Evidence that the endometrial microbiota has an effect on implantation success or failure

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BACKGROUND: Bacterial cells in the human body account for 1−3% of total body weight and are at least equal in number to human cells. Recent research has focused on understanding how the different bacterial communities in the body (eg, gut, respiratory, skin, and vaginal microbiomes) predispose to health and disease. The microbiota of the reproductive tract has been inferred from the vaginal bacterial communities, and the uterus has been classically considered a sterile cavity. However, while the vaginal microbiota has been investigated in depth, there is a paucity of consistent data regarding the existence of an endometrial microbiota and its possible impact in reproductive function.

OBJECTIVE: This study sought to test the existence of an endometrial microbiota that differs from that in the vagina, assess its hormonal regulation, and analyze the impact of the endometrial microbial community on reproductive outcome in infertile patients undergoing in vitro fertilization.

STUDY DESIGN: To identify the existence of an endometrial microbiota, paired samples of endometrial fluid and vaginal aspirates were obtained simultaneously from 13 fertile women in prereceptive and receptive phases within the same menstrual cycle (total samples analyzed n = 52). To investigate the hormonal regulation of the endometrial microbiota during the acquisition of endometrial receptivity, endometrial fluid was collected at prereceptive and receptive phases within the same cycle from 22 fertile women (n = 44). Finally, the reproductive impact of an altered endometrial microbiota in endometrial fluid was assessed by implantation, ongoing pregnancy, and live birth rates in 35 infertile patients undergoing in vitro fertilization (total samples n = 41) with a receptive endometrium diagnosed using the endometrial receptivity array. Genomic DNA was obtained either from endometrial fluid or vaginal aspirate and sequenced by 454 pyrosequencing of the V3−V5 region of the *16S* ribosomal RNA (rRNA) gene; the resulting sequences were taxonomically assigned using QIIME. Data analysis was performed using R packages. The χ^2 test, Student *t* test, and analysis of variance were used for statistical analyses.

RESULTS: When bacterial communities from paired endometrial fluid and vaginal aspirate samples within the same subjects were interrogated, different bacterial communities were detected between the uterine cavity and the vagina of some subjects. Based on its composition, the microbiota in the endometrial fluid, comprising up to 191 operational taxonomic units, was defined as a *Lactobacillus*-dominated microbiota (>90% *Lactobacillus* spp.) or a non-*Lactobacillus*-dominated microbiota (<90% *Lactobacillus* spp. with >10% of other bacteria). Although the endometrial microbiota was not hormonally regulated during the acquisition of endometrial receptivity, the presence of a non-*Lactobacillus*-dominated microbiota in a receptive endometrium was associated with significant decreases in implantation [60.7% vs 23.1% ($P = .02$)], pregnancy [70.6% vs 33.3% ($P = .03$)], ongoing pregnancy [58.8% vs 13.3% ($P = .02$)], and live birth [58.8% vs 6.7% ($P = .002$)] rates.

CONCLUSION: Our results demonstrate the existence of an endometrial microbiota that is highly stable during the acquisition of endometrial receptivity. However, pathological modification of its profile is associated with poor reproductive outcomes for in vitro fertilization patients. This finding adds a novel microbiological dimension to the reproductive process.

Key words: assisted reproductive techniques, bacterial pathogens, embryo implantation, endometrial microbiota, endometrial receptivity array

Introduction

Bacteria present in the urogenital tract make up 9% of the total human microbiota,[1,2] and most of them are not easily culturable. The vaginal microbiota was first identified in 2002 by molecular methods used to detect nonculturable bacteria.[3,4] A normal vaginal microbiota is defined by the presence of bacterial species (spp.) of the *Lactobacillus* genus that are commonly associated with a healthy genitourinary status. The vaginal microbiota typically changes throughout the menstrual cycle, depending on factors such as vaginal hygiene, sexual activity, use of intimate products, and underwear composition; greater microbiota stability is associated with the estradiol peak at ovulation and progesterone rise in the midluteal phase.[5] However, alterations in the vaginal microbiota can lead to several pathologies. For example, bacterial vaginosis (BV) is a vaginal syndrome produced by the overgrowth of anaerobic bacteria such as *Atopobium vaginae*, *Gardnerella vaginalis*, *Mobiluncus curtisii*, and *Mycoplasma hominis* to the detriment of *Lactobacillus* spp.[3,6]

Further, the vaginal microbiota has been shown to be different in pregnant and nonpregnant women in terms of stability and composition,[7] demonstrating that the vaginal microbiota could have implications for reproductive[8] and obstetrical[9] processes. BV has been associated with obstetric complications including early and late miscarriage rates[10,11] and preterm birth.[12] Interestingly, a microbiological culture of the tip of the transfer catheter in patients undergoing in vitro fertilization (IVF) revealed that the presence of bacterial species in the uterine cavity at the time of embryo transfer negatively affects implantation and pregnancy rates. Indeed, *Enterobacteriaceae* spp., *Streptococcus* spp., *Staphylococcus* spp., *Escherichia coli*, and Gram-negative bacteria have been associated with decreased

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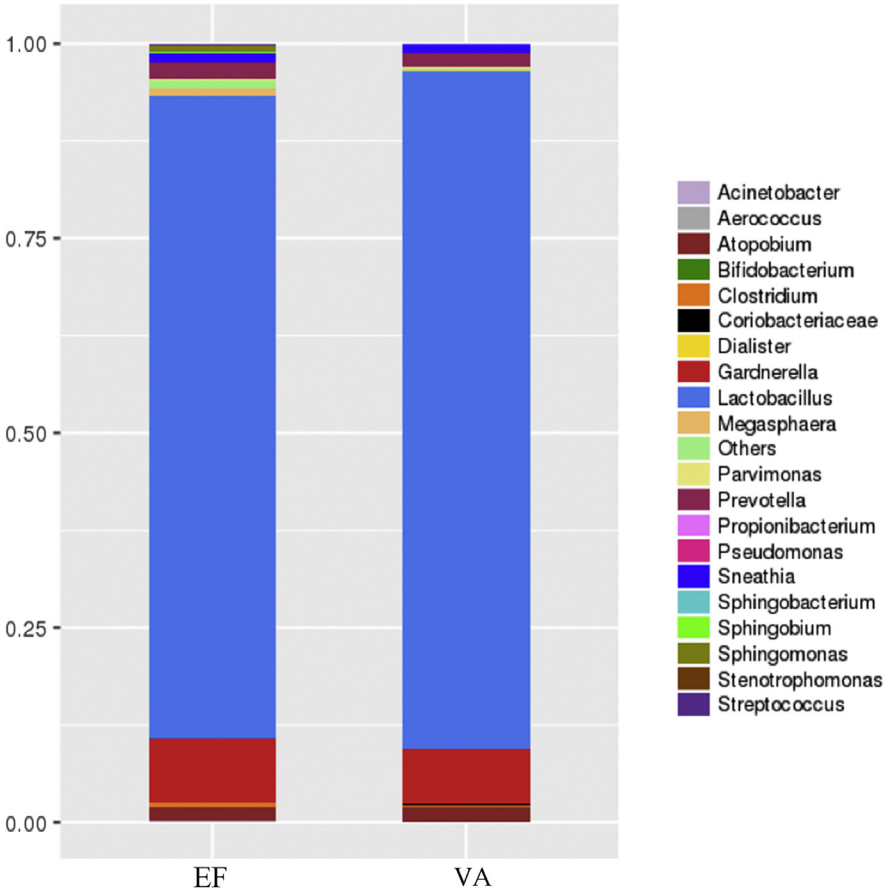
implantation rates and poor pregnancy outcomes,[13-17] but no consensus has been reached regarding the origin and genus of bacterial pathogens and the mechanisms by which they could interfere with embryonic implantation. Importantly, prior studies were limited by the number of bacterial species that can be isolated and identified following culture of the catheter tip, and the potential risk of contamination of the catheter tip in the vagina/ectocervix/endocervix.

Although the vagina has long been known to contain microbes, the uterine cavity was classically considered a sterile organ.[18] A report challenging this dogma suggested the existence of an endometrial microbiota comprising different microorganisms (*Lactobacillus* spp., *Mycoplasma hominis*, *Gardnerella vaginalis*, and *Enterobacter* spp.) isolated by classic microbiological culture techniques of endometrial samples obtained from hysterectomy.[19] Recently, the molecular identification of bacterial species in the endometrium of asymptomatic patients undergoing hysterectomy for benign indications confirmed that the uterine cavity is not sterile.[20] Although a pathological infection is not always produced by the host-microbiota interactions, a murine model of ascending bacterial infection supports the concept that the endometrium might not be as sterile as thought.[21] Given that changes in the human microbiota have been linked to various disease states,[22] the potential for an endometrial microbiota that contributes to reproductive health merits investigation.

Here, we investigated the existence of a differentiated endometrial microbiota using 16S *rRNA* gene pyrosequencing, and assessed its hormonal regulation and potential functional impact on reproductive outcome in patients undergoing IVF. The findings imply a role for the endometrial microbiota in reproductive outcomes.

FIGURE 1
Bacterial communities in endometrial and vaginal microbiota of fertile subjects



Bar charts showing mean values of 20 most abundant operational taxonomic units in endometrial fluid (EF) and vaginal aspirates (VA) of 26 paired samples from 13 fertile subjects. Technical filtering was performed on data produced by QIIME: ribosomal database project score <0.9 and ≤ 2 reads were filtered. Plot is based on filtered data. Others: *Bradyrhizobium*, *Desulfovibrio*, *Brachybacterium*, *Sporichthyaceae*, *Pseudoramibacter_Eubacterium*, *Lactobacillales*, *Facklamia*, *Scardovia*, *Rhizobium*, *Citrobacter*, JG30-KF-CM45, Micrococcaceae, *Cellulosimicrobium*, *Mycobacterium*, *Bacillus*, WCHB1-84, *Escherichia*, *Ruminococcus*, *Actinobaculum*, Rikenellaceae, Gemellaceae, [Weeksellaceae], *Coprococcus*, *Comamonas*, *Neisseria*, Clostridiales, *Dietzia*, *Varibaculum*, *Microbacterium*, *Ureaplasma*, *Faecalibacterium*, *Arcanobacterium*, *Streptophyta*, MLE1-12, *Parabacteroides*, *Granulicatella*, *Azospirillum*, *Fusobacterium*, 1-68, *Pseudoxanthomonas*, *Porphyromonas*, *Kocuria*, Clostridiales, *Methylobacterium*, *Ochrobactrum*, *Haemophilus*, *Listeria*, *Agrobacterium*, *Luteibacter*, *Peptostreptococcus*, *Actinomyces*, *Blastomonas*, [*Ruminococcus*], *Gemella*, WAL_1855D, *Micrococcus*, *Blautia*, *Veillonella*, *Actinobacillus*, *Staphylococcus*, *Finegoldia*, *Mycoplasma*, *Achromobacter*, *Anaerococcus*, *Paracoccus*, *Ralstonia*, *Corynebacterium*, *Enhydrobacter*, *Mobiluncus*, *Peptoniphilus*.

