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Towards a deeper understanding of the vaginal microbiota

Michael France[1,2], **Madeline Alizadeh**[1,2], **Sarah Brown**[1,3], **Bing Ma**[1,2], **Jacques Ravel**[*,1,2]

[1]Institute for Genome Sciences, University of Maryland, School of Medicine

[2]Department of Microbiology and Immunology, University of Maryland, School of Medicine

[3]Department of Epidemiology and Public Health, University of Maryland, School of Medicine

Abstract

The human vaginal microbiota is a critical determinant of vaginal health. These communities live in close association with the vaginal epithelium and rely on host tissues for resources. Although often dominated by lactobacilli, the vaginal microbiota is frequently comprised of a collection of facultative and obligate anaerobes. The prevalence of these communities with a paucity of *Lactobacillus* varies among women and epidemiological studies have associated them with an increased risk of adverse health outcomes. The mechanisms that drive these associations have yet to be described in detail with few studies establishing causative relationships. Here, we review our current understanding of the vaginal microbiota and its connection with host health. We center our discussion around the biology of the vaginal microbiota when *Lactobacillus* species are dominant versus when they are not, including host factors that are implicated in shaping these microbial communities and the resulting adverse health outcomes. We discuss current approaches to modulate the vaginal microbiota, including probiotics and vaginal microbiome transplants, and argue that novel model systems of the cervicovaginal environment that incorporate the vaginal microbiota are needed to progress from association to mechanism and this will prove invaluable for future research.

Introduction

The microbial communities that inhabit the human vagina are unique. Unlike the relatively diverse and even communities found at other body sites[1], the vaginal microbiota of reproductive-age cisgender women is often dominated by single species of *Lactobacillus*[2–4]. This *Lactobacillus*-dominant configuration was first reported in 1892 by Donderlein[5] and has long been considered to be a hallmark of vaginal health[6–9]. The production of lactic acid as a fermentation end-product by *Lactobacillus* spp. lowers vaginal pH (~4.0) and is thought to constrain the growth of many pathogenic microbes[10,11] and has a beneficial effect on the host epithelium, such as immune modulation[12,13]. However, around 25% of North American women have communities that are not dominated by *Lactobacillus* spp. and are instead comprised of a more proportionally even collection of obligate

[*]Corresponding author, address correspondence and requests for materials to: jravel@som.umaryland.edu.

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and facultative anaerobes (e.g. species in the genera *Gardnerella*, *Prevotella*, *Atopobium*, *Sneathia*, *Megasphaera*, *Peptoniphilus*)[2,4,14–17]. These women are often diagnosed with bacterial vaginosis (BV), a common vaginal condition poorly characterized as a dysbiosis of the vaginal microbiota[18,19]. Many of these women do not report experiencing adverse vaginal symptoms, for example odor and discharge, and appear to be otherwise "healthy" upon gynecological examination[2,14,20,21]. Epidemiological studies have linked the presence of these non-*Lactobacillus*-dominant communities with increased risk for adverse health outcomes including sexually transmitted infection (STI) acquisition[22–25] and spontaneous preterm birth[26–36], indicating that they may be less protective, hence non-optimal[37]. The mechanistic underpinnings of these epidemiological associations have yet to be described in detail. Here, we discuss our current understanding of the vaginal microbiota, how these communities interact with host tissues, and propose the next steps on the path towards a deeper understanding of their relationship to health.

This review is focused on the vaginal microbiota of cisgender female individuals, primarily of reproductive age. A brief discussion on the vaginal microbiota of premenarchal girls and postmenopausal women is included and highlights gaps in our knowledge of these age groups. We know comparably little about the vaginal microbiota of other individuals with a vagina, including transgender individuals. This topic was reviewed recently in Krakowsky, et al.[38]. More study is needed to comprehensively characterize these microbial communities and their relationships with health.

Composition of the vaginal microbiota

Advances in molecular biology and DNA sequencing have enabled the high throughput characterization of the taxonomic composition of the vaginal microbiota[2,39]. Composition is often established via sequencing of 16S rRNA gene amplicons, although others have utilized sequencing of *cpn60* gene amplicons[40], or a battery of taxon-specific qPCR assays[41]. Although the bulk of these data describe the vaginal microbiota of reproductive age North American women, a growing number of studies have examined women from other regions[15,25,42–48]. Most reproductive age women have a vaginal microbiota whose taxonomic composition resembles one of a limited number of configurations termed community state types (CSTs; also referred to as vaginotypes or cervicotypes, see McKinnon, et al.[37]). These configurations can be represented by five CSTs, four of which are dominated by single species of *Lactobacillus* (CST I-*L. crispatus*, CST II-*L. gasseri,* CST III-*L. iners*, CST V-*L. jensenii*). A fifth configuration, CST IV, represents the more proportionally even collection of facultative and obligate anaerobes. The phylotypes common to CST IV include, among others: *Gardnerella*, *Atopobium*, *Prevotella*, *Candidatus* Lachnocurva vaginae (formerly BVAB1[49]), *Sneathia*, *Peptoniphilus*, *Finegoldia,* and *Megasphaera*[39,50]. These are largely fastidious bacteria that are either difficult to cultivate, or so far uncultivatable (e.g. *Ca*. L. vaginae[49]). CSTs I, III, and IV are the most prevalent and account for around 90% of reproductive-age women[2]. Larger studies have employed finer resolution classification schemes that split the five CSTs into subtypes[39], most of which distinguish between variations of CST IV and describe uncommon communities (e.g. *Bifidobacterium* or *Streptococcus* dominated communities).

Although the CST approach does simplify community composition, it continues to be an important framework for the study of the vaginal microbiota.

The term “Community State Type” was originally meant to convey its representation of the taxonomic composition at a single timepoint[2]. This distinction is important because the vaginal microbiota of some women has been documented to vary, including shifts in CST[51,52]. Changes in composition are sometimes explicable, occurring at the onset of menstruation or following unprotected vaginal intercourse. Menstruation is accompanied by biophysical and hormonal fluctuations which impacts host physiology and thus the microbial communities present. Unprotected vaginal intercourse introduces semen into the vagina, an alkaline substance that temporarily raises vaginal pH[53], and has the potential to bring new microbial species and strains into the community from the penile microbiota[54]. Other changes in the vaginal microbiota cannot be obviously attributed to a specific factor and may be the result of fluctuations in host physiology, competitive interactions between members of the community, bacteriophage activity, ecological drift, or some other mechanisms[55]. The vaginal microbiota of some individuals, however, have been shown to not demonstrate temporal variation and instead maintain their community composition over several menstrual cycles[51]. It is not clear if this stability is a property of the microbiota, host physiology, or a combination of the two. Understanding the factors that drive temporal variation in the vaginal microbiota will be critical in the development of strategies to modulate these communities.

