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Vaginal dysbiosis and biofilms, volume II

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Vaginal dysbiosis and biofilms, volume II

Topic editors

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Table of contents

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Editorial: Vaginal dysbiosis and biofilms, volume II

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Editorial on the Research Topic
Vaginal dysbiosis and biofilms, volume II

The vaginal microbiota is a dynamic ecosystem essential for reproductive health, predominantly maintained by peculiar *Lactobacillus* species. These bacteria produce lactic acid, creating an acidic environment that inhibits pathogen colonization (Machado et al., 2013; Rodríguez-Arias et al., 2022; Morselli et al., 2024). However, disruptions in this balance lead to vaginal dysbiosis, associated with bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), and aerobic vaginitis (AV) (Salinas et al., 2018). Biofilms, structured microbial communities encased in an extracellular matrix, play a crucial role in vaginal dysbiosis (see Figure 1), such as BV, by enhancing pathogen persistence and resistance to antimicrobials (Muñoz-Barreno et al., 2021). Vaginal dysbiosis is associated with an increased risk of acquiring sexually transmitted infections, such as human immunodeficiency virus (HIV), Herpes simplex type 2, and *Chlamydia trachomatis*, as well as an increased frequency of reproductive complications (Parolin et al., 2015; Ceccarani et al., 2019; De Gregorio et al., 2020).

This second volume of the Research Topic expands on the complex interactions between microbial communities, host responses, and biofilms (Machado et al., 2022). The articles included explore novel diagnostic methods, targeted therapies, and probiotic interventions aimed at restoring microbial balance. Given the challenges posed by biofilm-associated infections, understanding their molecular mechanisms is essential for developing more effective treatments. By advancing research on vaginal dysbiosis and biofilms, this Research Topic seeks to improve diagnostic accuracy and therapeutic strategies (Henriques et al., 2012; Machado et al., 2015), ultimately enhancing women's reproductive health and preventing long-term complications (De Gregorio et al., 2020).

Zierden et al. explored the influence of the vaginal microbiome on cervicovaginal mucus (CVM) barrier properties during pregnancy. The authors collected CVM samples from pregnant participants and used multiple particle tracking (MPT) and *16S* rRNA sequencing to analyze barrier function and microbiome composition. Their findings revealed that *Lactobacillus crispatus*-dominated microbiota was associated with stronger

CVM barrier function, while polymicrobial communities increased permeability. This suggests that microbial composition plays a crucial role in preventing bacterial ascension, with potential implications for preterm birth risk. Understanding these interactions could aid in the development of vaginally delivered therapeutics to support reproductive health during pregnancy. Building on the role of beneficial microbes in vaginal health, Takano et al. evaluated how *Lactobacillus* species inhibit *Candida albicans* growth, biofilm formation, and epithelial adhesion. By analyzing 27 *Lactobacillus* strains, the study identified lactate as a key factor in suppressing *C. albicans* biofilms and hyphal transition, while some strains also significantly reduced fungal adhesion to epithelial cells. These findings highlight the potential of *Lactobacillus*-derived metabolites as alternative antifungal strategies, offering a promising avenue for preventing vulvovaginal candidiasis.

Given the importance of accurately characterizing vaginal microbiota for clinical and research applications, Short et al. compared two sampling techniques in pregnant women living with HIV-1: menstrual cups and high vaginal swabs. The study found no significant differences in bacterial load, composition, or diversity between methods, validating both for microbiota analysis. Menstrual cups, however, collected a larger sample volume, making them a practical alternative for self-sampling and expanded laboratory analysis. This research underscores the need for adaptable and efficient methodologies in vaginal microbiota studies, particularly for populations with increased reproductive health risks.

Furthermore, Mao et al. studied the association between vaginal and cervical microbiome dysbiosis and uterine fibroids. By analyzing microbial profiles from 29 women with uterine fibroids and 38 healthy controls, the study found no significant difference in overall microbial diversity. However, alpha diversity was negatively correlated with the number of fibroids, and an increased abundance of *Firmicutes* was observed in fibroid patients. Certain bacterial genera were significantly enriched or depleted, indicating microbial alterations linked to fibroid presence. The findings suggest that microbiome disruptions may contribute to fibroid pathogenesis, offering new insights for potential preventive and therapeutic strategies. The impact of vaginal microbiota on systemic health is further exemplified in the case study by Liu et al., which reported a rare instance of *Fannyhessea vaginae* bacteremia in a pregnant woman with bacterial vaginosis. Blood cultures confirmed *F. vaginae* as the causative agent, and the patient responded well to cefoperazone/sulbactam treatment. This study highlights the clinical significance of anaerobic vaginal pathogens, particularly in pregnant women, where microbial imbalances can lead to severe complications. A review of previous cases reinforced *F. vaginae*'s association with bacterial vaginosis, preterm birth, and systemic infections, emphasizing the need for heightened awareness of its pathogenic potential.

Beyond microbial composition, hormonal regulation plays a key role in shaping the vaginal microbiota, as explored by Rahman et al. Using a mouse model, they demonstrated that estrogen significantly influences *Lactobacillus* and *Gardnerella vaginalis* colonization. Mice treated with 17β-estradiol exhibited increased glycogen levels, which supported *Lactobacillus* colonization, whereas progesterone alone failed to restore microbial balance. These findings suggest that sex hormones modulate vaginal microbiota stability, offering potential therapeutic avenues for managing dysbiosis through hormonal interventions.

Zhang et al. reviewed the relationship between vaginal microbiota, human papillomavirus (HPV) infection, and cervical cancer. They highlighted how vaginal dysbiosis, characterized by

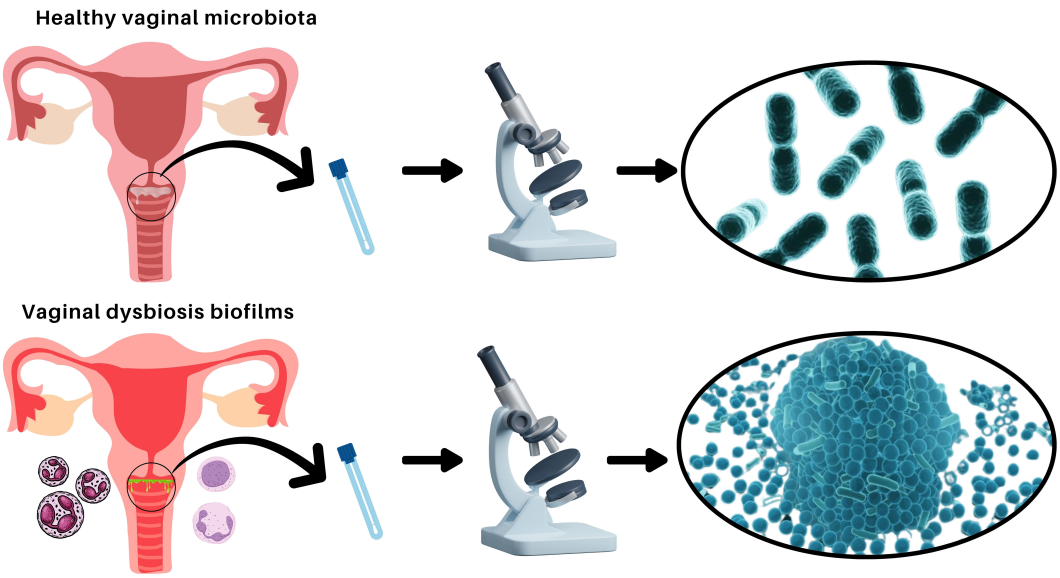

FIGURE 1
Comparison of healthy vaginal microbiota and vaginal dysbiosis with biofilm formation. Illustration depicting the differences between a balanced vaginal microbiota dominated by *Lactobacillus* species (top) and vaginal dysbiosis characterized by pathogenic biofilm formation (bottom). In a healthy state, the vaginal microbiota consists of beneficial bacteria that maintain an acidic pH and protect against infections. In dysbiosis, biofilms formed by opportunistic pathogens contribute to persistent infections and increased resistance to treatments.

reduced *Lactobacillus* abundance and increased microbial diversity, contributes to HPV persistence and cervical lesion progression. This review underscores the complex interactions between microbial communities and HPV, providing a basis for future research into personalized diagnostic and treatment strategies. Meanwhile, Chen et al. analyzed the pathogenic function of sialidases in BV. Sialidases produced by *Gardnerella vaginalis* and other anaerobes degrade the protective mucus layer of the vaginal epithelium, facilitating bacterial adhesion, biofilm formation, and immune evasion. The study reviewed sialidase-based diagnostic tools and therapeutic potential, suggesting that sialidase inhibitors could be promising pharmacological targets for BV treatment. On the other hand, Cao et al. evaluated the synergistic effects of Kangbainian (KBN) lotion and miconazole nitrate (MN) against drug-resistant *Candida albicans* biofilms. *In vitro* assays revealed that the combination of KBN and MN disrupted biofilm integrity, reduced fungal viability, and downregulated key biofilm-associated genes. This study highlights the growing need for novel antifungal strategies to combat drug-resistant biofilms in vaginal infections.

Finally, Himschoot et al. investigated the prevalence and clinical correlations of *Gardnerella* species, *Fannyhessea vaginae*, *Lactobacillus crispatus*, and *L. iners* in pregnant women in the Democratic Republic of the Congo. By analyzing samples from 331 pregnant women, they found that *L. iners* was the most prevalent species, while *G. vaginalis* was the most common *Gardnerella* species. Notably, *F. vaginae* was identified as the best molecular marker for bacterial vaginosis (BV), with a high diagnostic performance. The study also highlighted associations between microbial species and BV-related symptoms, as well as potential links between *L. iners* and preterm birth. Last, but not least, in the eleventh article, Zheng et al. assessed the role of reproductive tract microbiota in gynecological diseases. The review explored microbial alterations in conditions such as endometrial polyps, uterine fibroids, endometriosis, adenomyosis, and endometrial cancer, highlighting the potential of microbiota as both diagnostic markers and therapeutic targets. The review suggested that certain bacterial species, such as *F. vaginae*, may contribute to disease progression, while microbiota-targeted interventions, including probiotics and microbiome transplants, offer promising treatment avenues. The integration of microbiome research with other omics sciences like transcriptomics and proteomics could further refine diagnostic and therapeutic strategies.

Author contributions

AMac: Writing – original draft, Writing – review & editing. CF: Writing – review & editing. AMar: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cervicovaginal mucus barrier properties during pregnancy are impacted by the vaginal microbiome

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Introduction: Mucus in the female reproductive tract acts as a barrier that traps and eliminates pathogens and foreign particles via steric and adhesive interactions. During pregnancy, mucus protects the uterine environment from ascension of pathogens and bacteria from the vagina into the uterus, a potential contributor to intrauterine inflammation and preterm birth. As recent work has demonstrated the benefit of vaginal drug delivery in treating women's health indications, we sought to define the barrier properties of human cervicovaginal mucus (CVM) during pregnancy to inform the design of vaginally delivered therapeutics during pregnancy.

Methods: CVM samples were self-collected by pregnant participants over the course of pregnancy, and barrier properties were quantified using multiple particle tracking. 16S rRNA gene sequencing was performed to analyze the composition of the vaginal microbiome.

Results: Participant demographics differed between term delivery and preterm delivery cohorts, with Black or African American participants being significantly more likely to delivery prematurely. We observed that vaginal microbiota is most predictive of CVM barrier properties and of timing of parturition. Lactobacillus crispatus dominated CVM samples showed increased barrier properties compared to polymicrobial CVM samples.

Discussion: This work informs our understanding of how infections occur during pregnancy, and directs the engineering of targeted drug treatments for indications during pregnancy.

KEYWORDS

vaginal microbiome, drug delivery, mucus, pregnancy, preterm birth (PTB)

Introduction

The female reproductive tract is equipped with a cervicovaginal mucus (CVM) barrier which protects the underlying epithelium from foreign pathogens. As the first line of defense against infection, CVM is a complex mixture of mucin proteins, ions, lipids, cells, and bacteria (Cone, 2009). The mesh-like structure of CVM aids in sterically hindering large particulates, while the charged and hydrophobic regions of mucus facilitate adhesive barrier properties (Cone, 2009; Ensign et al., 2012). Mucus is continually secreted and cleared, eliminating materials that become trapped in the sticky gel from the body. Despite the benefits of CVM to reproductive health, it presents a challenge to effective local drug administration. Vaginal drug delivery is optimal for targeting the female reproductive tract, leading to increased drug concentrations in local tissues while decreasing off-target side effects. However, vaginally delivered therapeutics must be designed to overcome the steric and adhesive barrier properties of mucus. We previously engineered nano-sized formulations coated with a hydrophilic, net-neutral polymer to prevent preterm birth (PTB) in preclinical murine models of progesterone withdrawal and intrauterine inflammation with vaginal administration (Hoang et al., 2019; Zierden et al., 2021a). While these studies demonstrated the potential benefit of vaginally delivered therapies in obstetric indications, the barrier properties of human CVM during pregnancy have yet to be defined.

In addition to protecting the female reproductive tract, during pregnancy, CVM serves to protect the fetal compartment from ascending bacteria and inflammation. Indeed, a compromised CVM barrier has the potential to increase a woman's risk of adverse pregnancy outcomes, including PTB, the leading cause of infant mortality and morbidity (CDC, 2019a; CDC, 2019b). It is estimated that 25% of PTBs are due to intrauterine infection and inflammation (Cunningham et al., 2018; Zierden et al., 2020). One potential source of intrauterine inflammation is ascension of bacteria from the vagina into the uterine environment (Malaeb and Dammann, 2009; Norwitz and Caughey, 2011). Unlike most microbial communities in the human body, what is considered an optimal vaginal ecosystem in reproductive aged women consists of a low level of bacterial diversity, typically dominated by *Lactobacillus* species (Ravel et al., 2011). Conversely, ~30% of women in the United States are affected by bacterial vaginosis (BV), a state of dysbiosis where the microbial community is dominated by anaerobic bacteria, including *Gardnerella vaginalis* (Eschenbach, 1993; Kroon et al., 2018; Sobel, 2018). BV is associated with a higher risk for PTB (DiGiulio et al., 2015; Callahan et al., 2017), as well as a variety of other adverse obstetric and gynecologic outcomes (Bukusi et al., 2006; Koumans et al., 2007; Atashili et al., 2008; Nelson et al., 2015). Sonographic short cervix, a key risk factor for PTB, has been associated with vaginal colonization by *G. vaginalis* (Hassan et al., 2006; Gerson et al., 2019), potentially due to inflammation and altered cervical epithelial cell function (Anton et al., 2018; Sierra et al., 2018). Furthermore, it has been described that samples of the cervical mucus plug from women at high-risk of PTB showed decreased barrier function to particles and peptides *ex vivo* (Critchfield et al., 2013). In the context of BV, the production of mucin-degrading enzymes by BV-associated bacteria may lead to impaired protection of the uterine compartment from microbial ascension (Howe et al., 1999; Wiggins et al., 2001; Cauci et al., 2008; Lewis et al., 2013). Thus, the complex interplay between CVM barrier function and the vaginal microbiota plays a key role in pregnancy and neonatal health.

Multiple particle tracking (MPT) is a technique that has enabled characterization of the structural and barrier function of various human mucus secretions, including CVM (Suh et al., 2005; Lai et al., 2007; Lai et al., 2010; Schuster et al., 2015). By tracking the spatial location of various sizes of fluorescently-labeled probe nanoparticles with either mucoadhesive (conventional particles, CP) or mucoinert (mucus-penetrating particles, MPP) surface characteristics over time, both adhesive and steric interactions between particles and the mucus mesh can be observed. We previously employed MPT to characterize the impact of the vaginal microbiota composition on CVM barrier function in a cohort of non-pregnant participants. We observed decreased CVM barrier function to HIV virions and CP in polymicrobial samples from women with and without BV symptoms compared to CVM from women with vaginal microbiota dominated by *Lactobacillus* spp (Nunn et al., 2015; Hoang et al., 2020). Further, there was no restoration of barrier function after antibiotic treatment for BV (Zierden et al., 2020). In a small cohort of pregnant participants with healthy vaginal microbiota, we observed that there was a minor decrease in nanoparticle mobility in their CVM samples, suggesting that there may be an impact of hormones on pore size (Hoang et al., 2019). Here, we utilized MPT to probe the barrier properties and pore sizes of CVM samples collected from 92 pregnant participants in Baltimore City as a function of both weeks of pregnancy and the vaginal microbiota. Our work here suggests that the vaginal microbiome is more predictive of CVM barrier properties during pregnancy than other physical characteristics of CVM or weeks of pregnancy. By understanding the relationship between the vaginal microbiome and the mucus barrier over the course of pregnancy, we can both elucidate the mechanistic contribution to PTB risk and further define design criteria for effective vaginal therapeutics for obstetric applications (Mitchell and Marrazzo, 2014; Anton et al., 2018; Fettweis et al., 2019; Zierden et al., 2021a).

Materials and methods

Materials

Instead Softcups® were obtained from Evofem (San Diego, CA). Wiretrol® disposable micropipets were obtained from Drummond Scientific Co. (Broomall, PA). ESwabs™ were obtained from BD (Franklin Lakes, NJ) and hCG urine test strips were obtained from Clinical Guard (Atlanta, GA). A pH microelectrode was obtained from Microelectrodes, Inc. (Bedford, NH). D/L-lactic acid assay kits were obtained from R-Biopharm (Darmstadt, Germany). V-PLEX plates and assay kits were obtained from Meso Scale Discovery (Rockville, MD). DNeasy® Blood and Tissue kits were purchased from QIAGEN (Hilden, Germany). Lysozyme, gram staining kit (crystal violet, iodine, safranin), ethanol, acetone, Tris-Cl pH 8.0,

EDTA, Triton X, and *N*-Hydroxysulfosuccinimide (NHS) were obtained from MilliporeSigma (Burlington, MA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and FluoSpheres™ Carboxylate-Modified Microspheres (100, 200, and 500 nm) were purchased from ThermoFisher Scientific (Walthman, MA). 5 kDa molecular weight (MW) methoxy (MeO)-polyethylene glycol-amine (-NH_2) was obtained from Creative PEGworks (Chapel Hill, NC).

Participant enrollment and sample self-collection

All sample self-collection methods were approved by the Johns Hopkins University Institutional Review Board under IRB study IRB00099798, and written informed consent was obtained from each participant. The approved protocol granted access to medical history, which was used to confirm delivery dates, numbers of previous pregnancies, and history of sexually transmitted infections. Participants provided additional information about their medical history and behaviors on a written questionnaire (Supplementary Figure 1). Participants (n = 92) were between 18-45 years old and provided a total of n = 432 samples over the course of their pregnancies. In general, samples were collected approximately every 4 weeks during pregnancy. A post-partum sample was collected up to 8 weeks post-delivery. CVM samples were self-collected, as previously described (Boskey et al., 2003; Hoang et al., 2020). Participants were instructed to insert an Instead Softcup® (Evofem) into their vagina and twist while pulling out the cup (< 30 s total) to collect undiluted CVM. They placed the Softcup® into a 50 mL conical tube, which was stored on ice until processing within 12 h of collection. Additionally, participants self-collected a vaginal swab (BD Eswab) sample to be used for sequencing and a urine specimen to confirm pregnancy status with hCG urine test strips.

Cervicovaginal mucus (CVM) characterization

Conical tubes containing the Softcups were centrifuged at 1000 rcf for 2 min to separate CVM from the Softcup®. A Wiretrol® disposable micropipet (Drummond Scientific Co., Broomall, PA) was then used to transfer CVM, which was kept on ice for the duration of the characterization. The pH of the sample was measured using a standard pH microelectrode. A wet mount slide of the sample was imaged to observe bacteria morphology. Gram staining was performed by staining a vaginal smear first with crystal violet, then with iodine. Slides were destained using a mixture of 75% ethanol and 25% acetone, safranin was used as a counterstain (Nugent et al., 1991). Gram stained slides were used for Nugent scoring, which was performed on a color microscope at 100x magnification (Nikon, Tokyo, Japan) (Nugent et al., 1991). The Nugent score relies on gram staining and bacteria morphology to characterize samples as healthy (score 0-3), intermediate (score 4-6), or as having bacterial vaginosis (score 7-10). As described by others, *Lactobacillus*-dominated samples were marked by thick, gram-positive rods (Nugent et al., 1991; Zielinski et al., 2017). The polymicrobial samples (CST IV) showed few rod-shaped bacteria, and many gram-negative cocci. *L. iners* samples showed smaller, gram-variable rods. Lactic acid concentration in the sample was measured using a D/L-lactic acid assay kit, as previously described (Hoang et al., 2020). Briefly, approximately 10 mg of sample was diluted 1:50 with normal saline, and centrifuged at 1000 rcf for 10 min to pellet mucus solids. The supernatant was used in the assay kit according to the manufacturer's instructions. Cytokine concentrations were measured in one sample from each trimester, from each participant when possible. Samples were stored at -80°C for up to three years, but were freeze-thawed only once. Cytokines were measured using a custom V-PLEX plate, according to the manufacturer's instructions. IL-1β, IL-6, IL-10, and TNFα were validated to ensure no background interference with CVM. Samples were diluted 1:50 in the supplied sample buffer, and 50 µL of diluted sample was added to appropriate wells. Samples were run in duplicate. Plates were incubated at room temperature on a plate shaker for 2 h. After washing, 25 µL of detection antibody solution was added to each well, and the plate was incubated at room temperature on a plate shaker for 2 h. The plate was washed and 150 µL of read buffer was added to each well. The plate was immediately read using the SECTOR Imager 2400. Cytokine concentration was calculated based on a standard curve run on each plate.

16S sequencing and data analysis

DNA extracted from vaginal swab fluid was used to perform 16S rRNA gene sequencing, as previously described (DeLong et al., 2019). Briefly, 150 µL of amies liquid from each swab sample was diluted 5x in lysis buffer and treated with lysozyme for 1.5 h at 37°C prior to processing with a DNeasy® Blood and Tissue, used according to the manufacturer's instructions. Library preparation and sequencing was performed by the Deep Sequencing and Microarray Core at the Johns Hopkins Medical Institute. The Illumina 16S metagenomic library preparation protocol was used to amplify the V4 region of the 16S gene, and a MiSeq system was used to perform paired-end sequencing of the pooled library. QIIME 2™ was used to generate a table of amplicon sequence variants (ASV) and process 16S sequencing results, as previously described (DeLong et al., 2019). R was used to cluster samples into to community state types (CSTs) using Bray-Curtis distances between all samples and partitioning around the medoids as previously described (DiGiulio et al., 2015). Clustered CSTs were named in accordance with prior convention (Ravel et al., 2011). While we were able to establish delivery dates for all participants, in the case of attrition (n = 11), the last sampled CST was assumed to persist for that participant. In order to adjust for unequal sampling across participants, particle mobility data represents the average particle mobility for all samples within a given CST for a given participant.

Multiple particle tracking

Multiple particle tracking (MPT) was used to analyze probe particle motion in CVM samples, as previously described (Suh et al., 2005; Lai et al., 2007; Schuster et al., 2015; Hoang et al., 2020). Probe particles used in this study are listed in Supplementary Table 1. Replication defective HIV-1 was internally fluorescently labeled with mCherry-Gag, as previously described (Nunn et al., 2015). Fluorescent carboxylate polystyrene beads (conventional particles, CPs) were coated with 5 kDa MW MeO-PEG-NH2 *via* a carboxyl-amine reaction, as previously described (mucus penetrating particles, MPPs) (Nance et al., 2012). Dynamic light scattering was used to measure the size of particles diluted 1:1000 in water (Malvern Zetasizer Nano ZS, 173° scattering angle). Particle ζ-potential (surface charge) was measured at a 1:1000 dilution in 10 mM NaCl. Particles were diluted 10-400-fold depending on particle size in ultrapure water for MPT experiments. For MPT experiments, a 3-dimensional well was made with a small hole puncher (1.5 mm) and two layers of electrical tape on a glass slide. 5 μL of CVM was added to the well, and 0.3 μL of probe particle (HIV, CP, or MPP) was added to the CVM sample. A glass coverslip and superglue were used to seal the well. Particle motion was recorded in a 20 s video, taken at room temperature using an EM-CCD camera (Evolve 512; Photometrics) mounted on a Zeiss Axio Observer inverted epifluorescence microscope. Videos were taken using the 100x oil-immersion objective, had an image resolution of 25 nm/px, and a frame rate of 15 Hz. For each CVM sample, a minimum of 5 videos were taken for each particle type. Particle motion in CVM samples was quantified using a MATLAB image processing script, as previously described (Suh et al., 2005; Lai et al., 2007; Schuster et al., 2015; Hoang et al., 2020). The MATLAB output included a mean squared displacement (MSD) for each individual particle, which was used to generate a histogram of all particle MSDs at a time scale of $\tau = 1$ sec with the normalmixEM tool (R mixtools version 1.2.0). Using this histogram, R was used to find representative Gaussian distributions, indicative of a fraction of trapped particles (represented by lower MSDs) and a fraction of mobile particles (higher MSD). In cases where the Gaussian distribution revealed only one peak, a log(MSD) of -1.3 μm^2 was used as a cutoff to distinguish between trapped and mobile particles. The cutoff value was selected based on providing a clear separation between the two Gaussian curves for all particle types, and was in a similar range of values that separated the curves describing a slower and a faster moving particle population for a variety of nanoparticle types in human mucus secretions (Ensign et al., 2012; Schuster et al., 2014; Schuster et al., 2015). Average MSD at $\tau = 1$ sec data were used to calculate the pore size of each CVM sample, as previously described (Lai et al., 2010). Here, we utilized data from 100, 200, and 500 nm MPPs to calculate an average pore size for each sample.

Statistical analysis

Samples were excluded if they contained blood, or if a participant marked that they had engaged in unprotected vaginal intercourse in the three days prior to sample collection. GraphPad Prism 8 (San Diego, CA) was used for statistical analyses. Simple linear regressions were done on scatter plots to determine if the slope was significantly non-zero. For these analyses, both R^2 and *p*-value are reported. Student's t-tests (comparison of 2 groups) and one-way ANOVA corrected using Tukey's multiple comparisons test (for comparisons of 3 or more groups) were used to compare particle mobility and cytokine concentrations. Unless otherwise noted, significance is shown as $p < 0.05$. Relative risk, odds ratio, and 95% confidence intervals were calculated in GraphPad Prism 8. Data are presented as mean ± SD.

Results

Participant demographics differ between term delivery and preterm delivery cohorts

Participant demographics based on self-reported questionnaires and medical records are tabulated in Table 1. The median age of participants was 29, with a range from 18-41. Twenty-four (26%) participants identified as Hispanic or Latino, 40 (43%) were white, 25 (27%) were Black or African-American, 4 (4%) were Asian, and 23 (25%) self-identified as 'other'. Twenty-four (26%) of participants self-reported a diagnostic history of female reproductive tract conditions. Seventy-one (77%) participants self-reported a previous pregnancy, with 7 (8%) reporting a previous PTB, and 26 (28%) reporting a previous miscarriage. Based on patient electronic medical records (EMR), 26 (28%) participants had previously experienced homelessness, and 35 (38%) had experienced intimate partner violence. During their current pregnancies, 16 (17%) participants reported BV symptoms, while 9 of the 16 participants who reported BV symptoms reported having received treatment for BV. Fourty-eight (52%) participants were considered high-risk in the EMR system. Of high-risk participants, 85% (n = 41/48) were part of an addiction counseling program.

Table 1 also shows demographics for participants who delivered prematurely. We observed 10.8% (10/92) of participants delivering prematurely in this study, consistent with the US average of ~10% (Cunningham et al., 2018; Mod, 2019). The median age of participants who delivered prematurely was 28, with a range of 22-35. The median BMI was 28.8, and the median delivery time was 34.4 weeks. In contrast to the total cohort, 70% of participants who delivered prematurely were Black or African American ($p = 0.004$). While only 3/10 participants who delivered prematurely had experienced a prior pregnancy, 2/3 of these participants had experienced a prior premature delivery. Figure 1 compares weeks at delivery for participants determined clinically to be high- or low-risk (Figure 1A), participants who had or had not experienced homelessness (Figure 1B), and participants who had or had not experienced intimate partner violence (Figure 1C). Participants who were clinically high-risk had a significantly earlier delivery date than participants who were low-risk ($p = 0.012$). There were no significant differences in participants who had or had not experienced homelessness or intimate partner violence, although

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TABLE 1 Demographics and characteristics for participants enrolled in the longitudinal analysis of mucosal barrier properties and vaginal microbiome during pregnancy.

