How *Lactobacillus* and BV-associated bacteria affect HIV-1 and HPV susceptibility in the vaginal epithelium

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Abstract

Bacterial vaginosis (BV) is a dysbiotic state of the vaginal microbiota which is a common condition that occurs in women of reproductive age. BV is strongly associated with a higher susceptibility for sexual transmitted infections like Human Immunodeficiency Virus type 1 (HIV-1) and Human Papillomavirus (HPV). In a beneficial vaginal microbiome, the dominant *Lactobacillus spp.* maintain a low vaginal pH by secreting lactic acids and prevent HIV-1 and HPV infections through the production of lectins and other protective compounds. During BV, *Lactobacillus spp*. are replaced by anaerobic bacteria such as *Gardnerella* and *Prevotella spp*., disturbing this protective environment. Anaerobes produce enzymes that degrade the protective mucus layer and damage the vaginal epithelium, making it more vulnerable for viral infections. In addition, some of these bacteria induce a pro-inflammatory response, resulting in damaging of the vaginal epithelium and the recruitment of CD4+ T lymphocytes, the main target cells of HIV-1. This review discusses the molecular mechanisms underlying the increased susceptibility for HIV-1 and HPV infections during BV.

Plain language summary

Similar to other parts of the human body, there is a community of bacteria living in the vagina. Normally, these bacteria maintain a healthy environment that protect the vagina against infectious microorganisms like HIV-1 and HPV. Beneficial bacteria produce lactic acids that maintain the low vaginal pH and other important proteins (bacteriocins) to protect the vagina against harmful bacteria and viruses. In such a way, they block the binding and growth of pathogenic bacteria. Furthermore, the low vaginal pH is very important for immobilizing HIV-1 and inactivating HPV viruses. Some bacteria living in the beneficial vaginal microbiome may even directly bind to HIV-1 or HPV particles, thereby immobilizing them. The beneficial vaginal bacteria also stimulate the production of anti-inflammatory cytokines by the cells of the vagina, resulting in a more protective environment against viral infections. However, these beneficial effects can be lost when the healthy bacteria are replaced by harmful species during an imbalanced state of the vaginal microbiome called bacterial vaginosis (BV). BV is commonly characterized by smelly and more production of discharge, a more acidic vaginal pH and vaginal dryness. Women with BV are pointed out to be more susceptible for viral infections such as HIV-1 due to changes in the vaginal environment. These changes are caused by the harmful bacteria that have replaced the healthy vaginal bacteria. Because the harmful bacteria do not produce lactic acid, the vaginal pH will increase during BV and HIV-1 and HPV viruses can move more easily through the vagina, making the risk for viral infections higher. Besides that, harmful bacteria are known to produce multiple harmful compounds that damage the vagina. For example, some enzymes produced by specific harmful bacteria break down the mucus layer that protects the vagina against infections like HIV-1 and HPV. Some bacteria also produce enzymes that directly impair the connections between epithelial cells in the vagina, making it more susceptible to viral infections. In addition, BV is associated with the

inducement of inflammation in the vagina, also resulting in tissue damage and higher susceptibility for viral infections. In short, beneficial vaginal bacteria are important for maintaining a healthy vaginal environment which is protective against HIV-1 and HPV infections. The colonization of harmful bacteria during BV is known to increase the susceptibility for these viruses by affecting vaginal pH, inducing inflammation, degrading vaginal mucus and impairing the epithelial barrier..

Abbreviations

BV	Bacterial vaginosis
CVM	Cervicovaginal mucus
DCs	Dendritic cells
EMMPRIN	Extracellular metalloproteinase inducer
FRT	Female reproductive tract
H_2O_2	Hydrogen peroxide
HIV-1	Human Immunodeficiency Virus type 1
hMR	Human mannose receptor
HPV	Human Papillomavirus
IL	Interleukin
IL-1RA	Interleukin 1 receptor antagonist
IP-10	Interferon-γ induced protein 10
LCs	Langerhans cells
Llp1	Lectin-like protein 1
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
MMP-9	Matrix metalloproteinase 9
MUCs	Mucins
NETs	Neutrophil extracellular traps
NK	Natural killer
PBMCs	Peripheral blood mononuclear cells
PrEPs	Pre-exposure prophylaxis
SCFA	Short chain fatty acids
Th1	T helper cell type 1
Th2	T helper cell type 2
TLR	Toll-like receptor
TER	Transepithelial electrical resistance

Introduction

The female reproductive tract (FRT) consists of an upper and lower part differing in the type of epithelial cells and the composition of microorganisms living in this environment. The cervicovaginal epithelium of the lower FRT consists of stratified squamous nonkeratinized epithelium covered in cervicovaginal mucus (CVM) and cervicovaginal fluid (1,2). This gel-like mucosal layer serves as a protective barrier against pathogenic bacteria and viruses, lubricates the vaginal epithelium and contains the vaginal microbiome (2,3). The vaginal microbiome is a complex and dynamic system and is important for maintaining a healthy vaginal environment in the lower FRT. Besides, the types of bacteria living in the CVM change during a woman's life based on fluctuating hormonal levels (1,2). For example, the bacterial composition changes drastically during puberty when estrogen levels are changing and its overall diversity is commonly reduced in pregnant women due to lower estrogen concentrations (1).

A beneficial vagina in reproductive women is dominated by *Lactobacillus* species of which *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii* are mostly predominant (1,4,5). These *Lactobacillus* bacteria adhere to the vaginal epithelium, thereby reducing the adhesion of pathogenic bacteria and viruses (2). Most *Lactobacillus spp*. produce multiple compounds supporting a healthy vaginal ecosystem that protects the vaginal epithelium from infectious microorganisms. An impaired state of the vaginal environment is known as bacterial vaginosis (BV) which is associated with a loss of *Lactobacillus spp.* and the rise of anaerobic bacteria like *Gardnerella, Atopobium* and *Prevotella* species (1,4–6). During BV, women are more susceptible to sexually transmitted infections such as Human Immunodeficiency Virus type 1 (HIV-1) and Human Papillomavirus (HPV) infections (1,2). This review describes the molecular mechanisms underlying the protective role of *Lactobacillus* species and increased susceptibility to HIV-1 and HPV infections during BV.

