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The Vaginal Microbiome: I. Research Development, Lexicon, Defining "Normal" and the Dynamics Throughout Women's Lives

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Objective: This series of articles, titled The Vaginal Microbiome, written on behalf of the International Society for the Study of Vulvovaginal Disease, aims to summarize the current findings and understanding of the vaginal bacterial microbiota, mainly regarding areas relevant to clinicians specializing in vulvovaginal disorders.

Materials and Methods: A database search of PubMed was performed, using the search terms "vaginal microbiome" (VMB) with "research," "normal," "neonate," "puberty," "adolescent," "menopause," and "ethnicities," as well as "human microbiome project." Full article texts were reviewed. Reference lists were screened for additional articles.

Results: In the last 2 decades, many studies applying molecular techniques were performed, intending to characterize the vaginal microbiota. These studies advanced our understanding of how vaginal health is defined. The first article in this series focuses on the advancement of VMB research, technical definitions, the definition of "normal" VMB, and the dynamics of VMB throughout women's lives.

Conclusions: Understanding how microorganisms inhabiting the vagina interact with each other and with the host is important for a more complete understanding of vaginal health. The clinical application of microbial community sequencing is in its beginning, and its interpretation regarding practical clinical aspects is yet to be determined.

Key Words: vaginal microbiome research, normal vaginal microbiome, dynamics of vaginal microbiome

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The vagina is a highly versatile organ that profoundly affects women's health as well as the health of their newborns. As microorganisms play a critical role in determining the vaginal environment in terms of biochemical and inflammatory properties, the characteristics of the vaginal environment may not only relate to genital symptoms but also impact conception, the ability to carry a fetus to term, the risk of acquiring sexually transmitted infections (STIs), and the risk for gynecological malignancies.

In the last 2 decades, many studies using molecular techniques were performed, aiming to characterize the vaginal microbiota. These studies identified vaginal bacteria that had been previously overlooked by culture-based methods, showing that bacterial communities in the vagina are more complex than previously thought. Although many different vaginal microbiome (VMB) reviews have been published recently, we aim to integrate the most recent knowledge regarding topics relevant to clinicians specializing in vulvovaginal disorders. This series of articles seeks to describe the current concepts regarding normal VMB and dysbiosis, VMB findings in different genital conditions, and discuss future therapeutic options. In this series of articles, we focused on the vaginal bacterial microbiota and did not describe the fungal, viral, archaeal, and protozoan diversity due to the paucity of published molecular surveys.

The first article in this series focuses on the evolution of VMB research, technical explanations, the definition of "normal" VMB, and the dynamics of VMB throughout women's lives. Subsequent articles in this series discuss VMB variations and dysbiotic conditions (II), the VMB in various urogenital disorders, including vulvovaginal candidiasis, urinary tract infections, STIs, and vulvodynia (III). The fourth part (IV) focuses on 2 distinct areas: the role of VMB in various aspects of human reproduction, including infertility, pregnancy, preterm birth, and miscarriages, and in sharp contrast, the association between the VMB and gynecologic cancers. The last article (V) discusses therapeutic modalities and the challenges facing the research of VMB.

Historical Aspects and Development of Vaginal Microbiome Research

Investigation of the microbes inhabiting the human body has been conducted for more than 350 years, since van Leeuwenhoek described the discovery of "animalcules," looking at teeth plaque with his handmade microscope in 1683. The investigation was done primarily by microscopic methods that allow inspection of individual microbes and later by culture-based methods, enabling growth and isolation of individual microorganisms. Over time, these methods became an integral part of clinical practice. For example, wet mount microscopy is used for office diagnosis of vaginitis, and Gram staining is used to diagnose bacterial vaginosis (BV), using the Nugent score.[1]

In the past 2 decades, molecular methods have been developed to characterize microbial diversity. The first study using molecular methods to characterize the VMB was published in 2002.[2] Since then, phylogenetic analyses of vaginal samples (mostly bacterial 16S ribosomal RNA gene sequencing, see hereinafter) have shown that bacterial communities in the vagina are more complex than previously thought.[3,4]

Molecular methods enable comprehensive and quick analysis of microbial communities. In contrast, older, classical, culture and isolation requirements vary greatly for different bacteria, resulting in inability to culture many human-associated microbes 5, known as 'fastidious' microbes.[5] Polymerase chain reaction

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(PCR) can detect the presence and abundance of specific microbes using a designated primer. Polymerase chain reaction assays are being used for assessment of specific microorganisms, such as *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Mycoplasma genitalium*, and *Trichomonas vaginalis*. Nevertheless, these assays are restricted to detect specific microbes, for which primers were designed.

