Diversity of Vaginal Microbiota Affects Epithelial Barrier Permeability Among African Pregnant Women

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Research

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Abstract

Background

Women with diverse genital anaerobic bacterial communities, are at over 4-fold higher risk of HIV acquisition than women with a *Lactobacillus*-rich vaginal microbiome. The mechanisms underlying this are poorly understood. We set out to examine how vaginal microbiota diversity affects epithelial integrity in HIV sero-negative pregnant women. We also investigated how HIV infection alters mucosal integrity within the prevalent genital microbiome diversity.

Methods

We assessed epithelial permeability by measuring the concentrations of tight junction proteins, claudin-1 and zonula occludens- (ZO)-1, in cervico-vaginal lavages (CVL) by enzyme-linked immunosorbent assay (ELISA). Cytokines in the vaginal fluids were measured by multiplex magnetic bead assay to establish the inflammatory state.

Bacterial cell-free supernatants were used to treat vaginal epithelial cells and tissues, mimicking the *in vivo* vaginal milieu. Gene and protein expression levels of tight junctions of vaginal epithelial cells and tissues in response to treatment were quantified by QuantiGene™ Plex Gene Expression Assay and by western blot respectively. The cytokine response of vaginal epithelial cells, VK2 (E6/E7, ATCC® CRL-2616™), to bacterial cell-free supernatants was measured by ELISA method.

Results

Among women with CT3 cervicotype, HIV sero-negative pregnant women had significantly higher claudin-1 in their CVL than the HIV-infected pregnant women (p=0.0011). IL-8 (p=0.0028), IL-1beta (p<0.0001) and TNF-alpha (p=0.0283) were significantly higher among HIV-negative pregnant women with a non-*Lactobacillus* dominant vaginal microbiota than those with a *Lactobacillus*-dominant vaginal microbiota. Bacterial cell-free supernatants from *Lactobacillus* elicited low levels of pro-inflammatory cytokines IL-1alpha, IL8, IL-6 and IL-1 beta in comparison to media and *G. vaginalis*. Treatment with *G. vaginalis* supernatant lowers claudin-1 and claudin-4 expression yet presence of either *L. crispatus* or *L. iners* mitigates this effect of *G. vaginalis* as observed by immuno-staining of treated vaginal cells.

Conclusions

Pregnant women with a non-*Lactobacillus* dominant microbiome had a disrupted epithelial barrier and elevated pro-inflammatory cytokines, making them at a higher risk of HIV acquisition than women with a *Lactobacillus*-dominant microbiome. Targeting vaginal microbiota and/or its effects on the vaginal epithelium and cervico-vaginal milieu can potentially lower rates of HIV acquisition and transmission.

Introduction
There is consensus that the optimal vaginal microbiome is predominantly rich in *Lactobacillus* species \(^1,^2\) and is of low pH (below 4.5). Alteration of this environment leads to bacterial vaginosis (BV), which is characteristically a depletion of *Lactobacillus* coupled with an overgrowth of anaerobic bacterial species such as *Sneathia, Atopobium vaginae*\(^3^,^4\). Women with BV are at a higher risk of HIV acquisition \(^5^,^6\). A cohort study of young, healthy South African women showed that women with predominantly anaerobic vaginal commensal bacteria other than *Gardnerella* had a 4-fold higher risk of acquiring HIV relative to those with a *Lactobacillus*-dominant microbiome \(^7\).

BV is also associated with increased viral shedding in the genital fluids of HIV sero-positive women \(^8^,^9\) which may explain the increased risk of HIV transmission to their male partners \(^10\). Black women especially in sub-Saharan Africa are more likely to have a BV-associated microbiome than Caucasian women in North America \(^2^,^11^,^12\) which may explain why sub-Saharan Africa is the epicenter of the HIV pandemic. Suggested mechanisms by which BV enhances host susceptibility to HIV infection include increased recruitment and activation of HIV target cells, CCR5+CD4+ cells \(^12\) and enzymatic disruption of the mucosal epithelial barrier \(^13^,^14\). The metabolites of BV-associated microbiota, such as succinate, also elicit a pro-inflammatory response \(^15\) that is associated with increased risk of sexually transmitted infections such as HIV and HPV. The role of commensal bacteria in modulating host susceptibility to HIV infection is not fully known.

