

# Relevance of assessing the uterine microbiota in infertility

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Technical advances in massive parallel sequencing have allowed the characterization of the whole reproductive tract microbiome in all the compartments beyond the vagina. The microbiota in the uterine cavity seem to be a continuum from the microbiota in the vagina, but several works have reported significant differences between vaginal and endometrial microbiota, highlighting the relevance of assessing the upper genital tract microbiota to better understand the potential roles of bacteria in the physiological and pathological processes taking place in the uterine cavity, including embryo implantation, pregnancy maintenance, and other gynecological diseases. However, the study of the endometrial microbiota, as with other low-biomass microbiota, presents important hurdles because, due to the small amount of starting material, they are easily contaminated by exogenous bacterial DNA. For this reason, careful and appropriate investigation of the endometrial microbiota is of outstanding importance to detect uterine dysbiosis that may impact the reproductive function. (Fertil Steril® 2018;110:337–43. ©2018 by American Society for Reproductive Medicine.)

**Key Words:** Endometrial microbiota, 16S rRNA sequencing, bacterial pathogens, chronic endometritis, low-biomass microbiome

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The study of the female reproductive tract microbiota has been long focused on the vagina, while the presence of microorganisms in the upper genital tract (UGT) was mainly associated to infectious conditions. The first hint of endometrial colonization by commensal bacteria comes from the isolation of *Lactobacilli* along with *Enterobacter*, *Mycoplasma hominis*, and *Gardnerella vaginalis* from endometrial samples collected transcervically with a double-lumen catheter (1). Then the existence of an endometrial microbiome was further supported by the microbial growth of bacteria from uterine pieces obtained after hysterectomy, which avoids potential contamination of endometrial samples through the cervicovaginal canal in patients operated for benign conditions (2). Also using microbial culture, bacteria belonging to *Lactobacillus*, *Actinomyces*, *Bifidobacterium*,

*Propionibacterium*, *Staphylococcus*, and *Streptococcus* genera have been identified in the follicular fluid. Moreover, the microbiota composition in ovarian follicular fluid shows an association with reproductive outcomes after IVF (3).

The most plausible way of UGT colonization is the ascension of microorganisms from the vagina. This hypothesis has been demonstrated by the presence of polymicrobial biofilms, containing *G. vaginalis*, attached to the endometrium and fallopian tubes of women with bacterial vaginosis (4). However, other modes of UGT seeding have been proposed, such as migration of gastrointestinal, airways, or oral bacteria via hematogenous spreading (5). These types of colonization are supported by previous findings showing similarities between oral and placental microbiomes in pregnant women (6) and the higher prevalence of cervical *Lactobacillus iners* in

obese reproductive-age women in whom dysbiosis of the digestive tract is a characteristic (7). Finally, the vertical transmission of the maternal microbiome to the newborn has been suggested to influence microbial health throughout life (5).

The advent of highly sensitive molecular techniques, especially next-generation sequencing, has opened up new possibilities to explore the microbiota of body sites that were previously unexplored or considered sterile and has broadened our view of the UGT microbiota. Recently, a study has reported the microbiota across the female reproductive tract (including lower vagina, posterior fornix, cervical mucus, endometrium, fallopian tubes, and peritoneal fluid obtained from the pouch of Douglas) in women being operated for benign and noninfectious conditions (8). In addition, to confirm the viability of the bacteria found, fresh samples of peritoneal fluid, the most distal part from the vagina, were subjected to standard microbial culture, showing bacterial isolates from *Lactobacillus*, *Actinomyces*, and *Staphylococcus* genera in one third of the samples

Received March 31, 2018; revised April 19, 2018; accepted April 26, 2018.

I.M. has nothing to disclose. C.S. has nothing to disclose.

