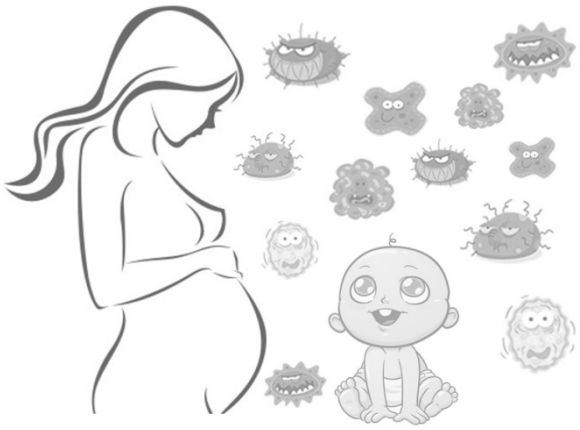
UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXIX CICLO DEL DOTTORATO DI RICERCA IN SCIENZE DELLA RIPRODUZIONE E DELLO SVILUPPO

MICROBIOTA & INFERTILITY:
MICROORGANISMS AND IMMUNE FACTORS IN THE IDIOPATHIC INFERTILITY

MED/07



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RIASSUNTO

Nel presente lavoro di tesi viene indagata la possibile correlazione tra l'infertilità *sine causa* (o idiopatica) e l'alterazione sia del microbiota vaginale che della risposta immunitaria locale. Lo studio del microbiota è stato effettuato mediante la piattaforma di sequenziamento di nuova generazione Ion Torrent PGM mentre il dosaggio di fattori immunitari solubili è stato effettuato mediante piattaforma Luminex.

A seguito dell'analisi, le donne affette da infertilità idiopatica hanno mostrato un microbiota vaginale simile a quello osservato nelle donne affette da vaginosi batterica (Capitolo 1), in particolar modo a quelle appartenenti al gruppo con un Nugent Score intermedio (Capitolo 2).

Tra i *Lactobacilli*, *L. iners*, *L. crispatus* e *L. gasseri* mostrano un profilo peculiare nelle donne affette da infertilità idiopatica. Inoltre, attori secondari come *P. bivia*, *U. parvum* e *E. fergusonii* contribuiscono al disequilibrio microbico. Simultaneamente, le donne affette da infertilità *sine causa* mostrano una risposta immunitaria locale alterata.

SUMMARY

In the present work, the possible relationship between the idiopathic infertility, the alteration of the vaginal microbiota and that of the local immune response is investigated. The next-generation Ion Torrent PGM sequencing platform allowed the survey of the vaginal microbial composition, while the Luminex assay permitted the dosage of the soluble local immune mediators.

After the analysis, the women affected with idiopathic infertility showed a vaginal microbiota similar to that of women affected with bacterial vaginosis (Chapter 1) and, especially, to those women who belong to the intermediate Nugent score group (Chapter 2).

Among *Lactobacilli*, *L. iners*, *crispatus* and *gasseri* have a peculiar pattern in women affected with idiopathic infertility. Furthermore, secondary players such as *A. vaginae*, *P. bivia*, *U. parvum*, and *E. fergusonii* contribute to the affected microbiome. Simultaneously, an altered immune response is detected in the vaginal niche.

INTRODUCTION

The term “microbiota” refers to the total collection of organisms (bacteria, archaea, fungi, and viruses) of a geographic region or a time period. In the context of human health, the term human microbiota, within which bacteria are the most represented organisms, was first introduced to describe the gingival crevice[1] and, later, for the description of the biggest accumulation of bacteria within the human body: the gastrointestinal microbiota[2]. The term “microbiome” instead is used to refer to the collection of the genomes of the microbes in a particular ecosystem and termed by Nobel laureate Joshua Lederberg[3].

Human microbiome meaningfully affects the physiologic function of every organ where bacteria are present[4]. The human body harbors an order of magnitude more bacteria than human cells[5].

The female reproductive tract exhibits a complex microbiome[6]. Considering the influence which the microbiome has in every organ, alteration of the microbial structure within genital tract may provide insight into the reproductive issues.

The female reproductive organs are the ovaries, fallopian tubes, uterus, cervix, vagina, and vulva. Since the combined primary function of these organs is the reproduction, disorders affecting them, including microbiome alteration, can result in infertility.

Infertility is defined as more than one-year-long timespan of unwanted non-conception with unprotected intercourse in the fertile phase of the menstrual cycle[7], according to the guidelines from the National Institute for Health and Clinical Excellence, 2004.

Primary and secondary infertility affects a range from 2% to 11% of women of reproductive age (20–44 years old)[8]. Infertility may depend on a combination of congenital and hormonal disorders, lifestyle, and environmental risks.

Many therapies successfully solve infertility issues, nonetheless the 15-30% of couples do not benefit from them. Couples that do not benefit from the infertility treatments, after the assessment of tubal patency, normal ovulatory and sperm parameters, have a diagnosis of idiopathic or unexplained infertility[9] (Figure 1). Couples affected with idiopathic infertility likely undergo *in vitro* fertilization (IVF) procedures.

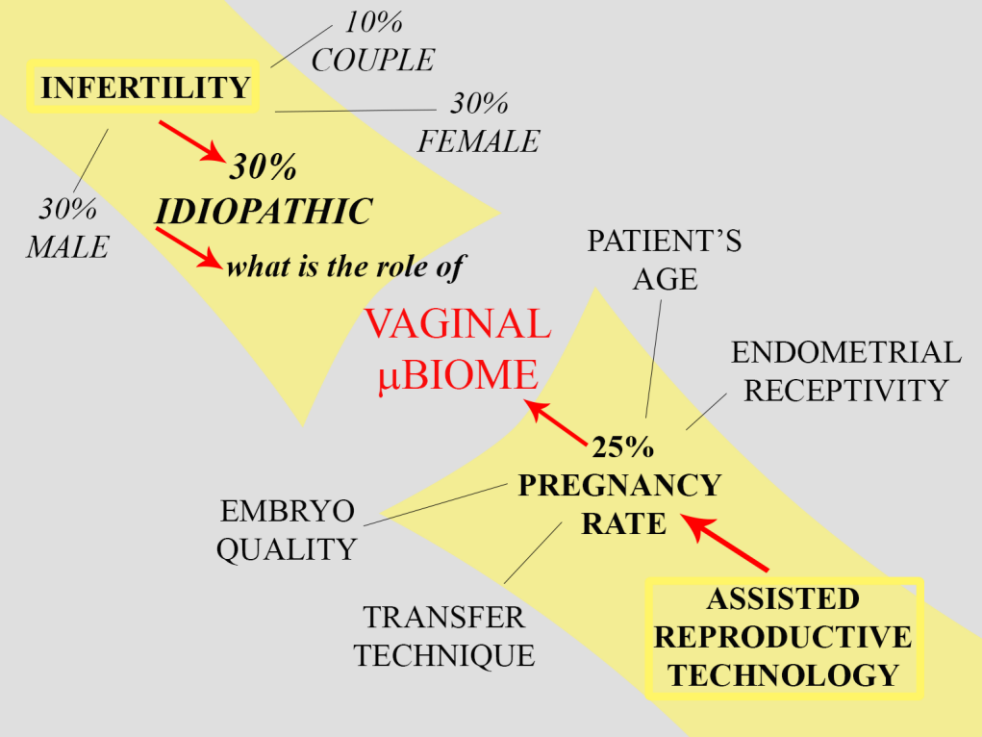

Figure 1. The involvement of the vaginal microbiome in the idiopathic infertility and in the assisted reproductive technology outcome.

Considerable progresses have been afforded in the assisted reproduction field but the implantation rate of embryos remains still low. The per cycle success rate is around 25%

and the chance for a successful outcome decreases with subsequent attempts[10]. For instance, after seven IVF cycles, the cumulative live birth rate is around 60%. Variables such as the patient's age, endometrial receptivity, embryo quality[11], and embryo transfer technique itself[12] affect the outcome.

IVF embryo transfer allows the transfer of embryos by a catheter through the cervix into the uterus. The bacterial displacement from the cervix to the uterus during the embryo transfer is possible and the vaginal microbiome on the day of embryo transfer affects pregnancy outcome[13].

For instance, the presence of *Chlamydia trachomatis* DNA in the endocervix of asymptomatic women during IVF procedures correlates with the decrease of implantation and ongoing pregnancy rates[14]. Also, the presence of *Enterobacteriaceae* and *Staphylococcus* correlates with a decreased pregnancy rate[15]. On the contrary, colonizing the transfer-catheter tip with *Lactobacillus crispatus* at the time of embryo transfer seems to increase the implantation and live birth rates and to decrease the rate of infection[16].

The exact features that contribute to the overall success rate of the embryo transfer are not yet clarified. The decreased pregnancy rates could be heavily influenced by the inoculum of harmful microorganisms into the uterine cavity. Bacteria are able to alter both biochemical and structural properties of the endometrium[17]. Furthermore, the high load of harmful microorganisms can determine a subclinical chronic endometritis, which, in turn, determines a lower uterine receptivity[18]. Likely, a bacterial contamination of the embryos during transcervical embryo transfer could affect their ability to implant.

Lactobacillus species dictate a healthy vaginal environment (Figure 2). In almost all the women, *Lactobacilli* comprise 90–95% of the total bacterial count in the vagina[19].

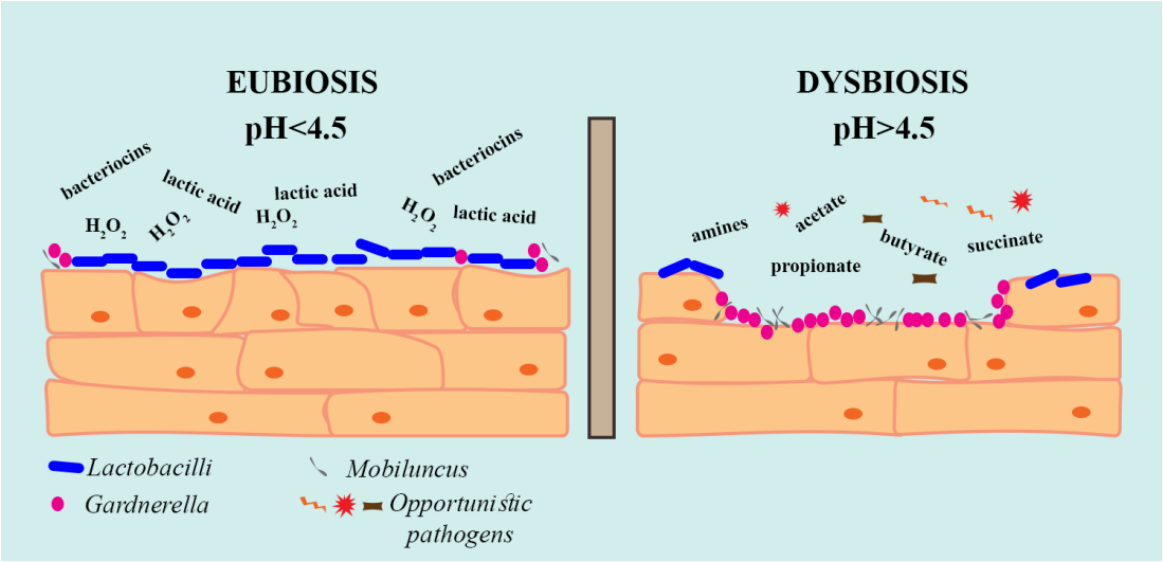

Figure 2. Eubiosis: the normal vaginal microflora. Dysbiosis: the affected vaginal microflora. For instance, the bacterial vaginosis.

Lactobacilli appear after menarche, due to the hormonal changes, while during the pre-menarcheal period the microbiota is composed by low numbers of strict and facultative anaerobes, with most species belonging to bacteria that are likely of enteric origin[20,21].

Lactobacilli prevent the growth of potentially virulent bacteria in the vagina by producing lactic acid and hydrogen peroxide (H_2O_2). Lactic acid assures a vaginal pH of 4.5[20], which is inhibitory to the growth of most microbes. H_2O_2 inhibits microbes with low levels of H_2O_2-scavenging enzymes, such as catalase[22]. Also, H_2O_2 combined with halide ions and peroxidase (present in the vagina)[23] constitutes a potent system of killing of bacteria and viruses. H_2O_2-producing *Lactobacilli* in the vagina appear to positively influence the live-birth rate among women undergoing IVF[24].

The surveillance for vaginal microbial composition of both commensal and pathogenic microbes is generally performed by a number of immune-related cells and receptors in order to help sense the microbial environment[25]. The microbial sensing is based on the

microbial motif pattern recognition by pattern recognition receptors (PRRs), such as toll-like receptors (TLRs)[26]. Microbial stimulation of PRRs initiates the cascades of the cytokine/chemokine signaling, for example secretion of interleukin IL-1β, IL-6, IL-8 and tumor necrosis factor-α (TNF-α), in order to recruit or activate specialized cells, such as NK cells, macrophages, CD4+ helper T-cells, and CD8+ cytotoxic T-cell lymphocytes and B lymphocytes[27,28]. Thus, vaginal bacterial community drives the immune response within the reproductive tract (Figure 3).