The vaginal microenvironment

The estrogenized vaginal epithelium consists of several squamous layers, with a superficial outermost layer overlying an intermediate, parabasal, and basal layer beneath[56] (Fig. 1). The upper layer is composed of flattened, dead cells that have undergone cornification, offering a physical protective barrier[57]. This barrier also serves as an immune junction, separate from that of the cervix. While immune cells are present at the transformation zone of the cervix[58], vaginal mucosal tissue harbors few T cells and antigen presenting cells (APCs) under normal conditions but displays increased numbers in response to inflammatory triggers. Additionally, the vaginal mucosal immune profile fluctuates with hormonal cycles, such that the highest levels of IgA and IgG are present just prior to ovulation, with lower levels at the time of menses[59].

The vaginal epithelium itself also responds to hormonal fluctuations, undergoing cyclic proliferation throughout the menstrual cycle with a peak at ovulation (Fig. 1), though changes are not as drastic as those of the uterus[60]. The vaginal epithelium is coated in a cervical mucus layer that is subject to regulation by hormonal fluctuation, with progesterone associated thickening seen in the peri-ovulatory period[61]. Although the vagina doesn’t produce its own mucus, cervical mucus is produced in high enough abundance to flow down and coat the vaginal epithelium[62]. The mucus is composed primarily of proteins, lipids, water, and glycoproteins referred to as mucins[63,64]. Every mucin is rich in sequences of repeating serines and threonines, with the repeat regions serving as the location for O-linked glycosylations chains comprised of N-acetylgalactosamine, galactose, and N-actylglucosamine and capped with fucose or sialic acid[65,66]. These glycosylation

chains play a key role in mucin function, and alterations to these patterns are associated with several adverse health conditions including spontaneous preterm birth[67]. Mucins are hypothesized to play a protective role for the vaginal epithelium[68,69] and they may also serve as a source of nutrition for the vaginal microbiota[70,71]. Mucin levels vary throughout the menstrual cycle; for instance, the amount of MUC5B peaks mid-cycle at ovulation[72] and is accompanied by an increase in the glycosylation of several mucins[73]. Glycogen made by the vaginal epithelium is also thought to be a nutrient source for vaginal bacteria[74,75]. Vaginal epithelial cells, in particular, contain an overabundance of glycogen relative to other epithelial tissues[76]. Higher free glycogen concentrations are associated with lower progesterone levels[77], while intracellular concentrations are associated with higher estrogen levels[78]. Both free and intracellular glycogen fluctuate throughout the menstrual cycle.

Many characteristics of vaginal physiology are altered following hormonal changes associated with the onset of menopause. The predominant cell type of the parabasal layer changes from stratum spinosum to predominantly basophilic stratum granulosum with clear cell nuclei[79,80]. Cycles of epithelial cell proliferation no longer occur due to the reduction in circulating estrogen levels, and vaginal atrophy is common[81]. Additionally, there are decreases in cervical mucus production[82], and changes in mucus composition[83], concomitant with the decline in estrogen and testosterone levels observed in this period. Free and intracellular levels of glycogen also decline[84]. Additionally, an increase in vaginal pH to ≥ 4.7 has been found to be one of the more sensitive markers of menopause[85]. Altogether, these changes contribute to a vastly different microenvironment for the microorganisms residing in the vagina. These differences are thought to be responsible for menopause-associated changes in vaginal microbiota composition[86] and the genitourinary syndrome of menopause (GSM)[87]. Hormonal replacement therapy is often used to treat GSM and may also impact the vaginal microbiota via its effect on the vaginal microenvironment.

Lactobacillus spp. and the vaginal microbiota in reproductive age women

It is well accepted that a vaginal microbiota dominated by *Lactobacillus* offers a greater degree of protection to their host compared to a more diverse microbiota. Recent work has highlighted that populations of *Lactobacillus* are typically not comprised of a single strain and display a substantial amount of intraspecies diversity[88]. Considering the continual supply of new mutants originating from each genetic background[89], these populations might best be thought of as clouds of related genotypes rather than single entities. This intraspecies diversity could be a critical determinant of community stability by buffering the dominant *Lactobacillus* population against perturbations[90]. There is consensus that the *Lactobacillus* species common to the human vagina are likely not equivalent with respect to their positive impacts on the host. Communities dominated by *L. crispatus* are thought to offer the most protective benefits and those dominated by *L. iners*, the least. It could be that *L. jensenii* and *L. gasseri* are equivalent to *L. crispatus* as they are more similar to this species in their metabolic capabilities than to *L. iners*[91], but their rarity impedes the investigation of their relationships to host health. Many hypotheses exist to explain the associations between *Lactobacillus* dominance and vaginal health, and they have varying degrees of evidential support[9,92,93]. In this section, we review our current understanding of the mechanistic explanations for these associations and discuss the ecology of the vaginal microbiome when

Lactobacillus species are abundant. We focus our discussion on the biology of *L. crispatus* in the vaginal microenvironment (Fig. 2), followed by a brief examination of *L. iners* and how it differs from the other vaginal lactobacilli.

L. crispatus is a Gram positive, facultative, anaerobic bacterium that produces both the L- and D-lactic acid isomers as its primary fermentation end-products[94] (Fig. 2). Although originally thought to lack the intrinsic ability to degrade glycogen without the help of host amylases[95,96], studies have now confirmed and described this metabolic capability in *L. crispatus,* including the identification of PulA homologs[97–99]. As the human vaginal epithelium is glycogen-rich[100], *L. crispatus* likely derives the majority of its carbon and energy through the fermentation of glycogen, converting it ultimately into lactic acid. Lactic acid production lowers vaginal pH, often to level less than pH 4.2[11,39], and this acidification of the vaginal microenvironment is one hypothesized means by which *L. crispatus* benefits the host. *In vitro* studies have demonstrated that acidic conditions can preclude or inhibit the growth of less beneficial bacterial species including *Gardnerella*, *Prevotella*, *Mobiluncus*, and *E. coli*[101–103]. Lactic acid may also have direct effects on host tissues by modulating the immune system and gene expression. For example, D-lactic acid, which is produced by *L. crispatus* (as well as *L. gasseri* and *L. jensenii*) but not *L. iners*[91,104], has been associated with differential expression of immune factors by host tissues[105,106]. A study by Hearps et al., on the other hand, found that the ionization status of lactic acid, which is a function of pH, had a larger impact on its ability to suppress inflammation than the isomer form[13]. Lactic acid more readily diffuses through epithelial cell membranes when in the non-ionized form[107]. It is clear that the relationship between lactic acid and vaginal health is multifaceted and its effects extend beyond lowering vaginal pH.

