpha diversity was higher in the endometrium (Shannon $p = 0.0139$, Simpson $p = 0.046$); differences were also observed in beta diversity ($p = 0.001$). Compared to the endometrium, the vagina showed a greater relative abundance of *Lactobacillus* spp. (83.17% vs 84.82%, $p < 0.0001$), *Streptococcus* spp. (1.59% vs 7.74%, $p = 0.014$) and *Ureaplasma* spp. (0% vs. 0.89%, $p = 0.006$), and a lower abundance of *Delftia* spp. (0.95% vs 0%, $p = 0.0003$), *Anaerobacillus* spp. (1.59% vs 0%, $p = 0.0004$), and *Ralstonia* spp. (3.17% vs 0%, $p = 0.0006$). We also observed differences in both alpha diversity (Shannon $p = 0.0206$, Simpson $p = 0.0206$) and beta diversity between groups, along with differences for *Ralstonia* spp. (0.09% study group and 0.73% control, $p = 0.0012$). Finally, the relative abundance of *Lactobacillus* spp. differed between patients that did not versus did achieve pregnancy (91% vs 99%, $p = 0.0445$ visit 1, 94.63% vs. 97.69%, $p = 0.0268$ visit 2, 97.73% vs 99.74%, $p = 0.0492$ visit 3). The relative abundance of *L.reuteri* was also different between groups (0.39% vs 0.17%, $p = 0.0397$ visit 1, 0.15% vs 0.30%, $p = 0.0491$ visit 3).

Conclusions:

The vaginal and endometrial microbiome pattern correlates with the pregnancy rate, and it is different in patients who do versus do not have repetitive implantation failures. No significant differences in the composition of the microbiome were observed through the different visits. The lack of dynamism in the microbiome pattern of repetitive implantation failure patients might reflect an impaired adaptation to endometrial changes. A greater relative abundance of *Lactobacillus* spp. and *L. reuteri* is correlated with higher chances of pregnancy.

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Background

For over a century, conventional wisdom held that the cervical plug maintained the sterility of the uterine cavity. Nevertheless, technological advances in mass sequencing over the last decade have begun to uncover different microbial communities, including in the vagina and the endometrial cavity. These

microbiota have proven to be involved in reproductive health and disease, interacting with host cells along the female reproductive tract and generating the physical, chemical and biological environment that the embryo encounters during the peri-implantation period and throughout the pregnancy (1).

Endometrial implantation is the single most important event determining the success of embryo transfer in assisted reproductive therapy (ART) (2). Other factors include the presence of microbial colonization of the upper genital tract (3) and possibly utero-cervical microbial colonization (4), which has been shown to be an independent and significant factor determining the success of assisted reproductive treatments (5).

The main bacteria at vaginal and endometrial level belong to the genus *Lactobacillus*, producers of lactic acid that maintain the acidic pH of the vagina, which acts as a barrier against pathogens (6). The association between vaginal flora and fertility has been widely studied for years. The normal flora of the reproductive tract includes a variety of *Lactobacillus* spp., which provides a healthy environment for the embryo during the pre-implantation period and also promotes successful implantation. The livebirth rate is correlated with the production of H_2O_2 by *Lactobacillus* spp. and inversely correlated with the existence of bacterial vaginosis. Thus, alterations of the vaginal flora such as bacterial vaginosis (provoked by *Gardnerella vaginalis*) are associated with an increased risk of miscarriage (7, 8). Other pathogenic microorganisms such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Mycoplasma tuberculosis* have also been associated with a lower gestation rate, causing subclinical alterations related to risk factors for subfertility (9).

Studies on the vaginal microbiome and female infertility are scarce and recent. The first longitudinal study analyzing the microbiome of healthy pregnant women with on-term delivery versus healthy non-pregnant women showed differences between the two groups, with the microbiome of pregnant women showing greater stability (10).

At the moment, the microbiome can be studied by analyzing samples taken either from the endometrium or vagina, but all up-to-date studies have collected their specimens from different locations. Indeed, the best sample site for providing a prognosis of ART success has yet be determined. A recent paper published by Moreno et al. concluded that the uterine microbiota appears to be a continuum of the vaginal one. By contrast, other authors have reported significant differences between the vaginal and endometrial microbiota, highlighting the importance of evaluating the upper genital tract in order to understand the role of its microbiota in the physiological and pathological processes that take place in the uterine cavity, including embryo implantation, pregnancy maintenance and gynecological diseases. With regard to the endometrial microbiota, however, there are several significant obstacles. As in the case of any other low biomass microbiota, the small amount of the initial sample makes it vulnerable to contamination with exogenous bacterial DNA. For this reason, careful and appropriate investigation of the endometrial microbiota is exceptionally important in detecting uterine dysbiosis, which may affect reproductive function (11).

Within the framework of this study, we assumed that alterations in the vaginal microbiome reduce women's fertility through their negative impact on embryo implantation. Determining what makes a microbiome normal or which microorganisms can further limit female fertility may be the key to improving the prognosis of fertility treatments.

Our primary aim was to identify the vaginal and endometrial microbiome patterns associated with the rate of gestation in women undergoing assisted reproduction treatments.

Results

Description Of The Studied Variables

The study included 48 participants, who provided 273 samples, of which 264 were analyzed. Figure 1 details the number of patients and samples initially included in the study, losses and net inclusion data and analysis. Sociodemographic characteristics and clinical outcomes of the patients included in the study were recorded for the whole sample and according to achievement of an evolutionary pregnancy (Table 1), as well as patients with and without repeated implantation failures (RIF and NO RIF). At the level of the graph analysis, the "NO RIF" group is labeled as a control group for methodological issues, though it was not treated as such. Participants' mean age was 39.44 years, and their mean weight was 63.41 kilos. Smokers made up 14.29% of the sample, while 0.67% had had previous pregnancies—58.33% of which ended in miscarriage. In addition, 43.75% had carried out the treatment with donated oocytes. All the patients underwent endometrial preparation under hormonal replacement therapy. In terms of clinical data, 54.2% of the patients had a positive pregnancy test. The clinical gestation rate was 43.8%, with an evolutionary pregnancy rate of 37.5%. The rate of biochemical miscarriage was 10.4%, and of clinical miscarriage, 14.28%. Of the patients who achieved pregnancy, 38.9% had a history of miscarriages, compared to 70.0% of those who did not conceive ($p = 0.034$).

Table 1
Patients socio-demographic characteristics and clinical outcomes, by pregnancy outcome

Variables	Total sample N = 48	Pregnancy N = 26	No pregnancy N = 22	P value
Age, years, mean \pm SD	39.44 \pm 3.82	38.28 \pm 3.39	40.13 \pm 3.95	0.09235
Weight, kilos, mean \pm SD	63.41 \pm 9.79	59.57 \pm 7.98	64.69 \pm 10.16	0.1944
Height, cm, mean \pm SD	162.33 \pm 6.89	160.25 \pm 10.41	163.09 \pm 5.22	0.4806
Tobacco user (%)	14.29	16.7	11.1	0.5975
N previous pregnancies, mean \pm SD	0.67 \pm 0.60	0.56 \pm 0.51	0.73 \pm 0.64	0.2955
Previous miscarriages (%)	58.33	38.90	70.0	0.0343*
N of previous miscarriages, mean \pm SD	1.44 \pm 1.80	1.11 \pm 1.79	1.63 \pm 1.81	0.3343
Previous treatments (%)	77.08	72.20	80.0	0.5348
Semen analysis, normozoospermia (%)	77.08	83.30	73.30	0.6164
Donated semen (%)	20.83	22.20	20.0	0.8544
Endometrial thickness, mm, mean \pm SD	8.40 \pm 2.04	8.54 \pm 2.01	8.32 \pm 2.09	0.7254
B-HCG + (%)	54.2	—	—	—
Clinical gestation (%)	43.8	—	—	—
Evolutionary pregnancy (%)	37.5	—	—	—
Biochemical miscarriage (%)	10.4	—	—	—
Clinical miscarriage (%)	14.28	—	—	—