To provide a comprehensive description of the microbiome, high-throughput sequencing methods are being used, providing fast, accurate, and comprehensive descriptions of whole microbial communities. The 3 main community sequencing methods used are: amplicon sequencing, shotgun metagenomic sequencing, and metatranscriptomic sequencing (for a review of the various methods, see Berman et al.[6]). Community sequencing has quickly become the preferred research method for describing the composition of the VMB, as it allows a description of an entire microbial community with a single measurement.[6] To date, amplicon sequencing has been the most common method used because of its cost advantage; however, metagenomic and metatranscriptomic sequencing are being used more frequently.

Microbiome studies also aim to study microbial factors in a variety of health conditions, as will be described in this review. However, the clinical application of microbial community sequencing is in its early stage of development, and its interpretation regarding practical clinical aspects, including diagnostics and interventions, is yet to be determined.

Definitions

In the following section, we have included a lexicon used to describe different aspects of microbiome research.

- Microbiota: the collection of microorganisms present in a defined environment.[7]
- Microbiome: the entire habitat, including the microbiota, their genomes, and the surrounding environmental conditions.[7]
- Metagenome: the collection of genomes and genes from the members of a microbiota, studied using "shotgun sequencing" of DNA present in each sample.[7] The method provides strain-level resolution through sequencing and bioinformatic assembly of microbial genomes, which can then be mapped and annotated using reference databases.
- Metataxonomics: the high-throughput process used to characterize the entire microbiota and produce a metataxonomic tree, which demonstrates the connections between all sequences obtained.[7] Metataxonomics involves amplification and sequencing of specific, often short-length regions of microbial taxonomic marker genes. The bacterial 16S ribosomal RNA gene (16S rRNA) is most targeted for bacteria. It is composed of highly conserved regions and 9 "hypervariable" regions (V1–V9). Polymerase chain reaction primers designed to bind to the conserved regions enable amplification of the variable regions, which are generally distinctive and diverse. This facilitates the classification of bacterial taxonomy to species and, in some cases strain level, by mapping the resulting sequences to 16S rRNA gene database.[8]
- Metabolomics: the analytical methods used to determine the metabolite profile(s) of each strain or single tissue. The resulting census of all metabolites present is called metabolome.[7]
- Alpha diversity: the diversity of microbiome profile within a sample.
- Beta diversity: the diversity/dissimilarity of microbiome profile among samples.

THE NORMAL MICROBIOME

Determining what is normal in the context of the human microbiome is challenging. Within the framework of the Human Microbiome Project (HMP, see below), a "reference microbiome" has been defined in what were considered "relatively healthy" adults,[9] whereas a "healthy microbiome" has been defined elsewhere as the microbiome "in the absence of overt disease."[10] Hence, these and similar translational definitions merely formalize the assumption that the health of a host organism equals "health" or "normality" of its associated microbiome communities. This also reflects our current inability in defining "normal" or "healthy" microbiome ecologies, for example, through metabolic phenotyping.[11]

