Cervicovaginal microbiota, women's health, and reproductive outcomes

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The human microbiome project has shown a remarkable diversity of microbial ecology within the human body. The vaginal microbiota is unique in that in many women it is most often dominated by *Lactobacillus* species. However, in some women it lacks *Lactobacillus* spp. and is comprised of a wide array of strict and facultative anaerobes, a state that broadly correlates with increased risk for infection, disease, and poor reproductive and obstetric outcomes. Interestingly, the level of protection against infection can also vary by species and strains of *Lactobacillus*, and some species although dominant are not always optimal. This factors into the risk of contracting sexually transmitted infections and possibly influences the occurrence of resultant adverse reproductive outcomes such as tubal factor infertility. The composition and function of the vaginal microbiota appear to play an important role in pregnancy and fertility treatment outcomes and future research in this field will shed further translational mechanistic understanding onto the interplay of the vaginal microbiota with women's health and reproduction. (Fertil Steril® 2018;110:327–36. ©2018 by American Society for Reproductive Medicine.)

Key Words: Sexually transmitted diseases, pelvic inflammatory disease, bacterial vaginosis, in vitro fertilization, contraception

This review addresses recent advances into our understanding of the microbial ecosystem in the human vagina and its role on women's health and reproductive outcomes. We have summarized the most recent knowledge, in the context of prior understanding of how the vaginal microbiota is influenced by menstrual cycle and sex hormones, contraceptives and influences the risk of infections and disease, adverse pregnancy and fertility treatment outcomes.

LACTOBACILLUS SPP. OFTEN UNIQUELY PREDOMINATE THE HUMAN VAGINAL MICROBIOTA

It is now well accepted that microbes present in or on the human body can impact immunity, nutrition, and physiology (1–3). The human vagina is unique in that, in healthy states, it is most often characterized by reduced bacterial diversity and the dominance of *Lactobacillus* spp. ($\sim 10^7$–10^9 per gram vaginal fluid in reproductive aged healthy women) compared to other microbiota (4). The presence of *Lactobacillus* spp., known to produce copious amount of lactic acid, is directly correlated with vaginal pH <4.5. Lactic acid driven acidity (low pH) has been strongly correlated with protection against cervico-vaginal infections, including HIV and other sexually transmitted infections (5–8).

Lactobacillus spp. dominated vaginal microbiota have been intrinsically linked to estrogen production and the accumulation of glycogen in the upper layers of the stratified vaginal epithelium (9, 10). Beyond lactic acid, *Lactobacillus* spp. beneficial properties are associated with the production of bacteriocins (antimicrobial compounds), adherence to the vaginal epithelia (competitive exclusion of other bacteria), and ability to competitively use available nutrients (11, 12). The physiology of the vaginal *stratum corneum* (*SC*), consisting of loosely associated cells with glycogen stores, and innate defense mediators (13), is thought to contribute to this site being a niche for *Lactobacillus* spp. However, the exact reason for *Lactobacillus* spp. dominance in the human vagina remains to be fully elucidated. Interestingly, other mammals do not harbor *Lactobacillus* spp. in their vaginal microbiota, and consequentially their vaginal pH is not acidic. However, while the composition of the vaginal microbiota is different, it is hypothesized that it could perform the same functions (14). Factors such as diet and unique environmental exposures have been proposed as potential reasons for these compositional differences (14).

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The development of novel and high-throughput culture-independent methods to characterize the composition and structure of microbiota, supported by advances in next generation sequencing technologies and their reduced cost, have enabled a more in depth characterization of microbiota. In the vaginal microbiota (as discussed further in the next section) these advances have enabled the identification of strong correlations between different states of the vaginal microbiota and risk of infections (15). As a result, an improved understanding of the complexities of the microbial environment of the female reproductive tract is available. Approaches that do not rely on amplifying and sequencing specific taxonomically informative genes (i.e., 16S rRNA gene, *cpn60* [16]), such as metagenomics (sequencing of all genes and genomes in a microbial community) (17) or metatranscriptomics (sequencing all gene transcripts expressed in a microbial community) (18) are contributing to the functional characterization of the microbiota and its interaction with the human host.