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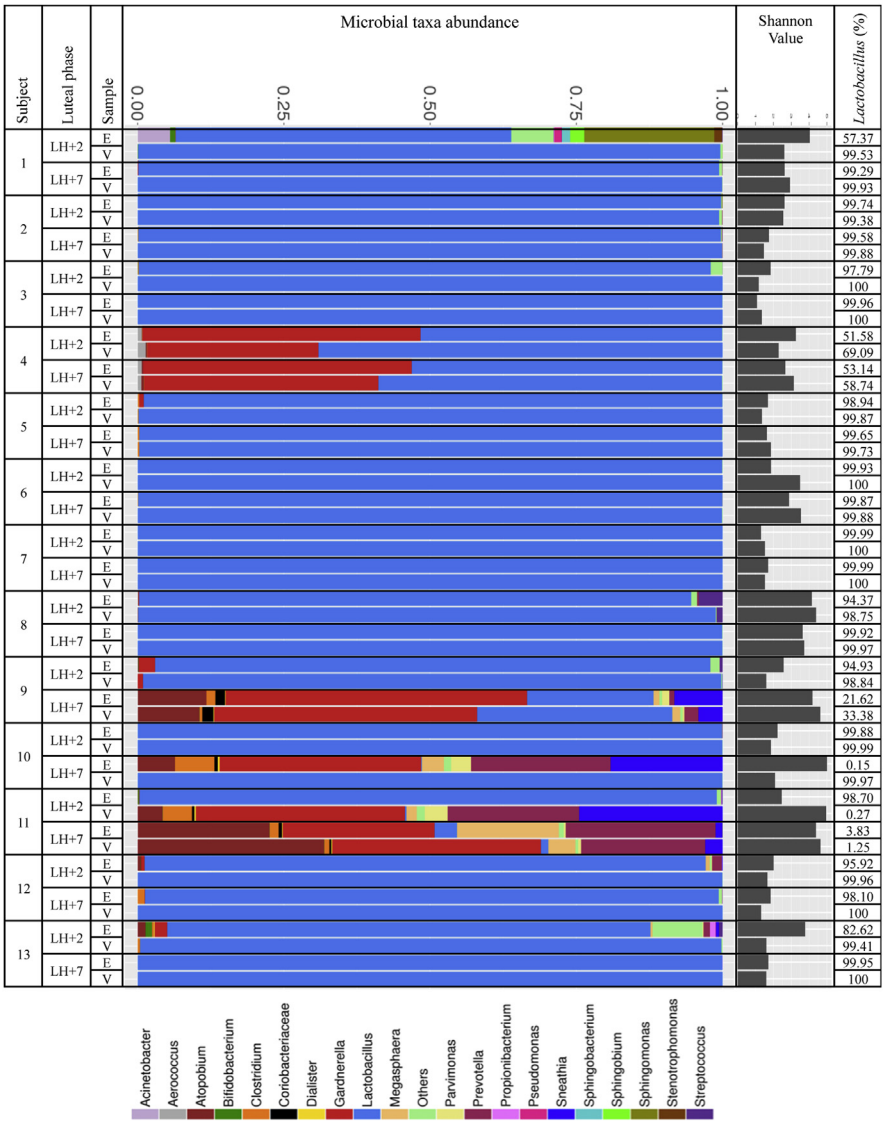
Materials and Methods

Study design

Three separate prospective pilot studies were performed. First, to analyze the existence of a differential endometrial microbiota, paired samples of endometrial fluid (EF) and vaginal aspirates (VA) were obtained simultaneously from 13 fertile women in their prereceptive (two days after the luteinizing hormone surge, known as LH+2) and receptive (seven days after the uteinizing hormone surge, also known as LH+7) phases in the same menstrual natural cycle (total samples analyzed n = 52). To investigate the hormonal regulation of the endometrial microbiota, EF samples (n = 44) were collected at LH+2

FIGURE 2
Endometrial vs vaginal microbiota in asymptomatic subjects



Microbiotic profiles showing microbial taxa composition, relative abundance of endometrial fluid (E) and vaginal aspirate (V) samples, and alpha diversity index represented as Shannon value, assessed simultaneously in prereceptive and receptive phase of 13 fertile subjects. Twenty most representative operational taxonomic units are shown. Technical filtering was performed on data produced by QIIME: ribosomal database project score <0.9 and ≤ 2 reads are filtered. Plot is based on filtered data. Others: *Bradyrhizobium*, *Desulfovibrio*, *Brachybacterium*, Sporichthyaceae, *Pseudoramibacter_Eubacterium*, *Lactobacillales*, *Facklamia*, *Scardovia*, *Rhizobium*, *Citrobacter*, JG30-KF-CM45, Micrococcaceae, *Cellulosimicrobium*, *Mycobacterium*, *Bacillus*, WCHB1-84, *Escherichia*, *Ruminococcus*, *Actinobaculum*, Rikenellaceae, Gemellaceae, [*Weeksellaceae*], *Coprococcus*, *Comamonas*, *Neisseria*, *Clostridiales*, *Dietzia*, *Varibaculum*, *Microbacterium*, *Ureaplasma*, *Faecalibacterium*, *Arcanobacterium*, *Streptophyta*, MLE1-12, *Parabacteroides*, *Granulicatella*, *Azospirillum*, *Fusobacterium*, 1-68, *Pseudoxanthomonas*, *Porphyromonas*, *Kocuria*, Methylobacteriaceae, *Methylobacterium*, *Ochrobactrum*, *Haemophilus*, *Listeria*, *Agrobacterium*, *Luteibacter*, *Peptostreptococcus*, *Actinomyces*, *Blastomonas*, [*Ruminococcus*], *Gemella*, *WAL_1855D*, *Micrococcus*, *Blautia*, *Veillonella*, *Actinobacillus*, *Staphylococcus*, *Finegoldia*, *Mycoplasma*, *Achromobacter*, *Anaerococcus*, *Paracoccus*, *Ralstonia*, *Corynebacterium*, *Enhydrobacter*, *Mobiluncus*, *Peptoniphilus*.

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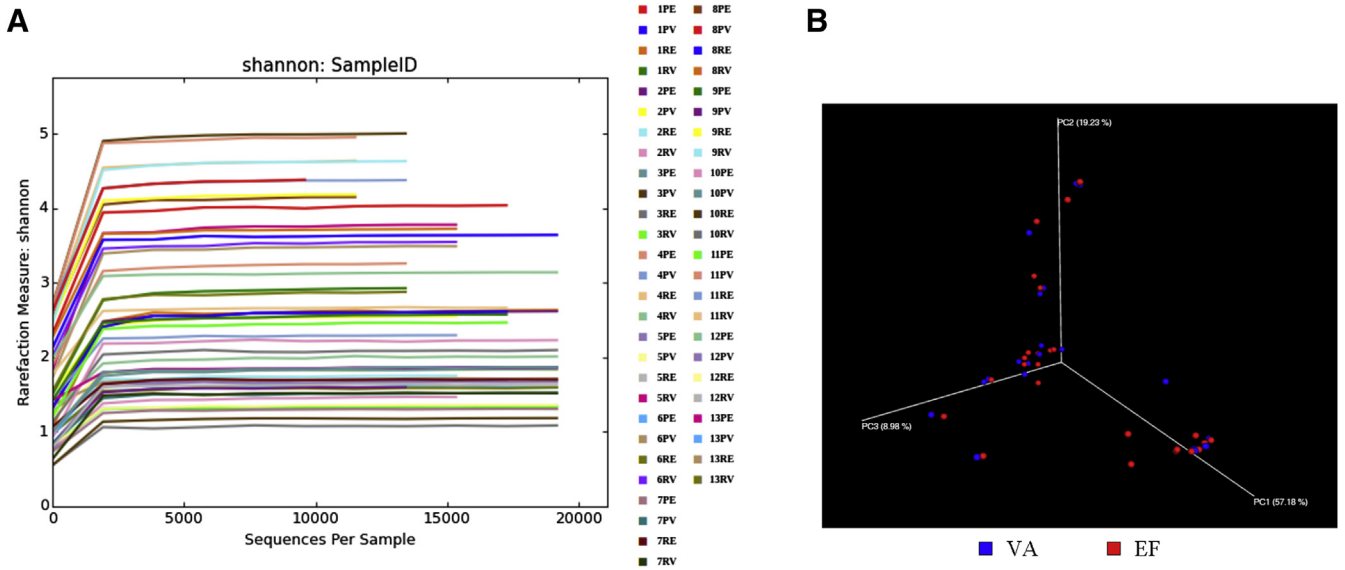
FIGURE 3
Bacterial community in pilot study 1



A, Shannon index rarefaction curves for each sample. Prereceptive (two days after the luteinizing hormone surge, LH+2). Receptive (seven days after the luteinizing hormone surge, LH+7). **B**, Principal coordinate analysis plot calculated based on Bray-Curtis distances.

EF, endometrial fluid; *VA*, vaginal aspirate.

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and LH+7 within the same natural cycle from 22 fertile women. Finally, the reproductive impact of an altered endometrial microbiota in EF was assessed by implantation, miscarriage, ongoing pregnancy, and live birth rates in infertile subjects undergoing IVF (n = 35) in whom a receptive endometrium was diagnosed using the endometrial receptivity array (ERA). EF samples were obtained in the cycle before embryo transfer. Approval for this study was obtained from the IVI Valencia Ethical Committee (project: 1404-FIVI-015-CS), and subjects provided written informed consent.

Subjects

For comparison between vaginal and endometrial bacterial communities and the study of hormonal regulation of the endometrial microbiota, samples were obtained within the natural cycles of women from the ovum donation program at IVI Valencia, Spain. The functional impact of the endometrial microbiota on reproductive outcome was explored in infertile subjects undergoing IVF treatment in whom a receptive endometrium was diagnosed by ERA (Igenomix SL, Valencia, Spain). A more detailed description is provided in Supplementary Methods.

EF aspiration

EF was obtained in all subjects as previously described.[23]

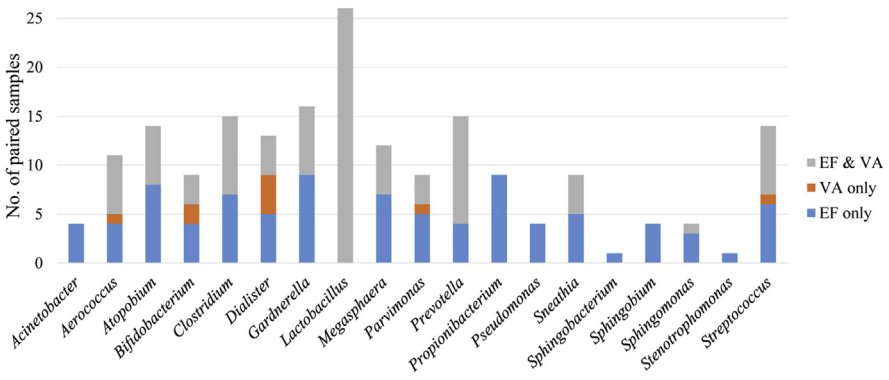
VA collection

In the first pilot study, 20−80 μL of VA were collected from the posterior vagina before EF aspiration using a sterile catheter under direct vision.

Endometrial receptivity diagnosis

Endometrial receptivity was diagnosed using the ERA as previously described.[24]

FIGURE 4
Distribution of endometrial and vaginal microbiota in paired samples



Number of pair samples with detection of most abundant operational taxonomic units in vaginal aspirate alone (VA), endometrial fluid (EF) alone, and in both VA and EF.

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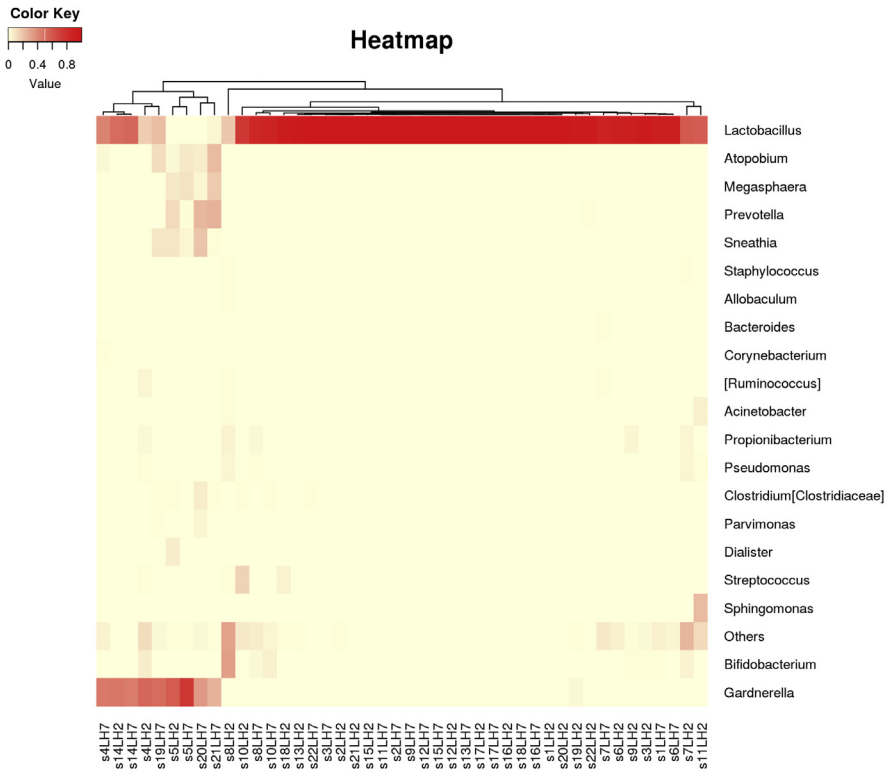
FIGURE 5
Endometrial microbiota distribution during the acquisition of endometrial receptivity



Clustering of individual samples showed 2 groups depending on abundance of *Lactobacillus* OTUs. Technical filtering was performed on data produced by QIIME: ribosomal database project score <0.9 and ≤2 reads are filtered. All plots are based on filtered data. "Others" are filtered OTUs + remaining OTUs.