Chapter 1: Role of the beneficial vaginal microbiota on HIV and HPV infections

A beneficial vaginal microbiome is known to be protective against HIV-1 infections through competition for adhesion molecules and by the production of multiple protective compounds (1,4,5). While it is well established that the vaginal microbiome composition impacts cervical cancer development upon HPV infection (5,7), the effect of the vaginal microbiome on HPV susceptibility is less studied. However, multiple studies describe a strong association between the colonization of *Lactobacillus* bacteria and HPV clearance (8,9) or HPV negativity (10,11), indicating that *Lactobacillus spp.* protects the vaginal epithelium against HPV infection. Both HIV-1 and HPV particles must cross the mucus layer containing protective compounds before reaching the epithelium for further diffusion to eventually reach their target cells. This chapter describes the mechanisms by which a beneficial microbiota protects the vagina against HIV-1 and HPV infections (**Figure 1**).

1.1 Mucus layer and epithelial barrier integrity

The first protective barrier against infectious microorganisms in the vagina consist of the mucus layer covering the vaginal epithelium. The mucus layer is a gel-like layer mostly consisting of highly *O*-glycosylated mucous glycoproteins called mucins (MUCs) expressed by epithelium cells in the lower FRT (3,12). The layer consists of both secreted gel-forming mucins that lubricates the vaginal epithelium and transmembrane mucins at the apical surface of vaginal epithelial cells which are important for cell signaling, cell-cell and cell-extracellular matrix interactions amongst others (2,3,13). Cervical cells secrete MUC5AC, MUC5B and MUC6 mucins into the cervix, which will flow into the vagina where it will combine with products of vaginal epithelial cells and bacteria to form the CVM in which the vaginal microbiome lives (13). The CVM can serve as a physical barrier against infectious agents by capturing and reducing diffusion of infectious microorganisms (12). Multiple studies show that the CVM reduces HIV-1 mobility (14,15), thereby greatly decreasing the number of viral particles reaching the vaginal epithelium. It has been shown that an acidic CVM (pH~4) traps a 1.000-fold more HIV-1 virions compared to neutralized CVM (pH~6) (14). Trapping of HIV-1 virions is a result of non-specific interactions between the lipid membrane of the HIV-1 virion and mucins of in the acidic CVM (15). Lactic acid in acidic CVM have been demonstrated to abolish the negative surface charge of HIV-1 virions (14), which might be essential for the mucoadhesion interactions that drive HIV-1 virion trapping. Since HPV is a non-enveloped virus (16), HPV virions may not be trapped by the CVM in the same extent as HIV-1 virions are.

Viral particles that manage to pass through the protective mucus layer get in contact with the vaginal epithelium. Epithelial cells from the basal layer are tightly connected to each other through tight junctions, thereby forming a physical barrier for diffusion of small compound to underlying tissues, including viral particles (1,17,18). When the epithelial integrity is impaired, viral susceptibility will be affected since more viral particles will be able to reach their target cells, CD4+ T lymphocytes for HIV-1 (1) and basal keratinocytes for HPV (19). The epithelial integrity can be disrupted as a result of direct action of viral proteins. For example, it has been proven that binding of HIV-1 gp120 to human mannose receptor (hMR) on the vaginal epithelium increases matrix metalloproteinase 9 (MMP-9) production by epithelial cells, which is a proteolytic enzyme that potentially contribute to the degradation of junctional and extracellular matrix (20). Therefore, inhibition of HIV-1 gp120 binding to hMR could potentially reduce HIV-1 susceptibility. The CMPG5300 protein expressed by vaginal *L. plantarum* showed significant binding to the gp120 and gp14 HIV-1 proteins in an ELISA-based assay (21), suggesting that this protein may protects against the degradation of the epithelial barrier by inhibiting HIV-1 adhesion to hMR in the vagina. Some *Lactobacillus spp*. have also been shown to improve the epithelial integrity in multiple studies (22,23). For example, *L. rhamnosus* GR-1 and *L. reuteri* RC-14 improve the vaginal epithelial integrity by increasing transepithelial electrical resistance (TER) indicates and decreasing FITC-dextran leakage in a monolayer of primary human endometrial cell system after administration (23). Furthermore, *in vitro* experiments with vaginal epithelial cells showed stimulation of re-epithelialization and vascular endothelial growth factor A production after co-culturing with *L. crispatus* (22), indicating that this *Lactobacillus* strain can be important for restoring damaged epithelium. These findings underline the importance of several *Lactobacillus spp.* in strengthening and maintaining epithelial integrity, thereby protecting the vaginal epithelium against viral infections.

1.2 Competition and viral capturing through bacterial lectins

Some of the symbiotic bacteria living in the vaginal mucus compete with pathogenic microorganisms for binding to receptor-binding sites on the vaginal epithelium (1). It is known that HIV-1 virions interact with the vaginal epithelium through several receptors including HIV-1 env protein gp120 (20). This interaction have been shown to increase the amount of cell-associated virus in vaginal epithelial cells *in vitro* (24), indicating that gp120 binding to these cells induce viral sequestration. In fact, the vaginal epithelium may serve as a transient reservoir for HIV-1 particles before infecting CD4+ T lymphocytes (24). *Lactobacillus spp.* might block HIV-1 binding to the vaginal epithelium by competing for glycans that serve as viral receptor-binding sites. For example, *L. gasseri* and *L. jensenii* showed binding to vaginal epithelial cells based on glycoprotein or carbohydrate adherence (25). *L. rhamnosus* GR-1 also shows tissue-specific adhesion via lectin-like protein 1 (Llp1) (26) and another vaginal *L. plantarum* strain via the lectin-like protein CMPG5300 (21). Since HIV-1 particles are also known to bind glycans like hMR on epithelial cells of the vagina (20), carbohydrate-binding proteins like Llp1 and CMPG5300 may block the glycan-based adhesion of HIV-1 particles. Additionally, Llp1 binding to sugars on vaginal epithelial cells showed blocking of the binding of urogenital pathogens like *Escherichia coli* and *Staphylococcus aureus* and increases biofilm formation of several vaginal *Lactobacillus* strains by 1.25-fold or more (26), giving this carbohydrate-binding protein an overall protective role against pathogenic microorganisms and the development of BV. *Lactobacillus spp*. are thus not only responsible for preventing vaginal infections by HIV-1 and HPV viruses but also prevent the colonization and growth of BV-associated bacteria.

HPV virions also bind basal keratinocytes via glycoproteins (27). If the epithelial integrity is damaged and basal keratinocytes are exposed, the carbohydrate-binding proteins expressed by

Lactobacillus species may thus also prevent HPV binding but this potential is not yet examined. Other carbohydrate-binding proteins expressed by *Lactobacillus* species are not yet determined so further identification of such proteins is needed to determine their importance in blocking adherence of HPV virions. Overall, bacterial lectin-like proteins responsible for glycan-binding on the vaginal epithelium have only demonstrated to compete for HIV-1 binding motifs but more research is needed, especially in the context of HPV infection.