There are other mechanisms by which *L. crispatus* is thought to exert beneficial effects on vaginal health. *L. crispatus* (and *L. gasseri* and *L. jensenii*) have long been known to produce hydrogen peroxide in the presence of oxygen[108] (Fig. 2). It was thought their production of H_2O_2 also served to inhibit the growth of anaerobic bacteria in the vaginal microenvironment[109,110]. Observational studies found associations between the presence of H_2O_2 producing lactobacilii and vaginal health[7,110,111]. We now know that only *L. iners* does not produce hydrogen peroxide[3] confounding this observation with other factors that distinguish *L. iners* from the other lactobacilli[104,112]. *In vitro* studies have shown that *Lactobacillus*-produced H_2O_2 can inhibit the growth of many of these less beneficial bacteria[113], although *Gardnerella* spp. seem to have the capability to resist H_2O_2[114]. These studies do not necessarily have relevance to the *in vivo* production of H_2O_2 by *Lactobacillus*. The reactions require molecular oxygen, which is likely rare in the microaerobic vaginal microenvironment where O_2 concentrations are 1/10 to 1/5 that of atmospheric concentrations[115]. Further, any H_2O_2 that is produced can be quenched through reactions with various non-microbial components of vaginal fluid[116]. If H_2O_2 production does play an inhibitory role in the vaginal microenvironment it is likely limited to localized interactions between the lactobacilli and their competitors. *L. crispatus* and other vaginal lactobacilli may also have other means of inhibiting the growth of competitors including the production of bacteriocins[117,118].

In addition to its thick cell wall, *L. crispatus* also produces a proteinaceous outer surface layer, called the S-layer[119,120] (Fig. 2). The S-layer, and its associated proteins, is thought to contribute to the species' ability to adhere to host cells[119,120] and its' immunomodulatory capabilities[121,122]. The adherence of *L. crispatus* to vaginal epithelial cells is thought to block adhesion of pathogens[123,124], although the role of adhesion to a rapidly shedding vaginal epithelium is unclear. Vaginal microbiota that are dominated by *L. crispatus* have been associated with lowered vaginal inflammation[28,125], although a complete mechanistic explanation of the immunomodulatory capacity of the species has not been described. It is likely that proteins in the S-layer contribute. Efforts to further characterize the biology of *L. crispatus* and many other vaginal bacteria have been severely hampered by a lack of tools to manipulate the species' genetics. Methods to generate targeted gene knockout mutants of these species will prove critical in future research.

One final aspect of *L. crispatus* biology that is often overlooked but may be relevant to vaginal health is the dominance of *L. crispatus* in the vaginal microbiota and therefore the low proportion of other bacteria. *L. crispatus*, and the other vaginal *Lactobacillus*, can dominate the vaginal niche, often accounting for 99% of the sequences in 16S rRNA gene amplicon data[2,39]. Some women also maintain *L. crispatus* dominance over several menstrual cycles, indicating the dominance of these populations can be fairly stable[51]. By dominating the vaginal niche, *L. crispatus* reduces and precludes the growth of other, potentially harmful bacteria. This concept, termed "pathogen resistance", is certainly a benefit provided by a *L. crispatus*-dominant vaginal microbiota[126]. Ecological theory predicts that a more complex community utilizes more resources in an environment than a simple community, due to the non-overlapping portions of the constituent's niches[127]. A community that is mostly comprised of a single species should therefore not exploit the vaginal environment to the same extent as the more proportionally even CST IV community. For example, *L. crispatus* is not predicted to be a substantial degrader of host protective mucus as it is not known to be capable of removing terminal sialic acid and fucose residues from mucin glycosylation chains[128,129]. This is in contrast with some of the other, non-*Lactobacillus* species that are capable of these metabolic feats[128–133]. *L. crispatus* can therefore preserve this critical barrier that protects the vaginal epithelium. Additionally, *L. crispatus* does not produce a cytolysin that would allow it to liberate resources through the lysis of host cells[134,135], nor does it appear capable of producing many of the biogenic amines thought to be responsible for vaginal odor[136] (e.g. trimethylamine, cadaverine).

L. iners is perhaps the most common vaginal bacteria and is unique among the *Lactobacillus*[2,39]. The species was first identified as the vaginal lactobacilli that did not produce hydrogen peroxide[109,111]. Compared to other vaginal *Lactobacillus*, *L. iners* has a smaller genome[104,112], produces a cytolysin[134], and does not produce the D isomer of lactic acid[91,105]. Its relevance to vaginal health has been a topic of much discussion[137]. The dominance of *L. iners* in the vaginal microbiota is associated with low vaginal pH (<4.5). due to its production of L-lactic acid as a fermentation end-product[2,39]. Longitudinal studies have also found that *L. iners* dominated communities are less stable than those dominated by other lactobacilli, and often transition to CST IV, which may contribute to its limited association with vaginal health[51,138]. In line with this, *L. iners* is sometimes found in low to moderate relative abundances in CST IV communities[2,39,109,111]. This species has been

shown to vary its gene expression when found within CST IV communities, including higher expression of its cytolysin[139,140]. These results suggest that the impact of *L. iners* on vaginal health may be community composition dependent. Although more study is needed to define the relationship between *L. iners* and vaginal health, all indications are that *L. iners* offers fewer benefits to its host than *L. crispatus*, or the other vaginal lactobacilli, although strain-level variations might modulate these benefits. A study by Bloom et al., has indicated that metabolic differences between *L. iners* and the other vaginal lactobacilli could be leveraged to selectively inhibit *L. iners*[141].

The vaginal microbiota when *Lactobacillus* does not dominate

Many women have a vaginal microbiota that is comprised of other facultative and obligate anaerobic bacteria[2,4,14–17] (Fig. 3). These communities are associated with a higher vaginal pH (>4.5) and with symptoms such as abnormal discharge and/or odor, although many are asymptomatic[2,14,20,21]. It is estimated that somewhere between 23–29% of reproductive-age women have BV[18,19], which is diagnosed on the basis of a high vaginal pH, a paucity of *Lactobacillus*, an increased abundance of odorific biogenic amines, and the presence of clue cells (shed vaginal epithelial cells coated in bacteria)[142]. In research settings, BV is typically identified using a Gram-stain procedure that produces a Nugent score[143]. Standard of care treatment for BV includes the use of metronidazole (topical or systemic) or clindamycin (topical)[144] and often fails to produce a lasting resolution of the condition[145,146]. The connections between BV and CST IV are clear—both are defined by a lack of lactobacilli and a higher vaginal pH. However, CST IV communities are not always associated with vaginal symptoms and this is often described as asymptomatic BV. The question of whether to treat remains controversial as epidemiological studies have linked asymptotic BV with increased risk to adverse health outcomes[147]. Understanding which, if any, CST IV communities do not cause vaginal symptoms and/or do not increase risk to adverse health outcomes will go a long way toward understanding when treatment is necessary.