Generalized Mixed Model (logistic Function)

A generalized mixed model was constructed to determine the evolution of the vaginal microbiome pattern and its association with the gestation rate. The variation in alpha diversity (Shannon index) at different times of the cycle was assessed, and there were no statistically significant differences in the evolution of the vaginal microbiome patterns between visits, according to the treatment, or based on the achievement of clinical gestation ($p = 0.412$). Analyzing this evolution by RIF versus NO RIF groups ($p = 0.019$), we observed a variation in the vaginal microbiome pattern over time in the NO RIF group. Specifically, these patients showed a decrease in alpha diversity from the follicular to the luteal phase. In contrast, the RIF

group showed a stable microbiome pattern across different timepoints. This lack of dynamism in the pattern of the vaginal microbiome in RIF patients could entail a lack of adaptation to endometrial physiology and preparation, and therefore a worse prognosis for embryo implantation (Fig. 2).

Vaginal And Endometrial Microbial Patterns At Visit 1

We observed statistically significant differences alpha diversity between endometrial and vaginal samples ($p = 0.0139$ for Shannon index and $p = 0.046$ for Simpson index), with higher values in endometrial samples (Fig. 3a).

Using PERMANOVA, the matrices with beta diversity measures showed statistical differences in composition according to the type of sample ($p = 0.001$). The unweighted UniFrac PCoA revealed a clear pattern of separation between vaginal and endometrial samples (Fig. 3b). The endometrial samples are grouped at the extreme right of the graph. The percentage of the variance explained by each component is shown on the axes (Principal component (PC), PC1: 34.16%, PC2: 20.31%, PC3: 8.58%). The first and second components would together explain more than 50% of the variability between our samples.

Regarding the taxonomic characterization in all the samples of the study, there was a clear dominance of the genus *Lactobacillus* in both the vaginal and the endometrial microbiome. The bar chart of the relative frequency of the most abundant genera grouped by sample type (vaginal/endometrial) is shown in Fig. 3c.

Microbiome profiles showed relative differences in genera and species present in the vaginal and endometrial samples. The univariate analysis reached statistical significance for *Lactobacillus* spp., *Streptococcus* spp., *Ureaplasma* spp., *Delftia* spp., *Anaerobacillus* spp. and *L. Helveticus* spp. Several genera were more abundant in the vagina than in the endometrium: *Lactobacillus*, 84.82% versus 83.17% ($p < 0.0001$); *Streptococcus*, 7.74% versus 1.59% ($p = 0.014$), and *Ureaplasma*, 0.89% versus 0% ($p = 0.0062$). The other genera showing significant differences were more abundant in the endometrium: *Delftia* 0.95% versus 0% in the vagina ($p < 0.0003$); *Anaerobacillus*, 1.59% versus 0% (p -value = 0.0004), and *Ralstonia* 3.17% versus 0% ($p = 0.0006$) (Table 2).

Table 2
Differences in the genera present in microbiome profiles in vaginal and endometrial samples

Genus	Endometrium	Vagina	p-value
<i>Lactobacillus</i> spp.	83.17%	84.82%	< 0.001
<i>Delftia</i> spp.	0.95%	0.00%	0.0003
<i>Anaerobacillus</i> spp.	1.59%	0.00%	0.0004
<i>Ralstonia</i> spp.	3.17%	0.00%	0.0005
<i>Ureaplasma</i> spp.	0.00%	0.89%	0.0062
<i>Streptococcus</i> spp.	1.59%	7.74%	0.0187

Figure 3d shows the relative frequency of the most abundant species for each sample type. *Lactobacillus iners* presents a higher relative abundance in endometrial samples (64% versus 40% in vaginal samples; $p > 0.05$). There was a significant difference in the abundance of *L. helveticus* spp.: 28% in the endometrium versus 47% in the vagina ($p = 0.0001$).

Microbiome patterns by diagnosis of implantation failure

Vaginal microbiome pattern

Regarding the vaginal microbiome pattern in the samples obtained at visit 1, we found no differences in alpha diversity between the RIF and NO RIF groups according to either the alpha Shannon or Simpson indices (Fig. 4a). A more detailed analysis showed that the results were statistically significant only for the Faith index. The box diagram for the alpha Faith phylogenetic diversity index (phylogenetic analogue of taxon richness expressed as the number of tree units which are found in a sample) yielded a p value of 0.027, representing a significantly lower Faith alpha diversity index in the RIF group compared to the NO RIF group.

In relation to beta diversity, no statistically significant differences were observed between the two groups (Fig. 4b). Likewise, the univariate analysis showed no statistically significant results. In relation to the taxonomic allocation, the RIF group had a lower relative abundance of the genus *Streptococcus*, and a higher abundance of *Prevotella* spp., *Ureaplasma* spp., and *Dialister* spp. The NO RIF group presented a higher relative abundance of *Streptococcus* spp., *Veionella* spp., and *Aerococcus* spp. As for the genus *Lactobacillus*, no differences were observed between groups (Fig. 4c). At the species level, we observed a higher relative abundance of *L. helveticus* in the RIF patients, and of *L. iners*, *L. jensenii*, *L. gasseri* and *L. agalactiae* in the NO RIF group patients (Fig. 4d).

Endometrial microbiome pattern

The alpha diversity of the endometrial microbiome at visit 1 was significantly higher in the NO RIF group (Fig. 4e; $p = 0.0206$ for both Shannon and Simpson indices). There were also statistically significant differences in beta diversity, as seen in the PCoA graph (Fig. 4f). There is a clear pattern of separation between the RIF group and the NO RIF group: the RIF samples fall in the top center of the graph and those collected in the NO RIF group are clustered in the centre. A larger sample size would help us to corroborate this difference. On the axes, there is the percentage of the variance, explained by each component (PC1: 31%, PC2: 14.1%, PC3: 8.9%). The results for the first and second component explain more than 45% of the variability between our samples.

The taxonomic assignment in the frequency table represents the relative abundance of the different taxa present in the samples for the RIF and NO RIF groups. A greater abundance of the genus *Prevotella* is observed in the RIF group (Fig. 4g). In the univariate analysis we found statistically significant differences for the genus *Ralstonia*, observing a much higher relative abundance in the NO RIF group compared to the RIF group (0.73% versus 0.09%; $p = 0.0012$).

Figure 4h shows the differences in relative abundance at the species level. *L. iners* and *L. jensenii* were more abundant in the NO RIF group, while *L. helveticus* and *Sneathia amnii* had a larger presence in the RIF group.

Evolution Of The Vaginal Microbiome (whole Sample)

Diversity analysis

There are no statistically significant differences in alpha or beta diversity between the samples over the different visits.

Taxonomic characterization

We did not observe any statistically significant difference between the visits for either the composition of genera or species (Fig. 5). There were some apparent changes in abundance of the genera *Lactobacillus*, *Streptococcus* and *Prevotella*: both *Lactobacillus* and *Streptococcus* were more abundant on visits 1 and 2, showing a decrease on visit 3. *Prevotella* shows a higher abundance on visit 1 and 3, especially on the latter timepoint. In the univariate analysis there were no statistically significant differences.

