Vaginal microbiota and susceptibility to HIV

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Bacterial vaginosis, characterized by the replacement of the *Lactobacillus*-dominant microbiota with anaerobic bacteria and facultative Gram-negative rods, has been associated with adverse reproductive health outcomes including HIV acquisition. With the advent of newer molecular techniques, the vaginal microbiota can be investigated in more detail and the association with HIV examined more thoroughly. This review examines recent evidence suggesting that vaginal dysbiosis with increased microbial diversity, specific vaginal bacterial communities, and the presence and concentrations of some individual bacterial species, may increase HIV susceptibility. Potential mechanisms through which vaginal microbiota could impact HIV susceptibility are discussed. On the basis of the available data, this review finds that there is a modest, but growing, body of evidence linking vaginal microbiota to HIV susceptibility in women. The evidence could be strengthened through two main pathways. First, laboratory studies such as *ex-vivo* or animal experiments are needed to move from plausible mechanisms towards proven mechanisms that explain an effect of the vaginal microbiota on HIV susceptibility. Second, experimental evidence could directly test the hypothesis that sustaining optimal microbiota reduces HIV risk, though there are important obstacles to conducting such studies. Finally, this review examines strong evidence from a recent publication suggesting that deviations from an optimal vaginal microbiome, and particularly the presence of some bacterial communities with high relative abundance of *Gardnerella vaginalis*, reduces the efficacy of vaginal tenofovir-based microbicides.

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Introduction

Women in sub-Saharan Africa continue to bear a greater burden of HIV compared with men [1]. Disruptions of the vaginal microbiota could play a key role in mediating HIV susceptibility in African women.

In the early 1900s, *Doderlein bacillus*, later classified as *Lactobacillus*, was linked with vaginal health in married white women [2]. In this population, deviation from a microbiota dominated by *Lactobacillus* species was associated with vaginal discharge. As the vaginal microbiota has been characterized in a wider range of populations, it is evident that non-*Lactobacillus*-dominant vaginal microbial communities are common [3], can occur with and without symptoms [2], and may be associated with a range of adverse reproductive health outcomes including HIV acquisition [4–9].

Heterosexual transmission of HIV is inefficient [10]. Co-factors including sexually transmitted infections (STIs) and vaginal dysbiosis likely contribute to increased HIV

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transmission efficiency [4,11–23]. Bacterial vaginosis, the most common type of vaginal dysbiosis, is a condition in which *Lactobacillus*-dominant microbiota is replaced by complex bacterial communities with anaerobic bacteria and facultative Gram-negative rods [2]. Bacterial vaginosis has been associated with HIV acquisition in women [4–9]. As bacterial vaginosis often persists [24,25], and frequently recurs even when treated [26,27], this condition may contribute substantially to the population attributable risk (PAR) of HIV infection. Two studies suggest that bacterial vaginosis contributes substantially more to HIV PAR than any genital condition other than herpes simplex virus-2 (HSV-2) [28,29].

Beginning in 2005, advances in molecular evaluation of the vaginal microbiota [30], have enabled examination of vaginal microbial communities in much finer detail. This article summarizes recent literature using molecular characterization to explore the possible role of vaginal microbiota in mediating women's susceptibility to HIV infection.

Laboratory methods for characterizing vaginal microbiota

There are two commonly used criteria to diagnose bacterial vaginosis. Amsel criteria includes three or more of four clinical signs including clue cells on wet mount microscopy, 'fishy' amine odor, vaginal pH greater than 4.5, and thin, homogenous vaginal discharge [31]. The criteria developed by Nugent and Hillier (Nugent criteria) define bacterial vaginosis based on Gram stain enumeration of bacterial morphotypes [32].

In addition to the appearance of bacteria on Gram stain, the vaginal microbiota has been characterized using culture-based methods. One advantage is that culture can identify some minority species more easily than through newer molecular techniques [33]. Culture also allows for antimicrobial sensitivity testing. However, many key bacterial taxa associated with bacterial vaginosis are difficult to cultivate [34].