Within the lower reproductive tract, the vaginal mucosal epithelium, a multi-layered non-keratinized squamous epithelial physical barrier \(^16\), provides a first-line defense against HIV. The apical layers of the vaginal epithelium are devoid of tight junctions \(^17\) implying that HIV infection occurs within the more basal layers of this mucosa. Intercellular junctions including desmosomes, tight and adherens junctions maintain the morphology and physiological integrity of the epithelial barrier. Tight junctions, comprising of claudins, and occludins, act as seals between adjacent epithelial or endothelial cells, controlling paracellular permeability \(^18\). Structurally, claudins interact with ZO-1 through its C-terminus using the PDZ domain, anchoring the junction to the cell cytoskeleton \(^19\). Claudins maintain cell homeostasis by regulating the passage of small ions and nutrients \(^20\).

BV-associated bacteria such as *Gardnerella* disrupt genital epithelial barrier integrity \(^6^,^21\), a critical factor in host susceptibility to HIV infection. Cell-free supernatants of *G. vaginalis* \(^21\) and *Mobiluncus mulieris* \(^22\) increase the permeability of ectocervical and endocervical cells in vitro. Vaginolysin, a cholesterol-dependent cytolysin, produced by *G. vaginalis* has been shown to initiate blebbing of vaginal and cervical epithelial cells \(^23\). *Prevotella bivia* (*P. bivia*), another BV-associated bacterium produces fibrinolysins and collagenase that enzymatically slough off vaginal epithelial cells hence weakening barrier integrity \(^4\). The physical disruption of the genital epithelia coupled with the inflammatory response elicited by BV-associated vaginal microbiota magnify the risk of HIV acquisition among women with a diverse vaginal microbiome.
To further explore the relationship between vaginal barrier integrity and microbiota, we investigated how diversity of vaginal microbiome affects genital mucosal sturdiness. Concentrations of tight junction proteins, claudin-1 and ZO-1, in cervico-vaginal lavages of HIV-negative pregnant mothers of different cervicotypes were measured as proxies for barrier permeability. We also investigated how HIV infection alters mucosal integrity within the prevalent genital microbiome diversity, which had been previously determined by 16S rRNA sequencing. We used this in-vivo data to create an ex-vivo experimental bacterial milieu to mimic the vaginal microbiota observed in pregnant Ugandan women to assess its effect on gene expression levels of junctional proteins, claudin-1 and -4, zonula occludens-(ZO)-1 (ZO-1), occludin, F11R, and pro-inflammatory cytokines in vaginal tissues and epithelial cells (VK2 (E6/E7, ATCC® CRL-2616™)).

Materials And Methods

Study population: The HIV and Microbiome (HM) study enrolled a total of 360 pregnant women with a gestation age >12 weeks, attending routine antenatal clinic at Mulago National Referral Hospital. All study participants were aged 18 years and older, with the average age of 26 years (SD± 5.14 years). The average gestational age of all women enrolled in the study was 27.9 weeks (SD±6.4 weeks). Of the 360 pregnant women enrolled, we included only the 179 who had had their vaginal microbiome sequenced. We included 79 (44%) HIV-infected (all receiving antiretroviral therapy) and 100 (56%) were HIV-negative pregnant women. Participants gave written informed consent to participate in the study and their vaginal microbiome was determined by 16S rRNA sequencing. Four cervicotypes (CT) were identified; CT1 (the least diverse) which was predominantly non-iners Lactobacillus, CT2 which was dominated by L. iners, CT3 that was Gardnerella dominant and CT4 (most diverse), a mixed CT co-dominated by L. iners, Gardnerella and Atopobium. Approval for this study was obtained from the Institutional Review Board of School of Biomedical Sciences, Makerere University College of Health Sciences and Uganda National Council for Science and Technology (study reference number: HS 2257).