At the species level, the bar chart shows some differences in relative abundance for the following species: *L. helveticus*, *L. iners*, *L. gasseri* and *L. jensenii* (Fig. 5). *L. helveticus* was most abundant on visit 2; *L. iners*, on visit 1; and *L. gasseri*, on visit 3. At that timepoint, results showed a smaller proportion of *L. jensenii*. However, these differences were not statistically significant.

Association of the vaginal sample taken at different visits with the gestation rate

Diversity analysis

Analyzing diversity as a function of the gestation rate, we observed a greater alpha diversity in patients who do not achieve pregnancy, obtaining a trend without reaching statistically significant values (Shannon $p = 0.0748$ and Simpson $p = 0.0856$). Regarding the beta diversity, no statistically significant differences were found at visit 1 according to gestation rate. For the samples collected at visit 2, the differences in alpha diversity were not statistically significant; however, there is a trend suggestive of a negative correlation between the gestation rate and alpha diversity ($p = 0.1518$). For beta diversity, we found no difference in relation to visit 2 and the pregnancy rate. The samples taken at visit 3 show no difference in alpha or beta diversity.

Taxonomic characterization

At visit 1, participants who achieved pregnancy presented a significantly greater abundance of *Lactobacillus* spp. than those who did not, while *Streptococcus* spp. and *Prevotella* spp. were more abundant in the latter group (Fig. 6a). *Streptococcus* and *Prevotella* may thus be associated with a poor prognosis with regard to gestation. On the other hand, an abundance of *Lactobacillus* spp. could be indicative of more favorable conditions. The differences were observed at the genus level for *Lactobacillus* spp. (91% with no gestation vs 99% with gestation; $p = 0.0445$) and at the species level for *L. reuteri* (0.39% vs 0.17%; $p = 0.0397$; Fig. 6b).

Similar results were obtained at visit 2. Those who achieved pregnancy presented a greater relative abundance of *Lactobacillus* spp. than those who did not (97.69% versus 94.63%; $p = 0.0268$; Fig. 6c-6d). The opposite was true for the case of *Streptococcus* spp. (Fig. 6c).

Findings at visit 3 were similar (Fig. 6e). The univariate analysis showed statistically significant differences ($p = 0.0492$) for the genus *Lactobacillus* spp. (99.74% with gestation versus 97.73% without) and the species *L. reuteri* (0.30% versus 0.15%, respectively; $p = 0.0591$; Fig. 6f).

Discussion

The role of the vaginal microbiota in reproduction and assisted reproductive technology procedures is an active field of research, and while there is a growing body of evidence supporting its relevance, many questions remain unanswered. Our analysis shows that the pattern of the vaginal and endometrial microbiome are not comparable. However, the results do suggest stability between visits, meaning that the sample could be taken at any timepoint to obtain similar findings. The vaginal samples obtained at

visit 1 showed differences only for the Faith index between the patients belonging to the RIF and NO RIF group, with a higher alpha diversity in participants with a history of implantation failure.

At the taxonomic level, we observed that the genus *Streptococcus* presents a lower relative abundance in the RIF group. The genera *Prevotella*, *Ureaplasma* and *Dialister* show a higher relative abundance in RIF patients; and *Streptococcus*, *Veillonella* and *Aerococcus* in the NO RIF group. The alpha diversity of the endometrial samples was significantly higher in the NO RIF group. With regard to beta diversity, statistically significant differences were seen between the two groups. The NO RIF group presented a significantly higher relative abundance of the genus *Ralstonia*. Overall, there was less diversity in the pattern of both vaginal and endometrial microbioma in the RIF group.

A relevant question when investigating the association between infertility and the microbiome is whether the uterus, where embryonic implantation takes place, has a specific microbiome or if it is an extension of the vaginal one. The widespread belief that the uterus was sterile was overturned only in 2016, when NGS studies began to shed light on its microbiome in non-pregnant women, showing that it is mainly constituted by a diverse community of *Lactobacillus* spp., specifically *L. iners* and *L. crispatus* (12). In this sense, we intended to assess the correlation between the vaginal and endometrial microbiome in order to simplify the study of the female reproductive tract. We found that the vaginal and the cervical microbiome are concordant with the endometrial one, although the relative proportions of the microorganisms may vary.

A recent pilot study retrospectively analyzed 65 samples collected from the reproductive tract of 31 women by sequencing the 16S rRNA gene (16 controls and 15 cases). The dominant members of the microbial community were constant in the vagina and the cervix, although the relative proportions varied. Also, a tendency was observed for infertile women to have *Ureaplasma* spp. in the vagina and *Gardnerella* spp. in the cervix more frequently than fertile women (13). In relation to the above-mentioned study, our analysis of alpha and beta indices for the microbial diversity of vaginal and endometrial samples revealed significant differences.

Similarly, the taxonomic characterization showed differences in the microbial profiles between the vaginal and endometrial samples in terms of genera and species. The univariate analysis carried out on the relative abundance of the different genera for sample type reached statistical significance for *Lactobacillus* spp., *Streptococcus* spp., *Ureaplasma* spp., *Delftia* spp., *Anaerobacillus* spp., *Ralstonia* spp., and the species *L. helveticus*. Taken together, our results suggest that vaginal and endometrial samples are different both in terms of microbial diversity and the composition of the taxa.

In terms of which sample type would be preferable for studying the microbial composition at a given point in treatment, our results suggest that vaginal samples can provide sufficient evidence to correlate the diversity and taxonomic composition of endometrial samples both with pregnancy rates and for patients with RIF (in fact, both are equally valid because of their association with pregnancy). Vaginal samples are also more convenient, less invasive, less risky in terms of complications like endometrial tearing, and easier to collect. In addition, processing the samples coming from the endometrium using

NGS technique presents greater difficulties in the analysis, especially in terms of DNA extraction and the quality of the sequences obtained, so a single endometrial sample could be insufficient. As both types of samples are similarly predictive of the result for IVF, as far as the pattern of the microbiome is concerned, the vaginal sample is preferable.

Another important aspect of the present study is the assessment of both diversity and taxonomic characterization according to participants' history of repeated implantation failures. Previously, was characterized the microbiota in endometrial fluid (EF) and vaginal secretions (VS) in 28 infertile women with a history of RIF and 18 infertile controls without, who underwent their first attempt at embryo transfer and IVF. The microbiota in the EF presented a higher alpha diversity and higher quantity of bacterial species than the microbiota in the VS in both the RIF and the control group. The analysis of the UniFrac distance matrices between EF and VS also revealed a significantly different grouping. Moreover, the microbiota detected in the EF showed significant variation in the composition of the bacterial community between the RIF group and the control group, which was not observed in the VS. *Burkholderia* spp. were not detected in the microbiota of the EF in any sample in the control group, but they were in a quarter of the RIF patients (14).

In our study, alpha diversity was higher at the endometrial level in the NO RIF patients. This did not occur in vaginal samples, where no differences were observed. In the above-mentioned study they did find differences in EF in both groups. When we analyzed the taxonomic characterization, we also observed clear differences in the relative abundances of the different genera and species in the different groups. For the RIF group at the vaginal level, the genus *Streptococcus* presented lower relative abundance, whereas *Prevotella*, *Ureaplasma* and *Dialister* were more prominent. In contrast, the genera *Streptococcus*, *Veionella* and *Aerococcus* showed a higher abundance in the NO RIF group. As for *Lactobacillus* spp., we did not observe differences in the relative abundance between groups.