Pregnancy demographics	Total Cohort (n=92)	Term (n=82)	Preterm (n=10)	Relative Risk	Odds Ratio	95% CI	p Value
	Median (range)						
Age	28.8 (18-41)	28.9 (18-41)	28.3 (22-35)				
BMI	29.9 (18-64)	30 (18-64)	28.8 (22-38)				
Weeks at delivery	38.7 (27-43)	39.3 (37-43)	34.4 (27-37)				
Ethnicity	Number (%)						
Hispanic or Latina	24 (26)	22 (27)	2 (20)	0.7083	0.6818	0.14 to 3.3	>0.999
Race	Number (%)						
White	40 (43)	39 (48)	1 (10)	0.144	0.123	0.011-0.84	**0.039**
Black or African American	25 (27)	18 (22)	7 (70)	6.25	8.296	0.032-0.53	**0.004**
Asian	4 (4)	4 (5)	0 (0)	0	0	0.0-9.55	>0.999
Other	23 (25)	21 (26)	2 (20)	0.781	0.726	0.146-3.5	>0.999
History of conditions	Number (%)						
None	68 (74)	63 (77)	5 (50)	0.353	0.302	0.085-1.09	0.12
Yes, specified (see below)	24 (26)	19 (23)	5 (50)	2.833	3.32	0.919-11.77	0.12
HIV	2 (2)	1 (1)	1 (10)	5	9	0.429-172.1	0.21
Herpes	6 (7)	5 (6)	1 (10)	1.59	1.71	0.132-11.75	0.51
Gonorrhea	3 (3)	1 (1)	2 (20)	7.42	20.25	2.03-294.8	**0.03**
Chlamydia	10 (11)	7 (9)	3 (30)	3.51	4.59	1.074-19.81	0.075
Bacterial vaginosis (BV)	13 (14)	10 (12)	3 (30)	2.6	3.09	0.758-11.82	0.147
Syphilis	1 (1)	1 (1)	0 (0)	0	0	0.0-73.80	>0.999
Trichomoniasis	9 (10)	8 (10)	1 (10)	1.03	1.03	0.0841-7.19	>0.999
History of experiences	Number (%)						
Previous pregnancy	71 (77)	68 (83)	3 (30)	0.127	0.088	0.038-0.419	**0.0010**
Previous PTB	7 (8)	5 (6)	2 (20)	3.04	3.85	0.67-20.98	0.166
Previous miscarriage	26 (28)	23 (28)	3 (30)	1.09	1.10	0.29-4.63	>0.999
Previous BV	18 (20)	16 (20)	2 (20)	2.60	3.09	0.76-11.82	0.147
Previous short cervix	1 (1)	1 (1)	0 (0)	0	0	0.0-73.8	>0.999
Previous low birth weight	4 (4)	4 (5)	0 (0)	0	0	0.0-9.55	>0.999
Previously homeless	26 (28)	22 (27)	4 (40)	1.69	1.82	0.53-6.37	0.460
Experienced intimate partner violence	35 (38)	30 (37)	5 (50)	1.62	1.73	0.50-5.94	0.496
Current pregnancy experiences	Number (%)						
BV Symptoms	16 (17)	15 (18)	1 (10)	0.5278	0.4963	0.04263-3.762	>0.9999
BV Treatment	9 (10)	9 (11)	0 (0)	0	0	0.0-3.669	0.5897
High risk	48 (52)	43 (52)	5 (50)	0.9167	0.907	0.2661-3.096	>0.9999
Addiction counseling	41 (45)	38 (46)	3 (30)	0.5331	0.4962	0.1332-2.026	0.5029
Smoking	40 (43)	38 (46)	2 (20)	0.325	0.2895	0.05965-1.339	0.1774
Male baby	47 (51)	40 (49)	7 (70)	2.234	2.45	0.6000-9.109	0.3167

Significance is indicated by a bolded p Value.

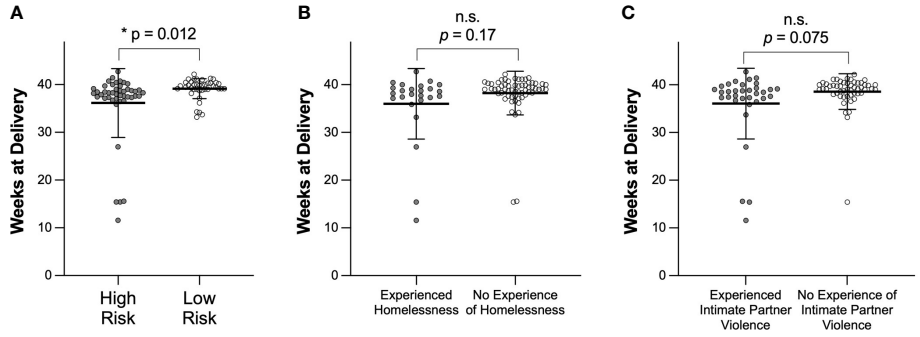

FIGURE 1
Comparison of weeks of pregnancy at time of delivery based on **(A)** clinical designation of high or low risk, **(B)** experience of homelessness, or **(C)** experience of intimate partner violence. Data are shown as mean ± SD. Significance was determined using a t test with Welch's correction. n.s. indicates not significant.

both groups of participants who had experienced homelessness and those who had experienced intimate partner violence trended towards shorter median weeks of pregnancy at delivery than participants who had not.

Participants in this study provided a total of 432 samples. Based on exclusion criteria, 377 samples were used in analyses for mucus changes during pregnancy, where 17 participants provided 1 sample each, 15 participants provided 2 samples each, 12 participants provided 3 samples, 8 participants provided 4 samples, 13 participants provided 5 samples, 11 participants provided 6 samples, 6 participants provided 7 samples, 4 participants provided 8 samples, 4 participants provided 9 samples, 1 participant provided 10 samples, and 1 participant provided 11 samples.

Weeks of pregnancy does not predict cervicovaginal mucus (CVM) barrier properties

We sought to investigate how pregnancy may impact mucus barrier properties. Using MPT, we quantified probe particle motion in CVM samples self-collected by participants approximately every 4 weeks from study enrollment. We first compared particle mobility in all CVM samples collected in this study. Supplementary Figure 2 shows differences in HIV, as well as CP and MPP mobility for 100, 200, and 500 nm particles. HIV showed 15.7 ± 21.35% mobility across all samples. CPs are both adhesively and sterically hindered by interactions with mucus, and MPPs are trapped only *via* steric interactions. Consistent with what previous observations in CVM, CP were less mobile than MPPs across 100 (18% v 78%), 200 (15% v 77%), and 500 nm (8% v 65%) particle sizes. Additionally, we observed a significant decrease in 500 nm CP and MPP mobility as compared to 100 and 200 nm CP and MPP, respectively. Figure 2 demonstrates that there were no significant trends in overall particle mobility over the course of pregnancy. Further, we demonstrate in Supplementary Figure 3 and Supplementary Table 2 that using MPP mobility as an indicator of the surrounding mesh structure, the average pore size in the CVM was 309 nm, consistent with our previous findings in CVM from non-pregnant women (Lai et al., 2010).

Pregnancy outcomes are linked to composition of the vaginal microbiome

We next determined the composition of the vaginal microbiome for each sample collected in this study. We utilized 16S rRNA gene sequencing (75,363 ± 1,729 reads per sample) to assign each sample into one of five previously defined community state types (CSTs): CST I was marked by a dominance of *L. crispatus*; CST II by a dominance of *L. gasseri*; CST III by *L. iners*; CST IV included samples which were polymicrobial; and CST V by *L. jensenii* (Ravel et al., 2011). A heatmap depicting the relative abundance of bacteria species measured in each sample can be found in Supplementary Figure 4. Of the 432 samples collected in this study, 29% were characterized as CST I (n = 125); 4% CST II (n = 16); 32% CST III (n = 138); 28% CST IV (n = 122); 7% CST V (n = 30). One sample was unable to be sequenced due to lack of sample. Figure 3 depicts samples from each individual participant, grouped by whether the participant delivered at full term or delivered prematurely, and shows the breakdown of CSTs for samples collected over time. Consistent with what was previously described, many participants in our study experienced shifts in CST over time, with shifts from CST III to CST I, and CST III to CST IV being the most common (Figure 3).

After assigning each sample to a CST, we analyzed PTB risk based on composition of the vaginal microbiome across pregnancy, as previous work demonstrated that CST IV was associated with an increased risk of PTB (Romero et al., 2014a; DiGiulio et al., 2015; Koullali et al., 2016; Callahan et al., 2017; Kindinger et al., 2017; Amabebe and Anumba, 2018; Elovitz et al., 2019). Figure 4 shows the percentage of all samples collected that were classified in each CST for participants who delivered at term (Figure 4A), or preterm (Figure 4B). In participants who delivered at term, 45% of all samples collected were CST I, 6.2% CST II, 30% CST III, 9.4% CST IV, and 9.4% CST V. In participants who delivered prematurely, 19.2% of samples were CST I, 0% CST II, 50% CST III, 23.1% CST IV, and 7.7% CST V.

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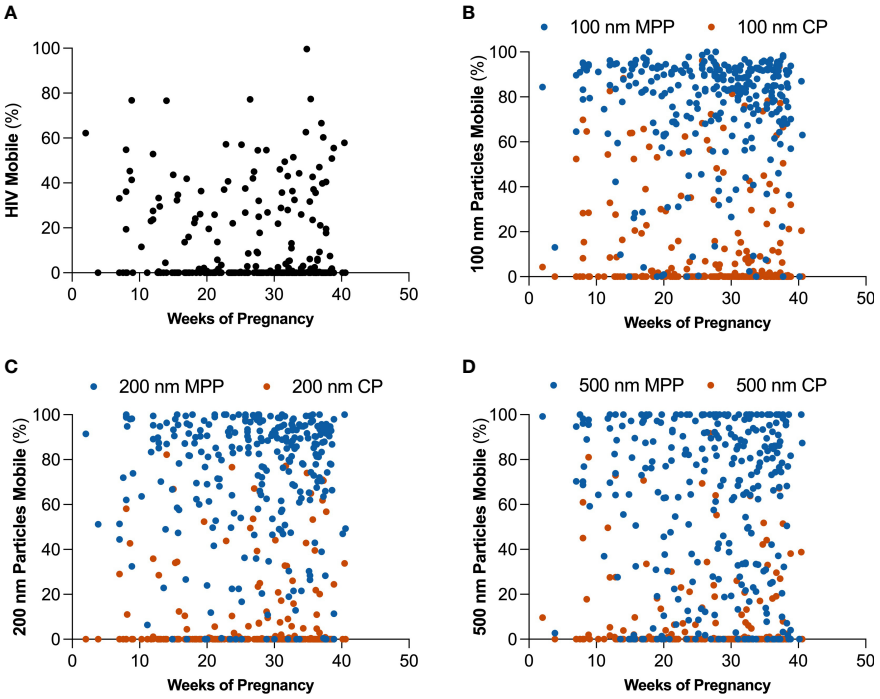

FIGURE 2
Changes in estimated percentage of mobile particles at τ = 1 s over the course of pregnancy for **(A)** HIV virion, **(B)** 100 nm mucus penetrating particles (MPP) in blue and 100 nm conventional particles (CP) in orange, **(C)** 200 nm MPP in blue and 200 nm CP in orange, and **(D)** 500 nm MPP in blue and 500 nm CP in orange.

The composition of the vaginal microbiome is the dominant factor associated with changes to the physical properties of CVM during pregnancy

We previously observed that the physical properties of CVM from non-pregnant participants are affected by the composition of the vaginal microbiome (Zierden et al., 2020). Supplementary Figure 5 shows the pH, Nugent score, total lactic acid, and total D-lactic acid for samples collected from our pregnant cohort classified in each CST. These data are consistent with what we have previously observed in samples from non-pregnant participants, where CST IV was characterized by an increased pH, increased Nugent score, and decreased lactic acid content. Similarly, CST III (*L. iners* dominated) showed decreased D-lactic acid, consistent with previous reports that *L. iners* produces only L-isoform of lactic acid. We further demonstrate, in Supplementary Figure 6, that the pH of the sample was significantly correlated with Nugent score, total lactic acid, and D-lactic acid.

Figure 5 shows particle mobility in samples classified in each CST. These data are represented as $\log_{10}$(MSD) at τ = 1 s in Supplementary Figure 7. Across all particle types, CST I showed most trapping, and CST II showed least trapping. Consistently, CST IV showed reduced barrier properties as compared to CST I. CST I showed almost complete trapping of HIV (1.1 ± 6.6% mobile), and the average percentage of mobile particles increased from CST III < CST V < CST II < CST IV up to 34.3 ± 23.7% (Figure 5A). The percentage of mobile HIV particles in CST I samples was significantly reduced compared to CST II ($p < 0.014$) or CST IV ($p < 0.0001$) samples, and the percentage of mobile HIV particles was significantly increased in CST IV samples compared to CST III ($p < 0.0001$), and CST V ($p = 0.0001$) samples. Figure 5B shows mobility of 100 nm CP. CST I showed the most trapping at 4.0 ± 7.5% mobility, whereas CST II showed the least trapping at 54.3 ± 35.1% mobility (Figure 5B). 100 nm CP were significantly less mobile in CST I samples than in CST II ($p = 0.0018$) or CST IV ($p < 0.0001$), whereas CST IV samples showed decreased hindrance of 100 nm CP as compared to CST III ($p < 0.0001$), and CST V ($p = 0.0002$). The estimated percentage of 100 nm MPP in each CST is shown in Figure 5C. CST I showed the most trapping at 72.5 ± 16.7% mobility, whereas CST II showed 93.7 ± 4.0% mobility. CST IV showed 87.1 ± 12.5% mobility. 100 nm CP were significantly less mobile in CST I samples than in CST IV ($p = 0.012$). Figure 5D shows mobility of 200 nm CP. CST I showed the least mobility at 3.2 ± 8.9%, whereas CST II showed the most mobility at 35.5 ± 44.4%. 100 nm CP were significantly less mobile in CST I samples than in CST IV ($p < 0.0001$), and CST IV samples showed decreased hindrance of 200 nm CP as compared to CST III ($p = 0.0012$), and CST V ($p = 0.0026$). The 200 nm MPP mobility for each CST is shown in Figure 5E. CST I showed 62.8 ± 22.6% mobility, followed by CST V, CST III, CST IV, and finally CST II with 97.4 ± 2.0% mobility. 200 nm MPP were significantly more mobile in CST IV than in CST I ($p < 0.0001$) and CST V ($p = 0.049$). Figure 5F shows mobility of 500 nm CP where CST I showed 0.31 ± 0.86% mobility. CST I < CST V < CST III < CST IV < CST II at 23.5 ± 21.2% mobility. CST IV samples showed decreased hindrance of 500 nm CP as compared to CST I ($p < 0.0001$), CST III ($p = 0.0004$), and CST V ($p =$

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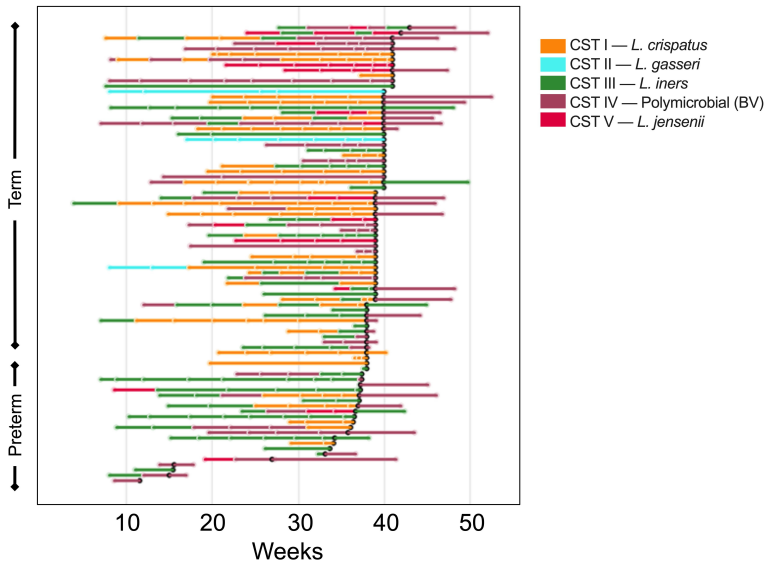

FIGURE 3
Changes in vaginal microbiome CST for each participant in the study over the course pregnancy. The lines are colored to correspond to the CST of the prior sampling, and each break in the line indicates a new sample. The bar color indicates the CST as shown in the key, where orange represents CST I, blue CST II, green CST III, purple CST IV, and pink CST V. The black dot represents time of delivery, and the lines following the black dot represent the postpartum sample.

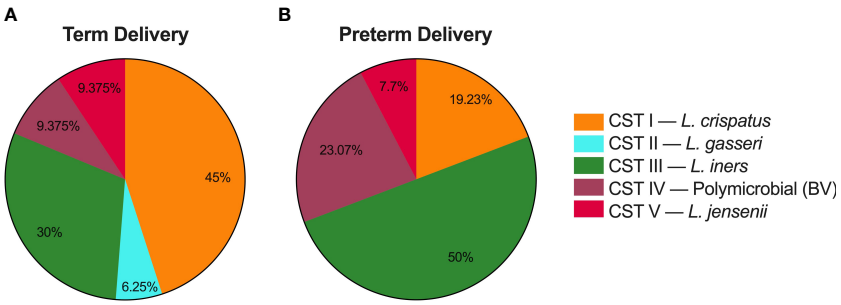

FIGURE 4
Delivery times based on CVM microbial composition for samples collected as part of this study. **(A)** Percentage of samples from participants who delivered at term that were characterized as community state type (CST) I, CST II, CST III, CST IV, and CST V. **(B)** Percentage of samples from participants who delivered prematurely that were characterized as each CST.

0.0052). Finally, 500 nm MPP estimated mobility is shown in Figure 5G. CST I showed 52.6 ± 25.5% mobility. Particle mobility was the highest in CST II at 87.7 ± 8.1%. 100 nm CP were significantly less mobile in CST I samples than in CST IV samples ($p < 0.0001$). As expected, particle mobility is significantly positively correlated with sample pH (Supplementary Figure 8). Furthermore, we observed a significant difference in average pore size between CST I and CST IV samples (Supplementary Figure 9). Consistent with Figure 2, there were no significant differences in particle mobility over the course of pregnancy when samples were separated by CST (Supplementary Figure 10).

Cytokine concentrations in CVM did not correlate with weeks of pregnancy or CST

We next investigated cytokine concentration in the five CSTs across pregnancy. Figure 6 shows changes in IL-10 (Figure 6A), IL-1β (Figure 6C), IL-6 (Figure 6E), and TNF-α (Figure 6G) over the course of pregnancy. We observed no significant trends in the changes of cytokine levels over the course of pregnancy. Figure 6 also shows differences in cytokine levels between the five CSTs. We observed no significant differences in IL-10, IL-1β, IL-6, or TNF-α in CVM samples across the CSTs.

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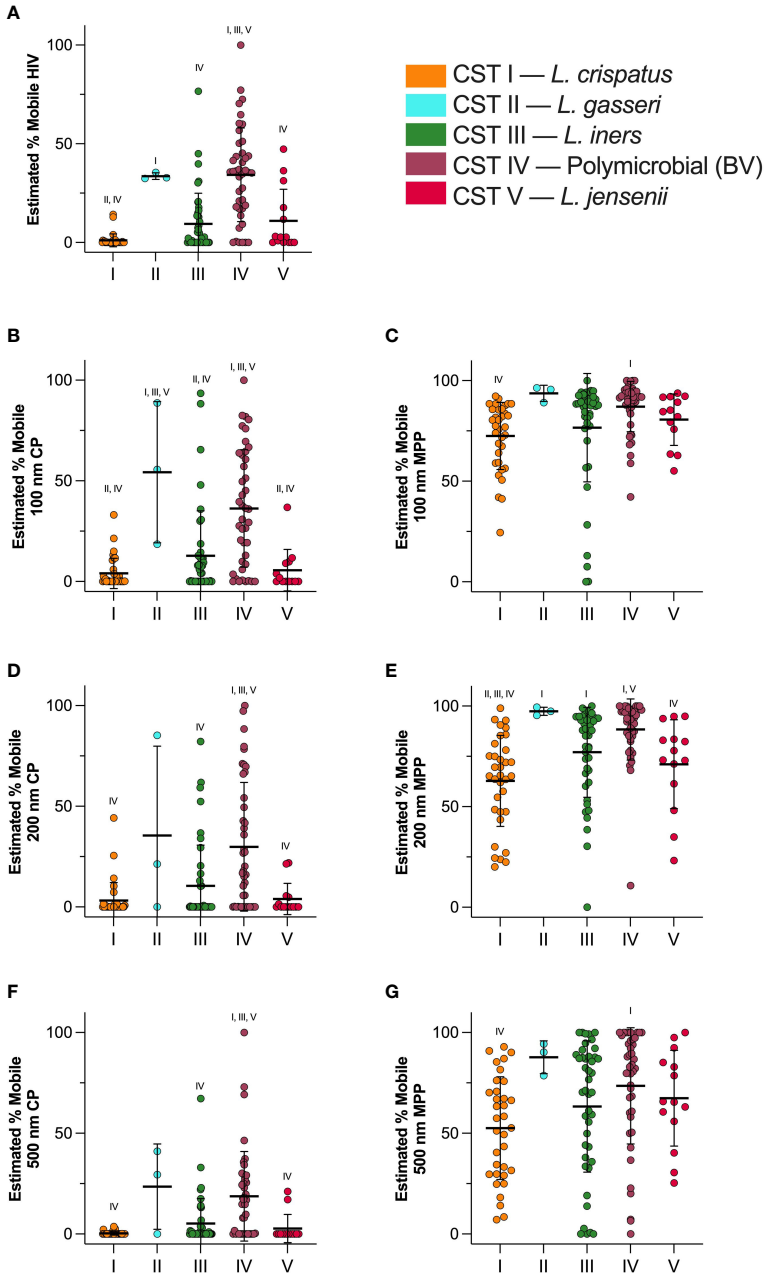

FIGURE 5
Estimated percentage of mobile particles at τ = 1 s in each CST. **(A)** HIV virion mobility, **(B)** 100 nm CP mobility, **(C)** 100 nm MPP mobility, **(D)** 200 nm CP mobility, **(E)** 200 nm MPP mobility, **(F)** 500 nm CP mobility, **(G)** 500 nm MPP mobility. Data are shown as mean ± SD. Significant differences are represented by CST groups listed above each dataset ($p < 0.05$). Significance was determined using ANOVA with Tukey's multiple comparison test.

Discussion

The human body is equipped with a mucus barrier that protects exposed epithelial tissues (Cone, 2009). Mucus is comprised of crosslinked glycoproteins that form a net-like structure capable of trapping and eliminating foreign pathogens from the body (Cone, 2009). This mucus mesh has small pores that sterically hinder bacteria and large pathogens from reaching the underlying epithelium (Lai et al., 2010). Additionally, mucin proteins are comprised of hydrophobic and negatively charged regions that serve to adhesively trap particulates (Cone, 2009). Mucus is continuously secreted, facilitating the clearance of foreign entities that may have been trapped either sterically or adhesively by the mucus mesh (Cone, 2009). In the female reproductive tract, CVM protects women from sexually transmitted infections, such as herpes simplex virus (HSV) and HIV-1, and from bacteria that can lead to urinary tract infections (UTIs), pelvic inflammatory disease (PID), and intrauterine infection (Cone, 2009; Lai et al., 2009a; Nunn et al., 2015; Hoang et al., 2020). In the context of pregnancy, CVM further serves to protect the developing fetus from infection that may lead to inflammation, resulting in adverse fetal outcomes and PTB (Howe et al., 1999; Cone, 2009; Koullali et al.,

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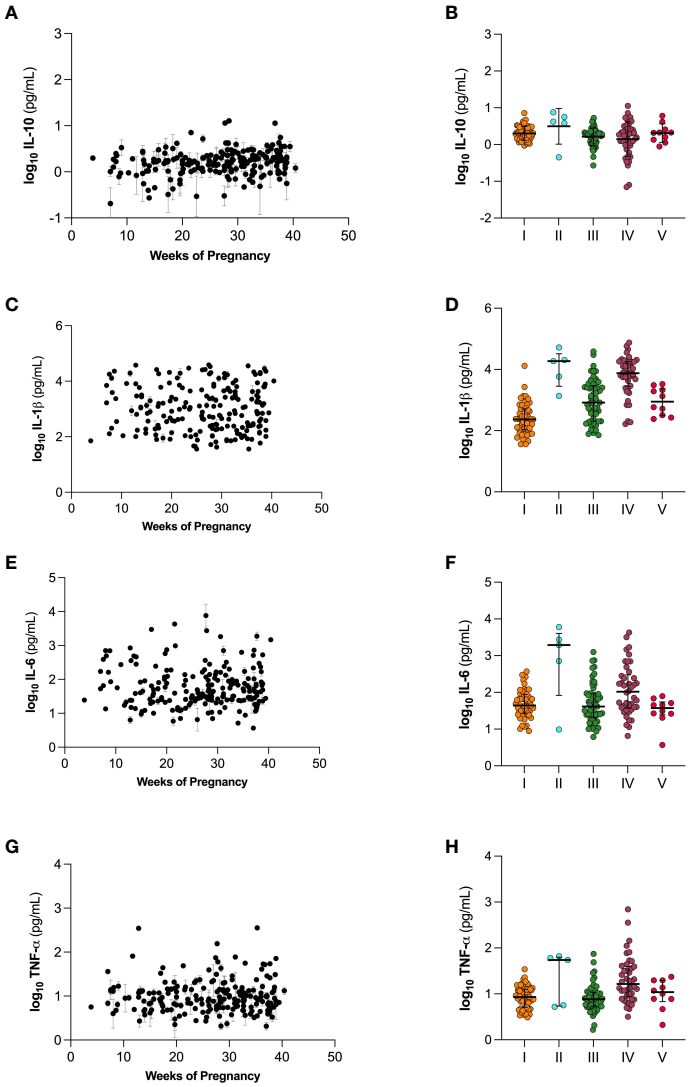

FIGURE 6
Cytokine levels over the course of gestation **(A, C, E, G)** and separated by vaginal microbiome CST **(B, D, F, H)**. **(A, B)** IL-10, **(C, D)** IL-1β, **(E, F)** IL-6, and **(G, H)** TNF-α. Individual datapoints in **(A, C, E, G)** are shown as mean ± SEM based on technical replicates. Data in **(B, D, F, H)** are shown as mean ± SD.

2016; Amabebe and Anumba, 2018). Despite these benefits, the vaginal mucus barrier also serves as a challenge to effective vaginal drug administration. We have demonstrated that vaginal drug delivery is optimal for targeting the female reproductive tract and preventing PTB in preclinical mouse models (Hoang et al., 2019; Zierden et al., 2021a). Here, we characterized CVM barrier properties and the vaginal microbiome over the course of pregnancy in order to define design criteria for vaginally administered therapeutics for obstetric indications. Importantly, the participants included in this study represent a wide range of race and ethnicities, as well as socioeconomic backgrounds.