During the past 15 years, advances in molecular microbiology have contributed substantially to our understanding of the vaginal microbiota by providing a complementary approach [35]. Many of these novel methods begin with broad-range PCR amplification targeting highly conserved 16S rRNA gene sequences. Additional steps are then employed to identify bacteria. These techniques include denaturing gradient gel electrophoresis (DGGE) [36], terminal restriction fragment length polymorphism analysis (T-RFLP) [37], cloning and Sanger sequencing [38], amplified ribosomal DNA restriction analysis (ARDRA) [39], and pyrosequencing [40,41]. Taxon-directed quantitative PCR (qPCR) can been used to measure quantities of individual bacterial taxa [42,43]. In addition, fluorescent in-situ hybridization (FISH) is a nonamplified method that uses fluorescently labeled 16S rRNA probes to detect bacterial taxa, characterize their morphology, and localize species into microniches [30]. In combination, newer methods have allowed for a simplified approach to identifying bacteria, including cultivation-resistant bacteria. Bacterial quantities can be evaluated in terms of their relative abundance (e.g. pyrosequencing data) and concentration (e.g. qPCR). Relative abundance data have been used to identify vaginal bacterial community types that provide more differentiation than the traditional Amsel and Nugent criteria [44].

Application of molecular methods has considerably advanced our understanding of both healthy and disrupted vaginal microbiota. Women with bacterial vaginosis have more diverse vaginal microbiota compared with women without bacterial vaginosis [41,45,46]. Diversity has been defined in a variety of ways including the Shannon Diversity Index, operational taxonomic units, and number of positive probes [41,45,46]. Multiple bacterial taxa have been associated with bacterial vaginosis, including bacterial vaginosis-associated bacteria 1 (BVAB1), BVAB2, *Mageeibacillus indolicus* (previously BVAB3), *Gardnerella vaginalis, Atopobium vaginae*, *Eggerthella*-like uncultured bacteria, *Leptotrichia* spp., *Megasphaera* spp., *Prevotella* spp., *Mycoplasma hominis*, *Bifidobacterium* spp., and *Dialister* spp. [30]. In contrast, *Lactobacillus crispatus* has been associated with a healthier vaginal microbiome [47–49].

Ethnic and geographic variations in vaginal microbiota

Since the 1990s, studies using Amsel or Nugent criteria have detected differences in bacterial vaginosis prevalence by race and ethnicity [3,50–54]. Nonwhite women generally have higher rates of bacterial vaginosis compared with white women. In 2011, Ravel *et al.* [44] used broad-range PCR amplification of 16S rRNA genes with pyrosequencing to explore this question. Compared with Hispanic and black women in North America, Asian and white women were more likely to have vaginal communities dominated by *Lactobacillus* species. Vaginal pH was lower in Asian and white women compared with black and Hispanic women, an effect the authors hypothesized was related to lactic acid production by *Lactobacillus* spp. Understanding the diversity of the vaginal microbiome across different populations is critical, because associations between the vaginal microbiota and susceptibility to HIV infection may vary by race, ethnicity, and geography.

Studies of bacterial vaginosis as a risk for HIV infection in women

Bacterial vaginosis, diagnosed by Amsel or Nugent criteria, has been associated with HIV prevalence and incidence in numerous studies. Beginning in 1995, multiple studies have found associations between bacterial vaginosis and prevalent HIV infection [4–6,9,28,29,55–74]. Stronger evidence is provided by numerous prospective cohort studies and meta-analyses, which have consistently shown that both intermediate vaginal microbiota and bacterial vaginosis are associated with ~1.5-fold higher risk of HIV acquisition [7,8,75]. As detailed above, bacterial vaginosis and abnormal microbiota diagnosed by Gram stain are microbiologically heterogeneous. Women with similar vaginal Gram stains often have distinctly different vaginal bacterial communities [76]. Taken together, the data on vaginal dysbiosis as a risk factor for HIV, combined with an evolving understanding of the complexity of the vaginal microbiome, raise a question about the specificity of the relationship between bacterial vaginosis and HIV. In particular, vaginal dysbiosis on Gram stain may be a nonspecific marker for individual bacteria or bacterial communities, which are the true drivers of increased HIV susceptibility.

Molecular studies of vaginal microbiota and HIV acquisition

Three studies published or presented during the past year have employed molecular microbiological approaches to expand our understanding of the vaginal microbiota and HIV acquisition [77–79] (Table 1). Last year, Gosmann *et al.* [79] published a prospective cohort study of 236 HIV-uninfected South African adolescent women (18–23 years). Cervical and vaginal samples were characterized using nucleic acid extraction, amplification with 16S rRNA V4 primer constructs, and sequencing with Illumina MiSeq [80]. The authors defined four vaginal bacterial community groupings (cervicotypes). Cervicotype 1 (CT1) was characterized by high relative abundance of *L. crispatus,* CT2 had a high relative abundance of *Lactobacillus iners,* CT3 was dominated by *G. vaginalis,* and CT4 was a diverse bacterial community not dominated by *L. crispatus, L. iners,* or *G. vaginalis.* In an analysis that excluded women with *Chlamydia trachomatis*, CT4 was associated with significantly higher risk of HIV acquisition compared with CT1. Two subtypes of *Prevotella bivia, Prevotella melaninogenica, Veillonella montpellierensis, Mycoplasma* spp., and *Sneathia sanguinegens* were significantly more abundant in 31 women who acquired HIV compared with 205 women who remained HIV-uninfected. Non-*iners Lactobacillus* species were associated with protection against HIV.