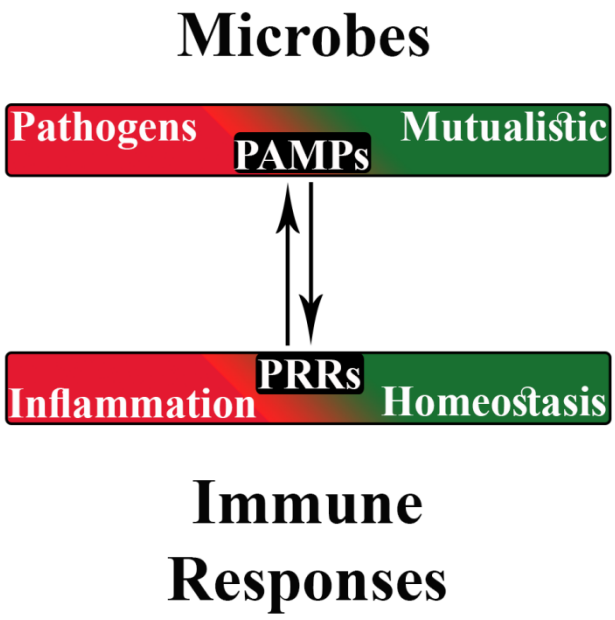

Figure 3. The surveillance for the microbial composition within the vaginal niche.

Perturbation of the microbial vaginal environment often naturally occurs, leading to a dysbiosis. Bacterial vaginosis (BV) is the most spread cause of vaginal dysbiosis. BV consists of a reduction of *Lactobacilli* and an overgrowth of anaerobes, and it remains asymptomatic in half of the cases[29] (Figure 2). Most of the anaerobes involved in the BV onset, e.g. *Veillonella*, *Prevotella*, *Escherichia*, produce large quantities of short chain fatty

acids (SCFAs)[30,31]. SCFAs (e.g., acetic, butyric, and propionic acids) exert a considerable role in a wide array of immune responses. SCFAs influence the immune responses by interfering with the cascade of pro-inflammatory cytokines, by inhibiting the immune cell migration and phagocytosis, and by inducing apoptosis in various cell types including neutrophils[32,33].

BV is strongly associated to reproductive failure[34]. Several studies showed that BV is particularly prevalent in patients with infertility[35,36], though it is not clear what risks infertile patients affected with BV incur for pregnancy outcome.

The main spread diagnostic tool for BV is the Nugent score. Nugent et al. developed a numerical score based on semi-quantization of Gram-positive rods, Gram-variable coccobacilli forms and curved Gram-variable rods. Namely, the morphotypes included in the Nugent score are *Lactobacillus* spp., *G. vaginalis* and *Mobiluncus* spp., respectively. The score ranges from 0 to 10: score 0-3 is a normal vaginal microflora where *Lactobacilli* are dominant; 4-6 is an intermediate microflora; 7-10 is consistent with BV where *Lactobacilli* are strongly decreased or absent and the two other morphotypes are dominant[37].

To restore a healthy vaginal microenvironment, physicians exploit antimicrobials drugs. The antimicrobials employed during the IVF procedures probably provide little inhibition to the great number of bacteria that have the potential to adversely affect the outcome[38,39]. Alongside, probiotics are widely used to resolve gynaecological dysbiosis, including BV and candidiasis. To improve the vaginal health, probiotic bacteria need to successfully colonize the female genital tract, hence to adhere to the vaginal epithelial cells, to produce hydrogen peroxide, bacteriocins and biosurfactant, to restore vaginal pH, and to inhibit potential pathogens associated with BV[40].

Probiotics are often administered alongside prebiotics that selectively support the growth of probiotic microbiota in order to increase their persistence[41]. Commonly used prebiotics are short-chain carbohydrates, oligosaccharides and pyrodextrins[42,43].

Studies have demonstrated that less than 10% of the microorganisms encompassing the human microbiome are cultivable[4]. Molecular techniques have the potential to reveal heretofore hidden features of the human microbiome that contribute to health and disease. The possibility of amplifying the bacterial 16S rRNA gene directly from samples, both human and environmental, allows to identify all bacteria species present in a sample, by-passing the cultivation step[44]. The amplification and sequencing of variable regions of the 16S rRNA gene, by exploiting primer sequences that target the conserved regions of this gene, assure the identification of a broad phylogenetic spectrum of bacteria (Figure 4).

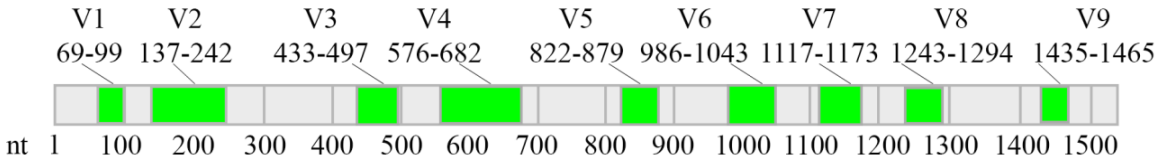

Figure 4. The 16S rRNA gene structure (1541 nucleotides, nt). Grey: conserved regions, Green: variable regions (V1-V9).

The advent of the next-generation sequencing (NGS) techniques, because of their high-throughput, cost and time saving features, enabled several surveys of the human microbial communities, including the uncultivable microbes.

Thanks to the NGS techniques, five main clusters of vaginal microbiome were identified basing on the ethnicity. Four clusters are dominated by *Lactobacilli*, namely *L. crispatus*, *L. iners*, *L. jensenii* and *L. gasseri*. The fifth cluster is not dominated by *Lactobacilli* rather

by species usually linked to BV, such as *G. vaginalis* and *Prevotella* spp.[45]. Many of the newly identified bacteria associated with BV are not considered in the Nugent score, highlighting its non-specificity.

Thus, microbial profiling is useful to provide an overall view of the microbial community, microbial diversity, and insight into the metabolic processes occurring in a given ecosystem. Furthermore, microbial profiling also has the potential applicability in monitoring efforts, such as the results of the attempts to restore a healthy vaginal microenvironment.

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 tools can help researchers to shed the light on unexplained/idiopathic infertility thanks to their high efficiency in profiling the microbiome. Highlighting the vaginal microbiome structure can extend the knowledge of other possible causes of failed conception.

AIM

The aim of the research is to highlight the possible relationship between the vaginal microbiota structure, the local immune mediators and the unexplained (or idiopathic) infertility status.

The survey of the microbiota and the soluble immune factors can spot bacteria and molecules suitable as markers of vaginal milieu healthiness and of prognostic/diagnostic utility during the *in vitro* fertilization procedures.

In order to pursue the aim, the vaginal microbiota and the local immune response of women affected with idiopathic infertility is compared to that women affected with a diagnosed cause of infertility, fertile healthy women, and fertile women affected with bacterial vaginosis.

MATERIALS AND METHODS

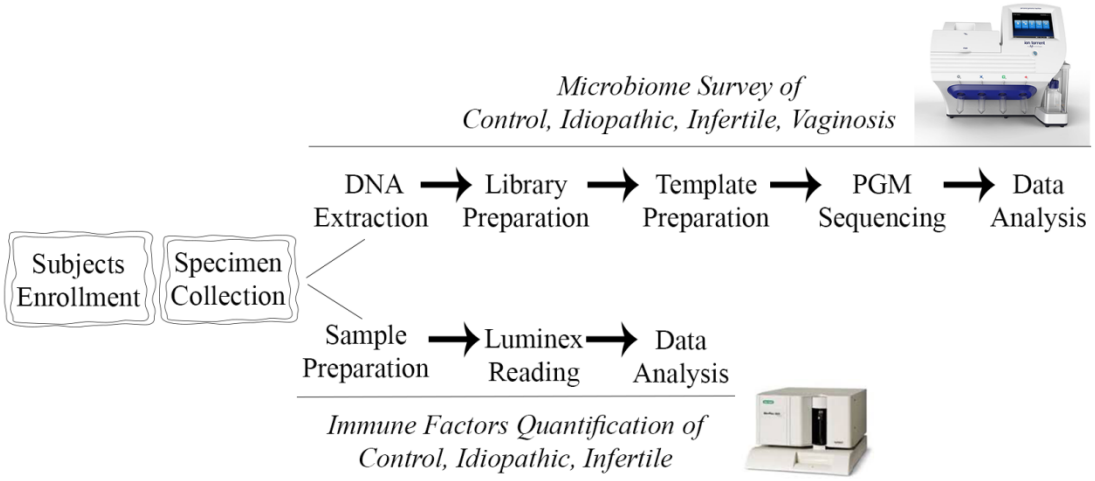

Figure 5. Overview of the experimental workflow.

Subjects enrollment, specimen collection and ethical approval

Ninety-two cervical-vaginal samples were obtained from women that full-filled the inclusion eligibility criteria of the study.

All the women were of Caucasian origin[46], non-pregnant, of reproductive age (range, 32-40 years), had no current use of tobacco, alcohol, and contraceptive methods, had no hospitalization or use of systemic medication for chronic diseases or antibiotics/probiotics (oral or topic) within the 6 months previous to samples collection.

Among the enrolled subjects, 4 cohorts were identified.

1. The cohort of women diagnosed with idiopathic infertility (Idiopathic) includes 14 subjects. The diagnosis is supported by the assessment of the tubal patency by hysterosalpingogram or laparoscopy and normal ovulatory function, including mid-luteal progesterone, basal body temperature and cervical mucus changes.

2. The cohort of infertile women (hereafter referred to as Infertile) refers to 13 subjects who showed an impairment of the reproductive tract; the clinical exams identified the endometriosis as the most frequent cause, followed by tubaric and ovulatory disorders.

3. The cohort of healthy women (Control) identifies 30 subjects who performed the periodic check-up.

4. The cohort of women affected with bacterial vaginosis (hereafter referred to as Vaginosis) includes 35 women who obtained a Nugent score consistent with altered microflora (Nugent score 4-10).

All the samples were collected in Mother and Child Health Hospital - IRCCS Burlo Garofolo, Trieste. Idiopathic and Infertile subjects attended the Assisted Reproductive Technology (ART) clinic, while Control and Vaginosis attended the Gynecology clinic.

The sampling was performed 5-7 days before the menstrual period and before programmed *in vitro* fertilization procedure. Cervical-vaginal samples were collected using the cervex brush device (Rovers Medical Devices B.V., The Netherlands) and dissolved in three mL of sterile water. After a centrifugation step (5000 x g, 20 min), aliquots of 500 µL were immediately prepared and stored at -80°C.

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

Sample processing and library construction

The previously prepared aliquots of cervical-vaginal samples were used for DNA extraction, using the NucliSENS® easyMAG® system (BioMèrieux, Gorman, North Carolina, USA), setting the final elution volume at 50 µl. All DNA samples were stored at

-20°C prior to further processing steps. A real time EvaGreen® dye (Fisher Molecular Biology, Waltham, USA) PCR was carried out 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 of 16S rRNA gene, spanning 500 bp).

A subsequent nested PCR was performed with the primers B338F_P1-adaptor (B338F 5′-ACTCCTACGGGAGGCAGC-3′) and U534R_A-adaptor_barcode (U534R 5′-ATTACCGCGGCTGCTGG-3′) to prepare a 200 bp long libraries for final V3 region sequencing, in association with the IonXpress Barcode Adapter[47].

The PCR reactions were performed using the Kapa 2G HiFi Hotstart ready mix 2X (Kapa Biosystems, Massachusetts, USA), 0.5 µM of each primer, and 400 ng/µL of Bovine Serum Albumin (BSA). The thermal cycling profile was: 5 min at 95°C, 30 sec at 95°C, 30 sec at 59°/57°C, 45 sec at 72°C and a final elongation step at 72°C for 10 min.

To assure the validity of the results, negative controls including no template and no bacterial DNA were processed with clinical samples, starting from the pre-analytic phase of samples manipulation. A total absence of amplification signal at the end of PCR runs (I and II step of PCR) was successfully obtained.

The size of the amplicon (560 bp for the I PCR and 260 bp for the II PCR) was checked on a 2% agarose gel. The amount of dsDNA within the libraries was quantified with a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, California, USA) using the Qubit® dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Ion Torrent Sequencing

An equal amount (60 ng) of all the libraries was pooled into a single batch. Then, 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, USA) 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, Waltham, MA, USA). A negative control was sequenced to remove the sequencing contaminants from the analyses of the samples.

NGS data processing

QIIME 1.8.01[48] was used to analyze the sequencing data. High quality (Q>20) sequences were demultiplexed and filtered by quality using split_libraries_fastq.py with default parameters, retaining sequences with a minimum length of 150 bp.

Operational taxonomic units (OTUs) were picked at 97% similarity and clustered against the Vaginal 16S rDNA Reference Database constructed by Fettweis *et al.*[49] with the open-reference OTU picking[50] script, using a uclust clustering tool[51].

Then, singleton OTUs and samples with low sequencing depth were removed (less than 10,000 reads). Rarefaction analysis was done both on separate and pooled samples (according to cohorts) by the Chao1 index[52]. Equitability and Simpson reciprocal index were used to assess alpha diversity (within-sample diversity). Beta diversity (between sample diversity comparison) was surveyed by weighted and unweighted UniFrac distance matrices[53,54] and presented with the principal coordinate analysis (PCoA). Robustness of the identified clusters was investigated using jackknifing (randomly resampling sequences without replacement).

Statistical analyses

Differences in the microbial community composition between cohorts were investigated using analysis of similarity (ANOSIM), Kruskal-Wallis test and similarity percentage (SIMPER) analysis. QIIME was used for ANOSIM basing on the UniFrac distance matrices and for Kruskal-Wallis using the biom tables as inputs. The vegan package[55] for R software[56] was used for the SIMPER analysis using taxonomy biom tables as inputs.