Vaginal drug delivery increases local drug levels, while minimizing unwanted, off-target side effects (Ensign et al., 2014). Additionally, the uterine first pass effect facilitates recirculation of drug around the uterus, before reaching systemic circulation (De Ziegler et al., 1997). However, as an adhesive net designed to trap foreign pathogens, CVM also serves as a barrier to effective vaginal drug delivery (Cone, 2009). While many studies explore the relationship between the vaginal microbiome and pregnancy outcomes for both mother and neonate, few studies describe changes to mucus structure over the course of pregnancy. During the normal 28-day menstrual cycle, CVM properties change based on fluctuating levels of progesterone and estrogen (Moreno-Escallon et al., 1982). As estrogen increases, and the body prepares for ovulation, the water content of cervical mucus increases and the average pore size increases, to facilitate sperm transport through mucus (Mihm et al., 2011). As progesterone increases in the luteal phase, mucus thickens, and the effective pore size decreases (Moreno-Escallon et al., 1982; Mihm et al., 2011). During pregnancy, endogenous progesterone levels increase until the point of delivery. Characterization of cervical mucus collected from the external cervical os, using shear rheology and particle permeability experiments revealed that samples collected from high-risk patients showed increased spinnbarkeit and weakened

crosslinking (Critchfield et al., 2013). Furthermore cervical mucus collected from participants considered high-risk was more permeable to 200 nm particles (Critchfield et al., 2013). These data are important for potentially identifying patients at risk for PTB, but do not consider vaginal microbiota, which we show plays a role in mucus barrier properties. Here, we used multiple particle tracking (MPT) with both CPs and MPPs in fresh, undiluted CVM samples, to probe adhesive properties and pore size of CVM over the course of pregnancy and in the context of the vaginal microbiome. MPPs are engineered to be coated with a high density of low-molecular weight polyethylene glycol (Nance et al., 2012). This coating results in a net neutral surface charge, and shields the hydrophobic polystyrene nanoparticle from adhesive interactions with the mucin proteins, so while conventional particles (CPs) are trapped adhesively by mucins, tracking MPPs allows us to measure steric interactions between the mucus mesh and particles of various sizes (Lai et al., 2009b; Lai et al., 2010). We previously used this technique to demonstrate that the average pore size of CVM self-collected by reproductive age, non-pregnant participants was ~340 ± 70 nm, much larger than the previous estimate of ~100 nm average size (Lai et al., 2010).

We previously demonstrated that utilizing MPP technology, we can engineer drug-loaded nanoparticles for effective vaginal drug delivery that provide improved drug delivery to the female reproductive tract compared to clinically used formulations (Ensign et al., 2013; DeLong et al., 2019). In a second study, we demonstrated that effective drug delivery can reveal mechanisms of inflammation-induced PTB, and help to identify therapeutics capable of preventing inflammation-induced PTB (Zierden et al., 2020; Zierden et al., 2021a). In a murine model of inflammation-induced PTB, we demonstrated that a novel combination of vaginally delivered drug-loaded MPPs was able to prevent PTB, whereas systemic administration of the same drug combination was ineffective (Zierden et al., 2021a). Here, using probe nanoparticles, and fluorescently labeled virions, we observed how particle mobility was affected by the heterogenous network of mucin proteins (Lai et al., 2009b). Consistent with studies using samples from non-pregnant participants, we observed that the mobility of 500 nm MPP was significantly more hindered than either 100 nm MPP or 200 nm MPP. However, we observed no significant impact of the longitudinal time throughout pregnancy on the barrier properties of CVM. Rather, we observed that the composition of the vaginal microbiome was more impactful. We used 16S rRNA gene sequencing to assign each sample to a community state type (CST) (DiGiulio et al., 2015; Gosmann et al., 2017; DeLong et al., 2019). As we previously observed in a non-pregnant cohort, particles and virions were more trapped in samples with a dominance of *L. crispatus* (CST I), as compared to polymicrobial samples (CST IV). Using MPP mobility to calculate the distribution of pore sizes in the surrounding environment, we observed that the average pore size in CVM from pregnant participants did not change over the course of pregnancy, and that the pore size was similar to that observed in prior studies characterizing CVM from non-pregnant participants (Lai et al., 2010). However, CVM from women with microbiota classified as CST I showed a smaller average pore size than that of CST IV, consistent with particle mobility data.

We have previously described that *ex vivo* particle mobility was predictive of vaginal particle distribution *in vivo* (Ensign et al., 2012). Here, our data demonstrate that for a vaginally delivered nanoformulation to be most effective, taking into consideration the most stringent case (CST I, first trimester), the particles should be mucus penetrating and smaller than 500 nm in size (Ensign et al., 2012; Ensign et al., 2013). Furthermore, as microbiota have the greatest effect on CVM barrier function, formulations should not perturb the vaginal environment and the microbiota. This includes having a low pH (~4.0), and not being hypertonic, which would cause irritation and inflammation in the vaginal compartment (Ensign et al., 2013; Zierden et al., 2021a; Zierden et al., 2021b). Vaginal formulations incorporating these criteria are likely to provide more efficient drug delivery to treat indications during pregnancy, including the prevention of PTB.

Many studies have investigated the human vaginal microbiome during pregnancy, and its implications in PTB (Romero et al., 2014a; Romero et al., 2014b; Elovitz et al., 2019; Gerson et al., 2019; Serrano et al., 2019). Generally, *Lactobacillus* spp. are regarded as beneficial, and are associated with term deliveries. In contrast, patients with CST IV (polymicrobial, BV) vaginal microbiota are more likely to experience PTB than patients with vaginal microbiota dominated by *L. crispatus* (DiGiulio et al., 2015). It is hypothesized that BV leads to decreased barrier function of CVM, allowing the ascension of bacteria or other inflammatory stimuli from the vaginal environment into the uterine compartment. Clinically, patients with BV often report an excess of thin, watery discharge. Indeed, others measured a decrease in mucus viscosity associated with BV using macrorheology (Chappell et al., 2014). Here, we demonstrated that, regardless of timing of pregnancy, CST IV samples showed decreased barrier properties as compared to CST I samples. We previously observed that CST III samples show decreased barrier properties (Zierden et al., 2020). Clinically, women with CST III vaginal microbiota are also at an increased risk for PTB (Kindinger et al., 2017). While CST III did not show significant differences in HIV and CP mobility as compared to CST I, we observed a significant increase in mobility of 200 nm MPP and 500 nm MPP. Increased mobility of 200 nm and 500 nm MPPs in CST III is indicative of larger pore sizes, which are ultimately less protective than smaller pores which have the ability to immobilize a wider range of pathogens, fitting with our previous findings regarding *L. iners*-dominated CVM (Hoang et al., 2020).

One potential mechanism by which CVM barrier properties are diminished is *via* degradation of mucins by BV-associated bacteria. In mice, it has been shown that *G. vaginalis*, a BV-associated species, is associated with an increase in sialidases and other enzymes capable of breaking down the CVM barrier (Howe et al., 1999; Wiggins et al., 2001; Cauci et al., 2008; Gilbert et al., 2013; Lewis et al., 2013). In mice, vaginal inoculation with *G. vaginalis* led to increased levels of IL-6, IL-10, IL-1β, and TNF-α (Anton et al., 2018; Sierra et al., 2018). *Mobiluncus mulieris*, another species highly associated with BV, has been shown to upregulate IL-6 and IL-8 in culture (Elovitz et al., 2019; Dude et al., 2020). While we

observed no significant differences in the cytokine concentration of CVM samples in this cohort, we hypothesize this may be attributed to differences in the method of sample collection. Further, this may be related to molecular characterization of CST IV, rather than characterization *via* Amsel's criterion or clinical diagnosis with symptomatic BV.

While our study established a connection between the vaginal microbiota and altered mucus barrier properties during pregnancy, incomplete longitudinal data and participant attrition limited the ability to find longitudinal associations. The diversity of the participant demographics was a strength of the study, but the sample size was too small to make conclusions about how participant demographics may affect PTB rates. Previous work associated race and ethnicity with risk for PTB, where Black and African American patients, as well as Hispanic patients, are at a higher risk for adverse obstetric outcomes (Alcendor, 2016; Callahan et al., 2017). Similarly, Black and African American women have an increased for being diagnosed with BV (Alcendor, 2016). Additionally, there is evidence that socioeconomic status, intimate partner violence, and stress affect the composition of the vaginal microbiome and pregnancy outcomes, further compounding observed trends from a human dataset (Turpin et al., 2021). Several larger studies integrated data from thousands of samples to identify important associations between vaginal microbiota, mucosal cytokines, demographics, and timing of parturition (Fettweis et al., 2019; Serrano et al., 2019).

The work here quantifies a mucus pore size in relation to time of pregnancy, as well as the vaginal microbiome. Our data demonstrate that the vaginal microbiome was the best predictor of CVM barrier properties. These results support what has been shown in recent studies, that not having a *L. crispatus* dominated vaginal microbiome is a risk factor for PTB (Kindinger et al., 2017). PTB is a global issue, impacting 15 million pregnancies each year (Cunningham et al., 2018). Infants born prematurely experience a wide range of health burdens, including necrotizing enterocolitis, retinopathy of prematurity, cerebral palsy, hearing impairments, among others. Currently, a sonographic short cervix, and a history of premature delivery, are the only two risk factors used to identify a woman at risk for PTB. It is hypothesized that the bacteria from the polymicrobial environment can ascend into the uterine environment, leading to inflammation and prematurely initiating biological mechanisms of parturition. Here, we use multiple particle tracking (MPT) to demonstrate that polymicrobial CVM samples from pregnant participants have significantly diminished cervicovaginal mucus (CVM) barrier properties, as compared to participants with *L. crispatus* dominated microenvironments. Our findings contribute to the potential for analyzing CVM properties to identify patients at risk for PTB, and further define design criterion for engineering therapeutics for vaginal delivery during pregnancy.

Data availability statement

The data presented in the study are deposited in the Sequence Read Archive (SRA) repository, accession number PRJNA942697, found at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA942697. All other relevant data are included within the paper and supplemental information.

Ethics statement

The studies involving human participants were reviewed and approved by Johns Hopkins University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

Author contributions

HZ, KD, and LE conceived and designed the study. HZ, FZ, VL, SB, NH, JO, and TH performed experiments. KD conducted statistical analyses. SL provided HIV-1 fluorescently labeled with mCherry-Gag. AB, JO, and HZ enrolled participants. HZ, KD, JH, and LE interpreted results. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The mucus penetrating particle technology is licensed and in clinical development for ocular indications by Kala Pharmaceuticals. JH is a founder of Kala Pharmaceuticals and serves as a consultant. JH, LE, and Johns Hopkins own company stock. Under a licensing agreement between Kala Pharmaceuticals and the Johns Hopkins University, LE, JH, and the University are entitled to royalty distributions related to the technology. LE is a co-founder and chair of the scientific advisory board of Freya Biosciences. These arrangements have been reviewed and approved by the Johns Hopkins University in accordance with its conflict of interest policies.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1015625/full#supplementary-material

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Inhibitory effects of vaginal *Lactobacilli* on *Candida albicans* growth, hyphal formation, biofilm development, and epithelial cell adhesion

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Introduction: Antifungal agents are not always efficient in resolving vulvovaginal candidiasis (VVC), a common genital infection caused by the overgrowth of *Candida* spp., including *Candida albicans*, or in preventing recurrent infections. Although lactobacilli (which are dominant microorganisms constituting healthy human vaginal microbiota) are important barriers against VVC, the *Lactobacillus* metabolite concentration needed to suppress VVC is unknown.

Methods: We quantitatively evaluated *Lactobacillus* metabolite concentrations to determine their effect on *Candida* spp., including 27 vaginal strains of *Lactobacillus crispatus, L. jensenii, L. gasseri, Lacticaseibacillus rhamnosus*, and *Limosilactobacillus vaginalis*, with inhibitory abilities against biofilms of *C. albicans* clinical isolates.

Results: *Lactobacillus* culture supernatants suppressed viable fungi by approximately 24%-92% relative to preformed *C. albicans* biofilms; however, their suppression differed among strains and not species. A moderate negative correlation was found between *Lactobacillus* lactate production and biofilm formation, but no correlation was observed between hydrogen peroxide production and biofilm formation. Both lactate and hydrogen peroxide were required to suppress *C. albicans* planktonic cell growth. *Lactobacillus* strains that significantly inhibited biofilm formation in culture supernatant also inhibited *C. albicans* adhesion to epithelial cells in an actual live bacterial adhesion competition test.

Discussion : Healthy human microflora and their metabolites may play important roles in the development of new antifungal agent against *C. albicans*-induced VVC.

KEYWORDS

***Candida albicans*, *Lactobacillus* species, biofilm, probiotics, cell adhesion**

1 Introduction

Fungal diseases cause considerable morbidity and mortality, resulting in a high economic burden (Drgona et al., 2014; Bongomin et al., 2017). Vulvovaginal candidiasis (VVC), a common genital infection, is commonly caused by *Candida albicans*, with a lifetime prevalence of up to 78% in women (Yano et al., 2019; Willems et al., 2020). Eight percent of women with VVC experience recurrent VVC (RVVC), which relapses more than four times a year due to the low response to antifungal treatment, including the use of azoles such as fluconazole (Denning et al., 2018; Cooke et al., 2022). The highest prevalence of RVVC occurs among 25-34-year-olds, and it has an annual economic burden of US$14-39 billion in developed countries because it reduces the quality of life (Denning et al., 2018). The emergence and spread of antimicrobial resistance (AMR) have become a global concern, and fungal infections have been excluded from the AMR program (Fisher et al., 2022). However, as with bacterial infections, the use of antifungal drugs is strongly implicated in the occurrence of pathogenic fungi, and thus new methods of prevention or treatment of RVVC that are not dependent on antifungal use are required (Matsubara et al., 2016).

C. albicans is a dimorphic fungus that can transform from yeast to an invasive filamentous hyphal form (Sudbery, 2011; Willems et al., 2020). Biofilm formation by *C. albicans* commonly consists of four major stages: yeast cells adhere to a substrate to form a yeast basal layer; initiation of propagating cells where the hyphae are formed; hypha formation and extracellular matrix accumulation with extracellular polysaccharides, structural proteins, cell debris, and nucleic acids; and dispersion of yeast cells from the biofilm to initiate biofilms at new locations (Chandra and Mukherjee, 2015). These biofilm structures are intrinsically resistant to antifungals, making VVC difficult to combat (Silva et al., 2017).

The vaginal microbiota of humans is known to be less complex than the intestinal microbiota and is usually dominated by the genus *Lactobacillus* (Matsumoto et al., 2018). The disruption of this vaginal microbiota promotes colonization by pathogenic microorganisms that leads to bacterial vaginosis and subsequent VVC (Ravel et al., 2013). With recent progress in sequencing technology, the presence of certain lactobacilli has been found to be associated with vaginal health. *Lactobacillus crispatus*-and *L. jensenii*-dominated vaginal microbiota are strongly associated with vaginal health (Chee et al., 2020). Furthermore, a *L. iners*-dominated environment could be affected by vaginal dysbiosis (Chee et al., 2020). These species contribute to vaginal homeostasis mainly by producing metabolites, including lactate and hydrogen peroxide, although their production abilities vary among isolates of the same species (Witkin et al., 2013).

In previous studies, production of lactate and hydrogen peroxide has been evaluated using a qualitative method (Matsubara et al., 2016; Ribeiro et al., 2017; Wang et al., 2017; Aarti et al., 2018; Rossoni et al., 2018). However, studies on quantitative evaluation of the metabolite are insufficient. This study aimed to quantitatively evaluate the metabolites of lactobacilli to determine the effects of lactobacilli on *C. albicans* growth, hyphal formation, biofilm development, and epithelial cell adhesion.

2 Materials and methods

2.1 Strains

Forty-five *C. albicans* strains, which were clinically isolated from the vagina and provided by Microskylab Inc. (Tokyo, Japan), were used in this study. All 27 *Lactobacillus* strains were previously obtained from vaginal swabs of healthy Japanese women at Aichi Medical University (Matsumoto et al., 2018). These strains belonged to five species: *L. crispatus*, *L. jensenii*, *L. gasseri*, *Lacticaseibacillus rhamnosus*, and *Limosilactobacillus vaginalis*. The characteristics of these bacterial strains are listed in Supplementary Table 1.

2.2 Biofilm formation and quantification

A 96-well microtiter plate-based method was used in this study (Lohse et al., 2018). *C. albicans* strains were cultured for 24 h in Yeast Peptone Dextrose (YPD) agar (Difco, Detroit, MI, USA) at 30°C under aerobic conditions. A single colony was inoculated into the YPD broth medium and incubated overnight for 16 h at 30°C, accompanied with shaking at 160 rpm under aerobic conditions. Under these conditions, *C. albicans* strains grew to the budding yeast forms (blastospores). The cells were centrifuged at 3,500 ×g for 10 min and re-suspended in RPMI 1640 medium buffered with morpholinepropanesulfonic acid (MOPS) at a concentration of 10^7 cells/mL, and 100 µL of the inoculum was seeded into a 96-well microtiter plate. The biofilms formed on the surface of the wells were gently washed twice with phosphate-buffered saline (PBS) after 48 h of incubation at 37°C. The yeast cells were not washed immediately after the initial adhesion, and thus, the final time point (48 h) reflects the total biomass that could not be initially adhered

to. Crystal violet (CV) (Merck KGaA, Darmstadt, Germany) and water-soluble tetrazolium salts (WST-1) (TaKaRa, Shiga, Japan) were used in this study (Mukherjee et al., 2005; Weber et al., 2008). CV stained the whole biomass, including dead cells and polysaccharides, whereas WST was converted to a colored formazan in the presence of metabolic activity. To quantify the total biomass, washed biofilms were stained with 0.1% (w/v) CV solution for 1 min. Each well was washed twice with PBS and dried for 30 min. The bounded CV was eluted using 99.5% (v/v) ethanol. The burden of viable cells was estimated using WST-1 based on the reduction of tetrazolium salt. To each well, we added 100 µL of PBS and 10 µL of premixed WST-1, and the mixture was incubated at 37°C for 3 h under shade conditions. The absorbance (Abs) of CV and WST-1 was measured at 570 nm and 440 nm, respectively. Eight replicate wells were used for each strain, and experiments was repeated three times, independently.

2.3 Quantitative reverse transcription polymerase chain reaction

ISOGEN II (Nippon Gene, Co., Ltd., Tokyo, Japan) was used for total RNA extraction from the *C. albicans* HB-10 strain. The RNA concentrations were measured using a Qubit® RNA Assay Kit (Promega, WI, USA). To prepare complementary DNA, the PrimeScript™ RT reagent kit (TaKaRa, Shiga, Japan) was used in accordance with the manufacturer's instructions. Moreover, qRT-PCR analysis was performed using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Shiga, Japan) in accordance with the manufacturer's protocol. Briefly, PCR was performed in a reaction mixture of TB Green Premix Ex Taq II (2 ×) 12.5 µL, PCR forward primer 1 µL, PCR reverse primer 1 µL, and RNase free dH_2O 8.5 µL added to 2 µL of each reverse transcription reaction solution. Primers used in this study are listed in Supplementary Table 2. The amplification conditions were as follows: 40 cycles under heat treatment at 95°C for 30 s, heat denaturation at 95°C for 5 s, and annealing at 55°C for 30 s, which is the optimum temperature for the primer. Melting curves were used to verify the quality of qRT-PCR, and the fold expression was calculated using the delta-delta Ct method.

2.4 Supernatants produced by *Lactobacillus*

Cell-free culture supernatants were extracted from *Lactobacillus* species. A single strain each was inoculated in de Man, Rogosa, and Sharpe (MRS) broth (Merck KGaA, Darmstadt, Germany) and incubated at 37°C for 72-h under anaerobic conditions (10% H_2, 10% CO_2, and 80% N_2) in an anaerobic chamber. Growth at the sampling point (72-h) was determined by measuring the optical density (OD) at 600 nm using a microplate reader (SH-9000Lab, HITACHI). The culture medium was then centrifuged at 3,500 ×*g* for 10 min and filtered through a 0.22-µm membrane filter (Sarutorius AG, Gettingen, Germany). Each collected culture supernatant was stored at −80°C until use.

2.5 High-performance liquid chromatography analysis of culture supernatants

HPLC (SHIMADZU, Kyoto, Japan) equipped with a conductivity detector was used to measure levels of lactate and short-chain fatty acids, such as acetate, propionate, and butyrate, in culture supernatants, as previously described (Hagihara et al., 2021). Briefly, the mobile phase required 5 mM p-toluenesulfonic acid (KANTO Chemical, Tokyo, Japan). The reaction buffer was made of 5 mM p-toluenesulfonic acid, 100 µM ethylenediaminetetraacetic acid (KANTO Chemical, Tokyo, Japan), and 20 mM bis (2-hydroxyethyl) aminotris (hydroxymethyl) methane (Tokyo Chemical Industry, Tokyo, Japan). The flow rate, oven temperature, and detector cell temperature were set at 0.8 mL/min, 40°C, and 48°C, respectively. The samples contained in 1.0 mL disposable vials (SHIMADZU Co., Kyoto, Japan) were held at 4°C in a sample cooler (SHIMADZU, Kyoto, Japan), and 10 µL was applied to tandemly arranged two columns (SHIMADZU, Kyoto, Japan) to measure lactate levels. The calibration curve solution adjusted with lithium DL-lactate (FUJIFILM Wako Pure Chemical, Co., Ltd., Osaka, Japan) was dissolved in deionized water. The quantification analyses for HPLC were performed using LabSolutions version 5.90 (SHIMADZU Co., Kyoto, Japan), and the peak area was used as the signal intensity.

2.6 Detection of hydrogen peroxide in culture supernatants

Hydrogen peroxide production was estimated using a hydrogen peroxide assay kit (ab102500, Abcam, MA, USA) according to the manufacturer's instructions. The stored culture supernatant was neutralized to pH 7.0, and 100 µL of the adjusted supernatants were reacted for 10 min in the presence of horseradish peroxidase. Duplicate wells were measured for each sample with absorbance at 595 nm, and experiments were repeated three times, independently.

2.7 Detection of pH in culture supernatants

The pH of cell-free culture supernatants and buffered RPMI 1640 medium, supplemented with culture supernatant, were measured promptly using a glass electrode-style hydrogen-ion concentration meter (Laqua, Horiba, Ltd., Japan). MRS (8%) was added to RPMI instead of *Lactobacillus* supernatant to achieve final concentrations of lactate and hydrogen peroxide standard of 4–64 mM and 4 nM–64 nM, respectively.

2.8 Measurement of minimum inhibitory concentration

C. albicans sHB-10 were cultured for 24 h in YPD agar at 30°C under aerobic conditions. A single colony was inoculated into the YPD broth medium and incubated overnight for 16 h at 30°C, accompanied with shaking at 160 rpm under aerobic conditions.

The cells were centrifuged at 3,500 ×g for 10 min and re-suspended in RPMI 1640 medium at a concentration of 10^6 cells/mL. Lactate and hydrogen peroxide were added to the RPMI broth with 8% MRS and adjusted to a concentration ranging from 0.5 to 1024 mM and 0.5 nM to 1024 mM, respectively. After 24 hours of incubation at 37°C, the turbidity of all well broth was visually observed, and the lowest concentration of lactate or hydrogen peroxide that suppressed the increased growth was determined as the MIC.

2.9 Effect of *Lactobacillus* culture supernatants on preformed *C. albicans* biofilm

The efficacy of the anti-biofilm activities of lactobacilli was determined by adding the culture supernatant of *Lactobacillus* to the preformed biofilm. *C. albicans* HB-10, which formed a mature biofilm in the assay described above, was selected and used for subsequent inhibition assays. A mature biofilm of *C. albicans* HB-10 was formed in a 96-well microtiter plate after 24 h of incubation under the same conditions as the biofilm formation and viability assay. The planktonic cells were aspirated from each well and washed twice with PBS. Cell-free supernatant extracted from a single *Lactobacillus* strain was added to each well at a final concentration of 8% (v/v) and incubated for 24 h. Culture supernatants were aspirated from each well and washed twice with PBS. Biofilm formation was quantified using CV and WST-1, as described above. The metabolic activity of the residual biofilm after the exposure of the *Lactobacillus* culture supernatant was quantified using WST-1 as described above. Eight replicate wells were used for each strains, and experiments was repeated three times, independently.

2.10 Effect of *Lactobacillus* culture supernatants on *C. albicans* hyphal formation

According to the hyphal formation method in the RPMI broth described previously (Wang et al., 2017), we estimated the effect of hyphal formation inhibition of *C. albicans* yeast-to-hyphal transition in the presence of *Lactobacillus* culture supernatants. *Lactobacilli* with strong inhibition of biofilm formation and those with low inhibition were selected. *C. albicans* HB-10 cells from overnight culture were washed with PBS and re-suspended at approximately 10^6 CFU/mL in RPMI 1640 medium buffered with MOPS. The yeast cell suspensions were then incubated with or without *Lactobacillus* culture supernatant at 37°C for 3 h. Quantification of the inhibitory effect of *Lactobacillus* on hyphal formation was performed using a light microscope (AxioCam MRc5; Carl Zeiss, Jena, Germany). The percentage of hyphal formation was calculated by obtaining the ratio of total number of *C. albicans* cells with hyphae to the total number of *C. albicans* cells counted. The number of yeast and hyphae (total cells) was counted using a hemocytometer (Supplementary Table 3).

2.11 Adhesion assay of *C. albicans* and *Lactobacilli*

Human cervical cancer HeLa cells (RCB0007; Riken BRC Cell Bank, similar to ATCC CCL2) were grown in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, MA, USA) supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher Scientific, MA, USA) and 1% (v/v) penicillin and streptomycin (FUJIFULM Wako Pure Chemical, Co., Ltd., Osaka, Japan) at 37°C under 5% CO_2 and humidity. HeLa cells were seeded into a 12-well plate (AGC Techno Glass Co., Ltd., Shizuoka, Japan) at approximately 1.0×10^5 cells per well and grown to confluence. After 90% confluency, each well was washed twice with PBS. *C. albicans* HB-10 and lactobacilli were grown under the conditions described above. Briefly, a single colony of *C. albicans* HB-10 and lactobacilli was inoculated into YPD and MRS broth, respectively. After 48 h of incubation, both *C. albicans* HB-10 and lactobacilli cells were collected using centrifugation at 3,500 ×*g* for 10 min and re-suspended in DMEM. The suspension of *C. albicans* HB-10 and lactobacilli contained approximately 1.0×10^7 CFU/mL. To the HeLa cell culture well, 100 µL of 10-fold serial dilutions of lactobacilli suspensions was added and incubated at 37°C for 1 h under 5% CO_2. Subsequently, 100 µL of 10-fold serial dilutions of *C. albicans* HB-10 suspensions was added to each well and incubated for 1 h under the same conditions to allow *C. albicans* HB-10 to adhere to cells. After incubation, each well was washed twice with PBS to remove non-adherent *C. albicans* cells and then treated with 0.05% trypsin-EDTA (Nacalai Tesque, Inc., Kyoto, Japan). The inhibitory rate of adhesion was calculated as the number of *C. albicans* cells that adhered to HeLa cells with *Lactobacillus* pre-treatment, per the number of *C. albicans* cells that adhered to HeLa cells in the absence of lactobacilli.

2.12 Statistical analysis

Statistical analyses were performed using R and RStudio (versions 4.0.3 and 1.4.1106, respectively). Mann–Whitney U-test was used to determine significant differences between DMEM control and different lactobacilli. One-way analysis of variance was used to compare multiple groups. Statistical significance was set at p values <0.05. Correlations between growth and metabolites (lactate and hydrogen peroxide) were determined using Spearman's rank correlation coefficient.

3 Results

3.1 Biofilm formation abilities of *C. albicans*

Biofilm formation by *C. albicans* clinical isolates was assessed using the WST-1 formazan dye (Figure 1A).