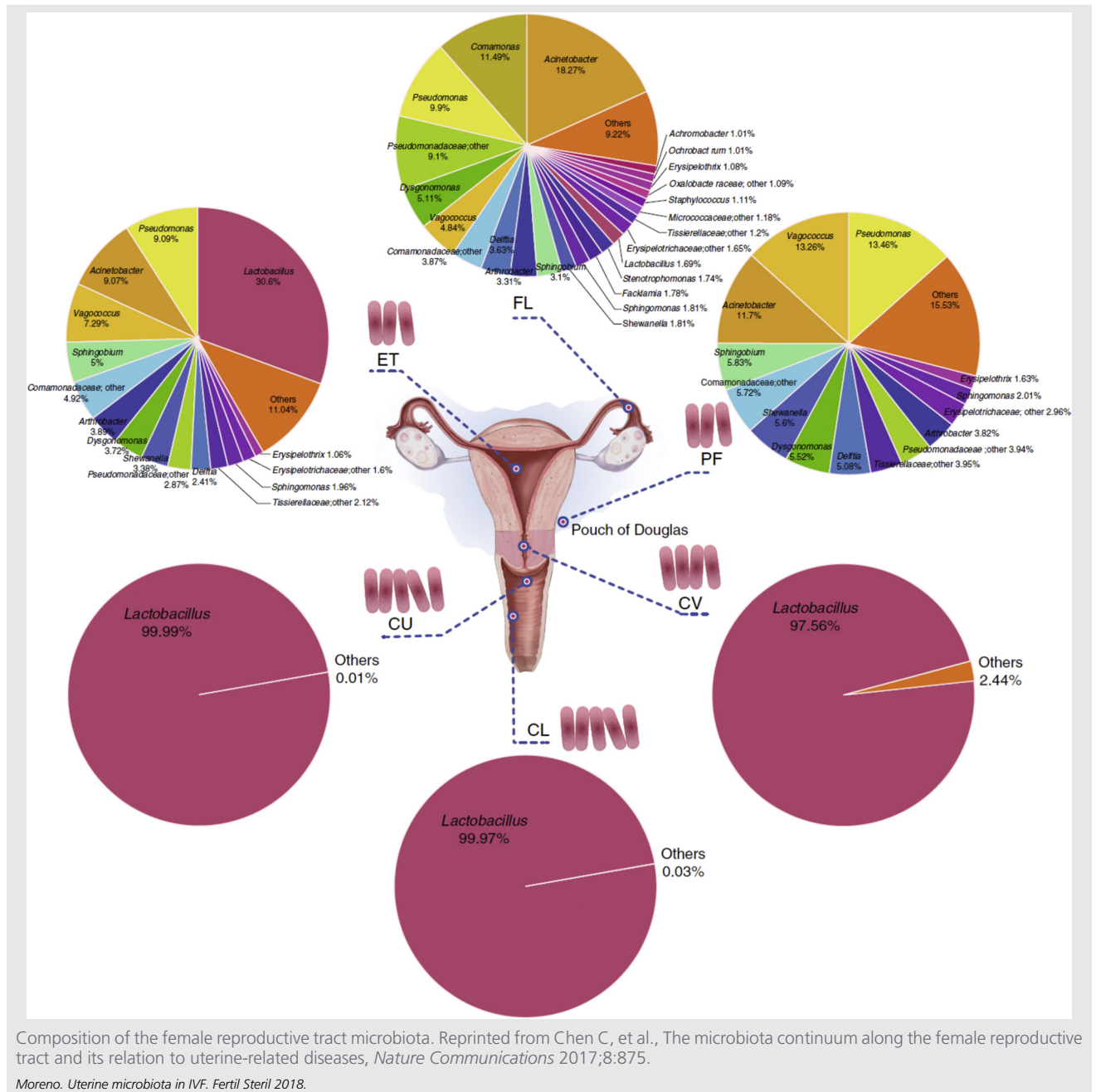
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Fertility and Sterility® Vol. 110, No. 3, August 2018 0015-0282/\$36.00

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<https://doi.org/10.1016/j.fertnstert.2018.04.041>

**FIGURE 1**



analyzed, confirming the existence of active bacterial microbiota throughout the UGT in reproductive age women (8). The results of this work show that there is a continuum of slightly different microbiota expanding gradually from the vagina to the ovaries (Fig. 1).

Once the existence of the whole reproductive tract microbiota is fully demonstrated, knowledge of the microbial communities inhabiting the different niches in physiological conditions will help to determine the pathological shifts that may be responsible for reproductive failure at different levels (from gamete formation in the gonads to implantation

failure in the uterus and/or pregnancy complications), as well as other gynecological conditions (9, 10).