Besides the competition for receptor-binding sites, lectin-like proteins expressed by the vaginal bacteria may also capture the virus directly. Since the envelope of HIV-1 particles consists of highly glycosylated proteins with mostly mannose type glycans (1), bacterial lectins and lectin-like proteins may bind, leading to viral capturing. In such a way, lectin-like protein CMPG5300 expressed by *L. plantarum* demonstrated specific binding to HIV-1 gp120 and gp41 proteins (21), suggesting that this lectin-like protein have a potential to capture HIV-1 particles. Although the earlier described Lpl1 expressed by *L. rhamnosus* GR-1 is also responsible for glycan-based adherence (26), direct binding of this protein to HIV-1 particles is not yet determined. Further experiments are needed to confirm whether bacterial lectins from symbiotic vaginal bacteria can capture viral particles.

1.3 Lactic acid production and vaginal pH

Lactobacilli spp. produce lactic acid to maintain a low pH of 4.0, characterizing the healthy vaginal environment (1,4). Both lactic acid and a low pH have been documented to decrease HIV-1 and HPV susceptibility through multiple mechanisms. Lactic acid produced by *L. iners, L. crispatus* and *L. jensenii* showed upregulation of tight junction genes claudin-1 and claudin-4 *in vitro* (17), thus enhancing the epithelial barrier integrity and reducing the paracellular passage of HIV-1 and HPV virions. Accordingly, the abundance of *L. crispatus* is strongly associated with HPV negativity among 41 women (8), indicating that *L. crispatus* plays a major role in the protection against HPV infections which may be due to the low vaginal pH and increased epithelial integrity.

Lactic acid has two isoform structures (D- and L-) that contribute to the acidification of the vaginal environment (1). The crucial E5 protein from HPV-16 is vulnerable to a low pH (28), indicating a protective role of lactic acid against HPV infections. Besides the effect of lactic acid on HPV infection, lactic acid has been shown to inhibit HIV-1 replication (29), inactivate HIV-1 virions (30,31) and enhance HIV-1 trapping (15). These antiviral and virucidal effects were dependent on the acidity of the environment (29–31) but could not be mimicked by other acids (29,30), suggesting that it is specific for the uncharged protonated form of lactic acid produced by some *Lactobacillus spp*. Supporting this idea, a recent study showed higher HIV-1 mobility in CVM samples from women with a BV-dominated microbiota which was also correlated to lower levels of lactic acid and a higher pH (32). In summary, *Lactobacillus*-produced lactic acid is crucial for the acidification of the vaginal environment which results in the inactivation and trapping of HIV-1 and HPV virions.

L-lactic acid concentrations and D/L-lactic acid ratios have been associated with an increase expression of the vaginal extracellular metalloproteinase inducer (EMMPRIN) which induces the production of the collagenase MMP-8 (33). Several studies showed that the increased MMP-8 levels resulted in an impaired epithelial integrity in the blood-testis barrier (34) and blood-brain barrier (35,36). Interestingly, these studies show that MMP-8 decrease the expression of tight junction proteins (occludin and zonula occludens-1) in endothelial cells (36) and mediated occludin degradation in the blood-testis barrier (34), indicating that MMP-8 may play a major role in impairing the vaginal epithelial barrier. An increase in D-lactic acid concentration relative to L-lactic acid positively correlates with vaginal EMMPRIN concentrations (37). As a consequence, decreased levels of L/D-lactic acid ratios

may be beneficial for both HPV and HIV-1 infection since basal keratinocytes and CD4+ T lymphocytes can more easily enter an impaired vaginal tissue. Vaginal samples dominated by G. vaginalis and L. iners have been shown to have extremely low levels of D-lactic acids, resulting in a significantly decreased L/D-lactic acid ratio compared to *L. crispatus*-dominated samples (37). This suggests that *G. vaginalis* and *L. iners* may be responsible for impaired vaginal integrity through higher MMP-8 activity and extracellular matrix degradation.

1.4 Other secretion products from Lactobacillus spp.

Lactobacillus bacteria are also known for secreting other compounds like hydrogen peroxide (H_2O_2) and bacteriocins (1,4). H_2O_2-producing lactobacilli have been shown to enhance antibacterial activity of metabolites like muramidase and lactoferrin secreted by vaginal epithelial cells against the pathogenic bacteria *E. coli* and *S. aureus* (38). In such a way, S.J. Klebanoff and R.W. Coombsin found a decreased viral replication of HIV after incubation with *L. acidophilus* producing H_2O_2 (39). However, a direct virucidal effect of H_2O_2 is under discussion since recent studies found contradicting results. It has been demonstrated that the growth of multiple pathogenic bacteria, including BV-associated bacteria, were not inhibited by H_2O_2, but lactic acid (40). In general, the direct antiviral effect of H_2O_2 is thus not yet clear, and the antiviral activity of H_2O_2-producing lactobacilli may be obtained through mechanisms other than H_2O_2 production.

Apart from H_2O_2, *Lactobacillus* species in the vagina produce other products that may influence HIV-1 and HPV infections. Bacteriocins do not directly affect viral infectivity but since these secretions inhibit the growth of pathogenic species they also contribute to the prevention of BV development. In addition, supernatants of *L. crispatus*, *L. jensenii* and *L. gasseri* have an inhibitory effect on HPV E6 and E7 oncogenes (41), indicating that these strains secrete products, although yet undetermined, that affect HPV protein expression.

1.5 Immunomodulation by Lactobacillus-species

The vaginal microbiota is also in close contact with immune cells that protect the cervicovaginal epithelium from infections. In such a way, neutrophils are mostly present in the CVM, thereby serving as the first innate immune cells against infectious microorganisms (12). On the other hand, natural killer (NK) cells, T lymphocytes and antigen-presenting cells including dendritic cells (DC), Langerhans cells (LCs) macrophages immune cells are found in both suprabasal layers of the epithelium and lamina propria (1,2,12). Cell-mediated immunity is driven by the presentation of pathogen-derived antigens by DCs or macrophages to naïve T lymphocytes. This antigen-presentation will lead to the activation and differentiation of T lymphocytes into effector and memory T cells of which the CD4+ and CD8+ effector T cells will attack and clear the pathogen. Alongside this cell-mediated immunity, humoral immunity is also present in the mucus layer of the vagina where high concentrations of IgG-antibodies and low concentrations of IgA-antibodies are present (1). This cellular and humoral immunity does not only protect the vaginal epithelium against pathogenic bacteria but is also important for the clearance of viruses like HIV-1 and HPV.