Among these 45 strains, representative strains that reproduced well and showed significant differences in the CV assay were selected as high and low biofilm-producing strains and renamed

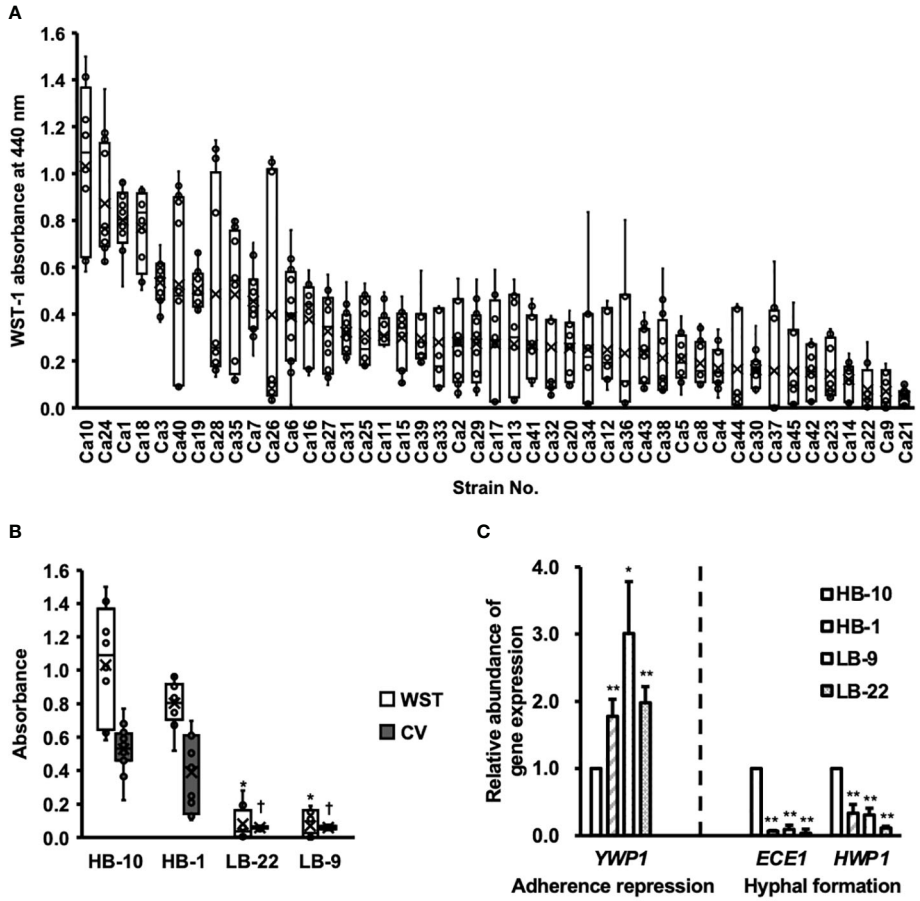

FIGURE 1
Clinical isolates of *Candida albicans* with different abilities to form biofilms. CV, crystal violet; WST, water-soluble tetrazolium salts. **(A)** The biofilm formation of 45 clinical isolates of *C albicans* were used to measure WST-1 and are exhibited by the box whisker plots. Box plot shows the median (horizontal thick blank line), mean (cross), and first and third quartiles (box). **(B)** Biofilm formation by different *C albicans* strains was estimated using both WST-1 reduction and CV staining. *$p < 0.05$ by U-test compared with WST-1 absorbance of *C albicans* HB-10. †$p < 0.05$ by U-test compared with crystal violet (CV) absorbance of *C albicans* HB-10. **(C)** Relative quantitation of genes associated with adherence repression (*YWP1*), or hyphal formation (*HWP1* and *ECE1*) normalized to the β-actin gene. The *C albicans* HB-10 strain was used as the reference to depict the difference among the four *C albicans* clinical isolates. Bars represent the standard deviation from the mean values. *$p < 0.05$ and **$p < 0.01$ by U-test.

as *C. albicans* HB-1 and HB-10 and *C. albicans* LB-9 and LB-22, respectively (Figure 1B). The expression levels of the three genes, *ECE1*, *HWP1*, and *YWP1*, which regulate different stages of biofilm formation in *C. albicans*, were determined using qRT-PCR (Figure 1C).

Four *C. albicans* strains with gene expression of *HWP1*, *ECE1*, *and YWP1* exhibited bar plots normalized by *C. albicans* HB-10 gene expression levels. These gene expression levels were calculated according to *ACT1* gene expression levels. Similar to the phenotypic biofilm-forming analysis, the relative gene expression levels of *ECE1* and *HWP1* in the HB-10 strain were significantly higher than those in the LB-9 (10.53-fold and 3.21-fold, respectively) and LB-22 strains (31.21-fold and 8.72-fold, respectively) ($p < 0.05$). Interestingly, the HB-1 strain, which was a high biofilm producer and did not show such a large difference in biofilm-forming ability, had significantly lower expression levels of these genes than the HB-10 strain. In contrast, *YWP1*, which suppressed initial adhesion, was the lowest in the HB-10 strain.

3.2 Characterization of culture supernatant

Vaginal lactobacilli produce various metabolites that exhibit antifungal activity. Cell-free culture supernatants extracted from 27 strains of *Lactobacillus* belonging to five species after 48-h incubation were characterized (Figure 2).

The lactate and hydrogen peroxide production of lactobacilli used in this study ranged from 42.1 to 201.7 mM and 38.7 to 170.8 nM, respectively. There was no correlation between lactate production and hydrogen peroxide production ($r = 0.426$; $p = 0.217$). In contrast, OD corresponding to the growth of *Lactobacilli* and lactate levels at the 72-h sampling point showed moderately positive correlations ($r = 0.667$; $p < 0.001$), while OD and hydrogen peroxide levels showed no correlation ($r = 0.265$; $p = 0.181$) (Supplementary Figure 2). In *L. jensenii*, the average hydrogen peroxide production was the highest compared to other species (mean 146.0 nM), although lactate production was not as high (ranging from 81.3 to 126.5 mM).

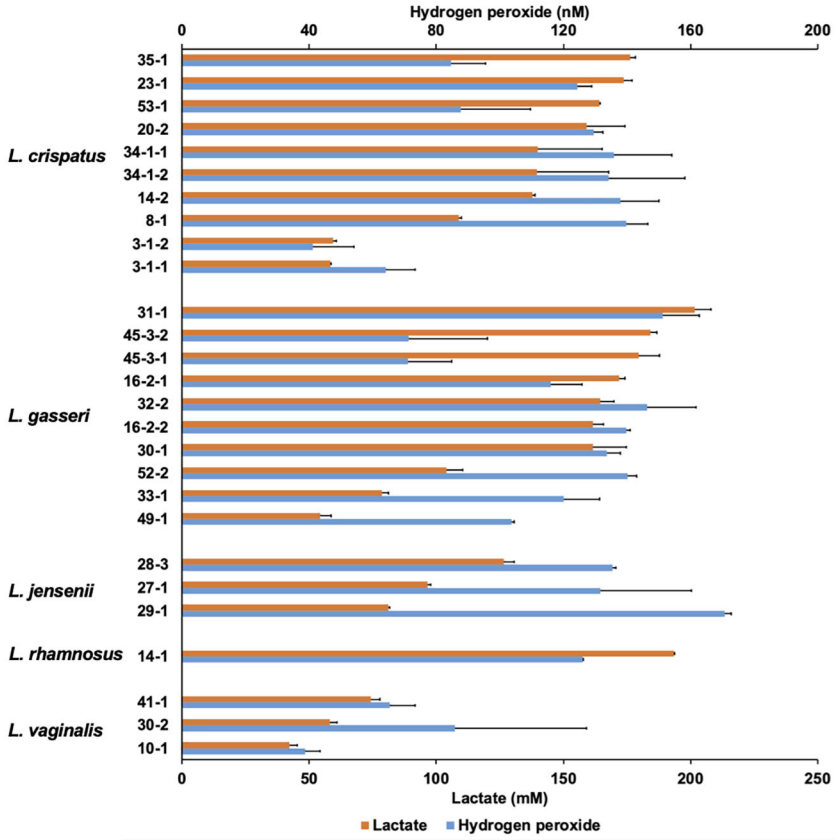

FIGURE 2
Lactate and hydrogen peroxide production by 27 *Lactobacillus* clinical isolates. Twenty-seven *Lactobacillus* clinical isolates were cultured in de Man, Rogosa, and Sharpe (MRS) broth for 72 h, and cell-free culture supernatants were collected. Lactate level was measured quantitatively by high-performance liquid chromatography (HPLC), and hydrogen peroxide was measured quantitatively using a hydrogen peroxide assay kit. Data are represented by the mean across the three replicates.

3.3 Effect of *Lactobacillus* culture supernatant on preformed biofilm

In a typical experiment using 96 well plates, biofilm formation takes 24 h to reach confluency. We investigated the effects of *Lactobacillus* culture supernatants on preformed biofilms (Figure 3).

The addition of *Lactobacillus* culture supernatant resulted in 24.3%-91.8% relative WST-1 readings compared with those of non-added control. The culture supernatants of *L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. vaginalis* with average relative WST-1 readings were approximately 52.5%, 43.1%, 57.2%, and 58.9%, respectively. The effect of different lactobacilli species on preformed biofilms of *C. albicans* HB-10 did not differ significantly. A moderate negative correlation was found between *Lactobacillus* lactate production and WST-1 readings (r = −0.625; p <0.001), but no correlation was observed between hydrogen peroxide production and WST-1 readings (Supplementary Figure 3).

We added several concentrations of the standards to the biofilm to reproduce lactate and hydrogen peroxide as metabolites in the culture supernatant (Supplementary Figure 4). The results showed that lactate concentrations had lower WST values than controls at all concentrations except 4 mM and a concentration-dependent effect on WST values (r = -0.930; p = 0.001). In contrast, hydrogen peroxide had no concentration-dependent effect on the preformed biofilm, with WST values not significantly different from the control at all concentrations. Lactate and hydrogen peroxide further showed no additive or synergistic effects on the preformed biofilms. A strong effect on the preformed biofilm was observed when the culture supernatant of the *L. crispatus* 35-1 strain was added (final concentration 14.1 mM), which was consistent with the WST-1 values for the biofilm when the 16 mM lactate standard was added (Figure 3 and Supplementary Figure 4).

3.4 Effect of *Lactobacillus* culture supernatant on the growth of planktonic cultures

The inhibitory effect of *Lactobacillus* spp. on *C. albicans* yeast cell growth was also evaluated. Significant growth inhibition was shown in 4/27 (14.8%) of the strains with the addition of culture supernatant of each *Lactobacillus* as follows: *L. crispatus* strain 23-1 and 20-2, *L. gasseri* strain 31-1 and *L. rhamnosus* strain 14-1. Interestingly, three of these strains showed lactate and hydrogen peroxide production above 165 mM and 120 nM, respectively (Supplementary Figure 7). The MICs for lactate and hydrogen peroxide standard for *C. albicans* HB-10 samples were 512 mM and 20 mM, respectively.

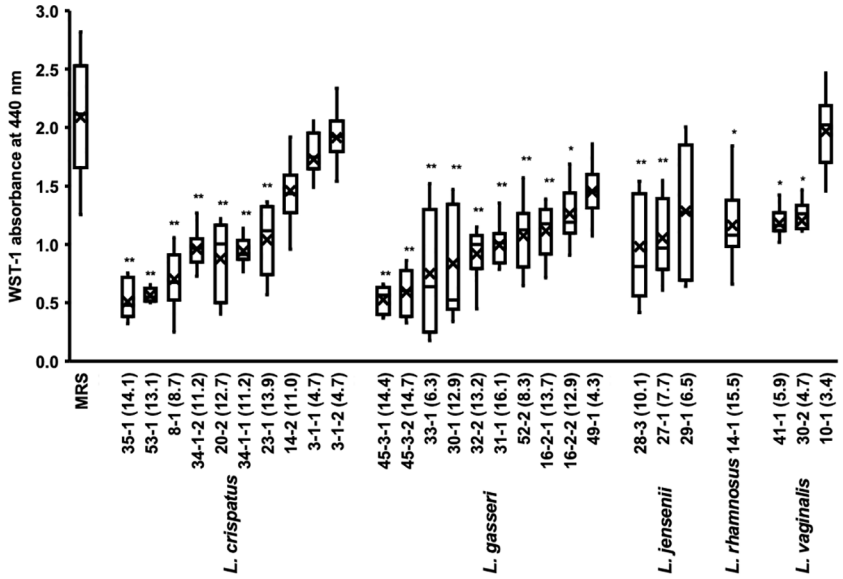

FIGURE 3
Metabolic activity of the biofilm of *C. albicans* HB-10 treated with culture supernatants of 27 different *Lactobacillus* clinical isolates. The x-axis indicates the strain number and lactic acid concentration (mM). The burden of viable cells of preformed biofilm treated after culture supernatants of 27 different *Lactobacillus* clinical isolates was measured using the WST-1 reduction reaction. MRS broth was used as the control. WST, water-soluble tetrazolium salts; MRS, de Man, Rogosa, and Sharpe. Box plot shows the median (horizontal thick blank line), mean (cross), and first and third quartiles (box). Bars represent the standard deviation from the mean values. *$p < 0.05$ and **$p < 0.01$ by U-test.

3.5 Effect of *Lactobacillus* culture supernatant on the hyphal formation

The effect on the rate of hyphal formation was compared for *Lactobacillus* culture supernatants that exhibited significant differences in their WST values to the preformed biofilm and for those that did not (Figure 4).

Yeast, hyphae, and pseudohyphae were identified under a microscope (Supplementary Figure 8). The addition of MRS medium control resulted in 54.95 ± 9.61% of the hyphae, and pseudohyphae were identified after 3 h of incubation. Compared to MRS control alone, lactate standards showed a concentration-dependent decrease in hyphal formation at 16 to 64 mM ($p<0.05$), while only 64 nM of hydrogen peroxide showed a

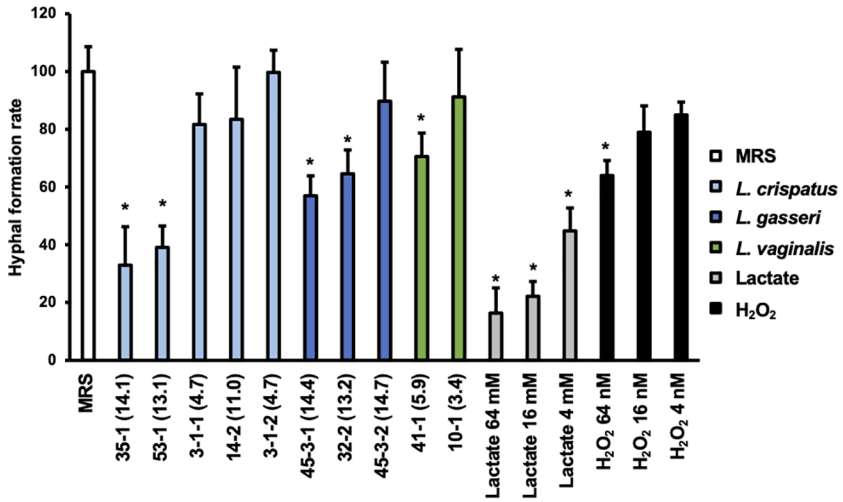

FIGURE 4
Hyphal formation rate of *C. albicans* HB-10 strain treated with *Lactobacillus* culture supernatants, lactate, or hydrogen peroxide. The x-axis indicates the strain number and lactic acid concentration (mM) or lactate concentration or hydrogen peroxide concentration. Relative hyphal formation in *C. albicans* HB-10 treated with culture supernatants of 10 different *Lactobacillus* clinical isolates, lactate, or hydrogen peroxide. Bars represent the standard deviation from the mean values. MRS broth was used as the control. MRS, de Man, Rogosa, and Sharpe. *$p < 0.05$ by U-test.

significant difference. The percentage of hyphal formation by *Lactobacillus* culture supernatants ranged from 18.11 to 54.77%. In terms of the percentage of a hyphal formation relative to untreated MRS, significant decreases were observed with the addition of culture supernatant in *L. crispatus* strain 35-1 (32.97 ± 13.29%) and 53-1 (39.10 ± 7.40%), *L. gasseri* strain 45-3-1 (56.99 ± 6.90%) and 32-2 (70.75 ± 4.15%), and *L. vaginalis* strain 41-1 (70.60 ± 8.12%). All of these showed final lactate concentrations >50 mM. In contrast, although there were no differences in lactate and hydrogen peroxide metabolite profiles between *L. gasseri* strains 45-3-1 and 45-3-2, only strain 45-3-1 showed significantly lower hyphal formation than the MRS control.

3.6 Effect of *Lactobacillus* bacterial cell on the initial adhesion

The inhibition of *C. albicans* yeast adhesion to human epithelial cells by *Lactobacillus* bacterial cells was assessed (Figure 5).

L. crispatus strain 35-1 showed a more efficient inhibition of *C. albicans* HB-10 (adhesion rate: 68.29 ± 6.90%). In contrast, *L. crispatus* strain 3-1-2 showed no statistically significant difference (adhesion rate: 93.90 ± 25.87%). Interestingly, *L. gasseri* strain 45-3-1, which showed inhibition of hyphal formation, also showed a significant reduction in *C. albicans* HB-10 adhesion (80.95 ± 3.17%), whereas strain 45-3-2 showed no inhibitory effect on initial adhesion (89.68 ± 2.38%).

4 Discussion

This study presents the steps of *C. albicans* biofilm formation that are affected by clinical isolates of lactobacilli. The analysis focuses on lactate and hydrogen peroxide among the metabolites (culture supernatants), and adhesion analyses were performed using viable bacteria.

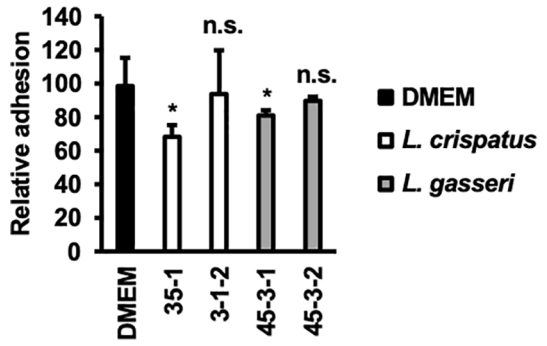

FIGURE 5
Adhesion of *C. albicans* HB-10 strain to HeLa cells according to the presence or absence of lactobacilli. Relative adherence of the *C. albicans* HB-10 strain to HeLa cells pretreated with DMEM or different lactobacilli. Bars represent the standard deviation from the mean values. *$p < 0.05$ by U-test. DMEM, Dulbecco's modified Eagle's medium. n.s., not significant.

The virulence of *C. albicans* in VVC is complexly related to multiple factors such as adhesion to cell surfaces and inert surfaces, cell damage by hydrolases and candidalysin, and subsequent active hyphal invasion, biofilm formation, and phenotypic switching (Berman and Sudbery, 2002; Moyes et al., 2016; Czechowicz et al., 2022). *C. albicans* yeast cells express adhesin and adhere to the host cell surface. HWP1 and a group of eight glycosylated proteins (*ALS1-ALS7* and *ALS9*) associated with the ALS gene are important adhesins (Rodríguez-Cerdeira et al., 2020). HWP1 is important as a component of the hyphal cell wall and may stabilize biofilms by adhering to yeast cells and hyphae in biofilms, making them highly pathogenic to the host (Zhu and Filler, 2010; Talapko et al., 2021). In addition to adhesion to cell surfaces, HWP1 is involved in adhesion to inert surfaces (Nobile et al., 2006). *C. albicans* yeast cells are transferred to hyphae by various environmental factors such as pH, CO_2 concentration, temperature, and N-acetylglucosamine (Sudbery, 2011). *C. albicans* invades cells from the cell surface in two ways: passive invasion by endocytosis and active invasion by disrupting the cell surface with hydrolases and candidalysin (Moyes et al., 2016; Maza et al., 2017). In particular, candidalysin, encoded by the ECE1 gene, directly disrupts epithelial cells by acting as a cytolytic peptide toxin (Moyes et al., 2016). *C. albicans* secretes candidalysin into the hyphal entry pocket, effectively destroying the tissue and establishing a mucosal infection with *C. albicans* (Mogavero et al., 2021). YWP1 inhibits adhesion of *C. albicans* yeast cells to the cell surface. Furthermore, YWP1 may express mannoproteins on the outer layer of the yeast cell wall, which may cover the epitope β-1,3-glucan and allow it to escape the immune system (Granger, 2012; Granger, 2018).

In this study, *C. albicans* biofilms are formed on inert surfaces (microtiter plates). For this reason, we evaluated the expression level of the HWP1 gene, which is important for inert surface attachment and is also associated with hyphal formation (Nobile et al., 2006). Candidalysin is encoded by *ECE1* and is important for active invasion of *C. albicans* by disrupting the host cell surface (Moyes et al., 2016). Since *ECE1* is an important gene for the invasion of C. albicans hyphae into HeLa cells, which are biotic surfaces, we evaluated the gene expression of *ECE1*. HB-10, which formed the highest amount of biofilms on inert surfaces (microtiter plates), was found to express high levels of HWP1. In contrast, HB-1 formed high biofilms, although the gene expression levels of *HWP1* and *ECE1* were low, indicating a dissociation between phenotype and gene expression. This may be because hyphal formation and invasion of epithelial cell, which is important for biofilm formation, are composed of multiple signal transduction pathways (Sudbery, 2011). For the *C. albicans* HB-10 biofilm, *C. albicans* biofilm formations were initiated using a 96-well plate in this study.

A strong effect on the preformed biofilm was observed when the culture supernatant of the *L. crispatus* 35-1 strain was added (final concentration, 14.1 mM), which is consistent with the WST-1 values for the biofilm when the 16 mM lactate standard was added. A healthy human vaginal environment is maintained at a low pH. In this acidic pH environment, *C. albicans* is less likely to undergo a morphological yeast-fungus transition (Davis et al., 2000). The pH of the buffered RPMI 1640 medium used in this study, supplemented with culture supernatant of the *L. crispatus* 35-1 strain (final

concentration 14.1 mM) and 16 mM lactate standard, had similar levels (at a pH range of 4.3 to 4.6) This suggests that direct pH reduction due to lactate might be responsible for the anti-*C. albicans* activities. This is confirmed by the fact that the inhibition of biofilm and hyphal formation disappears when the 4–64 mM Lactate standard is neutralized using NaOH (Supplementary Figures 5, 6). The MIC values for planktonic yeast were 512 mM for lactate and 20 mM for hydrogen peroxide. For the preformed biofilm in Figure 3, the concentration range for 8% *Lactobacillus* supernatant addition was 3.4 - 16.1 mM for lactate and 3.1 - 13.7 nM for hydrogen peroxide. In Supplementary Figure 6, regarding lactate and hydrogen peroxide standard samples, lactate inhibited preformed biofilms at 16 mM-64 mM; however, hydrogen peroxide did not inhibit at all in any concentration. Therefore, at sub-MIC concentrations, lactate (both supernatant and standard samples) may show fungistatic activities, whereas hydrogen peroxide may not. Fluconazole, a therapeutic agent for VVC, shows fungistatic activities against *C. albicans*, and increased susceptibility to fluconazole has been reported in biofilms in the presence of lactate (Alves et al., 2017). This could lead to the development of a new *C. albicans* treatment by combining *Lactobacillus* and fluconazole.

Hyphal formation and growth are associated with *C. albicans* virulence (Jang et al., 2019; Roselletti et al., 2019). HB-10 used in this study expresses the *ECE1* gene; Ece1p, a protease encoded by *ECE1*, causes inflammation in epithelial cells and allows *C. albicans* hyphae to adhere to and invade the cell epithelium (Moyes et al., 2016). To inhibit biofilm formation, it is important to prevent *C. albicans* adhesion to the epithelial cells. In the present study, lactate inhibited hyphal formation in a concentration-dependent manner. Despite the similar metabolic profiles of lactate and hydrogen peroxide in *L. gasseri* strains 45-3-1 and 45-3-2, only strain 45-3-1 significantly inhibited hyphal formation. Similarly, among the *L. gasseri* strains 45-3-1 and 45-3-2, only strain 45-3-1 significantly inhibited *C. albicans* adhesion to epithelial cells. This suggests that metabolites other than lactate and hydrogen peroxide inhibit hyphal formation. Indeed, recent studies have suggested that small molecules produced by *Lactobacillus* may inhibit *C. albicans* biofilm formation and growth as antimicrobial compounds (Lee et al., 2021; MacAlpine et al., 2021). *L. crispatus* strain 35-1 and *L. gasseri* strain 45-3-1 showed a significant reduction in *C. albicans* HB-10 adhesion (adhesion rates: 68.29 ± 6.90% and 80.95 ± 3.17%, respectively) to HeLa cells. Thus, different *Lactobacillus* strains showed different rates of inhibition of *C. albicans* HB-10 adhesion to HeLa cells. In the experimental setup of this study, *Lactobacillus* first adhered to HeLa cells before *C. albicans* was added. Although an accurate count of lactobacilli, which adhere to HeLa cells could not be obtained in this study, our findings reveal the *Lactobacillus* strain with high attachment ability to HeLa cells, which may preferentially adhere to a limited number of epithelial cell surfaces, indicating that *C. albicans* was physically unable to adhere to these cells. In this study, *L. crispatus* 35-1 and *L. gasseri* 45-3-1 strains inhibited *C. albicans* adhesion but failed to reduce it to less than 50%. Thus, it should be noted that in terms of multiplicity of infection, the inhibitory effect of *Lactobacillus* used in this study on *C. albicans*'s epithelial cell attachment is not so strong.

Several studies using clinical isolates and deposited strains in biofilm formation inhibition testing have been reported. Culture supernatant of a clinically isolated strain, *Lactobacillus crispatus* BC1-BC8, inhibited biofilm formation (Itapary Dos Santos et al., 2019). Compared to no cell-free culture supernatants, culture supernatants of deposited strains, *Lactobacillus fermentum* ATCC 23271 and *L. rhamnosus* ATCC 9595, inhibited biofilm formation by more than 40% in the CV and 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide assays (Itapary Dos Santos et al., 2019). On the other hand, the addition of cell-free culture supernatants of *Lactobacillus* iners ATCC 55195 significantly increased hyphal and biofilm formation of *C. albicans* compared to the control (Sabbatini et al., 2021). Matsuda et al. reported no inhibitory effect when 7.5% *L. crispatus* JCM 1185 and *L. gasseri* JCM 1131 culture supernatants were added to the *C. albicans* preformed biofilm (Matsuda et al., 2018). In our study, culture supernatants of 8% *L. crispatus* JCM 1185 and *L. gasseri* JCM 1131 exhibited no inhibitory effect on the biofilm (Residual biofilm 78.2% and 66.7% compared with no cell-free culture supernatants control), suggesting that the reproducibility of previous reports has been achieved. However, *L. crispatus* 35 -1 and *L. gasseri* 45 -3 -1 in this study significantly inhibited the pre-formed biofilm. The hyphae formation rate of *L. crispatus* JCM 1185 was 28.0%, which was not significantly different from that of clinical isolates. In contrast, that of *L. gasseri* JCM 1131 was 6.6%, which is interesting because it has a higher inhibitory effect than clinical isolates.

A worrisome trend is that VVC caused by non-*albicans Candida* species (NAC), *C. tropicalis, C. krusei*, and *C. glabrata*, has been increasing (Zhou et al., 2010; Ravel et al., 2011). In particular, *C. tropicalis* is frequently isolated in Asia and is known to have high hyphal budding ability and form strong biofilms that are resistant to treatment (Bizerra et al., 2008; Ravel et al., 2011; Kawai et al., 2017). Although *C. tropicalis* has good *in vitro* drug susceptibility to azoles, candins, and polyenes, the poor clinical prognosis may be related to biofilm formation (Yamagishi et al., 2009; Sakagami et al., 2019). Visualization of biofilm formation has shown that candin- and polyene-based drugs are suitable for biofilm-forming NAC (Kawai et al., 2015; Kawai et al., 2017). In the actual human vaginal environment, glycogen is digested by α-amylase to produce maltose, maltotriose, and maltotetraose (Spear et al., 2014). *Lactobacilli* are known to consume glycogen-breakdown products to produce lactate. However, *in vitro* experiments have not fully mimicked the vaginal environment with respect to nutrient sources for *Lactobacillus* development, which may have affected their growth and metabolite production (Spear et al., 2014; Nunn et al., 2020). Thus, classically defined bacterial aerobes and anaerobes form a community of microaerophilic environments in the mucosa lining the vaginal lumen. In this study, optimal growth environments for *Lactobacillus* and *C. albicans* were selected (anaerobic and aerobic conditions, respectively). However, it is difficult to reproduce the complex vaginal ecosystem under a single culture condition in an *in vitro* experimental system; thus, it is necessary to set *Lactobacillus* and *C. albicans* in aerobic, microaerobic, and anaerobic conditions to evaluate biofilms. The results of this study suggest that *Lactobacillus* metabolites other than lactate and hydrogen peroxide may also affect *C. albicans*, although they have not been evaluated in detail. In future, the effects of various metabolites

produced by *Lactobacillus* on *C. albicans* need to be evaluated under conditions that are more similar to the human vaginal environment. In this study, the effects of lactate and hydrogen peroxide on *C. albicans* HB10 biofilm and hyphal formation are investigated using lactate and hydrogen peroxide standard samples. However, metabolites other than lactate and hydrogen peroxide are possibly involved in *C. albicans* biofilm formation. Therefore, it is necessary to consider the effects of the absence of lactate and hydrogen peroxide, while considering the effects of various metabolites using *Lactobacillus* strains that cannot synthesize lactate and hydrogen peroxide. This study has not been able to evaluate this issue. The effect of *Lactobacillus* supernatant on *C. albicans* biofilm formation and the change from yeast to hyphal form could be better understood by imaging evaluation using electron microscopy. However, due to equipment limitations, electron microscopic evaluation was not available for this study. In future studies, evaluation with images should also be considered.