At the endometrial level, we found a higher abundance of the genus *Prevotella* and the species *L. helveticus* and *S. amnii* in the RIF group. As for the patients in the NO RIF group, we observed a greater abundance of *L. iners*, *L. jensenii* and the genus *Ralstonia*. Unlike Kitaya et al. (14), we found no differences for the genus *Burkholderia*. The generalized linear model showed that the microbiome pattern in the NO RIF group changed between visits, while that of the RIF patients remained stable. This could be due to a possible adaptation of the microbiome pattern as a result of physiological changes occurring from visit 1 to visit 3. The possible causes and mechanisms involved in these modifications are still unknown and unstudied, but this could be a valuable line of future research.

In relation to the importance of the genus *Lactobacillus* and its relationship with gestation, Franasiak et al. used 16S RNA sequencing (15): after the transfer of euploid embryos, the most distal portion of the transfer catheter, of 5 mm in length, was placed in a DNA-free tube under sterile conditions. NGS sequencing was performed on 35 samples from 33 patients: 18 (54.5%) had ongoing pregnancies and 15 (45.5%) did not. *Lactobacillus* spp. were the main species for both groups. These data show that the microbiome at the time of embryo transfer can be successfully characterized without altering standard

clinical practice. This new approach is the first step towards the goal of determining the physiological microbiota at the time of embryo transfer and its impact on pregnancy outcomes (15). In contrast to this study, in our work, vaginal samples were taken with a dry swab and endometrial samples by Tao Brush, in order to discern the microbiome coming from both locations and avoid possible contamination. Like Franasiak et al., we also observed a greater relative abundance of *Lactobacillus* spp. in patients who achieved gestation.

Lactobacillus spp. are the most abundant bacteria in the vaginal samples. They inhibit the attachment of other bacteria to epithelial cells and produce lactic acid that kills or inhibits the growth of many other bacteria. Lactic acid blocks histone deacetylases, thus improving gene transcription and DNA repair. Moreover, it induces autophagy in epithelial cells in order to degrade intracellular microorganisms and promote homeostasis. Lactobacilli are well tolerated by vaginal epithelial cells and inhibit the induction of pro-inflammatory cytokines. The ability of lactobacilli to inhibit infection without inducing inflammation can maximize fertility and favour pregnancy outcomes (16). The presence of a non-*Lactobacillus*-dominated microbiome (NLDM) is associated with a lower rate of implantation, pregnancy rate, pregnancy progression and live birth (17). Moreno et al. reported that a relative abundance of lactobacilli of less than 90% in endometrial fluid was predictive of adverse pregnancy outcomes. In this study, patients classified as NLDM and showing a relative abundance of more than 80% *Lactobacillus* spp. in the endometrium showed good pregnancy outcomes, suggesting that this threshold could be considered sufficient for embryo implantation (17). In addition, even if classified as NLDM, the endometrium with a dominant quantity of Bifidobacteria could also be an acceptable environment for implantation (18).

Another pilot study aimed to analyze both the endometrial and vaginal microbiome in the infertile Japanese population via sequencing and to assess the impact of the endometrial and vaginal environment on embryo implantation. In total, 102 infertile patients (79 IVF and 23 non-IVF patients) and 7 healthy volunteers were recruited. Endometrial and vaginal discharge samples were collected for sequencing using an intrauterine insemination catheter. The bacterial status of the endometrium and vagina was analyzed, showing *Lactobacillus*-dominated microbiota (> 90% *Lactobacillus* spp.) in the endometrium of 38% of IVF patients, and in the vagina of 44.3%. In non-IVF patients, these figures were 73.9% and 73.9%, respectively, and in healthy volunteers, 85.7% and 85.7%. The percentage of endometrial *Lactobacillus* spp. in healthy volunteers was highly stable within the same menstrual cycle and even in the next cycle. The main taxonomies were *Gardnerella* spp., *Streptococcus* spp., *Atopobium* spp., *Bifidobacterium* spp., *Sneathia* spp., *Prevotella* spp. and *Staphylococcus* spp. Fifteen patients achieved pregnancy in a single transfer of devitrified blastocysts during this study (18.9%). The mean percentage of *Lactobacillus* spp. in pregnant women was $96.45 \pm 33.61\%$. A considerably high percentage of NLDM was found in the endometrium of infertile Japanese women. Increasing the endometrial level of lactobacilli to more than 90% could favor the outcome of implantation of infertile patients with NLDM (18).

A subsequent pilot study from the same group aimed to analyze pregnancy outcomes in IVF patients who had either *Lactobacillus*-dominated microbiota (LDM) (> 90% *Lactobacillus* spp) or NLDM (< 90% *Lactobacillus* spp.) in their endometrium. They also aimed to report cases that were treated for NLDM simultaneously with antibiotics and prebiotic/probiotic supplements in an infertile Japanese population. Ninety-two patients undergoing IVF were recruited, and endometrial fluid samples were collected for sequencing using an intrauterine insemination (IUI) catheter. The bacterial composition of the endometrium and pregnancy outcomes were analyzed. For cases with NLDM, antibiotics and prebiotics/probiotics were administered according to their individual microbial conditions. Forty-seven cases (51.1%) presented LDM, and 45 cases (48.9%) NLDM, in the initial analysis. Nine patients with NLDM were treated with antibiotics and prebiotics/probiotics and successfully converted to LDM. The results of this study did not demonstrate a clear benefit for establishing a *Lactobacillus*-dominant endometrium in terms of pregnancy outcomes, but knowledge of the endometrial microbial status of infertile patients is important since recovery of the *Lactobacillus*-dominant endometrium could benefit implantation (18). In this preliminary study, the predominance of *Lactobacillus* was favorable in terms of the pregnancy rate; however, the results were not as significant as in the previous pilot study (17); the reasons for this may be due to the limited number of cases, short follow-up period, or ethnic differences. In another study also concluded that women with an abnormal vaginal microbiota are approximately 1.4 times less likely to become pregnant after in vitro fertilization treatment, compared to women with a normal microbiota pattern (19).

With reference to the taxonomic characterization in our study, we found that the relative abundance of bacteria of the genus *Lactobacillus* spp. is higher in patients who achieve pregnancy after ART. Likewise, the greater presence of this genus was detected in the first and third visit (secretory phase of the previous cycle and day of the negative HCG test). Since no statistically significant differences were obtained when analyzing the differences in relative abundance between samples taken in the three consecutive visits during the treatment, we could opt for any of the three options (secretory phase of the previous cycle, proliferative phase of the cycle of the cryotransfer or the day of the embryo transfer). We concluded that they are comparable in terms of the results of the microbiome pattern, and their extrapolation is justified. Samples can thus be taken when considered most appropriate during the fertility treatment.

Conclusions

When analyzing the pattern of the vaginal and endometrial microbiome, we observed differences between the two types of samples. Our analysis of the vaginal samples taken at different visits, in contrast, indicated no differences in the microbiome pattern according to assessment timepoint. A greater relative abundance of *Lactobacillus* spp. and *L. reuteri* correlated with a higher pregnancy rate.

When comparing changes in the microbiome pattern between the RIF and NO RIF group, we found a lack of adaptability and variation in the RIF group compared to the NO RIF group. In the RIF group, we found lower alpha diversity at the endometrial level along with a lower relative abundance of *Streptococcus* spp., *Aerococcus* spp. and *Ralstonia* spp. This group presented a higher abundance of *Prevotella* spp.,

Ureaplasma spp., and *Dialister* spp. Further studies are needed to confirm our findings and to clarify the role of antibiotic and/or probiotic treatment in the normalization of the microbiome pattern and its consequences on clinical outcomes.