MOLECULAR, CULTURE, AND SEQUENCING CONTRIBUTIONS TO UNDERSTANDING THE ECOLOGY OF THE HUMAN VAGINA

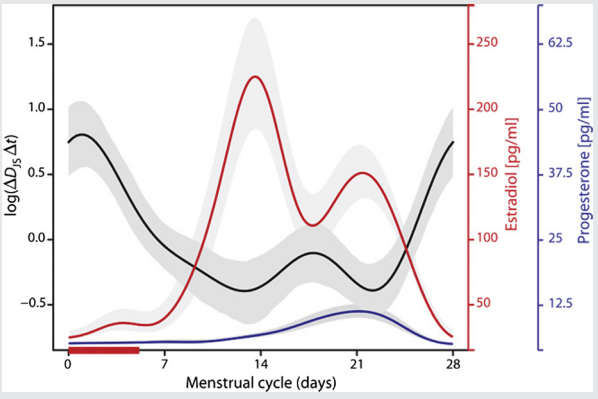
High-throughput 16S rRNA gene sequencing studies examining vaginal bacterial species composition and abundance in reproductive-aged women have shown that there are at least five major types of vaginal microbiota, termed community state types (CST) (19, 20). Four of these CSTs are dominated by either *Lactobacillus crispatus* (CST I), *L. gasseri* (CST II), *L. iners* (CST III), or *L. jensenii* (CST V). Additionally, CST IV does not contain a significant species or quantity of *Lactobacillus* but instead comprised of a polymicrobial mixture of strict and facultative anaerobes including species of the genera *Gardnerella*, *Atopobium*, *Mobiluncus*, *Prevotella,* and other taxa in the order *Clostridiales* (19–21). Further examination of CST IV has revealed distinct clusters within this polymicrobial community type, which have since been denoted subgroups CST IV-A and CST IV-B (20). Subgroup IV-A can contain moderate amounts of *Lactobacillus* spp. (typically *L. iners*) as well as strict anaerobes including *Corynebacterium*, while conversely CST IV-B contains a higher proportion of species associated with bacterial vaginosis (BV). The frequency of these CSTs has been shown to differ in different ethnic backgrounds (19, 22), with CST I more common in Caucasian women and CST IV more common (~40%) in African-American and Hispanic women. The frequency of these CSTs differs not only by ethnicity but also by geographical origins (22–24).

Daily (or frequent) fluctuations in the composition of the vaginal microbiota have been documented by microscopy and cultivation studies (25–27). These findings were confirmed and extended in longitudinal culture-independent analyses performed on vaginal swabs collected twice weekly for 16 weeks (20, 28), or daily for 10 weeks (29) or 4 weeks (4). It was observed that some vaginal microbial communities transitioned in and out of CST IV. The amount of time spent in a particular CST could vary individually as some women experienced consistent and stable CST longitudinal patterns, while others frequently transitioned between CSTs, most frequently to CST IV (20, 29). In some cases, CST transitions were triggered by menstruation or sexual behaviors, but in other cases they seem to be driven by uncharacterized factors (20). In another longitudinal study, presence of *Gardnerella* was found to be predictive of an impending CST change (30). Phase in the menstrual cycle greatly affects community stability. During ovulation, when estradiol production peaks, stability is highest, while during menstruation, *Lactobacillus* spp. tend to decrease in relative abundance (31), with the exception of *L. iners* (20). In general, molecular and culture-based methods are somewhat in agreement that menses significantly alters the composition of the vaginal microbiota (27,32–34), but change appears to depend on the initial CST present, as well as other factors (20) such as the use of menstrual pads or tampons (20, 35). Figure 1 shows the interplay of microbiome status throughout the menstrual cycle, (20). These longitudinal studies highlight the highly dynamic nature of vaginal microbial communities during the menstrual cycle and emphasize the need to better understand the underlying biological factors modulating fluctuations in composition and functions that affect host physiology. Bayesian network analysis was used to further understanding of the complex interplay between behaviors in menstrual hygiene and microbiota (36). The study highlighted that despite the relatively reduced complexity of the vaginal microbiota, novel approaches integrating more elements of the complex biological system will ultimately improve our understanding of the interactions that drive the vaginal ecosystem and ultimately women's health.

IMPACT OF HORMONAL CONTRACEPTION ON VAGINAL MICROBIOTA

Because estrogen cycling appears to be linked to vaginal microbiota stability and to some extent composition, several

FIGURE 1



Vaginal microbiota stability and sex hormone levels during the menstrual cycle. The highest stability correlates with high estrogen or progesterone levels, but can be affected by the community state type of the vaginal microbiota, behaviours, and other host factors. Reproduced with permission from AAAS (20).