Similar to lactobacilli, host-produced glycogen is likely to be a major source of carbon and energy for CST IV bacteria. *Gardnerella* and many of the other species common to CST IV have been shown to have genes associated with glycogen degradation[98,140,148]. Expression levels of predicted glycogen debranching enzymes are high in these communities and similar to that observed in *Lactobacillus* dominant communities[140]. Studies have shown a positive association between free glycogen in vaginal fluid and *Lactobacillus*[75,149]; however, we argue that this does not conflict with the observation that CST IV bacteria also utilize glycogen. The CST IV vaginal microbiota, which is often higher in bacterial load and more diverse, might simply consume more of the host-produced glycogen. The species common to CST IV have at least two other metabolic capabilities that likely allow them to access more host-produced resources (Fig. 3). First, various *Gardnerella* spp. and *Prevotella* spp. are known to produce sialidase and fucosidase enzymes capable of degrading mucin glycan chains[128–133]. Second, *Gardnerella* (and other species) produce a cholesterol-dependent cytolysin that is capable of lysing epithelial cells, thereby liberating their intracellular contents for use by the microbiota[135,150,151]. Damage to the vaginal epithelium likely activates proinflammatory signaling pathways, drawing leukocytes to the area[152]. These two metabolic feats, mucin degradation and host cell lysis, might act synergistically to

damage the vaginal epithelium: removing the mucin layer would give the cytolysin better access to epithelial cells. While mature vaginal epithelium cells are regularly shed, the CST IV microbiota is likely capable of actively depleting the vaginal epithelium (Fig. 3). Consistent with this hypothesis is the observation that women with symptomatic BV experience higher cell shedding while those with asymptomatic BV shed fewer, but more immature epithelial cells[153]. We argue that these results indicate that the vaginal epithelium of some women with CST IV microbiota is damaged and might require repair before a *Lactobacillus* dominant microbiota can re-establish. This hypothesis may explain the frequency of recurrence following treatment of BV.

The metabolic activities of the microorganisms that comprise the CST IV vaginal microbiota also impact the vaginal metabolome. One prominent example is that these communities are associated with an increased abundance of biogenic amines including: putrescine, cadaverine, and tyramine[136,154] (Fig. 3). Biogenic amines are hypothesized to explain the connection between BV and vaginal odor. However, their role in the vaginal microenvironment likely extends beyond this symptom. Production of biogenic amines is a mechanism of acid tolerance which could be necessary for these bacteria to survive in the vagina[155]. Several biogenic amines have also been shown to either increase the lag time or decrease the growth rate of the vaginal *Lactobacillus*, suggesting that they may drive the establishment and maintenance of the CST IV microbiota[156]. *Gardnerella* is not thought to be a primary producer of these metabolites; species within the *Prevotella*, *Mobiluncus*, *Dialister*, *Parvimonas*, *Megasphaera*, and *Peptostreptococcus* genera are instead suspected to be responsible[136]. The metabolic pathways that microorganisms use to produce biogenic amines are generally not well characterized so other bacteria could also be involved in their generation. For example, it is not known how trimethylamine (TMA), the compound thought to be responsible for the fishy odor symptom of BV, is produced in the vagina. *Mobiluncus* spp. are capable of producing TMA[157] but it seems unlikely that this is the only source as these bacteria are not common in the vaginal microbiota.

It is critical to also recognize that the CST IV microbiota is not monolithic. A unifying characteristic of these communities is that they are not dominated by lactobacilli but their composition can take a number of forms. While the presence of *Gardnerella*, *Atopobium*, and various *Prevotella* spp. is a common motif, some women have CST IV communities that also include high proportions of *Ca*. L. vaginae, *Sneathia*, *Mobiluncus*, and even *L. iners*[39,41,158]. It could be that a subset of species common to CST IV are responsible for the majority of its association with adverse health outcomes, or that these associations could be strengthened by looking at subtypes of these communities. Compositional characterizations of the vaginal microbiota have largely been derived from 16S rRNA gene amplicon survey data, which has likely underestimated diversity within CST IV communities. *G. vaginalis*, for example, has long been known to be a diverse species[159] and has recently been split into multiple genomospecies[160]. Most women who are colonized by *Gardnerella* have several of these species in their vaginal microbiota[88,159]. Over the years many genomic and *in vitro* phenotypic comparisons of *Gardnerella* strains have been conducted, some of which suggest that there is variation in pathogenic potential within *Gardnerella* (e.g. not all *Gardnerella* genomes encode a known sialidase)[159,161–163]. Shotgun metagenomic studies are necessary to disentangle diversity within *Gardnerella* and many of the other species common to CST

IV communities. Disentangling the diversity of CST IV will prove critical for resolving the connection between these communities and vaginal health and will lead to improved targeted treatments.

Host factors affecting vaginal microbiota composition

Early epidemiology studies observed that vaginal microbiota composition exhibits variation that is dependent on a woman's ethnicity or race. Some studies found Black women in North America and Europe were less likely to have a vaginal microbiota dominated by *Lactobacillus* than white women in these populations[2,16]. For example, in a study of 396 North American women, 10.3% of those who identified as white or Caucasian had a CST IV vaginal microbiota, compared to 40.4% of those who identified as Black or African American[2]. Another study identified a subtype of CST IV, defined by the presence of *Ca. Lachnocurva vaginae*, that was not prevalent in North American women who identified as Asian[39]. Given that race is a social construct, the factors that drive these differences are multifaceted and it has been hypothesized that socioeconomic, cultural, genetic, and/or behavioral factors, as well as inequalities in healthcare, are responsible[164]. However, it is important to note that these differences have largely not been found to extend within CSTs. The taxonomic composition of a vaginal microbiota assigned to CST IV, or any other for that matter, does not appear to depend on race or ethnicity. One exception is that *Prevotella* spp. may be more abundant in CST IV communities from women in African populations[25,43]. An in-depth comparison of African women and women with African ancestry living on other continents is necessary to confirm this observation.

Moreover, it is important to recognize and discuss the concordance in the composition of the human vaginal microbiota among reproductive-age women from around the world. *L. iners*, *L. crispatus*, and *G. vaginalis* are three of the most prevalent bacterial species in the vaginal microbiota of women from every population examined thus far, including: North American[39], South American[165], European[166], African[25,46], and Asian[15,42] populations. A study of Amerindian women living a pre-agricultural lifestyle found that their vaginal microbiota was commonly comprised of *L. iners* or *G. vaginalis* but *L. crispatus* was less prevalent than in other populations[48]. All indications are that the taxonomic composition of the vaginal microbiota is a shared distinguishing trait of humanity. *Lactobacillus* do not dominate the vaginal microbiota of any other known mammal[167], and many species common to our vaginal microbiota have not been identified in the vaginal microbiota of other mammals, including non-human primates. *Gardnerella* have been identified in rhesus macaques, but less frequently than in humans and at lower relative abundances[168]. It remains to be seen if these *Gardnerella* species are distinct from those found in humans. The driving factors behind the development of our unique vaginal microbiota are not known.