ELISAs for claudin-1 and ZO-1

We used the human tight junction protein ZO-1 and claudin 1 ELISA kits (abbexa®️, Cambridge, UK) to measure the concentrations of the afore-mentioned proteins in CVL. Briefly, standards and samples were added to 96-well plate pre-coated with antibodies to either ZO-1 or claudin-1, incubated and thereafter washed as per the manufacturer's instructions. A detection antibody that is biotin labelled and specific to either ZO-1 or claudin-1 was added, incubated then followed by incubation with avidin-conjugated horse radish peroxidase (HRP). TMB was catalyzed by HRP to give a blue product that changed to yellow upon addition of an acidic stop solution. The absorbance was read using a spectrophotometer set at 450nm.

Preparation of bacterial cell-free supernatants

Based on the cervicotypes previously identified among pregnant mothers in a Ugandan study, we chose the bacterial species of interest; L. crispatus (L. crispatus, ATCC 33820) representing CT1, L. iners (L.
iners, ATCC 55195) representing CT2, G. vaginalis (G. vaginalis, ATCC 14018) representing CT3 and combinations of G. vaginalis with either L. crispatus or L. iners representing CT4. L. crispatus was grown in Lactobacilli MRS broth (BD 288130) while L. iners and G. vaginalis were grown in NYC III media with 10% v/v heat inactivated horse serum at 37°C in a 5% CO₂ incubator for an initial 48 hours. Subsequently, 1 ml of each bacterial culture was inoculated into 100ml of NYC III media and grown for a further 20 hours to OD₆₀₀ 0.7 at 37°C in a 5% CO₂ incubator. The cultures were centrifuged two times for 10 min each at 2,500 rpm at 4°C to remove the bacteria. The resulting supernatants were filter-sterilized through a 0.22 μM membrane filter (EMD Millipore, Darmstadt, Germany) to remove any remaining bacterial components or debris. Cell-free supernatants and NYC III media (negative control to determine the baseline measurements of background growth media) were then used for in vitro cell and tissue culture experiments. For each treatment, experiment was done in duplicate or triplicate.

Cell culture

Vaginal epithelial (VK2/E6E7, ATCC® CRL-2616™) cell lines (American Type Culture Collection, Bethesda, MD, United States) were cultured in keratinocyte serum-free medium (K-SFM) supplemented with 0.1 ng/ml human recombinant epidermal growth factor (EGF) and 50 μg/ml bovine pituitary extract (ScienCell Research Laboratories, Carlsbad, CA, United States), 44.4mg/L calcium chloride, 100 U/mL penicillin, and 100 μg/mL of streptomycin at 37°C in a 5% CO₂ humidified incubator. The cells were grown to an 80% confluence before use in subsequent experiments.

In vitro tissue model (VEC tissue)

A human organotypic vaginal tissue model, EpiVaginal™ VEC-100-FT, was used to study the effects of bacterial cell-free supernatants on gene expression of tight junctions of fully differentiated vaginal epithelium. This model is reconstructed from primary human vaginal epithelial cells and fibroblasts and produced by the MatTek Corporation, Ashland, Massachusetts, USA. The EpiVaginal™ VEC-100-FT tissue is a three-dimensional tissue model reconstructed using normal vaginal ectocervical epithelial cells and is well stratified, containing differentiated basal, suprabasal, intermediate, and superficial cell layers similar to in vivo tissue.

Treatment of vaginal epithelial cells and tissues

To model the vaginal epithelium, vaginal epithelial cells (VK2/E6E7) and epithelial tissues (MatTek VEC-100-FT Epivaginal™, VEC) were used. VEC was continuously treated on the apical surface with 50uL (50% v/v) of bacterial cell-free supernatants for 18 hours at 37°C, 5% carbon dioxide. VK2/E6E7 cells were plated at 1.0 × 10⁶ cells/ml into culture plates and treated with 1mL (50%v/v) of bacterial cell-free supernatants for 18 hours at 37°C, 5% carbon dioxide. To mimic a diverse microbiota, we used combinations of G. vaginalis and L. crispatus or G. vaginalis and L. iners supernatants at final concentrations of 25% (v/v) of each supernatant. At the end of each experiment, cell culture media were
collected for multiplex cytokine assays and/or the cells and tissues were collected in RNAlater for RNA extraction. Experiments were performed in triplicate or duplicate.