The effect of *Lactobacillus spp.* on inflammatory responses by vaginal epithelial cells have been examined in multiple studies. *L. jensenii, L. crispatus*, *L. mucosae*, *L. vaginalis* and *L. gasseri* showed an overall little cytokine production by ectocervical epithelial cells and an increased production of IL-1 receptor antagonist (IL-1RA) (42), suggesting that beneficial vaginal microbiota maintain an anti-

inflammatory environment. In addition, another study demonstrate that pre-incubation with several *Lactobacillus spp.* (*L. crispatus*, *L. vaginalis*, *L. mucosase*, *L. gasseri*, *L. jensenii LJ2* and *L. jensenii LJ5*) significantly reduced the expression of multiple proinflammatory cytokines by ectocervical epithelial (CaSki) cells upon *G. vaginalis* infection (42). Four of the six tested *Lactobacillus* strains significantly inhibited the growth *of G. vaginalis* in acidic culture media, which was not influenced by H_2O_2 reduction or bacteriocins degradation (42), indicating that the growth inhibition of *G. vaginalis* by *Lactobacillus spp*. is mostly acid-dependent. Also, *L. crispatus* SJ-3C-US showed DC activation and an increased level of interleukin (IL)-10, resulting in T cell polarization towards a regulatory phenotype (43). Such an anti-inflammatory effect is also associated with *Lactobacillus*-secreted L-lactic acid. Multiple studies found an reduced production of pro-inflammatory cytokines (IL-6 and IL-8) and increased IL-1RA production by cervicovaginal epithelial cells (44), ectocervical cells (45) and vaginal epithelial cells (45) treated with L-lactic acid. Interestingly, the protective effects of L-lactic acids were not altered by metabolite mixtures from eubiotic vaginal microbiota, indicating that *Lactobacillus*-bacteria sustain this anti-inflammatory effect. A *Lactobacillus*-dominated vaginal microbiota is thus associated with an anti-inflammatory environment that maintains the epithelial barrier. This is protective against HIV-1 and HPV infections since an anti-inflammatory environment decreases viral passage through tissue and reduces contact with target cells (T lymphocytes and keratinocytes, respectively).

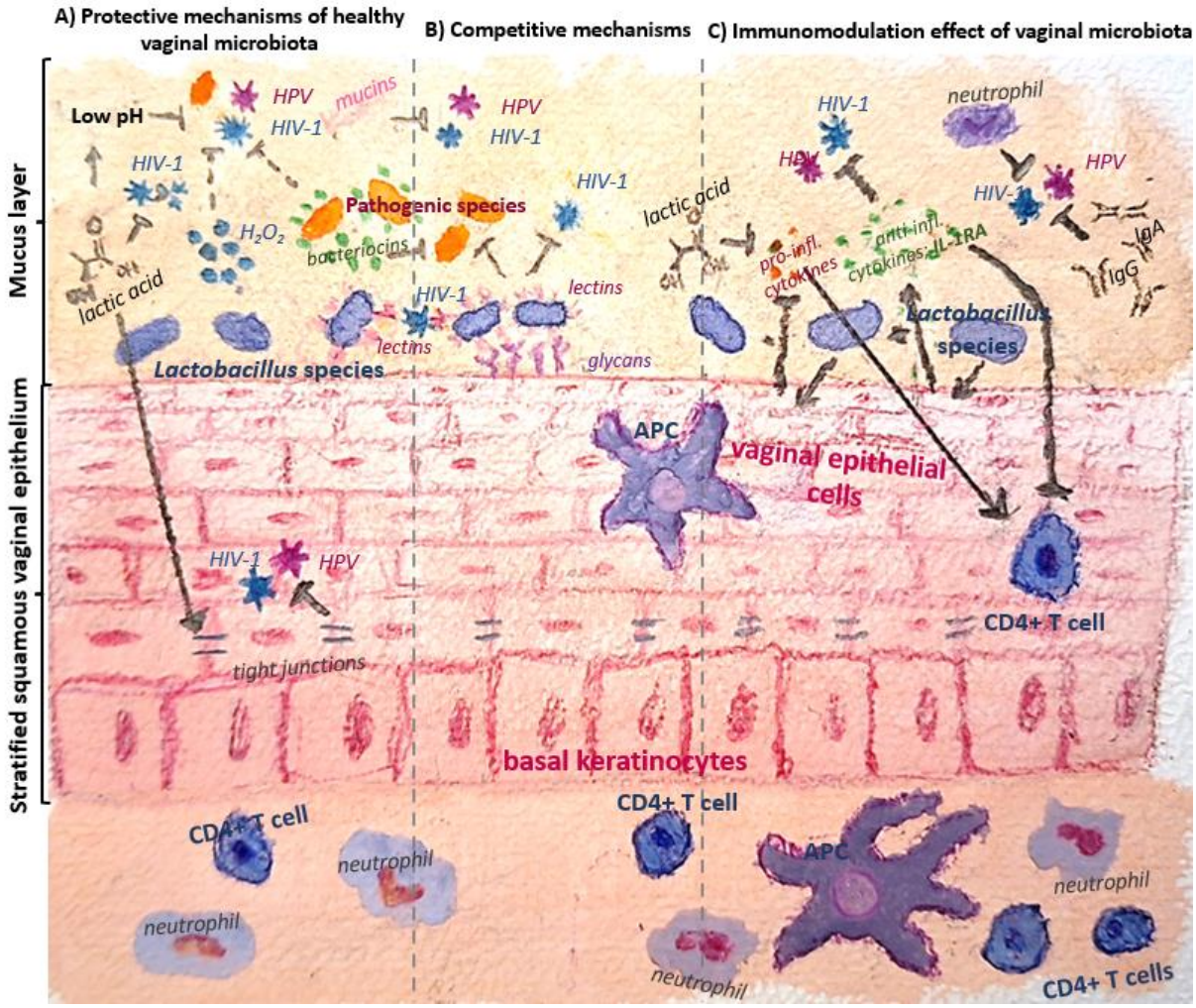

Figure 1: Overview of molecular mechanisms in which vaginal *Lactobacillus* species protect against HIV-1 and HPV infections. (A) *Lactobacillus* species secrete lactic acid, H_2O_2 and bacteriocins that have a protective effect against viral and pathogenic bacteria infections. Lactic acid directly inhibits HIV-1 replication and maintains the low pH in the CVM, leading to

inactivation of HPV and HIV-1 and the capturing of HIV-1 in the CVM through mucoadhesion. Although H_2O_2 and bacteriocins do not directly protect the vaginal epithelium from viral infections, they are associated with less viral infectivity and protection of the vaginal epithelium against BV-bacteria. (B) Different *Lactobacillus* species express several lectin-like molecules that capture HIV-1 virions and compete for HIV-1 adhesion to glycans on the vaginal epithelium. (C) The beneficial vaginal microbiota dominated by *Lactobacillus* species have an anti-inflammatory via the secretion of lactic acid effect (increased IL-1RA secretion by vaginal epithelial cells) and other unknown mechanisms.. This anti-inflammatory environment results in less CD4+ T lymphocyte recruitment which is favorable for a decreased HIV-1 susceptibility and maintains the epithelial integrity, protecting the vaginal epithelium to both HPV and HIV-1 infections.