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

Diagnosis of Bacterial vaginosis

Gram staining of vaginal secretions, Nugent's criteria and microorganisms isolation were performed to assess the diagnosis of bacterial vaginosis.

The Nugent score takes into account the presence of large gram-positive rods (*Lactobacilli* morphotypes; decrease in *Lactobacilli* scored as 0 to 4), small gram-variable rods (*G. vaginalis* morphotypes; scored as 0 to 4), and curved gram-variable rods (*Mobiluncus* spp. morphotypes; scored as 0 to 2). It can range from 0 to 10. A score ranging 4-10 is consistent with an altered vaginal flora.

Culture and identification of clinical isolates were carried out on agar plates, including Horse blood agar plates as non-selective growth medium, MacConkey agar plates for Gram negative bacteria and Mannitol Salt agar plates to discern coagulase positive or negative aerobically or anaerobically incubated *Staphylococcus*.

Dosage of the soluble immune factors

The quantification of cytokines and growth factors was performed on a platform based on magnetic bead multiplex immunoassays (Bio-Plex, BIO-RAD Laboratories, Milano, Italy).

Luminex multiplex panel technology simultaneously measures a panel of 48 analytes including cytokines, chemokines and growth factors (Table 1).

Fifty μL of cervical-vaginal fluid and standards were added in duplicate to a 96 multiwells plate containing analyte beads. After incubation for 30 minutes at room temperature and washing, the antibody-biotin reporter was added and incubated for 10 minutes with streptavidin-phycoerythrin. The concentrations of the cytokines were determined using the Bio-Plex array reader (Luminex, Austin, TX). The Bio-Plex Manager software optimized the standard curves automatically and returned the data as Median Fluorescence Intensity (MFI) and concentration (pg/mL). This assay has a reported limit of detection of 1–20 pg/ml, depending on the cytokine target (Table 2).

Table 1. Cytokines, chemokines and trophic factors detected by Luminex multiplex technology.

	Cytokines	Chemokines	Trophic factors
21-plex	IL-1α; IL-2Rα; IL-3; IL-12(p40); IL-16; IL-18; IFN-α2; LIF; MIF; SCF; TNF-β; TRAIL/TNFSF10	CTACK/CCL27; GRO-α/CXCL1; MCP-3/CCL7; MIG/CXCL9; SDF-1α/CXCL12	HGF; M-CSF/CSF1; β-NGF; SCGF-β/CLC11
27-plex	IL-1β; IL-1Ra; IL-2; IL-4; IL-5; IL-6; IL-9; IL-10; IL-12(p70); IL-13; IL-15; IL-17; IFN-γ; TNF-α	IL-8/CXCL8; Eotaxin/CCL11; MCP-1/CCL2; IP-10/CXCL10; MIP-1α/CCL3; MIP-1β/CCL4; RANTES/CCL5	IL-7; FGF-b; G-CSF; PDGF-BB; VEGF; GM-CSF

Table 2. Luminex sensitivity for the analyzed cytokines, chemokines, and growth factors.

CYTOKINES	Assay sensitivity (pg/ml)
IL-1β: Interleukin 1, beta	0.6
IL-1ra: Interleukin 1 receptor antagonist	5.5
IL-2: Interleukin 2, TCGF, lymphokine	1.6
IL-4: Interleukin 4, B cell growth factor 1	0.7
IL-5: Interleukin 5, B-cell differentiation factor I; T-cell replacing factor	0.6
IL-6: Interleukin 6, B-cell differentiation factor; B-cell stimulatory factor 2	2.6
IL-9: Interleukin 9, T-cell growth factor p40; cytokine P40	2.5
IL-10: Interleukin 10, T-cell growth inhibitory factor; cytokine synthesis inhibitory factor	0.3
IL_12(p70): Interleukin 12 subunit p70, CLMF, NKSF	3.5
IL-13: Interleukin 13, ALRH, BHR1	0.7
IL-15: Interleukin 15	2.4
IL-17A: Interleukin 17, CTLA-8; cytotoxic T-lymphocyte-associated antigen 8	3.3
IFN-γ: Interferon gamma	6.4
TNF-α: Tumor necrosisf actor, alpha	6
IL-1α: Interleukin 1 alpha	0,5
IL-2ra: Interleukin receptor alpha	2.1
IL-3: Interleukin 3	4.8
IL-12(p40): Interleukin 12p40	23.3
IL-16: Interleukin 16	0.4
IL-18: Interleukin 18	0.2
IFN-α2: Interferon alpha 2	4.3
LIF: Leukemia inhibitory factor	5.5
MIF: Macrophage migration inhibitory factor	1.5
SCF: Stem cell factor	1
TNF-β: Tumor necrosis factor-beta	0.3
TRAIL: TNF-related apoptosis inducing ligand	2.1

CHEMOKINES	Assay sensitivity (pg/ml)
IL-8: Interleukin 8, CXCL8, alveolar macrophage chemotactic factor I	1
Eotaxin: C-C motif chemokine 11; eosinophil chemotactic protein	2.5
MCP-1: CCL2, C-C motif chemokine 2; monocyte chemoattractant protein 1	1.1
IP-10: CXCL10, 10 kDa interferon gamma-induced protein	6.1
MIP-1α: CCL3, C-C motif chemokine 3; G0/G1 switch regulatory protein 19-1	1.6
MIP-1β: CCL4, C-C motif chemokine 4; CC chemokine ligand 4	2.4
RANTES: CCL5, C-C motif chemokine 5; SIS-delta	1.8
cTACK: Cutaneous T cell attracting chemokine	3.4
GROα: Growth related oncogene alpha	6.3
MCP-3: Monocyte chemotactic protein 3	1
MIG: Monokine induced by interferon	1.2
SDF-1α: Stromal cell derived factor alpha 1	8.7

GROWTH FACTORS	Assay sensitivity (pg/ml)
IL-7: Interleukin 7	1.1
FGF basic: Basic fibroblast growth factor; heparin-binding growth factor 2	1.9
G-CSF: Granulocyte colony-stimulating factor	1.7
PDGF-bb: Platelet derived growth factor, isoform b	2.9
VEGF: Vascular endothelial growth factor	3.1
GM-CSF: Granulocyte macrophage colony stimulating factor	2.2
HGF: Human growth factor	4.9
M-CSF: Macrophage colony stimulating factor	0.9
β-NGF: Nerve growth factor beta	0.2
SCGF-β: Stem cell factor growth factor -beta	45.4

GraphPad Prism (v. 5) was used for statistical data analysis. Comparisons between the groups were performed by the Kruskal-Wallis one-way analysis of variance. When a significant p-value was found ($p<0.05$), a multiple comparison test was used to determine which groups were different.

CHAPTER 1

RESULTS

Demographics of the study cohort

Table 3 shows the features of the patients enrolled in the study. The series comprises 92 women, within which the 4 identified cohorts are Idiopathic, Infertile, Control, and Vaginosis.

Table 3. Demographics.

	Idiopathic	Infertile	Control	Vaginosis
Number of women	14	13	30	35
Median age (range)	38 (36-40)	38 (36-40)	34 (32-36)	35 (33-37)
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian
Duration of infertility (> 5 years)	9	6	-	-
Infertility factors				
Tubaric	-	5	-	-
Ovulatory	-	2	-	-
Endometriosis	-	6	-	-
In vitro fertilization (1-4 cycles)	3	3	-	-
Embryo				
transfer (mean number/range)	1.5/0-3	1.4/0-3	-	-
quality (I grade)	42.8%	36.8%	-	-
Pregnancy outcome				
Negative	10	8	-	-
Biochemical	4	4	-	-
Positive	-	1	30	35
Sexually transmitted infections	Neg	Neg	Neg	Neg
Nugent Score	0-3	0-3	0-3	4-10
Menstrual cycle length (days)	28-35	28-35	28-35	28-35

Data processing

After the quality (Q ≥20) and length (reads ≥150 bp) filtering, the sequencing produced a total output of 6,665,606 reads, generated from the V3 variable region of the 16S rDNA. All the sequences were classified against the Vaginal 16S rDNA Reference Database to species level at the 97% of identity. To assure a sufficient coverage of the bacterial species identified in the samples during the OTU picking step, only the samples reaching at least 10,000 reads were retained; the residual number of reads was 6,649,314, eliminating four samples: 3 from Vaginosis and 1 from Control.

α-diversity analysis

Table 4 shows the list of the biodiversity estimators. Chao1 showed the highest richness in Idiopathic and the lowest in Control.

Table 4. Equitability, species richness (Chao1), and α-diversity measures.

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

Data are shown as mean value ± standard deviation

In Control, the low Equitability index value suggested that the species are not uniformly distributed, as expected by the characteristic dominance of *Lactobacilli* in the healthy condition. In the other three cohorts, the equitability index increased, likely because the dominance of *Lactobacilli* drops.

Simpson's reciprocal index showed that Vaginosis had the highest value compared with the other three groups, suggesting that it has the most heterogeneous microbial composition among the studied groups.

Chao1 confirmed the significant ($p<0.01$) difference between Control and Idiopathic. 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 ($p<0.01$).

β-diversity analysis

The following analyses, used to compare the composition of the microbial communities (β-diversity) between groups, fall into the category of the exploratory techniques.

Mainly, the presence/absence of selected bacterial species (unweighted UniFrac) accounts for the differences between groups rather than a different relative abundance (weighted UniFrac) of the shared species.

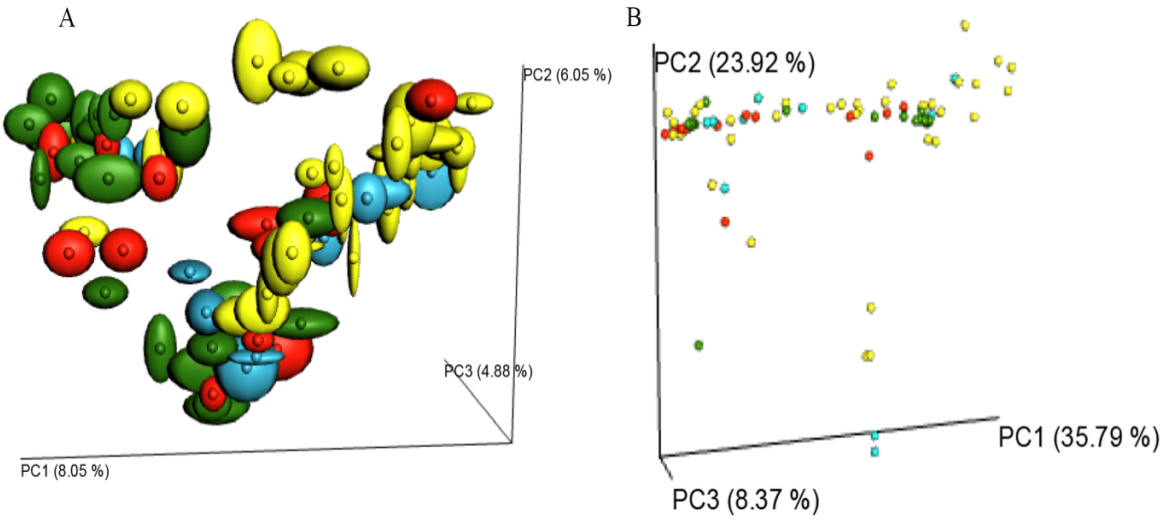

Figure 6. PCoA showing results based on unweighted (A) and weighted (B) UniFrac distances. Idiopathic (Red), Infertile (Light blue), Control (Green), and Vaginosis (Yellow).

Figure 6 shows PCoA drawn basing on both weighted (A) and unweighted (B) UniFrac distance matrices. The PCoA from unweighted UniFrac (Figure 6A) shows that Vaginosis has a divergent microbial composition than Control, these cohorts have different spatial coordinates within the 3D graph; Infertile and Idiopathic have not a specific clustering. Conversely, the comparison of the bacterial communities, basing on weighted UniFrac distance (Figure 6B), suggests no graphical evident separation between groups.

Exploring significant differences in cohorts' structure

Table 5 shows the output of the ANalysis Of SIMilarity (ANOSIM) statistic R, based on unweighted and weighted UniFrac distance matrices. ANOSIM statistically evaluates whether there is a significant difference among groups and operates directly on a distance matrix[57].

ANOSIM confirmed that groups statistically differ when accounting for presence/absence of bacterial species ($p<0.001$), although the effect of grouping is mild ($R = 0.16$). The comparison of the bacterial communities, based on weighted UniFrac distance, suggests a non-significant separation between the 4 groups.

Table 5. ANOSIM test on Unweighted and Weighted UniFrac distance matrices.

	UNIFRAC	
	Unweighted	Weighted
p value*	0.001***	0.5
R value	0.16	0

*p-values were calculated on 999 possible permutations.
***significant p-value ≤0.001

Microbial profiling within cohorts

The taxonomic assignment of OTUs against the Vaginal 16S rDNA Reference Database identified the bacterial species present in the studied samples.

At the highest taxonomic level (phylum) (Figure 7), cohorts exhibit a different microbial milieu. *Gammaproteobacteria* (False Discovery Rate (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.