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studies have evaluated the effect of contraception (oral, injected, and implanted) methods on the composition of the microbiota. A large cohort study of 266 healthy women initiating contraception and aged 18–35 years in Harare, Zimbabwe, used quantitative polymerase chain reaction (PCR) measurement of vaginal bacteria. No significant impacts of most hormonal contraceptives were found on vaginal microbiota composition, including on the abundance of *Lactobacillus* spp. Interestingly, copper intrauterine devices (IUDs) were associated with a significant increase of BV-associated bacteria (assessed by species specific quantitative PCR) over the 180-day study ($P=.005$) (37). This finding contradicts a study using Nugent and microscopic analysis of vaginal microbiota in Thai HIV-positive women, which found no association with these BV microbial indicators and IUDs (38). In another study of 682 women using contraceptive measures in the United States, combined oral contraceptives (COC) (progestin and estrogen) (39) users were more likely to be colonized by *Lactobacillus* spp. and less likely to harbor BV-associated bacteria than when using other forms of barrier (condoms) or hormonal contraceptives (depot medroxyprogesterone acetate [DMPA], or the levonorgestrel-releasing intrauterine system [LNG-IUS]) (adjusted odds ratio [OR]1.94, 95% confidence interval [CI] 1.25–3.02). A systematic review of HIV acquisition studies that include microbiota and contraceptive usage in women identified that there is some (limited evidence) that the combined oral contraceptive may pre-dispose to candidiasis, which may in turn be a risk factor for HIV acquisition (40). Other studies have reported the LNG-IUS can increase *Candida* colonization and temporally decrease *Lactobacillus* dominance (41), enhance susceptibility to herpes simplex virus infection (42) or delay clearing *Chlamydia trachomatis* infection (43). Mitchell et al. (44), in a small study of 32 women have reported that after 12 months of use, DMPA was associated with a decreased in vaginal *Lactobacillus* phenotype by culture as producing H_2O_2, a surrogate for non-*Lactobacillus iners* species. In that study, DMPA did not increase vaginal mucosal CCR5+ HIV target cells but did decrease CD3+ T lymphocytes. Borgdorff et al. (22), found that contraceptive use was not associated with vaginal microbiota composition, but they did find sexual behavior (inconsistent sexual partner OR 3.2 CI 1.0–9.9) and ethnicity correlate with a polymicrobial BV-like microbiota when compared to a *Lactobacillus*-dominated microbiota. Bassis et al. (45), analyzed the vaginal microbiota before, at 6, and 12 months following insertion of copper (n=36) and progesterone (n=40) IUDs, and found no correlation with the device and microbiota changes over this relatively large time frame (45). Interestingly, the literature is not always in agreement on the effect of contraception on the composition of the vaginal microbiota or susceptibility to sexually transmitted diseases. A major factor often not considered in several of these studies is ethnicity. Further studies are needed to evaluate the effect of contraceptive methods on disease susceptibility and the composition of the vaginal microbiota, while considering the previously reported association between ethnicity and vaginal microbiota (19).

VAGINAL INFECTIONS, DISEASE, AND THE MICROBIOTA

We have chosen to include a section on how the vaginal microbiota interplays with infections and disease, because these can result in infertility or adverse pregnancy outcomes and hence are important in the context of reproduction. Using microscopic observation, the composition of the vaginal microbiota has long been linked to disease risk, with the presence of *Lactobacillus* spp. providing protection while a paucity in *Lactobacillus* spp. and the presence of a diverse set of Gram-negative anaerobic species associated with increased risk to disease. The latter is often defined as bacterial vaginosis, a conditions present in 29% women aged 14–49 years in the general U.S. population, in over 50% of African-American women (46), and in over 70% of women attending sexually transmitted infection clinics (47). High-throughput molecular analyses afford a more in-depth and precise characterization of the vaginal microbiota and insight into the role of specific species or clades in disease risk. In this section, we address how these high-resolution analyses have advanced our understanding of disease risks for BV, pelvic inflammatory diseases (PID), and sexually transmitted infections.