The South African Study also explored potential mechanisms through which vaginal microbiota might impact HIV susceptibility. Women with CT4 vaginal communities had 17-fold higher number of activated $CD4^+$ HIV target cells on cervical cytobrushes compared with women with CT1 communities. Strengths of this study included a prospective cohort design, a subset

Table 1. Studies using molecular techniques to examine the vaginal microbiome and HIV acquisition.

Author (year)	Country/ region	Study population	*N* (number of HIV events)	Method	Main findings	Measure of association	Limitations
Gosmann *et al.* (2017) [79]	South Africa	Women in Females Rising Through Education, Support, and Healthy (FRESH) Study	236 (31)	16S rRNA V4 gene sequencing	Vaginal bacterial communities dominated by *Gardnerella vaginalis* (CT3) and by taxa other than *Lactobacillus* spp., *L. iners,* and *G. vaginalis* (CT4) associated with HIV acquisition[a]. *Prevotella bivia 1, Prevotella melaninogenica, Veillonella montpellierensis, Mycoplasma* spp., *Prevotella bivia 2*, and *Sneathia sanguinegens* associated with HIV	CT3: hazard ratio 4.22 (95% CI 1.06–16.88), $P=0.042$ CT4: hazard ratio 4.03 (95% CI 1.14–14.27) $P=0.031$	HIV incidence analyses stratify by the presence of *Chlamydia* spp., but do not adjust for other potential confounding factors including for sexual risk behaviors
Passmore and Williams (2016) [77]	South Africa	Women in CAPRISA 004 Trial	119 (49)	V1-V3 rDNA sequencing	Vaginal communities defined by *Prevotella bivia* (CST4) associated with inflammation and HIV acquisition[a]. *P. bivia* associated with HIV acquisition	aOR 12.7, 95% CI 2.1–77.8, $P=0.006$	Relatively small sample size Data described in abstract showed only unadjusted ratios.
McClelland *et al.* (2018) [78]	Eastern and Southern Africa	Female sex workers, pregnant/ postpartum, and HIV-negative women in discordant couples	349 (87)	Broad range PCR with deep sequencing and quantitative PCR	HIV seroconversion with more vaginal microbial diversity *Eggerthella* species type 1, *Gemella asaccharolytica*, *Leptotrichia/Sneathia*, *Megasphaera* spp., and *Mycoplasma hominis* significantly associated with HIV acquisition	Shannon Diversity Index median 0.9, (IQR 0.4–2.3) in HIV sero-converters compared with controls with median 0.7, (IQR 0.1–1.4), $P=0.03$	Did not explore mechanistic explanations for the associations

aOR, adjusted odds ratio; CI, confidence interval; CST, community state types; CT, cervicotypes; IQR, interquartile range.
[a]Although cervicotypes and CSTs both describe vaginal communities by relative abundance, the specific processes for defining these communities are different.

analysis excluding women with chlamydia, and parallel investigation of plausible mechanisms. One limitation of this study is that analyses examining the association between vaginal community types and HIV acquisition did not control for sexual risk behavior. Although the authors did not find statistically significant differences in condom use, frequency or type of sexual acts, or number of sexual partners across cervicotypes, this does not exclude possible confounding by these variables. Second, there were modest numbers of incident HIV infections. Third, this relatively homogenous population of adolescent South African women may not be generalizable to other regions or age groups. Despite these limitations, this study provides the first published evidence that certain vaginal communities and individual bacterial species may shape vaginal mucosal HIV susceptibility.

The next study, by Passmore and Williams [77], was presented at the 2016 International AIDS Conference in Durban, South Africa. This analysis included 119 South African women from the CAPRISA 004 trial, a Phase IIb clinical trial assessing the effectiveness and safety of 1% tenofovir vaginal gel for preventing HIV infection. Using 16S rRNA V1-V3 variable region primers, then sequencing amplified DNA, 1368 species were identified from cervicovaginal samples. A cluster analysis was used to identify eight broad community state types (CSTs), which differed from those described by Gosmann *et al.* Community state types 1–3 were all dominated by *Lactobacillus* species. In contrast, CSTs 4–8 were dominated by species other than *Lactobacillus,* with *P. bivia* defining CST4, and distinguishing CST4 from other CSTs. Community state type 4 was associated with an inflammatory vaginal cytokine profile and with HIV acquisition. Women with high relative abundance of *P. bivia* in vaginal samples were 19 times more likely to have a pro-inflammatory vaginal cytokine profile [adjusted odds ratio (aOR) 19.2, 95% CI 4.0–92.4, $P < 0.001$], and nearly 13 times more likely to acquire HIV (aOR 12.7, 95% CI 2.1–77.8, $P = 0.006$), compared with other women.