In this study, quantitative evaluation of lactobacilli metabolite (lactate and hydrogen peroxide) concentrations revealed that the inhibitory effects of lactate and hydrogen peroxide on *C. albicans* might be acting through multiple stages, such as *C. albicans* growth, hyphal formation, biofilm development, and adhesion to epithelial cells. Therefore, combining antifungal drugs with lactobacilli as a live biotherapeutic product, with anti-biofilm development activity, may lead to the development of new treatment strategies. Future studies are required to evaluate how lactobacilli affect both *C. albicans* and NAC, to promote the global use of lactobacilli.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

Conceptualization: TT and HaK. Methodology: HaK, KO, and TK. Software: SE. Validation: TT, AM, and HaK. Formal analysis: SE. Investigation: TT, SE, AM, and HaK. Resources: TT. Data curation: HaK. Writing-original draft preparation: TT, SE, AM, and HaK. Writing-review and editing: TT. Visualization: ES. Supervision: YukiY, MT, YukaY, HT, HM, and HiK. Project administration: TT. Funding acquisition: MT and HiK. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

HaK, SE, AM, KO, and MT are employees of Miyarisan Pharmaceutical Co., Ltd.; however, they have no conflicts of interest to declare regarding this study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1113401/full#supplementary-material

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Comparative analysis of vaginal microbiota sampling using menstrual cups and high vaginal swabs in pregnant women living with HIV-1 infection

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Background: Menstrual cups (MCs) are increasingly used to collect cervicovaginal secretions to characterise vaginal mucosal immunology, in conjunction with high vaginal swabs (HVS) for metataxonomics, particularly in HIV transmission studies. We hypothesised that both methods of collecting bacterial biomass are equivalent for 16S rRNA gene sequencing.

Material and Methods: Cervicovaginal fluid (CVF) samples from 16 pregnant women with HIV-1 (PWWH) were included to represent the major vaginal bacterial community state types (CST I-V). Women underwent sampling during the second trimester by liquid amies HVS followed by a MC (Soft disc™) and samples were stored at -80°C. Bacterial cell pellets obtained from swab elution and MC (500 µL, 1 in 10 dilution) were resuspended in 120 µL PBS for DNA extraction. Bacterial 16S rRNA gene sequencing was performed using V1-V2 primers and were analysed using MOTHUR. Paired total DNA, bacterial load, amplicon read counts, diversity matrices and bacterial taxa were compared by sampling method using MicrobiomeAnalyst, SPSS and R.

Results: The total DNA eluted from one aliquot of diluted CVF from an MC was similar to that of a HVS (993ng and 609ng, p=0.18); the mean bacterial loads were also comparable for both methods (MC: 8.0 log10 16S rRNA gene copies versus HVS: 7.9 log10 16S rRNA gene copies, p=0.27). The mean number of sequence reads generated from MC samples was lower than from HVS (MC: 12730; HVS:14830, p=0.05). The α-diversity metrices were similar for both techniques; MC Species Observed: 41 (range 12-96) versus HVS: 47 (range 16-96), p=0.15; MC Inverse Simpson Index: 1.98 (range 1.0-4.0) versus HVS: 0.48 (range 1.0-4.4),

p=0.22). The three most abundant species observed were: *Lactobacillus iners, Lactobacillus crispatus* and *Gardnerella vaginalis*. Hierarchical clustering of relative abundance data showed that samples obtained using different techniques in an individual clustered in the same CST group.

Conclusion: These data demonstrate that despite sampling slightly different areas of the lower genital tract, there was no difference in bacterial load or composition between methods. Both are suitable for characterisation of vaginal microbiota in PWWH. The MC offers advantages, including a higher volume of sample available for DNA extraction and complimentary assays.

KEYWORDS

menstrual cup, swab, cervicovaginal fluid, sampling, microbiota, metataxonomics, HIV

Introduction

The importance of microbiota in shaping health and disease states is increasingly appreciated. In the lower reproductive tract, a cervicovaginal microbiota dominated by one or relatively few *Lactobacillus* species is thought to promote reproductive health by inhibiting inflammation and preventing overgrowth of certain pathobionts implicated in several genital tract pathologies including bacterial vaginosis, vaginitis (including aerobic, inflammatory and atrophic) (Donders et al., 2017; Stewart et al., 2022), pelvic inflammatory disease, cervical intraepithelial neoplasia (Norenhag et al., 2020), vulval dermatological conditions (Brunner et al., 2021), menstrual disorders (Chen et al., 2021) and poor pregnancy outcomes such as recurrent miscarriage (Grewal et al., 2022) and preterm birth (Gudnadottir et al., 2022). A loss of *Lactobacillus* species from this niche is also a key risk factor for sexual transmission of infections including *Chlamydia trachomatis (*Ceccarani et al., 2019) and HIV (Bayigga et al., 2019).

There are now many techniques for study of the vaginal microbiota, in addition to standard traditional Gram staining and bacterial culture, both of which still play important clinical roles. Molecular techniques enable identification of organisms that are difficult to culture and include nucleic acid amplification of species-specific genes, often used for diagnostic purposes, and qPCR for total bacterial load or species-specific abundance. Sequencing techniques enable characterisation of bacterial communities from amplified DNA libraries of varying fragment lengths, either targeted to regions of the bacterial 16S rRNA genes (metataxonomics) (Marchesi and Ravel, 2015), multiple loci, or non-targeted whole genome sequencing (Malla et al., 2018). Metataxonomics has a low cost and error rate, but it can only accurately characterise to the genus level whereas multiple gene loci or whole genome sequencing can offer species and strain level and functional gene information including antimicrobial resistance.

Several sampling techniques are available to collect cervicovaginal secretions for onward assays, but there is no consensus on the optimum method for microbiota characterisation. High vaginal swabs (HVS) are widely used to sample secretions from the posterior fornix and vaginal wall (MacIntyre et al., 2015; Short et al., 2020). These swabs can be made from several materials such as cotton, polyester, rayon, nylon and even ophthalmic grade sponge with potential differences in absorption and elution (Castle et al., 2004; Dezzutti et al., 2011). Alternative sampling techniques historically used for sampling the lower female genital tract include saline lavage and absorbent wicks and tampons (Snowhite et al., 2002; van de Wijgert et al., 2006; Dezzutti et al., 2011; Jespers et al., 2011). Other sampling devices used for the characterisation of vaginal microbiota include cervical brushes (Mitra et al., 2017), some of which have been developed for self-sampling (Virtanen et al., 2017). The choice of sampling method may depend on what complementary assays are planned and pre-processing requirements (Jespers et al., 2011). For example, cytobrushes have been compared with HVS and have been demonstrated to be an effective method for sampling the cervical surface for metataxonomic, with the advantage of also providing host cellular material for flow cytometry (Mitra et al., 2017).

Menstrual cups (MCs) can collect large volumes of un-diluted cervicovaginal fluid (CVF), which can also be used for diagnostic vaginal microscopy. They provide the option of self-sampling and are increasingly used for studying the microbiota functional and immune interactions (Masson et al., 2019; Short et al., 2020; Wu et al., 2022) but have not been compared head to head with the more commonly used HVS for metataxonomic and bacterial load estimation. In this paper we compare a MC (Soft disc^{TM}) with a polyester HVS for DNA yield and metataxonomics to identify different vaginal microbiota taxa and community structures in a group of pregnant women with HIV-1 infection. We hypothesise that the two methods are equivalent, and both are valid for characterisation of the lower genital tract microbiota.

Materials and methods

Paired samples from a subgroup of sixteen pregnant women living with HIV from the Immunological Basis of Preterm Delivery Study were used for these analyses, on the basis that all major community state types (CSTs) would be represented, as identified,

from previous metataxonomic analysis, from the same samples (Short et al., 2020). The size of sample set was chosen on the basis of both the breath of CSTs, as well as the number of available paired samples and as such no formal sample size calculation was performed. This study was approved by the Southeast Coast RES Committee (13/LO/0107). Written informed consent was obtained enabling clinical data and sample collection. Inclusion criteria were: known HIV-1 antibody status; confirmed singleton pregnancy (by ultrasound); age > 18 and ability to provide informed consent. Exclusion criteria were: multiple or *in-vitro* fertilization pregnancy, injecting drug use and CD4 count < 350 cells/μL. Data on medical, obstetric and drug history were recorded including smoking status, antiretroviral drug exposure and recent antibiotic use. The practice of vaginal douching, recent sexual intercourse and vaginal pH were recorded. Screening for *Syphilis*, *Gonorrhoea* and *Chlamydial* infection was routinely offered as per national guidelines (Gilleece et al., 2019).

Sample collection

During attendance at routine second trimester antenatal appointments women were invited to donate both MC and HVS samples. The sampling procedure was clinician or self-taken HVS of the high lateral vaginal wall with a Liquid Amies Swab (BBL™ CultureSwab™, BD) followed by clinician or self-insertion of a MC (Soft disc™, The Flex Company, previously manufactured as Instead Soft cup™) for a minimum of five minutes, transferred to a sterile 50mL plastic conical tube. Samples were immediately transferred to the laboratory on wet ice and were stored at -80°C. CVF was removed from the MC prior to further processing by thawing on ice for a maximum of 30 minutes and centrifugation at 4°C for 15 mins at 400 x *g* to separate the CVF from the MC into the base of the conical tube, as previously described (Cosgrove et al., 2016; Short et al., 2018). CVF was divided into 100-200μL aliquots in 1.5mL microtubes, using a positive displacement pipette (Rainin c10–100™, Mettler Toledo) for the handling of high viscosity fluids. For DNA extraction, CVF was diluted 1 in 10 with an extraction buffer consisting of 1X protease cocktail I (Calbiochem™, 539131, Merk), 10μL 10% Sodium Azide solution, 0.75 g NaCl, final volume made to 50mL with 1X phosphate buffer solution (PBS) filter sterilised (Castle et al., 2004; Short et al., 2018). Bacterial biomass from swabs were extracted and pelleted as previously described (MacIntyre et al., 2015).

DNA extraction

Bacterial cell pellets obtained from both swab and 500 μL of diluted CVF were resuspended in filter sterilised PBS to a volume of 120 μL. DNA extraction was performed using a combination of enzymatic digestion and mechanical disruption of cell membranes and QIAamp Pathogen mini kits (Qiagen), eluted in AVE buffer to a final volume of 100 μL, as previously described (MacIntyre et al., 2015). DNA concentrations were measured using a Qubit™ high sensitivity kit (Thermo Fisher Scientific).

Quantitative polymerase chain reaction

qPCR was carried out for quantification of 16S rRNA gene copy number to compare the bacterial load collected by each technique. qPCR was performed with universal BactQUANT 16S rRNA gene primers (Forward primer:5′-CTACGGGAGGCAGCA, Reverse primer: 5′-GGACTACCGGGTATCTAATC) (Sigma) with the FAM labelled BactQUANT probe ((6FAM) 5′-CAGCAGCCGCGGTA-3′ (MGBNFQ)) (Liu et al., 2012; Mitra et al., 2017) on a CFX Real Time PCR system (Bio-Rad). A tenfold standard curve (3030 to 303,039,700 copies) of *Escherichia coli* genomic DNA (Sigma, D4889) was generated, each reaction contained 5 μL of DNA sample or standard, 10 μL Platinum PCR Super mix UDG containing Rox (Life Tech, 11730-017) and primers and probe. Thermal cycling was performed at 3 min at 50°C for UNG incubation,10 min at 95°C for Taq activation, then 40 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for annealing and extension. Cycle threshold (Ct) value for each reaction were obtained using CFX Maestro software version 1.1 (Bio-Rad) after application of fluorescence drift correction, background subtraction using the 'curve fit' option and automatic Ct baseline definition. Ct values were converted to 16S rRNA gene copy number from the generated standard curve. Samples were run in duplicate; a negative control of molecular grade water was included to eliminate contamination.

Metataxonomics

DNA concentrations were unadjusted prior to library preparation (range 1ng/μL to 36ng/μL, total volume 20μL). The V1-V2 hypervariable regions of the 16S rRNA gene were amplified with a fusion primer set that includes four different 28F primers chosen to improve detection of *Bifidobacteriales* (including the *Gardnerella* genus) and a 388R primer (Frank et al., 2008). The 28F-YM forward primer (5′-GAGTTTGATCNTGGCTCAG-3′) was mixed in a ratio of 4:1:1:1 with 28F Borrellia (5′-GAGTTTGATCCTGGCTTAG-3′), 28F Chloroflex (5′-GAATTTGATCTTGGTTCAG-3′), and 28F Bifido (5′-GGGTTCGATTCTGGCTCAG-3′) (RTL Genomics Amplicon Diversity Assay List). The forward primers included an Illumina i5 adapter (5′-AATGATACGGCGACCACC GAGATCTACAC-3′), an 8-base-pair (bp) bar code and primer pad (forward, 5′-TATGGTAATT-3′). The 388R reverse primer (5′-TGCTGCCTCCCGTAGGAGT-3′) was constructed with an Illumina i7 adapter (5′-CAAGCAGAAGACGGCATACGAGAT-3′), an 8-bp bar code and a primer pad (reverse, 5′-AGTCAGTCAG- 3′). The pair end multiplex sequencing was performed on an Illumina MiSeq platform (Illumina Inc.) at Research and Testing Laboratory (Lubbock, TX, USA).

The 16S rRNA sequences were analysed using the MiSeq SOP pipeline with the MOTHUR software package (Kozich et al., 2013). Highly similar amplicons were clustered into operational taxonomic units (OTUs) using the kmer searching method and the Silva bacterial database (www.arb-silva.de/) (Quast et al., 2013). All OTUs had a taxonomic cut-off of ≥97%. Classification was

performed using the Ribosomal Database Project (RDP) reference sequence files and the Wang method (Wang et al., 2007). The RDP MultiClassifier script was used for determination of OTUs (phylum to genus) and species level taxonomies were determined using USEARCH (Edgar, 2010). OTUs with <10 reads across the dataset were considered rare taxa and were grouped (taxonomy_species X). Diversity indices (Inverse Simpson index and species observed (SObs) were calculated using the Vegan package within R (Dixon, 2003).

Statistical analyses

The total DNA concentration extracted by sampling method was calculated by multiplying the original sample DNA concentration in ng/μL by the eluted volume of 100 μL. The total bacterial load extracted by sampling method was calculated by multiplying the number of 16S rRNA gene copies in a reaction volume of 5μL by a factor of twenty and is reported as total copies/100μL. The total DNA quantity, bacterial load and unrarefied OTU read sequences, sample richness (SObs) and α diversity (Inverse Simpson Index) were compared by sampling method using the paired t test in SPSS (Version 28.0, IBM). A p-value less than 0.05 was considered statistically significant.

Bacterial taxon data visualisation and statistical analyses were performed in MicrobiomeAnalyst and STAMP packages (Parks and Beiko, 2010; Chong et al., 2020). Beta diversity profiling of sampling method dissimilarity was explored using a Principal Coordinate Analysis plot of rarefied taxon data with Bray-Curtis dissimilarities distances and PERMANOVA. Community State Types (CSTs) were compared in individuals by method on unrarefied Centred Log Ratio transformed data using Ward hierarchical clustering with average distances using the top 25 species observed, accounting for >95% of the total reads. CSTs were compositionally consistent with those originally described by Ravel (Ravel et al., 2011) and Gajer et al. (2012): CST I: *L. crispatus* dominance; CST II: *L. gasseri* dominance; CST III *L. iners* dominance; CST IV-A: Moderate *L. iners* with mixed anaerobes; CST IV-B: Mixed anaerobes including higher proportions of genus *Atopobium* and BV associated bacteria and CST V: *L. jensenni* dominance (Gajer et al., 2012). Taxon specific abundance by method was compared to identify any species or genus with significant over representation with linear discriminant analysis (LDA) effect size (LEfSe) analysis. A logarithmic LDA score cut off of 2 was used to determine any discriminative features.

Results

The median age of the 16 participants was 34 years (IQR 30-37). Fourteen were of Black race (88%), two were White. Median gestational age at sampling was 24 weeks (IQR-21-28). All, but one woman, who delivered at 36.9 weeks, went on to have term deliveries. Median CD4 count was 550 cells/mcL (IQR 411-631) and median HIV viral load at baseline was < 40 copies reflecting the fact that 11/16 women conceived on antiretroviral therapy (ART). Two of the 5 women who initiated ART during pregnancy had started prior to the second trimester sampling timepoint. Most women received Non-Nucleoside Reverse Transcriptase Inhibitor based ART, 4 received Integrase Strand Transfer Inhibitor based ART, 2 received Protease Inhibitor based ART and 2 initiated triple Nucleoside Reverse Transcriptase Inhibitor based ART.

Swabs and menstrual cup yield similar DNA concentrations and bacterial load

Total DNA extracted by each collection method was comparable with similar mean DNA concentration extracted from the cell pellet from one aliquot of diluted MC CVF compared to a HVS (993ng and 609ng, p=0.18), see Table 1. The mean total bacterial load was similar for both methods (MC: 8.0 log10 16S rRNA gene copies/100μL versus HVS: 7.9 log10 16S rRNA gene copies/100μL, p=0.27). The number of sequence reads from swab samples were higher than diluted MC samples (median HVS sequence reads: 14830 (range 9572-21793) vs. MC: 12730 (range 7738-18295), p=0.05).

Swabs and menstrual cups provide comparable 16S rRNA gene sequencing results

Sample richness was similar in the number of species observed (SOb) by sampling method (mean HVS: 47 (range 16-96) versus MC: 41 (range 12-96), p=0.15). Inverse Simpson Index scores were also similar for samples obtained through each technique (mean HVS: 1.9 (range 1.0-4.4) versus MC: 1.8 (IQR 1.0-4.0), p= 0.22), see Figure 1.

The three most abundance species were in order: *L. iners*, *L. crispatus* and *G. vaginalis*, see Figure 2. Hierarchical clustering of relative abundance data showed that bacterial profiles obtained from the same individual via different samples methods clustered together in the same CST group, see Table 1 and Figure 3. Visualisation of the dissimilarity matrix with PCoA plots for the different sampling methods revealed near identical vaginal community structures with diversity and composition clustering according to patient, PERMANOVA F-value: 0.11861; R-squared: 0.0039381; p-value: 0.998, see Figure 4. No differentially abundant taxa were identified in either sampling method by LEfSe analysis, data not shown.

Discussion

We have demonstrated that MC sampling of the lower female genital tract secretions for DNA extraction and 16S rRNA gene amplicon sequencing provides comparable results to polyester HVS sampling. Whilst these analyses are drawn from a small sample set of pregnant women with HIV, these data are reassuring that MC

TABLE 1 Comparison of DNA concentration, 16S rRNA gene copies, sequence reads and metataxonomic profiles by collection method.

Method	High vaginal swab	Menstrual cup	P value
Total DNA concentration eluted/ng (mean (range))	**609 (10-1740)**	**993 (10-3550)**	**0.18**
Total Bacterial load by method/log10 16S rRNA gene copies/100µL (mean(range))	**7.9 (6.3-8.3)**	**8.0 (6.8-8.9)**	**0.27**
Total high-quality sequence reads (mean (range))	**14830 (9572-21793)**	**12730 (7738-18295)**	**0.05**
a diversity matrices (mean (range)) **Species observed** **Inverse Simpson Index**	**47 (16-96)** **1.9 (1.0-4.4)**	**41 (12-96)** **1.8 (1.0-4.0)**	**0.15** **0.22**
Community state type (n(%)) **I** **II** **III** **IV-A** **IV-B** **V**	**3 (19)** **1 (6)** **4 (25)** **3 (19)** **3 (19)** **2 (12)**	**3 (19)** **1 (6)** **4 (25)** **3 (19)** **3 (19)** **2 (12)**	**1.0**

and other similar methods for collecting undiluted cervicovaginal secretions should perform as well as HVS for metataxonomic and potentially metagenomic techniques and should be generalisable to vaginal sampling from all women.

The trend towards a slightly higher total DNA concentration with the MC method is likely to be the result of a greater starting volume of undiluted CVF collected with the Soft disc™ compared to swab samples which usually require elution. The 1 in 10 dilution of CVF was selected from our group's previous work with immunoassays as it enabled ease of sample handling, concentrations of related cytokine to be accurately measured as well as producing similar sized cell pellets to the swab method

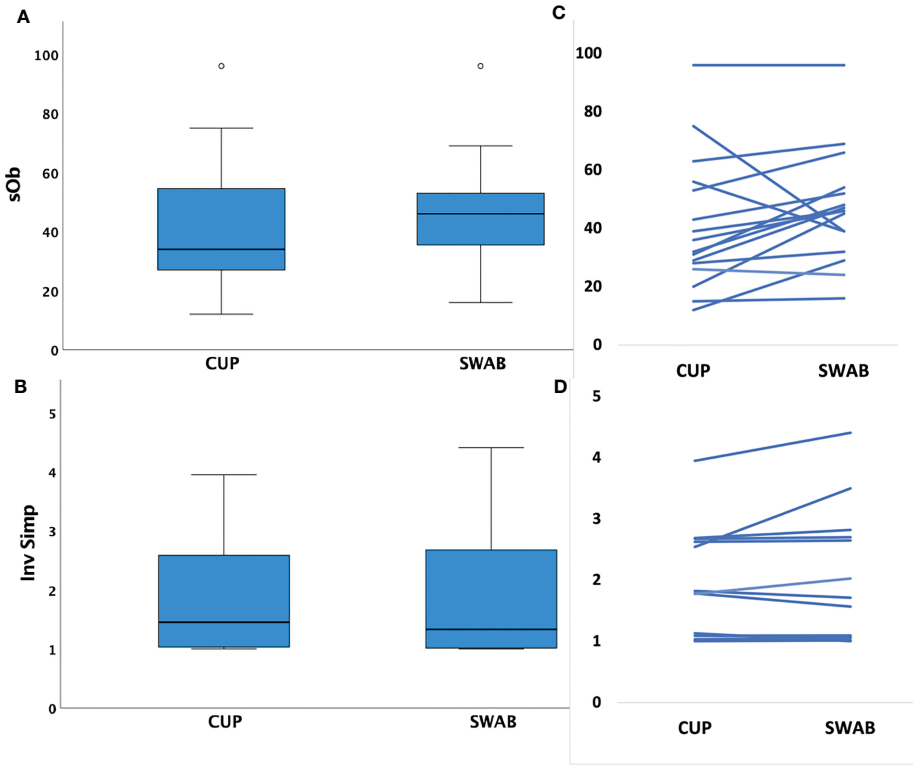

FIGURE 1
Box plots of mean species richness and alpha diversity indices by method and paired values for individuals. **(A)** Mean species observed (richness, SOb) did not differ by the two different sampling methods (p=0.15, paired t test); **(B)** paired samples showing similar SOb by method in individuals **(C)** Mean Inverse Simpson index (alpha diversity metric) did not differ by collection method (p=0.22, paired t test); **(D)** paired samples showing similar Inverse Simpson indices by method in individuals.

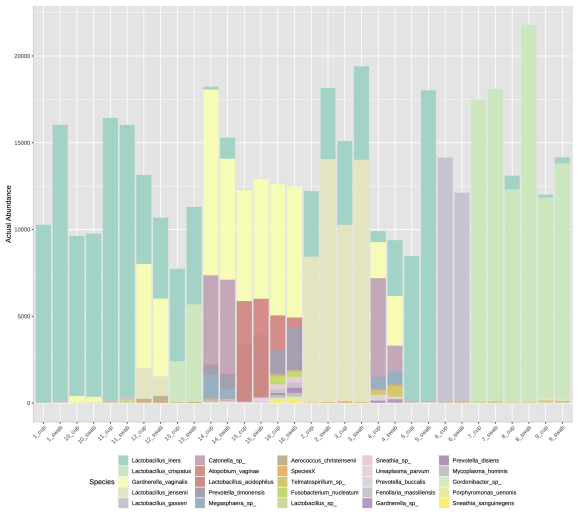

FIGURE 2
Stacked bar chart of species abundance by sample collection method in individuals 1-16. The top abundant species were *L. iners, L. crispatus* and *G. vaginalis* with no difference on species composition by sampling type.

(Short et al., 2018; Short et al., 2020). In a previous study of HIV negative pregnant women we found that MC samples, *in situ* for a minimum of 5 minutes and a maximum of 1 hour, yield a median weight of 0.5g of undiluted CVF which is equivalent to 500µL (Short et al., 2018), similar to others experience in non-pregnant women (Boskey et al., 2003; Jaumdally et al., 2017).

Undiluted CVF is very viscous and can require a positive displacement pipette for direct processing. Some groups have previously been deterred by this handling issue (Chappell et al., 2014), however, with the correct pipette, or with direct addition of elution buffer into the conical tube containing the MC (Jaumdally et al., 2018), the final volume can be significant. For example, 500µL of CVF diluted 1 in 10 would give a 5mL starting volume from which DNA could be extracted. This could potentially yield ten-fold the quantity of DNA albeit with the limitations of the yield of the chosen DNA purification procedure (Mattei et al., 2019). This dilution is easily adjusted, DNA concentration standardised prior to library preparation, with significant remaining available material for additional assays.

The Soft disc™, which opens inside the vagina to its 7cm diameter (Boskey et al., 2003), also collects CVF from a larger surface area of vaginal epithelium than a HVS. In spite of the potential for the MC samples to be more representative of the total bacterial biomass of lower genital tract, the lack of difference found between the methods may indicate the similarity in microbiota between the surface of cervix, high and middle vagina, replicating the finding of others (Chen et al., 2017).

In our experience, the MC was acceptable and well-tolerated by study participants and can be used to self-sample the lower female genital tract, potentially away from a healthcare facility which could offer some logistical benefits. Self-sampled vaginal swabs have been previously shown to be comparable to physician taken swabs for vaginal microbiota sequencing studies, which makes such methods ideal for large scale field studies (Forney et al., 2010). However, time taken to store sample should always be minimised and standardised to reduce any impact on sample integrity. Quick storage into freezers is particularly important for metabolomic work, yet there is data from faecal microbiota samples that shows that with storage at ambient temperatures, interpatient variability is maintained at forty-eight hours, including in the *Lactobacillaceae* and

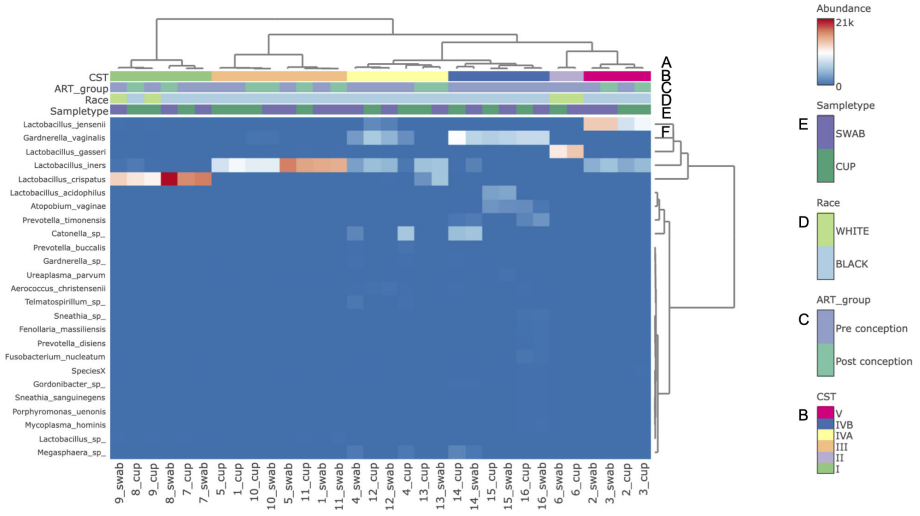

FIGURE 3
Bacterial species composition of vaginal CSTs from individual pregnant mothers with HIV during the second trimester by sampling method. **(A)** Hierarchical clustering analysis using ward linkage and maximum distances of microbial species data show both methods identified that there were 6 major vaginal microbiota groups; **(B)** CST types: I- *Lactobacillus crispatus* dominant, II- *L. gasseri* dominant, III- *L. iners* dominant, IV-A- moderate *L. iners* with mixed anaerobes and IV-B- mixed anaerobes including higher proportions of genus Atopobium and bacterial vaginosis associated bacteria; **(C)** ART exposure in relation to conception; **(D)** Race; **(E)** Sample type; **(F)** Heat map of relative species abundance of vaginal bacteria with top 25 abundant species shown.