Bacterial Vaginosis

Diagnosis of BV in a clinical setting relies on the Amsel criteria (48) and in research settings on the Nugent scoring system (49). Interestingly, despite the use of molecular analysis to define BV states (50, 51), no one taxa has been confirmed as the etiological agent of the condition, and BV remains ill-defined microbiologically as a polymicrobial state, basically characterized by the lack of predominant *Lactobacillus* spp. That said, several bacteria, such as *Gardnerella* (*G.*) *vaginalis*, have been shown to be associated with the condition in some studies but not others (52). Early studies failed to reproduce the disease after direct vaginal inoculation of *G. vaginalis* isolated from women with BV, while inoculation with whole vaginal secretions did (53, 54), supporting that the condition is either polymicrobial or other factors contribute. It is highly likely that the pathogenic potential of *G. vaginalis* might differ depending on the specific strain of *G. vaginalis* colonizing and possibly the vaginal immune state or ethnicity (55). Interestingly, it appears that *G. vaginalis* can be transferred sexually. A longitudinal study of young women in Australia found that *Gardnerella* was more likely to be found in those having penile sex (OR 11.82, 95% CI 1.87–74.82; $P=.009$) (24). *Gardnerella* was also found in approximately a third of girls (aged 10–12 years) in a pre-menarche vaginal microbiota study, indicating that this organism is not only acquired/facilitated by sexual activity, potentially could be transfer at birth from mother to daughter (56). Yet, *G. vaginalis* colonization increased after sexual activity in a cohort of young women who were monitored pre- and post-sexual debut, ($P=.02$) indicating sexual activity is a factor in the transmission of this bacteria (57). In support of the polymicrobial nature of BV, in a longitudinal study of women who have sex

with women, BV incidence was associated with sexual behaviors, and most strongly correlated with a new sexual partner with BV-associated symptoms (adjusted hazard ratio 2.8, 95% CI 1.30–4.82) (58). Furthermore, a large cohort study of 1,093 women in general practice care in Australia found that either a recent new female sexual partner or multiple male partners were significantly associated with prevalent or incident BV cases (59). Interestingly, in this study estrogen contraceptives were protective (adjusted OR 0.6, 95% CI 0.4–0.9) (59). A similar study in the U.S. found that new sexual partners and oral vulvovaginal sex were both significant risk factors for BV, while a *L. crispatus*-dominated (CST I) microbiota was protective (hazard ratio 0.18, 95% CI 0.08–0.4) (60). The lack of a clear definition for BV makes studying its etiology challenging. One study attempted to improve the definition of BV using molecular methods, combining bacterial composition (16S rRNA gene amplicon sequencing), eukaryotic composition (ITS sequencing), and *Trichomonas* characterization (sequencing of the *tvk* loci) (61), but it failed because of limitations associated with the study such as the lack of speciation of *Lactobacillus* spp., highlighting the need for further development. An improved definition of BV would have major implications in women's clinical management and women's health as a whole.

While new antibiotics are being developed or tested to treat BV (62–64), leveraging the vaginal microbiota for the development of live biotherapeutic formulations to modulate the microbiota is also considered (65) to restore a *Lactobacillus*-dominated protective vaginal microbiota. As drug-based treatment failure can be high, with antibiotic resistance appearing (66), and recurrence very common (67, 68), alternative approaches are needed. Vaginally delivered live biotherapeutics are safe and can be used in combination therapy after antibiotic treatment (69–71), however success has been limited, certainly because formulations do not take into account the ecology of the vaginal microbiota and often rely on one strain of *Lactobacillus*, mostly *L. crispatus* or non-vaginal *Lactobacillus* strains (70–73). Nonetheless, further work is needed to develop and optimize an efficacious formulation.

The majority of trials in this space have been in the context of BV. An analysis of several trials of probiotics, orally administered with presumed rectal transfer, or vaginally distributed supported that greater than 10^8 cfu of leading probiotic strains for more than 2 months helped some participants resolve BV (74). However, a trial comparing metronidazole treatment with combined metronidazole and a vaginal probiotic of *Lactobacillus acidophilus* with estrogen did not find a significant impact on BV recurrence (69). One interesting approach used an *ex vivo* model to provide further evidence of *Lactobacillus* defence against HIV (75), further supporting the potential for developments in this field to live biotherapeutics.

Alternative strategies, such as metabolite or receptor competitive molecules, along with precision medicine approaches are likely to emerge. One example that has been proposed for family members with a genotypically driven dectin-1 deficiency linked to recurrent vulvo-vaginal candidiasis was supplementation with dectin-1 (76, 77). Also, antimicrobial proteins and peptides that are mimics of those already produced as innate defense and used as vaginal supplements are possible future therapeutic options (reviewed [76]).