**Multiplex cytokine assay**

To establish the inflammatory state within the vaginal micro-environment, we measured cytokines in CVL and within the supernatants from the *in-vitro* experiments. For the CVLs, the cytokines IL-1β, IL-1RA, IL-6, IL-8, IL-10, and TNF-α were measured. All samples were run in duplicate as per the manufacturer's instructions. Vaginal epithelial cells and tissue were treated with bacterial cell-free supernatant as described above. A 6-plex human cytokine magnetic bead panel (PPX-06-MXKA3DC, ProcartaPlex, ThermoFisher Scientific, Vienna, Austria) was run on apical and basal tissue culture supernatants and cell culture supernatants after 18 hours of treatment. We measured the concentrations of IL-1α, IL-1β, IL-1RA, IL-6, IL-8 and MCP-1.

**Multiplex gene expression assay**

A multiplex gene expression assay, QuantiGene™ Plex Gene Expression Assay (Affymetrix), was used to determine the changes in expression of cell junction proteins of the epithelial tissue namely; claudin-1 and 4, occludin, JAM-A, ZO-1 in response to treatment with the bacterial supernatants. The QuantiGene™ Plex assay is a probe hybridization-based method of target-specific RNA capture and quantification that utilizes branched DNA signal amplification and Luminex multi-analyte profiling technology. MatTek™ tissues were lysed using the QuantiGene™ sample processing kit for cultured cells (QS0100) as per manufacturer’s instructions. 10ul of Proteinase K was added per 1ml of lysis buffer. One part of this mixture was further diluted by adding it to two parts RNAse-free water. MatTek™ tissues were removed from their inserts and cut up before adding to the lysis buffer. Lysis of the tissue was achieved by vortexing for 1 minute and then incubating the tissue in lysis buffer at 50-55°C for 30 minutes with gentle agitation. The lysed samples were kept on ice and used immediately for the QuantiGene™ Plex Gene Expression Assay (Affymetrix Inc., Santa Clara, USA) or stored at -80°C for later use, as per the manufacturer’s instructions. Output was initially analyzed using QuantiGene Plex Data Analysis application available from ThermoFisher.

**Western blots for claudin-1 and e-cadherin**

To quantify the changes in barrier permeability due to bacterial cell-free treatment, tight junction protein expression by VK2 cells was quantified by western blot. VK2 cells were plated in a 6-well tissue-culture treated plate at a concentration of 0.7x10^6 cells/mL in 1mL volume. The next day, the media was aspirated, and cell-free bacterial supernatants were added, diluted 50% (v/v) in K-SFM. After an 18-hour incubation, supernatants were collected and cells were lysed using 0.5mL of RIPA Lysis Buffer (Santa Cruz Biotechnology Inc, Santa Cruz, California, USA). Lysates were quantified by BCA and 15ug of total protein per sample was loaded and run on a 4-15% polyacrylamide gel (Bio-Rad, Hercules, California, USA). Proteins were transferred to a PVDF membrane and stained with antibodies at the following concentrations: rabbit anti-human claudin-4 at 1ug/mL (Thermo Fisher, Rockford, IL, United States,
cat#51-9000), mouse anti-human e-cadherin at 1ug/mL (ThermoFisher cat#33-400), and rabbit anti-human beta-tubulin (Novus Biologicals, Colorado, USA, cat#NB600-936) at 1:1000. Anti-rabbit or anti-mouse HRP antibodies were used as secondary antibodies and the membrane was visualized using Pierce ECL Western Blotting Substrate (ThermoFisher cat#32106) on a luminescent imager (FujiFilm LAS-4000). Restore Western Blot Stripping Buffer (ThermoFisher cat#21059) was used to strip the membrane before re-staining with a different antibody. Protein expression was quantified using the gel analyzer tool in the Fiji distribution of ImageJ (version 1.52p) 25.