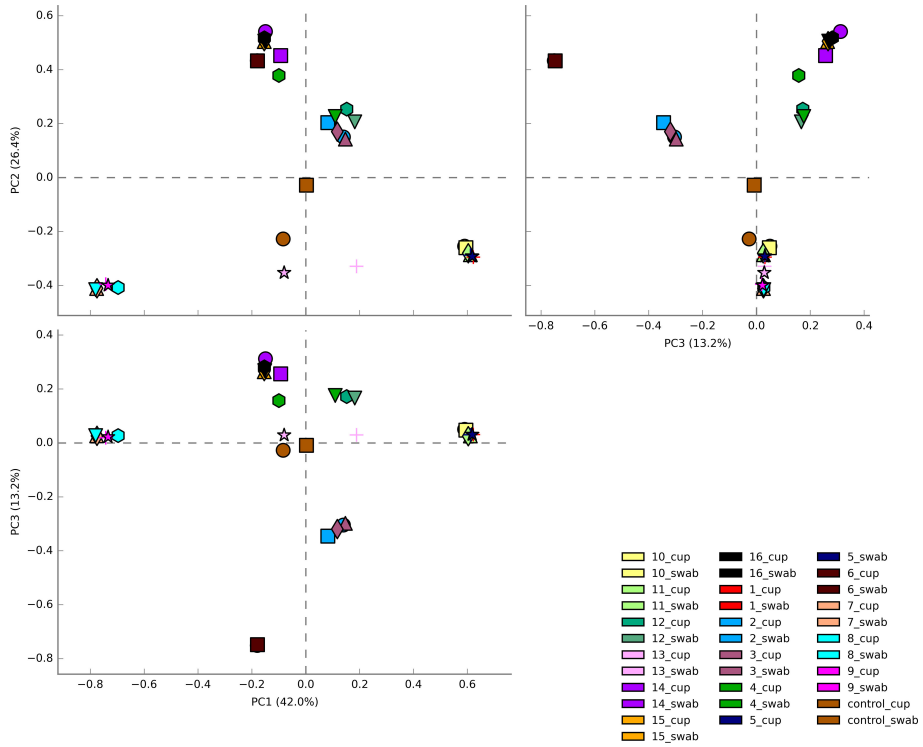

FIGURE 4
Principal Coordinate analysis (PCoA) plots of beta diversity of all vaginal microbiota samples from HIV-1 infected pregnant women. Samples are coloured by individual patient and sample type are represented by different shapes. Vaginal community diversity and composition largely clustered according to the individual patient with component 1 explaining 42% of variation and component 2 explaining 26% and component 3 explaining 13%.

Bifidobacteriaceae bacterial families commonly found in the vagina (Nel Van Zyl et al., 2020).

Our understanding of the importance of vagina microbiota to health and disease is expanding rapidly with the use of culture independent DNA assays. Key to this is our understanding of how bacteria interact with their human host through allied techniques such as: flow cytometry of local immune cell populations (Byrne et al., 2021); multiplex immune assays of cytokines and other immune proteins (Short et al., 2020; Short et al., 2021); RNA sequencing of the bacterial transcriptome (Mohd Zaki et al., 2022); mass-spectrometry of bacterial metabolomic signatures and their glycan binding proteins (Pruski et al., 2021; Wu et al., 2022) in addition to a variety of microarrays (Li and Feizi, 2018).

To date, MC sampling of CVF has been widely applied in the study of HIV infection to assess genital HIV viral load measurement (Jaumdally et al., 2017), HIV-1 diffusion (Shukair et al., 2013), microbicide drug concentrations (Price et al., 2011) and to quantify mucosal immunoglobins (Archary et al., 2015; Cosgrove et al., 2016) and cytokines (Archary et al., 2015; Jaumdally et al., 2018). Its utility as a collection method is increasingly recognised in the study of microbiota host interactions (Masson et al., 2019; Short et al., 2020; Wu et al., 2022) but it can now be considered valid for metataxonomic characterisation. This method's utility may increase as the importance of studying how vaginal microbiota increase HIV transmission risk and risk of preterm birth in women living with HIV are gaining wider recognition and have the potential to be modified through modulating these microbial interactions.

Conclusion

Ultimately the choice of CVF collection method in trial design will depend on the assays required, their processing requirements, as well as cost, but if MCs are used then additional HVS sampling is not required for DNA based characterisation of the microbiota.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ebi.ac.uk/ena/browser/view/PRJEB60624, accession number PRJEB60624.

Ethics statement

The studies involving human participants were reviewed and approved by Southeast Coast RES Committee (13/LO/0107). The patients/participants provided their written informed consent to participate in this study.

Author contributions

C-ES and GT conceived and designed the study. Patient recruitment and sample collection were undertaken by C-ES and

RQ. Experiments and data collection were performed by C-ES, RQ and VP. Data processing, analyses, and interpretation were performed by C-ES, AS, GT and DM. All figures and tables were generated by C-ES. C-ES wrote the first draft of the manuscript and all authors contributed critical revisions to the paper, interpretation of the results and approved the final version.

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Conflict of interest

PB reports personal fees and shares and stock ownership in ObsEva Pharmaceuticals, personal fees from GlaxoSmithKline that are both outside the submitted work. DM and PB have a patent for the use of *Lactobacillus crispatus* CTV-05 in the prevention of preterm birth US 63/151,474. DM has received consultancy fees from Freya Biosciences and Kean Health by Psomagen. No other authors had any commercial or financial relationships that could be construed as a potential conflict of interest. JM has received consultancy fees from EnteroBiotix and Cultech Ltd.

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Dysbiosis of vaginal and cervical microbiome is associated with uterine fibroids

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Dysbiosis of the female reproductive tract is closely associated with gynecologic diseases. Here, we aim to explore the association between dysbiosis in the genital tract and uterine fibroids (UFs) to further provide new insights into UF etiology. We present an observational study to profile vaginal and cervical microbiome from 29 women with UFs and 38 healthy women, and 125 samples were obtained and sequenced. By comparing the microbial profiles between different parts of the reproductive tract, there is no significant difference in microbial diversity between healthy subjects and UF patients. However, alpha diversity of UF patients was negatively correlated with the number of fibroids. Increased Firmicutes were observed in both the cervical and vaginal microbiome of UF patients at the phylum level. In differential analysis of relative abundance, some genera were shown to be significantly enriched (e.g., *Erysipelatoclostridium*, *Mucispirillum*, and *Finegoldia*) and depleted (e.g., *Erysipelotrichaceae* UCG-003 and *Sporolactobacillus*) in UF patients. Furthermore, the microbial co-occurrence networks of UF patients showed lower connectivity and complexity, suggesting reduced interactions and stability of the cervical and vaginal microbiota in UF patients. In summary, our findings revealed the perturbation of microbiome in the presence of UFs and a distinct pattern of characteristic vaginal and cervical microbiome involved in UFs, offering new options to further improve prevention and management strategies.

KEYWORDS

uterine fibroids, vaginal microbiome, cervical microbiome, dysbiosis, microbial interaction

1 Introduction

Uterine fibroids (UFs), also known as uterine leiomyomas or myomas, are the most common nonmalignant neoplasms of the reproductive tract among reproductive-age women and are composed of smooth muscle cells and fibroblasts (Stewart, 2001; Stewart et al., 2016). It is estimated to affect more than 25% of women worldwide, particularly black

women (Baird et al., 2003). Careful pathological examination of hysterectomy histopathologic specimens showed that the incidence of UFs was as high as 77% (Cramer and Patel, 1990; Harris et al., 2022). Although UFs are often asymptomatic, reasonable therapy is still needed for many women who have significant symptoms and consequences that include heavy or prolonged bleeding, anemia, pelvic pain, even infertility, and adverse pregnancy outcomes (Donnez and Dolmans, 2016; Zepiridis et al., 2016). In addition to the significant impacts on quality of life, UFs also incur heavy individual and societal costs, including surgery, hospital admissions, outpatient visits, psychological distress, and medications (Al-Hendy et al., 2017; Millien et al., 2021). The pathophysiology of UFs is still under investigation, including genetic susceptibility, sex steroid hormones, and abnormal stem cell transformation (Moravek et al., 2015; El Sabeh et al., 2021).

The human microbiome has co-evolved with the human as a unity called holobiont (Postler and Ghosh, 2017). The microbial communities play a fundamental role in human health and diseases, performing key functions in digestion, metabolism, mood and behavior, development and immunity, and a range of acute and chronic disorders (Ottman et al., 2012). Furthermore, the existence and invasion of microbiota inhabited along the female reproductive tract have long been known to impact female reproductive health and the onset of gynecological diseases (e.g., gynecological cancers, infertility, preterm birth, polycystic ovary syndrome, cervical intraepithelial lesions, and/or endometriosis) (Nieves-Ramirez et al., 2021; Salliss et al., 2021; Zhu et al., 2022). Generally, the vaginal microbiome in healthy women is dominated by *Lactobacillus*, but this ecosystem could be disrupted in the presence of some vaginal microorganisms, such as *Atopobium vaginae*, *Mycoplasma*, and *Prevotella bivia*, as well as a decrease in the proportion of *Lactobacillus* spp. Exogenous pathogen infections, such as parasites (*Trichomonas vaginalis* and *Schistosoma*) and viruses (HPV, HIV, and HSV), also alter the microbiota of the female reproductive tract by influencing the host's immune response and metabolism (Martin et al., 2013; Lebeau et al., 2022; Bongiorni Galego and Tasca, 2023). Given that a vast community of indigenous microorganisms colonize the reproductive tract and interact with the host in a symbiotic relationship, the microbiome also can alter among populations depending on the host's diet, ethnicity, geographical environment, health status, etc. (Gupta et al., 2017; Song et al., 2020).

Previous studies demonstrated that the reproductive tract microbiota was involved in reproductive tract diseases through multiple approaches, including local immune responses and metabolic regulation, and we hypothesized that the reproductive tract microbiota might play a role in UFs. Emerging evidence suggests the dysbiosis of the female genital tract (FGT) microbiome in patients with UFs, while these findings are not comprehensive and uniform among studied populations. Prior clinical studies that used self-reported questionnaire data showed an association between an increased risk of fibroids and bacterial vaginosis (BV). BV is characterized by vaginal microbial community alteration in which the microbiome normally dominated by *Lactobacillus* switches to anaerobes like *Gardnerella vaginalis* (Moore et al., 2015; Moore and Baird, 2017). However, qPCR analysis of BV-associated bacteria and *Lactobacillus* found no powerful evidence to support the hypothesis that BV could increase the risk of leiomyoma incidence or growth in subsequent prospective studies (Moore et al., 2021; Moore et al., 2022). Lately, a study employing high-throughput 16S rRNA taxonomic profiling found that *Lactobacillus* spp. were more abundant in the vaginal and cervical samples of individuals without UFs, while *Lactobacillus iners* was more abundant in the cervix of UF patients (Chen et al., 2017). Moreover, the abundance and diversity of vaginal bacterial taxa were significantly higher in recurrent vaginitis patients with underlying uterine diseases (UFs, adenomyosis, and endometrial polyps) than those without (Kim et al., 2022). In summary, there are still some limitations in these studies of the correlation between reproductive tract dysbiosis and UFs, including confounding factors, lack of suitable controls, and insufficient sample size, so there is no consistent conclusion on the relationship between microecological imbalance in the reproductive tract and UFs, and further studies are necessary.

In this work, we collected 125 specimens from 29 patients with UFs and 38 healthy subjects to analyze vaginal and cervical microbiome. By comparing the microbial profiles between UF patients and healthy individuals, we revealed the perturbation of the genital tract microbial community in the presence of UFs. Moreover, we highlighted the pivotal role of genital tract microbial interplay in patients with UFs and healthy women.

2 Materials and methods

2.1 Study design and sample collection

Samples from the vagina and cervix of patients with UFs and healthy people were taken for this experiment during the same period. Volunteers who were not in the pre- or post-menopausal period were recruited between December 2020 and May 2021 at the Third Xiangya Hospital of Central South University. Patients with UFs all met clinical diagnostic criteria and all subjects underwent ultrasound within 1 month to identify the presence/absence of UFs (Stewart, 2015). None of the participants received recent vaginal medication or cervical treatment or had performed douching within the 7 days before sample collection. Exclusion criteria include the following: administration of exogenous estrogens, progestins, and antibiotics within 3 months; history of endocrine or autoimmune disorders, gynecological cancer, and adenomyoma; pregnant and lactating women; episodes of vaginitis and pelvic inflammatory disease (PID); HPV infection of the genital tract; and placement of intrauterine device (IUD). The samples were collected by swabs, including leucorrhea (drawn from the mid-vagina), and cervical mucus (drawn from the cervical canal) in the follicular phase of the menstrual cycle. The collected fresh samples were immediately stored in sampling tubes with the preservative solution and stored at −80°C until further processing.

For experiments involving human swab samples whose donors were identifiable, written informed consent was obtained from each study participant, according to protocols approved by the Institutional Review Board (IRB) of Third Xiangya Hospital, Central South University (under permit number 22224).

2.2 DNA extraction

Total genomic DNA was extracted with the OMEGA Soil DNA Kit (M5636-02) (OMEGA Bio-Tek, Norcross, GA, USA) according to the following steps. Weigh 500 mg of glass beads and 0.25–0.5 g of sample in a 2-ml centrifuge tube, add 0.7 ml of Buffer SLX Mlus and 70 µl of Buffer DS, and mix by vortexing. Then, incubating at 70°C for 10 min and centrifuging at 13,000×*g* for 5 min were performed. After transferring 500 µl of the supernatant into new 2-ml tubes and adding 170 µl of Buffer SP2, add 170 µl of HTR Reagent to samples and mix thoroughly by vortexing for 10 s. After incubating on ice for 5 min and centrifuging at 13,000×*g* in a microcentrifuge for 5 min, we transferred 450 µl of the cleared supernatant to new 1.5-ml tubes, added 450 µl of Buffer XP5 and 40 µl of MagSi Particles to the samples, and shook 60 s to mix well. Then, incubate at room temperature for 2 min and place the tube or plate on a magnetic separation device suitable for 2-ml tubes to magnetize the MagSi particles. We added 500 µl of Buffer XP5 to the test tube, then placed the tube on a magnetic separation device and carefully removed and discarded the cleared supernatant. After adding 800 µl of Buffer PHB into the tube, place the tube onto the magnetic separation device and carefully remove and discard the cleared supernatant. We added 800 µl of SPM Wash Buffer diluted with ethanol into the tube, placed the tube onto the magnetic separation device to magnetize the MagSi particles, and carefully removed and discarded the cleared supernatant. Wash MagSi particles with SPM one more time. After removing the supernatant, air-dry the magnetic beads by inverting the tube on absorbent paper for 15 min. Remove any residue liquid from the tube with pipettor. After adding 50–100 µl of Elution Buffer or water to the tube, incubate the tube and resuspend MagSi particles by vortexing at 65°C for 10 min. We transferred the cleared supernatant containing purified DNA to new 1.5-ml tubes and stored it at −20°C before analysis. The quantity and quality of extracted DNA were measured respectively using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis.

2.3 16S rRNA gene amplicon sequencing

PCR amplification of the V3–V4 region of the bacterial 16S rRNA gene was performed using the forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Sample-specific 7-bp barcodes were integrated into the primers for multiplex sequencing. The components of PCR contained 5 µl of buffer (5×), 0.25 µl of fast Pfu DNA polymerase (5 U/µl), 2 µl (2.5 mM) of dNTP, 1 µl (10 µM) of each forward and reverse primer, 1 µl of DNA template, and 14.75 µl of ddH_2O. Thermal cycling consisted of initial denaturation at 98°C for 5 min, followed by 25 cycles including denaturation at 98°C for 30 s, annealing at 53°C for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 5 min. PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2×250 bp sequencing was performed using the Illumina NovaSeq platform with NovaSeq 6000 SP Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China).

2.4 Sequence analysis

After sequencing, the reads were de-multiplexed into samples according to the barcodes and the sequence was imported to the QIIME2 (version 2022.2) (Bolyen et al., 2019). The raw data were filtered to eliminate the adapter pollution and low-quality reads to obtain clean reads. Sequences were clustered at the 97% similarity level using the Vsearch plugin (Rognes et al., 2016). Taxonomic classifiers use classify-consensus-blast of the plugin feature-classifier (Camacho et al., 2009), based on the Silva 138 reference sequence (MD5: a914837bc3f8964b156a9653e2420d22) and taxonomy files (MD5: e2c40ae4c60cbf75e24312bb24652f2c) (Holgersen et al., 2021). Non-bacterial sequences and mitochondrial chloroplast contamination were removed by the taxa plugin.

2.5 Statistical analysis

All statistical analyses were performed by using the R environment (V4.2.1) (R Core Team, 2022). The statistical results were visualized using the "ggplot2" package without special instructions (Wickham and Wickham, 2016). The package "vegan" was used to calculate alpha diversity based on flat taxonomy table and obtain the differential expression matrix and the *p*-value matrix of bacterial composition at the genus level (Oksanen et al., 2022). The Gini–Simpson diversity index was obtained by subtracting the value of the classical Simpson index from 1. Beta diversity was analyzed using constrained principal coordinate analysis (CPCoA) by the "amplicon" package (Zhang et al., 2019; Liu et al., 2021), and bacterial community composition across all samples was based on Bray–Curtis distances. Venn was performed by the "ggvenn" package (Yan, 2021). Differential analysis of the relationship between the number of fibroids and Gini–Simpson was performed by the function "summary" in the R environment. Phylum-level Manhattan plot was computed using the "edgeR" package based on taxonomic information, and the *p*-value was corrected by the Benjamin and Hochberg false discovery rate (FDR) (Robinson et al., 2010). The taxonomic composition of microbiota at the phylum and genus level was completed using the "ggplot2" package. The package "DESeq2" was used to analyze abundance difference and marker genus based on the rules that the significance level was adjusted p-value < 0.05 and absolute foldchange value was greater than 1 (Love et al., 2014). The co-occurrence networks of microbiome within four groups were performed using the relative abundance table of genus level, and

it was established based on Spearman correlation matrix and corrected *p*-value matrix using the "igraph" package (Csardi and Nepusz, 2006). FDR was used to correct the *p*-value. Genera with relative abundance lower than 0.1% were filtered out before analysis, and the thresholds of Spearman correlation coefficient and corrected *p*-values were 0.8 and 0.05, respectively. Network topology properties and hub networks were calculated using Gephi software (Bastian et al., 2009). Properties of the co-occurrence networks of microbiome were compared by Mann–Whitney *U* test in R environment.

Clinical data analyses were performed using SPSS 26.0 software (IBM Corp, Armonk, NY, USA). Continuous variables were presented as median with range (minimum–maximum) or mean ± standard deviation (SD) and appropriately analyzed with the Wilcoxon or *t* test. Categorical variables were described as percentages and evaluated using the Chi-squared or Fisher exact test. In addition, the Mann–Whitney *U* test was used to compare the differences in nonparametric data between the groups. *p*-value less than 0.05 (2-sided) was considered statistically significant.

3 Results

3.1 Demographic characteristics of the study population

In total, 29 patients with UFs and 38 healthy subjects were enrolled in this study. All participants were fully informed about the study protocol. The clinical information of the participants, including age, body mass index (BMI), menstrual history, and reproductive history, is shown in Table 1. No statistical significance for these characteristics was found between UFs and the healthy group. In addition, the clinical characteristics of the UF patients are also shown in Supplementary Table S1.

3.2 The microbial diversity of the female genital tract

To explore whether the microbiome in the genital tract was altered in patients with UFs, we collected samples from the vagina and cervix. We collected a total of 50 samples from patients with UFs and 75 samples from healthy people. We divided samples into four groups according to different sampling sites in the genital tract and whether they had UFs: healthy individuals' vaginal microbiome (HV, n = 38), UF patients' vaginal microbiome (FV, n = 29), healthy women's cervical microbiome (HC, n = 37), and cervical microbiome of patients with UFs (FC, n = 21) (Supplementary Figure S1). Upon analysis, the rarefaction curves of all samples were parallel to the *X*-axis (Supplementary Figure S2), indicating that all samples were sequenced to sufficient depth for subsequent analysis.

We performed analysis of alpha diversity in four groups to present the changes of numbers, abundance, and evenness of the microbiome in the genital tract. The results of the Gini–Simpson index showed that the alpha diversity of UF patients was lower than that of healthy people at different sampling points, but there was no significant difference (Figure 1A). The Pielou index also showed no difference, suggesting no significant difference in the evenness of OTUs among the four groups. (Figure 1C). Interestingly, we observed a significant difference in the Richness index between the groups of healthy people sampled from the vagina and cervix, respectively (p = 0.0171) (Figure 1B). We then compared beta diversity based on Bray–Curtis distances between the groups of patients and healthy people using constrained principal coordinate analysis (CPCoA) and found that there was no significant difference in Bray–Curtis distances among four groups, suggesting that the compositional differences of samples from four groups were relatively similar (Figure 1D). In addition, the relationship between alpha diversity and clinical variables (location and

TABLE 1 Characteristics of the study subjects.

Variables	Control group (n = 38)	Uterine fibroid group (n = 29)	*p*-value
Age [years, median (range)]	34 (23–54)	40 (24–49)	0.065
BMI [kg/m^2, (mean ± SD)]	22.6 ± 3.60	22.7 ± 3.38	0.923
Number of gravidities	2 (0–8)	3 (0–7)	0.355
Number of parities	1 (0–3)	1 (0–4)	0.803
Number of abortions	1 (0–5)	1 (0–5)	0.389
Caesarean section delivery, *n* (%)	9 (23.7%)	6 (20.7%)	0.771
Menstruation [day, median (range)]			
Duration	7 (5–13)	7 (4–13)	0.072
Frequency	28 (22–40)	28 (25–45)	0.079
Increased flow volume, *n* (%)	19 (50.0%)	9 (31.0%)	0.119

BMI, body mass index; SD, standard deviation.

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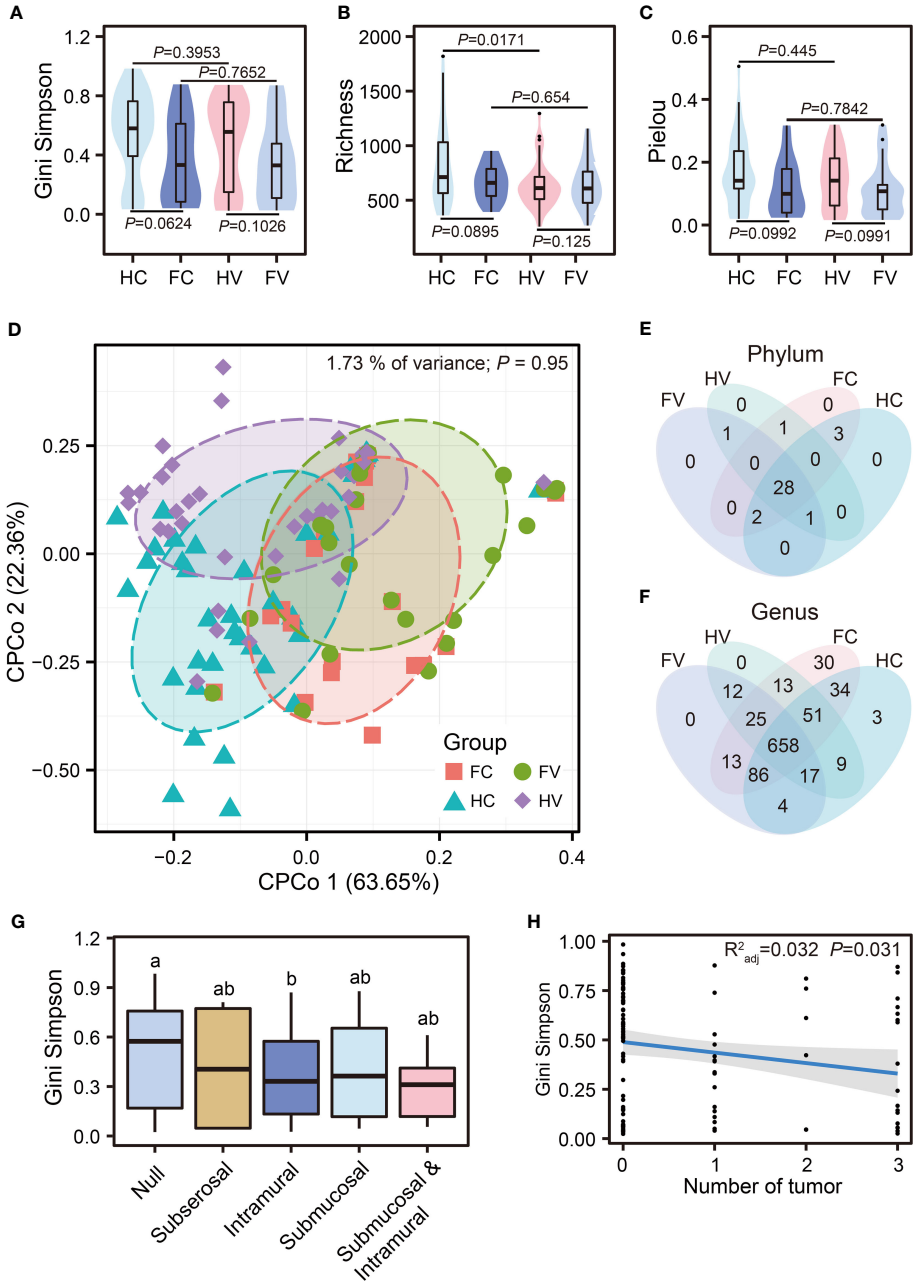

FIGURE 1
The diversity of microbial communities. Analysis of alpha diversity was presented by Gini–Simpson **(A)**, Richness **(B)**, and Pielou **(C)** among four groups. Differential analysis was performed by the Wilcoxon test. **(D)** Beta diversity was presented by CPCoA (Constrained Principal Coordinate Analysis) analysis based on Bray–Curtis distance. The difference of composition in phylum **(E)** and genus **(F)** levels between four groups. **(G)** Comparison of Gini–Simpson among different locations of tumor occurrence. The letters such as "a" and "b" indicate whether there is a significant difference between different groups. The same letters indicate no significant difference between different groups (Kruskal–Wallis test). **(H)** Regression analysis of tumor numbers and Gini–Simpson.

number of fibroids) was explored. No difference in the Gini–Simpson index was found in UF patients at different locations (Figure 1G). The number of tumors showed a strong negative correlation with the Gini–Simpson index of vaginal and cervical microbiome in patients with UFs (R_{adj}^2 = 0.032, p = 0.031, Figure 1H). As the number of tumors increased, the alpha diversity decreased significantly.

3.3 The composition and biomarkers of the female reproductive tract

To explore alterations in the composition of bacterial communities located in different sites of the genital tract, we compared the differences in bacterial abundances among the four groups at the phylum and genus levels.

10.3389/fcimb.2023.1196823

The bacterial composition of the four groups had both commonalities and differences. At the phylum level, there were 31 phyla in the HV group, 32 phyla in the FV group, 33 phyla in the HC group, and 34 phyla in the FC group (Figure 1E). At the genus level, there were 785 genera in the HV group, 815 genera in the FV group, 862 genera in the HC group, and 910 genera in the FC group, of which 658 genera were common to all groups (Figure 1F). The differences in the bacterial genus level composition of the four groups were greater than at the phylum level. To further specify the bacterial differences, we then analyzed the composition of the flora at the phylum and genus levels. From the results of the phylum, the four groups were dominated by Firmicutes (Figure 2A). We analyzed bacterial alterations at the phylum level in UF patients and healthy people. Compared with the groups of healthy people, the abundance of Firmicutes showed a highly significant upward trend in the groups of UF patients ($p < 0.0001$) (Figure 2B). According to the compositional analysis at the genus level, *Lactobacillus* spp. was the dominant bacterium in the vagina and cervix (Figure 2C).

In addition, we analyzed differentially abundant bacteria in the vagina and cervix based on diversity of biomarker signature differences. Compared to the group of healthy people, the volcano plot showed that one biomarker was found to be depleted (marked by blue) and two were enriched (marked by red) in patients with UFs in the vagina (Figure 3A). In the cervix, 11 distinct genera were found in the UF group relative to the healthy control, with 1 genus (highlighted in red) upregulated and 10 genera downregulated (highlighted in blue) (Figure 3B).