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A more detailed protocol is provided in Supplementary Methods.

Genomic DNA isolation from EF and VA samples

Isolation of DNA from frozen EF or VA was performed following the MagNa Pure compact nucleic acid isolation kit I (Roche, Madison, WI) protocol with modifications. A more detailed protocol is provided in Supplementary Methods.

Polymerase chain reaction and 16S rRNA sequencing

For sequencing and barcoding, the V3-V5 region of the *16S rRNA* gene was amplified using key-tagged eubacterial primers, as previously described.[25] Unidirectional pyrosequencing was carried out on a 454 Life Sciences GS FLX+ instrument (Roche) following the Roche Amplicon Lib-L according to manufacturer's protocol. A more detailed protocol for polymerase chain reaction is described in Supplementary Methods.

Taxonomic assignment and bioinformatics

Taxonomic classifications

Sequences were treated for quality control prior to taxonomic classification (Supplementary Methods). QIIME[26] was utilized to produce operational taxonomic units (OTU) clusters and classifications. All the processed *16S rRNA* sequences were clustered into OTUs based on their sequence similarity using UCLUST[27] algorithm, setting the sequence similarity threshold to 0.97. For each OTU, a representative sequence was selected for downstream analysis. Taxonomy was assigned to each representative sequence using the ribosomal database project classifier method v2.2.[28-30] The taxonomic assignments that resulted in no more than 2 sequence reads assigned to a genus, and with a mean ribosomal database project score of <0.9, were considered to be low-quality and were excluded from the community structure analysis. This filtering eliminated a total of 94 low-quality genus assignments for pilot 1, 164 for pilot 2, and 147 for pilot 3. All sequence data were deposited in http://www.ncbi.nlm.nih.gov/sra/ under the SRP078557 accession.

Alpha diversity

QIIME was used to calculate alpha diversity and rarefaction curves before filtering. Shannon[29] and Simpson[30] methods were employed to analyze the biodiversity within a group of samples. Plots were generated by QIIME, with the number of sequences on the x-axis and the corresponding alpha diversity index on the y-axis. In rarefaction curves, if the lines for some categories do not extend, that means that at least one of the samples in that category does not reach that number of sequences. The shape on the horizontal axis plot serves as an indicator of richness: greater y-axis values indicate more species richness and lower values indicate the opposite.

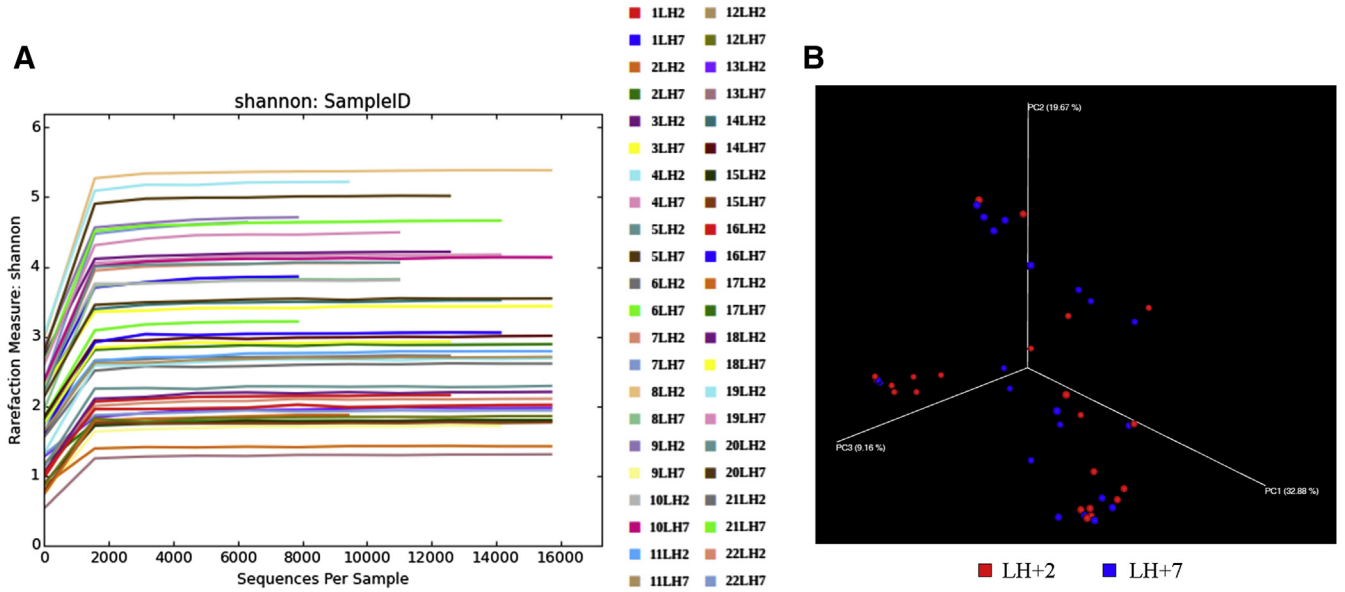
Community clustering analysis

The clustering of communities was done using Bray-Curtis distance and hierarchical clustering with R vegan package.

Principal component analysis of microbial communities

Principal component analysis (PCA) was generated using the prcomp routine in the R package on a data set consisting of the percentage abundances of taxa in each community. The 2 principal components explained 88% of the variance. Principal coordinates analysis (PCoA) plots were generated using Bray-Curtis distances.

FIGURE 6
Bacterial community in pilot study 2



A, Shannon index rarefaction curves for each sample. **B**, Principal coordinate analysis plot calculated based on Bray-Curtis distances. Prereceptive (LH+2); Receptive (LH+7).

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Statistical analysis

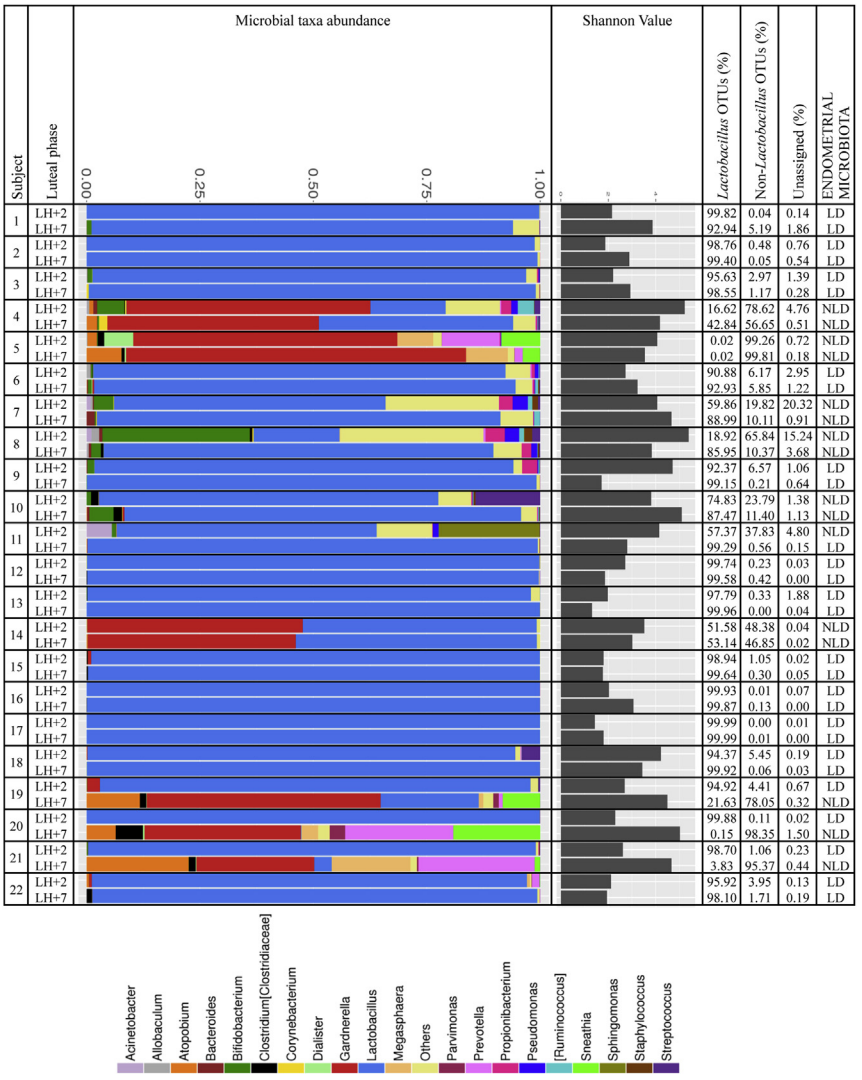
Statistical analysis on bacterial taxonomic identification was performed using R version 3.1.1. Supervised machine learning models were performed in R (http://www.R-project.org) using different packages from CRAN, ROCR for receiver operating characteristic curves, rpart for classification and regression tree (CART), and generalized linear model for logistic regression. A comparison of quantitative variables was done using Student *t* test or analysis of variance test for independent samples (depending on whether ≥2 groups were compared). The Adonis function in the R package vegan was used to conduct nonparametric multivariate analysis of variance. For comparing categorical data, χ^2 test was performed. $P < .05$ was considered statistically significant.

Results

Endometrial and vaginal microbiota differ in some asymptomatic subjects

To assess any potential experimental bias due to the primer set used in our study, the microbial communities identified in VA using the V3−V5 hypervariable regions of the *16S rRNA* gene (Figure 1, right column) were compared to the bacterial taxa previously reported in vaginal samples by other groups using the V1-V2 hypervariable regions.[6] The results demonstrate the identification of the same bacterial OTUs in vaginal samples independent of the set of primers used for sequencing (OTU list is detailed in Table S1). Then, to test the existence of a differential endometrial microbiota, paired samples of EF (n = 26) and VA (n = 26) from 13 fertile subjects were obtained and their bacterial communities investigated by pyrosequencing of the variable regions V3-V5 of the bacterial *16S rRNA* gene. Different OTUs were identified between endometrial and vaginal samples (Figures 1, 2, and 3, A, and Table S1). From them, 9 samples were colonized only by *Lactobacillus* spp., while the rest of the samples were colonized by combinations of different OTUs in addition to *Lactobacillus*. Only 2 of 26 pairs of samples showed the presence of the same bacterial OTUs in endometrium and vagina (Table S1). In the remaining 24 pairs of samples, small differences between endometrial and vaginal microbiota were found with OTUs present in endometrium but not in the vagina, and vice versa, although some of those differences were nearly imperceptible and only contributed small percentages (Figure 4). Interestingly, in 6 paired samples the bacterial communities in the endometrium and vagina were completely different, with a high proportion of potential pathogens belonging to *Atopobium*, *Clostridium*, *Gardnerella*, *Megasphaera*, *Parvimonas*, *Prevotella*, *Sphingomonas*, or *Sneathia* genera found in the endometrium but not in the vagina (n = 5: subject 1 at LH+2, subject 4 at LH+2, subject 9 at LH+7, subject 10 at LH+7, and subject 13 at LH+2), or potential bacterial pathogens (*Gardnerella*, *Clostridium*, *Sneathia*, or *Prevotella* spp.) in the vagina that were not present in the endometrium (n = 1: subject 11 at LH+2) (Figure 2). Combinations of up to 54 bacterial OTUs formed the microbial endometrial communities, while in vagina we detected up to 20 genera. *Lactobacillus* was found as the major

FIGURE 7
Endometrial microbiota stability during acquisition of endometrial receptivity