Staining for tight junctions

To assess effect of bacterial soluble factors on barrier integrity, we stained VK2 (E6/E7) cells treated with bacterial cell-free supernatants to localize tight junctions claudin-1 and claudin-4. Slides with VK2 (E6/E7) cells were fixed in acetone and rehydrated in Tris-Buffered saline (TBS, Biocare Medical Immunocare, Pacheco, California, USA, diluted to 1x with distilled water) prior to staining. Vaginal epithelial tissues were embedded in wax and sliced into 5-micron thick sections, thereafter rehydrated in decreasing concentrations of ethanol and subjected to heat-mediated antigen retrieval prior to staining. Slides were then washed liberally and subsequently blocked with 10% normal donkey serum in TBS (Jackson ImmunoResearch, West Grove, Pennsylvania, USA, cat#017-000-001) for tissues sections and DAKO serum-free protein block (Agilent Technologies Inc, Santa Clara, California, USA, cat#X0909) for cells for 30 minutes. Rabbit anti-human monoclonal antibodies for claudin-1 and claudin-4 were diluted to 5ug/mL ThermoFisher catalog# 51-9000 and cat#PA5-16875) in DAKO antibody diluent with background reducing components (Agilent Technologies Inc, Santa Clara, California, USA, DAKO cat#S3022) were added to each slide and incubated for one hour in a wet box with gentle shaking. Slides were then washed in TBS and thereafter, secondary antibody, Cy3 diluted 1:1000 or FITC diluted 1:200 in TBS was added to each tissue. Stained cells were incubated at room temperature for one hour in a wet box under darkness and with gentle shaking. Vectashield anti-fade mounting medium with DAPI (Novus Biologicals, Colorado, USA, cat#H-1200) were added to stained cells after washing with TBS.

Statistical analysis

Statistical analysis and visualization of data was done in GraphPad Prism version 8.2.1. Comparisons between cytokine or tight junction proteins levels between two unpaired groups were made using Mann-Whitney test and statistical significance was set at p<0.005.

Results

Diverse vaginal microbiota alters vaginal epithelial integrity and is associated with inflammation

We sought to describe mucosal barrier integrity markers, claudin-1 and ZO-1, among pregnant women with different vaginal microbiota cervicotypes CT1-CT4. Cervicotypes CT1 and CT2, were categorized as the Lactobacillus (LAB) dominant and CT3 and CT4 were the non-Lactobacillus (non-LAB) dominant vaginal microbiota profiles. We measured the concentrations of tight junction proteins, claudin-1 and ZO-
1 in cervico-lavage among HIV-negative pregnant mothers (n=79) whose vaginal microbiota profile had been previously characterized.

CT1 had the lowest concentration of both tight junctions (Figure 1A) and the difference in claudin-1 concentrations between CT1 and CT2 was statistically significant, p=0.0484 (Figure 1A) as determined by Mann Whitney test. In general, claudin-1 levels (mean=0.8038, SD + 0.5927) were higher than ZO-1 levels (mean=0.3603, SD + 0.0889). There was no correlation between ZO-1 and claudin-1 levels in CVL of HIV-negative women (Spearman r= -0.03885, p=0.6056, data not shown). There was a trend of increasing claudin-1 levels with increase in vaginal microbiota diversity i.e. shift from CT1 to CT4 although this was not statistically significant (Kruskal-Wallis test p=0.0685, see figure S1A, supplemental digital content 1).

Levels of ZO-1 protein in cervicovaginal lavages of HIV-negative pregnant women tended to decrease with increasing vaginal microbiota diversity (Kruskal-Wallis test p=0.2292, see figure S1B, supplemental digital content 1). Pro-inflammatory cytokines IL-8 (figure 1B), IL-1beta (figure 1D) and TNF-alpha (figure 1E) were statistically significantly higher among HIV-negative pregnant women with a non-*Lactobacillus* dominant vaginal microbiota than those with a *Lactobacillus*-dominant vaginal microbiota. There was no statistical difference in the levels of IL-10 (figure 1F) and IL-1RA (figure 1G) among HIV-negative women with versus those without a *Lactobacillus*-dominant vaginal microbiota.