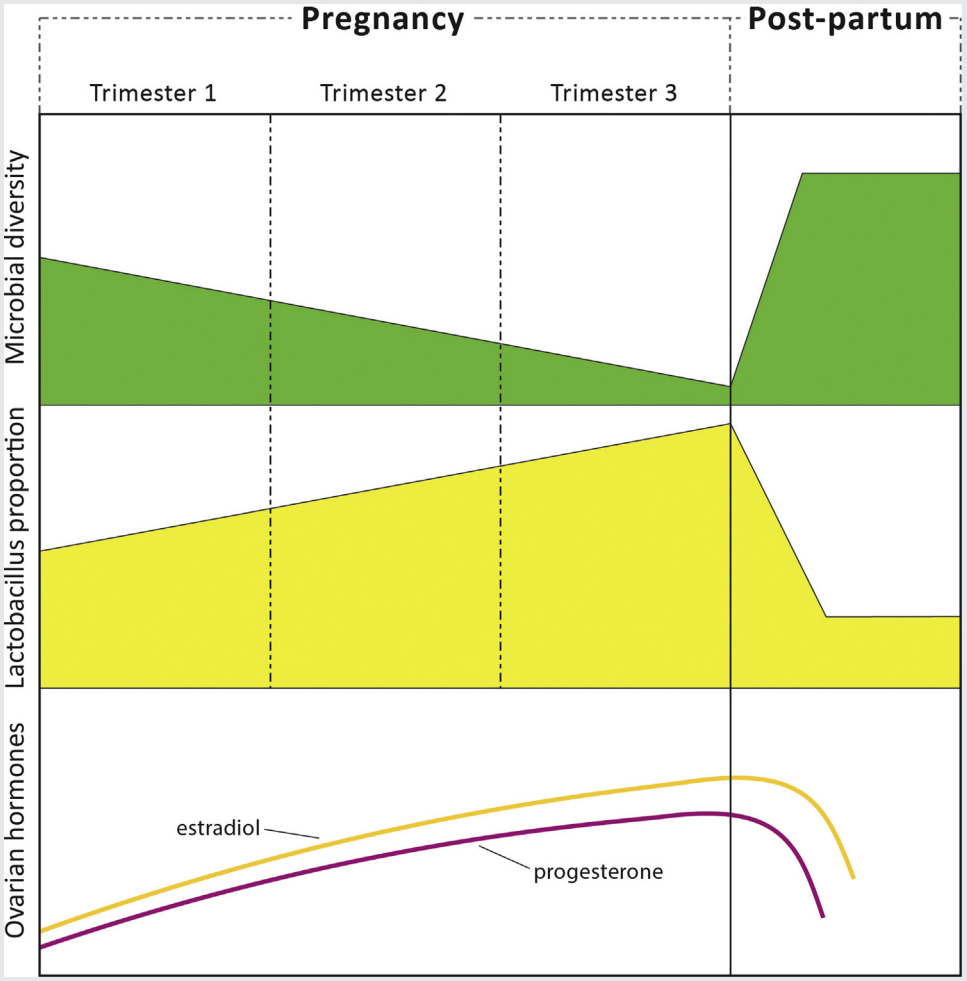
Pelvic Inflammatory Disease

The composition of the vaginal microbiota appears to play a role in the development of another important disease, PID. PID is associated with inflammation in the upper reproductive tract in women, characterized by sudden onset of pain along with cervical, adnexal, or uterine tenderness. Risk factors for PID include those that also affect the composition of the vaginal microbiota, such as history of multiple sexual partners, or early age of commencement of sexual activity (78). Microbial risk factors for PID include sexually transmitted infections and bacterial vaginosis (79, 80). In addition to the endometrial presence of sexually transmitted pathogens such as *C. trachomatis*, *Mycoplasma genitalium,* and *Neisseria gonorrhoeae*, PCR testing for BV-associated bacteria in endometrial samples identified bacteria such as *Sneathia sanguinegens*, *Sneathia amnionii*, *Atopobium vaginae* and BV-associated bacteria 1 (BVAB1) in women with PID (81). It is common to fail to identify known pathogens in women with PID, although frequently many other organisms are detected in the upper reproductive tract (82). Hence, it is likely that a *Lactobacillus*-dominated vaginal microbiota could be protective for PID. However, as yet there are no reports of extensive molecular analyses of the vaginal microbiota in the context of PID and the question remains open.