In what is arguably the most cited study of microbiome communities, Ravel et al.[12] distinguished in a cohort of healthy, reproductive-aged US women between five VMB "community state types" (CST), based on composition and abundance of vaginal bacterial species. Specifically, CST-I, CST-II, CST-III, and CST-V referred to an abundance of *Lactobacillus crispatus*, *L. gasseri*, *L. iners*, *and L. jensenii*, respectively, whereas CST-IV was characterized by a combination of diverse facultative anaerobes with low abundances of lactobacilli. As a heterogeneous group of lactobacilli depleted, high-diversity community states, CST-IV was further subdivided in a subsequent study into 2 substates CST IV-A and CST IV-B; CST IV-A comprises of species of genera *Anaerococcus*, *Peptoniphilus*, *Corynebacterium*, *Prevotella*, *Finegoldia*, and *Streptococcus*, whereas CST IV-B is characterized by *Atopobium*, *Gardnerella*, *Sneathia*, *Mobiluncus*, *Megasphera*, and other taxa of order *Clostridiales.*[13] As the cohort consisted of asymptomatic women, it was considered by the authors as representing a healthy state. Unfortunately, there are many examples in medicine in which asymptomatic states and exposures are not quite "normal" and pose defined risks to human health. In this respect, it was observed that CST-IV represents Nugent scores 4–10 and hence also BV[3,14] and was reported predominantly in Black and Hispanic women.[12,13] While such communities display lactic acid production, as contended by the authors, this occurs to a much lower extent relative to *Lactobacillus* species dominated communities,[15] consistent with an overall dissimilar functional assembly and metabolome profile.

Overall, although widely used, the validity and the resolution of the vaginal CST model remain unsettled.[3]

Systematic appraisal of published microbiome data[4] revealed that in a majority of reproductive-aged women and for a majority of points in time, the VMB is essentially dominated by 1 of 4 niche-specific[16] *Lactobacillus* taxa, specifically *L. crispatus*, *L. jensenii*, *L. gasseri*, and *L. iners*, consistent with the CST model. The remarkable community structure of the VMB with a single taxon largely dominating an ecological niche[17] presumably is consistent with microbial communities having coevolved with humans throughout evolution to serve critical host functions.[3,16,17] This, in turn, likely accords with unique metabolic profiles observed with the VMB under differing conditions, e.g., gross differences are observed with metabolomic profiling between *Lactobacillus*-dominated and BV communities,[18–20] whereas metabolome divergence also occurs in the setting of vaginal infections with *Candida* and STI, such as *Chlamydia trachomatis.*[20–22]

Longitudinal studies of the VMB have further highlighted the dynamic nature of the VMB.[13,14,23] Temporary, mostly short-lived, deviations from a *Lactobacillus*-dominated ground state and accompanying metabolome shifts are common in reproductive-aged women, typically observed in association with menses and sexual activity. Studies consistently suggest that menses is the major disturbing factor to VMB during the menstrual cycle, with large reductions in lactobacilli,[13,24,25] shifts from *L. crispatus* to *L. iners*,[24,26] or the appearance of BV-associated bacteria.[26,27] However, longitudinal studies also suggest a "dynamic stability" wherein most women retain their CST or alternate between certain CSTs, mostly in correspondence to menses.[13,24,27,28]

Overall, the distinct *Lactobacillus*-dominated community states are thought to be the most optimal[3,4] with, as further discussed,

numerous associations with reproductive health. These may, therefore, cautiously be considered as representative of the normal VMB. The future understanding of "normality" of the VMB should define microbe community states through functional and ecological indices that transcend taxonomic description. The "healthy" VMB should not only be defined by symptoms at the time of sampling but also be defined by considering the long-term health of women, their partners, and their offspring.

The Role of Lactobacilli in Vaginal Health

Over 20 species of lactobacilli have been detected in the vagina; however, in most women, the vagina is dominated by a single species of lactobacilli[12] and characterized by a pH level less than 4.5. *Lactobacillus* dominance and low pH are also unique compared with other mammals, in which lactobacilli hardly encompass more than 1% of the VMB, and which present a mixture of bacterial species and neutral pH.[29,30] It was hypothesized that the protective role of lactobacilli in humans has evolved in association with continuous sexual receptivity throughout the menstrual cycle, pregnancy, and the postpartum period, exposing to risks of STIs and obstetric complications.[31]

The protective role of lactobacilli in the vagina is exerted by several mechanisms, counteracting overgrowth of other microorganisms by competition for nutrients and tissue adherence, reduction of the vaginal pH by production of organic acids, mainly lactic acid, modulation of local immune system, and production of antimicrobial substances, such as bacteriocins.[32]