## DIFFERENCES BETWEEN ENDOMETRIAL AND VAGINAL MICROBIOTA

Because embryo implantation occurs in the uterine cavity and not in the vagina, reproductive medicine remains primarily interesting in the endometrial microbiota and their impact on pregnancy establishment and maintenance.

Molecular identification of bacterial species using 16S rRNA-targeted polymerase chain reaction has allowed the detection of at least one of the 12 surveyed bacterial taxa in 95% of the endometrial samples (11). Interestingly, bacterial DNA was also detected in endometrial samples collected at hysterectomy from asymptomatic patients with no signs of endometrial inflammation or a positive diagnosis of bacterial vaginosis, showing a different microbial profile to their vaginal counterparts in some cases, meaning that these identified bacteria should constitute the endometrial microbiota (11). Consistent with this work, 16S NGS has been used to compare the endometrial and vaginal microbiota from asymptomatic and fertile subjects showing bacterial detection in all the samples analyzed (12). As expected, *Lactobacillus* spp. was the most abundant operational taxonomic unit in the endometrium, but other taxa such as *Atopobium*, *Bifidobacterium*, *Gardnerella*, *Megasphaera*, *Prevotella*, *Sneathia*, *Streptococcus*, and other dysbiotic agents previously found in the vagina were also detected; however, in approximately 20% of the subjects analyzed, the endometrial and vaginal microbial populations differed in either the bacterial taxa identified or the relative abundance in which they were represented in both types of samples (12). These differences have been also reported by Wee and collaborators in a case-control study comparing endometrial versus vaginal and cervical samples in fertile and infertile women (13). Again, the intraindividual similarities between the upper (endometrial) and lower (vaginal) microbiota have been observed in the general population with discrete and gradual transitions between community state types at different sites (8). It is important to highlight that differences between the endometrial and vaginal flora have been observed regardless of the method of collection of endometrial samples—transcervically (8, 12, 13) or at uterine surgery (8, 11)—which confirms the existence of indigenous endometrial microbiota and supports the vaginal-cervical canal as a safe route for sampling the uterine cavity for microbiome analysis.

Together, these results show that the endometrial and vaginal microbiota are similar, consistent with the uterine colonization from bacteria ascending from the vagina, but not always identical in every woman. The endometrial and vaginal microbiome are mainly dominated by *Lactobacillus* spp. (unless at different proportions along the reproductive tract), while the presence/dominance of other bacterial taxa may lead to a dysbiotic/pathological microbial state responsible for subfertility or other conditions.

## ENDOMETRIAL MICROBIOTA IN FEMALE REPRODUCTION

The role of endometrial microbiota at the embryo-maternal interface in the onset of pregnancy is of great interest in reproductive medicine, and a better understanding of what a healthy uterine environment is, and how to achieve it, would benefit not only women undergoing IVF but also every woman wishing to conceive.

Uterine infection is a known risk factor for infertility, as this pathogenic environment may entail inflammation and immune activation in the endometrium, impairing embryo

implantation and the onset of a successful pregnancy. During the 1990s and 2000s, the association of endometrial infection with reproductive failure of IVF treatments was reported by several groups assessing the endometrial flora at the time of ET by microbial culture of the distal tip of the transfer catheter. In all these studies, the reproductive outcome was consistently poor upon isolation of endometrial pathogens as *Streptococcus* spp., *Staphylococcus* spp., *Enterococcus* spp., *Escherichia coli*, *Klebsiella pneumoniae*, and Gram-negative bacteria (14–18) compared with cases with negative culture. On the other hand, isolation of *Lactobacillus* spp. was associated with increased implantation and pregnancy rates per transfer and lower miscarriage rates (15, 16). Also, a comprehensive study conducted by Egbase and collaborators in 1999 showed the relevance of a healthy microbial environment at the window of implantation by culturing endometrial samples at the time of oocyte retrieval (when a prophylactic antibiotic was prescribed) and at ET, which was performed 48 hours later. In this study, patients with endometrial pathogens at the time of ET presented decreased clinical pregnancy rates per transfer (18.7%) compared with those women with negative cultures or those who had responded to antibiotic therapy previous to ET (41.3% and 38.1%, respectively), supporting the concept that reversibility of endometrial infections could improve reproductive outcomes in IVF patients (19).