Subject	Luteal phase	*Lactobacillus* OTUs (%)	Non-*Lactobacillus* OTUs (%)	Unassigned (%)	ENDOMETRIAL MICROBIOTA
1	LH+2	99.82	0.04	0.14	LD
	LH+7	92.94	5.19	1.86	LD
2	LH+2	98.76	0.48	0.76	LD
	LH+7	99.40	0.05	0.54	LD
3	LH+2	95.63	2.97	1.39	LD
	LH+7	98.55	1.17	0.28	LD
4	LH+2	16.62	78.62	4.76	NLD
	LH+7	42.84	56.65	0.51	NLD
5	LH+2	0.02	99.26	0.72	NLD
	LH+7	0.02	99.81	0.18	NLD
6	LH+2	90.88	6.17	2.95	LD
	LH+7	92.93	5.85	1.22	LD
7	LH+2	59.86	19.82	20.32	NLD
	LH+7	88.99	10.11	0.91	NLD
8	LH+2	18.92	65.84	15.24	NLD
	LH+7	85.95	10.37	3.68	NLD
9	LH+2	92.37	6.57	1.06	LD
	LH+7	99.15	0.21	0.64	LD
10	LH+2	74.83	23.79	1.38	NLD
	LH+7	87.47	11.40	1.13	NLD
11	LH+2	57.37	37.83	4.80	NLD
	LH+7	99.29	0.56	0.15	LD
12	LH+2	99.74	0.23	0.03	LD
	LH+7	99.58	0.42	0.00	LD
13	LH+2	97.79	0.33	1.88	LD
	LH+7	99.96	0.00	0.04	LD
14	LH+2	51.58	48.38	0.04	NLD
	LH+7	53.14	46.85	0.02	NLD
15	LH+2	98.94	1.05	0.02	LD
	LH+7	99.64	0.30	0.05	LD
16	LH+2	99.93	0.01	0.07	LD
	LH+7	99.87	0.13	0.00	LD
17	LH+2	99.99	0.00	0.01	LD
	LH+7	99.99	0.01	0.00	LD
18	LH+2	94.37	5.45	0.19	LD
	LH+7	99.92	0.06	0.03	LD
19	LH+2	94.92	4.41	0.67	LD
	LH+7	21.63	78.05	0.32	NLD
20	LH+2	99.88	0.11	0.02	LD
	LH+7	0.15	98.35	1.50	NLD
21	LH+2	98.70	1.06	0.23	LD
	LH+7	3.83	95.37	0.44	NLD
22	LH+2	95.92	3.95	0.13	LD
	LH+7	98.10	1.71	0.19	LD

Microbiotic profiles showing microbial taxa composition, relative abundance of endometrial fluid samples in prereceptive (luteinizing hormone (two days after the luteinizing hormone surge, LH+2) and receptive (seven days after the luteinizing hormone surge, LH+7) phase of 22 fertile subjects, and alpha diversity index calculated as Shannon value. Depending on percentage of *Lactobacillus* operational taxonomic units (OTUs), *Lactobacillus*-dominated (LD) or non-LD (NLD) endometrial microbiota profile was assigned to each sample. Twenty most representative OTUs are shown. Technical filtering was performed on data produced by QIIME: ribosomal database project score <0.9 and ≤ 2 reads are filtered. Plot is based on filtered data. Others: *Scardovia*, *Brachybacterium*, Sporichthyaceae, *Alloiococcus*, Oxalobacteraceae, *Dokdonella*, JG30-KF-CM45, *Arcanobacterium*, Veillonellaceae, S085, *Mucispirillum*, SMB53, Ellin6075, *Burkholderia*, *Alicyclobacillus*, *Cellulosimicrobium*, *Hydrogenophaga*, *Leucobacter*, *Pseudoramibacter_Eubacterium*, *Facklamia*, *Rhodovibrio*, *Inquilinus*, Ellin6529, *Mycobacterium*, *Balneimonas*, MLE1-12, *Azospirillum*, *Varibaculum*, *Abiotrophia*, *Tepidimonas*, [*Eubacterium*], [*Weeksellaceae*], Methylobacteriaceae, Porphyromonadaceae, Sphingobacteriales, *Eikenella*, *Dermabacter*, *Chryseobacterium*, *Carboxydocella*, *Comamonas*, *Capnocytophaga*, KSA1, *Propionimicrobium*, iii1-15, *Ochrobactrum*, *Georgenia*, *Bilophila*, *Moraxella*, Cytophagaceae, *Alistipes*, *Luteibacter*, *Moryella*, *Odoribacter*, RB41, *Rikenella*, *Salinicoccus*, Caldilineaceae, *Desulfovibrio*, *Enhydrobacter*, *Thermoanaerobacterium*, *Propionicimonas*, *Granulicatella*, *Cloacibacterium*, *Porphyromonas*, *Marinobacter*, *Pseudoclavibacter*, *Bartonella*, *Actinobaculum*, WAL_1855D, *Microbacterium*, *Agrobacterium*, *Rhodoplanes*, *Parabacteroides*, *Phascolarctobacterium*, *Peptostreptococcus*, *Variovorax*, *Diaphorobacter*, *Flavisolibacter*, *Methylobacterium*, *Thermicanus*, *Proteus*, *Coprococcus*, *Hymenobacter*, *Dorea*, *Sutterella*, *Lachnospira*, *Nitrospira*, *Collinsella*, *Fusobacterium*, *Petrobacter*, *Novosphingobium*, *Micrococcus*, *Lactobacillales*, *GMD14H09*, *Citrobacter*, *Actinobacillus*, *Ureaplasma*, *Haemophilus*, 1-68, *Anoxybacillus*, *Microbispora*, Rubrobacteraceae, *Mycoplasma*, *Clostridium* [*Lachnospiraceae*], *Bradyrhizobium*, *Neisseria*, Flavobacteriaceae, *Gemella*, *Lautropia*, *Actinomyces*, *Achromobacter*, *Ralstonia*, *Mobiluncus*, *Streptophyta*, S24-7, *Roseomonas*, *Paracoccus*, *Rubrobacter*, *Ruminococcus*, Lachnospiraceae, *Janthinobacterium*, *Klebsiella*, *Roseburia*, Ruminococcaceae, *Cyanothece*, *Finegoldia*, *Lactococcus*, *Peptoniphilus*, *Sphingobacterium*, *Stenotrophomonas*, *Blautia*, Aerococcaceae, *Anaerococcus*, *Aerococcus*, Rikenellaceae, *Faecalibacterium*, *Halorhodospira*, *Sphingobium*, *Oscillospira*, *Rothia*, Coriobacteriaceae, *Bacillus*, Gemellaceae, *Veillonella*, Clostridiales.

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TABLE 1
Endometrial microbiota is related to pregnancy outcomes for in vitro fertilization patients

Sample	Endometrial receptivity (d)	Shannon index	*Lactobacillus* OTUs, %	Non-*Lactobacillus* OTUs, %	Unassigned, %	Endometrial microbiota	Pregnancy	Ongoing pregnancy
1	R (P+5)	3.090	90.85	5.35	3.80	LD	Yes	Yes
2	R (P+5)	3.079	95.10	2.13	2.77	LD	Yes	Yes
3	R (P+5)	4.257	66.48	31.28	2.24	NLD	Yes	Yes (VTOP)
4	R (P+5)	1.510	99.57	0.12	0.32	LD	Yes	Yes
5	R (P+7)	4.216	93.37	6.16	0.47	LD	Yes	Yes
6	R (CD21)	2.681	91.12	7.11	1.77	LD	Yes	Yes
7	R (LH+7)	NA	No data			NA	Yes	Yes
8	R (P+5)	NA	No data			NA	Yes	No
9	R (P+7)	1.550	3.27	94.36	2.37	NLD	Yes	No
10	R (P+5)	0.557	0.07	98.26	1.66	NLD	Yes	No
11	R (P+5)	1.316	0.26	85.43	14.31	NLD	Yes	Yes
12	R (LH+6)	1.527	99.54	0.32	0.15	LD	Yes	Yes
13	R (P+5)	3.288	97.35	2.40	0.25	LD	Yes	No
14	R (P+3.5)	5.834	36.11	58.24	5.65	NLD	Yes	No
15	R (P+5)	0.964	98.73	1.14	0.12	LD	Yes	Yes
16	R (CD21)	1.153	99.35	0.50	0.15	LD	Yes	No
17	R (CD20)	2.443	99.91	0.07	0.03	LD	Yes	Yes
18	R (P+6)	2.282	99.93	0.01	0.06	LD	Yes	Yes
19	R (P+5)	0.550	99.77	0.22	0.01	LD	Yes	Yes
20	R (P+4.5)	NA	No data			NA	Yes	Yes
21	R (P+5)	1.168	0.29	99.34	0.36	NLD	No	NA
22	R (P+5)	2.799	7.68	90.53	1.79	NLD	No	NA
23	R (P+5)	2.504	88.96	10.39	0.65	NLD	No	NA
24	R (P+5)	3.499	4.26	95.66	0.07	NLD	No	NA
25	R (P+5)	2.886	87.86	7.19	4.96	LD	No	NA
26	R (P+5)	4.954	3.43	95.31	1.26	NLD	No	NA
27	R (P+5)	1.900	92.70	5.74	1.55	LD	No	NA
28	R (P+5)	4.699	66.82	29.83	3.35	NLD	No	NA
29	R (P+7)	0.469	1.23	98.63	0.14	NLD	No	NA
30	R (CD22)	1.503	99.70	0.09	0.22	LD	No	NA
31	R (P+5)	4.639	73.89	23.81	2.30	NLD	No	NA
32	R (P+5)	1.927	4.62	95.22	0.16	NLD	No	NA
33	R (P+5)	0.805	98.00	1.96	0.05	LD	No	NA
34	R (P+5)	0.696	99.89	0.11	0	LD	No	NA
35	R (P+5)	3.202	87.70	12.20	0.11	NLD	No	NA
36	NR (P+5)	2.297	39.64	58.98	1.38	NLD	No ET	NA
37	NR (P+5)	5.274	16.34	82.92	0.74	NLD	No ET	NA
38	NR (P+5)	3.609	96.43	3.26	0.31	LD	No ET	NA

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(continued)

TABLE 1
Endometrial microbiota is related to pregnancy outcomes for in vitro fertilization patients *(continued)*

Sample	Endometrial receptivity (d)	Shannon index	*Lactobacillus* OTUs, %	Non-*Lactobacillus* OTUs, %	Unassigned, %	Endometrial microbiota	Pregnancy	Ongoing pregnancy
39	NR (P+3)	4.651	22.38	63.72	13.91	NLD	No ET	NA
40	NR (P+5)	4.026	14.07	84.30	1.63	NLD	No ET	NA
41	NR (P+4)	2.013	97.94	1.77	0.29	LD	No ET	NA

Shannon diversity values, OTUs abundances, and microbiotic profiles from in vitro fertilization patients. Technical filtering was done from data coming from QIIME: ribosomal database project score <0.9 and ≤2 reads are filtered. All plots are based on these filtered data.

CD, cycle day; *ET*, embryo transfer; *LD*, *Lactobacillus* dominated; *LH*, luteinizing hormone; *NA*, not applicable; *NLD*, non-*Lactobacillus* dominated; *NR*, nonreceptive; *OTU*, operational taxonomic unit; *P*, progesterone; *R*, receptive; *VTOP*, voluntary termination of pregnancy.

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genus in all EF and VA analyzed, but in different percentages between women. Bacterial genera such as *Atopobium*, *Gardnerella*, *Prevotella*, or *Sneathia* were also commonly identified in both endometrial and vaginal samples. With the present design we demonstrated that the endometrial microbiota is not a carryover from the vagina, because some bacterial genera present in the endometrium were not in the vagina of the same subject, and vice versa. Thus, although endometrial and vaginal microbiota were not statistically different in the pool of healthy and fertile women ($P = .733$) (Figure 3, B), we identified the existence of vaginal and endometrial bacterial communities that, although closely related in most of the subjects tested, are not identical in every woman; these differences were detected in approximately 20% of the women tested.

The endometrial microbiota

After validating EF aspiration as an acceptable method to assess the structure of endometrial bacterial communities, EF was obtained from 22 fertile subjects during the acquisition of receptivity at LH+2 and LH+7 (total samples n = 44). A total of 166 different OTUs were identified. The most represented genus was *Lactobacillus* (71.7% of identified bacteria); while *Gardnerella* (12.6%), *Bifidobacterium* (3.7%), *Streptococcus* (3.2%), and *Prevotella* (0.866%) were the other most common genera. The bacterial communities found in EF samples from fertile subjects were clustered according to the bacterial OTUs identified and their abundances. The resulting heatmap showed 2 sets of samples classifying depending on the percentage of *Lactobacillus* OTUs identified (Figure 5). The first set of samples included those with a high abundance of *Lactobacillus* (>90%) and very low or nonexistent other OTUs. The second set of samples was formed by lower *Lactobacillus* abundances that coexisted with bacteria represented by other OTUs. According to the criteria used in a recent study in which the vaginal microbiota of pregnant women was analyzed during gestation,[31] the EF samples were classified in terms of the microbiota into 2 different groups: (1) a *Lactobacillus* dominated (LD)-microbiota for those samples in which >90% of the detected bacteria belonged to *Lactobacillus* OTUs, and (2) a non-LD (NLD) microbiota when <90% of the OTUs identified in the sample belonged to *Lactobacillus* OTUs and thus presented >10% of bacterial OTUs including pathogenic or dysbiotic bacteria. This classification is in agreement with previous evidence demonstrating that gonococcal adherence to in vitro cultured endometrial epithelial cells is significantly reduced at a 1:10 ratio (gonococci:lactobacilli).[32] Using this classification, a correspondence between the community state types (CSTs) used by other authors[5,8,31] and our classification of LD and NLD microbiota could be established by comparing CSTs 1, 2, 3, and 5 with the LD microbiota, and CST 4 with the NLD microbiota. This classification was used to identify the microbiota status of EF in subjects during the acquisition of endometrial receptivity, and in IVF subjects in the next pilot studies. Thus, of the 44 endometrial microbiota analyzed from fertile subjects, 28 were assigned to the LD group, and the remaining 16 to the NLD group. When the endometrial microbiota of IVF subjects' samples were classified, 18 were assigned to the LD group and 20 to the NLD group; the remaining 3 did not produce sequencing data and were excluded from the analysis.