Sexually Transmitted Infections

Risk for contraction of sexually transmitted pathogens has been associated with the composition of the vaginal microbiota. The most insights have come from studies into incidence and prevalence of *C. trachomatis*. *Chlamydia* is one of the most common sexually transmitted bacterial pathogens and has been found in three independent studies to be more likely detected in association with *L. iners* (CST III) (e.g. OR 2.6–4.4) and/or CST IV (OR 4.2) (83–85). This may relate to availability of metabolites produced by these types of microbiota that benefit the pathogen, as found in one study (86). On the other hand, the vaginal microbiota, in particular *L. crispatus* (CST I), may have specific anti-chlamydial, anti-gonococcal, and immune enhancing properties, as evidenced in vitro and on a porcine epithelial model (6,87,88). *N. gonorrhoeae* infections are less common (compared to *Chlamydia*) in women. Whilst there is little information on the composition of the vaginal microbiota in the context of gonococcal infections, it has been shown in vitro that *Lactobacillus* spp.(especially *L. gasseri* (CST II), and *L. jensenii* (CST V)) can directly compete with *N. gonorrhoeae* for epithelial binding (89, 90). A large nested case-control study in African women identified that there are several taxa within the vaginal microbiota that are associated with increased risk of HIV acquisition (91). Bacterial vaginosis, sexually transmitted infections such as *Chlamydia* and Herpes, and vaginal washing have also been associated

FIGURE 2



Vaginal microbiota decreases in diversity during pregnancy, often with an increased relative abundance in *Lactobacillus* spp. In the post-partum phase an immediate increase in vaginal microbiome diversity and decrease in *Lactobacillus* spp. has been observed.

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with increased risk of transmission and/or acquisition of HIV (92, 93). Altogether, these data support mechanisms such as competition, low pH, specific anti-bacterial molecules (bacteriocin) (94, 95), through which the vaginal microbiota is a major driver of protection to infectious agents.

Pregnancy Outcomes and the Cervicovaginal Microbiota

In pregnancy the lack of menses and the increase in circulating estrogen are associated with a microbiota characterized by an increased dominance of *Lactobacillus* spp. as gestation progresses (96). This is a feature of the vaginal microbiota in pregnancy that has been established by several studies of varied power and sampling intensities, both in the U.S. and in Europe (23, 96–99). Interestingly, this inherent stability of the microbiota in pregnancy was also true at other body sites (96). Post-partum, and up to a year after delivery, the vaginal microbiota was characterized by a paucity of *Lactobacillus* spp. (CST IV), even in pregnancies where *Lactobacillus* spp. were dominant during gestation (23,96). While these findings support the hypothesis that adverse postpartum outcomes such as endometritis and sepsis might be mediated by vaginal microbes, its biological and reproductive implications remain unknown. Overall summary of this data is presented in Figure 2, showing that generally *Lactobacillus* spp. abundance increases, and community diversity decreases during pregnancy, with a shift post-partum to high diversity.

Several studies have documented the composition of the vaginal microbiota associated with adverse pregnancy outcomes such as pre-term birth (96, 100–108). While certain studies found association with a few bacteria (mostly anaerobes), others did not, and no consistent signature has been identified. Deciphering cause from effect remains a challenge in these studies and it is likely that most suffered from a low number and poor phenotype (not all preterm births were spontaneous) of preterm birth cases.

Preterm premature rupture of membranes (PPROM) is one adverse pregnancy outcome that has strongly correlated with the cervicovaginal microbiome in distinct studies. In one study, and consistent with several others, women who experienced PPROM were less likely to have *Lactobacillus*, or lower abundance of *Lactobacillus* in the vaginal microbiome composition and high diversity of the microbiota (106). Furthermore, the presence of *Mollicutes* such as *Mycoplasma* or *Ureaplasma* (although these are also common in healthy vaginal microbiomes) have been found to be more frequently present in the vaginal microbiome of women who experienced PPROM (106). Early and late miscarriages have long been associated with BV or the presence of specific flora in the vagina (109–112). Chorioamnionitis or intra-amniotic infections have also been associated with the vaginal microbial community state, including a recent history of BV (113–115). Further research is desperately needed and would involve well-powered prospective study designs, include ethnically diverse populations, and aim at identifying predictive signatures in the cervico-vaginal microbiota or its products, thereby ultimately providing novel strategies to restore a protective vaginal microbiota.