To explore this association, *P. bivia's* metagenome was characterized. There was an enrichment of lipopolysaccharide (LPS) biosynthesis, indicating production of this immunostimulatory molecule, in women with CST4. The increased inflammation associated with LPS is a possible mechanism for increased HIV risk. To date, this study has only been presented in a conference symposium, so it is not possible to fully characterize strengths and limitations. Nonetheless, one important strength was the pairing of epidemiological data showing an association with laboratory analyses aimed at uncovering the mechanism of increased HIV susceptibility. There were also some limitations noted. First, it appears that there were only 10 women in the *Prevotella*-dominant CST4, resulting in wide confidence intervals, and potential for selection bias. Second, it is not clear whether any adjustment was undertaken for sexual risk behaviors. Third, the study population was restricted to younger South African women. It is unclear if these results can be generalized to other populations. Despite the limitations, these data provide potentially important evidence of a strong association between inflammation and *P. bivia,* possibly mediated by LPS.

At the same conference, McClelland *et al.* presented a nested case–control study of diverse populations of women from six countries in Eastern and Southern Africa, including female sex workers, pregnant and postpartum women, and HIV-seronegative women in discordant couples [78]. There were 87 vaginal microbiota samples from case women who seroconverted to HIV and 262 samples from HIV-seronegative controls. Deep sequencing of broad-range 16S rRNA gene PCR products and highly sensitive taxon-directed qPCR assays were used to characterize the vaginal microbiota. Women who acquired HIV had greater vaginal bacterial community diversity compared with women who remained HIV-seronegative (mean Shannon Diversity Index 1.3 versus 0.9, $P = 0.02$). Using qPCR, five bacterial taxa showed significant associations with HIV acquisition; *Eggerthella* species type 1, *Gemella asaccharolytica*, *Leptotrichia/Sneathia* spp., *Megasphaera* spp., and *M. hominis*. These associations remained significant after adjustment for age, pregnancy, contraceptive use, sex partner number, sex frequency, and recent unprotected intercourse. A strength of this study was inclusion of a diverse population. In addition, the relatively large sample size facilitated adjustment for multiple potential confounders. Data from both deep sequencing data and qPCR assays were included, facilitating examination of concentration-dependent associations between bacteria and HIV acquisition. This study also had limitations. The epidemiological analyses were not paired with data exploring potential mechanisms linking vaginal bacteria to HIV susceptibility. In addition, this study explored associations with multiple bacterial taxa, and it is possible that some associations were observed by chance. Nonetheless, these data highlight potential biological gradients by showing concentration-dependent associations between several types of bacteria and women's risk of HIV acquisition.

Mechanisms through which vaginal microbiota may influence HIV susceptibility

Numerous studies have focused on mechanisms through which the vaginal microbiota may influence HIV susceptibility. An exhaustive review of this literature is beyond this article's scope. Nonetheless, understanding the possible mechanistic pathways is important for establishing biological plausibility. Readers are directed

to excellent recent reviews by Mirmonsef *et al.* [81], Petrova *et al.* [82], Mirmonsef and Spear [83], Cone [84], and Murphy and Mitchell [85], for detailed reviews of mechanisms through which the vaginal microbiome may influence HIV susceptibility. A brief summary of key mechanisms is presented here.

Vaginal microbiota associated with bacterial vaginosis can recruit mucosal immune cells. In Gosmann *et al.* [79], women with diverse non-*Lactobacillus*-dominant communities had 17-fold more activated $CD4^+$ HIV target cells compared with women with *Lactobacillus crispatus*-dominant vaginal communities. The same laboratory used transcriptional profiling to illustrate that epithelial cells and antigen-presenting cells (APCs) sense high-diversity vaginal communities associated with bacterial vaginosis [80]. These APCs use Toll-like receptor-4 signaling to respond to LPS, which activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), leading to inflammation and recruitment of lymphocytes. Another recent study points to an alternative mechanism. In this study of US women, numbers of cervical gamma delta 1 (GD1) cells that were protective against HIV were higher in women with normal versus abnormal microbiota by Gram stain [86]. In contrast, vaginal GD2 cells, acting as targets for HIV entry into cells, were associated with abnormal vaginal microbiota.