Chapter 2: BV-associated bacteria negatively influence HIV-1 and HPV susceptibility in the vaginal epithelium

The vaginal microbiome can undergo a switch in its composition in which anaerobic bacteria such as *Gardnerella* and *Prevotella* species become more abundant and overgrow the beneficial *Lactobacillus spp.*, leading to BV (1,6). Recent epidemiology studies suggested that switching from a healthy vaginal microbiome to BV is caused by sexual intercourse with men carrying BV-associated bacteria (46,47) and BV is further associated with risk factors such as new sexual partners, douching and smoking (47). Accordingly, several studies have reported that *G. vaginalis* (41,43) and *P. bivia* (49) are present in the semen of men. BV-associated bacteria affect the vaginal ecosystem by increasing the vaginal pH and impairing the mucosal and epithelial barrier (1,6), thereby influencing HIV-1 and HPV susceptibility. This chapter describes how BV-associated bacteria in the vaginal mucosa increase HIV-1 and HPV susceptibility through multiple molecular mechanisms (**Figure 2**).

2.1 Bacterial adhesin to the vaginal tissue and mucus degradation

Introduction of BV-associated bacteria in the vaginal mucosa is suggested to be acquired via sexual transmission and influenced by multiple risk factors (46,47). Although multiple studies propose that the primary BV-associated pathogen is *G. vaginalis* since this bacterium can adhere to the vaginal epithelium and has a good ability to form biofilms (46,50), this bacterium is also found in women without BV (4,46,50). This characteristic might be obtained by the tolerance for *Lactobacillus*-produced H_2O_2 and lactic acids of *G. vaginalis* biofilms (46). *G. vaginalis* is thought to have synergistic characteristics together with other BV-associated bacteria. For example, amino acids produced by proteolysis of *G. vaginalis* serve as building blocks for the growth of another virulent bacterium, *P. bivia,* which itself produces ammonia stimulating the growth of *G. vaginalis* (51). Other *Prevotella spp.* such as *P. timonensis* is also commonly found in women with BV (52,53). This pathogenic bacterium can adhere to vaginal and endocervical cells, similarly to *G. vaginalis* (unpublished data[1]), indicating that this strain might also play an initial role in biofilm formation characterizing BV.

Certain BV-bacteria are known to produce mucin-degrading enzymes, such as sialidases and fucosidases (51,53–55) that degrade CVM by the removal of sialic acids and fucoses, respectively (51,56). Since the CVM is known to be protective against HIV-1 and HPV infections through virion inactivation and immobilization, the degradation of mucins will increase viral susceptibility. S.S. Olmsted and L.A. Meyn, et al. (2001) found higher concentrations of bacteria producing mucin-degrading enzymes in women with BV compared to healthy women, which was also correlated with BV-associated symptoms (55). Accordingly, researchers examined the mucin-degrading ability of individual bacteria in the vaginal epithelium. *G. vaginalis* is known to express three different sialidase

[1]Perez C.S. et al (2023). Prevotella timonensis degrades vaginal epithelial glycal landscape through high fucosidase and sialidase activities. [Unpublished manuscript]

genes, *NanH1*, *NanH2* and *NanH3*. Although *NanH2* and *NanH3* sialidase genes have shown mucin-degrading activity *in vitro*, *NanH1* have been thought to be important for bacterial adherence to the vaginal epithelium and biofilm formation (56). Interestingly, production of NanH3 by *G. vaginalis* is found to be higher during persistent HPV infections, even significant for HPV16 (57), indicating that this gene plays a role in HPV persistence and progression.

G. vaginalis was the first BV-associated bacteria reported to have sialidase activity *in vitro* (58) and *in vivo* (59). In mice, infection with *G. vaginalis* showed higher vaginal sialidase activity compared to non-infected mice (59), indicating that *G. vaginalis* sialidase activity can be responsible for mucin degradation in women with BV. A follow-up study found that not only mice infected with *G. vaginalis,* but also *P. bivia*, shows vaginal sialidase activity (54). Remarkably, *G. vaginalis* showed exfoliation of vaginal epithelial cells in both mice studies (54,59), unlike *P. bivia* (54), suggesting that *G. vaginalis* is particularly responsible for this hallmark of BV. However, several other *Prevotella spp*. also show expression of different sialidase proteins that are active against mucous glycoproteins. Accordingly, five *Prevotella* sialidases proteins purified from *P. bivia*, *P. amni*, *P. denticola* and *P. timonensis* showed *in vitro* activity against the fluorescent 4-methylumbelliferyl N-acetyl-α-D-neuraminic acid and different sialic acid-containing substrates (unpublished data[2]). In another study, *P. timonensis* displayed higher sialidase activity in a 3D human endometrial epithelial cell model compared to other *Prevotella spp.*, including *P. bivia* (53). Further investigation showed both fucosidase and sialidase activity by *P. timonensis* leading to *O*-glycan degradation at the vaginal epithelial surface in an *in vitro* monolayer of vaginal epithelial cells (unpublished data[3]). These findings suggests that not only *G. vaginalis* but also *P. timonensis* may be a major contributor to the degradation of mucins in women with BV.

Fucosidase enzymes expressed by some BV-associated bacteria are also known to contribute to the degradation of mucins in the CVM (51,56). *L. crispatus, P. timonensis, P. bivia* and *Bacteroides fragilis* showed high fucosidase activity against the fluorescent 4-Methylumbelliferyl α-L-fucopyranoside substrate, unlike *G. vaginalis*. However, only *P. timonensis* and *G. vaginalis* significantly reduced UEA-1 fucose staining after incubation with vaginal epithelial cells in this study (unpublished data[3]). This remarkable finding shows that mucin-degrading enzyme activity via fluorescence substrate does thus not always correlate to the degradation of sialic acids or fucoses of vaginal epithelial cells. Moreover, another study showed that different sialidase purified from different *Prevotella* strains exhibited variable activity towards different mucous glycoproteins (unpublished data[2]). These findings underline the importance to test the mucin-degrading enzymes of different BV-bacteria in the context of vaginal epithelial cells to determine their ability.