**Vaginal epithelial integrity among HIV-infected pregnant mothers**

We described claudin-1 and ZO-1 (surrogate markers of epithelial damage) among HIV-negative and HIV-infected pregnant mothers with the diverse vaginal microbiota cervicotypes CT1-CT4. Given that claudin-1 is a transmembrane protein, we postulated that increased claudin-1 levels in cervico-vaginal lavage would imply a more intact epithelium. On the other hand, ZO-1 is intracellular so we postulated that its abundance in cervico-vaginal fluid would indicate epithelial cell degradation. In general, HIV-negative pregnant women had higher claudin-1 levels than the HIV-infected pregnant women across all cervicotypes (figure 2A). For women with CT3 cervicotype, HIV-negative pregnant women had significantly higher Claudin-1 in their cervico-vaginal lavages than the HIV-infected pregnant women (p=0.0011, Figure 2A). Claudin-1 levels were similar between HIV-negative and HIV-infected pregnant women with *Lactobacillus*-dominant vaginal microbiota (CT1 and CT2), see figure 2A.

HIV-infected women had higher levels of ZO-1 protein in their cervico-vaginal lavages compared to the HIV-negative pregnant women across cervicotypes. For CT3, HIV-infected pregnant women had significantly higher levels of ZO-1 proteins in their cervico-vaginal lavages than HIV-negative pregnant women, p=0.0028.

We further evaluated differences in concentrations of cytokines TNF-alpha, IL-6, IL-8, IL-10, IL-1beta, and IL-1RA in the cervico-vaginal lavages of pregnant women with versus those without HIV (figure 2C-2H). The cytokine concentrations were statistically similar among HIV-infected pregnant women and HIV-negative pregnant women except for IL-1RA that was interestingly higher among the HIV-infected women, p=0.0004 (figure 2H).
Innate Immune responses of vaginal epithelium to cell-free bacterial supernatants

We set out to determine the immune responses of vaginal epithelium to soluble factors secreted by commensal bacteria observed among Ugandan pregnant women. We selected the bacterial strains for these experiments based on the vaginal microbiota profile of pregnant mothers attending antenatal care at Mulago National Referral Hospital as mentioned earlier. Four cervicotypes were identified in this study population as previously described. Cervicotype 1 (CT1) was predominantly *Lactobacillus* other than *L. iners* and this was represented by *L. crispatus*. CT2 was dominated by *L. iners* and CT3 was *G. vaginalis* dominated CT3. CT4 was the most diverse cervicotype with *G. vaginalis, L. iners* and *Atopobium vaginae* co-dominating this cervicotype. Therefore we selected *L. crispatus, L. iners* and *G. vaginalis* (ATCC™) to represent CT1, CT2 and CT3 respectively. Cervicotype 4 (CT4) was represented by combinations of *G. vaginalis* with *L. crispatus* or *L. iners*.

*G. vaginalis* supernatant elicited the highest concentration of IL-1RA (figure 3A) and IL-1alpha (figure 3B) amongst all the bacterial cell-free supernatants. Cell-free bacterial supernatants elicited higher amounts of IL-1RA (Figure 3A) and IL-1alpha (figure 3B) than media. *G. vaginalis* elicited concentrations of IL-1alpha, IL-8, IL-1beta and IL-6 that were comparably similar to those elicited by *L. crispatus, L. iners* and the combinational supernatants. Generally, bacterial cell-free supernatants from *Lactobacillus* elicited low levels of pro-inflammatory cytokines IL-1alpha, IL8, IL-6 and IL-1 beta in comparison to media and *G. vaginalis*.

To visualize the effects of bacterial cell-free supernatants on the expression of tight junctions claudin-1 and claudin-4, we stained VK2 cells after 18-hour treatment with the bacterial supernatants previously mentioned. Images of stained cells were captured by a digital camera (Olympus DP73) attached to an Olympus microscope fitted with fluorescence imaging. Figure 4 shows representative images of VK2 cells treated with keratinocyte serum-free media (KSFM), see figure 4A and bacterial cell free supernatants, figure 4B-F. Treatment with *G. vaginalis* supernatant lowers claudin-1 and claudin-4 expression (figure 4D) yet presence of either *L. crispatus* or *L. iners* mitigates this effect of *G. vaginalis* as seen in figure 4E and 4F respectively. We also evaluated the changes in junctional protein expression, claudin-4 and E-cadherin, in response to bacterial cell-free supernatants by western blot and the changes were quantified by Image J (see figure S2A, Supplemental Digital Content 2). We further looked at the gene expression of the tight junctions in response to treatment of vaginal epithelial tissues with bacterial cell-free supernatants. Lysed tissue was assessed for gene expression using Quantigene™ Plex Gene Expression assay as per the manufacturer’s instructions. We evaluated fold changes in genes encoding tight junctions claudin-1 and 4, occludin, ZO-1 and JAM-A with respect to the house-keeping gene GAPDH. When compared to media treatment, bacterial cell-free supernatants did not significantly alter expression of genes for tight junctions (see figure S2B-F, Supplemental Digital Content 2).