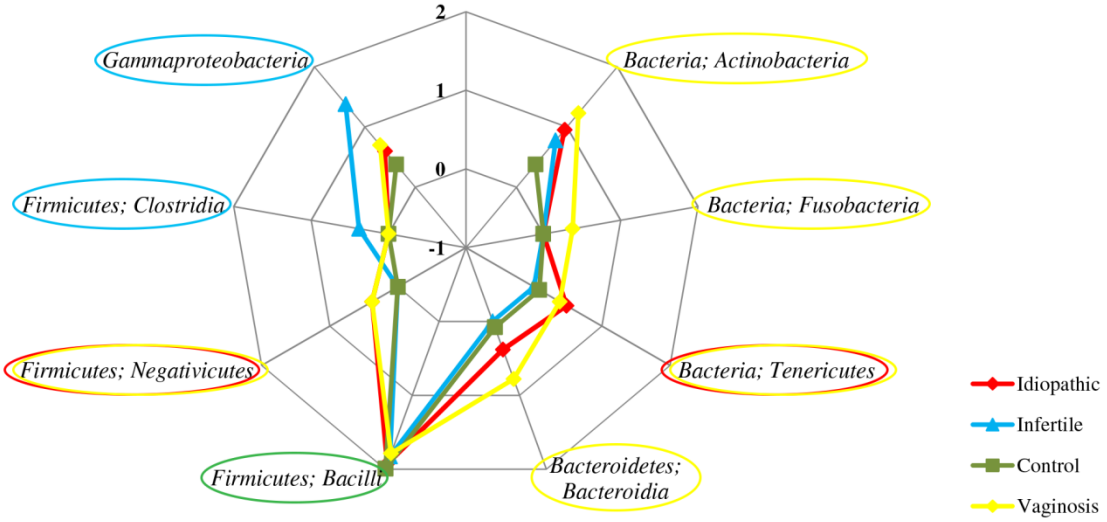

Figure 7. Taxonomic distribution at the phylum-taxonomic level. The circles highlight similarities between groups when more colors are combined or a distinctive feature when one color is used. Data are normalized to sum to 100 and $\log_{10}$ transformed.

Figure 7 illustrates the microbial distribution of the phyla within the groups. *Lactobacilli* was the dominant phylum in all the cohorts. Specifically, Vaginosis and Infertile showed a reduced relative abundance of *Lactobacilli* than Idiopathic and Control.

Idiopathic shared a similar bacterial pattern with Vaginosis, but lacked *Fusobacteria*. *Fusobacteria*, together with *Tenericutes*, *Bacteroidia*, and *Negativicutes*, were also absent

in Control and Infertile. *Gammaproteobacteria* and *Actinobacteria* were common to both infertility groups (Idiopathic and Infertile) and Vaginosis.

The microbial taxonomic composition was also gained at the species level. Figure 8 shows the list of the 25 most abundant bacterial species identified in the samples. Among the *Firmicutes*, *Lactobacilli iners*, *crispatus*, and *gasseri* were the most abundant species identified within the 4 groups. *Lactobacilli vaginalis*, *johnsonii*, *delbrueckii*, and *acidophilus* showed a different pattern of presence/absence among cohorts. Idiopathic and Vaginosis showed the presence of *Veillonella montpelliierensis*, while the remaining species belonging to *Firmicutes* were present only in Vaginosis, except for *Staphylococcus cohnii* and *Streptococcus anginosus* which were present also in Infertile.

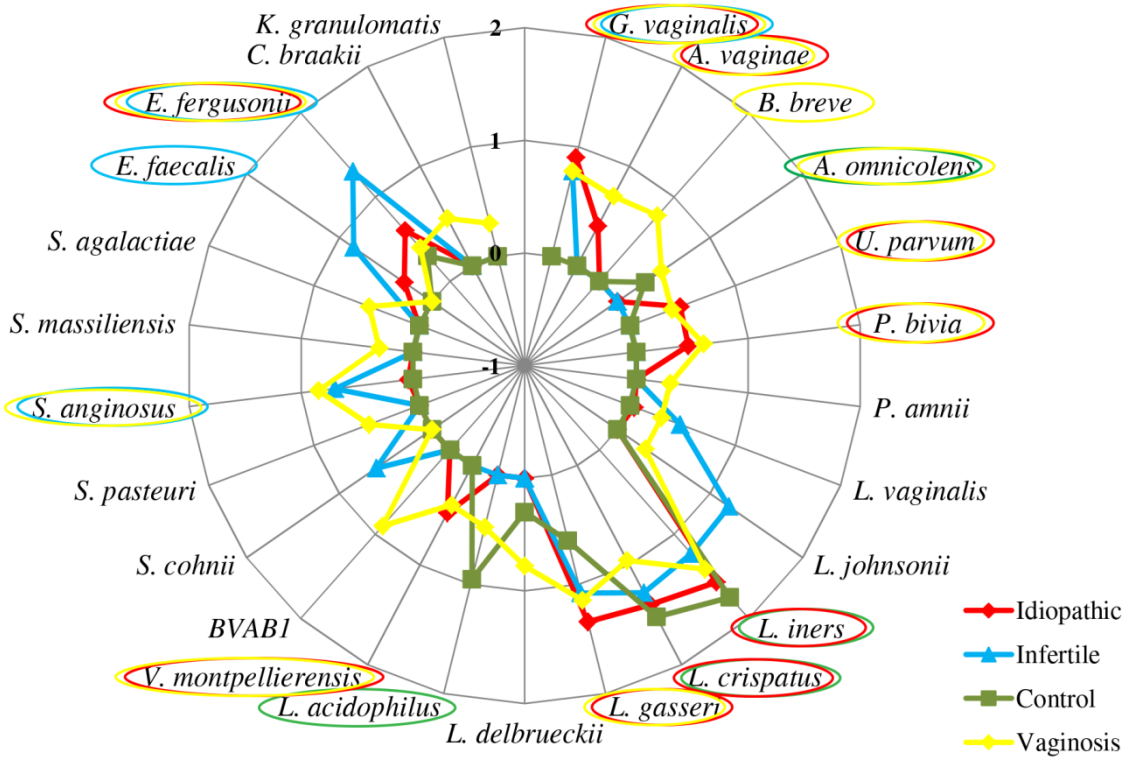

Figure 8. Taxonomic distribution at the species-taxonomic level. The circles highlight similarities between groups when more colors are combined or a distinctive feature when one color is used. Data are normalized to sum to 100 and $\log_{10}$ transformed.

Different other species were common mainly to Idiopathic and Vaginosis. They shared, among *Actinobacteria*, *Gardnerella vaginalis* and *Atopobium vaginae*; among *Tenericutes* and *Bacteroidia, Ureaplasma parvum* and *Prevotella bivia*, respectively. Among *Gammaproteobacteria*, Idiopathic and Vaginosis shared *Escherichia fergusonii*, which was also present in Infertile.

Species contributing to the differences in microbial community structure

The following analysis was carried out with the aim to identify the taxonomy of the microbial species that contribute to the observed differences between groups. The SIMilarity PERcentages (SIMPER) procedure[57] accounts for the average percent contribution of each species to the dissimilarity between groups in a Bray-Curtis dissimilarity matrix.

Table 6 shows the output of SIMPER analysis. The taxa that contribute to the greatest dissimilarity between groups are the *Lactobacilli iners*, *crispatus*, and *gasseri*. The cumulative sum of the dissimilarity explained by upper-mentioned *Lactobacilli* is the 80% between Idiopathic and Control, the 54% between Idiopathic and Vaginosis, and the 63% between Idiopathic and Infertile. All the remaining taxa account for the residual dissimilarities, each with a modest average contribution.

Table 6. SIMPER analysis.

Idiopathic *vs* Control	**Ave-Abn**		**Ave-Cntr**	**Sd**	**Ratio**	**Cumsum**
	Idiopathic	Control				
L. iners	29	51	25	21	1.15	35
L. crispatus	31	36	21	20	1.05	65
L. gasseri	21	4	11	16	0.7	80
G. vaginalis	6	0.3	3	6	0.5	85
E. fergusonii	2	2	2	6	0.3	88
L. acidophilus	0	3	1	6	0.2	90
V. montpellierensis	3	0	1	5	0.3	92
A. vaginae	2	0	1	3	0.3	93
U. parvum	1	0	0.7	1	0.4	94
P. bivia	1	0	0.6	2	0.3	95

Idiopathic *vs* Infertile	**Ave-Abn**		**Ave-Cntr**	**Sd**	**Ratio**	**Cumsum**
	Idiopathic	Infertile				
L. crispatus	31	25	20	19	0.9	24
L. iners	29	18	18	20	0.9	47
L. gasseri	21	13	13	15	0.9	63
E. fergusonii	2	16	8	17	0.5	74
G. vaginalis	6	5	4	7	0.6	80
L. johnsonii	0	6	3	1	0.3	83
E. faecalis	0	3	2	4	0.4	85
V. montpellierensis	3	0	1	5	0.3	87
P. mirabilis	0	0	1	1	0.3	89
A. vaginae	2	0	1	3	0.4	90
S. cohnii	0	2	1	2	0.3	91
S. anginosus	0	1	0.7	2	0.3	92
S. macedonicus	0	1	0.7	2	0.3	93
P. bivia	1	0	0.6	2	0.3	94
U. parvum	1	0	0.6	1	0.4	95

Idiopathic *vs* Vaginosis	Ave-Abn		Ave-Cntr	Sd	Ratio	Cumsum
	Idiopathic	Vaginosis				
L. iners	29	15	17	19	0.9	20
L. crispatus	31	6	16	19	0.8	38
L. gasseri	21	14	13	16	0.8	54
G. vaginalis	6	7	5	7	0.7	60
A. vaginae	2	6	3	8	0.4	64
B. breve	0	6	3	9	0.3	68
L. delbrueckii	0	4	2	6	0.4	70
P. bivia	1	3	2	5	0.4	73
E. fergusonii	2	2	2	4	0.4	75
V. montpellierensis	3	1	2	5	0.3	77
S. agalactiae	0	3	1	8	0.2	79
L. acidophilus	0	2	1	5	0.3	80
S. anginosus	0	2	1	5	0.2	82
S. pasteuri	0	2	1	7	0.2	83
BVAB1	0	2	1	7	0.2	85
C. braakii	0	2	1	7	0.2	86
S. massiliensis	0	2	1	6	0.2	87
A. omnicolens	0	2	1	4	0.2	88
K. granulomatis	0	2	1	5	0.2	90
U. parvum	1	1	1	2	0.5	90
S. cohnii	0	1	0.6	3	0.2	91
P. amnii	0	1	0.6	2	0.2	92
S. simiae	0	1	0.6	3	0.2	93
P. timonensis	0	1	0.5	2	0.3	93
L. johnsonii	0	1	0.5	2	0.3	94
D. micraerophilus	0	1	0.5	1	0.3	94
L. vaginalis	0	0.7	0.5	1	0.6	95

Ave-Abn = average abundance of the species in Idiopathic *vs* Control, Idiopathic *vs* Infertile and Idiopathic *vs* Vaginosis.
Ave-Cntr = average contribution of feature to the overall dissimilarity between groups.
Sd = standard deviation.
Ratio = ratio of average contribution to standard deviation of contribution.
Cumsum = cumulative contributions.
The list of features is not complete so percent values do not sum to 100%.

Immune mediators in infertility

Idiopathic group showed a peculiar immunological pattern. Among the 48 measured immune mediators, the observed differences were not all statistically significant, likely because of the limited number of samples analyzed.

The molecules that exhibited a different trend across Control, Idiopathic, and Infertile can be divided into two groups: the first group involves the interaction among IL-1β, IL-1α, and IL-1ra; the second group includes G-CSF and LIF.

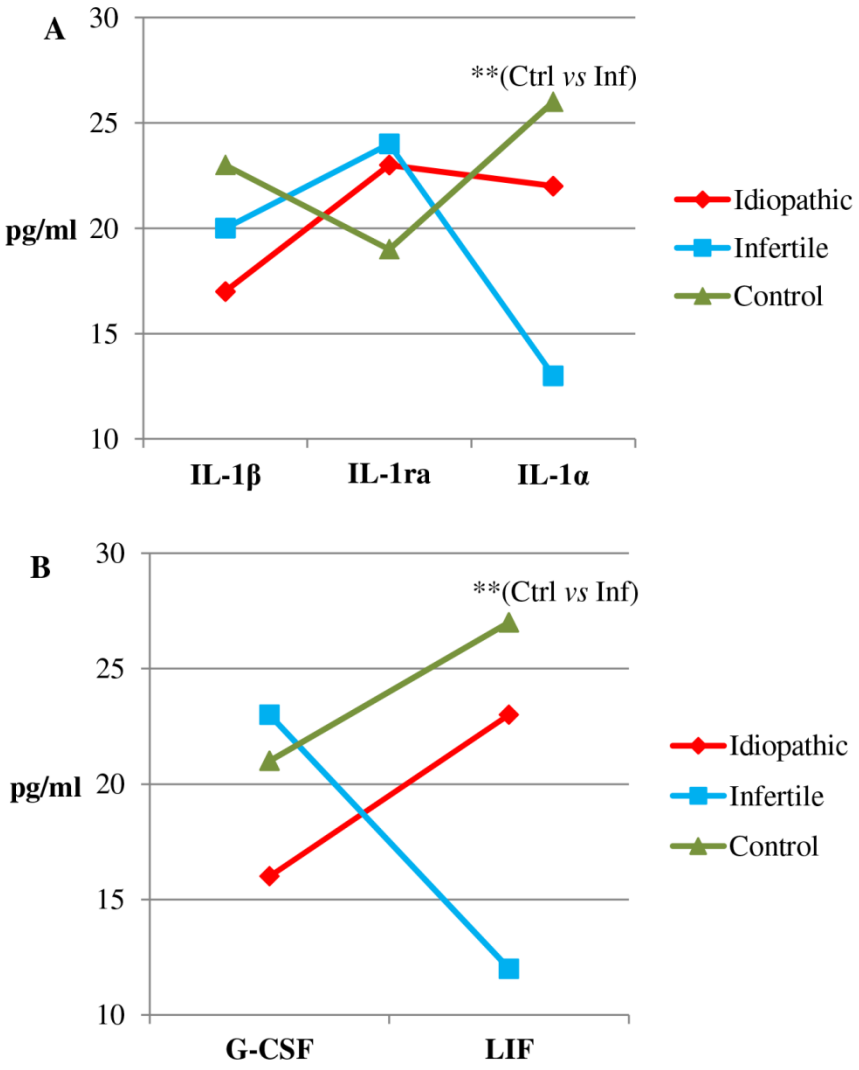

Figure 9. Immune mediators influenced by the clinical status.