Age is also known to impact the vaginal microbiota. Less is known about the communities that reside in the vagina during and prior to puberty or during/following menopause. This lack of knowledge should not be interpreted as a reflection of the vaginal microbiota's relative importance to health in these populations. For example, the vaginal microbiota is thought to play a role in urinary tract infections during childhood, which afflict 3–7% of premenarchal girls[169,170]. For post-menopausal women, the vaginal microbiota

is thought to contribute to atrophic vaginitis and associated sexual dysfunction[171,172]. Both premenarchal girls and postmenopausal women are less likely to have communities dominated by *Lactobacillus*, although their composition is also somewhat distinct from the CST IV communities commonly found in reproductive age women[173,174]. One commonality between these two age groups is their propensity to have lower levels of circulating estrogen than reproductive-age women[100]. Low estrogen levels are thought to result in a thinner vaginal epithelium that is not as glycogen rich[175]. It could be that without this glycogen, the environment is less conducive for the growth of lactobacilli and other species common to the reproductive age vaginal microbiota. The number of bacteria in these communities is typically several logs lower than that found in reproductive-age women, which could be driven by lower nutrient levels[176]. Additional studies are needed to define the relationship between the vaginal microbiota and health in these age groups (see Laniewski and Herbst-Kralovetz for more in depth discussion on the menopause and vaginal microbiota[177]).

Although often overlooked, the vaginal microbiota of premenarchal girls is of particular interest as it may influence the future composition of these communities. At birth, neonatal estrogen levels are high due to their mother's circulating hormones. However, the estrogen levels decline during the first weeks of life, and normally remain low until the initiation of puberty[178]. A recent study examined the vaginal microbiota of 4–6-year-old Chinese girls and found their communities were comprised of a diverse collection of *Peptoniphilus*, *Porphyromonas*, *Prevotella*, *Pseudomonas*, and *Escherichia coli*[179]. The timing of the transition towards a vaginal microbiota that resembles that at reproductive-age is not well characterized. A study by Hickey et al. found that the adolescent vaginal microbiota (aged 10–13) resembled that of a reproductive-age woman prior to their first menses, indicating the transition must happen earlier in life[180]. If we generalize the results of these two studies, we can posit that the transition must occur sometime between the ages of 6 and 12. Estrogen levels begin to rise during this time period indicating that it may be a driving force behind the transition. The source of the species that gain dominance in the vaginal microbiota during reproductive ages (e.g. *L. crispatus*, *L. iners*, *G. vaginalis*) is also not clear. It could be that these species are vertically transmitted from mother to offspring during the birthing process or early in life. Under this scenario the species would need to persist in the vagina throughout early childhood and then increase in abundance during adolescence. However, the vaginal microbiota might experience more frequent influxes of new strains and species through another mechanism and transmission happens later in life.

Epidemiologic associations between the vaginal microbiota and adverse health outcomes

The results from epidemiological studies have described associations between the composition of the vaginal microbiota and adverse health outcomes (Table 1). In this section, we will refer to a community with lower proportion or abundance of *Lactobacillus* and a higher proportion or abundance of facultative and obligate anaerobes (e.g. *Gardnerella*, *Prevotella*, *Atopobium*, *Sneathia* etc.) as a "non-optimal vaginal microbiota". Note that this definition includes women with asymptotic and symptomatic BV. There is strong and consistent evidence from longitudinal studies linking this non-optimal microbiota to an increased risk of acquiring and transmitting human immunodeficiency virus (HIV)[181–185]. Similar associations have been identified between these communities and an increased

risk for acquiring other STIs, including gonorrhea, chlamydia, trichomonas, herpes simplex virus 2 (HSV-2), and syphilis[22,186–189]. The non-optimal microbiota has also been linked to both incidence and prevalence of human papillomavirus (HPV), as well as the associated development and progression of cervical intraepithelial neoplasia and increased risk for cervical cancer[190–195]. Again though, there are contrary reports[196–199]. The composition of the vaginal microbiota has also been associated with increased risk for non-sexually transmitted infections, including urinary tract infections[200,201], vulvovaginal candidiasis[202–204], and pelvic inflammatory disease[205–207]. There is evidence supporting an association between the composition of the vaginal microbiota and reproductive health including risk for spontaneous preterm birth[33,208–210]. Studies that utilized sequence-based methodologies have found associations between specific vaginal bacteria, and bacterial community structures and preterm birth, spontaneous preterm birth, and preterm premature rupture of fetal membranes; however, results were heterogeneous across studies, with some finding no association[27–29,211–214].

Despite the volume of work establishing associations between the vaginal microbiota and health, we are still lacking descriptions of the causal mechanisms and pathways. It is particularly difficult to determine whether the associations are driven by the microbiota influencing host physiology or by changes in host physiology impacting the composition of the microbiota. Parsing these tripartite associations will require the development of animal and cell culture model systems that incorporate the vaginal microbiota (Box 1).

Efforts to modulate the vaginal microbiota

Efforts to impart lasting change in the composition of the vaginal microbiota have largely proven unsuccessful. Standard of care antibiotic treatment for BV often yields only temporary resolution of the condition[215–217]. Other methods to repress the growth of BV-associated anaerobes and/or support the growth of lactobacilli include estrogen therapy[172] and treatment with lactic[218] or boric acid[219] (Fig. 4). Many have also suggested probiotics for the modulation of the vaginal microbiota, either following antibiotic treatment or primary treatment. Several vaginal probiotics containing *Lactobacillus* species have been designed and tested, largely yielding mixed results[220–227]. There are a number of reasons why the efficacies of these probiotics fell short of expectations. In some cases, the probiotic formulations did not utilize species that are common to the human vagina, opting instead to use those that were already in gut probiotics[222,227]. Other probiotics were given to women in the form of oral tablets with the expectation that such a probiotic might influence host physiology, creating a vaginal environment favorable for *Lactobacillus*[221,227]. A recent randomized, double-blind, placebo controlled clinical trial, was conducted to test the efficacy of a vaginally delivered *L. crispatus* probiotic called Lactin-V. The probiotic was provided to women with BV, following metronidazole treatment, and resulted in a difference of 15% in the rate of BV recurrence between the treatment and placebo groups (30% versus 45% recurrence)[228]. This result is encouraging but still over a quarter of treated women experienced BV recurrence within 12 weeks. Identifying the factors that drive treatment failure will prove critical for the development of more effective vaginal probiotics.