Regulation of endometrial microbiota during the acquisition of endometrial receptivity

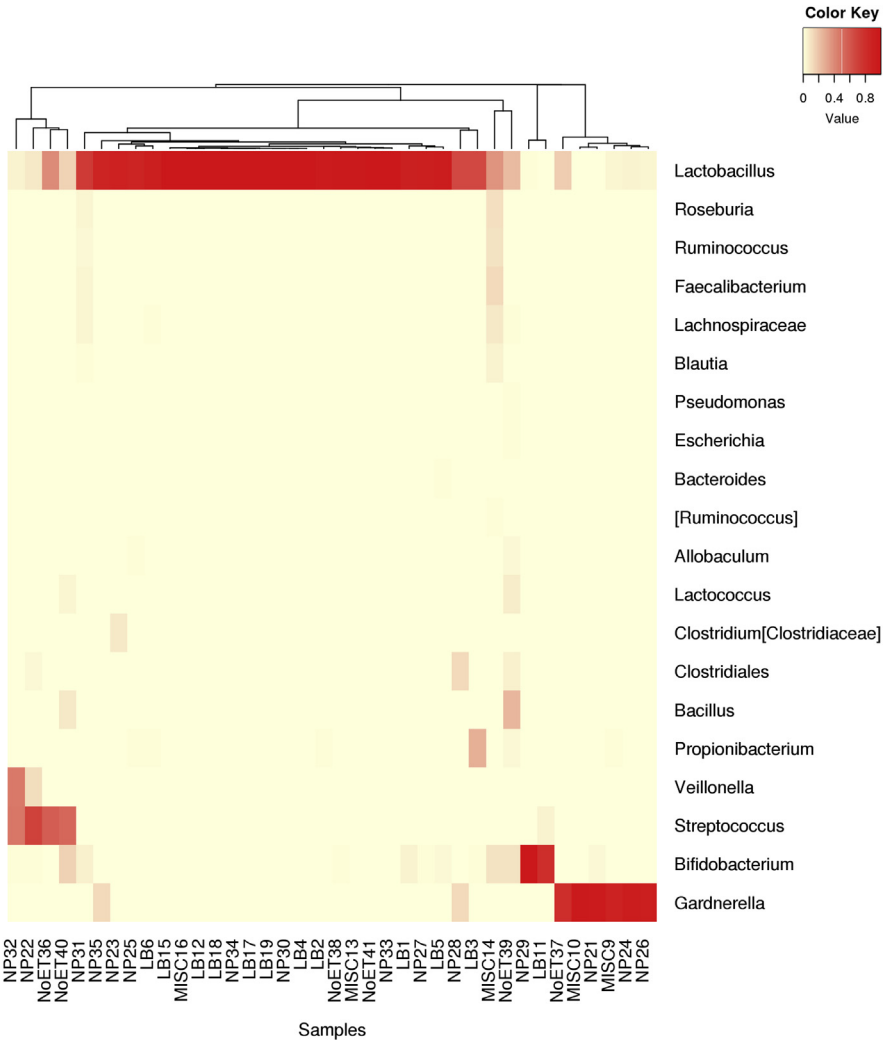
The endometrium is hormonally regulated throughout the menstrual cycle by ovarian steroids to induce the characteristics necessary for implantation and pregnancy. However, pyrosequencing data suggested a remarkable stability of the endometrial microbiota in the prereceptive vs the receptive phase (Figure 6, A, and Table S2). From the 22 subjects analyzed, 18 showed stable microbiota profiles during the transition from the prereceptive to the receptive phase (12 of them were LD, but 6 subjects had NLD microbiota). However, in 4 of the 22 subjects differences were observed during the acquisition of endometrial receptivity (from LD at LH+2 to NLD microbiota at LH+7 in 3 subjects, and from NLD at LH+2 to LD microbiota at LH+7 in 1 donor) (Figure 7). Interestingly, we found that bacterial community diversity did not vary significantly during the acquisition of endometrial receptivity in most cases

($P = .221$) (see PCoA plot in Figure 6, B), as shown by microbial taxa relative abundance and alpha diversity index, measured as Shannon value, in paired samples. Altogether, these results suggest that the endometrial microbiota is not hormonally regulated during the acquisition of endometrial receptivity, despite the tight hormonal regulation affecting endometrial epithelial cells within this period. Moreover, the endometrial microbiota profile of EF (defined as LD or NLD) in the prereceptive state coincided with that of the receptive phase in 81.8% of the cases (18 of 22 paired samples), but was variable over short time periods in a small number of subjects.

Functional impact of the endometrial microbiota composition on reproductive outcome in patients undergoing IVF

We sought to determine the functional impact of different types of endometrial microbiota on reproductive outcome. EF samples (n = 41) were obtained from 35 subjects undergoing IVF just before collecting an endometrial biopsy for the diagnosis of endometrial receptivity using ERA. Genomic DNA was extracted from EF samples and subjected to pyrosequencing and bacterial taxonomical assignment (Table S3). Three of the 41 samples presented poor DNA quality and did not amplify properly. The bacterial communities found in EF samples were clustered according to the bacterial OTUs identified and their abundances. According to the groups observed in the clustering, 4 variables (percentage of *Lactobacillus*, *Bifidobacterium*, *Gardnerella*, and *Streptococcus*) were selected to predict the target classes named live birth or no live birth (including miscarriage and nonpregnancy). To classify the different samples, 2 supervised machine learning models were applied, a CART and a generalized linear model by logistic regression. Both models provided similar conclusions, ascribing the percentage of *Lactobacillus* as the only significant variable in these 2 models. In the case of CART, the rule obtained was: (1) if *Lactobacillus* percentage is $\geq$0.9 the

FIGURE 8
Endometrial microbiota distribution on 35 in vitro fertilization patients



Clustering of individual samples showed 2 groups depending on abundance of *Lactobacillus* OTUs and other coexisting OTUs. Twenty most representative OTUs are shown. Data for each sample are detailed in Table 1. Technical filtering was performed on data produced by QIIME: ribosomal database project score <0.9 and $\leq$2 reads are filtered. All plots are based on filtered data. Others: *Cloacibacterium*, *Ethanoligenens*, *Akkermansia*, Acidimicrobiales, Cytophagaceae, Haliangiaceae, *Halomonas*, *Hydrogenophaga*, *Brochothrix*, *Ureaplasma*, *Bilophila*, *Actinobaculum*, *Diaphorobacter*, *Phascolarctobacterium*, SMB53, *Oligella*, WCHB1-84, Ellin6075, *Cellvibrio*, *Balneimonas*, *Ochrobactrum*, *Petrobacter*, *Chryseobacterium*, *Carnobacterium*, WAL_1855D, *Thermoanaerobacterium*, *Anoxybacillus*, *Hymenobacter*, *Leucobacter*, *Eggerthella*, *Propionimicrobium*, [*Mogibacteriaceae*], *Ralstonia*, 1-68, *Rubrobacter*, *Methylobacterium*, *Brevibacterium*, *Pyramidobacter*, Flavobacteriales, *Pediococcus*, *Sphingopyxis*, *Alcanivorax*, *Clostridium* [*Lachnospiraceae*], *Parabacteroides*, *Neisseria*, *Peptoniphilus*, *Stenotrophomonas*, *Varibaculum*, *Novosphingobium*, *Pseudoramibacter_Eubacterium*, *Actinomyces*, *Butyricicoccus*, *Klebsiella*, *Sphingomonas*, *Lactobacillales*, *Acinetobacter*, *Dialister*, *Finegoldia*, *Lachnospira*, Streptococcaceae, *Anaerococcus*, MND1, *Micrococcus*, Aerococcaceae, *Owenweeksia*, *Halorhodospira*, *Anaerostipes*, *Bartonella*, *Coprococcus*, *Paracoccus*, *Fusobacterium*, *Bacteroidales*, *Peptostreptococcus*, Erysipelotrichaceae, *Swaminathania*, *Collinsella*, *Corynebacterium*, *Streptophyta*, *Prevotella*, *Staphylococcus*, *Rothia*, *Dorea*, *Oscillospira*, Gemellaceae, *Atopobium*, S24-7, Ruminococcaceae.

LB, live birth; *MISC*, miscarriage; *NP*, nonpregnant.

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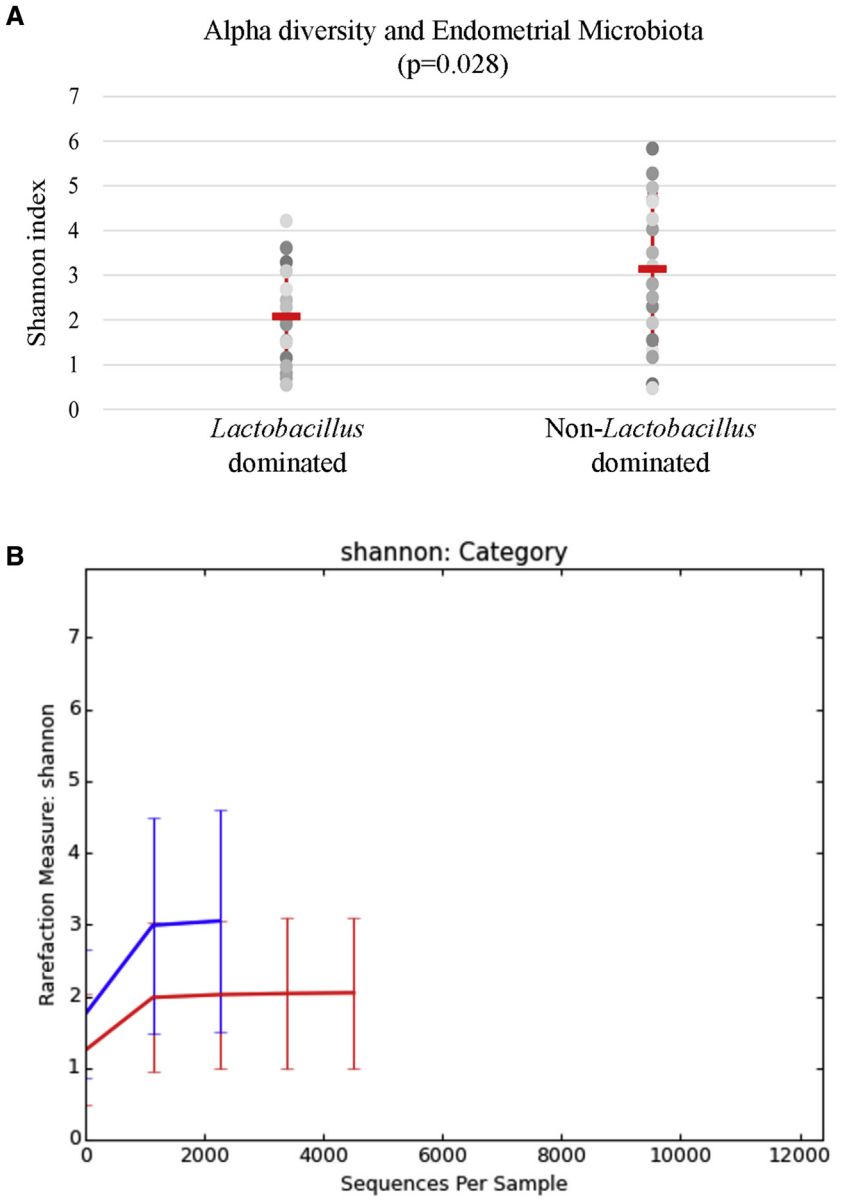
FIGURE 9
Community diversity in *Lactobacillus* dominated (LD) and non-LD (NLD) microbiota groups



A, Bacterial community diversity assessed by Shannon value, in endometrial fluid of in vitro fertilization patients classified as LD or NLD microbiota. Each individual is represented separately. Individual samples (gray dots). Mean of all subjects (red). **B**, Shannon index rarefaction curves show statistical significance (Shannon *t* test, $P = .0239$) of separation between 2 groups: LD (red) and NLD (blue).