Discussion
Vaginal commensal bacteria have been postulated as possible contributors to the disproportionately higher risk of HIV acquisition and transmission among women than men in the sub-Saharan HIV epidemic. Commensal bacteria, especially anaerobic species, have been implicated in promoting inflammation, which is a key driver of activation and recruitment of HIV target cells, CCR5+CD4+ cells, to the genital mucosa. Vaginal microbiota also produces enzymes such as vaginolysin, sialidases, prolidases that degrade the epithelial barrier, making it more permeable to HIV entry. In this study, we explored the association of vaginal microbiota with markers of genital mucosal integrity, as proxy indicators of host susceptibility to HIV infection.

We report that among HIV-negative pregnant women, those with a diverse microbiota i.e. CT3 and CT4 that were predominantly *G. vaginalis* and mixed anaerobic bacteria respectively, had more soluble claudin-1 in their cervico-vaginal lavages than women who had *Lactobacillus*-dominant vaginal microbiota. We postulate that the increasing levels of claudin-1 coupled with high pro-inflammatory cytokines among women with diverse microbiota is due to increased sloughing off of epithelial cells. This agrees with previous studies that have shown increased shedding of epithelial cells in women with BV in comparison with women with a *Lactobacillus* vaginal microbiota. *G. vaginalis*, *Atopobium vaginae* and other BV-associated bacteria are known disruptors of the mucosal membrane. *G. vaginalis* produces sialidase which enzymatically degrades mucus by breakdown of sialic acid from glycoproteins hence weakening the epithelial barrier. *G. vaginalis* also produces pore-forming vaginolysin that further decimates the epithelial barrier. The low ZO-1 proteins in the cervico-vaginal lavages of HIV-negative pregnant women with a diverse vaginal microbiota i.e. CT3 and CT4 could possibly be due to the decreased cell proliferation and differentiation as has been previously reported. Women with a non-*Lactobacillus* dominant vaginal microbiota have a higher number of immature epithelial cells which have lower expression of tight junction proteins. We show that both inflammation and poor barrier integrity are associated with increased diversity of the vaginal microbiota. Given that inflammation and barrier function are known drivers of establishment of HIV infection, women with a diverse vaginal microbiota are likely at increased risk of HIV acquisition. We therefore postulate that modifying the vaginal microbiota profile from non-*Lactobacillus* dominant to *Lactobacillus*-dominant would confer protection against HIV acquisition to these pregnant women.

We further examined the effect of HIV on the barrier integrity in the genital tract of pregnant mothers. HIV-infected pregnant mothers had lower claudin-1 concentrations in cervico-vaginal lavages than HIV-negative pregnant women. Lower claudin-1 levels could possibly be due to disruption of epithelial junctions by HIV; mediated by the envelope protein gp120. Interaction of HIV gp120 with epithelial cells activates the MAPK, PI3K and TLR signaling that elicits pro-inflammatory cytokines such as TNF-alpha, which activates apoptosis and the caspase-mediated destruction of junctional proteins, including claudin-1. It has also been shown that HIV-1 tat activates MAPK signaling in epithelial cells leading to down-regulation of tight junction protein expression. Lower claudin-1 proteins among HIV-infected pregnant women could also be due to the internalization of the claudins as a result of myosin light chain kinase/myosin light chain (MLCK/MLC) activation and contraction of actin cytoskeleton when epithelial
cells interact with HIV-1 gp120 \(^{37}\). Our results show that the disruption of the epithelial barrier by HIV is exacerbated by having a non-\textit{Lactobacillus} dominant vaginal microbiota as evidenced by markedly decreased levels of claudin-1 in the cervico-vaginal lavages of women with CT3 and CT4 cervicotypes (figure 2A). This is possibly due to the pro-inflammatory cytokines elicited by vaginal microbiota in CT3 and CT4 which activate the disruption of epithelial junctional proteins by apoptosis or internalizations.

