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Original
Subclinical alteration of the cervical–vaginal microbiome in women with idiopathic infertility / Campisciano, G., Florian, F., D'Eustacchio, A., Stankovic, D., Ricci, G., De Seta, F., Comar, M.. - In: JOURNAL OF CELLULAR PHYSIOLOGY. - ISSN 1097-4652. - STAMPA. - 232:7(2017), pp. 1681-1688. [10.1002/jcp.25806]

Availability:
This version is available at: 11368/2896094 since: 2017-03-20T09:30:01Z
Publisher:

Published
DOI:10.1002/jcp.25806
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13 November 2026

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Archivio della ricerca – postprint

Subclinical alteration of the cervical–vaginal microbiome in women with idiopathic infertility

Accepted: 17 January 2017

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Funding information
IRCCS Burlo Garofolo, Grant number: RC 26/13

Biomarkers have a wide application in research and clinic, they help to choose the correct treatment for diseases. Recent studies, addressing the vaginal microbiome using next generation sequencing (NGS), reported the involvement of bacterial species in infertility. We compared the vaginal microbiome of idiopathic infertile women with that of healthy, including bacterial vaginosis affected women and non-idiopathic infertile women, to identify bacterial species suitable as biomarkers. Information on microorganisms was obtained from the V3-16S rDNA sequencing of cervical–vaginal fluids of 96 women using the Ion Torrent platform. Data were processed with QIIME and classified against the Vaginal 16S rDNA Reference Database. The analysis revealed a significant beta-diversity variation ($p < 0.001$) between the four groups included in the study. *L. iners*, *L. crispatus*, and *L. gasseri* distinguished idiopathic infertile women from the other groups. In these women, a microbial profile similar to that observed in bacterial vaginosis women has been detected. Our results suggest that the quantitative assessment and identification of specific microorganisms of the cervical–vaginal microflora could increase the accuracy of available tools for the diagnosis of infertility and improve the adoption of therapeutic protocols.

KEYWORDS
anaerobes, idiopathic infertility, ion torrent, microbiome, 16S sequencing

1 | INTRODUCTION

The complex interaction between the vaginal microbiome and host physiology plays a pivotal biological role in women (Peterson et al., 2009); its perturbation influences all the phases of a woman's reproductive life, including infertility (Walther-Antonio et al., 2014).

Female infertility is a complex health issue caused by single or multiple factors that impair the reproductive function. Infertility, according to guidelines from the National Institute for Health and Clinical Excellence, 2004, is described as 2-year-long timespan of unwanted non-conception with unprotected intercourse in the fertile phase of the menstrual cycle (Evers, 2002).

A range of 2–10.5% of women in the reproductive age (20–44 years old) is affected by primary and secondary infertility (Mascarenhas, Flaxman, Boerma, Vanderpoel, & Stevens, 2012), which may depend on a combination of congenital and hormonal disorders, lifestyle, and environmental risks.

Although there are many effective therapies to treat infertility, 15–30% of couples do not benefit from them. The couples that do not benefit from infertility treatment, after the assessment of tubal patency, normal ovulatory, and sperm parameters, have a "diagnosis" of idiopathic or unexplained infertility (Ray, Shah, Gudi, & Homburg, 2012). An efficient treatment for infertility depends on the identification of a vast range of biomarkers, in order to select the best existing therapy among the many possible options; thus, a correct identification of the ongoing alterations is fundamental for selecting a suitable therapy.

Recent studies associate opportunistic pathogens in the lower female reproductive tract with adverse pregnancy outcomes after both natural and in vitro fertilization (IVF) conceptions (Fox and Eichelberger, 2015; Franasiak et al., 2016; Sirota, Zarek, & Segars, 2014). Opportunistic pathogens frequently and transiently colonize or infect the female upper genital tract without symptomatic inflammatory disorders (Cottell et al., 1996; Viniker, 1999).

Some sexually transmitted viruses have a possible impact on reproductive processes (Kapranos, Petrakou, Anastasiadou, & Kotronias, 2003; Souho, Benlemlih, & Bennani, 2015), while it is widely accepted that bacterial infections with *Neisseria gonorrhoeae*, *Treponema pallidum*, *Chlamydia trachomatis*, and *Mycoplasma* can alter fertility (Gibbs, Romero, Hillier, Eschenbach, & Sweet, 1992; Jamalizadeh Bahaabadi et al., 2014; Malik, Jain, Hakim, Shukla, & Rizvi, 2006).