2.2 BV-associated bacteria impair the vaginal epithelial barrier

Some BV-associated bacteria produce compounds that directly damage vaginal epithelial cells. Women with BV, dominated by *G. vaginalis,* show significant higher levels of the cytotoxic vaginolysin in comparison to women with a *Lactobacillus*-dominated microbiome (60). Vaginolysin is a human-specific hemolysin that damages vaginal epithelial cells by forming pores in the membranes (50,61,62). The formation of these membrane pores by *G. vaginalis* expressed vaginolysin have been show to induce membrane blebbing in human vaginal and cervical epithelial cells (63,64), suggesting that *G.*

[2]Pelayo P. et al (2023). Prevotella are major contributors of sialidases in the human vaginal microbiome. [Unpublished manuscript]
[3]Perez C.S. et al (2023). Prevotella timonensis degrades vaginal epithelial glycal landscape through high fucosidase and sialidase activities. [Unpublished manuscript]

vaginalis impair the vaginal epithelial integrity through vaginolysin expression. Besides, FITC-dextran movement, an indicate for cell permeability, was significantly increased for ectocervical, endocervical and vaginal cells after co-culturing with live *G. vaginalis* which was not observed for *L. crispatus* (64). This effect was maintained when only *G. vaginalis* supernatant was added to the vaginal cells (64), indicating that secretions from *G. vaginalis* such as vaginolysin may contribute to the impaired cell permeability. An increased permeability may enable viral particles to reach deeper within the vaginal tissue, thereby affecting viral susceptibility since HPV might easier enter basal keratinocytes, while HIV-1 may encounter its target cells.

L. iners produces a similar cytolysin, inerolysin, that is also able to induce pores in epithelial cells (62,65). This cholesterol-dependent cytolysin is extremely pH-dependent with its optimal at an acidic pH and is not species-specific (62,65), indicating that it may also affect the vaginal epithelium negatively and could contribute to the pathogenesis of BV, even in a healthy *Lactobacillus*-dominated microbiome. Since the direct cytotoxic effect of *L. iners* or *L. iners* supernatants have been performed yet, the potential cytotoxicity of *L. iners* and its cytolysin should be further investigated to determine the effect of this bacterium on HIV-1 and HPV susceptibility. Nonetheless, multiple studies showed that inerolysin expressed by *L. iners* results in the upregulation of several pro-inflammatory genes and signaling pathways (62,65,66), thereby contributing to a pro-inflammatory vaginal environment that increases viral susceptibility as described later on. The role of lactic acid produced by *L. iners* regarding the protection against viral infections is also controversial. Although lactic acid produced by *L. iners* have been shown to upregulate several tight junction genes (17), this effect may be contradicted by the decreased L/D-lactic acid ratio that is associated with higher EMMPRIN levels (37) which might induce degradation of tight junction proteins. Overall, despite that *L. iners* is part of the beneficial vaginal microbiome, this bacterium thus also has BV-characteristics, unlike other lactobacilli, which may contribute to the pathogenesis of BV and increased susceptibility for HIV-1 and HPV infections.

2.3 Direct effect of BV-associated bacteria on viral uptake, transmission and infection

The direct effect of vaginal bacteria on viral uptake, transmission or infection has not been extensively studied, even though there is some evidence for HIV-1. Multiple studies have shown inhibition of HIV-1 replication by metabolites from *L. crispatus*, *L. gasseri* and *L. vaginalis* (29) and through IFN type 1 signaling by some gram-negative commensal bacteria (*E. coli*, *Neisseria mucosa* and *Veillonella parvula*) (67). In contrast, multiple *Prevotella spp.* including *P. bivia* and *P. melaninogenica* showed an enhanced HIV-1 expression after exposure to vaginal epithelial cells *in vitro* (67), indicating that these anaerobic bacteria stimulate HIV-1 proliferation. In another study, the expression of HIV-1 was strongly upregulated upon stimulation of monocyte cells with bacterial lysates of *P. bivia*, *Peptococcus asocharolyticus, G. vaginalis* and *M. hominis* compared to untreated cells (68). Moreover, *P. asaccharolyticus* and *P. anaerobius* showed an upregulation of HIV-1 expression in T lymphocytes, unlike *P. bivia*, *L. acidophilis* and *Bacteroides ureolyticus* (68). These findings suggest that some *Prevotella spp*. living in the vaginal mucus may induce HIV-1 expression and enhance HIV-1 replication in CD4+ T lymphocytes. HIV-1 virions have also showed to be internalized into intercellular vesicles of vaginal and epidermal LCs upon *P. timonensis* infection, making HIV-1 resistant against langerin-mediated degradation (69). Subsequently, vaginal LCs might serve as a temporary pool of HIV-1 that may later be transmitted to HIV-1 target cells. Overall, BV-associated bacteria induce HIV-1 expression in monocytes and lymphocytes and help HIV-1 to evade the immune system to reach its target cells, but such mechanisms are not yet discovered for HPV.

2.4 Disruption in immune profile by BV-associated bacteria

The immune profile is affected by the colonization of BV-associated bacteria in the CVM. Multiple studies proved that high levels of short chain fatty acids (SCFAs) associated with BV induce a pro-inflammatory response in the vagina (40,45,70). For example, high concentration of acetic and butyric acid induced the secretion of pro-inflammatory cytokines IL-8, IL-6 and IL-1β by peripheral blood mononuclear cells (PBMCs) and neutrophils *in vitro,* which was demonstrated to be T-like receptor (TLR)-mediated response (70). Interestingly, the lipopolysaccharide (LPS)-mediated IL-8 production by TLR4 was not enhanced by high levels of SCFA (70). This may be due to an immune evasion mechanism of BV-associated bacteria or suggest that SCFA produced by BV-associated bacteria affect LPS-mediated immunity against gram-negative bacteria that are associated with BV. Concentrations of butyrate, propionate and acetate in women with BV induce phenotypic changes in human blood neutrophils affecting neutrophilic migration, maturation and aging (71), indicating an impairment in neutrophilic function, which might lead to lower viral clearance in the vaginal epithelium. Moreover, neutrophils treated with high SCFA concentrations upon HIV-1 administration showed a delayed formation of neutrophil extracellular traps (NETs) (71), suggesting that high vaginal SCFA levels secreted by BV-associated bacteria lead to a higher HIV-1 susceptibility. BV-associated bacteria can also have a direct effect on neutrophilic activity. R.K. Cheu and A. Mohammadi, et al. (2022) demonstrated a higher fractions of CD62LhighCD16low neutrophils, a marker for an active neutrophil population. Besides that, higher numbers of non-apoptotic neutrophils were found in vaginal swabs from women with BV compared to samples cultured with *L. crispatus* and *L. iners*, due to the direct promotion of neutrophilic survival by *G. vaginalis*, *P. bivia*, and *A. vaginae* (72). In conclusion, these findings indicate that SCFA production by BV-associated bacteria may 1) inactive and impair the ability of neutrophils of viral clearance or 2) over-activate neutrophils delaying NET formation which may also decrease HIV-1 and HPV clearance.