Materials And Methods

Design And Study Population

We designed a longitudinal, descriptive, observational cohort study. People presenting to the Instituto Bernabeu fertility clinic (Alicante, Spain) for frozen embryo transfer (FET) of euploid embryos from May 2017 to May 2019 were eligible. Inclusion criteria were: aged 18 to 50 years, using either their own or donated oocytes, and indication to use the intracytoplasmic sperm injection method to generate embryos. Participants transferred one euploid embryo that had undergone preimplantation genetic testing for aneuploidies and was frozen in the blastocyst stage (Veriseq, Illumina). Exclusion criteria were: use of antibiotics in the three months preceding the fertility treatment, unwillingness to sign informed consent, uterine malformations, untreated hydrosalpinx or known implantation failure factors.

This study is considered a pilot study. Given the sample size (48 patients and 264 samples), a pregnancy rate of 50% can be estimated with an accuracy of 15% and a confidence level of 95%. In our work, the diversity is estimated by grouping the sequences in operational taxonomic units (OTUs), with a percentage of similarity of 97%.

Sample Collection

Three samples were collected in the different stages of *in vitro* fertilization (IVF) treatment: (1) the secretory phase of in the cycle preceding the treatment (vaginal and endometrial samples); (2) the embryo transfer day (vaginal sample); and (3) the pregnancy test day (vaginal sample).

Vaginal samples

We used a dry swab to collect the vaginal discharge from the bottom of the posterior sac by means of direct visualization with vaginal speculum and in the lithotomy position. In order to avoid contamination, we did not use lubricant or gel on the speculum. The second sample was always collected before preparing the embryo transfer, ensuring sure that no intervention could interfere. All samples were stored at -80°C until further analysis.

Endometrial samples

Endometrial samples were collected using the Tao Brush IUMC Endometrial Sampler in secretory phase (day 18–22 of the cycle) in the cycle preceding the frozen embryo transfer. This device minimizes the risk of contamination during the collection of the endometrial sample through the sheath that closes prior withdrawing from the uterus. All samples were stored at -80°C until further analysis.

Sample Analysis

We used metagenomics for sample analysis, studying the 16S rRNA gene marker of the included samples with next generation sequencing (NGS). Analyses took place in the molecular genetics laboratory of IB Biotech, Instituto Bernabeu, Alicante, Spain.

DNA extraction

DNA extraction was performed using the PureLink microbiome DNA purification kit (ThermoFisher, PureLinkTMMicrobiome DNA Purification Kit, Darmstadt, Germany and/or its affiliates). The DNA was quantified using fluorometry with Qubit 2.0 (ThermoFisher). The extracted DNA was stored at -20°C for later use.

Amplification of region V3V4 of 16S rRNA gene

PCR amplification of the variable region V3V4 of the 16S rRNA gene was performed with Taq DNA polymerase ($2 \times$ KAPA HiFi HotStart, Roche) in the presence of dNTPs, oligonucleotides 357F and 806R at a final concentration of $1 \mu\text{M}$ and an average of 100 ng of DNA, and at a final reaction volume of $25 \mu\text{L}$, following the recommendations of Illumina (16S Metagenomic Sequencing Library Preparation). PCR was carried out in the thermal cycler (Verity, Applied Biosystems). For the validation of the PCR technique, all amplification reactions included positive and negative controls without DNA template. The PCR products were visualized using agarose electrophoresis, verifying that the amplified DNA band was the correct size (449 base pairs). All products of amplification were stored at -20°C for subsequent sequencing.

Sequencing of region V3V4 of 16S rRNA gene

Once the V3V4 amplicon was obtained and purified, we generated the library with the identifying indexes of each sample using the Nextera XT sequencing kit (Illumina). After the purification of the libraries, the samples were quantified using Qubit 2.0 (ThermoFisher), which were previously diluted to a concentration of 4 nM before being mixed and prepared for sequencing. The final concentration of the library was 15 pM . The library was sequenced using Miseq Reagent kit v3 (Illumina) reagents. We used Miseq (Illumina) as the sequencing equipment and metagenomics for the workflow.

Bioinformatic Analysis Of The Sequences

Once the sequencing was finished, the primary analysis of the obtained sequences consisted of demultiplexing, using MiSeqReporter software (Illumina). The unindexed paired-end sequences of each sample were exported from MiSeq for their analysis in fastq format.

The bioinformatic analysis of the sequences was carried out using the QIIME2 package. In addition, for further data analysis we worked with the MicrobiomeAnalyst program. Deblur was used to filter and

denoise the sequences with QIIME2. The sequences were grouped in OTUs with a similarity percentage of 97%.

In order to estimate alpha diversity, a rarefaction analysis was performed at 1000 sequences per sample, followed by an alpha diversity analysis. Different indexes were used: Shannon, Simpson and Faith. Since these indices did not follow a normal distribution, the non-parametric Mann-Whitney U method was used.

The results for beta diversity were visualized with QIIME2 using the graphics generated by principle coordinate analysis (PCoA), obtained with EMPeror. We carried out the analysis of beta diversity using the unweighted UniFrac index. UniFrac is a measure of beta diversity that uses phylogenetic information to compare samples belonging to the interest groups, in this case four. The unweighted version is qualitative. Therefore, UniFrac measures concordance based on the abundance of OTUs in each sample, including also phylogenetic distances. The matrices with beta diversity measurements were analysed for differences in composition according the group they belong to (type of sample) by PERMANOVA.

The taxonomic assignment was made using a classification based on a filtering of the 99_otus sequence from the Greengenes database to the V3V4 region. Finally, we performed the univariate analysis for each specified taxon or group according to the results we obtained using the correction for multiple testing. For quantitative variables, we used the parametric student's T test or the non-parametric Mann-Whitney U test, as appropriate.

A descriptive analysis of all variables was performed by calculating frequencies for the qualitative parameters and minimum, maximum, mean as well as standard deviation (SD) for quantitative variables. Microbiome patterns were analyzed by estimating the prevalence and variability of types of bacteria at both vaginal and endometrial levels.

Microbiome patterns were compared between groups according to implantation and gestation outcomes as well as vaginal and endometrial levels, using double-entry tables for qualitative variables and the Chi-squared test. For quantitative variables, the student's T test was used to assess the association between microbiome patterns and gestation outcome. The analyses was performed using R v.3.5.1.

Declarations

- Ethics approval and consent to participate:

The Clinical Research Ethics Committee (CEIC) of the University Hospital San Juan de Alicante evaluated the study protocol on 29 November 2016, and its secretary, Domingo Orozco Beltrán, signed the approval on 13 January 2017 (Committee code: 16/318).

- Consent for publication:

All included patients signed informed consent prior to participating in the study.

- Availability of data and material:

The datasets generated and/or analysed during the current study are not publicly available because they contain patients' personal data and the results of their microbioma pattern analysis in addition to sociodemographical parameters, but they are available from the corresponding author on reasonable request.

- Competing interests:

The authors declare they have no competing interests.

- Funding:

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- Authors' contributions:

Conceptualization: MCDM*, ABG*, BLLB, MCCM, JAQR; Design: MCDM*, ABG*, BLLB, MCCM, JAQR; Validation: MCCM, JAQR; Data analysis: BLLB, JAQR; Research: MCDM*, ABG*, BLLB; Data management: BLLB, JAQR; Drafting: MCDM*, ABG*,; Revision and editing: MCDM*, ABG*, BLLB, MCCM, JAQR; Supervision: MCDM*, ABG*, BLLB, MCCM, JAQR. All authors read and approved the final manuscript.