Recent findings investigated the role of Bacterial Vaginosis (BV) causing species in the subclinical alteration of the vaginal microbiome and their relationship with women's infertility (van Oostrum et al., 2013). BV is a polymicrobial disorder characterized by a local pro-inflammatory environment, a decrease of protective *Lactobacilli*, and the overgrowth of anaerobic species (Brotman, 2011), which produce polyamines and other compounds. The alteration of vaginal environment may facilitate the ascending dissemination of the infecting species that could finally lead to tubal factor infertility (Mastromarino et al., 2014). In addition, the presence of BV during pregnancy is responsible for certain clinical outcomes, including late abortion (Donati et al., 2010), premature rupture of the amniotic membrane (Fredricks, Fiedler, & Marrazzo, 2005; McDonald, Brocklehurst, & Gordon, 2007), chorio-amnionitis (Fahey, 2008), and post-partum endometritis (Hillier et al., 1996; Pellati et al., 2008; Sweet, 1995).

Recent studies, addressing the vaginal microbiome composition using the next generation sequencing (NGS) techniques, reported the broad involvement of bacterial species in infertility. The NGS analysis identified a new bacterium of the *Coriobacteriaceae* family, the *Atopobium vaginae*, as a key factor contributing to the failure of IVF and embryo transfer procedures in women with asymptomatic anaerobic infections (Fredricks et al., 2005; Livengood, 2009). The same sequencing approach established that previously unappreciated species of *Lactobacilli*, belonging to the normal vaginal microflora, promote a supportive and protective local environment for pregnancy (Tarnberg, Jakobsson, Jonasson, & Forsum, 2002).

The aim of the present study was to characterize the cervical–vaginal microbiota of women diagnosed with idiopathic infertility. The resulting microbial composition was compared with that of demographically matched women including fertile, non-idiopathic infertile and BV affected subjects. Information on the vaginal microbiome was obtained from the analysis of cervical–vaginal fluid using the V3-16S rDNA Ion Torrent NGS technique.

2 | MATERIALS AND METHODS

2.1 | Subjects and specimen collection and ethical approval

Cervical–vaginal samples, which were collected from January 2015 to June 2015 from 96 women that fulfilled the inclusion eligibility criteria of the study, were investigated for vaginal microbiome composition.

The studied population comprised 27 infertile women attending the Assisted Reproductive Technology (ART) clinic and 69 fertile women attending the Gynecology clinic of the Mother and Child Health Hospital —IRCCS Burlo Garofolo, Trieste, Italy for regular check-up (n = 30) or due to vaginal complaints (discharge, itching, burning, dysuria) (n = 39). All the women were of Caucasian origin, non-pregnant, of reproductive age (range, 32–40 years), had no current use of tobacco, alcohol, and contraceptive methods, and had no hospitalization or use of systemic medication for chronic diseases or antibiotics/probiotics (oral or topic) within the 6 months previous to sample collection.

Biological samples were collected 5–7 days before the menstrual period and before programmed in vitro fertilization practice. After a centrifugation step (5000g, 20 minutes), aliquots of 500 µl were immediately prepared and stored at −80°C.

The study's protocol was approved by the Ethics Committee of the IRCCS Burlo Garofolo Institute, Trieste (RC 26/13). All of the participants provided a written consent and gave permission to access medical records in order to obtain their reproductive history and IVF outcome.

2.2 | Sample processing

DNA extraction from the aliquots of 500 µl was carried out using the NucliSENS® easyMAG® system (BioMèrieux, Gorman, NC), optimizing the working volume for NGS to a final elution of 50 µl. All DNA samples were stored at −20°C prior to Ion Torrent sequencing.