Concentrations of ZO-1 in the cervico-vaginal lavages of HIV-negative pregnant women were lower than those in HIV-infected women. This could possibly be due to the increased epithelial shedding observed in HIV infection hence increased degradation of tight junctions and subsequently higher concentrations in CVL. The cytokine milieu of HIV-infected pregnant women was comparably similar to that of HIV-negative women. This can be attributed to antiretroviral therapy which the HIV-infected women were actively taking.

\textit{In vitro} experiments on the effect of bacterial cell-free supernatants on immune response of vaginal epithelial cells of tissue models, showed that \textit{Lactobacillus} supernatants elicited very low concentrations of pro-inflammatory cytokines IL-1beta, IL-6, IL-8 and IL-1 alpha in comparison to \textit{G. vaginalis} cell-free supernatant. This is probably due to the lactic acid produced by Lactobacilli. These findings are consistent with previous reports that lactic acid, a metabolite of Lactobacillus, elicits an anti-inflammatory response from cervico-vaginal cells \(^{38}\). Treatment of vaginal epithelial cells (VK2 (E6/E7)) with \textit{G. vaginalis} cell-free supernatant showed a decreased expression of claudin-1 and claudin-4 in comparison to cells treated with \textit{L. crispatus} or \textit{L. iners}. Treatment of VK2 cells with a combination of \textit{G. vaginalis} with either \textit{L. crispatus} or \textit{L. iners} mitigated the effect of \textit{G. vaginalis} on claudin-1 and claudin-4 expression. The \textit{G. vaginalis} mediated inflammation and disruption of epithelial barrier has been previously reported by Anton et al., 2018 \(^{21}\). They reported increased permeability of ectocervical and endocervical cells and pro-inflammatory immune mediators in response to treatment with \textit{G. vaginalis} cell-free supernatant. Diverse vaginal microbiota may increase host susceptibility to HIV infection due to the disruptions in epithelial barrier and the inflammation which attracts CCR5+CD4+ cells to the mucosa. Similarly, the leaky epithelial barrier in HIV-infected pregnant women demonstrated by the disruption of tight junctions of vaginal epithelium, makes the pregnant women susceptible to HIV re-infection that could lead to high HIV viral load in acute HIV infection and the associated increase in HIV transmission risk to the unborn baby. This work supports the epidemiological studies that show higher HIV acquisition risk among women with a BV-like vaginal microbiota.

**Conclusion**

Vaginal microbiota diversity in African pregnant women is associated with altered barrier integrity and elevated pro-inflammatory cytokines in cervico-vaginal lavage. Efforts to modify diverse BV-associated vaginal microbiota to a healthier Lactobacillus-dominated are likely to complement pre-exposure prophylaxis (PrEP) and prevention of mother-to-child transmission (PMTCT) efforts among HIV-negative and HIV-infected women respectively.
Abbreviations
PrEP, pre-exposure prophylaxis; PMTCT, prevention of mother-to-child transmission; CT, cervicotypes; ZO-1, zonula occludens; CVL cervico-vaginal lavage; ELISA enzyme-linked immunosorbent assay

Declarations

Ethic approval and consent to participate
Approval for this study was obtained from School of Biomedical Sciences Research and Ethics Committee, Makerere University College of Health Sciences and Uganda National Council for Science and Technology (study reference number: HS 2257). Each study participant gave written consent to participate in the study.

Consent for publication
Not applicable

Availability of data and materials
Data and materials are available on request form the authors.

Competing interests
The authors declare that they have no competing interests.” If you are unsure whether you or any of your co-authors have a competing interest please contact the editorial office.

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Author Contributions
LB, EM, DPK, MS, DJA, and DN conceived the study and participated in its design and coordination. LB, EM and RN performed the experiments and data analysis. LB and DN drafted the manuscript. All authors reviewed and approved the final manuscript for publication.

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