Comparing to Control, both Idiopathic and Infertile showed (Figure 9A) a higher amount of IL-1ra. In idiopathic the ratio between IL-1β and IL-1ra decreased, with the decrease of IL-1 β and the increase of IL-1ra. The difference in the ratio between IL-1β and IL-1ra is

also observed in Infertile but it is less pronounced. In Infertile, also the ratio between IL-1β and IL-1α differed from Control and Idiopathic, with IL-1α downregulated ($p<0.01$).

Regarding the second group of cytokines, namely G-CSF and LIF, Idiopathic showed a decrease of G-CSF and an increase of LIF (Figure 9B) comparing to Control. Infertile showed an inverted G-CSF/LIF ratio, with the increase of G-CSF and the decrease of LIF, comparing both to Control and Idiopathic.

DISCUSSION

Previous studies on infertility afforded useful findings about the mechanisms of the fertilization but still one-third of infertility cases remains unexplained[9]. The comparison between the vaginal microbiota of women suffering with idiopathic infertility and that of women affected with bacterial vaginosis, non-idiopathic infertility, and healthy women extended the knowledge of unappreciated microbe-host interactions.

1.1 Microbiome within cohorts

Species biodiversity can indicate the status of health of a biological niche. *Lactobacilli* normally dominate the vaginal environment in terms of relative abundance[6]. An even microbial distribution, with the loss of a dominant bacterial genus, suggests a disturbance in the vaginal ecosystem.

BV always shows a drop of *Lactobacilli* and a polymicrobial structure[29]. Both infertility cohorts seem to exhibit the same feature, especially Idiopathic that accounts for a number of observed species (Chao1) similar to that of Vaginosis.

Chao1 estimates the species richness of the cohorts; equitability index assumes a value between 0 and 1, with 1 being complete evenness in the distribution of species within the microbial community; Simpson's reciprocal index (α-diversity) starts with 1 as the lowest possible value: the higher the value, the greater the diversity. All those indexes showed (Table 4) a more even distribution of the species present in the vaginal microbiome of Idiopathic, Infertile, and Vaginosis, when compared to Control.

Hence, infertility issues seem to arise from the same microbial alteration which is observed in BV. Mainly, the loss of the protective *Lactobacilli* and the overgrowth of other bacteria could have a key role in the onset of the microbial imbalance.

1.2 Microbiome across cohorts

The clinical condition influences the vaginal microbial environment. Quantitative and qualitative β-diversity measures (Figure 6) provided different perspectives on the factors that affect microbial community structure. Both results of α- and β-diversity suggested that a transient alteration in relative abundances of some resident bacteria lays the foundation for the entry of opportunistic pathogens and/or the overgrowth of resident species, usually detected at low levels, which alter the normal community structure.

Qualitative measure (Figure 6A) suggested a significant effect ($p < 0.001$, ANOSIM) of distinct microbial population whose presence strengthen the differences between groups. Quantitative measures (Figure 6B) showed that the relative abundances of microbial species had lesser impact on the observed differences.

1.3 Profiling the microbiome

The results of the taxonomic assignment at the phylum level (Figure 7) supported the previous discussed results. Idiopathic shows a large number of low-abundance taxa, then being more similar to Vaginosis than to Control and to Infertile. Compositional changes in the vaginal microbiota was mostly evident at high taxonomic level (phylum).

Three phyla - *Tenericutes*, *Bacteroidia*, and *Negativicutes* - were shared only between Idiopathic and Vaginosis. *Firmicutes* was the dominant phylum in all the cohorts and *Fusobacteria* was present only in Vaginosis. *Actinobacteria* and *Gammaproteobacteria*

were more relevant in Vaginosis and in both infertility groups. This general picture of the vaginal microbiome is yet potentially an excellent marker and could be used as target for clinical evaluation of infertility cases. In both infertility cohorts, together with a slight decrease of *Lactobacilli*, other phyla increase and their relative abundances can link to the observed clinical condition.

Basing on the observation of the microbiome composition, it is not possible to state if the vaginal microbiome alteration is a driving process or a consequence in all the idiopathic infertility cases. Nonetheless, spotting specific mechanisms across the microbial interplay can help to shed the light on the idiopathic infertility status.

According to the previous hypothesis, a survey of the vaginal microbiota at the species level was performed. The vaginal ecosystem showed differences between the cohorts (Figure 8), although no single bacterium could be identified as a specific marker for Idiopathic. The lack of a "unique specific marker bacterium" of Idiopathic suggests that the picture of the microbial composition has to be considered as a whole, taking into account the interaction among the residing bacteria in a specific given moment.

1.4 The link between *Lactobacilli* and idiopathic infertility

Considering the vaginal microbiome of the cohorts, data suggested a particular mechanism of dysbiosis in Idiopathic, which could cause an asymptomatic and subclinical alteration. Subclinical issues are difficult to identify and to treat.

Figure 10 shows the summary of the possible interplay between different microbial species within vaginal niche.

A decrease of *L. crispatus* and *L. iners* and an increase of *L. gasseri* seem to justify the altered *Firmicutes* structure in Idiopathic. Although, the total amount of *Firmicutes* is very

similar between Idiopathic and Control, the dysmicrobism depends on the relative equilibrium among *Lactobacilli* species. Since Idiopathic has a regular total amount of *Lactobacilli*, the Nugent score (Table 3), which only accounts for the total amount of *Lactobacilli*[58], was not able to identify the alteration.

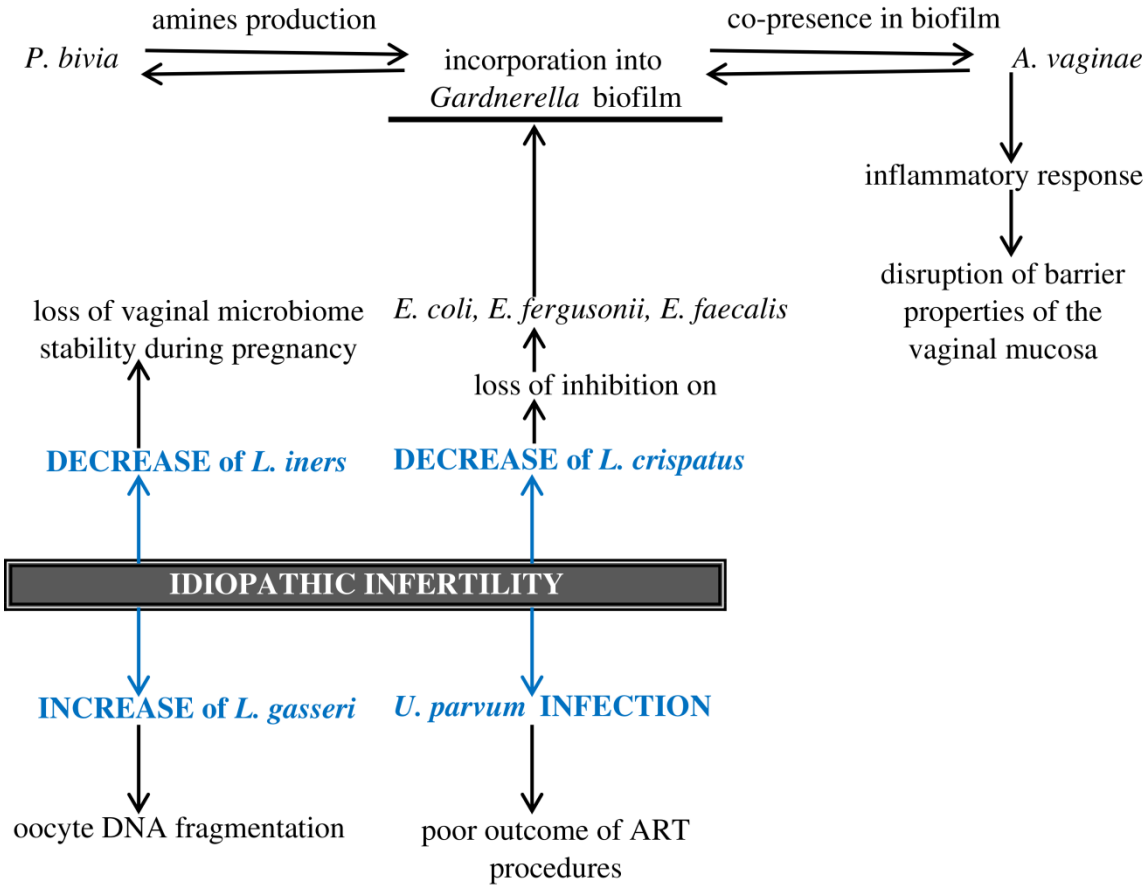

Figure 10. The main players of the affected vaginal microbial profile in idiopathic infertility.

Lactobacilli gasseri, *iners*, and *crispatus* showed a great impact on the cumulative sum of the dissimilarity between Idiopathic and the other cohorts (Table 6). Overall, Idiopathic and Vaginosis have a similar microbial profile. In fact, the different proportion of *Lactobacilli* accounted more for the differences between Idiopathic and both Infertile (63%) and Control (80%) rather than Vaginosis (54%).

Results suggest a link between the increase of *L. gasseri* and the rate of negative outcome of IVF procedures. Idiopathic had a higher presence of *L. gasseri* in the cervical-vaginal niche. Studies reported that *L. gasseri*, *iners,* and *crispatus* have an involvement on infertility, although to a different extent and with different mechanisms. *L. gasseri* plays a pivotal role in early embryo demise and in low rate of pregnancy, since high-load of this bacterium triggers oocyte DNA fragmentation[59].

L. iners is suitable as a marker of healthiness of the vaginal niche and as a supportive clinical tool in order to predict the ART procedures outcome. *L. iners* and *L. crispatus* support the vaginal microbiome stability during childbearing period[60]. *L. iners* showed the highest relative abundance in Control (51%) comparing to Idiopathic (29%), Infertile (18%), and Vaginosis (15%).

The relative abundance of *L. crispatus* is lower in Infertile and Vaginosis (25% and 6%, respectively) than Idiopathic (31%) and Control (36%). *L. crispatus*, when dominant in the vaginal niche, has an inhibitory activity against *E. coli*[61]. *E. coli* and *E. faecalis* can incorporate into a pre-formed *G. vaginalis* biofilm[62]; once incorporated in biofilms, they provide a protective niche for a further potential urinary tract infection[63]. In Idiopathic, *L. crispatus* could exert a partial protective role against *E. coli* and create a subclinical alteration. Where the level of *L. crispatus* is lower, the level of *E. fergusonii*, which is on the basis of 16S rRNA sequences closely-related to *E. coli* (99.8% of identity)[64], is not directly affected, instead *G. vaginalis* seems to be upregulated (Figure 8).

Therefore, according to the previous explained findings, *Lactobacilli* favor a healthy environment for the onset of pregnancy and are suitable markers in monitoring the transitional phase of vaginal flora during the estrogen treatment of *in vitro* fertilization[65].

Especially, the depletion of *Lactobacilli* is linked to the inability to inhibit the colonization by specific harmful microorganisms that increase early miscarriage rates[66-68].

1.5 Secondary players

Results spotted some species as secondary players in the observed differences. Idiopathic and Vaginosis shared the presence of *A. vaginae* and *U. parvum*. Within an imbalanced vaginal ecosystem, regardless their low abundance, they could contribute to the adverse outcome of the ART procedures.

G. vaginalis cooperates with *A. vaginae* in biofilm formation[69] and together they are responsible for the complete or partial failure of antibiotic therapy[70]. Furthermore, *A. vaginae* elicits a robust inflammatory response that alters the physicochemical barrier properties of the vaginal mucosa[71].

A symbiotic relationship exists also between *G. vaginalis* and *P. bivia*. *G. vaginalis* needs ammonia to growth, which is provided by *P. bivia*. In turn, *G. vaginalis* produces amino acids that further stimulates the growth of *P. bivia*. This interaction leads to an increase of both bacteria[72].

U. parvum is strictly correlated to ART procedures; standard ART washing protocols do not assure the removal of this infectious agent from semen[73]. The presence of *U. parvum* in semen influences pregnancy rate per embryo transfer[74], pregnancies onset[75], and the abortion rate[76].

1.6 Impact of the vaginal immune status on idiopathic infertility

The immunological profile within the vaginal niche can affect the IVF outcome. The measurement of 48 immune mediators revealed a different pattern between Idiopathic, Infertile, and Control. The main factors involved in fertility issue are listed in Table 7.