A real time quantitative EvaGreen® dye (Fisher Molecular Biology, Waltham, MA) PCR was performed with the degenerated primer 27FYM (5′-AGR GTT YGA TYM TGG CTC AG-3′) to construct richer libraries and with the U534R primer (primers target the V1–V3 region, spanning 500 bp). A nested PCR was performed with the primers B338F_P1-adaptor (B338F 5′-ACTCCTACGGGAGGCAGC-3′) and U534R_A_barcode (U534R 5′-ATTACCGCGGCTGCTGG-3′) to prepare a 200 base template for final V3 region sequencing, in association with the Ion Xpress Barcode Adapter (Sundquist et al., 2007). Negative controls, including no template and no bacterial DNA, were processed with clinical samples. The PCR reactions were performed using the Kapa 2G HiFi Hotstart ready mix 2X (Kapa Biosystems, MA) and BSA 400 ng/µl, under the following conditions: 5 minutes at 95°C, 30 seconds at 95°C, 30 seconds at 59°/57°C, 45 seconds at 72°C, and a final elongation step at 72°C for 10 minutes.

The size of the amplicon (260 bp) was checked on a 5% acrylamide gel. The subsequent step of DNA normalization of the purified PCR products, carried out using the MagBind Normalizer 96 Kit (OMEGA Bio-Tek, GA) according to the manufacturer's instructions, yielded a predicted DNA concentration of 4 ng/µl in a final elution volume of 40 µl. The amount of DNA after normalization was quantified with a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA).

2.3 | Ion Iorrent sequencing

The pooled library was diluted to a concentration of 26 pM. Template preparation was performed using the Ion PGM Template OT2 200 kit on Ion OneTouch™ 2 System (Thermo Fisher Scientific, Waltham, MA) and the subsequent quality control was carried out on Qubit® 2.0 Fluorometer. The templates were sequenced on the Ion PGM™ System machine, using the Ion PGM sequencing 200 KIT V2 (Thermo Fisher Scientific).

2.4 | Data analyses

QIIME 1.8.01 (Caporaso et al., 2010) was used to process the sequence data. High quality (Q>25) sequences were demultiplexed and filtered by quality using split_libraries_fastq.py with default parameters, except for the length parameter (150 bp). Operational taxonomic units (OTUs) were defined at 97% similarity and clustered against the Vaginal 16S rDNA Reference Database constructed by Fettweis et al. (2012) using open-reference OTU picking (Rideout et al., 2014) with a uclust clustering tool (Edgar, 2010). Before further analysis, singleton OTUs and samples with low sequencing depth were removed (less than 10,000 reads). Rarefaction analysis on separate and pooled samples (according to cohorts) was done for the Chao1 index (Chao, 1984). Equitability and Simpson reciprocal index were used to assess alpha diversity (within-sample diversity), while beta diversity (between sample diversity comparison) was assessed with weighted and unweighted UNIFRAC distance matrices (Lozupone and Knight, 2005; Lozupone, Hamady, & Knight, 2006) and presented with scatter plots matrices. Robustness of the identified clusters was investigated using jackknifing (randomly resampling sequences without replacement). Differences in community composition between cohorts were investigated using analysis of similarity (ANOSIM) and similarity percentage (SIMPER) analysis. QIIME was also used for ANOSIM using UNIFRAC distance matrices and OTU biom tables as inputs and for the Kruskal–Wallis test using OTU biom tables as inputs, while the vegan package (Jari Oksanen et al., 2016) for R software (Team, 2009) was used for the SIMPER analysis using taxonomy biom tables as inputs.

The dataset was deposited in NIH Short Read Archive (SRA: SRP073429).

2.5 | Diagnosis of BV

The diagnostic routine protocol of BV was based on laboratory Gram staining of vaginal secretions, Nugent's criteria and microorganisms isolation. The Nugent score is calculated by assessing for the presence of large gram-positive rods (*Lactobacilli* morphotypes; decrease in *Lactobacilli* scored as 0–4), small gram-variable rods (*G. vaginalis* morphotypes; scored as 0–4), and curved gram-variable rods (*Mobiluncus* spp. morphotypes; scored as 0–2) and can range from 0 to 10. Culture and identification of clinical isolates were performed on agar plates, which included Horse blood agar plates as a non-selective growth medium, MacConkey agar plates for Gram negative bacteria, and Mannitol Salt agar plates for discerning coagulase positive or negative aerobically or anaerobically incubated *Staphylococcus*.