Vaginal dysbiosis is associated with an enhanced pro-inflammatory response (1). Elevated pro-inflammatory cytokines in BV, such as cytokines IL-1β and IL-22 (11), may lead to an increased viral susceptibility because of the subsequent vaginal tissue damage and enhanced recruitment of viral target cells such as CD4+ T lymphocytes in the case of HIV-1. In addition, upregulation of several pro-inflammatory-associated genes (*IRF1, NFKBIA, TNFα* and *IRAK2*) was showed after *A. vaginae* and *L. iners* infections in a 3D vaginal epithelial cell colonization model, and infection with *A. vaginae* significantly increased the secretion of IL-6, IL-8 and TNFα by these cells (66). Co-culturing of live G. vaginalis with ectocervical, endocervical and vaginal epithelial cells also showed an increased TLR2-mediated production of IL-6 and IL-8 (64). Furthermore, the cytolysins, vaginolysin and inerolysin, produced by *G. vaginalis* and *L. iners,* both induce p38 MAPK signaling in epithelial cells of the vagina (61,65), thereby contributing to the pro-inflammatory environment. DC activity and maturation has is also influenced by BV-associated bacteria. *P. timonensis* and *M. elsdenii* show higher expression of multiple pro-inflammatory cytokine (IL-1β, IL-6, IL-8, Il-12p40 and TNFα) and DCs maturation *in vitro* (52). Accordingly, *P. timonensis* showed a significant pro-inflammatory T helper type 1 (Th1) maturation and inhibition of T helper type 2 (Th2) skewing after co-culturing with *P. timonensis*-stimulated DCs (52), suggesting that some *Prevotella spp.* could indeed have a pro-inflammatory effect through DC maturation and Th1 skewing. The pro-inflammatory immune profile associated with BV could be due to elevated concentrations of metabolic products observed during BV (45). Supporting this idea, higher acetic and butyric SCFA concentrations induce the secretion of pro-inflammatory cytokines by PBMCs and resulted in an enhanced TLR2 and TLR7 response *in vitro* (71). This hypothesis is supported by the finding that the anti-inflammatory effects of L-lactic acid were significantly reduced after addition of metabolite mixtures from BV bacteria containing SCFAs and succinic acid (45). Another explanation could be that *G. vaginalis* induce changes in the cytoskeleton protein vimentin, resulting in bacterial

internalization into vaginal epithelial cells (73), may resulting in the inducement of proinflammatory cytokine secretions in the vaginal epithelium. Overall, multiple BV-associated bacteria play an important role in the induction of a proinflammatory vaginal immune profile that negatively influences susceptibility for HIV-1 and HPV infections.