The higher amount of IL-1ra observed in Infertile and Idiopathic correlates to an adverse effect on embryo implantation. Studies demonstrated that repeated injections of the IL-1ra into mice, two days before the onset of implantation, result in an apparent failure of blastocyst to implant[77]. Furthermore, an appropriate ratio of both IL-1β to IL-1ra in human endometrial stromal cells is relevant to embryo implantation. The ratio remains constant even in the presence of increasing concentrations of IL-1β, suggesting that IL-1 plays a crucial role in embryo-maternal interaction[78].

Table 7. The main immune players in fertility issues.

Amount	Role
↑	**IL-1ra** correlates to adverse effect on embryo implantation (Huang, 2006)
↓	**IL-1ß** negatively correlates to pregnancy outcome after ICSI (Rehman, 2015)
↓	**IL-1α** negatively correlates to the production of prostaglandins (Tanikawa, 2008)
↑	**G-CSF** enhances endometrial development, ongoing pregnancy and implantation rates. The positive role might result from the ability of G-CSF in increasing **[LIF]** (Eftekhar, 2014)

A high amount of serum IL-1β positively correlates to pregnancy outcome after intracytoplasmatic sperm injection (ICSI)[79]. Idiopathic showed the lowest amount of IL-1β in the vaginal niche comparing to Control and Infertile. The amount of IL-1β, both in serum and vagina, can be an important predictive factor for the implantation of fertilized oocyte and for the positive pregnancy outcome after ICSI.

IL-1α decreased from Control to Infertile. IL-1α regulates the production of uterine prostaglandins in endometrial stromal cells[80]. Prostaglandins, derivatives of arachidonic acid, absolve to a primary role in embryo implantation, increasing vascular permeability, stromal decidualization, blastocyst growth and development, leukocyte recruitment, embryo transport, trophoblast invasion, and extracellular matrix remodeling during implantation[81]. Thus, both altered prostaglandins synthesis and actions result in implantation failure. In fact, IL-1α is one of the earliest embryonic signals, exerts a direct impact on the receptive endometrium, and induces molecular changes that are fundamental for embryo implantation[82]. Furthermore, *in vitro* cultured human embryos secrete high concentrations of IL-1α and IL-1β, and the presence of these cytokines correlates with successful implantation after the transfer to the uterine cavity[83]. Thus, monitoring the level of IL-1α can be a useful tool to monitor the outcome of IVF procedures.

The relative amount of G-CSF and LIF showed a different pattern only in Infertile comparing to Control and Idiopathic. Studies demonstrated that transvaginal endometrial perfusion with G-CSF enhances endometrial development, ongoing pregnancy, and implantation rates. The positive role on pregnancy outcome might result from the ability of G-CSF in increasing the amount of LIF[84]. A deregulation of LIF production in the endometrium during both the proliferative and the secretory phases of the cycle correlates to multiple failures of implantation and idiopathic infertility[85].

The deregulation of endometrial LIF secretion throughout the menstrual cycle can be a possible cause of unexplained infertility and repetitive failures of implantation. A dose-dependent effect of G-CSF and LIF can explain the apparently normal amount observed in Idiopathic. In fact, in Idiopathic, the amount of LIF is higher than G-CSF, thus suggesting

a stimulation of G-CSF on LIF production, but the concentration of both G-CSF and LIF are lower in Idiopathic than Control.

1.7 The immune-microbiome network

Alteration of the vaginal microbial milieu and changes in the genital tract mucosal immune environment could exert a primary role in both pregnancy establishment and outcome (Figure 11).

For instance, genital tract secretions from healthy non-pregnant women absolve to a variable bactericidal activity against *E. coli*[86].

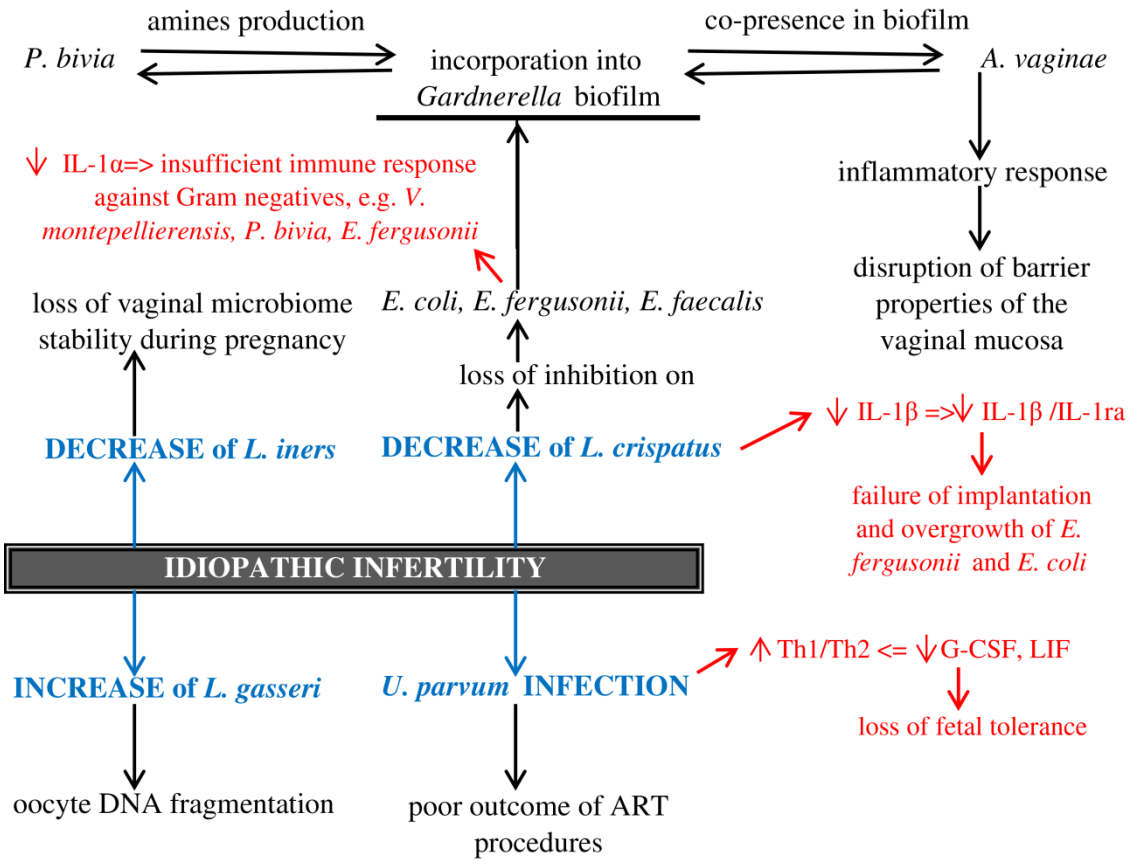

Figure 11. The immune-microbial network in the vaginal niche of Idiopathic group.

Idiopathic showed a higher level of *E. fergusonii* (99.8 % of DNA sequence similarity to *E. coli*) than Control and Vaginosis, together with a slightly decreased level of IL-1β comparing to Control. *Lactobacilli,* which normally dominate the vaginal microbiota in healthy women, can inhibit *E. coli* growth and vaginal colonization[87]. In particular, *L. crispatus*, when dominant in the vaginal niche, has an inhibitory activity against *E. coli*[61]. The inhibitory activity of *Lactobacilli* against *E. coli* reflects cumulative interactions between multiple mediators secreted by genital tract epithelial cells, immune cells, and vaginal microbiota. The loss of protective role absolved by *Lactobacilli* is accompanied with the decrease of IL- 1β[88]. The presence of IL-1β positively affects the implantation rate[89]. Thus, the decrease of IL-1β and the colonization of *E. coli* observed in Idiopathic can be considered as a peculiar feature of a compromised mucosal immune environment.

In Idiopathic, the presence of *U. parvum* stimulates the shift of Th1/Th2 ratio to a Th1 dominant immunity, elucidating why *Ureaplasma* spp. has the potential of being harmful in pregnancy establishment[90].

In Idiopathic, the shift toward the Th1 immunity is also proved by the decrease of G-CSF.

In fact, the maintenance of fetal tolerance and pregnancy depend on T cells mucosal-associated immune responses, including that of the genital tract[91]. The adequate balance for Th1/Th2 immunity, slightly shifted to Th2-type immunity, is suitable for the maintenance of pregnancy[92]. Among the immune mediators, G-CSF alters T-cell function and induces Th2 immune responses[93].

IL-1α showed different amount across the cohorts, especially between Control and Infertile ($p<0.01$). IL-1α mediates many of the biological effects of LPS, which induces histopathological alterations in the various reproductive organs of pregnant animals[94]. In Infertile an inadequate stimulation of the immune response by the LPS is presumable,

considering the high presence of Gram-negative (LPS-releasing) bacteria such as *E. faecalis* and *E. fergusonii*. In Idiopathic, *E. fergusonii, V. montepellierensis,* and *P. bivia* are the main Gram-negative bacteria. Despite of the presence of gram-negative bacteria, the level of IL-1α is downregulated in Idiopathic comparing to Control. Thus, comparing to Control, an overexpression of IL-1α would be deleterious for embryo implantation, considering that the upper-regulation of IL-1α negatively affects the pregnancy rate[95], and a downregulation can account for an inadequate immune response to harmful bacteria in the vaginal environment.

In addition, anaerobic bacteria, e.g. *Veillonella*, *Prevotella*, *Escherichia*, produce large quantities of short chain fatty acids (SCFAs) in mucosal sites such as the female genital tract[30,31]. SCFAs include acetic, butyric, and propionic acids and play an important role in a wide array of immune responses. SCFAs modulate the immune responses by inhibiting the production of pro-inflammatory cytokines, by affecting immune cell migration and phagocytosis, and by inducing apoptosis in various cell types including neutrophils[32,33].

CONCLUSIONS

The vaginal microbiome screened by NGS suggested the presence of an affected microbiome in women with idiopathic infertility. Results from this study suggested that, in the Idiopathic group, the decrease of *L. crispatus* and *L. iners*, and the increase of *L. gasseri* synergize with the action of different anaerobic bacteria, including *Atopobium*, *Prevotella*, *Veillonella*, *Ureaplasma*, and *Escherichia*[96], in creating an adverse environment for pregnancy establishment. After the drop of *Lactobacilli*, the production of acid lactic decreases and the metabolic byproducts of the anaerobic bacteria can cause an increase of normal vaginal pH (normal pH is 3.8 to 4.5), favoring a more hospitable niche for opportunistic pathogens. The alteration across *Lactobacilli* species and the consistent action of secondary players is a possible guide for intervention in idiopathic infertility. Bacteria could influence the outcome of ART procedure by different mechanisms. For instance, bacteria influence the local immune system[71].

A dysregulation of the local immune mediators is observed in the vaginal niche of Idiopathic. The dysregulation depends on the altered amount of some cytokines that associate to an adverse pregnancy outcome and/or establishment.

Numerous cytokines and growth factors are involved in maternal-embryo cross-talk[97]. In Idiopathic, the alteration of cytokines that are fundamental for the embryo implantation, such as IL-1β, IL-1α and IL-1ra, and for the maintenance of an adequate Th1/Th2 ratio, such as G-CSF and LIF, can affect fertility.

The combined effect of the microbial structure alteration and the immune profile dysregulation dictates an adverse vaginal environment that seems to strongly affect fertility.

Identifying candidate species and immune mediators as prognostic markers can be a primary goal to monitor the outcome of the attempts in improving the likelihood of pregnancy.

CHAPTER 2

Working with idiopathic diseases is challenging. When no evident cause or mechanism are identified, treatments are empirically administered basing on symptoms.

The comparisons between women affected with idiopathic infertility with those who showed an identified cause of infertility, bacterial vaginosis and healthy condition highlighted similarities between Idiopathic and Vaginosis. Thus, a deeper study of Vaginosis could account for additional information on idiopathic infertility.

Vaginosis cohort was analyzed by diving the total number of women into two cohorts according to the Nugent score: women who had a Nugent score ranging from 4 to 6 (Intermediate) and women who had a Nugent score ranging from 7 to 10 (Vaginosis).

RESULTS

α-diversity analysis

Within Vaginosis, only the samples reaching 10,000 reads were retained (n=32) for the analyses. Basing on the Nugent score, Vaginosis comprised two groups: Intermediate (n=15) and Vaginosis (n=17).

Both Intermediate and Vaginosis cohorts exhibited a polymicrobial structure.

The absolute number of species, assessed by Chao1, within Intermediate and Vaginosis is not explicative for the difference with respect to Control (Table 8). Instead, Intermediate and Vaginosis cohorts show an uneven microbial distribution (Equitability index), which is

significantly different from Control ($p<0.01$). Accordingly, the α-diversity (Simpson index) is higher in Intermediate and Vaginosis ($p<0.01$) than Control.

Table 8. Equitability, species richness (Chao1), and α-diversity measures.

	Control	Intermediate	Vaginosis
Equitability	0.16±0.07	0.32±0.1	0.32±0.1
Chao1	419±121	519±155	449±153
Simpson's reciprocal Index	1.5±0.5	3.5±1.8	3.6±2.3

Data are shown as mean value ± standard deviation

β-diversity analysis

The comparison between the cohorts suggests a markedly separation between Control, Intermediate, and Vaginosis.

PCoA (Figure 12) shows the comparison between Control, Intermediate, and Vaginosis.