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classification is live birth; while (2) if *Lactobacillus* percentage is <0.9 the classification is no live birth. In the case of the logistic regression (after stepwise procedures) the probability of an IVF outcome resulting in live birth followed the equation: P (live birth) = $\exp(\times)/[1+\exp(\times)]$, where $\times = \mathrm{Ln}(p/1-p) = -2.359 + 2.554$ * (percentage of *Lactobacillus*). The areas under the curve, using the original data for CART and logistic regression, were 0.76 and 0.75, respectively, showing that these 2 models could predict the pregnancy outcome based on relative abundance of *Lactobacillus* in EF. Then, based on these classifications, an endometrial microbiota profile (LD ≥ 90% *Lactobacillus*; NLD < 90% *Lactobacillus*) was assigned to each subject (Table 1). The comparison of our data with the existing literature suggested that the LD group in our cluster is comparable to CSTs 1-3 and 5 reported by others, while the groups dominated by *Gardnerella*, *Streptococcus*, and *Bifidobacterium* mostly resemble CST 4. For this reason, we consider that there are 2 general profiles depending on *Lactobacillus* dominance, LD and NLD. The NLD group could be subdivided depending on the different OTUs present, but due to the small number of patients investigated we preferred to group them as NLD (Figure 8).

The analysis of the resulting microbiota reflected significant differences in the bacterial diversity, with the NLD group showing higher diversity than those in the LD group, as assessed by Shannon diversity indexes (Figure 9). In contrast with those with LD microbiota, subjects with NLD microbiota had significantly lower implantation (60.7% vs 23.1%, $P = .02$), pregnancy (70.6% vs 33.3%, $P = .03$), ongoing pregnancy (58.8% vs 13.3%, $P = .02$), and live birth (58.8% vs 6.7%, $P = .002$) rates, as well as higher miscarriage rates, although this was not statistically significant (16.7% vs 60%, $P = .07$) (Figure 10, A, and Table 2). This adverse effect on pregnancy outcomes was more evident in subjects presenting high percentages of bacteria from the *Gardnerella* and *Streptococcus* genera (Figures 10, B, and 11). Bacterial community diversity did not correlate with IVF outcome (Figure 12).

Lactobacillus spp. present in the vagina produce lactic acid and short-chain fatty acids that decrease pH values (pH ≈ 4.5); thus, we hypothesized that a healthy endometrial microbiota, mainly comprising *Lactobacillus* spp., would produce lower pH values in EF samples compared to NLD microbiota. Therefore, the pH was measured in a series of EF samples (n = 14) before genomic

FIGURE 10
Lactobacillus abundance in endometrial samples is associated with reproductive outcome

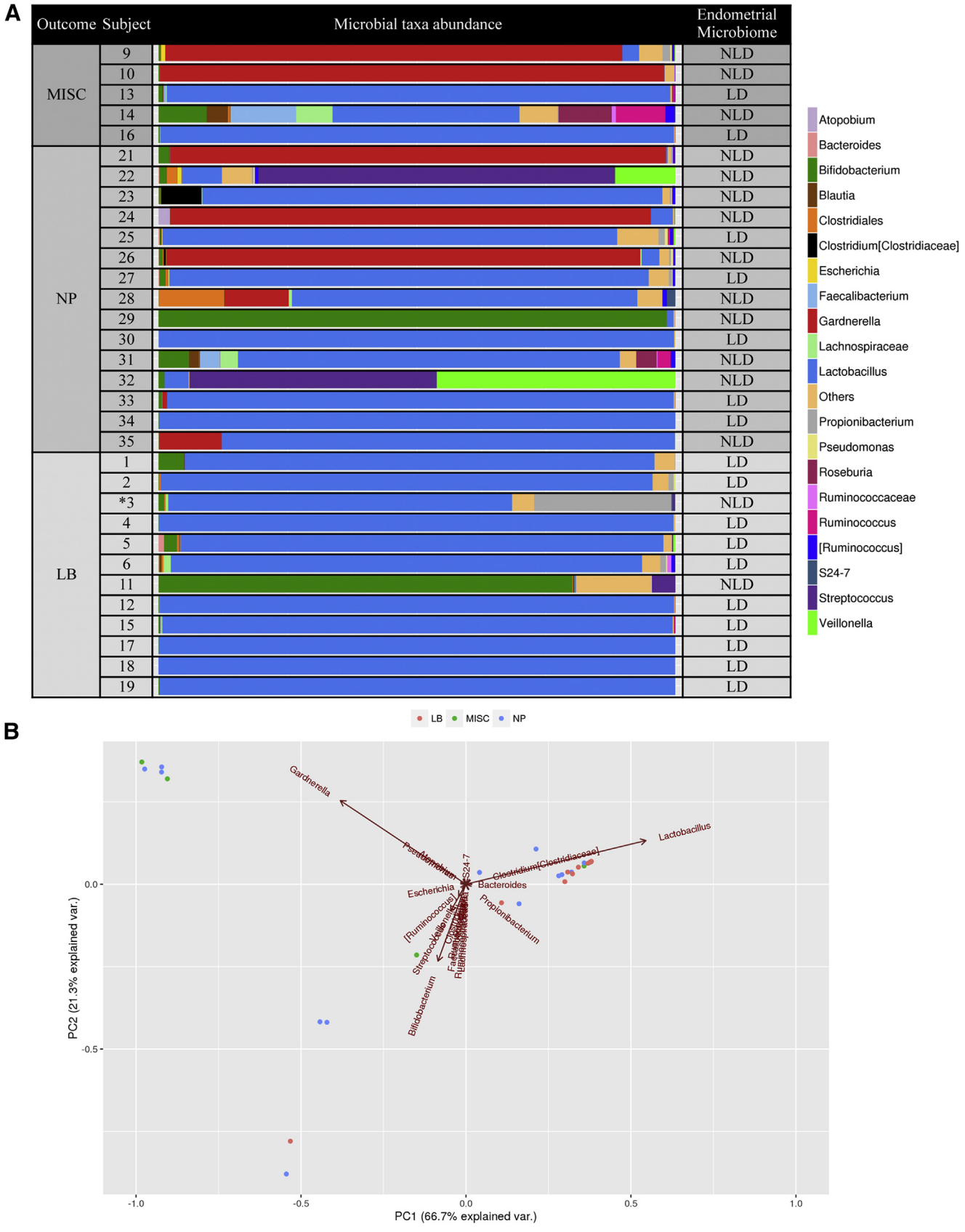

TABLE 2
Descriptive characteristics of subjects, cycles, transfers, and outcome results

Characteristics and outcomes	LDM, n = 17	NLDM, n = 15	*P* value
Age, y	40.06 ± 3.47	39.00 ± 5.09	.49
BMI, kg/m^2	24.18 ± 5.18	22.45 ± 4.02	.30
Previous pregnancies	1.71 ± 2.44	1.53 ± 2.32	.84
Previous miscarriages	1.53 ± 2.21	1.14 ± 1.56	.58
Metaphase II oocytes/cycle	11.94 ± 4.27	10.20 ± 4.81	.28
Fertilization rate/cycle	157/203 (77.34%)	118/153 (77.12%)	.62
Transferred embryos/cycle	1.65 ± 0.49	1.73 ± 0.59	.65
Time between EF and transfer, mo	2.82 ± 2.55	1.80 ± 1.08	.16
Pregnancy rate/transfer	12/17 (70.6%)	5/15 (33.3%)	.03[a,b]
Implantation rate/transfer	17/28 (60.7%)	6/26 (23.1%)	.02[a,b]
Ongoing pregnancy/transfer	10/17 (58.8%)	2/15 (13.3%)	.02[a,b]
Miscarriage rates	2/12 (16.7%)	3/5 (60%)	.07
Live birth rate/transfer	10/17 (58.8%)	1[c]/15 (6.7%)	.002[a,b]

Values are mean ± SD unless otherwise noted.

BMI, body mass index; *EF*, endometrial fluid; *LDM*, *Lactobacillus*-dominated microbiota; *NLDM*, non-*Lactobacillus*-dominated microbiota.

[a] χ^2 test and Student *t* test were performed; [b] *P* value < .05; [c] Voluntary termination of pregnancy.

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DNA extraction. A high variability was found in the EF samples, with pH values between 6.6−8.51, independent of their bacterial composition and the percentage of *Lactobacillus* OTUs in those samples. Therefore, the pH of EF cannot be used as a predictor of endometrial microbiota status (Figure 13).

Finally, to evaluate the impact of the endometrial microbiota in IVF outcomes, a follow-up of those subjects previously diagnosed with nonreceptive endometrium (n = 5) was performed by assessing endometrial receptivity and endometrial microbiota simultaneously until their window of implantation was achieved. Only subjects showing endometrial receptivity and LD microbiota in the same cycle succeeded in their IVF treatment, while subjects with NLD microbiota presented adverse implantation rates and pregnancy outcomes despite receiving personalized embryo transfer (Table 3).

Comment

The uterine cavity has been traditionally considered to be sterile, but potentially susceptible to be affected by vaginal bacteria. The impact of BV in reproductive outcome remains controversial: 1 study correlated it with a decrease in pregnancy rates in IVF patients,[33] while others, including a recent meta-analysis, reported no correlation between pregnancy outcomes and BV in patients undergoing IVF.[34,35]

Principal findings of the study

To our knowledge, this work is the first comparative study between endometrial and vaginal microbiota using next-generation sequencing. The results show that endometrial and vaginal microbiota can differ in structure and composition in some women. This finding supports the concept that the uterine cavity is not a sterile site, challenging the current dogma. Also, our results show evidences that NLD endometrial microbiota is associated to negative reproductive outcomes in IVF patients when compared to those with LD endometrial microbiota.

Is there an endometrial microbiota different that is from the vaginal microbiota?

The detection of bacterial DNA in 100% of EF samples is consistent with the identification of bacteria in 95% of the hysterectomy specimens analyzed by

A, Bar charts showing individual microbial taxa composition and relative abundance of endometrial fluid samples of 35 IVF patients and their reproductive outcomes. **B**, Principal component analysis plot showing contribution of 20 most representative operational taxonomic units to reproductive outcome in IVF patients. *Voluntary termination of pregnancy.

LB, live birth; *LD*, *Lactobacillus* dominated; *MISC*, miscarriage; *NLD*, non-*Lactobacillus* dominated; *NP*, nonpregnant.

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fingerprinting of the *16S rRNA* gene for 12 bacterial species.[20] Our results also show that aspiration of EF under aseptic conditions is a safe and effective method to evaluate the endometrial microbiota. Further, these findings highlight the importance of endometrial investigation to improve pregnancy outcomes in those patients with differential vaginal and endometrial microbiota, since the bacterial structure and composition of the vagina does not accurately mirror, in every woman, the bacteria colonizing the endometrium, where embryonic implantation occurs.

Clinical implications

We also defined the endometrial microbiota profile as LD or NLD according to the identity and relative abundance of the bacteria identified in EF. This classification enabled the diagnosis of the endometrial microbiological health of IVF patients and its correlation with their reproductive outcome. A NLD microbiota strongly correlated with adverse outcomes, when compared to subjects presenting a LD endometrial microbiota. Interestingly, these

Chryseobacterium, *Carnobacterium*, WAL_1855D, *Thermoanaerobacterium*, *Anoxybacillus*, *Hymenobacter*, *Leucobacter*, *Eggerthella*, *Propionimicrobium*, [*Mogibacteriaceae*], *Ralstonia*, 1-68, *Rubrobacter*, *Methylobacterium*, *Brevibacterium*, *Pyramidobacter*, Flavobacteriales, *Pediococcus*, *Sphingopyxis*, *Alcanivorax*, *Clostridium* [*Lachnospiraceae*], Parabacteroides, *Neisseria*, *Peptoniphilus*, *Stenotrophomonas*, *Varibaculum*, *Novosphingobium*, *Pseudoramibacter_Eubacterium*, *Actinomyces*, *Butyricicoccus*, *Klebsiella*, *Sphingomonas*, Lactobacillales, *Acinetobacter*, *Dialister*, *Finegoldia*, *Lachnospira*, Streptococcaceae, *Anaerococcus*, MND1, *Micrococcus*, Aerococcaceae, *Owenweeksia*, *Halorhodospira*, *Anaerostipes*, *Bartonella*, *Coprococcus*, *Paracoccus*, *Fusobacterium*, *Bacteroidales*, *Peptostreptococcus*, Erysipelotrichaceae, *Swaminathania*, *Collinsella*, *Corynebacterium*, Streptophyta, *Prevotella*, *Staphylococcus*, *Rothia*, *Dorea*, *Oscillospira*, Gemellaceae, *Atopobium*, S24-7, Ruminococcaceae.