Classical microbial culture provides a limited and biased view of the uterine environment, as not every bacterium is able to grow in standard laboratory conditions. For this reason, molecular technologies able to provide a 360° view of the uterine microbiota have been used to unravel the composition and impact of endometrial flora in patients subjected to assisted reproductive technologies. Taking advantage of 16S rRNA gene sequencing, the endometrial microbiome of infertile patients with repeated implantation failure (RIF) or recurrent pregnancy loss (RPL) has been investigated, showing that the uterine microbiome of these patients is predominantly made of bacteria from the phyla Firmicutes, Bacteroidetes, and Proteobacteria (20). These phyla contain several genera previously reported not only in the female reproductive tract, but specifically in endometrial samples: Firmicutes contains *Lactobacilli*, *Streptococci*, *Staphylococci*, among others; Bacteroidetes contains *Prevotella*; and Proteobacteria contains Enterobacteria as *E. coli* and *K. pneumoniae*.

The impact of endometrial microbiota in reproductive outcomes was first reported by Franasia and collaborators in 2016. In that study, 16S rRNA sequencing was applied to the tip of the transfer catheter in 33 patients undergoing IVF with euploid embryos. Using this approach, *Lactobacillus* and *Flavobacterium* were the most abundant genera identified, followed by other genital tract bacteria, which was consistent with previous findings. However, no statistically significant correlation was found between the endometrial microbiome profile and the reproductive outcome on these patients (21).

Later, a second study on the impact of endometrial microbiome on reproductive outcomes revealed that low abundance of *Lactobacillus* in the endometrial fluid is associated

with poor reproductive success in patients with RIF even if they have a receptive endometrium at the time of ET (12). Consistent with previous studies, *Lactobacillus* spp. was the most represented genus in the endometrial samples of these patients, followed by *Gardnerella*, *Streptococcus*, and *Bifidobacterium*. However, only the percentage of *Lactobacilli* was predictive of the reproductive success or failure of IVF in those 35 patients receiving personalized ET. In this regard, patients with a *Lactobacillus*-dominated microbiome (more than 90% of *Lactobacilli*) presented significantly increased implantation, pregnancy, ongoing pregnancy, and live birth rates (60.7% vs. 23.1%,  $P=.02$ ; 70.6% vs. 33.3%,  $P=.03$ ; 58.8% vs. 13.3%,  $P=.02$ ; and 58.8% vs. 6.7%,  $P=.002$ , respectively) compared with patients with a non-*Lactobacillus*-dominated microbiome, especially when *Gardnerella* and *Streptococcus* were identified in endometrial fluid (12). Although further studies are required to fully understand the role of endometrial bacteria in reproductive function, these results suggest an important role of microbial communities in embryo implantation and pregnancy.

## CLINICAL IMPORTANCE OF ENDOMETRIAL DYSBIOSIS

Several studies reported to date using classical or molecular techniques have pointed at endometrial dysbiosis as an emerging cause of implantation failure and pregnancy loss (12,14–19). For this reason, the impact of endometrial microbiota on several causes of infertility is being investigated to improve clinical management of infertile patients with altered uterine microbiota.

One example is endometriosis, a severe disease characterized by the growth of endometrial tissue outside the uterine cavity leading to the formation of endometriomas. This condition affects up to 10% of woman of reproductive age, producing serious symptoms including pelvic pain and infertility (22). Currently, the cause of endometriosis is still unknown, and so is its treatment, but several investigations has linked the presence of endometrial pathogens to endometriosis, providing a potential role of bacterial pathogens in the onset of the disease. The evidences supporting this hypothesis come from the increased isolation of *Actinomyces*, *Corynebacterium*, *Enterococcus*, *E. coli*, *Fusobacterium*, *Gardnerella*, *Prevotella*, *Propionibacterium*, *Staphylococcus*, and *Streptococcus* to the detriment of *Lactobacillus* spp. in endometrial samples and menstrual blood of patients with endometriosis, while bacteria from the Staphylococcaceae and Streptococcaceae families have been identified at the molecular level in ovarian endometrioma fluid (23–25). Recently, the description of the reproductive tract microbiota by NGS has showed that patients with and without endometriosis-related infertility present a different microbiome (8), supporting the correlation of endometriosis with the presence of endometrial infections that may impair contractility of the uterus, facilitating the retrograde seeding of endometrial cells (26, 27).