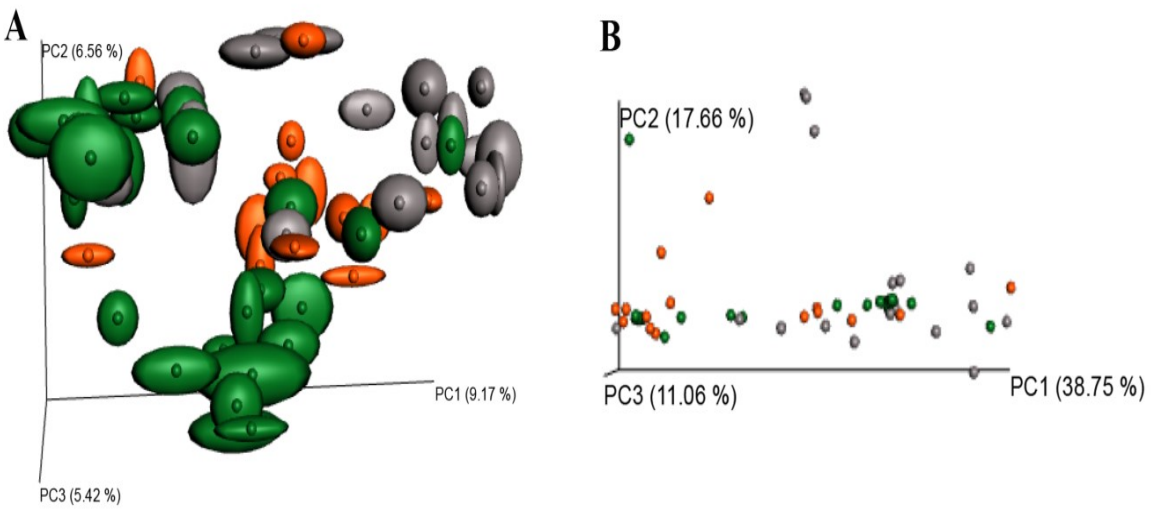

Figure 12. PCoA showing results based on unweighted (A) and weighted (B) UniFrac distances. Control (Green), Intermediate (Orange), and Vaginosis (Grey).

The unweighted UniFrac PCoA (Figure 12A) highlights three clusters, corresponding to the clinical groups. The distribution of the groups suggests a transition from Control to Vaginosis, where Intermediate seems the transitional phase. The weighted UniFrac PCoA (Figure 12B) partially retains the same clustering, although it is graphically less evident. Vaginosis still locates in a separated area of the 3D graph comparing to Control and Intermediate, whose localization is shared.

Exploring significant differences in cohorts' structure

ANOSIM test statistically compared the Intermediate and Vaginosis cohorts (Table 9). The statistic supported the previous results. Intermediate and Vaginosis statistically differ according to the unweighted UniFrac ($p<0.05$), even though the effect of grouping is small (0.12). Groups also differ according to weighted UniFrac ($p<0.001$), with an R value of 0.16.

Table 9. ANOSIM test on unweighted and weighted UniFrac distance matrices.

	UNIFRAC	
	Unweighted	Weighted
p value*	0.009**	0.001***
R value	0.12	0.16

*p-values were calculated on 999 possible permutations.
**significant p-value ≤0.05
***significant p-value ≤ 0.001

Microbial profiling within Intermediate and Vaginosis cohorts

The microbial composition at the phylum level (Figure 13) exhibits a characteristic pattern within Intermediate and Vaginosis cohorts, although not significantly different. *Tenericutes* and *Gammaproteobacteria* are predominant in Intermediate, while BVAB1

and *Negativicutes* in Vaginosis. *Actinobacteria*, *Bacteroidia*, and *Bacilli* are shared between groups but their relative abundance is different.

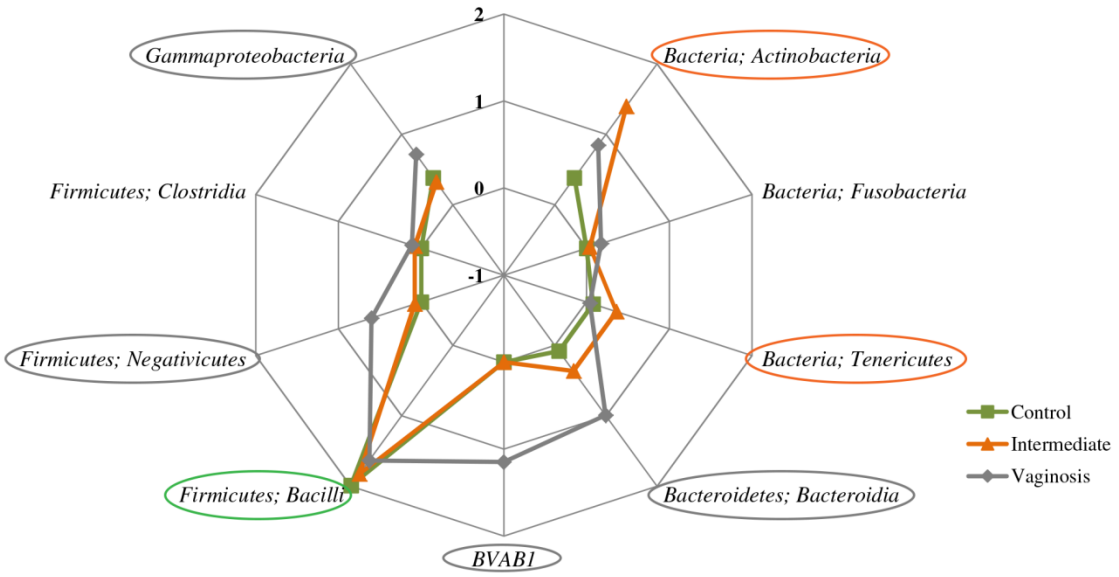

Figure 13. Taxonomic distribution at the phylum-taxonomic level. The circles highlight similarities between groups when more colors are combined or a distinctive feature when one color is used. Data are normalized to sum to 100 and $\log_{10}$ transformed.

The species within each phylum also exhibit a different pattern between cohorts (Figure 14). Among *Actinobacteria*, *Gardnerella vaginalis* and *Atopobium vaginae* were shared between the Intermediate and Vaginosis groups, *Bifidobacterium breve* was only present in Intermediate. *Alloscardovia omnicolens* (*Fusobacteria*), *Citrobacter braakii* (*Gammaproteobacteria*), and *Klebsiella granulomatis* (*Gammaproteobacteria*) were identified only in Vaginosis. Although with different relative abundance, *Ureaplasma parvum* (*Tenericutes*), *Prevotella bivia* and *amnii* (*Bacteroidia*), and *Escherichia fergusonii* (*Gammaproteobacteria*) were present both in Intermediate and Vaginosis. Among *Firmicutes*, *Lactobacilli* spp. were underrepresented in Vaginosis which exhibited

the presence of opportunistic pathogens, such as BVAB1, *Streptococcus pasteuri*, *Streptococcus anginosus*.

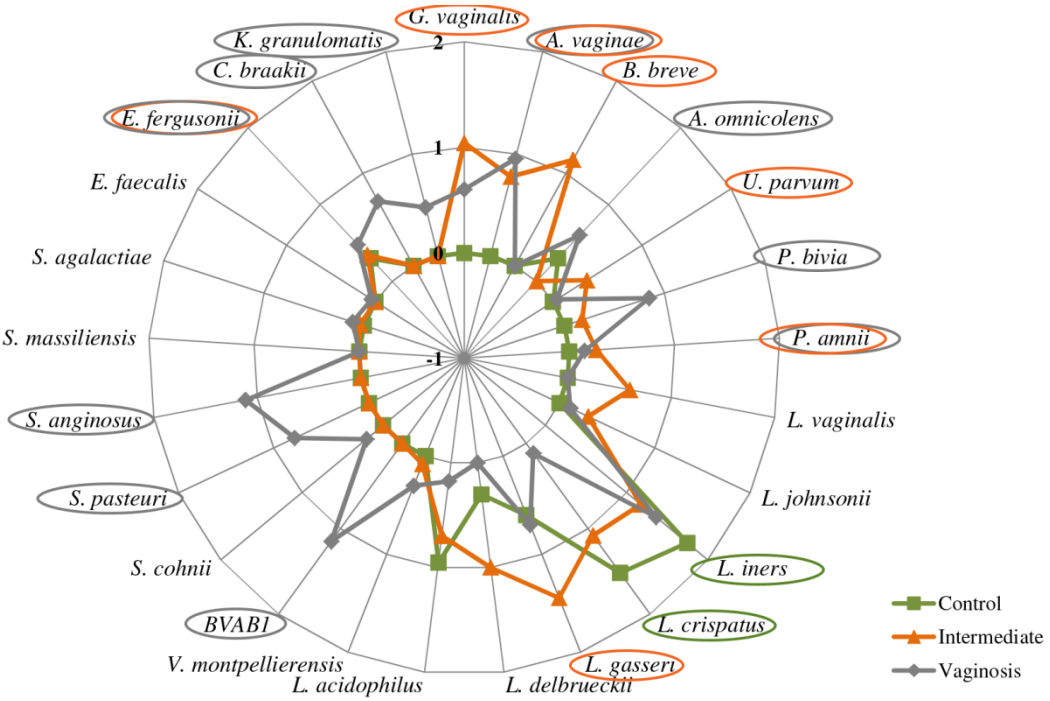

Figure 14. Taxonomic distribution at the species-taxonomic level. The circles highlight similarities between groups when more colors are combined or a distinctive feature when one color is used. Data are normalized to sum to 100 and $\log_{10}$ transformed.

Species contributing to the differences in microbial community structure

SIMPER test accounted for the species which contribute to the dissimilarities between cohorts. Table 10 compares Intermediate to Vaginosis. The main species which determine the observed differences are the *Lactobacilli gasseri*, *iners*, *crispatus* together with *B. breve*, *G. vaginalis*, *A. vaginae*, and *P. bivia*. Their contribution accounts for about the 60% of the cumulative sum of the differences.

Table 10. SIMPER analysis

Intermediate *vs* Vaginosis	**Ave-Abn**		**Ave-Cntr**	**Sd**	**Ratio**	**Cumsum**
	Intermediate	Vaginosis				
L. gasseri	26	5	14	14	0.9	15
L. iners	11	17	12	16	0.7	27
B. breve	14	0	7	13	0.5	35
G. vaginalis	9	7	6	8	0.7	42
A. vaginae	4	8	5	10	0.5	48
L. crispatus	9	0	5	7	0.7	53
P. bivia	1	6	3	6	0.5	57
L. delbrueckii	6	0	3	7	0.4	60
S. anginosus	0	5	3	7	0.3	63
L. acidophilus	5	0	3	7	0.4	66
S. pasteuri	0	5	3	10	0.2	69
BVAB1	0	5	3	10	0.2	71
C. braakii	0	5	2	9	0.2	74
S. massiliensis	0	5	2	9	0.2	76
A. omnicolens	0	4	2	6	0.4	78
E. fergusonii	2	3	2	4	0.5	81
K. granulomatis	0	4	2	7	0.3	83
P. amnii	2	1	1	4	0.3	85
S. simiae	0	2	1	4	0.3	86
S. cohnii	0	2	1	4	0.3	87
P. timonensis	0	2	1	3	0.4	88
L. johnsonii	1	1	1	3	0.4	89
U. parvum	2	0	1	2	0.6	90
L. vaginalis	2	0	1	1	0.7	92
S. sanguinegens	1	0	1	3	0.3	93
D. micraerophilus	0	2	1	1	0.7	93
V. montpellierensis	0	1	1	1	0.5	94
B. scardovii	1	0	1	2	0.3	95
F. magna	0	0	1	2	0.3	95

Ave-Abn = average abundance of the species in Intermediate *vs* Vaginosis.
Ave-Cntr = average contribution of feature to the overall dissimilarity between groups.
Sd = standard deviation
Ratio = ratio of average contribution to standard deviation of contribution.
Cumsum = cumulative contributions.
The list of features is not complete so percent values do not sum to 100%.

Microbial profiling within Idiopathic, Intermediate and Vaginosis cohorts

The microbial profile of Idiopathic was compared to Intermediate and Vaginosis cohorts (Figure 15).

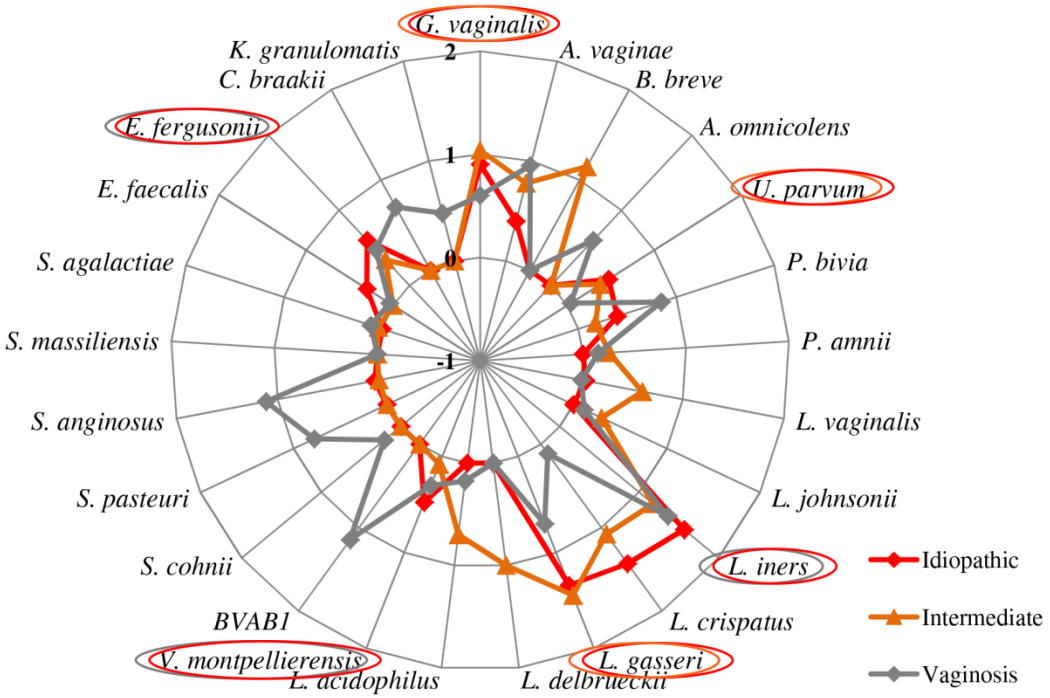

Figure 15. Taxonomic distribution at the species-taxonomic level. The circles highlight similarities between groups when more colors are combined or a distinctive feature when one color is used. Data are normalized to sum to 100 and $\log_{10}$ transformed.