Promising results from studies reporting the efficacy of fecal microbiota transplants to treat recurrent *Clostridioides difficile* infections[229] have motivated the investigation of vaginal microbiota transplants (VMT) as a potential approach to treat recurrent BV (Fig. 4). The concept involves sampling vaginal secretions from an individual with a *Lactobacillus*-dominant vaginal microbiota and introduce it into the vagina with recurrent and/or recalcitrant BV[230]. An exploratory study on women with recurrent BV indicated the potential efficacy for this approach as long-term remission was achieved for four of the five recipients of the VMT[231]. It is not clear how VMT could be implemented at scale safely, as each donation requires extensive testing for vaginal pathogens and viruses (e.g. HSV or HPV) and contains a relatively small bacterial load[232]. However, studies on the mechanisms of VMT are likely to yield novel insights into the factors that influence the successful modulation of the vaginal microbiota. These insights could then be translated to traditional *Lactobacillus* probiotic formulations with increased safety and can be produced at scale.

Outlook

Over the past decade we have learned a great deal about the vaginal microbiome and how it relates to host health. Unfortunately, our reliance on observational studies and amplicon-based compositional survey data has stymied the progress towards a mechanistic understanding of these communities and their impact on host physiology. These observational studies have generated innumerable hypotheses that must be tested in the laboratory. Recent *in vitro* work has characterized aspects of the biology of individual bacteria (e.g. on glycogen debranching enzymes of the vaginal bacteria[97,99]) but these studies often do not include the microbiota and/or the host. A major barrier towards the development of a mechanistic understanding is the dearth of suitable model systems for *in vitro* experimentation. While it is true that no model is perfect, some models are certainly better than others and a cervicovaginal model that incorporates the vaginal microbiota is sorely needed. Progress must be made in the field of multi-omics as integration of metagenomic, metatranscriptomic, metabolomic, and immunology datasets could afford a detailed look into the biology of the microbiota-host relationship as it exists *in vivo*. Results from such *in vitro* and *in vivo* studies, along with interventional clinical trials will likely drive the development of advanced and innovative treatment options and preventative measures for the myriad of adverse health outcomes that impact individuals with a vagina and remain unaddressed.

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Box 1:

Model systems for studying the vaginal microbiota

A major obstacle in vaginal microbiome research is a lack of suitable animal and cell culture model systems. These model systems are needed to investigate and test mechanistic hypotheses generated through observational studies of the vaginal microbiota. Unfortunately, the uniqueness of the human vaginal microenvironment and the human vaginal microbiota means that routinely used animal model systems lack relevance. Mouse models, that have proven so useful for investigations of the intestinal tract microbiota[234,235], have also been used in studies of the vaginal microbiota[236–238]. However, because these animals do not naturally have a vaginal microbiota that resembles that of humans, it is difficult to interpret whether results are generalizable to humans. Animal models are more frequently used in STI research[239–242], but these studies have historically, and unfortunately, not considered the role of the microbiota in the host-pathogen interaction. Two- and three-dimensional cell culture models have been developed and used in vaginal microbiome research including: cellular hydrogels[243,244], self-assembled organoids[245,246], and microfluidic organ-on-a-chip models[247]. Notably, microfluidic organ-on-a-chip models offer the ability to place cells within defined geometries, can reproduce key microenvironment conditions, and can be maintained for longer durations. They also allow the integration of immune cells, the use of hormonal control, and the application of relevant mechanical forces[248]. An ideal organ-on-a-chip model would include cervical and vaginal tissues with a transition zone between them. The vaginal epithelium should be stratified in multiple layers and should shed superficial cells that contain glycogen stores and vaginal mucus, either produced by the cervical tissue or supplied from an external source, should coat the vaginal tissue. The application of spatial transcriptomics to such a model system would allow the researcher to characterize the local host response to the microbiota and would be critical for the multi-layered vaginal epithelium[249,250]. The development and use of such a model would be a major breakthrough for vaginal microbiota research and will enable mechanistic hypothesis testing.

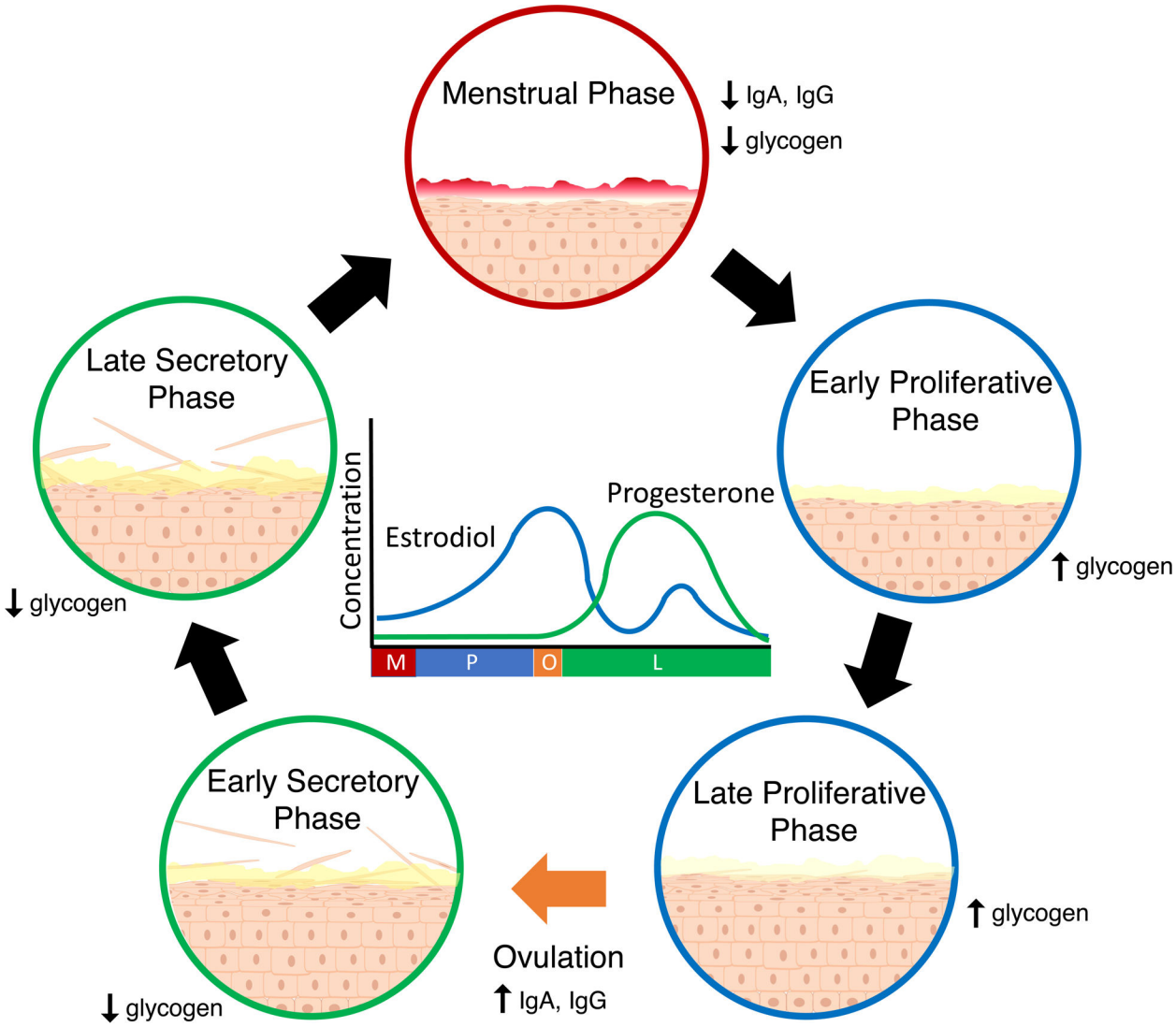

Fig. 1 |. Effect of the menstrual cycle on the vaginal microenvironment.