3 | RESULTS

3.1 | Participants

Table 1 shows the demographics of study participants. The study design identified four groups including women with idiopathic infertility (Idiopathic, *n* 14), women with a diagnosed infertility (Infertile, n 13), fertile women with BV (Vaginosis, *n* 39) and fertile healthy women (Control, *n* 30).

TABLE 1 Demographics of groups

	Infertility type		Control type	
	Diagnosed	Idiopathic	Healthy	Vaginosis
Number	13	14	30	39
Median age (range)	38 (36–40 y)	38 (36–40 y)	34 (32–36 y)	35 (33–37 y)
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian
Duration of infertility (>5 years)	6	9	–	–
Infertility factors				
Tubaric	5	–	–	–
Ovulatory	2	–	–	–
Endometriosis	6	–	–	–
IVF (1–4 cycles)	3	3	–	–
Embryo			–	–
Transfer (mean number/range)	1.4/0–3	1.5/0–3	–	–
Quality (I grade)	36.8%	42.8%	–	–
Pregnancy outcome				
Negative	8	10	–	–
Biochemical	4	4	–	–
Positive	1	–	30	39
Sexually transmitted infections	Neg	Neg	Neg	Neg
Nugent score	0–3	0–3	0–3	7–10
Menstrual cycle length (days)	28–35	28–35	28–35	28–35

Out of 27 infertile women, 14 were "diagnosed" with idiopathic infertility. The women affected by idiopathic infertility showed tubal patency by hysterosalpingogram or laparoscopy and normal ovulatory function, including mid-luteal progesterone, basal body temperature, and cervical mucus changes.

Among women with a diagnosed infertility, the most frequent cause was endometriosis.

In fertile women, Bacterial Vaginosis (BV) was diagnosed using the Nugent score criteria. In parallel, the diagnosis was assessed also by culture isolation. Cultures of vaginal fluids detected different microbial species, among which *Lactobacilli*, *Gardnerella*, and *Bacteroides* were the most prevalent.

3.2 | Biodiversity analysis

We excluded samples with fewer than 10,000 reads. These were samples 29a (Control) and 5a, 46a, and 52a (Vaginosis) (Table S1).

We expected and found a correlation between the clinical status and the microbial structure of vaginal niche. For instance, the affected groups (Vaginosis, Idiopathic, and Infertile) had higher α-diversity than Control, as their microbiota is not dominated by *Lactobacilli*.

The Idiopathic group showed the greatest number of observed OTUs, assessed by the species-richness analysis (Chao1 index) (Table 2). This was expected since by its nature it is an uneven cohort: the diagnosis is based on exclusion criteria of known causes.

The Simpson's reciprocal index (α-diversity) increased from Control to Vaginosis, suggesting that within Vaginosis the bacterial species are not in absolute number the most abundant ones (Chao1 index) but they are the most heterogeneous comparing to the other cohorts. The equitability index confirmed that the species are not uniformly distributed within groups.

According to Chao1, only Control and Idiopathic were statistically different ($p < 0.01$). Equitability and Simpson's reciprocal indexes both revealed that Control is different from Idiopathic ($p < 0.05$), Infertile ($p < 0.05$ and $p < 0.01$, respectively), and Vaginosis ($p < 0.01$).

3.3 | The microbial profiles within vaginal ecosystem

The microbial structure showed different patterns across cohorts.

At the highest taxonomic level, cohorts differ in their relative microbial composition. *Gammaproteobacteria* (FDR_$p < 0.05$), *Bacilli* (FDR_$p < 0.001$), *Bacteroidia* (FDR_$p < 0.05$), and *Actinobacteria* (FDR_$p < 0.01$) accounted for the main observed differences. Figure 1 shows the microbial distribution at the phylum level in the four groups.