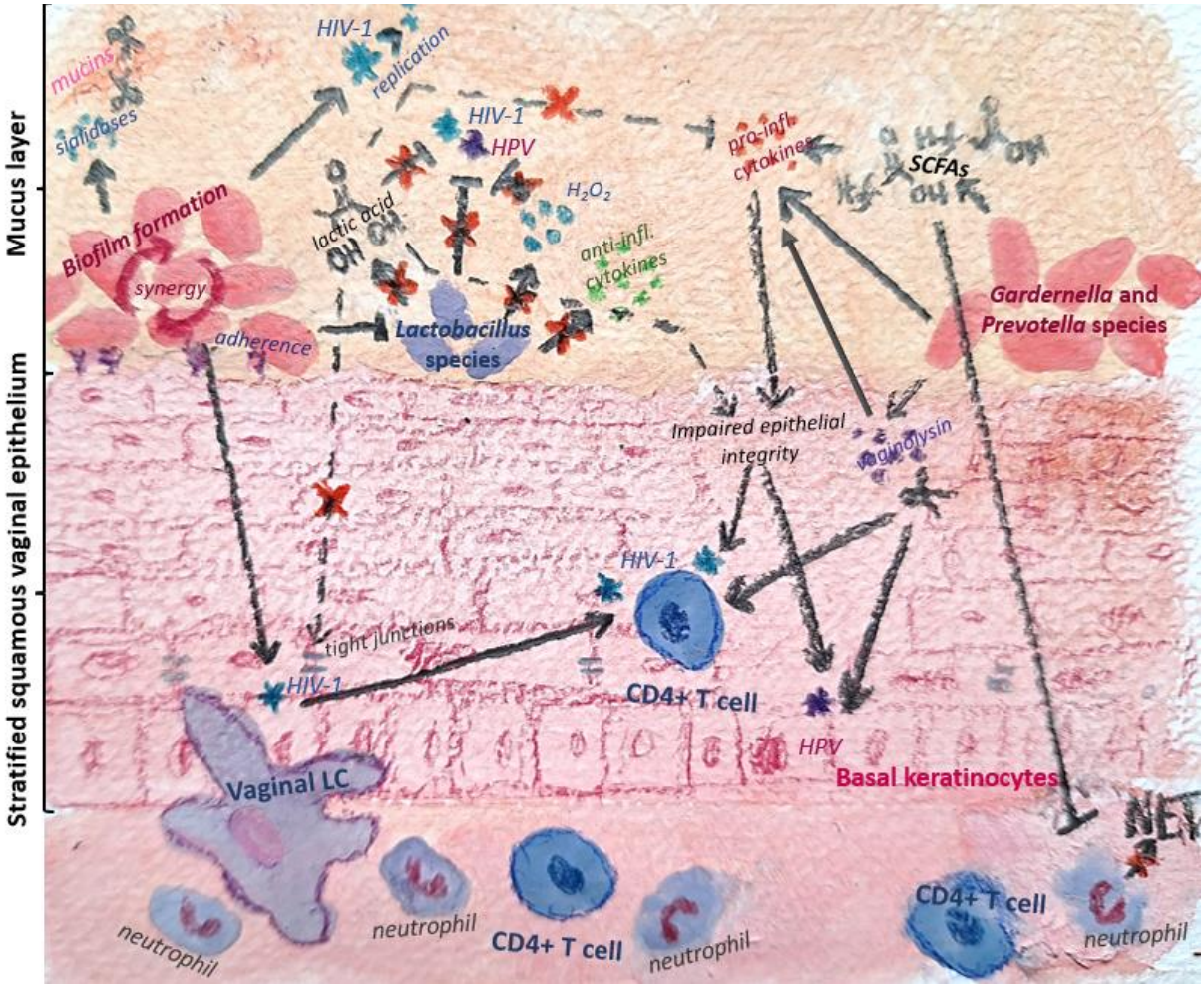

Figure 2: Overview of the molecular mechanisms in which BV-associated bacteria like *Gardnerella* and *Prevotella spp.* affect HIV-1 and HPV susceptibility in the vaginal epithelium. BV bacteria adhere to the vaginal epithelium and help each other grow in a synergistic way. They inhibit the growth of *Lactobacillus spp.*, resulting in an increased viral susceptibility because all protective characteristics of the *Lactobacillus*-dominated vaginal microbiota will be diminished (red crosses). Besides, BV-associated bacteria will produce SCFAs that stimulate a proinflammatory response and negatively influence neutrophilic function. The proinflammatory response will induce more tissue damage in the vaginal epithelium resulting in an increased HPV infectivity in basal keratinocytes and the recruitment of CD4+ T lymphocytes which may result in an increased HIV-1 infectivity. Lastly, the colonization of multiple BV-bacteria lead to an increased HIV-1 expression and infection in vaginal LCs, serving as a reservoir for later CD4+ T lymphocyte infections.

Discussion

This review discussed the molecular mechanisms by which vaginal microorganisms influence the susceptibility for HIV-1 and HPV infections. In general, more research regarding HIV-1 infections was found compared to HPV infections. Nonetheless, multiple studies have described a strong association between *Lactobacillus*-dominated vaginal microbiomes with higher HPV clearance (8,9), HPV negativity

(10,11) and risk for cervical cancer development upon HPV infection (5,7), indicating that *Lactobacillus spp.* also play an important role in HPV infection.

Some vaginal bacteria such as *G. vaginalis* and *L. iners* have been described to have a dual role in the protection against viral infections. For example, *L. iners* which is found in a beneficial vaginal microbiome (1,4,74) has also been shown to produce inerolysin, thereby impairing epithelial integrity (65) and negatively affecting viral susceptibility. Some *G. vaginalis* strains are also found in the CVM of healthy women (50) although *G. vaginalis* is commonly described in the context of BV. Moreover, non-BV associated *G. vaginalis* strains showed lower cytotoxicity and reduced adhesion to vaginal epithelial cells compared to BV-associated strains (50). No significant difference in gene expression of vaginolysin (50,75), sialidase (50,75) or phospholipase C (75) between non-BV and BV associated *G. vaginalis* isolates are found yet, suggesting that the strain variability of *G. vaginalis* may be caused by differential expression of other yet unknown virulence genes. These findings highlight the importance of bacterial strain distinction and the need for better understanding of which bacterial mechanisms are necessary for epithelial colonization, BV progression and an increased viral susceptibility.

Since *Lactobacillus spp.* have a huge impact on the protection of the vaginal epithelium, multiple studies have discussed the use of several lactobacilli as probiotics. The therapeutic use of lactobacilli against pathogenic bacteria was first documented in 1998 and years later more studies suggested that administration of specific *Lactobacillus* strains could recover the vaginal microbiota in women with BV (4). Adhesion to the epithelium may be a requirement for bacteria to block the adherence of pathogenic bacteria in the vagina. For example, *L. rhamnosus* GR-1, a well-studied probiotic strain (1), may block adherence of pathogenic bacteria by binding to glycans on the vaginal epithelium through the lectin Llp1 (26). BV is commonly accompanied by a pro-inflammatory response, which may lead to chronic inflammation, a major risk factor for cervical carcinogenesis (19,76). Several studies have shown that *Lactobacillus spp.* are protective against cervical carcinogenesis through the induction of apoptosis, regulating the cell cycle (41,76,77) and decreasing the expression of HPV E6/E7 oncogenes (41), thereby raising the possibility of using probiotics to prevent carcinogenesis (77). Since the therapeutic use of bacteria always implies several risks such as developing *Lactobacillus* bacteremia, we would like to highlight the importance of bacterial strain variability and the need for testing different probiotic candidate strains in human clinical trials.

Anti-retroviral drugs and vaccines against HIV-1 and HPV viruses could also help prevent or treat vaginal infections. Pre-exposure prophylaxis (PrEP) products such as vaginal gels containing 1% tenofovir and orally administrated tenofovir are widely used for preventing HIV-1 infection in women (78,79). PrEP adherence have been suggested to limit efficacy of both gel and oral PrEP products and vaginal dysbiosis have been shown to negatively impact the efficacy of only vaginally administrated PrEP products such as the tenofovir gel (78,79). For example, *G. vaginalis* showed over 50% depletion of tenofovir *in vitro*, unlike *L. iners* and *L. crispatus* (78,79), suggesting that BV-associated bacteria may negatively affect the efficacy of vaginally administrated PrEPs by metabolizing the product. The vaginal microbiome has no effect on orally administrated PrEP products because of the systemic delivery and high plasma concentrations of oral PrEPs (78,79). Viral infections can also be prevented by using vaccines. Multiple vaccine therapies are in use for HPV, mostly targeting E6/E7 oncogenes (5). Although these HPV vaccines can be protective against HPV infection and thereby also reduce the risk of cervical cancer, already infected individuals cannot be treated with these vaccines (5). Despite many vaccines targeting HIV-1 are currently being tested in clinical trials, they have mostly failed to obtain enough efficacy due to the complex immune evasion mechanism and genetic diversity of HIV-1 (80). Broadly neutralizing antibodies targeting heterologous HIV-1 strains are needed to obtain more efficacy, though the production becomes more complex and may be a limiting factor. In conclusion, there is need for

future research for the improvement of anti-retroviral drugs and viral vaccines since some BV-associated bacteria may negatively impact the efficacy of some existing anti-retroviral drugs against HIV-1 and vaccines targeting HPV and HIV-1 are not optimal yet.

Lactobacillus spp. are not only responsible for preventing the colonization and growth of BV-associated bacteria, but also for reducing the risk for HIV-1 and HPV infections. On the other hand, BV-associated bacteria have been shown to negatively influence viral susceptibility, underlining the importance of having a beneficial vaginal microbiome. However, every bacterial strain behaves and affects viral susceptibility differently, underlining the importance for strain-specific screening to diagnosticating BV disease in women, determining the effect of specific strains on viral infections and improving the use of probiotics for the prevention of viral infections. Although several studies found associations between BV and HPV infections, more research is needed to fully understand the mechanisms behind the increased risk for HPV infection in women with BV.

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