The vaginal microenvironment not only provides a tissue substrate on which bacteria may reside but also bathes these organisms in a complex milieu, originating from transudation of fluids into the lumen, shedding of epithelial cells, secretions from the cervix and inflammatory cells, introducing antimicrobial substances. The microbiota itself may contribute to the environment by degradation of macromolecules to make nutrients available or release metabolites. Vaginal fluid also contains amylases[33] that degrade glycogen releasing monosaccharides, disaccharides, and trisaccharides that support *Lactobacillus* growth.[34] The glycogen content of the vaginal epithelium covariates with estrogen levels and, in general, high levels of estradiol may favor a lactobacilli-dominant environment, especially *L. crispatus, L. gasseri*, and *L. jensenii*.[13]

Lactobacillus iners. *Lactobacillus iners* is one of the most frequently isolated bacteria in the vagina; however, its role in vaginal health is still unclear.[35] *Lactobacillus iners* can be detected in both dysbiotic and healthy conditions and presents unusual characteristics, including an unusually small genome compared with other lactobacilli, possibly indicative of a symbiotic or parasitic lifestyle,[35] and specific genes encoding unique proteins.

For many years, culture and microscopy-dependent approaches failed to identify this species. Only in 1999 was it named *L. iners*[35] and was described as a gram-positive, rod-shaped bacterium. However, it is not always clearly gram-positive, and at least some isolates seem to have a coccobacillary morphology (rather than bacillary), and this may be one of the reasons why *L. iners* has been overlooked by Gram staining of vaginal smears of women with vaginal dysbiosis.[36] This ambiguity in *L. iners* Gram staining properties and cell morphology is important because some diagnostic determinations of vaginal health depend on these characteristics, that is, the Nugent score.

A similar prevalence of *L. iners* was found in women with normal, intermediate, and BV Nugent scores.[37] Women with high abundances of *L. iners* could be either BV-negative or BV-positive and have either low or high pH levels.[38] It was also shown that *L. iners* presence and abundance inversely correlate with *L. crispatus*.[12,39] While *L. crispatus* seems to decline during menses, *L. iners* concentrations increase[24,25,27] along with *Gardnerella vaginalis* and is the dominant species during menses. These findings suggest that this species is very flexible and can easily adapt to the fluctuating vaginal niche.

The genome of *L. iners* encodes proteins (iron-sulfur proteins and unique s-factors) that optimally adapt the microbe to the vaginal niche. These proteins may be involved in resistance to oxidative stress, present where high levels of H_2O_2 are produced by other lactobacilli.[40] *Lactobacillus iners* also encodes stress resistance proteins, which might promote improved tolerance to vaginal environmental fluctuations (pH, mucus concentration, hormones, and infection),[40] and exhibits superior metabolic adaptation to the changing carbohydrate sources in this environment. *Lactobacillus iners* strains have also been shown to secrete inerolysin, a pore-forming toxin, which may contribute to the pathogenesis of BV.[35]

In summary, various genetic and functional studies point toward a remarkable environmental adaptation of *L. iners* to the vaginal niche. It remains to be clarified to what extent this represents pathogenicity or strong colonization capacity and to further clarify *L. iners* role in health and disease. Possibly, this organism may have clonal variants that in some cases promote a healthy vagina and in other cases are associated with dysbiosis and disease.[35]

Vaginal Microbiome Throughout Life: Childhood, Reproductive Years, and Menopause

The vagina is a dynamic ecosystem, undergoing a natural fluctuation in the composition of the VMB throughout a woman's life. Such changes are influenced by levels of sex hormones, glycogen content in the vaginal epithelium, menstrual cycle, vaginal pH, and immune responses. The primary driver of lactobacilli dominance in the vaginal niche is generally assumed to be the availability of glycogen,[41] which accumulates in the cervicovaginal environment, in an estrogen-dependent manner.[42,43] Much in accordance, the VMB is thought to relate essentially to reproductive health, which, arguably, also explains why the VMB has been primarily studied in reproductive-aged women.