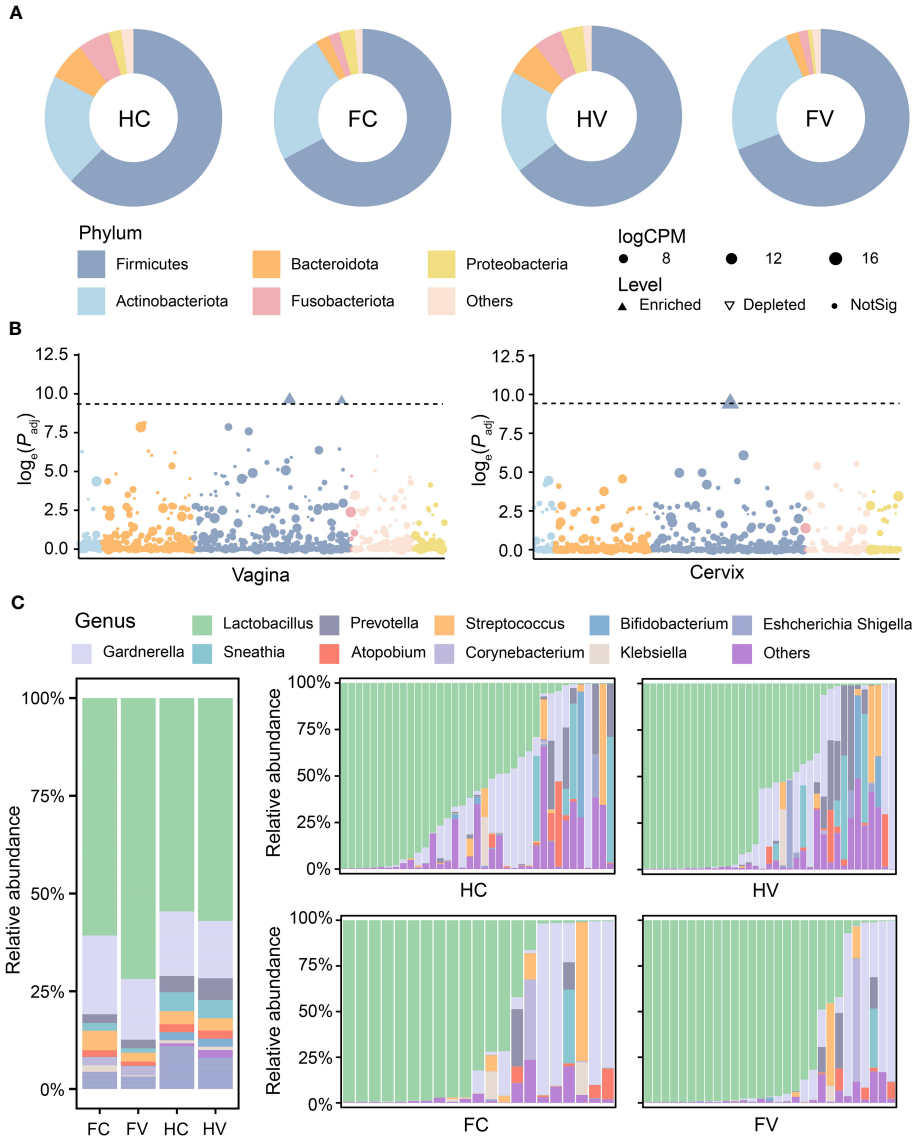

FIGURE 2
The composition of microbiome at the phylum and genus levels. **(A)** Microbial composition of four groups at the phylum level. **(B)** The difference analysis of microbiome in relative abundance at phylum levels between healthy and UF groups in the position of vagina and cervix. Dashed lines show that the threshold of *p*-value is 0.0001. Dots show that there is no significant difference in the relative abundance of phylum. Upwards filled triangles represented significant enrichment and downwards hollow triangles represented significant depletion in the relative abundance of phylum. **(C)** Microbial composition of four groups at the genus level.

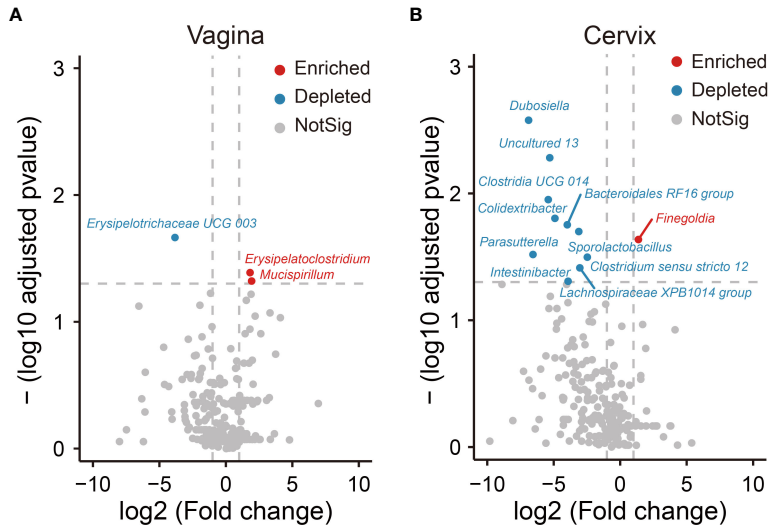

FIGURE 3
Differential analysis of relative abundance at the genus level. The analysis of volcano shows relative abundance of genera, which is significantly enriched and depleted in the UF groups compared to the healthy groups in the position of vagina **(A)** and cervix **(B)**. Dots in red show that the relative abundance of genera in UF groups enriched significantly compared to healthy groups. Dots in blue show that the relative abundance of genera in UF groups depleted significantly compared to healthy groups. Dots in gray show that there is no significant difference in relative abundance of genera between UFs and healthy groups.

3.4 Microbial interactions and networks between microbiome in the female genital tract

We performed co-occurrence networks analysis to reveal the relationships among microorganisms. Based on the same network construction parameters, the network showed 35 nodes and 176 edges for the HV group, 34 nodes and 51 edges for the FV group, 77 nodes and 272 edges for the HC group, and 65 nodes and 88 edges for the FC group (Figure 4A). We analyzed the network properties for each group of networks. The microbial average weighted degrees in the UF patients and healthy people showed significant differences (Figure 4C). The number of cervical and vaginal triangles was also significantly lower in the UF groups (Figure 4E), suggesting that the connectivity and complexity of the genital tract microbiota were significantly lower in patients with UFs. In the analysis of clusters, clusters of vagina and cervix were significantly higher in the UF groups, which indicated that the average "clustering property" of the whole microbial network in both the vagina and cervix was higher in UF groups than in the healthy groups (Figure 4D). Meanwhile, we extracted and presented hub networks of four microbiomes with the highest degree of clustering by group (Figure 4B) to gain insight into each network in the UFs and healthy groups at different locations. Overall, the hub networks of cervical microbiome in healthy people had the highest number of nodes and aggregation.

4 Discussion

In this study, we conducted microbial profiling for cervical and vaginal microbiome associated with UFs, and further highlighted the interplay between microbial communities. By comparing the microbial profiles between different parts of the reproductive tract among groups, we revealed the perturbation of microbial community in the presence of UFs. It was found that microbiome of vagina and cervix in patients with UFs were altered in composition and ecological network compared with healthy women. The alpha and beta diversities showed no statistical significance between UF patients and the healthy women. However, a significant negative correlation was observed between Gini–Simpson and the number of fibroids. Moreover, higher microbial Richness index was found in the HC group than the HV group. Samples from patients with UFs exhibited significant alterations in Firmicutes at the phylum level. In differential abundance analysis, enriched *Erysipelatoclostridium*, *Mucispirillum*, and *Finegoldia* and depleted *Erysipelotrichaceae* UCG-003 and *Sporolactobacillus* were observed in UF groups. Furthermore, more dispersed and lower node degree distribution were presented in the networks of the UF groups (both in the vagina and cervix), suggesting lower connectivity and complexity than the networks of healthy groups.

In our study, the microbial alpha and beta diversities of vaginal and cervical microbiome between UF patients and healthy women were not significant ($p > 0.05$). These results were also in line with previous research on female reproductive disorders, which found that endometriosis, intrauterine adhesion, and cervical intraepithelial neoplasia showed no alteration compared with healthy people (Ata et al., 2019; Lin et al., 2022; Liu et al., 2022). Interestingly, the alpha diversity (Gini–Simpson index) of the microbiome was negatively correlated with the number of fibroids according to our results. The correlation between microbial imbalance and increase in the number of tumors indicated an important role of microbiota in the development of disease. For example, gut dysbiosis-stimulated cathepsin K secretion mediated

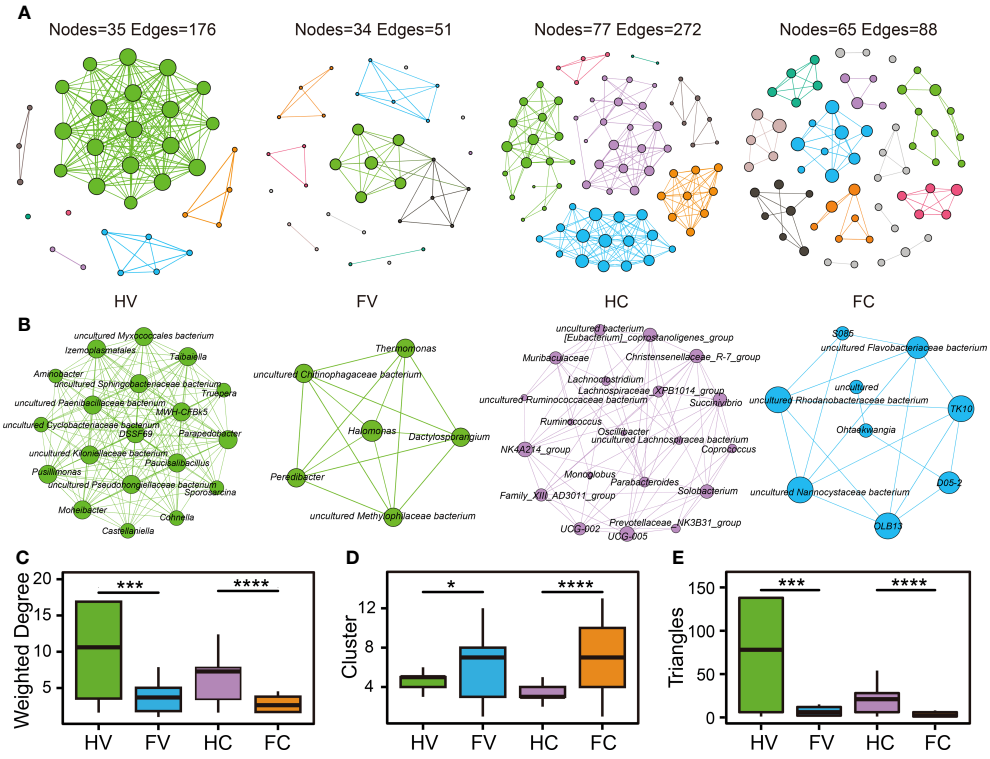

FIGURE 4
The co-occurrence network of microbiome in the four groups. The co-occurrence network **(A)** and hub network **(B)** of health and UF groups in the position of vagina and cervix. Comparison of network topology properties among four groups, containing weighted degree **(C)**, cluster **(D)**, and triangles **(E)**. Differential analysis was performed by Kruskal–Wallis test. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

TLR4-dependent M2 macrophage polarization and promoted tumor metastasis in colorectal cancer (Li et al., 2019). However, further experiments are needed to verify and explore possible mechanisms in benign UFs. Additionally, higher microbial Richness was observed in the healthy vagina than in the cervix, representing a unique ecological niche in cervical canal, as indicated by the heterogeneity of microbiota between this location and that of the vagina. On the other hand, highly cited research showed distinct biomass between vagina and cervix by qPCR test of *Lactobacillus* species, although no obvious difference was observed in microbial diversity (Chen et al., 2017).

Some gynecological diseases or organ malfunctions are marked by the presence of potential pathogenic microbes, whereas others are characterized by depletion of health-associated bacteria (Onderdonk et al., 2016; Brusselaers et al., 2019; Laniewski et al., 2020). In the vaginal microbiome, *Erysipelatoclostridium* and *Mucispirillum* were significantly enriched, while the *Erysipelotrichaceae* UCG-003 was depleted in UF patients. *Erysipelatoclostridium*, an opportunistic pathogen reported to be enriched in patients with allergic diseases and metabolic syndrome, modulates small intestinal glucose and lipid transport by altering intestinal barrier permeability (Woting et al., 2014; Shen et al., 2019; Labarta-Bajo et al., 2020; Li et al., 2022). *Mucispirillum*, observed more abundant in progression of hepatocellular carcinoma in mice driven by high cholesterol, may be reversed with atorvastatin treatment through bile acid biosynthesis pathway (Zhang et al., 2021). Moreover, *Erysipelotrichaceae* UCG-003 is one of the main bacteria that produce butyrate, which is considered to play an important role in maintaining the integrity of the colonic epithelium. The abundance of *Erysipelotrichaceae* UCG-003 was found to be decreased in patients with neurological disorders and lung cancer compared with healthy individuals (Singh et al., 2019; Zhao et al., 2021; Kaiyrlykyzy et al., 2022). Regarding the cervical microbiome, *Finegoldia* usually appears on the skin and mucous membranes and is associated with vaginosis, as well as infectious diseases and soft tissue abscesses. More importantly, *Finegoldia magna* was found to be involved in high-grade squamous intraepithelial neoplasia and cervical cancer (Murphy and Frick, 2013; So et al., 2020; Zhou et al., 2020). *Sporolactobacillus* is similar to *Lactobacillus* in metabolic function and has even been commercialized as a probiotic. Depleted *Sporolactobacillus* may indicate an imbalanced vaginal environment that promotes the growth of pathogenic bacteria (Wei et al., 2020). In brief, significant decreases in probiotics and increases in pathogenic bacterial species were shown among UF subjects, indicating their reduced ability to maintain homeostasis and the increased risk of disease.

Network analysis is a promising and increasingly used approach that involves analyzing and understanding complex systems and emerging phenomena, including understanding functions of the ecosystems, such as stability and resilience (Greenbaum et al., 2019). Analysis of the co-occurrence network of microbiome revealed that microbial networks were composed of tightly connected nodes and formed a kind of “small world” topology. As shown in previous studies, ecological dynamics of the vaginal microbiome play a crucial role in health and disease (Ma and Ellison, 2021). Our network analysis demonstrated differences in

the microbial interaction network between the patients and healthy people at different locations. In these two sites of the genital tract, the microbial network in patients with UFs presented lower connectivity and complexity, suggesting that the microbiome found in UFs could be less stable (Naqvi et al., 2010). Therefore, dysbiosis of the microbiome in the cervix and vagina is reflected not only in the changes of microbial relative abundance at the different taxonomic level, but also in the alteration of relationships within microbial interactions.

However, certain limitations of this study should also be considered. Several potential risk factors, such as unhealthy habits and genetic susceptibility, were not described in detail. More completed microbial community information and precise functional genes cannot be provided by 16S rRNA sequencing, although the method has the advantage of easy analysis and low cost. Thus, metagenomic, metatranscriptomics, and/or metabolomic technologies are supposed to conduct further study of microbial communities comprehensively and identify species and strains with higher resolution and confidence. Likewise, no precise mechanism is involved in this study, such as host hormone levels, metabolism, and immune regulation. Therefore, further studies are required to clarify whether the association is causal, whether dysbiosis of the female reproductive tract leads to UFs or whether the disease renders dysbiosis in the vagina and cervix. Moreover, microbial environment of the whole female reproductive tract may not be provided or reflected by vagina and cervical canal, which is closely related to the systemic status. However, it is health-threatening and unethical for healthy women and UF patients who choose minimally invasive treatment to take samples from uterine cavities. Given the ethnic variance, it is reasonable to further broaden the scope of the study population in different ethnic and racial groups to comprehensively identify the microbial alterations in the UFs, thus providing the possibility of geographically tailored microbiome-based therapeutic strategies.

In summary, our preliminary study can provide distinct evidence of the imbalance of vaginal and cervical canal microbiomes in UF patients and serve as a good starting point to narrow down the candidate pathogens for subsequent assays. Our results can lay the foundation for subsequent studies on the role of reproductive tract microbiome in the UF etiology. More studies are needed to analyze the mechanism of how pathogenic bacteria and microbial dysbiosis might affect UFs.

Data availability statement

The raw data reported in this paper have been deposited in the Gene Expression Omnibus 291 (https://www.ncbi.nlm.nih.gov/geo/), under accession number GSE197904.

Ethics statement

The studies involving human participants were reviewed and approved by The Third Xiangya Hospital of Central South University and performed under the relevant guidelines and regulations (IRB number 22224). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

DX and ZY conceived the study. XM and HC performed the experiments and analyzed the data. XM, HC, XP, and XZ wrote and edited the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1196823/full#supplementary-material

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A rare bacteremia caused by *Fannyhessea vaginae* in a pregnant woman: case report and literature review

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Bloodstream infection caused by anaerobic microorganisms continues to be associated with a high mortality risk, necessitating a rapid diagnosis and an appropriate treatment. As an anaerobic gram-positive organism associated with vaginal infections, *Fannyhessea vaginae* is a rare cause of invasive infections. In this case, a 32-year-old pregnant woman with bacterial vaginosis presented with bacteremia. The microbiological analysis of the blood cultures identified *F. vaginae*. The patient was treated empirically with 5 days of cefoperazone/sulbactam and recovered well. Here, we provide a review of the literature on *F. vaginae* infections, and the reported cases demonstrate the need for awareness of the different anaerobic species found in the vaginal tract and adaptation of empirical therapies, especially in pregnant women.

KEYWORDS

anaerobic bloodstream infections, bacteremia, case report, *Fannyhessea vaginae*, literature review

1 Introduction

Anaerobes are the dominant organisms of the normal human microbiome. They inhabit mucosal membranes such as those in the female reproductive tract, gastrointestinal system, and oral cavity. Generally, these organisms play a crucial role in sustaining normal homeostasis in the human body. However, they can also serve as pathogens that cause invasive infections in human populations (Watanabe et al., 2021). Anaerobic bloodstream infections are responsible for up to 20% of bacteremic episodes with a high mortality rate, even higher in patients who are of advanced age and lack appropriate treatment (Dien Bard et al., 2020).

Fannyhessea vaginae, previously known as *Atopobium vaginae*, is a strict anaerobe first isolated from the vaginal flora of a healthy Swedish woman in 1999 (Jovita et al., 1999). In 2018, it was reclassified as *F. vaginae* (Nouioui et al., 2018). *F. vaginae* is a Gram-positive, elliptical- or rod-shaped coccobacillus that can appear as single elements, pairs, clumps, or short chains and is a part of the human vaginal microbiome. Many studies have

emphasized that *F. vaginae* plays an important role in the pathophysiology of vaginal diseases (Mendling et al., 2019). *F. vaginae* is able to incorporate into *Gardnerella vaginalis* biofilms, a crucial marker of bacterial vaginosis (Castro et al., 2021). It has also been determined that high vaginal loads of *F. vaginae* in conjunction with *Gardnerella* spp. is linked to late miscarriage and preterm birth (Bretelle et al., 2015). Although *F. vaginae* can be detected in the normal vaginal microbiome (8%–25%), it is more prevalent in patients with bacterial vaginosis (50%–96%) (Mendling et al., 2019). However, *F. vaginae* is an uncommon cause of invasive infections.

Here, we describe a rare case of bacteremia caused by *F. vaginae* in a pregnant woman with bacterial vaginosis and hypothesize that an ascending infection of *F. vaginae* in the vagina caused this woman's bacteremia. We also provide a review of previously published cases related to *F. vaginae* infections. The reported cases demonstrate that if a patient is febrile and exhibits symptoms of bloodstream infection, anaerobic species that are prevalent in the vaginal tract should be considered, especially for pregnant women with vaginal infections

2 Case report

2.1 Case description

The patient, a 32-year-old woman with uterine fibroids and resistant hypertension, was admitted for the delivery of her second child at a gestational age of 40 + 4 weeks on March 4, 2022. At the time of admission, the fetal membranes had not ruptured, and her body temperature was 36.3°C. Abdominal B-mode ultrasound indicated singleton pregnancy and multiple uterine fibroids (the largest was 17 × 8 mm). Chills occurred after oxytocin administration at admission, and her body temperature was 36.7°C. A baby boy was delivered by vaginal delivery, and his birth weight was 4,000 *g*. She had a first-degree perineal tear. After delivery, she was observed for >1 h, and her body temperature increased to 39°C. Emergency blood culture, blood analysis, and procalcitonin test were carried out. Her 4-h postpartum vaginal blood loss was 850 mL. Respiratory disease, hematologic disease, and urinary tract infection were ruled out. Prenatal vaginal discharge evaluation (posterior fornix swab) with a direct microscopic examination found gram-negative or -variable rods, and her Nugent score was 7. Hence, the patient was diagnosed with bacterial vaginosis, and cefoperazone/sulbactam was used for anti-infective treatment. After 5 days of antibiotic treatment, the patient was discharged. The patient appeared well at subsequent visits and seemed to have recovered completely.

2.2 Test results

In bilateral dual-bottle blood culture, the left anaerobic bottle was positive after 50 h of culture. The liquid in the positive blood culture bottle was aspirated and inoculated on blood agar plates, which were cultured in an aerobic environment and an anaerobic environment at 35°C. After 48 h, no bacterial growth was observed in the blood agar plate from the aerobic environment, and small grayish-white colonies could be observed in the plate from the anaerobic environment (Figure 1A). The Gram staining smear was positive, and the bacteria were elliptical or short rods in shape (Figure 1B). A single colony was selected, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker, Germany, MALDI Biotyper 3.1) rapid identification result was *F. vaginae*, and the score was 2.010. Additionally, the 16S rRNA sequence (GenBank accession no. OR287194) analysis also indicated that this bacterium was *F. vaginae*.

2.3 Clinical efficacy

Cefoperazone/sulbactam was used as an anti-infective treatment when the patient's white blood cell count peaked at 34 × 10^9/L. The dose was 3.0 g, intravenous infusion for 8 h. At 5 days postpartum,

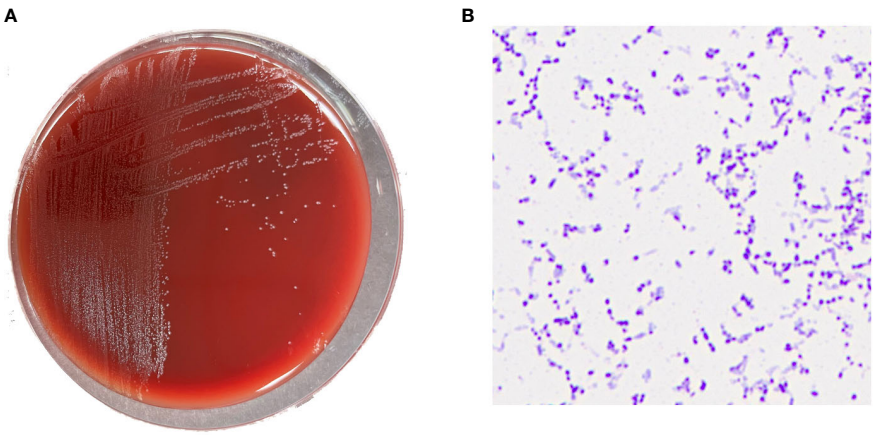

FIGURE 1
(A) Grayish white colonies of *Fannyhessea vaginae* after 48h of culture on blood agar plate under anaerobic conditions; **(B)** Gram staining showing gram-positive (*F*) *vaginae* bacteria appearing as elliptical or short rods.

antibiotic treatment was discontinued. The patient's white blood cell count and procalcitonin continuously decreased until they returned to normal, and her temperature gradually returned to normal. Figure 2 displays the variation trend of body temperature, white blood cell count, and procalcitonin concentration.

2.4 Drug sensitivity

The minimum inhibitory concentration (MIC) breakpoints for anaerobic bacteria in the Clinical and Laboratory Standards Institute M100-S31 and European Committee on Antimicrobial Susceptibility Testing (Version 10.0) were used as a reference, one McFarland turbidity solution was prepared, and the E test strip was used for the drug sensitivity test. The results are shown in Table 1.

3 Literature review

To the best of our knowledge, there are 10 previously published cases of *F. vaginae* infections, and a list of these cases is summarized in Table 2. These *F. vaginae* infections occurred in bloodstream infections (Knoester et al., 2011; Chan et al., 2012; Dauby et al., 2019; Taillandier et al., 2020), tubo-ovarian abscess (Geissdörfer et al., 2003), bacterial vaginosis (Burton et al., 2004), uterine endometritis (Yamagishi et al., 2011), subchorionic hematoma (Jacqmin et al., 2018), endocarditis (Mansell et al., 2018), and prosthetic joint infection (Massa et al., 2022). Our reported case revealed that anaerobic species found in the vaginal tract could cause bacteremia in pregnant women and cefoperazone/sulbactam was effective for antimicrobial treatment of *F. vaginae* infection.

4 Discussion

In the last 20–30 years, rapid and precise species-level identification of anaerobes has aided clinicians in providing the best care for their patients, resulting in dramatically lower morbidity and mortality rates and hospital stays (Kovács et al., 2022). However, anaerobic bacteria continue to be among the most neglected and unrecognized pathogens because their cultivation necessitates substantial microbiological experience, and many hospitals (particularly in developing nations) may lack the equipment necessary to achieve anaerobiosis (Nagy et al., 2018). As an anaerobic, *F. vaginae* is found in the normal vaginal microbiota but is increasingly linked to bacterial vaginosis (Mendling et al., 2019). Recently, a prospective investigation linked *F. vaginae* to salpingitis and infertility, indicating this microorganism's potential pathogenicity (Haggerty et al., 2016).

According to the Nugent score criteria for the identification of bacterial vaginosis via a direct microscopic examination, a Nugent score of 7 can be diagnosed as bacterial vaginosis as in our case report. This vaginal infection case allowed us to hypothesize that an ascending infection of *F. vaginae* from the vagina caused this woman's bacteremia. Similar ascending bacteremia of *F. vaginae* has also been reported in other cases. For instance, a case reported

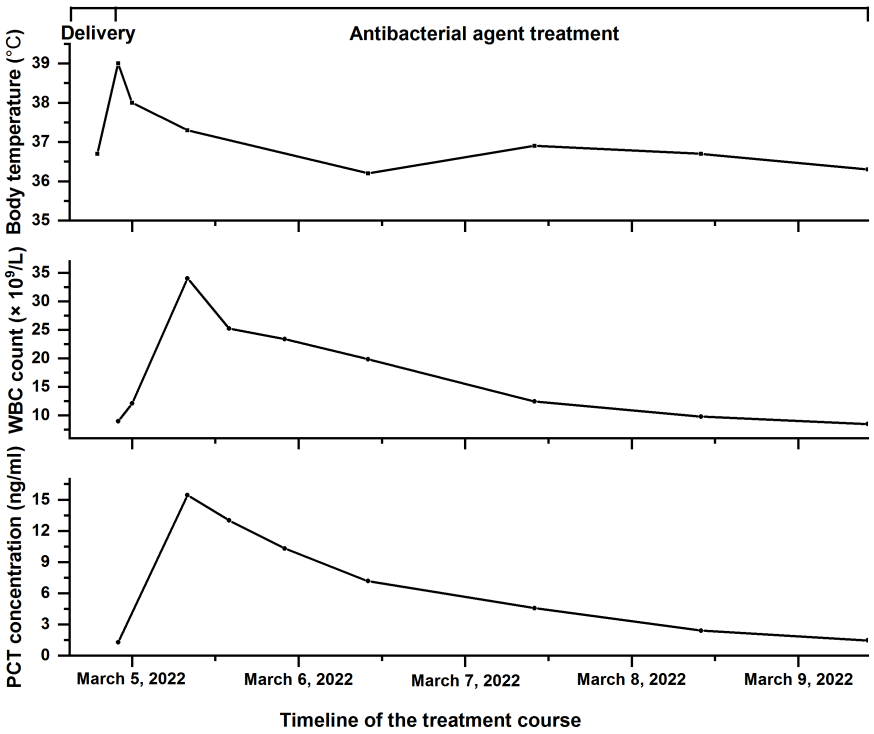

FIGURE 2
The timeline of the treatment course of the patient with *Fannyhessea vaginae* infection. Body temperature (top), white blood cell (WBC) count (middle), and procalcitonin (PCT) concentration (bottom) during hospitalization.