Humoral immune mediators, including pro-inflammatory chemokines and cytokines, have been studied as a mechanism to explain the association between vaginal dysbiosis and HIV acquisition. Abnormal vaginal microbiota (Nugent score 4–10) has consistently been associated with higher levels of interleukin-1β (IL-1β), a pro-inflammatory cytokine associated with toll-like receptor (TLR) signaling and tissue damage [87–100]. Utilizing data from the CAPRISA 004 trial, cervicovaginal samples from 58 women prior to HIV seroconversion were matched to 58 women who remained HIV-negative [101]. This study showed higher concentrations of interferon gamma inducible protein (IP-10), macrophage inflammatory protein-1alpha (MIP-1α), macrophage inflammatory protein-1beta (MIP-1β), and interleukin-8 (IL-8) in women who seroconverted to HIV compared with HIV-uninfected women. Additionally, MIP-1α and MIP-1β were associated with more diverse vaginal communities. These two chemokines, as well as IP-10, are chemotactic for T cells, monocytes, macrophages, and dendritic cells, all of which are potential HIV target cells [102,103].

Since the 1990s, it has been evident that bacterial vaginosis is associated with the presence of an HIV-inducing factor (HIF) in vaginal secretions [104]. This factor leads to increased HIV-1 replication in T cells and monocytes by activating AP-1 and NF-κB [105,106]. *Mycoplasma hominis*, a vaginal bacteria species frequently linked with bacterial vaginosis, has been significantly associated with HIF [107].

Lactobacillus-dominant vaginal microbiotas have generally been considered to reflect vaginal health, and have been associated with decreased risk of HIV acquisition [6,48,79]. Lactic acid, and associated low pH produced by glycogen metabolism by *Lactobacillus* species, can inactivate HIV [84,108,109]. A recent study also documented significant increases in IL-1RA, an anti-inflammatory cytokine, whenever human vaginal and cervical epithelial cell lines were treated with lactic acid [110]. This highlights a novel anti-inflammatory mechanism by which lactic acid may impact HIV susceptibility. In addition, some lactobacilli, including *Lactobacillus gasseri,* may exert direct anti-HIV effects through bacteriocins, antimicrobial compounds that kill other microorganisms [83,111].

Disruption of physical barriers, including cervicovaginal mucus and epithelium, may increase women's HIV susceptibility. Cervicovaginal mucus acts as a physical barrier to HIV [112,113]. In addition, the virus may diffuse more rapidly in cervicovaginal mucus with high concentrations of *L. iners* or *G. vaginalis* [114]. In contrast, there may be more virus trapping with *L. crispatus*-dominated microbiota. In a recent study by Borgdorff *et al.* [113], vaginal dysbiosis was associated with cytoskeleton alterations, increased proteolytic activity, and cell death, likely representing epithelial damage.

Do high-risk vaginal bacteria increase women's susceptibility to HIV infection?

In considering the question of whether vaginal microbiota influences women's susceptibility to HIV infection, it is useful to refer to a set of criteria first proposed by Hill [115] in 1965, and used extensively in epidemiology since that time. This list of conditions, often referred to as the Hill criteria, includes strength, consistency, specificity, temporality, biological gradient, plausibility, coherence, experiment, and analogy (Table 2). The Hill criteria are not a checklist to be fulfilled in their entirety to provide proof of causation, but do offer a helpful framework for examining causal inference.

Strength of association: multiple prospective studies and meta-analyses estimate an ∼1.5-fold higher risk of HIV acquisition with bacterial vaginosis compared with normal vaginal microbiota. Diverse non-*Lactobacillus*-dominant bacterial communities, higher relative abundance of some bacteria, and higher quantities of some bacteria, have been associated with increased risk of HIV acquisition. Effect estimates for significant associations range from an aOR of 2.59 (95% CI 1.26–5.34) for the

Table 2. Hill criteria for causation [115].