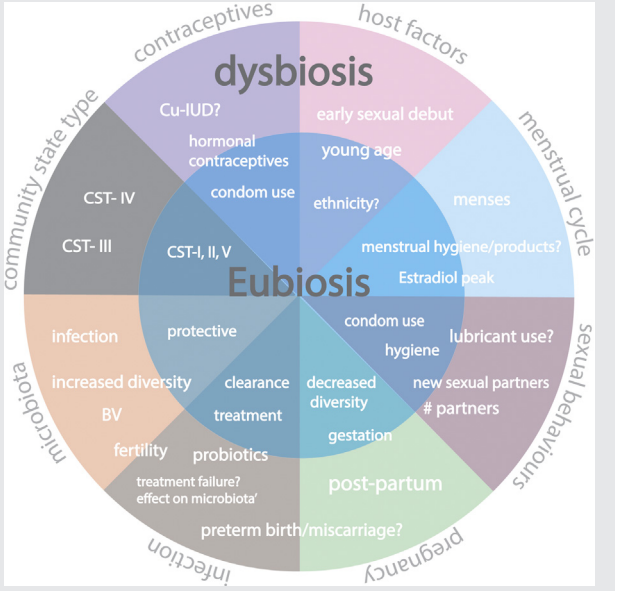
Cervico-vaginal Microbiota in Infertility and Fertility Treatment

The composition of the vaginal microbiota is thought to influence fertility and outcomes of fertility treatment. Most published studies were performed in the context of in vitro fertilization procedures, which could be well-controlled. In these studies, BV-associated bacteria in the vagina have been shown to be associated with a reduced pregnancy rate (116–118). The study by Haahr and colleagues (116) focused on using Nugent score and PCR detection of BV-associated bacteria in the vagina and comparing to IVF success in 130 women. They found that Nugent and PCR correlated highly, and that women with PCR detected BV-associated bacteria were significantly less likely to obtain a clinical pregnancy (9%) compared to the overall rate of 35% ($P=.004$) (116). Interestingly, women undergoing IVF with tubal factor infertility (a pathology associated with infections) were found to be more likely to have a vaginal microbiota consistent with BV by analysis of smears (118), supporting a connection between these etiologies. Although the authors acknowledge the lack of cause and effect in an infertility context, this finding supports that precision medicine approaches around fertility treatment and the vaginal microbiota could well inform practice in the fertility clinic. Further to this finding, using next generation sequencing of the vaginal microbiota, women with idiopathic infertility were found to have a microbiota profile consistent with BV compared to healthy women (119). Another study identified trends for distinct microbiota in women with a history of infertility compared to women with a history of fertility, albeit a retrospective study on a small sample size (120). A study of the composition of the vaginal microbiota on the day of embryo transfer in women undergoing IVF found that a lower vaginal microbiota diversity index correlated with a resultant live birth (121). However, this study did not profile the taxa present but rather compared the diversity index, providing a relatively limited insight into the role of the vaginal microbiota in reproductive outcomes. It is likely that analysis of the upper reproductive tract microbiota will shed further insights into fertility and fertility treatment outcomes, and this topic is reviewed in this issue (117,118,122–124). However, additional studies are needed to better understand fertility outside the controlled context of IVF procedures. Studies involving time to pregnancy and detailed monitoring of the composition and function of the vaginal microbiota would be extremely informative and would provide the evidence necessary to develop ways to improve natural conception outcomes.

FUTURE PERSPECTIVES

The vaginal microbiota critically interplays with women's health and reproduction. We have summarized the factors reviewed here which are thought to drive or be associated with vaginal microbiota dysbiosis or eubiosis in Figure 3. It is becoming critical to further our understanding of the cervicovaginal microbiota from a mechanistic and functional aspect, so that causal relationships can be established between the microbiome and adverse outcomes. These mechanistic understandings could be leveraged to develop improved protective and curative strategies, or to optimize the vaginal microbiome using rationally designed live biotherapeutic products or metabolites. To achieve these goals, improved study designs and sampling strategies are needed that would maximize power and frequency of sampling in prospective study design. The applications of advanced and high-resolution approaches such as metagenomics, metatranscriptomics, metabolomics, and/or proteomics, detailed immunological characterizations

FIGURE 3



Factors driving or associated with dysbiosis or eubiosis of the vaginal microbiota in reproductive age women.

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in combination with novel systems biology, modelling and statistical approaches will be critical to advancing the field and improving women's reproductive health.

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