But infertility may not be the only cause of altered endometrial microbiota, as pathological shifts of a balanced endometrial flora have been associated with different pathologies

of the genital tract. This is the case in patients with endometrial hyperplasia and endometrial cancer, which have been associated with decreased abundance of *Lactobacilli* and increased abundance of *Anaerostipes*, *Anaerotruncus*, *Atopobium*, *Bacteroides*, *Dialister*, *Peptoniphilus*, *Porphyromonas*, and *Ruminococcus* compared with healthy controls (28), pointing to an infectious role in the onset of some endometrial cancers. Patients with adenomyosis, which is the presence of endometrial tissue in the myometrium, present significant differences along their reproductive tract microbiome compared with subjects without the condition (8).

Also, alterations of the endometrial microbiome could be related to pregnancy complications involving infection and inflammation of the maternal or fetal membranes such as deciduitis, chorioamnionitis, and other obstetrical conditions. The most plausible link connecting endometrial infection with miscarriage or obstetrical complication relies on alterations of the immune and inflammatory response (29). In addition, it has been proposed that an unfavorable bacterial composition may activate antiangiogenic pathways during placental and embryo fetal development, resulting in altered trophoblast and endothelial function, which are characteristic of preeclampsia (30).

A clear scenario of altered endometrial microbiota is chronic endometritis, the persistent inflammation of the uterine lining mainly caused by bacterial pathogens. Because it is often asymptomatic, chronic endometritis is seldom suspected and diagnosed, leading to important inconsistencies in the estimated prevalence reported for this disease. This subclinical disease could affect up to 45% of infertile patients with RIF and RPL (31); however, other investigators, using the same techniques, report the diagnosis of chronic endometritis in 14% and 27% of patients with RIF and RPL, respectively (32). Supporting the relevance of chronic endometritis in reproductive outcomes of these patients, a couple of retrospective studies conducted by Cicinelli and collaborators have shown that antibiogram-driven treatment of chronic endometritis in RIF and RPL patients improves their reproductive outcomes (33, 34), but further prospective studies are needed to confirm the usefulness of antimicrobial therapy in IVF patients diagnosed with chronic endometritis. However, the current diagnosis of chronic endometritis still depends on the method used, as the three classical methods, histological examination of endometrial pieces, hysteroscopy of the uterine cavity, or microbial culture, in the same patient often offer contradictory results. A recent work has evaluated the accuracy of these three methods in diagnosing chronic endometritis in a set of 65 infertile asymptomatic patients in which the three methods were independently and blindly used (35). Interestingly, only 20% of the samples analyzed presented concordant results using the three techniques. In all the inconsistent results there was a general trend of histology to underdiagnose the disease, while hysteroscopy usually overdiagnoses chronic endometritis. However, the molecular detection of bacterial pathogens in the same endometrial samples, using either targeted polymerase chain reaction for chronic endometritis-causing pathogens or endometrial microbiota using 16S sequencing, presented 77% of accuracy with those cases with concordant classical results,



providing additional information about nonculturable microorganisms. The unequivocal diagnosis of chronic endometritis in infertile patients, using objective and reliable methods, could help to improve the clinical management of asymptomatic patients in whom chronic endometritis is not suspected or diagnosed (35).

## CHALLENGES OF ANALYZING THE ENDOMETRIAL MICROBIOTA

Despite the high similarities between the lower and UGT microbiota, several authors have observed small but still significant differences between paired endometrial and vaginal samples of the same subjects (8,11–13). These discrepancies between the endometrial and vaginal microbiota rationalize the importance of analyzing the uterine microbiota in infertile patients as this is the maternal environment in which the embryo must adhere, implant, and grow to develop a healthy pregnancy to term. However, there are some technical hurdles associated with the investigation of the endometrial microbiome. Consistent with the concept of a microbiota continuum along the reproductive tract, the vagina presents a high load of residing bacteria that gradually decreases from the most exposed to the most remote location. In this gradient, the vaginal microbiota have been estimated to harbor approximately  $10^{10}$ – $10^{11}$  bacteria, while the endometrium may contain four orders of magnitude fewer bacteria than the vagina, which is considered a low-biomass microbiome (8, 11). The differences observed in the bacterial colonization of the lower and UGT could be explained by the cervical mucus, a natural barrier for ascending bacteria that changes during the menstrual cycle, allowing the transit of microorganisms to the UGT. Once these bacteria have colonized the UGT, they are exposed to the immune system, which should prevent colonization of bacterial pathogens. However, some bacteria have gained the competence for