Criteria and explanation as described in Bradford Hill's original 1965 article [115]	Evidence from studies using classical methods for bacterial vaginosis diagnosis	Evidence from newer studies using molecular methods to characterize vaginal microbiome	Data satisfy criterion[a]
Strength: 'First upon my list I would put the strength of association [. . .] In thus putting emphasis upon the strength of association we must, nevertheless, look at the obverse of the coin. We must not be too ready to dismiss a cause-and-effect hypothesis merely on the grounds that the observed association appears to be slight.'	Multiple prospective studies and meta-analyses generally agree that both bacterial vaginosis and intermediate microbiota on vaginal Gram stain are associated with approximately 1.5-fold higher risk of HIV acquisition compared to normal microbiota.	Diverse non-*Lactobacillus*-dominant vaginal bacterial communities, relative abundance of some bacteria, and quantities of some bacteria have been associated with increased risk of HIV acquisition. Risk estimates vary across the three available studies	Partially
Consistency: 'Has it been repeatedly observed by different persons, in different places, circumstances and times?'	Of 15 prospective studies and meta-analyses, all but one found a statistically significant association between bacterial vaginosis and HIV acquisition	Across all three studies, more diverse bacterial communities were associated with HIV acquisition. Relationships between individual bacterial taxa and HIV acquisition are more consistent for some species (e.g. *Sneathia* spp. and *Mycoplasma* spp.) than for others (e.g. *Prevotella* spp., non-*iners Lactobacillus* species)	Mostly
Specificity: 'One reason, needless to say, is the specificity of the association [. . .] If as here, the association is limited to specific workers and to particular sites and types of disease and there is not association between the work and other modes of dying, then clearly that is a strong argument in favour of causation.'	Bacterial vaginosis is not a necessary exposure to acquire HIV	There is limited evidence, but it seems highly unlikely that a particular vaginal community or the presence or high concentrations of a specific type of bacteria is required for HIV acquisition	Not at all
Temporality: 'My fourth characteristics is the temporal relationship of the association—which is the cart and which the horse?'	Numerous prospective studies have demonstrated an association between bacterial vaginosis and subsequent acquisition of HIV	The three available molecular studies all captured data on the vaginal microbiome prior to or very shortly after, HIV infection	Fully
Biological gradient: '. . .if the association is one which can reveal a biological gradient, or dose-response curve, then we should look most carefully for such evidence.'	Prospective studies provide inconsistent evidence of a biological gradient for increased HIV risk with increasing Nugent score or numbers of Amsel criteria fulfilled	There is some evidence from recent studies of increasing HIV risk with increasing vaginal bacterial community diversity, and with higher relative abundance and absolute concentrations of some types of bacteria	Partially
Plausibility: 'It will be helpful if the causation we suspect is biologically plausible. But this is a feature I am convinced we cannot demand.'	Multiple biologically plausible mechanisms could explain the relationship between bacterial vaginosis and increased susceptibility to HIV	Studies at the molecular level suggest that more diverse vaginal bacterial communities, and the presence and concentrations of key bacteria, may increase HIV susceptibility	Mostly
Coherence: '[. . .] the cause-and-effect interpretation of our data should not seriously conflict with the generally known facts of the natural history and biology of the disease.'	Laboratory studies have mostly shown plausible mechanisms, as noted in the previous row. However, there is not a good animal model of the *Lactobacillus*-dominated healthy human microbiota associated with lower risk. Ex-vivo infection of biopsy specimens from women with different vaginal conditions could be helpful in providing additional data showing coherence between laboratory data and epidemiological studies		Minimally
Experiment: 'Occasionally it is possible to appeal to experimental, or semi-experimental, evidence. For example, because of an observed association some preventive action is taken. Does it in fact prevent?'	Clinical trials of bacterial vaginosis interventions have produced moderate reductions in bacterial vaginosis prevalence over periods up to one year. However, no effective interventions for reducing bacterial vaginosis have been evaluated in HIV prevention trials	One study has demonstrated that periodic presumptive treatment with metronidazole plus miconazole reduces detection and concentrations of several bacterial vaginosis-related bacteria. However, no effective interventions targeting the vaginal microbiome have been evaluated in HIV prevention trials	Not at all
Analogy: 'In some circumstances it would be fair to judge by analogy.'	Prospective studies and clinical trials provide some evidence for an analogous relationship between bacterial vaginosis and STIs other than HIV. However, these are also not proven causal relationships, and could be mediated through separate mechanisms	There is some evidence that the presence of key bacteria may increase susceptibility to STIs other than HIV. However, these are also not proven causal relationships, and could be mediated through separate mechanisms	Minimally

CST = community state types, HIV = human immunodeficiency virus, STIs = sexually transmitted infections.
[a]Overall degree to which the available evidence satisfies each of the nine Hill criteria on a scale that includes not at all, minimally, partially, mostly, fully.

highest concentrations of *Leptotrichia/Sneathia* spp. [78], to an aOR of 12.7 (95% CI 2.1–77.8) with *P. bivia* [77]. Most estimates fall toward the lower end of this range. Additional data will be essential to clarify the strength of these associations.