TABLE 2 Evenness, richness, and α-diversity estimators in control, idiopathic, infertile, and vaginosis

	Equitability	Chao1	Simpson's reciprocal index
Control	0.16 ± 0.07	419 ± 121	1.5 ± 0.5
Idiopathic	0.25 ± 0.11	579 ± 114	2.43 ± 1.19
Infertile	0.27 ± 0.1	508 ± 109	2.6 ± 1.3
Vaginosis	0.31 ± 0.1	480 ± 156	3.4 ± 2

Data are shown as group mean ± standard deviation

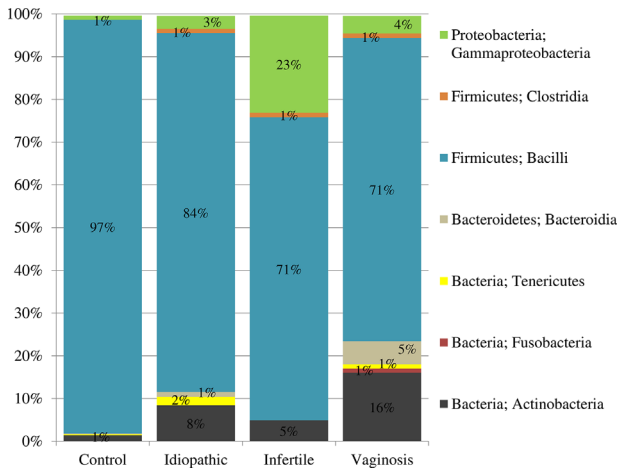

FIGURE 1 Comparison of microbiome taxonomic profiles. Comparison between cervical–vaginal microbiomes from the four groups included in the study. The phylum-level taxonomic classification was based on the relative abundance of normalized samples

In Idiopathic we observe a lack of *Fusobacteria*, which are present only in Vaginosis, and the presence of *Clostridia*, missing only in Control. Infertile has not *Bacteroidia* and *Tenericutes*, which are present in the other three groups, mainly in Idiopathic and Vaginosis. The remaining phyla are found in all the groups although they are distributed unevenly.

At the lowest taxonomic level (Figure 2, Table S2), among species belonging to *Lactobacilli* (*Firmicutes*), *Lactobacillus gasseri*, *Lactobacillus iners*, and *Lactobacillus crispatus* are the most abundant in Idiopathic.

L. gasseri is overrepresented in Idiopathic compared to the other three groups; *L. iners* is underrepresented in Idiopathic compared to Control, and its abundance decreases in Vaginosis and Infertile. *L. crispatus* is more abundant in Control than Idiopathic, while Vaginosis shows the lowest abundance. *Veillonella* (*Firmicutes*) and *Staphylococcus pasteuri/warneri* (*Firmicutes*) are common to Idiopathic and Vaginosis. Among *Actinobacteria*, *Gardnerella vaginalis* is underrepresented only in Control. *A. vaginae* (*Actinobacteria*) and *Prevotella bivia* (*Bacteroidetes*) are shared between Idiopathic and Vaginosis.

3.4 | Testing for significant differences in communities

We performed the ANOSIM test to compare the variation in species abundance and composition among cohorts (β-diversity). The groups differ more for presence/absence of many species rather than different abundance of the shared species. Unweighted UNIFRAC distances (Table 3) confirmed a mild but significant effect of grouping, which can also be graphically observed from the scatter plot matrices (Figures 3 and S1).

The scatter plot matrices from unweighted UNIFRAC (Figure 3) display a markedly different distribution between Vaginosis and Control; Infertile spreads mostly in the Vaginosis area and Idiopathic has not a specific clustering. The comparison of the bacterial communities, basing on weighted UNIFRAC distance (Figures 4 and S4), indicates a non-significant separation between the four groups.

Consequently, we performed the SIMPER test to determine the species that contribute to the differences in microbial community