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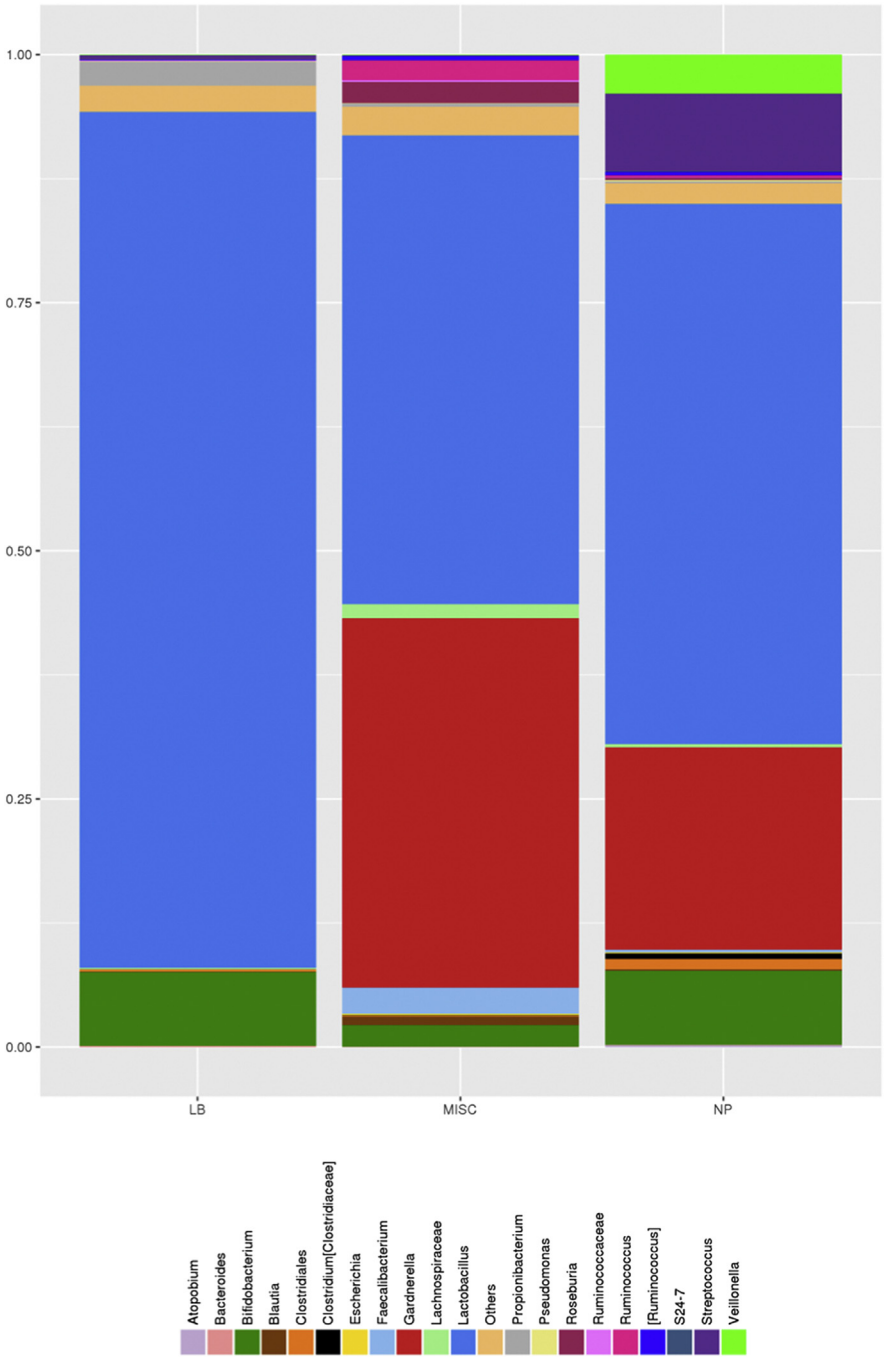
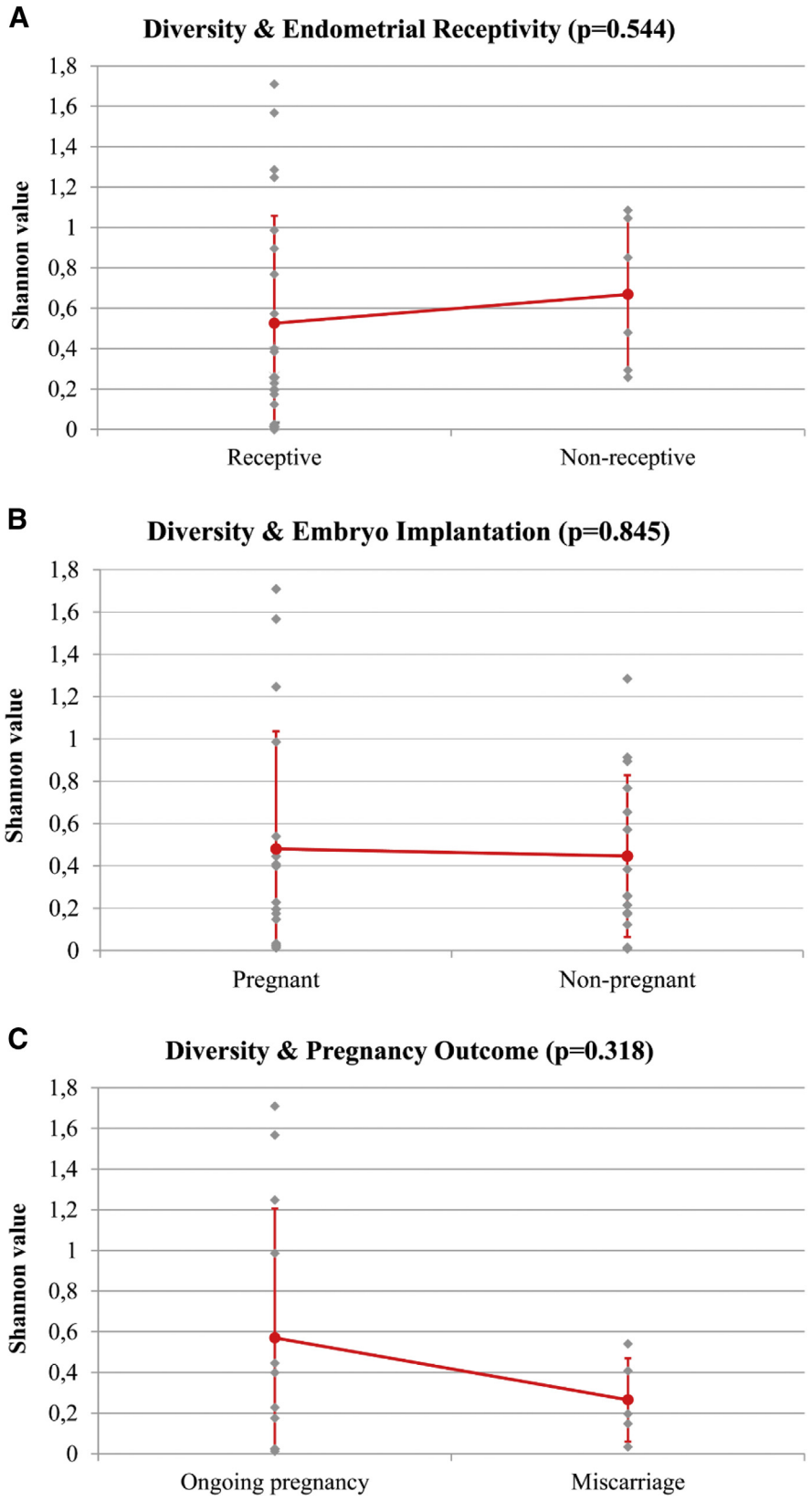
FIGURE 11
Low abundance of endometrial Lactobacillus is associated with poor reproductive outcome



Bar charts showing mean values of 20 most abundant operational taxonomic units (OTUs) in receptive subjects grouped by their reproductive outcomes: live births (LB) correspond to patients who became pregnant and successfully delivered; nonpregnant (NP) are patients who did not conceive; finally, miscarriage (MISC) applied for those patients who became pregnant but experienced either biochemical or clinical pregnancy. Twenty most abundant taxa are represented, while sum of remaining OTUs are included as "others" that comprise: *Cloacibacterium*, *Ethanoligenens*, *Akkermansia*, *Acidimicrobiales*, Cytophagaceae, Haliangiaceae, *Halomonas*, *Hydrogenophaga*, *Brochothrix*, *Ureaplasma*, *Bilophila*, *Actinobaculum*, *Diaphorobacter*, *Phascolarctobacterium*, SMB53, *Oligella*, WCHB1-84, Ellin6075, *Cellvibrio*, *Balneimonas*, *Ochrobactrum*, *Petrobacter*,

FIGURE 12
Endometrial community diversity is not predictor for reproductive outcome



correlations were much more evident when the non-*Lactobacillus* OTUs identified in the samples belonged to the *Gardnerella* or *Streptococcus* genera, as all subjects presenting high rates of these genera either did not become pregnant after embryo transfer or experienced a miscarriage. These results accord with previously published data of other groups that analyzed the impact of endometrial pathogens in IVF by using classic microbiological culture of the distal tip of the catheter used for embryo transfer.[13,14,16,17,36] However, a recent work published by Franasiak et al[37] using a similar technical approach analyzing the transfer catheter tip instead of EF for bacterial *16S rRNA* sequencing resulted in the identification of *Lactobacillus* as the most represented bacteria in endometrial samples. However, no association between *Lactobacillus* abundance and pregnancy outcome was shown in their IVF patients, which clearly differs with what is reported here.[37] The reason for this difference could reside in the quantitative dimension that we have introduced in our model to classify samples as LD or NLD depending on the percentage of *Lactobacillus* OTUs and that was not considered in the study by Franasiak et al.[37]

Research implications

Some authors suggest that the *Lactobacillus* genus produces lactic acid and short-chain fatty acids, acidifying the environment to pH $\leq$4.5 in the vagina and prohibiting the growth of other pathogenic or dysbiotic bacteria in healthy women.[38,39] Apparently, this is

Bacterial community diversity and/or stability, assessed by Shannon value, in different groups analyzed. **A**, Receptive vs nonreceptive subjects diagnosed by endometrial receptivity array (ERA). **B**, Implantation rates among receptive subjects. **C**, Pregnancy outcomes on ERA-based receptive patients who became pregnant. Values from each subject are represented separately (gray diamonds). Red dots and line represent average values of subjects within group.