Consistency: of the 15 prospective studies and meta-analyses [4–8,28,29,56,57,73–75,116–118], all but one species. These differences may be related to the biology of the vaginal mucosa in different populations, methodological differences, or could have resulted by chance. In general, there is high consistency in studies showing an association between bacterial vaginosis, diverse vaginal microbiota, and HIV acquisition. Further work is needed to clarify the relationships between individual bacterial taxa and HIV risk in different populations.

Specificity: the relationship between vaginal microbiota and HIV is not specific, as bacterial vaginosis is not required for HIV acquisition. Although there are few molecular studies to date, it seems unlikely that a particular bacterial community or species would be a necessary precursor to HIV infection. Importantly, specificity is not essential for determining the existence of a causal relationship.

Temporality: numerous prospective studies have demonstrated an association between bacterial vaginosis and subsequent acquisition of HIV. In addition, all three available molecular studies collected data on the vaginal microbiome prior to or very shortly after HIV infection.

Biological gradient: prospective studies provide inconsistent evidence for a biological gradient for increased HIV risk with increasing Nugent score [8,28], or numbers of Amsel criteria fulfilled [4]. There is some evidence from recent molecular studies, that increasing vaginal bacterial community diversity, relative abundance, and absolute concentrations of some bacteria may be associated with increased HIV risk.

Plausibility: numerous studies demonstrate possible mechanisms through which the vaginal microbiome could contribute to HIV susceptibility. Additional research that more directly links vaginal bacteria to mucosal markers of HIV susceptibility could further strengthen this link.

Coherence: laboratory studies have primarily addressed mechanisms to explain the association between vaginal microbiota and increased HIV susceptibility. These types of data would seem to pertain more directly to biological plausibility than to coherence between laboratory studies and epidemiological evidence. Future studies should aim to more directly explore the hypothesis that vaginal dysbiosis increases susceptibility. Unfortunately, there is not a suitable animal model of the *Lactobacillus*-dominant healthy human vaginal microbiota. As such, ex-vivo infection of biopsy specimens from women with different vaginal conditions might provide the strongest evidence for coherence.

Experiment: there is some evidence that periodic presumptive treatment (PPT) and suppression approaches can reduce bacterial vaginosis [119–122], leading to more optimal vaginal microbiota, increased frequency of hydrogen-peroxide-producing *Lactobacillus* species colonization [123], and reduced quantities of BVAB1, BVAB2, *A. vaginae, Leptotrichia/Sneathia* spp., and *Megasphaera* spp. [124]. However, no effective interventions for reducing bacterial vaginosis or modifying the vaginal microbiota have been evaluated in HIV-prevention trials. Such trials, if undertaken in the era of effective HIV prevention interventions including preexposure prophylaxis (PrEP) and treatment of HIV-positive partners, would likely require sample sizes in the tens of thousands.

Analogy: the association between the vaginal microbiome and women's susceptibility to HIV infection represents a unique biological relationship for which it is difficult to identify a closely analogous system. The relationship between bacterial vaginosis and other STIs has some parallels. However, the mechanisms of susceptibility to other STIs may not reflect the most important mechanisms mediating HIV susceptibility.

In conclusion, current data fulfill some of the Hill criteria for assessing whether the vaginal microbiome is causally related to HIV susceptibility. Additional epidemiological studies with prespecified hypotheses will be valuable in establishing which relationships between individual bacterial taxa and communities are consistent across multiple studies and populations. As many of the current microbiome studies include multiple comparisons, using a global *P* value for the entire vaginal microbiota, and delving deeper only if the overall *P* value is significant, or using a false discovery rate, may help to reduce the number of associations identified by chance.

Clinical trials of vaginal health interventions would provide the gold standard of evidence, and could be considered if the mechanistic data are sufficiently strong and the proposed interventions can be shown to impact the putative mechanisms driving HIV susceptibility.

Impact of the vaginal microbiome on microbicide and oral preexposure prophylaxis efficacy

In the past 10 years, there has been significant investment in both topical and oral PrEP to reduce women's HIV risk. Of the five major trials, three have shown a benefit

[125–127], with the other two citing low adherence as a reason for null results [128,129]. Of the microbicide trials, only the CAPRISA 004 trial demonstrated significant efficacy of intravaginal tenofovir PrEP [125].

Three recent studies have examined how the vaginal microbiome may impact topical and oral tenofovir-based PrEP. The first of these included data from the CAPRISA 004 trial [125]. Women were randomized to vaginal tenofovir 1% gel versus placebo. Cervicovaginal lavages were analyzed from women prior to acquiring HIV compared with samples from randomly selected HIV-seronegative women [130]. Using a metaproteomic analysis, 188 species were identified. Vaginal microbiomes were classified as *Lactobacillus*-dominant or non-*Lactobacillus*-dominant. There were no differences in demographics, sexual behaviors, clinical characteristics, or adherence between women with these two vaginal microbiome types. Women with *Lactobacillus*-dominant vaginal microbiota showed significantly reduced risk of HIV acquisition with vaginal tenofovir gel compared with placebo (hazard ratio 0.39, 95% CI 0.20–0.83). In contrast, women with a non-*Lactobacillus*-dominant microbiota treated with tenofovir gel had no difference in HIV acquisition compared with placebo (hazard ratio 0.82, 95% CI 0.40–1.65).