SIMPER test between Idiopathic both *vs* Intermediate and Vaginosis cohorts was also performed to ascertain whether Idiopathic shows a microbial structure which is more similar to one of the upper-mentioned cohorts.

Table 11 shows the comparison between Idiopathic and Intermediate. Here, the *Lactobacilli iners*, *crispatus*, and *gasseri* explain the 60% of the cumulative sum of the

differences. *B. breve* and *G. vaginalis* contribute for an additional 20% to the cumulative sum.

Table 11. SIMPER analysis.

Idiopathic *vs* Intermediate	**Ave-Abn**		**Ave-Cntr**	**Sd**	**Ratio**	**Cumsum**
	Idiopathic	Intermediate				
L. iners	29	11	16	19	0.8	20
L. crispatus	31	9	16	17	0.9	40
L. gasseri	21	26	16	15	1	60
B. breve	0	14	7	13	0.5	68
G. vaginalis	6	9	5	5	0.9	75
L. delbrueckii	0	6	3	7	0.4	79
A. vaginae	2	4	3	4	0.6	82
L. acidophilus	0	5	2	7	0.3	85
V. montpellierensis	3	0	2	5	0.3	87
E. fergusonii	2	2	1	4	0.4	89
U. parvum	1	2	1	2	0.7	90
P. amnii	0	2	1	4	0.3	92
P. bivia	1	1	1	2	0.4	93
L. vaginalis	0	2	1	1	0.7	94
S. sanguinegens	0	1	1	3	0.3	95

Ave-Abn = average abundance of the species in Idiopathic *vs* Intermediate.
Ave-Cntr = average contribution of feature to the overall dissimilarity between groups.
Sd = standard deviation
Ratio = ratio of average contribution to standard deviation of contribution.
Cumsum = cumulative contributions.
The list of features is not complete so percent values do not sum to 100%.

Table 12 compares Idiopathic and Vaginosis. Again, *Lactobacilli iners*, *crispatus*, and *gasseri* mainly contribute to the observed differences (63%), with *G. vaginalis* and *A. vaginae* bringing the cumulative sum to the 80%.

Table 12. SIMPER analysis.

Idiopathic *vs* Vaginosis	**Ave-Abn**		**Ave-Cntr**	**Sd**	**Ratio**	**Cumsum**
	Idiopathic	Vaginosis				
L. iners	29	17	20	19	0.9	24
L. crispatus	31	0	18	20	0.9	47
L. gasseri	21	5	13	15	0.9	63
G. vaginalis	6	7	8	17	0.5	74
A. vaginae	2	8	4	7	0.6	80
P. bivia	1	6	3	1	0.3	83
S. anginosus	0	5	2	4	0.4	85
S. pasteuri	0	5	1	5	0.3	87
BVAB1	0	5	1	1	0.3	89
C. braakii	0	5	1	3	0.4	90
S. massiliensis	0	5	1	2	0.3	91
E. fergusonii	2	3	0.7	2	0.3	92
A. omnicolens	0	4	0.7	2	0.3	93
K. granulomatis	0	4	0.6	2	0.3	94
V. montpellierensis	3	1	0.6	1	0.4	95

Ave-Abn = average abundance of the species in Idiopathic *vs* Vaginosis.
Ave-Cntr = average contribution of feature to the overall dissimilarity between groups.
Sd = standard deviation
Ratio = ratio of average contribution to standard deviation of contribution.
Cumsum = cumulative contributions.
The list of features is not complete so percent values do not sum to 100%.

DISCUSSION

Although bacterial vaginosis is worldwide the most common origin of vaginal discharge, the cause remains enigmatic despite numerous studies based on microbial cultures.
Results highlighted that the Vaginosis cohort, divided into two groups (Intermediate and Vaginosis) according to the Nugent score, are distinct entities.

2.1 Microbiome within cohorts

The microbiome of Vaginosis and Intermediate is highly heterogeneous, as confirmed by the α-diversity values (Table 8). BV is characterized by a polymicrobial disequilibrium[96]. Mainly, a loss of a dominant cluster of species is evident in the altered vaginal flora. The species within the microbial structure of affected cohorts are more evenly distributed than that of Control (Equitability index), which has *Lactobacilli* as the dominant phylum. In BV, the observed drop of *Lactobacilli*[96] accounts for the greater fragmentation across the microbial ecosystem. Nonetheless, the absolute number of species is not indicative of the healthiness condition. For instance, Chao1 was very similar between the Intermediate, Vaginosis and Control (Table 8).

2.2 Microbiome across cohorts

The graphical representation of the β-diversity (difference between cohorts) suggests a specific clustering for the affected cohorts. Intermediate and Vaginosis differ for the presence/absence of many bacteria (Figure 12A). Furthermore, the shared species between Intermediate and Vaginosis show different relative abundances (Figure 12B).

The unweighted UniFrac PCoA suggests that each cohort is characterized by the presence of specific bacteria (ANOSIM, $p<0.05$). The 12% of the differences is explained by the clinical grouping (Table 9).

The weighted UniFrac PCoA suggests that the shared species differ in their relative abundance (ANOSIM, $p<0.001$) between cohorts (Table 9), explaining a higher percentage of the observed diversity (16%) than unweighted UniFrac (12%).

2.3 Profiling the microbiome

The β-diversity analysis showed a different pattern between cohorts: PCoA suggested a markedly separation between Control and both Intermediate and Vaginosis groups.

At the highest taxonomic level (phylum), the identified phyla did not account for the graphically observed differences (Figure 13). Although *Actinobacteria* and *Gammaproteobacteria* are predominant in Intermediate, while BVAB1 and *Negativicutes* in Vaginosis, their distributions are not significantly different between cohorts.

The Intermediate and Vaginosis cohorts are distinguishable when accounting for the species level composition (Figure 14). SIMPER test showed the species which contribute to the observed differences between Intermediate and Vaginosis cohorts (Table 10).

The interplay between *Lactobacillus gasseri* and *Atopobium vaginae* explains some of the observed differences between the Intermediate and Vaginosis. Intermediate exhibits a higher level of *L. gasseri*, which shows a negative correlation with *A. vaginae*[98]. Thus, the high amount of *A. vaginae* characterizes the Vaginosis status, together with the low amount of *L. gasseri*.

Bifidobacterium breve is another marker suitable to characterize the Intermediate cohort. *B. breve* is higher in Intermediate than Vaginosis. The presence of *B. breve* can account for

the production of acid lactic[99], despite the general decrease of *Lactobacilli*. Furthermore, the Nugent score takes into account only the decrease of *Lactobacilli*. In the case of presence of *Bifidobacteria,* which can support the normal production of acid lactic in the vaginal environment, the Nugent score overstates the dysbiosis.

Ureaplasma parvum is specifically identified in the Intermediate group, although at low relative abundance. *U. parvum* does not seem to cause symptoms in females, but its role in the female urogenital tract remains unknown[100]. Since the vast majority of asymptomatic presence of bacteria possibly associates to adverse pregnancy outcomes, and taking into account the ascending capability of *U. parvum* to the upper reproductive tract, a deeper study of the role of *U. parvum* is needed[100].

Discerning among the relative abundances of the *Lactobacillus* spp. in the vaginal ecosystem is more useful than assessing the vaginal healthiness basing solely on the total amount of *Lactobacilli*. For instance, although high levels of *Lactobacillus crispatus* are linked to the inhibition of the growth of *Gardnerella vaginalis*[101], Intermediate has higher level of both bacteria. On the other hand, *L. iners* enhances the adhesion of *G. vaginalis* strain 101 to cervical epithelial cells and, on the contrary, does not affect the adhesion of strain 5-1[102]. Taxonomic assignment against the Vaginal 16S rDNA Reference Database spotted *G. vaginalis* ATCC 14019/317 strain. It is likely that *L. iners*, which is higher in Intermediate, can interfere with the inhibition activity of *L. crispatus,* and enhance the attachment also of the strain 317. In Control, despite the higher amount of both *L. crispatus* and *L. iners*, *L. acidophilus* exerts a protective role against the overgrowth of *G. vaginalis* by the secretion of a specific bacteriocin[103].

2.4 Comparison between Intermediate flora and Vaginosis

Basing on the composition at the specie level within Intermediate and Vaginosis, the two cohorts cannot be considered as two strictly sequential phases. Intermediate will not always evolve into a Vaginosis status.

Intermediate seems to arise from a dysbiosis of resident bacteria, while Vaginosis is sustained by opportunistic pathogens. Among the opportunistic pathogens, Vaginosis exhibits *BVAB1*, *Streptococcus anginosus*, *Prevotella bivia*, *Klebsiella granulomatis*, *Alloscardovia omnicolens*, and *Staphylococcus pasteuri*.

2.5 The link between idiopathic infertility and the affected microbiome

SIMPER test revealed the species that contribute to the differences between Idiopathic and both Intermediate and Vaginosis (Table 10).

Among *Lactobacilli*, Idiopathic shows more similarities with Intermediate. While in the comparison between Idiopathic and Intermediate, *Lactobacilli* exert each a 16% average contribution to the observed differences, in Vaginosis the contribution to the differences is slightly dominated by the decrease of *L. iners* and the absence of *L. crispatus*. Considering Vaginosis as the point of reference of an altered microbial vaginal environment, *L. iners* confirms its role as biomarker of healthiness, as previously discussed.

Furthermore, both Idiopathic and Intermediate exhibit a higher presence of *L. gasseri*, which is able to inhibit *Atopobium* and *L. iners*[98] but it is also involved in early embryo demise[59].

The secondary leading difference between Intermediate and Idiopathic is the presence of *B. breve*. *B. breve* is able to produce acid lactic and can increase the pregnancy rate in Intermediate, despite the high load of *L. gasseri*. In fact, as widely accepted, *Bifidobacteria*

exert health-promoting effects in the genito-urinary tract[99], where they can also help the positive IVF procedures outcome.

Intermediate and Idiopathic share the presence of *Ureaplasma parvum*. Since urogenital mycoplasmas can affect fertility[104], screening for asymptomatic individuals and treatment of infected ones looks necessary.

CONCLUSIONS

Bacterial vaginosis (BV) is particularly prevalent in patients with infertility[36,105].

A first step toward the comprehension of microbiome alteration in infertility consists in a deeper study of a better known clinical condition, such as BV.

Dividing the BV into two cohorts, according to the Nugent score, highlighted microbial differences between Intermediate and Vaginosis. Intermediate seems to be linked mainly to an imbalance across vaginal resident bacteria; Vaginosis is sustained by the alteration of the vaginal microbiota structure together with the action of opportunistic pathogens.

The comparison between vaginal microbiota of Idiopathic and both Intermediate and Vaginosis shed the light on a possible contribution of specific bacteria to infertility.

Mainly, the interplay among *Lactobacilli iners*, *crispatus*, and *gasseri* creates a favorable environment for pregnancy. An imbalanced equilibrium among *Lactobacilli* is linked to the loss of protection against opportunistic pathogens.

In Intermediate affected vaginal microbiome, *Bifidobacterium breve* can exert a supportive role to counteract the imbalance of *Lactobacilli*. Idiopathic lacks of species acting as health promoter of the vaginal niche when *Lactobacilli* drop.

CONCLUDING REMARKS

The importance of the physiologic role of microbiome in the human body is widely recognized. The female reproductive tract shows a complex microbiome, whose alteration may provide insight into previously unexplained infertility treatment failure.

Women affected with idiopathic infertility exhibit an altered vaginal microbial structure.

- Idiopathic shows a decrease of normally dominant *Lactobacilli* and the presence of bacteria which can act as secondary players in the context of an altered vaginal microbiome.
- Taking into account the impact that the microbiome can exert on IVF procedures, further studies on large cohorts are needed to highlight strong correlations between idiopathic infertility and the affected microbiome.
- The microbial species present in the vaginal environment impact the modulation of local immune responses, for instance, by the production of short-chain fatty acids (SCFAs). The amount of anaerobic bacteria, identified in vaginal milieu of Idiopathic, suggests their involvement in the alteration of a proper immune response, directly linked to the loss of fetal tolerance and to the inability of pregnancy establishment.
- The ultimate aim is to introduce the microbial and immune profiling of infertile couples as a diagnostic tool in order to select a tailored IVF protocol.

Idiopathic means “without an evident explanation”. The inability to figure out the causes that lead to idiopathic infertility does not mean that a cause is not present. Extensive research should be conducted to better define biological mechanisms affecting conception.

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