evading the immune response of the host in several niches and may remain in the endometrium after being eliminated from the vagina; others are able to grow inside the uterine cavity due to the different physiological conditions compared with the vagina.

Low-biomass microbiomes (endometrial, urine, blood) may play important roles in microbial homeostasis and physiology, but their study is often biased by potential contaminations coming from bacteria or bacterial DNA present in the air, the laboratory equipment, and reagents that are inadvertently incorporated into the samples during sample collection, processing, and analysis. One of the most relevant sources of bacterial DNA contamination comes from DNA extraction kits and laboratory reagents, even if they are sterile. Research performed by analyzing the resulting microbiome of serial dilutions of a single bacterium *Samonella bongori* has allowed the identification of the most common bacterial genera arising from bacterial DNA contamination (Table 1) (36). This contaminant DNA may affect the results of both 16S rRNA gene sequencing and shotgun metagenomic analysis. For this reason, it is of outstanding relevance that researchers working with low-biomass samples follow strict protocols to avoid misleading conclusions about the sequencing results. These measures would include the prevention of contamination before sequencing by maximizing the protective equipment, during sequencing by including sufficient negative and blank controls, and after sequencing by developing bioinformatic pipelines to track and subtract potential contaminants from the bona fide microbiome coming from the sample (37).

## CONCLUSIONS

Implantation is a complex process that requires the orchestration of several biological functions at the cellular and molecular levels. The technical advances in massive parallel sequencing have allowed for the identification of