In stark contrast, little, if anything, is known on the VMB in prepubertal girls. At birth, it is believed that the VMB is established when the neonate is exposed to the vaginal tract during vaginal delivery or the skin bacteria after cesarean section.[44] The VMB of the neonate delivered through the vaginal tract resembles that of her mother, dominated by either *Lactobacillus* species, *Prevotella* species, or *Sneathia* species.[44] Later, with estrogen withdrawal, the vaginal epithelium becomes thinner and contains less glycogen, resulting in neutral vaginal pH due to *Lactobacillus* species diminution.[45]

Vulvovaginitis in preadolescent children is common, typically attributed to the anatomical immaturity of the vulva in fending off bacterial invasion of the prepubertal vagina.[46] The vagina in prepubertal girls is a hypoestrogenic, alkaline environment, which presumably does not provide the colonization resistance observed in adults.[46] It is not known, however, to which extent the prepubertal VMB is a cofactor to the common occurrence of vulvovaginitis.

Two longitudinal studies have documented the transition of the vaginal microbiota throughout peripuberty, through gram-stained smears[47] and 16S rRNA sequencing.[48] Although both studies are not entirely concordant, the emerging picture is that around the menarche, a BV-like microbiome gradually shifts toward an adult-like *Lactobacillus*-dominated microbiome, which occurred, at least in the latter study,[48] well before the onset of menarche, probably corresponding to the dynamics of circulating prepubertal sex hormones.[49] Hence, although an adult-like *Lactobacillus*-dominated microbiome is presumably established in at least a majority of adolescents, it remains elusive whether the adolescent VMB at sexual debut

might affect mucosal immune homeostasis and susceptibility to STIs, including human papillomavirus and HIV.[50,51]

The role of the VMB in reproductive health is more apparent. The peculiar VMB community structure has likely evolved to preserve reproduction in response to the unique human lifestyle.[52] First, although we presumably understand only a small part of the broad array of antimicrobial defense mechanisms at play in the cervicovaginal environment, it is clear that the *Lactobacillus*-dominated microbiome—and this is most apparent in the case of *L. crispatus*—confers several mechanisms directed toward invading and colonizing pathogens,[17,53–55] termed "colonization resistance." This, in turn, may at least in part relate to continuous sexual receptivity, which is unique to humans and some other hominids. Conversely, vaginal dysbiosis predisposes to an increased risk of STI acquisition, as most extensively documented for HIV and human papillomavirus. Furthermore, although, hardly explored, the cervico VMB may also be of undefined importance to conception and fertility.[56,57] Albeit difficult to document in human populations, the VMB may also affect fetal development.[58] During pregnancy, the VMB tends to evolve as an even more stable community with advancing pregnancy, with increased lactobacilli abundance and even lower alpha diversity.[14,59–61] The latter phenomenon has been aptly referred to as "pregnancy's stronghold on the VMB."[59] Dysbiotic states in turn and BV in particular are associated with adverse pregnancy outcomes and most notably with spontaneous preterm birth. Study of the association between the VMB and preterm birth was a major objective of the recently completed second or integrative HMP project.[62,63] After birth, there is a defined increase in VMB diversity and a shift toward *Lactobacillus* species depleted community structures that have been reported to persist for up to 1 year postpartum.[61]

The initial human neonatal microbiota across all body habitats after vaginal delivery were found to originate primarily from the maternal VMB.[44,64] Disturbance of this vertical transmission route has recently been proposed to have potentially profound effects on offspring development and adult health, including disruption of neonate-microbe interactions necessary for immune education, metabolic programming, and neurodevelopment.[58]

With advancing age, the marked decrease of circulating estrogen in menopause contrasts with the premenopausal state by a reduction of *Lactobacillus* species dominance and a concomitant increase in diverse anaerobes.

Gliniewicz et al[65] documented 6 ecological clusters in a cross-sectional study involving postmenopausal women with and without hormonal therapy, specifically a cluster dominated by *L. crispatus* (A), by *L. iners* (C), and by *L. gasseri* (F), respectively, whereas further communities belonged to a cluster dominated by *G. vaginalis* (B), a cluster dominated by *Bifidobacterium* (E), and a more diverse cluster codominated by several taxa (D). Although a small study, there was no obvious relation between menopausal state and hormonal therapy with community clusters. Indeed, although a deviation might be expected from lactobacilli dominance with menopause, such transitioning is neither abrupt nor predictable at present. The association between the VMB in menopause and vulvovaginal symptoms is also equivocal, as some studies did not find such an association,[66] whereas other studies did suggest a role for the VMB, possibly mediating symptoms of the "genitourinary syndrome of menopause."[67–69]

Comparison Between Races and Ethnicities—Is "Normal" Universal?