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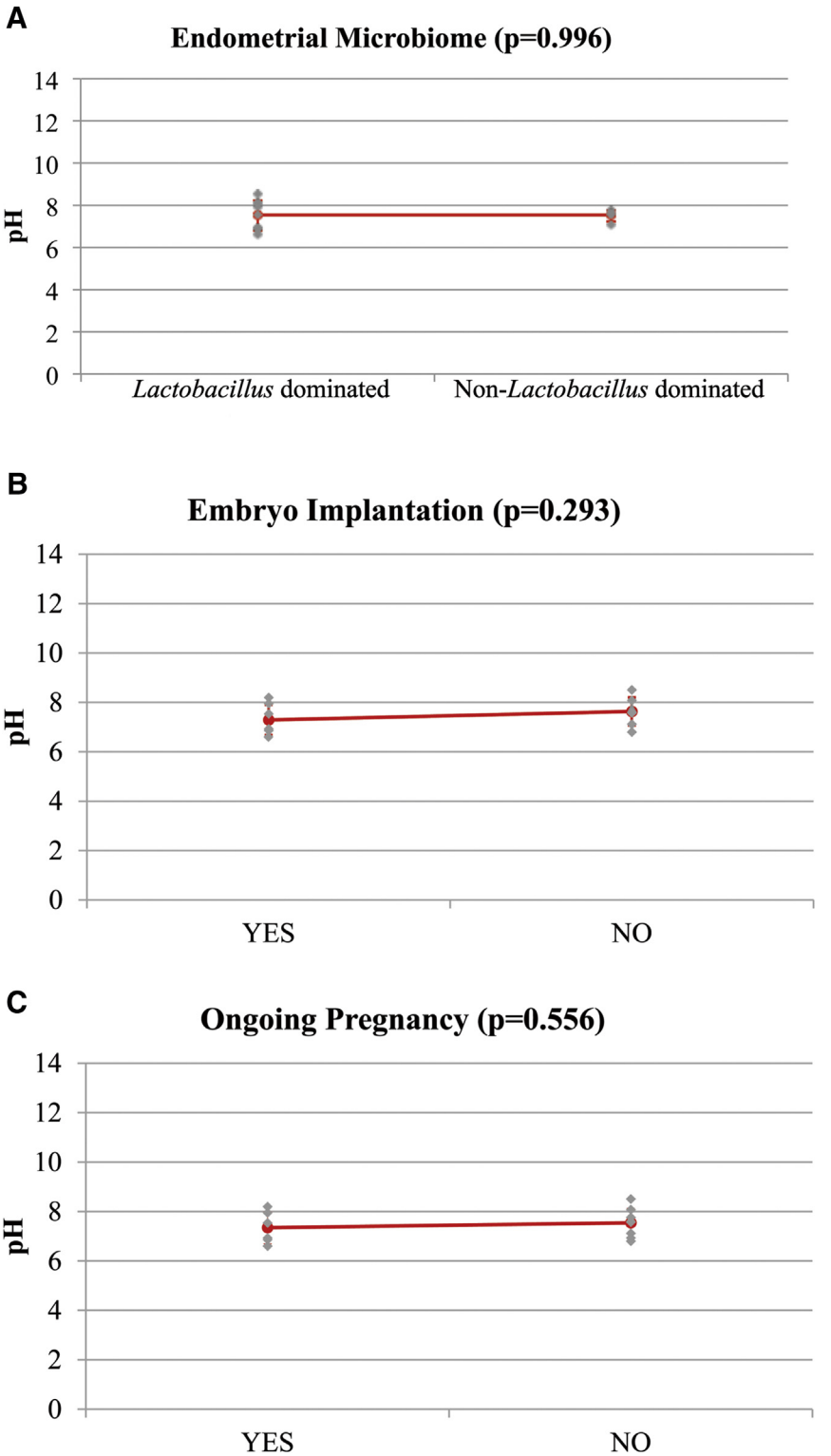
not the case in the endometrium because when pH levels were measured in EF samples, no correlation was observed between pH values and the endometrial microbiota, suggesting that other biochemical effects occur in the endometrium where the embryo will adhere and develop. In this sense, it is important to notice that NLD microbiota may trigger an inflammatory response in the endometrium that affects the success of embryo implantation, as inflammatory mediators are tightly regulated during the adhesion of the blastocyst to the epithelial endometrial wall.[40,41] Also, some other mechanisms of action related with the direct production of microbial metabolites and/or enzymes that are able to produce relevant compounds able to induce key cellular pathways in the endometrial epithelium need to be considered. In any case, a fascinating time for the study of the "dialogue" between the endometrial microbiota and the endometrial epithelium is beginning. These experiments will require new approaches most probably based on systems biology approaches.

Strengths and limitations

An important strength of our work is that endometrial receptivity was analyzed by ERA and embryo transfer performed in those subjects with receptive endometrium, avoiding any interference of the endometrial factor in this study. Additionally, in those subjects presenting a nonreceptive endometrium, a second sample of EF was obtained and analyzed until receptivity was confirmed. Only subjects who acquired endometrial receptivity with LD endometrial microbiota presented successful ART outcomes upon personalized embryo transfer.

A limitation of these prospective pilot studies is the time between EF collection and embryo transfer because the consistency of the endometrial microbiota in IVF subjects is unknown. Surprisingly, the endometrial microbiota is not regulated during the shift from the prereceptive (LH+2) to the receptive (LH+7) phase of the menstrual cycle in nearly 82% of the subjects when the

FIGURE 13
Endometrial pH



pH of endometrial fluid does not predict **A**, endometrial microbiota; **B**, implantation rates; and **C**, pregnancy outcomes for in vitro fertilization patients. pH from individual samples (gray diamonds). Red dots and line represent average values among compared groups.

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TABLE 3
Follow-up of patients with nonreceptive endometrium

Patient	EF sample in Table 1	Sample (d)	ERA test	Embryo transfer	*Lactobacillus* OTUs, %	*Non-Lactobacillus* OTUs, %	Unassigned, %	Microbiomic profile	Pregnancy	Ongoing pregnancy
1	36	1A (P+5)	NR (P+5)	No	39.64	58.98	1.38	NLD	NA	NA
	5	1B (P+7)	R (P+7)	P+7	93.37	6.16	0.47	LD	Yes	Yes
2	37	2A (P+5)	NR (P+5)	No	16.34	82.92	0.74	NLD	NA	NA
	9	2B (P+7)	R (P+7)	P+7	3.27	94.36	2.37	NLD	Yes	No
3	39	3A (P+3)	NR (P+3)	No	22.38	63.72	13.91	NLD	NA	NA
	14	3B (P+3.5)	R (P+3.5)	P+3.5	36.11	58.24	5.65	NLD	Yes	No
4	40	4A (P+5)	NR (P+5)	No	14.07	84.30	1.63	NLD	NA	NA
	29	4B (P+7)	R (P+7)	P+7	1.23	98.63	0.14	NLD	No	NA
5	38	5A (P+5)	NR (P+5)	No	96.43	3.26	0.31	LD	NA	NA
	41	5B (P+4)	NR (P+4)	No	97.94	1.77	0.29	LD	NA	NA
	20	5C (P+4.5)	R (P+4.5)	P+4.5	No amplification			NA	Yes	Yes

EF, endometrial fluid; *ERA*, endometrial receptivity array; *LD*, *Lactobacillus* dominated; *NA*, not applicable; *NLD*, non-*Lactobacillus* dominated; *NR*, nonreceptive; *OTU*, operational taxonomic unit; *P*, progesterone; *R*, receptive.

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endometrial microbiota is highly stable. This observation is in agreement with previous data reporting high stability of vaginal microbiome coinciding with the early and mid secretory phases in contrast with the high instability during the late secretory and menstrual phases of the cycle.[5] Another limitation is that, unless the molecular methods used in this work are widely accepted, no microbiological culture techniques were used in this study.

Conclusion

In conclusion, a human endometrial microbiota exists and is independent of hormonal regulation. The existence of non-*Lactobacillus* bacteria in the endometrium is correlated with negative impacts on reproductive function and should be considered as an emerging cause of implantation failure and pregnancy loss. The results presented herein expand the evaluation of endometrial receptivity not only at the morphological and molecular levels but also at the microbiological viewpoint. It is time to consider microorganisms not only as enemies but also as allies in reproductive medicine. ■

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Supplementary Methods

Subjects

For comparison between vaginal and endometrial bacterial communities, donors were fertile women recruited from the ovum donation program, aged 18−35 years, with normal body mass index of 19−29 kg/m^2, normal karyotype, and regular menstrual cycles. Paired samples of endometrial fluid (EF) and vaginal aspirates were obtained 2 and 7 days after their LH surge within the same natural cycle. For the study of hormonal regulation of the endometrial microbiota, samples were also obtained from ovum donors at IVI Valencia, Spain, with the same inclusion criteria. EF samples were obtained within the same natural cycle two and seven days after the luteinizing hormone surge (LH+2 and LH+7, respectively) of fertile women from the ovum donation program at IVI Valencia. The functional impact of the endometrial microbiota on reproductive outcome was explored in infertile subjects undergoing in vitro fertilization treatment in whom a receptive endometrium was diagnosed by endometrial receptivity array (ERA) (Igenomix SL, Valencia, Spain). Inclusion criteria encompassed reproductive-age women ages 25-40 years, presenting normal body mass index of 19-29 kg/m^2, normal karyotype, and intracytoplasmic sperm injection or oocyte donation treatments in whom at least 1 good-quality embryo was transferred. Some subjects with a nonreceptive endometrium result in the ERA test ($n = 5$) were tested again until receptivity was confirmed, and the EF microbiota was investigated. Personalized embryo transfer was performed according to ERA test results in all subjects. A general criterion in all studies excluded subjects who had used antibiotics or probiotics 1 month before the study.

EF aspiration

Briefly, with the patient in lithotomy position, the cervix was cleansed with a cotton swab and 20-80 μL of EF was aspirated using a catheter (Wallace, Smith Medical International Ashford, Reino Unido) transcervically introduced into the uterine cavity to avoid any contact with vaginal walls. To prevent contamination, cervical mucus was aspirated before EF recovery and suction was stopped at the entrance of the internal cervical os during catheter removal. The aspiration of EF is a painless and minimally invasive method that does not cause any risk for the patient and that can be safely used 24 hours before embryo transfer without altering implantation rates.[1] Therefore, the investigation of the endometrium does not increase the risk compared to vaginal investigation.

Endometrial receptivity diagnosis

Endometrial biopsies (~3 g of tissue) from patients undergoing in vitro fertilization treatment were collected from the uterine fundus with the use of a Pipelle catheter (Genetics, Belgium) under sterile conditions. Total RNA was extracted by Trizol method according to the manufacturer's protocol (Life Technologies Carlsbad, CA). RNA quality was assessed by loading samples into RNA Labchip and subsequently analyzed in an A2100 Bioanalyzer (Agilent Technologies). Sample preparation and hybridization were adapted from the Agilent technical manual (1 color). Briefly, first-strand cDNA was transcribed with T7-Oligo deoxythimine (dT) promoter primers. Samples were transcribed in vitro and Cyanine-3 labeled, all with the Low Input Quick Amp Labeling Lit (Agilent Technologies, Santa Clara, CA). The labeling reaction typically yielded 4−5 mg of complementary RNA with a specific activity >6. Fragmented complementary RNA samples were hybridized onto the customized ERA[2] by incubation at 65°C for 17 hours with constant rotation. After washing, hybridized microarrays were scanned in an Axon 4100A scanner (Molecular Devices, Sunnyvale, CA), and data were extracted with the use of Genepix Pro 6.0 software (Molecular Devices). Gene expression values were preprocessed and normalized, and subjected to the ERA computational predictor arrays.[3] The ERA test diagnoses the endometrial samples as receptive or nonreceptive with an associated diagnostic probability.

Genomic DNA isolation from EF and vaginal aspirate samples

Briefly, to obtain a complete digestion of the bacterial cell wall, an extra enzymatic lysis step was performed using 50 μL lysozyme (50 mg/mL) (Sigma, Dorset, United Kingdom) and bacteria lysis buffer (Roche, Madison, WI) with incubation at 37°C for 30 minutes. Subsequent steps (proteinase K, inactivation treatment, and purification) were performed according to manufacturer's protocol in a MagNa Pure compact (Roche). Total genomic DNA was measured using the Quant-iT PicoGreen DNA assay (Invitrogen) and photometric technology (Nanodrop, Waltham, MA).

Polymerase chain reaction and pyrosequencing

Polymerase chain reaction (PCR) was performed with 5 μL of DNA, 200 μmol/L each of the 4 deoxynucleotide triphosphates, 400 nmol/L of each primer, 2.5 U of FastStart HiFi polymerase (Roche, Madison, WI), 4% of 20 g/mL bovine serum albumin (Sigma), 0.5 mol/L betaine (Sigma), and the appropriate buffer with magnesium chloride supplied by the manufacturer (Roche). Thermal cycling consisted of initial denaturation at 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 20 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes. To obtain the appropriate amount of material, reactions were repeated in triplicate and pooled by running the PCR amplicons on 1% (wt/vol) agarose gels. Amplicons were combined in a single tube in equimolar concentrations. The pooled amplicon mixture was purified twice (AMPure XP kit, Beckman Coulter, Brea, CA) and the cleaned pool requantified using Quant-iT PicoGreen DNA assay (Invitrogen, Carlsbad, CA). This pool was then diluted in TE buffer to 108 molecules/μL and PCR was performed.

Quality control of the FASTQ files

Quality control of the FASTQ files was perfomed using Fastx tool kit version 0.013[4] to remove reads with quality less than Q20, once the sequences were clean

based on quality scores, we trimmed traces of the 16S rRNA primers and sequencing adapters using cutadapt version 1.2.[5] After primer removal, sequences with <300 nucleotides read length were trimmed using perl scripting. Clean FASTQ files were converted to FASTA files and UCHIME program version 7.0.1001[6] was used to remove chimeras that could arise during the amplification and sequencing step.

Receiver operating characteristic

A receiver operating characteristic (ROC) chart is a 2-dimensional plot with the proportion of false positives (1-specificity) on the horizontal axis and the proportion of true positives on the vertical axis (sensitivity) when using different cut-offs for a classifier score. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. A common measure for comparing the accuracy of various classifiers is the area under the ROC curve. It evaluates the method's ability to correctly classify. The closer to 1 the area under the ROC curve of a classifier is, the higher the accuracy. More details can be found in Fawcett.[7]

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