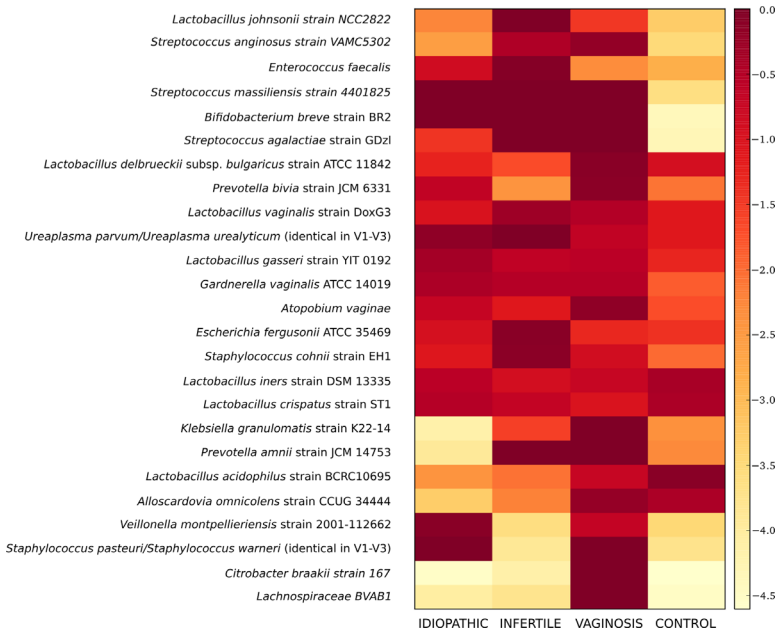

FIGURE 2 Heat map of the relative abundances of the top 25 species. The heat map shows the 25 most abundant bacterial species present in the cervical–vaginal milieu from the four groups analyzed in this study. Color keys are indicated on the right side, data are normalized to sum to 1 and log-transformed. The heat map was constructed with QIIME and species order matches UPGMA clustering of species. OTUs were classified against Vaginal 16S rDNA Reference Database constructed by Fettweis et al. (2012)

structure between the study groups. The results, provided in S3 Table, show that the major players are *L. iners*, *L. crispatus*, and *L. gasseri*. These *Lactobacilli* taken together are responsible for the 80% of the observed differences (cumulative sum) between Idiopathic and Control, for the 54% between Idiopathic and Vaginosis, and for the 63% between Idiopathic and Infertile.

G. vaginalis, normally expected in Vaginosis, is absent only in Control (0.3%) and common to both Idiopathic and Infertile.

Despite the low abundance, we suggest a role for *A. vaginae*, *P. bivia*, *V. montpellierensis*, *E. fergusonii*, and *U. parvum* as secondary players in Idiopathic infertility, where they could strengthen a previous imbalance in the healthy vaginal microbiome.

4 | DISCUSSION

This study extends the knowledge of female idiopathic infertility. We compared the vaginal microbiome of infertile women affected by different clinical/physiological conditions with that of healthy and bacterial vaginosis affected women.

Data from the present study are consistent with recent reports, which documented that infertility condition is accompanied by a compositional change in the vaginal microbiota. Local dysbiosis is frequently determined by the depletion of *Lactobacilli* and the increased amount of a variety of bacteria, mainly strict anaerobes and mostly resident in the gastrointestinal tract (*Enterobacteriaceae)* and in the urogenital (*Ureaplasma*) tract (Ghiasi, Fazaeli, Kalhor, Sheykh-Hasan, & Tabatabaei-Qomi, 2014).

We observed an uneven distribution of *Lactobacilli* among the studied cohorts of women (Table 4). A *Lactobacilli*-dominated microbiota is a suitable biomarker of a healthy vaginal ecosystem. *Lactobacilli* can act as a barrier against invasion of pathogens since the products of their metabolism, secreted in the cervical–vaginal fluid, play a fundamental role in contrasting both bacterial and viral

TABLE 3 ANOSIM analysis of the effect of grouping on bacteria dissimilarities based on unweighted and weighted UNIFRAC

	Unweighted UNIFRAC	Weighted UNIFRAC
p-value[a]	0.001***	0.5
R	0.16	0

[a]*p*-values were calculated on 999 possible permutations.
***Significant *p*-value ≤ 0.001

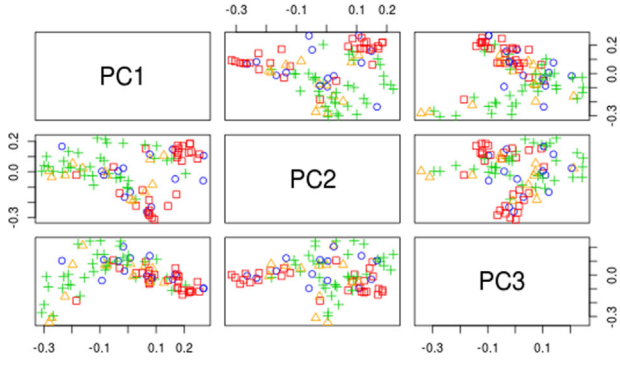

FIGURE 3 Scatter plot matrices showing results based on unweighted UNIFRAC distance. The scatter plot matrix displays a statistical association ($p < 0.001$) between the presence/absence of species and the effect of grouping for unweighted UNIFRAC distance. Healthy (red squares, □), Vaginosis (green crosses, +), Idiopathic (blue circles, ○), and Infertile (orange triangles, Δ)