During the menstrual phase (M; red), blood and the shed functional layer of the uterine endometrium flow through the vagina. During the subsequent proliferative phase (P; blue), higher estrodiol levels promote the growth and maturation of the vaginal epithelium. The mucus is thinner during this stage, which is thought to facilitate sperm penetration. Following ovulation (O; orange), progestrerone levels rise during the secretory phase (L; green), halting growth and maturation of the epithelium. Superficial cells of the epithelium are shed and the protective mucus layer is thicker.

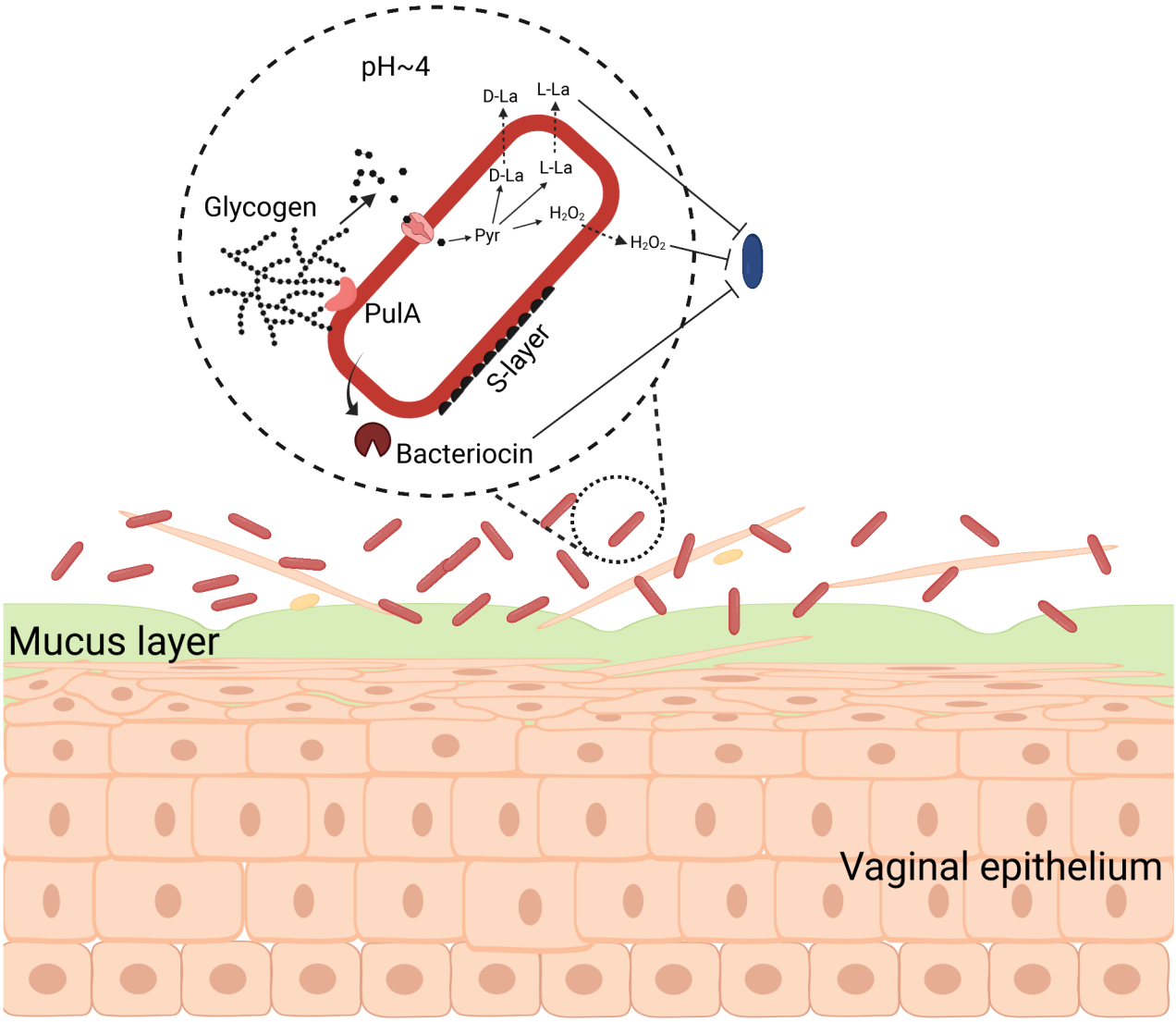

Fig. 2 |. The biology of *Lactobacillus crispatus* in the vaginal microbiota.

When lactobacilli (red) dominate the vaginal microbiota, less beneficial bacteria (blue) are lower in abundance. *Lactobacillus* spp. produce PulA, a glycogen-degrading enzyme that generates smaller glucose polymers that are then imported into the cell and fermented via pyruvate (Pyr), producing lactic acid isomers (D-La and L-La). This acidifies the microenvironment to a pH <4. Glycogen breakdown products can also be used to produce hydrogen peroxide (H_2O_2). Growth of less beneficial bacteria is suppressed by the low vaginal pH and bacterial products, such as lactic acid, bacteriocins and H_2O_2. D-lactic acid production and S-layer proteins can modulate host immune function in an anti-inflammatory manner.