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Figures

FIGURE 1

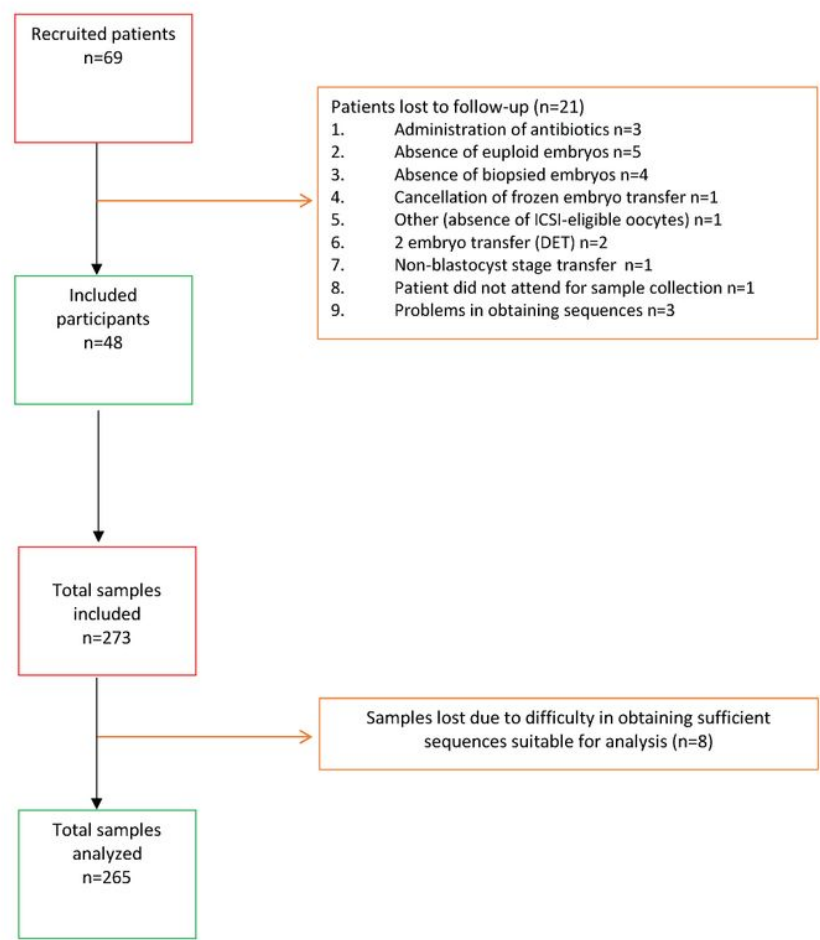


Figure 1

Description of the patients and total samples included in the study

FIGURE 2

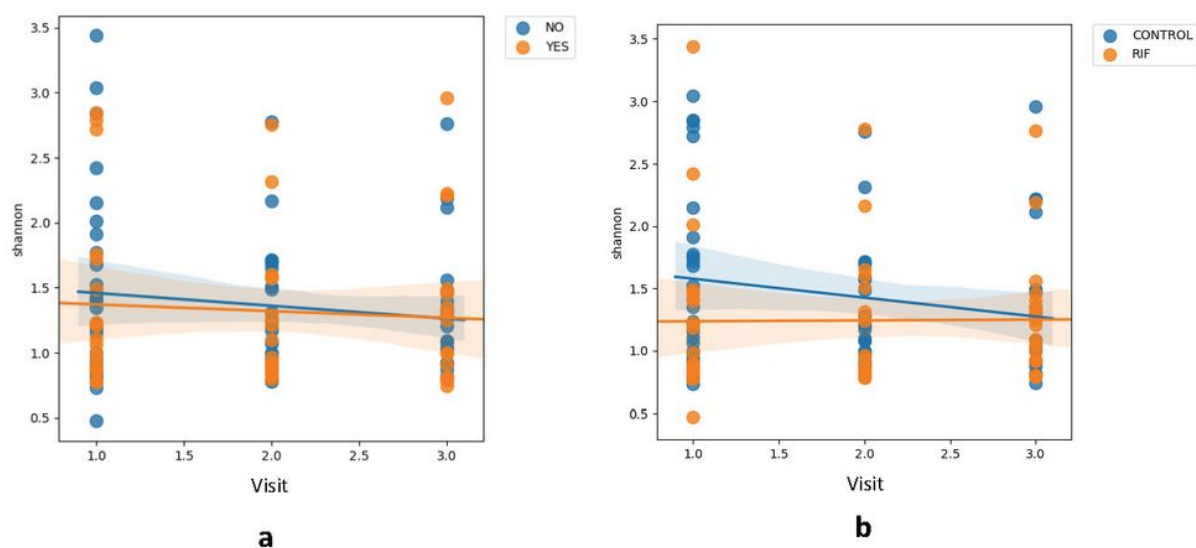


Figure 2

Linear mixed effects models test (a) for the gestation rate (b) for the study group