To explore the mechanism underlying these results, tenofovir was inoculated in media with *G. vaginalis, L. iners, L. crispatus,* and an abiotic control [130]. Mass spectrometry illustrated that *in vitro*, *G. vaginalis* was associated with a 67.4% depletion of tenofovir levels. This dramatic effect was not present whenever media was inoculated with *L. iners, L. crispatus,* or the abiotic control. Thus, metabolism of tenofovir by *G. vaginalis* could explain the findings of decreased efficacy of vaginal tenofovir in women with non-*Lactobacillus*-dominant microbiota. An abstract presented at the Conference on Retroviruses and Opportunistic Infections (CROI) in early 2017 by Hillier *et al.* [131] further illustrates this point. Lower cervical tissue tenofovir levels were observed in women receiving tenofovir 1% gel whenever they had bacterial vaginosis diagnosed by Nugent score, higher concentrations of *G. vaginalis*, and higher concentrations of *A. vaginae*.

In contrast to topical tenofovir, the efficacy of oral PrEP with tenofovir disproxil fumarate does not appear to be affected by the vaginal microbiome [132]. Heffron *et al.*'s study presented data from a secondary analysis from the Partners PrEP study [127]. Efficacy of daily oral tenofovir was the same for women with normal microbiota, intermediate microbiota, and bacterial vaginosis by Gram stain. Oral PrEP efficacy also did not differ by presence of *Gardnerella/Bacteroides* morphotypes on Gram stain.

In summary, recent studies provide strong evidence that the vaginal microbiome impacts the efficacy of topical PrEP, an effect not present with oral PrEP. These results are likely to dramatically change the way vaginal microbicides are developed and tested.

Current approaches for modulating the vaginal microbiome

Current regimens for symptomatic bacterial vaginosis provide modest cure rates with frequent recurrences [26,27,133,134]. New approaches to control bacterial vaginosis have included alternative drug regimens [135], probiotics [136], biofilm disruptors [137–143], risk factor modification (e.g. cessation of intravaginal practices) [144,145], and suppressive regimens administered as PPT and periodic directed treatment [119,120,123,146]. Many of these regimens have reduced bacterial vaginosis recurrences. Less is known about such regimens' impact on individual bacterial taxa. One study found that oral PPT with 2 g of metronidazole each month resulted in more frequent *Lactobacillus* colonization [123], and another found that monthly intravaginal metronidazole PPT reduced concentrations of BVAB1, BVAB2. *A. vaginae, Leptotrichia/Sneathia* spp., and *Megasphaera* spp. measured using quantitative PCR assays [124]. However, producing sustained changes in the vaginal microbiome remains a challenge only partially addressed by current regimens.

Conclusion

Prospective studies have consistently demonstrated an association between bacterial vaginosis and increased risk of HIV acquisition. Recently, data from studies using molecular methods have led to the hypothesis that high-risk vaginal microbial communities and the presence and concentrations of key bacterial taxa may be more predictive of women's HIV risk than a bacterial vaginosis diagnosis by traditional methods. One limitation of the current literature is a lack of attention to the potential role of vaginal fungi and viruses (other than herpes and human papilloma viruses) in mediating HIV susceptibility in women. In addition, recently presented and published data examining associations between the vaginal microbiome and HIV acquisition have come from observational studies, and may be most useful for generating new hypotheses about how the vaginal microbiome influences HIV susceptibility.

Given the high prevalence of vaginal dysbiosis, particularly in African women, the question of whether vaginal health interventions could reduce women's susceptibility to HIV infection is of great importance. Current research priorities should include identifying the vaginal

microbiota associated with increased risk, substantiating mechanisms linking vaginal bacteria to mucosal HIV susceptibility, and evaluating the efficacy of interventions for interrupting these mechanisms. If such data are sufficiently convincing, clinical trials of vaginal health interventions should be considered. Although clinical trials of new HIV prevention interventions will be challenging in an era where other effective HIV prevention strategies are available, such studies would provide the strongest evidence to prove or disprove the presence of a causal association, and could provide an important and novel approach to HIV prevention in women.

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Conflicts of interest

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