Comparison of VMB profiles of African American women and White women with and without a clinical diagnosis of BV showed differences between the 2 groups.[70] Of those without a clinical diagnosis of BV, African American women were more likely to be colonized by strict anaerobes, whereas White women were more likely colonized by *L. crispatus*, *L. gasseri*, and *L. jensenii*. These data suggest that even among healthy women, African American ethnicity is associated with a VMB that more closely resembles BV, characterized by an increase in species diversity and a decrease in lactobacilli. Another study found that 28 bacterial taxa were significantly associated with VMB in African American women than in White women without BV,[38] including *Leptotrichia amnionii*, *Atopobium vaginae*, and BVAB1. More African American women had VMB dominated by *L. iners*, whereas more White women had microbiota dominated by *L. crispatus*.[38] It is unclear whether these different profiles in women without BV lead to differences in risk for BV. Ravel et al.[12] also observed differences in the VMB associated with race; asymptomatic African American and Hispanic women in North America were more likely to have vaginal bacterial communities comprising diverse bacteria that mirrored bacterial communities typically seen with BV (CST IV).[12] However, examinations to document absence of vaginal discharge were not reported, Amsel criteria for BV were not assessed, and many of these women had BV diagnosed by gram stain of vaginal fluid.

Studies in Sub-Saharan African populations found *Lactobacillus* dominance in less than 40% of asymptomatic women,[71,72] further indicating the controversy around whether *Lactobacillus* dominance should be considered "normal" in all populations.

The factors driving racial and geographic differences of VMB are unknown but could include variation in hygienic practices, contraceptive use, sexual practices, rectal colonization, or host genetics. Nevertheless, it remains possible that VMB configurations, which are associated with elevated baseline genital inflammation, may be advantageous in specific contexts, contributing to the high prevalence of diverse VMB in certain populations.[17]

Main Findings of the Human Microbiome Project

The HMP, established in 2007, is a global project in which multiple research groups sought to characterize the microbiomes of 18 (15 in men) bodily habitats in healthy participants. Through the use of molecular sequencing methods over the past decade, the HMP and other studies have revolutionized the understanding of diversity and complexity of the human microbiome. Older classical culture and isolation methods have commonly failed to demonstrate this diversity and complexity discovered in the vagina and other human body environments.

The first of a 2-phase effort, frequently referred to as HMP1, ran from 2008 through 2013.

In the HMP,[73] 3 vaginal sites were considered, specifically, the vaginal introitus, midvagina, and posterior fornix. An overall little distinction among different vaginal sites was found, with *Lactobacillus* species dominating all 3 and correlating in abundance. As a matter of fact, of all human body sites targeted in the HMP project, the vaginal sites displayed the lowest within community or alpha diversity at the operational taxonomic unit (OTU) level (OTU refers to sequence similarity of the 16S rRNA gene amplicons and is used in settings as these as a proxy for bacterial species). Indeed, as the majority of community members are lactobacilli, the overall species diversity of the community tends to be low. Vaginal *Lactobacillus*-dominated communities further consistently included a variable assortment of low abundance community members, albeit with an overall OTU richness far below that observed with any other human microbiome site.[74] Between subject (or beta diversity) was still fairly low at the genus level (since in most women the dominant genus was *Lactobacillus*), yet very high among OTUs due to the presence of the presence of distinct *Lactobacillus* species dominance[73] (because of the dominance of different *Lactobacillus* species across women, as discussed previously).

The HMP project was also the first large-scale study to map the relative abundances of microbial metabolic and functional pathways in the microbiome communities under study, hence accounting for community phenotypes. An important finding was that the relative abundances of pathways in community metagenomes were much more constant and evenly diverse than were organismal abundances.[75] Protein families showed diversity and prevalence trends similar to those of full pathways, with an estimated number of approximately 16,000 unique families per community in the vagina (e.g., compared with almost 400,000 in the oral cavity), consistent with a more limited set of core functions. Of note in this respect is that increasing pH with a reduction in *Lactobacillus* species abundance was also accompanied by an increase in metabolic diversity.

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