**TABLE 1**

**List of contaminant genera most commonly detected in sequencing of low-biomass microbiome.**

Phylum	List of constituent contaminant genera
Proteobacteria	<p>Alphaproteobacteria:</p> <p><i>Afipia</i>, <i>Aquabacterium</i>,<sup>e</sup> <i>Asticcacaulis</i>, <i>Aurantimonas</i>, <i>Beijerinckia</i>, <i>Bosea</i>, <i>Bradyrhizobium</i>,<sup>d</sup> <i>Brevundimonas</i>,<sup>c</sup> <i>Caulobacter</i>, <i>Craurococcus</i>, <i>Devosia</i>, <i>Hoeflea</i>,<sup>e</sup> <i>Mesorhizobium</i>, <i>Methylobacterium</i>,<sup>c</sup> <i>Novosphingobium</i>, <i>Ochrobactrum</i>, <i>Paracoccus</i>, <i>Pedomicrobium</i>, <i>Phyllobacterium</i>,<sup>e</sup> <i>Rhizobium</i>,<sup>c,d</sup> <i>Roseomonas</i>, <i>Sphingobium</i>, <i>Sphingomonas</i>,<sup>c,d,e</sup> <i>Sphingopyxis</i></p> <p>Betaproteobacteria:</p> <p><i>Acidovorax</i>,<sup>c,e</sup> <i>Azoarcus</i>,<sup>e</sup> <i>Azospira</i>, <i>Burkholderia</i>,<sup>d</sup> <i>Comamonas</i>,<sup>c</sup> <i>Cupriavidus</i>,<sup>c</sup> <i>Curvibacter</i>, <i>Delftia</i>,<sup>e</sup> <i>Duganella</i>,<sup>a</sup> <i>Herbaspirillum</i>,<sup>a,c</sup> <i>Janthinobacterium</i>,<sup>e</sup> <i>Kingella</i>, <i>Leptothrix</i>,<sup>a</sup> <i>Limnobacter</i>,<sup>e</sup> <i>Massilia</i>,<sup>c</sup> <i>Methylophilus</i>, <i>Methyloversatilis</i>,<sup>e</sup> <i>Oxalobacter</i>, <i>Pelomonas</i>, <i>Polaromonas</i>,<sup>e</sup> <i>Ralstonia</i>,<sup>b,c,d,e</sup> <i>Schlegelella</i>, <i>Sulfuritalea</i>, <i>Undibacterium</i>,<sup>e</sup> <i>Variovorax</i></p> <p>Gamma-proteobacteria:</p> <p><i>Acinetobacter</i>,<sup>a,d,c</sup> <i>Enhydrobacter</i>, <i>Enterobacter</i>, <i>Escherichia</i>,<sup>a,c,d,e</sup> <i>Nevskia</i>,<sup>e</sup> <i>Pseudomonas</i>,<sup>b,d,e</sup> <i>Pseudoxanthomonas</i>, <i>Psychrobacter</i>, <i>Stenotrophomonas</i>,<sup>a,b,c,d,e</sup> <i>Xanthomonas</i><sup>b</sup></p>
Actinobacteria	<p><i>Aeromicrobium</i>, <i>Arthrobacter</i>, <i>Beutenbergia</i>, <i>Brevibacterium</i>, <i>Corynebacterium</i>, <i>Curtobacterium</i>, <i>Dietzia</i>, <i>Geodermatophilus</i>, <i>Janibacter</i>, <i>Kocuria</i>, <i>Microbacterium</i>, <i>Micrococcus</i>, <i>Microlunatus</i>, <i>Patulibacter</i>, <i>Propionibacterium</i>,<sup>e</sup> <i>Rhodococcus</i>, <i>Tsukamurella</i></p>
Firmicutes	<i>Abiotrophia</i> , <i>Bacillus</i> , <sup>b</sup> <i>Brevibacillus</i> , <i>Brochothrix</i> , <i>Facklamia</i> , <i>Paenibacillus</i> , <i>Streptococcus</i>
Bacteroidetes	<i>Chryseobacterium</i> , <i>Dyadobacter</i> , <i>Flavobacterium</i> , <sup>d</sup> <i>Hydrothalea</i> , <i>Niastella</i> , <i>Olivibacter</i> , <i>Pedobacter</i> , <i>Wautersiella</i>
Deinococcus-Thermus	<i>Deinococcus</i>
Acidobacteria	Predominantly unclassified Acidobacteria Gp2 organisms

Reprinted from Salter SJ, et al., Reagent and laboratory contamination can critically impact sequence-based microbiome analyses, *BMC Biology* 2014;12:87. Moreno. Uterine microbiota in IVF. *Fertil Steril* 2018.

microorganisms at the molecular level, adding a new microbiological dimension to human reproduction. The existence of a reproductive microbiota continuum has highlighted the importance of a healthy microbiome in all the steps of reproduction, from gamete formation, to implantation, to labor, involving all the locations within the reproductive tract. Therefore, pathological shifts of these reproductive tract microbiota may be the cause or consequence of diseased conditions in women's health. Based on the similarity between the lower and upper genital tract microbiome, the vaginal or cervical microbiome could correlate with the endometrial microbiome or even with the reproductive outcomes of patients, as reported by some investigators (38). Interestingly, in up to 20% of subjects, significant differences between the bacterial composition of vaginal and endometrial samples have been observed, pointing to the analysis of the uterine cavity as the most straightforward way to predict implantation (12).

Concretely, the endometrial microbiome seems to have an impact on embryo implantation and pregnancy maintenance, as an altered endometrial microbiome, characterized by a low abundance of *Lactobacillus*, has been associated with implantation failure and pregnancy loss. In this regard, not only the microbial profiles leading to reproductive success or failure are under investigation, but also the functional interactions between the community of microorganisms and their host are crucial to understand the effect of endometrial microbiota in infertility. However, the study of endometrial microbiota, as of other low-biomass communities, present some technical challenges that need to be overcome to avoid misleading conclusions.

From the clinical point of view, the identification of endometrial dysbiosis as a new cause of infertility opens a new microbiological field in the evaluation of endometrial factor, highlighting the relevance of assessing the uterine microbiota in infertile patients to restore a favorable endometrial flora in those patients with altered uterine microbiota to improve and personalize the clinical care of infertile patients.

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