FIGURE 3

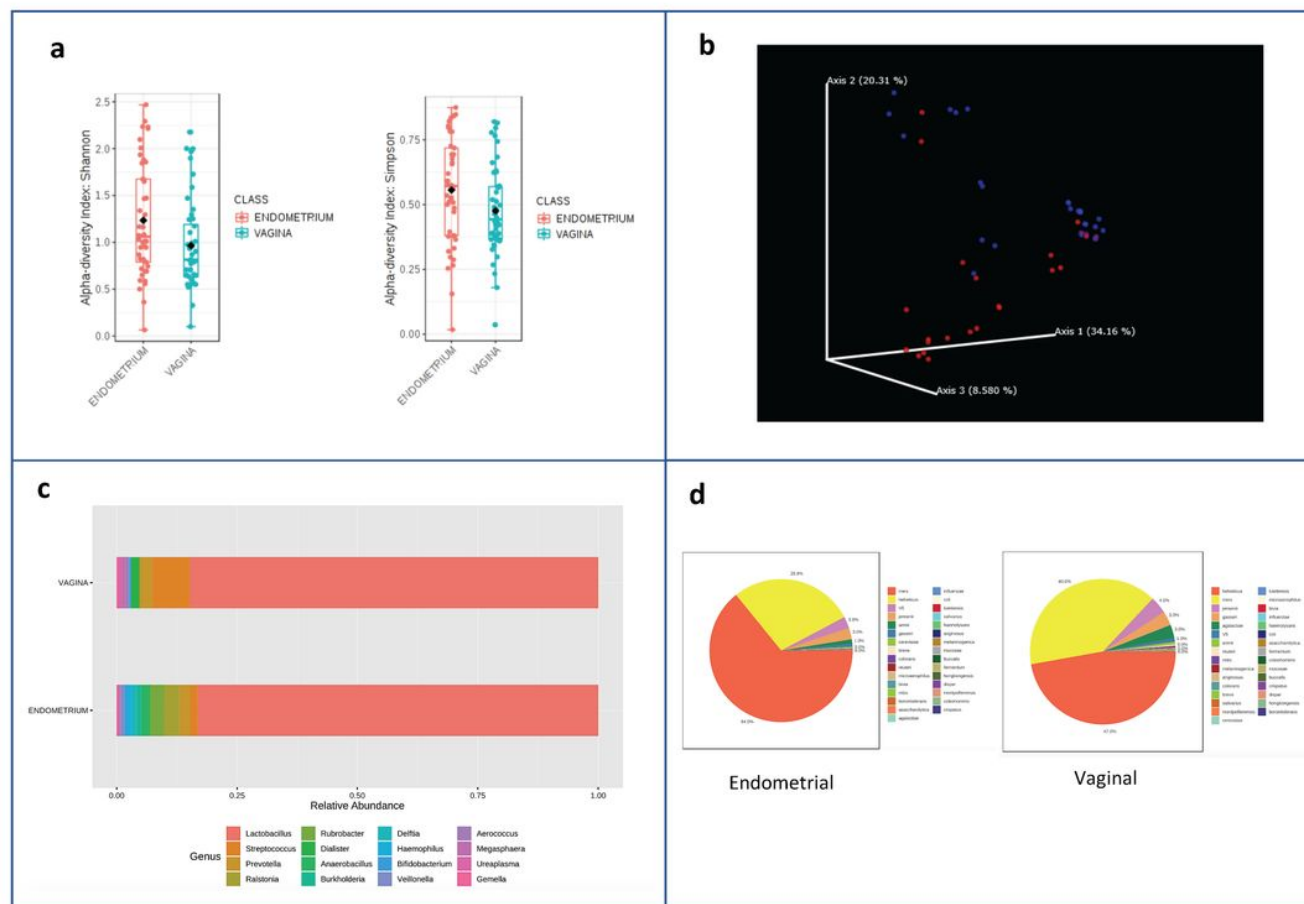


Figure 3

Vaginal and endometrial microbial patterns at visit 1 Legend: (a) Mann-Whitney U Test. Comparative analysis for the Shannon diversity index for the sample type in relation to the alpha diversity study ($p = 0.013859$) and comparative analysis for the Simpson diversity index and for the sample type in relation to the alpha diversity study ($p = 0.046019$) MicrobiomeAnalyst MDP (b) PCoA, constructed from the UniFrac distance matrix, which represents the study samples with different colors depending on the type of sample: vaginal (red) or endometrial (blue). QIIME2 (c) Bar chart of the relative frequency of the most abundant genders grouped by sample type (vaginal/endometrial) MicrobiomeAnalyst MDP (d) Pie chart showing the relative frequency of the most abundant species of the genus *Lactobacillus* spp. grouped by sample type (vaginal/endometrial).

FIGURE 4

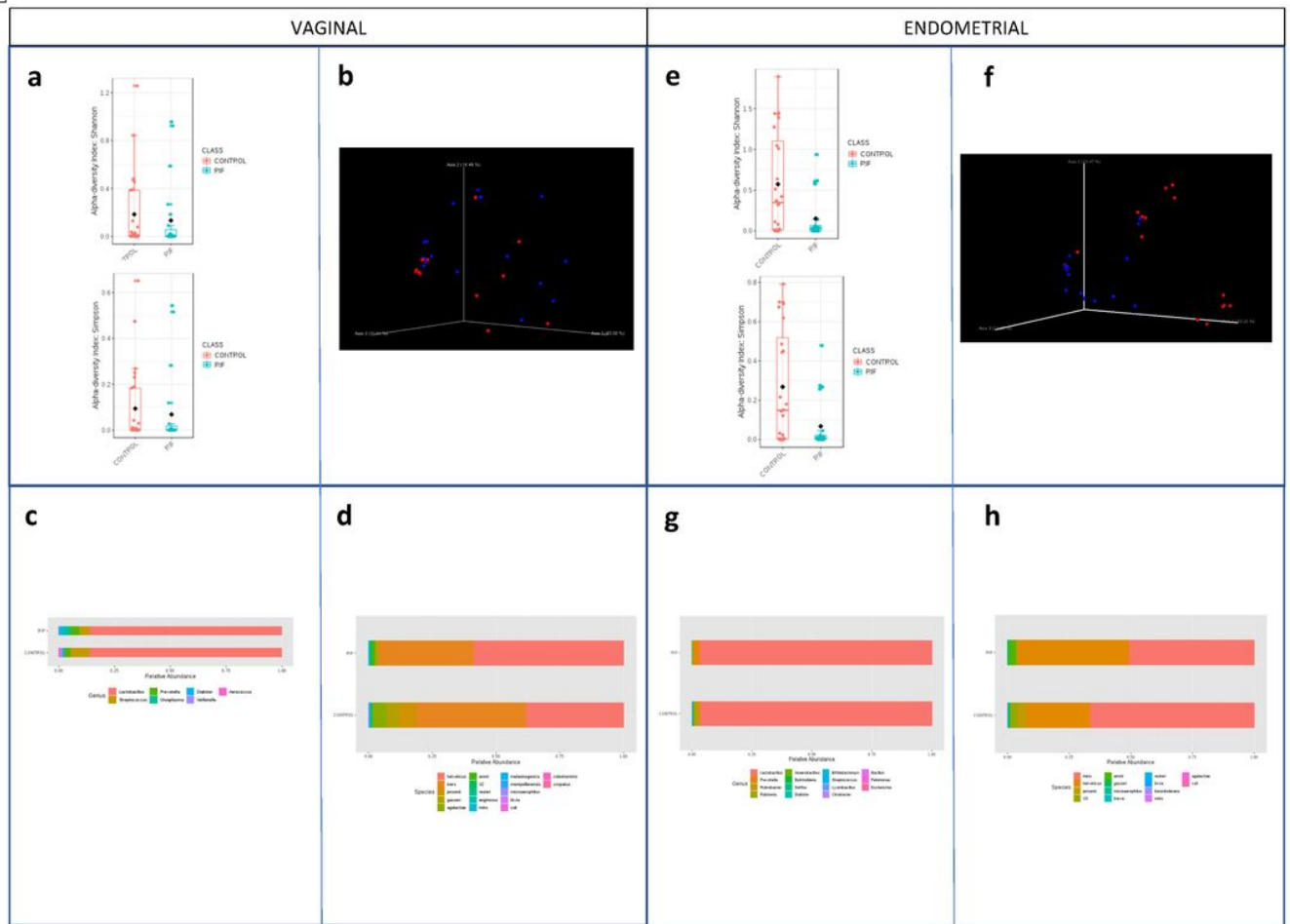


Figure 4

Microbiome patterns by diagnosis of implantation failure Legend: (a) Comparative analysis of the Shannon diversity index ($p = 0.28512$) and the Simpson diversity index ($p = 0.27578$) for the RIF and NO RIF groups in relation to the study of alpha diversity. MicrobiomeAnalyst MDP. (b) PCoA, based on the Unifrac distance matrix which represents the study samples with different colors depending on the RIF and NO RIF group: vaginal (red) or endometrial (blue). QIIME2 (c) Bar chart of the relative frequency of the most abundant genera grouped by study group in the vaginal samples. MicrobiomeAnalyst MDP (d) Bar chart of the relative frequency of the most abundant species grouped by study group in the vaginal samples. MicrobiomeAnalyst MDP (e) Univariate analysis represented with box plot showing the differences in the alpha diversity index for the study group in the endometrial samples. Shannon diversity index analysis, $p = 0.020647$ and Simpson, $p = 0.020647$; Mann-Whitney U. (f) PCoA, which represents the study group (RIF/NO RIF) with different colors: NO RIF (red) or RIF (blue). QIIME2 (g) Bar chart of the relative frequency of the most abundant genera grouped by study group MicrobiomeAnalyst MDP (h) Bar chart of the relative frequency of the most abundant species grouped by study group. MicrobiomeAnalyst MDP (L. iners, L. helveticus, L. jensenii, L. gasseri, S. amnii, V3 represents unidentified species)