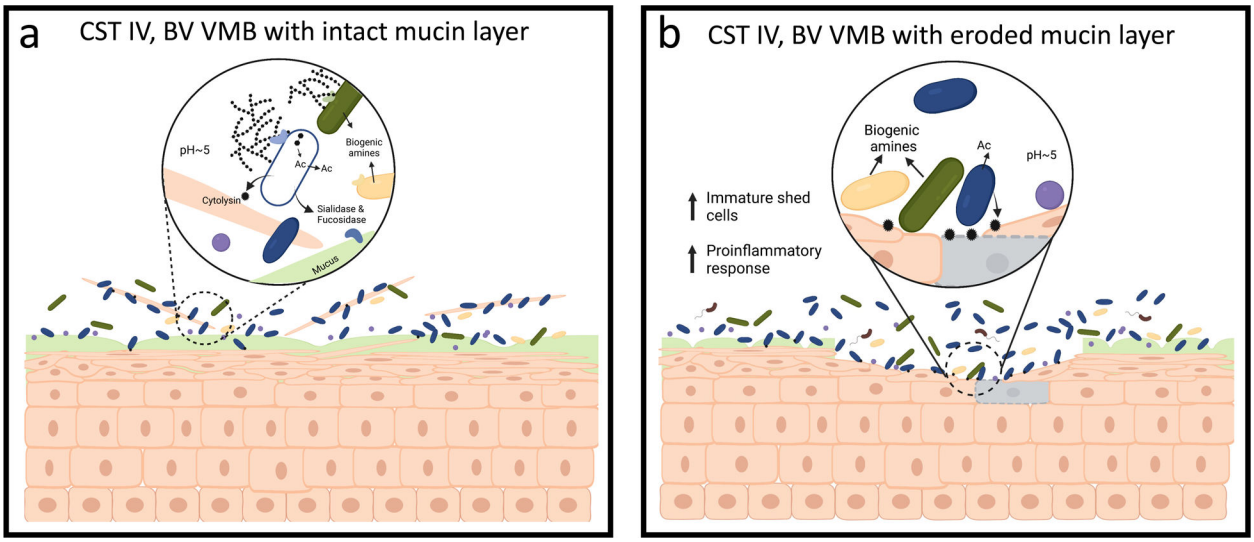

Fig. 3 |. The CST IV vaginal microbiota.

a, The CST IV vaginal microbiota is comprised of a more even collection of *Gardnerella* (Blue), *Prevotella* (Green, Yellow), *Atopobium* (Purple), *Ca*. L. vaginae (Brown, flagellated) and is associated with a higher pH (>4.5). These bacteria produce biogenic amines that raise vaginal pH, impact host physiology, and inhibit the growth of *Lactobacillus*. These species can break down glycogen produced by the host to produce acetate (Ac), for example, using sialidase and fucosidase enzymes. Production of cytolysin enzymes by *Gardnerella* and other species allow the community to liberate more resources through the lysis of host cells. **b**, A subset of CST IV communities has the potential to degrade host mucin glycochains due to their ability to produce sialidase and fucosidase enzymes. If the mucus layer is degraded faster than it can be replenished, the integrity of the protective mucus layer might become compromised, exposing the vaginal epithelium to further damaged by cytolytic activity.

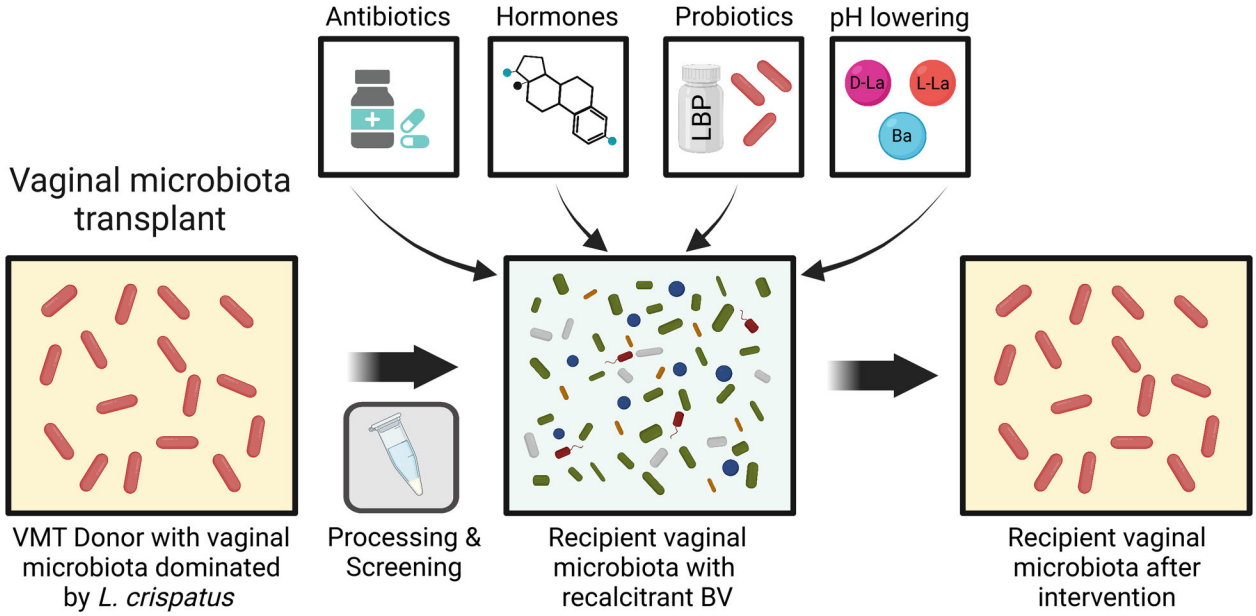

Fig. 4 |. Vaginal microbiota interventions to treat bacterial vaginosis.

Existing treatments include antibiotics such as metronidazole, estrogen therapy, lactic and boric acid, and vaginal lactobacilli probiotics. However, these interventions vary in their success and do not effectively prevent recurrent/recalcitrant BV. Vaginal microbiota transplants (VMTs) are a promising intervention for BV. A suitable donor with a *Lactobacillus* dominant vaginal microbiota is identified. Vaginal secretions are collected from the donor, screened for various STIs, and processed. The processed vaginal secretions are then introduced into the vagina of a recipient who is typically experiencing recurrent/recalcitrant BV. The recipient may or may not be treated with antibiotics prior to the transplant. Success is defined as a long-lasting resolution of the recipient's BV and a shift of their vaginal microbiota to the *Lactobacillus* dominant configuration.

Table 1:

Epidemiological associations between the composition of the vaginal microbiota and vaginal health

Outcomes	Summary of findings	Citations
STI acquisition (including HIV, gonorrhea, chlamydia, trichomonas, HSV, HPV, syphilis)	Results of studies vary, especially depending on the STI; presence or increased relative abundances of *Lactobacillus* spp. are generally associated with decreased risk; BV, a CST IV vaginal microbiota, and particular BV-associated phylotypes have been found to be associated with increased risk.	22,181–185,187–189,195–199
Vulvovaginal candidiasis	Results of studies vary with one finding no evidence for differences in vaginal microbiota of women with and without recurrent vulvovaginal candidiasis but another suggested risk of symptomatic candidiasis may be higher for a *Lactobacillus* dominant community.	202,203
Urinary tract infection (UTI)	UTIs were more common among women with vaginal *E. coli* colonization, and without H_20_2-producing *Lactobacillus*.	200
Pelvic inflammatory disease (PID)	Higher growth of several BV-associated bacteria was associated with increased risk for PID, while there was no association between carriage of non-BV-associated bacteria and PID risk.	205–207
Preterm delivery	Results of studies vary; increased relative abundances of *Lactobacillus* spp. have generally been shown to be associated with decreased risk; BV, a CST IV vaginal microbiota, and particular BV-associated phylotypes have been found to be associated with increased risk.	27–29,33,208,210–213,233