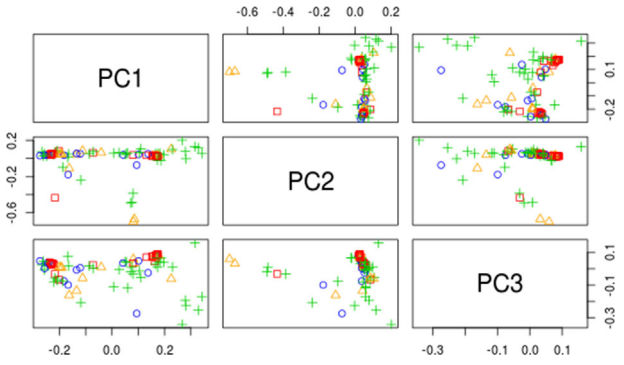

FIGURE 4 Scatter plot matrices showing results based on weighted UNIFRAC distance. The scatter plot matrix does not display statistical association between the relative abundance of species and the effect of grouping for weighted UNIFRAC distance. Healthy (red squares, □), Vaginosis (green crosses, +), Idiopathic (blue circles, ○), and Infertile (orange triangles, Δ)

infections (Lamont et al., 2011). The most frequently isolated species are *L. gasseri*, *L. iners*, and *L. crispatus* (Ravel et al., 2011).

We observed a higher presence of *L. gasseri* in the cervical–vaginal niche of Idiopathic, where it can adversely affect the ART outcome. Recent studies linked bacterial colonization of follicular fluid to early embryo demise and a reduced rate of ongoing pregnancy. In this context, *L. gasseri* plays a pivotal role, since high-load of this bacterium seems to trigger oocyte DNA fragmentation (Pelzer et al., 2013). The retrieval of *L. gasseri* in the Idiopathic suggests a negative correlation between an elevated amount of *L. gasseri* and the ART outcome.

Our study supports *L. iners* as a marker of microbiome healthiness, basing on the abundance of this bacterium in Control (51%) comparing to Idiopathic (29%), Infertile (18%), and Vaginosis (15%). Walther-Antonio et al. (2014) reported that *L. iners* and *L. crispatus* have positive role during childbearing period. For this reason, *L. iners* is a putative predictor of good/poor pregnancy or ART procedures outcome.

We suggest that, in Idiopathic, *L. crispatus* could absolve to a partial protective role against *E. coli* and, in turn, create a subclinical alteration of the reproductive tract. *L. crispatus* has been associated with inhibitory activity against *E. coli* (Ghartey et al., 2014). In our series, where the level of *L. crispatus* is lower, the level of *E. fergusonii* (which is on the basis of 16S rDNA sequences closely related to *E. coli* (99.8% of identity) (Wang, Ametaj, Ambrose, & Ganzle, 2013) is not directly affected, instead *G. vaginalis* is upregulated (Table S3). Both *E. coli* and *E. faecalis* can incorporate into a pre-formed *G. vaginalis* biofilm (Castro, Machado, & Cerca, 2016); uropathogens incorporated in these biofilms provides a protective niche for a further potential urinary tract infection (Swidsinski et al., 2014). In Infertile and Vaginosis, the relative abundance of *L. crispatus* is lower (25% and 6%, respectively) than Idiopathic (31%), where in turn it is underrepresented than Control (36%). Thus, the drop of *L. crispatus* correlates with a more susceptible urinary tract to uropathogens, revealing a link between lower genital and urinary tract infections.