FIGURE 5

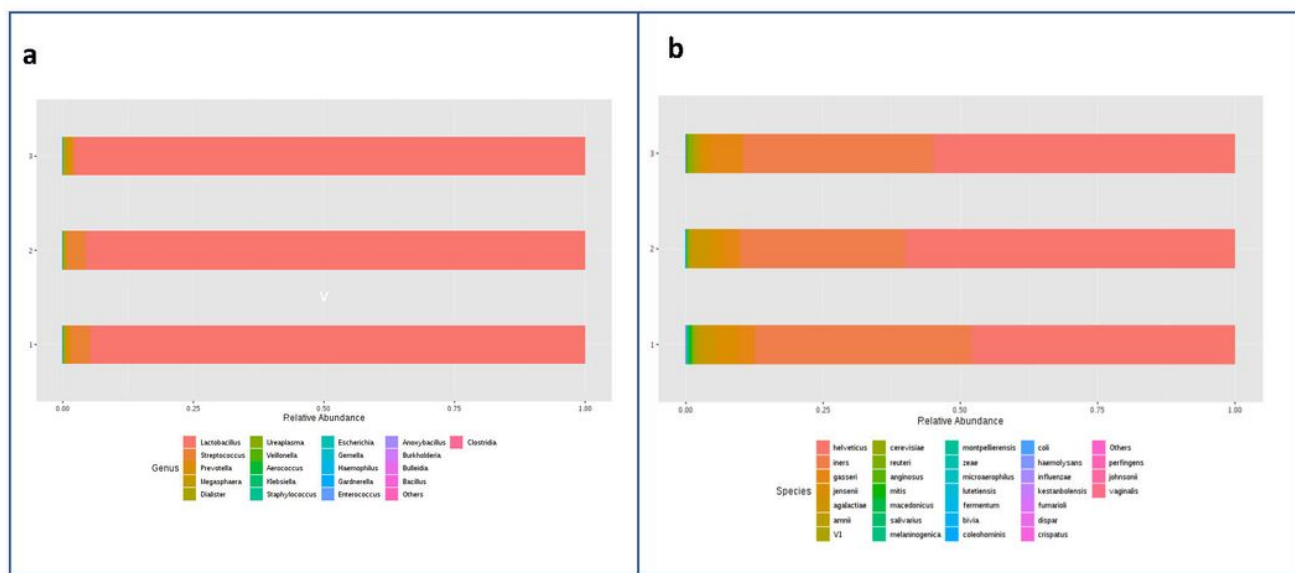


Figure 5

Bar chart of the relative frequency of the most abundant genera (a) and species (b) grouped by type of visit. MicrobiomeAnalyst MDP.

FIGURE 6

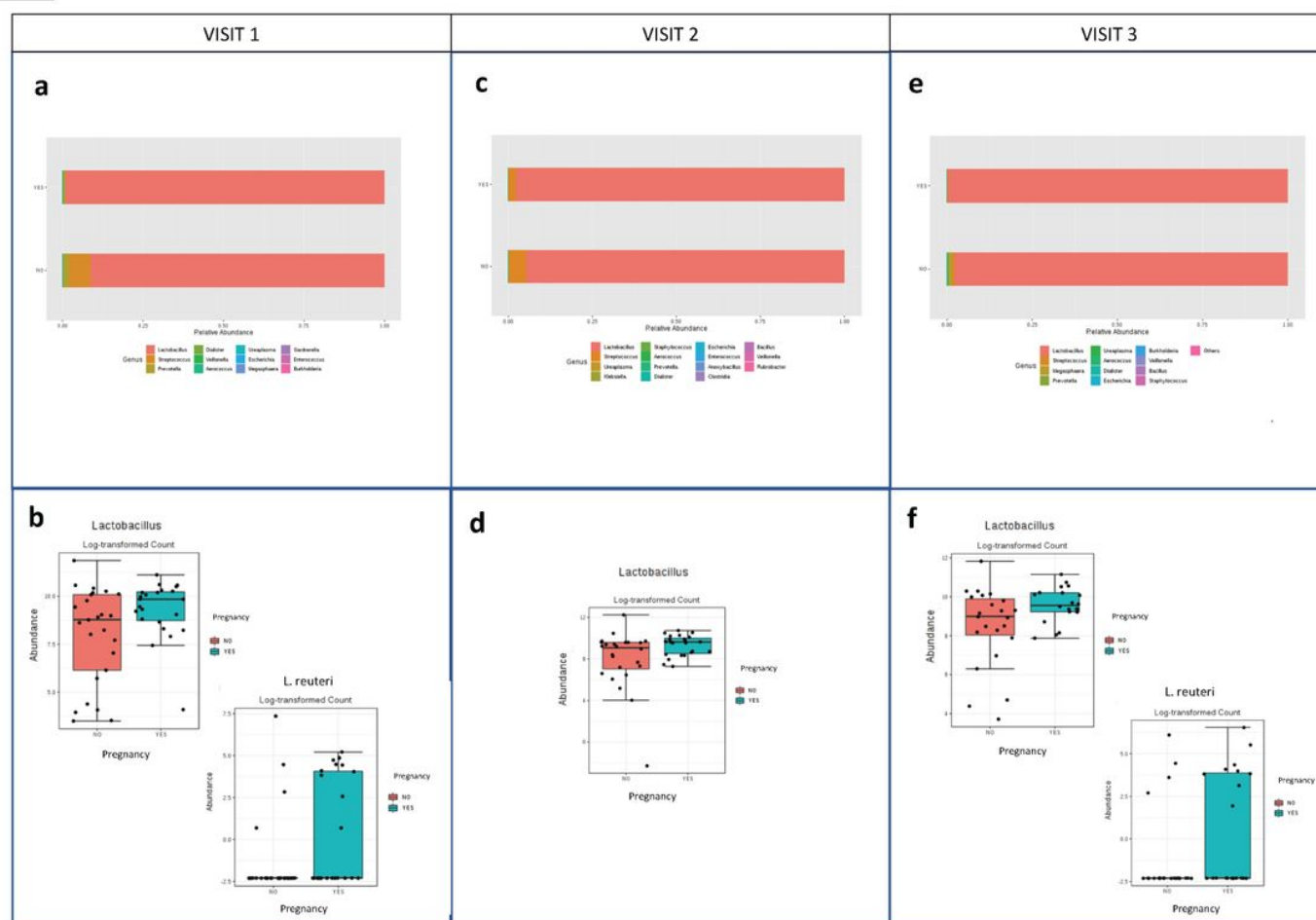


Figure 6

Association of the vaginal sample taken at different visits with the gestation rate Legend: (a) Bar chart of the relative frequency of the most abundant genera grouped by gestation rate for visit 1. MicrobiomeAnalyst MDP (b) Univariate analysis represented with box plot showing relative abundance of *Lactobacillus* spp. (0.0445), and *L. reuteri* ($p = 0.0397$) for the gestation rate for visit 1 (c) Bar chart of the relative frequency of the most abundant genera grouped by gestation rate for visit 2. MicrobiomeAnalyst MDP (d) Univariate analysis represented with box plot showing relative abundance of the genus *Lactobacillus* spp. (0.0268), for the gestation rate for visit 2 (e) Bar chart of the relative frequency of the most abundant genera grouped by gestation rate for visit 3. MicrobiomeAnalyst MD (f) Univariate analysis represented with box plot showing relative abundance of *Lactobacillus* spp. ($p = 0.0492$), and *L. reuteri* ($p = 0.0591$) for the gestation rate for visit 3.

Supplementary Files

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