TABLE 4 The relative abundances (%) of the *Lactobacilli* in the four cohorts of women

	Idiopathic	Control	Vaginosis	Infertile
L. iners	29	51	15	18
L. crispatus	31	36	6	25
L. gasseri	21	4	14	13
L. acidophilus	0	3	2	0
L. delbrueckii	0	4	0	0
L. johnsonii	0	1	0	6
L. vaginalis	0	0.7	0	0

Our study overall supports that *Lactobacilli* favor a healthy environment for pregnancy and are suitable marker in monitoring the transitional phase of vaginal flora during the estrogen treatment of in vitro fertilization (Jakobsson and Forsum, 2007). In addition, the depletion of *Lactobacilli* is linked to the inability to inhibit the colonization by specific harmful microorganisms that increase early miscarriage rates (Mangot-Bertrand et al., 2013; Petrova, Lievens, Malik, Imholz, & Lebeer, 2015; van Oostrum et al., 2013).

Among the secondary players in Idiopathic infertility, we spotted *A. vaginae*, *G. vaginalis*, and *Ureaplasma parvum*.

In our series, Idiopathic together with Vaginosis shared the presence of *A. vaginae* and *U. parvum*. It is likely that in the context of an altered microbial environment, even though at low abundance, *Atopobium* and *Ureaplasma* contribute to an adverse outcome of the ART procedures.

A. vaginae cooperates with *G. vaginalis* in biofilm formation (Hardy et al., 2016) and their co-presence before antibiotic therapy is associated with complete or partial failure of treatment itself (Ferris, Masztal, Aldridge, Fortenberry, & Martin, 2004).

Furthermore, the infection of *U. parvum* is particularly important in ART procedures, since the standard ART washing protocols may not remove this infectious agent from semen. Previous studies have reported a reduced pregnancy rate per embryo transfer (Montagut, Lepretre, Degoy, & Rousseau, 1991), no pregnancies (Shalika, Dugan, Smith, & Padilla, 1996), and an increased abortion rate (Kanakas, Mantzavinos, Boufidou, Koumentakou, & Creatsas, 1999) for couples after IVF treatment with *Ureaplasma*-infected semen compared with ART outcomes of uninfected couples.

Our results suggest that, in the Idiopathic, after a leading alteration caused by a decrease of *L. crispatus* and *L. iners*, and an increase of *L. gasseri*, a synergic action of different anaerobic bacteria, including *Atopobium*, *Prevotella*, *Veillonella*, *Ureaplasma*, and *Escherichia* (Biagi et al., 2009), rather than a predominant species, is involved in the pathogenesis of idiopathic infertility. The drop of some spp. of *Lactobacilli* corresponds to a decrease in acid lactic production and the metabolic byproducts of the anaerobic bacteria lead to an increase of normal vaginal pH (pH > 4.5), favoring a more hospitable niche for opportunistic pathogens.

5 | CONCLUSIONS

The results of this study suggest that women with repeated failed IVF cycles may benefit from microbial screening by NGS, due to its higher performance and throughput. Although NGS requires dedicated personnel and facility, it benefits from the ability to identify all the bacterial sequences within a specimen, including the low number and

uncultivable organisms. If compared to the routine cultivation methods, NGS reduces the cost of analysis per sample when the aim is that of performing the microbiome survey. NGS is also useful considering that the commonly used Nugent Score is not fully adequate to identify the microorganisms able to contribute to the healthy vaginal pH; thus, the score overstates or underestimates the dysbiosis condition (Hickey, Zhou, Pierson, Ravel, & Forney, 2012). The quantitative assessment of the abundance and composition of the cervical–vaginal microflora could increase the accuracy of available tools for the diagnosis of infertility and improve therapeutic protocols. The identification of specific micro-organisms colonizing the vaginal tract may provide support for the adoption of antimicrobial pre-treatment in women experiencing prolonged failure to conceive in the absence of a causal explanation.

NGS microbial screening can be more effective in evaluating the efficacy of antibiotic treatment on non-cultured microorganisms. Bacteria could modulate the local immune system playing the most critical role in pregnancy and ART outcomes. On the other hand, intervention attempts to improve the likelihood of pregnancy could be explored through the therapeutic alteration of microbiota, with the replacement of bacteria with beneficial profiles, as currently used with success for BV (Recine et al., 2016).

ACKNOWLEDGMENTS

We wish to acknowledge Prof. Alberto Pallavicini (University of Trieste, Trieste, Italy) for his assistance with the setting up of the experimental conditions and Dr. Roman Cheplyaka (Volunteer collaborator) for critically revising the manuscript. This study was supported by the grant RC 26/13, IRCCS Burlo Garofolo, Italy.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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