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TABLE 1 Retrospective antimicrobial testing of the *Fannyhessea vaginae* strain using the *E* test.

Antibacterial agent	MICs (mg/L)	Breakpoints		
		Susceptible	Intermediate	Resistant
Ampicillin	0.094	≤0.5[a]	1[a]	≥2[a]
Clindamycin	0.016	≤2[a]	4[a]	≥8[a]
Imipenem	0.016	≤4[a]	8[a]	≥16[a]
Penicillin	0.19	≤0.5[a]	1[a]	≥2[a]
Metronidazole	>256	≤8[a]	16[a]	≥32[a]
Vancomycin	1	≤2[b]	N/A	≥2[b]
Linezolid	0.38	N/A	N/A	N/A
Ampicillin/sulbactam	0.75	≤8/4[a]	16/8[a]	≥32/16[a]
Piperacillin/tazobactam	0.38	≤16/4[a]	32/4–64/4[a]	≥128/4[a]
Cefoperazone/sulbactam	0.38	N/A	N/A	N/A

[a]Clinical and Laboratory Standards Institute breakpoints.
[b]European Committee on Antimicrobial Susceptibility Testing breakpoints.
MIC, minimum inhibitory concentration; N/A, not available.

TABLE 2 Summary of 11 reported cases of *Fannyhessea vaginae* infections.

Reference	Age (years)/ sex	Country of origin	Disease	Antibiotic susceptibility testing (MICs mg/L)	Antimicrobial treatment	Outcome
Knoester et al. (2011)	40/ female (pregnant)	Netherlands	Bacteremia	PEN S (0.094), MTZ R (24), VAN S (1.5), CXM N/A 0.125, CLI S (<0.016)	AMP (1 g administered intravenously four times a day) for 2 weeks	Fetal death and the patient was cured
Chan et al. (2012)	33/ female (pregnant)	China	Bacteremia	PEN S (0.25), MTZ R (>256)	AMC (1.2 g administered intravenously three times a day) for 4 days	A neonate was delivered (cesarean section) and the patient was cured
Dauby et al. (2019)	29/ female (pregnant)	Belgium	Bacteremia	PEN S (0.03), AMP S (<0.0016), CLI S (0.016), MTZ R (>256)	AMC	A neonate was delivered (vaginal birth) and the patient cured
Taillandier et al. (2020)	57/female	France	Septic shock	MTZ R (N/A), CIP R (N/A), PEN S (N/A), AMC S (N/A), CTX S (N/A), CLI S (N/A), VAN S (N/A)	TZP and GEN	Cured
Geissdörfer et al. (2003)	39/female	Germany	Tubo-ovarian abscess	AMP S (0.032), PEN S (0.125), CXM S (0.19), FOX S (2), IMP S (2), MTZ R(>256)	FOX (2 g administered intravenously three times a day) and MTZ (0.5 g administered intravenously twice a day) for 5 days	Hysterectomy, bilateral salpingectomy, left-sided ovariectomy, appendectomy, and adhesiolysis
Burton et al. (2004)	48/female	Canada	Bacterial vaginosis	N/A	N/A	N/A
Yamagishi et al. (2011)	33/female	Japan	Uterine endometritis	AMP S (0.19), SAM S (0.125), PIP R (1.5), TZP S (1.0), MNO N/A (0.19), MEM S (1.5), IMP S (0.25), ERY N/A (<0.016), CLI S (<0.016), CHL S (1), CFP N/A (3), CSL N/A (1.5), CIP N/A (0.064), OFX N/A (0.19), MTZ R (>256)	AMX (0.5 g administered intravenously three times a day) for 4 days	Cured
Jacqmin et al. (2018)	38/ female (pregnant)	Belgium	Subchorionic hematoma	PEN S (0.016), MTZ S (1), AMC S (0.016), CLI S (<0.016)	AMC (1 g administered intravenously four times a day) for 4 days	Termination of pregnancy and the patient cured

(Continued)

TABLE 2 Continued

Reference	Age (years)/ sex	Country of origin	Disease	Antibiotic susceptibility testing (MICs mg/L)	Antimicrobial treatment	Outcome
Mansell et al. (2018)	18/female	United Kingdom	Endocarditis	N/A	VAN for 4 weeks	Surgical excision of tricuspid valve vegetation
Massa et al. (2022)	77/female	Belgium	Prosthetic joint infection	AMX S (0.047), CIP R (8), CLI S(<0.016), RIF S (<0.002)	TLC and then changed to AMX in combination with RIF on day 19 of admission	Cured
Present case	32/ female (pregnant)	China	Bacteremia	AMP S (0.094), CLI S (0.016), IMP S (0.016), PEN S (0.19), MTZ R (>256), VAN S (1), LNZ N/A (0.38), SAM S (0.75), TZP S (0.38), CSL N/A (0.38)	CSL (3 g administered intravenously three times a day) for 5 days	A baby boy was delivered (vaginal birth) and the patient was cured

AMC, amoxicillin/clavulanic acid; AMP, ampicillin; AMX, amoxicillin; CFP, cefoperazone; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; CSL, cefoperazone/sulbactam; CTX, cefotaxim; CXM, cefuroxime; ERY, erythromycin; FOX, cefoxitin; GEN, gentamicin; IMP, imipenem; LNZ, linezolid; MEM, meropenem; MNO, minocycline; MTZ, metronidazole; OFX, ofloxacin; PEN, penicillin; PIP, piperacillin; RIF, rifampicin; SAM, ampicillin/sulbactam; TLC, temocillin; TZP, piperacillin/tazobactam; VAN, vancomycin; MIC, minimal inhibitory concentration; R, resistant; S, susceptible; N/A, not available.

of a pregnant woman who had *F. vaginae* transferred from her cervix to her uterus during chorionic villus sampling, causing an intrauterine infection that resulted in fetal mortality and bacteremia of the mother (Knoester et al., 2011). Another case described an intrapartum *F. vaginae* bacteremia that occurred spontaneously without any prior surgical trauma to the female genital tract; the case was characterized by an imbalanced vaginal microbiota with the proliferation of *G. vaginalis* and *Candida albicans* (Chan et al., 2012). In addition to bacteremia, other diseases have been documented as a result of ascending *F. vaginae* infections. A 33-year-old woman with bacterial vaginosis was clinically diagnosed with uterine endometritis due to an ascending *F. vaginae* infection (Yamagishi et al., 2011). Similarly, an 18-year-old patient lanced a vaginal cyst herself with a subcutaneous insulin cannula, resulting in infective endocarditis due to an ascending *F. vaginae* infection (Mansell et al., 2018). Therefore, disturbed vaginal microbiota is a significant cause of a variety of diseases in women, and more attention should be given to the vaginal microbiome of female patients with vaginal dysbiosis.

F. vaginae infections in pregnant patients have a clinical consequence that affects both the mother and the fetus. Although no maternal deaths had been documented, major morbidities such as the need for extensive surgery and consequent infertility, as well as the emotional toll of losing the fetus, might still occur (Table 2). In light of the severe consequences associated with *F. vaginae* infections, appropriate treatment should be initiated once the diagnosis is made.

The European Committee on Antimicrobial Susceptibility Testing states that the sensitivity of anaerobic bacteria to antimicrobial agents is exclusively measured using the MIC technique. However, because commercial automated identification and susceptibility testing systems are not commonly available, treatment of infections caused by these anaerobic microorganisms remains a challenge. Metronidazole is the most commonly used antimicrobial agent against anaerobic bacterial species. However, the results of susceptibility testing on metronidazole for *F. vaginae* are variable because some strains have high MIC values (De Backer et al., 2006). In our reported case, this *F. vaginae* strain was resistant to metronidazole (MIC >256 μg/mL), although the pathogen can be inhibited by low concentrations of clindamycin (MIC of 0.016 μg/mL), another commonly used antimicrobial agent for treating anaerobes.

According to the review of the literature (Table 2), penicillin-based antibiotics, such as amoxicillin and piperacillin, were mostly used in the treatment of *F. vaginae* infections in the reported cases. We also performed the susceptibility testing of the *F. vaginae* strain to penicillin-based antibiotics and found that this strain can be inhibited by low concentrations of ampicillin (MIC of 0.094 μg/mL), penicillin (MIC of 0.19 μg/mL), ampicillin/sulbactam (MIC of 0.75 μg/mL), and piperacillin/tazobactam (MIC of 0.38 μg/mL). However, in our case report, based on the understanding that cefoperazone/sulbactam appears in low levels in human milk and are not expected to cause adverse effects in breastfed infants (Matsuda et al., 1985; Lai et al., 2018), the patient was treated empirically with cefoperazone/sulbactam, and the retrospective antimicrobial susceptibility testing confirmed that cefoperazone/sulbactam (MIC of 0.38 μg/mL) was effective at inhibiting pathogen proliferation. In addition to our case report, it has been reported that other cephalosporin antibiotics, such as cefoxitin, are efficacious against *F. vaginae* infections (Geissdörfer et al., 2003). These results demonstrate that cephalosporin antibiotics are also an option for treating *F. vaginae* infections.

5 Conclusions

Incidence, morbidity, and death rates due to anaerobic bloodstream infections should be given more attention in patients. As an anaerobic bacterium, *F. vaginae* is found in normal vaginal microbiota; however, under certain conditions, it may cause life-threatening infections. If a patient with bacterial vaginosis is febrile and exhibits bloodstream infection symptoms during the postpartum period, it is vital to be mindful of bacterial vaginosis associated anaerobic species such as *F. vaginae* and to adapt the empirical therapy, as was the case here.

Data availability statement

The datasets presented in this article are not readily available because of ethical/privacy restrictions. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (protocol code 2023-1-026). Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article. Written informed consent was obtained from the participant/ patient(s) for the publication of this case report.

Author contributions

PL: Writing – original draft. LW and RL: Data curation. XC: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Human vaginal microbiota colonization is regulated by female sex hormones in a mouse model

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Introduction: Clinically, a *Lactobacillus* rich vaginal microbiota (VMB) is considered optimal for reproductive outcomes, while a VMB populated by anaerobes is associated with dysbiosis and the clinical condition bacterial vaginosis (BV), which is linked to increased susceptibility to sexually transmitted infections and adverse reproductive outcomes. Mouse models that mimic eubiotic and dysbiotic VMB are currently lacking but could play a critical role in improving protective interventions.

Methods: In this study, probiotic, eubiotic, and dysbiotic models were developed in C57BL/6 mice, using probiotic strains *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14, eubiotic *Lactobacillus crispatus*, or dysbiotic *Gardnerella vaginalis* strains. Endogenous sex hormones were manipulated by either ovariectomizing (OVX) mice or administering 17β-estradiol or progesterone pellets in OVX mice. Hormone-altered mice were inoculated with probiotic *Lactobacillus* species, *L. crispatus*, or *G. vaginalis*, and colonization was tracked using quantitative plating assays. Glycogen and MUC-1 levels in hormone-treated mice were determined with ELISA and MUC-1 staining.

Results: Following a single administration, *L. rhamnosus* and *L. reuteri* persisted in the mouse vaginal tract for up to eight days, *L. crispatus* persisted for up to three days, and *G. vaginalis* persisted for up to two days, as measured by quantitative plating assays and qPCR. Colonization of *G. vaginalis* was facilitated by the presence of mucin. The lack of endogenous hormones in OVX mice dramatically decreased VMB bacterial load compared to normal mice. None of the exogenous bacteria including *Lactobacilli* could colonize OVX mice for more than 24 hours. Treatment with 17β-estradiol but not progesterone restored the endogenous VMB and colonization with *Lactobacilli* and *G. vaginalis*. Interestingly, 17β-estradiol treated mice had significantly increased levels of glycogen compared to OVX and progesterone-treated mice.

Discussion: Based on the results, we have shown that estrogen played a significant role in the ability for human VMB species to colonize in our mouse models, potentially through a glycogen mediated mechanism. These results suggest there is a dynamic interaction between sex hormones and the VMB, which can affect bacterial diversity and the ability for a VMB to colonize.

KEYWORDS

vaginal microbiota (VMB), *Lactobacillus*, bacterial vaginosis (BV), sex hormones, mouse models, female reproductive health

Introduction

The female reproductive tract (FRT) is colonized by an endogenous collection of microbes, termed the vaginal microbiota (VMB), which exists in a mutualistic relationship with the host (Chen et al., 2021). The VMB can be characterized into five different community state types (CST), with four of them being dominated by different *Lactobacillus* species whereas the fifth comprises of lower proportions of *Lactobacillus* and higher proportions of anaerobic organisms (Ravel et al., 2011). A eubiotic VMB is associated with CST I, which is characterized by low diversity and is *Lactobacillus crispatus* dominant (Ravel et al., 2011; Ghartey et al., 2014; Chee et al., 2020). A *L. crispatus* rich VMB has a low vaginal pH as a result of the large amount of lactic acid produced by the bacteria (Ravel et al., 2011). A low vaginal pH reduces pathogen viability and is associated with a protective outcome against pathogen exposure in the FRT (Miller et al., 2016). CST IV is considered to be a dysbiotic VMB and is composed of a diverse community structure. A dysbiotic VMB is comprised of strictly anaerobic bacteria such as *Gardnerella vaginalis*, a bacterial species that is commonly seen in patients with the clinical condition bacterial vaginosis (BV) (Morrill et al., 2020). *G. vaginalis* produces several virulence factors such as sialidase A and vaginolysin (Pleckaityte et al., 2012), and has been linked to biofilm formation (Patterson et al., 2010), allowing it to often outcompete *Lactobacillus* species in the vaginal tract. Crucially, women with BV are at an increased risk of acquiring sexually transmitted infections (STI) such as HIV-1 (Harold et al., 1999; Cherpes et al., 2003; Rebecca et al., 2010). The majority of data showing effects of the VMB on reproductive health comes from clinical studies and is correlative. There is a lack of VMB associated animal models in the literature that can be used for understanding the mechanism underlying the complex role the VMB plays in female reproductive health.

Many factors are known to affect VMB composition such as diet, ethnicity, antibiotic use, and importantly, sex hormones (Lehtoranta et al., 2022). A metanalysis examining the changes in the VMB throughout various phases of a women's gynecological lifecycle found distinct microbial profiles at different stages of a woman's reproductive life (Kaur et al., 2019). As the shifts between these gynecological stages are largely regulated by fluctuations in sex hormones, there is an underlying relationship between hormones and the changes in the VMB throughout a woman's life. At puberty and pregnancy, when there is a major shift in endogenous hormones, there is an increase in glycogen deposition in the vaginal walls, enabling glycogen degrading *Lactobacillus* species to grow, which is considered optimal (Amabebe and Anumba, 2018). There is a shift from a low diversity to high diversity VMB when circulating sex hormones decrease such as during menopause (Oliveira et al., 2022), which is also implicated in increased susceptibility to other diseases such as heart disease and stroke, gynaecological malignancies, osteoporosis, and various genitourinary conditions (Elias and Sherris, 2003). Given the critical role of sex hormones on the VMB, it is important to take this into consideration when developing models.

A few studies have attempted to colonize mice with human VMB (Wolfarth et al., 2020), or BV-associated bacteria (Gilbert et al., 2019), however comprehensive studies that colonize mice with eubiotic and dysbiotic human VMB species have not yet been published. In this study, we successfully colonized normal female mice with human derived VMB species. A eubiotic model was developed with *L. crispatus* and a dysbiotic model was developed with *G. vaginalis*. Dysbiosis is associated with a heterogenous VMB, however since *G. vaginalis* is the most common bacteria seen in BV patients (Chen et al., 2021), this was the primary focus of the current study. As a positive control, a model with probiotic *Lactobacillus* species was also developed. To attempt to improve upon colonization, bacteria were supplemented with the nutrient sources glycogen or mucin. Given the critical role of hormones in VMB colonization, hormones were altered in mice to determine if there are relationships between sex hormones and VMB colonization *in vivo*. We looked at the VMB throughout the mouse estrus cycle, depleted all sex hormones by ovariectomizing (OVX) mice, and determined the effects of individual sex hormones by treating OVX mice with 17β-estradiol, the primary form of estrogen circulating in women during reproductive years (Ryan, 1982), or progesterone. Estrogen was found to promote VMB colonization in mice. 17β-estradiol was associated with increased glycogen in the vaginal tract, a common nutrient source used by bacteria (Hertzberger et al., 2022), which could explain the relationship between increased VMB colonization in 17β-estradiol treated mice. Collectively, our study successfully developed novel *in*

vivo mouse models that harbor human-derived VMB species in hormone-unaltered and hormone-altered mice. These models will serve as invaluable tools in studying the relationship between the VMB and female reproductive health.

Materials and methods

Mice

Six–eight-week-old female C57BL/6 mice were obtained from Charles River Laboratories and housed in the Central Animal Facility at McMaster University. Mice were maintained under specific pathogen-free and standard temperature-controlled conditions that followed a 12h light/dark cycle and fed low-fat mouse chow. Mice were allowed one week after arrival to acclimate prior to experimental use. All mouse studies performed were approved by and were in compliance with the Animal Research Ethics Board at McMaster University in accordance with the Canadian Council of Animal Care guidelines.

Bacteria stock preparation

Lactobacillus crispatus SJ-3C-US (PTA10138) from American Type Culture Collection (ATCC) was provided by Dr. Nuch Tanphaichitr (University of Ottawa, Canada). Probiotic *Lactobacillus* species *L. reuteri* (RC-14) and *L. rhamnosus* (GR-1) were received in the form of stab-cultures from the laboratory of Dr. Gregor Reid (Western University, Canada). *Gardnerella vaginalis* ATCC 14019 was purchased from ATCC. *L. rhamnosus*, *L. reuteri*, and *L. crispatus* were grown in ATCC medium 416 (Lactobacillus MRS broth/agar) in anaerobic conditions using the GasPak™ EZ Anaerobe Container System (Becton, Dickinson and Company, USA, Cat #260001) at 37°C. *G. vaginalis* was grown in ATCC medium 1685 (NYC III medium) in anaerobic conditions using the GasPak™ EZ Anaerobe Container System at 37°C. Stocks suspended in 20% glycerol were prepared for each bacterium and stored at -80°C for future use. To determine the bacterial stock concentration, serially diluted stocks were plated onto MRS agar plates (*L. rhamnosus*, *L. reuteri*, and *L crispatus*) or tryptic soy agar supplemented with 5% sheep's blood *(G. vaginalis*) to determine Colony Forming Units (CFU)/mL by the Miles and Misra technique (Miles et al., 1938).

Collection of vaginal washes

Two 30 µL volumes of sterile PBS were pipetted in and out of the mouse vagina 5-7 times, resulting in a total of 60 µL being collected. If the samples were used to check for bacterial colonization in the mice or to stage the mice in their estrus cycle, they were used right away. If they were collected to isolate DNA, then the samples were stored at -80°C.

Colonization of mice

Bacteria were grown in their respective media for 24 h. After 24 h of growth, the bacteria were spun down at 4000 rpm for two minutes, washed with PBS, and spun down once again. After the wash, bacteria were resuspended in PBS at 10^7 CFU for one mouse in 25 µL volumes. Depending on the number of mice being colonized, a greater volume was prepared. To colonize the mice, 25 µL of the bacteria was pipetted into the vaginal canal. The mice were then held facedown to allow for the bacteria to persist in the vaginal canal for at least one minute. To mimic a eubiotic human VMB, mice were inoculated with *L. crispatus* at a concentration of 10^7 CFU. A probiotic model was also developed by inoculating *L. rhamnosus* GR-1 and *L. reuteri* RC-14 together in equal concentrations of 5 x 10^6 CFU to give a final concentration of 10^7 CFU. *Lactobacillus* species were supplemented with 5 µL of 20mg/mL glycogen (0.1 mg) as a nutrient source in some experiments. To create the dysbiotic model, mice were inoculated with *G. vaginalis* at 10^7 CFU. *G. vaginalis* was supplemented with 5 µL 10 mg/mL (0.05 mg) mucin in some experiments. To evaluate colonization, vaginal washes were collected from the mice and quantitative plating assays were performed as described below.

DNA extraction from vaginal washes

Vaginal washes were collected from mice and frozen at -80°C for DNA extraction. DNA was isolated from vaginal washes or from cultured bacteria using the DNeasy™ Blood & Tissue Kit (Qiagen, Netherland, Cat #69506). DNA was isolated as per the manufacturer's instructions, including a primary digestion step using lysozyme to target the gram positive bacteria cell wall (Gill et al., 2016; Martzy et al., 2019), the primary type of bacteria in the mouse VMB (Vrbanac et al., 2018). For the latter method, an enzymatic lysis buffer consisting of 20 mM Tris·Cl (pH 8.0), 2 mM sodium EDTA, and 1.2% Triton® X-100 was prepared, and immediately before use, 20 mg/ml of lysozyme was added. The wash or cultured bacteria was pelleted and resuspended in 180 µl enzymatic lysis buffer and incubated for 30 minutes at 37°C. Following enzymatic lysis, DNA extraction was completed as per the manufacturer's instructions for the DNeasy™ Blood & Tissue Kit.

Quantitative PCR

qPCR of the 16S rRNA gene was performed to assess overall bacterial load (Rezki et al., 2016), as well as species DNA present in a sample using species specific primers for *L. crispatus* (Zozaya-Hinchliffe et al., 2010), *L. rhamnosus* (Kim et al., 2020), *L. reuteri* (Kim et al., 2020), and *G. vaginalis* (Zozaya-Hinchliffe et al., 2010) (Table 1). Genus specific qPCR primers were also designed for *Staphylococcus*, *Enterococcus*, *Actinomyces*, and *Corynebacterium*. A master mix containing 12.5 µL RT^2 SYBR® Green qPCR master mix

TABLE 1 qPCR primers for the 16S rRNA gene, exogenous bacteria, and endogenous genera.

Target	Forward Primer (5′ → 3′)	Fwd. Primer Tm (°C)	Reverse Primer (5′ → 3′)	Rev. Primer Tm (°C)
16S rRNA gene qPCR	926f* (MS91) AAACTCAAAKGAATTGACGG	49.9	1062r (MS92) CTCACRRCACGAGCTGAC	55.9
L. crispatus qPCR	GATTTACTTCGGTAATGACGTTAGGA	54.5	AGCTGATCATGCGATCTGCTTTC	57.7
L. rhamnosus qPCR	GCCGATCGTTGACGTTAGTTGG	58.3	CAGCGGTTATGCGATGCGAAT	58.5
L. reuteri qPCR	GATTGACGATGGATCACCAGT	54.6	CATCCCAGAGTGATAGCCAA	54.1
G. vaginalis qPCR	GGAAACGGGTGGTAATGCTGG	58.6	CGAAGCCTAGGTGGGCCATT	59.9
Staphylococcus qPCR	CCTTGACTCTGTGCCA	51.5	GTGTTCCTCCATATCTCTG	49.3
Enterococcus qPCR	GATCTCTATCTCGAAGG	44.9	ACTCGTTGTACTTCCCA	49.9
Actinomyces qPCR	AACACCACCCCTGAGCG	58.0	GTGTGTACAAGGCCCGAG	55.8
Corynebacterium qPCR	GCAGCAGACACTTTAAGGCC	56.6	CTACACCTAGCGCCCAC	55.3

(Qiagen, Netherlands, Cat #330503), 0.25 µL forward primer (100 µM), 0.25 µL reverse primer (100 µM), and 7 µL water per well was prepared. 20 µL of the master mix was aliquoted to each well of a 96-well plate and 5 µL of template DNA was added accordingly. The annealing temperature was input as T_m – 5°C, where the lower of the two T_m from the forward and reverse primers was used. Primer sequences with their respective T_m can be found in Table 1. The reaction was run using the StepOne Plus™ Real-Time PCR System (ThermoFisher™, USA). Samples were run in triplicate and bacterial load or species load was assessed by analyzing the number of cycles required for the fluorescent signal to cross the threshold (ct value). This value was inversed in our figures to show a positive relationship for ease of readability.

Quantification of VMB by culture

Vaginal washes were collected from individual mice and serially diluted ten-fold from 10^{-1} to 10^{-6}. MRS plates were used to screen *Lactobacillus* species and tryptic soy plates supplemented with 5% sheep's blood were used to screen *G. vaginalis* and endogenous species. Plates were divided into 6 sections, and 10 µL of each dilution (sometimes including the undiluted sample) was pipetted dropwise onto the plate in duplicate. The plates were incubated in anaerobic conditions using the GasPak™ EZ Anaerobe Container System at 37°C. After 24 h, the number of colonies were counted using the dilution that isolated colonies could be identified and CFU/mL was calculated. Endogenous and exogenous colonies were identified by visually comparing colonies based on morphology from plating vaginal washes from untreated mice or from streaked bacterial stocks on plates, respectively.

Estrus cycle staging

10 µL of vaginal washes were pipetted on to a glass slide and viewed under a microscope. The cells were observed and compared to images of vaginal washes from mice under different stages of the estrus cycle (Ajayi and Akhigbe, 2020). Briefly, vaginal washes from mice in the estrus stage of their cycle were primarily composed of cornified epithelial cells and vaginal washes from mice in the diestrus stage were composed of primarily leukocytes.

Ovariectomy

Ovariectomies were performed to eliminate the effect of endogenous sex hormones in mice. Mice received the analgesic carprofen (5 mg/kg) subcutaneously and after 30 minutes, they were administered an intraperitoneal injection of ketamine (100-150 mg/kg) and xylazine (10 mg/kg). After the mice reached surgical plane, the surgical area was shaved, the mice received an intradermal injection of bupivacaine (4 mg/kg per incision site), and the surgical site was sterilized with iodine scrub and isopropyl alcohol. The ovaries were then removed through a small incision near the hind limbs and incision sites were sutured and stapled. 1 mL of saline was administered, and the mice recovered on a heat pad and were monitored until they were awake and able to move on their own. Mice received carprofen (5 mg/kg) for two days after surgery and were monitored for 5 days post-surgery. Staples were removed 7-10 days post-surgery and mice were allowed at least 1 week to recover before use.

Hormone treatments

Mice received the analgesic carprofen (5 mg/kg) subcutaneously and after 30 minutes, they were anaesthetized with isoflurane gas. After the mice reached surgical plane, the surgical area was shaved, the mice received an intradermal injection of bupivacaine (4 mg/kg), and the surgical site was sterilized with iodine scrub and isopropyl alcohol. 10 mg progesterone 21-day release pellets (Innovative Research of America, USA, Cat #P-131-10MG-25) or 0.01 mg 17β-estradiol 21-day release pellets (Innovative Research of

America, USA, Cat #E-121-0.01MG-25) were surgically inserted into the scruff of mice. These doses correspond to hormone levels measured during the estrous cycle (Bhavanam et al., 2008; Bagri et al., 2020). Mice received carprofen (5 mg/kg) for 2 days after surgery and were monitored for 5 days post-surgery. Staples were removed 7-10 days post-surgery and mice were allowed at least 1 week to recover before use.

Immunohistochemistry of vaginal tissue

Mouse vaginal tissue was collected, placed in cassettes, and fixed in methacarn (60% methanol, 30% chloroform and 10% glacial acetic acid) for 72 h. Cassettes were transferred to 70% ethanol and samples were taken to McMaster Immunology Research Center (MIRC) Histology Core Facility for processing. The tissue was embedded, and slides were cut and mounted on microscope slides and stained with Mucin-1 (MUC-1) antibody (Abcam, United Kingdom, Cat #ab15481). Slides were scanned using the Leica Aperio Scanscope XT and viewed using Aperio ImageScope software.

Statistical analysis

All statistical analysis was done using GraphPad Prism version 10.1.0 (GraphPad Software, San Diego, CA). Two-way ANOVA with Tukey's multiple comparisons was used to determine all statistical significance for quantitative plating assays. A one-way ANOVA with Tukey's multiple comparisons or t-test was used in all qPCR experiments and ELISAs.

Results

Normal female mice can be colonized by human VMB species

In order to develop a mouse model of human female microbiota, we first assessed if the vaginal tract of normal mice can be colonized by human VMB strains. We developed a eubiotic model with the physiologically relevant CST I bacteria *L. crispatus* and a dysbiotic model with the primary BV-associated bacteria *G. vaginalis*. A model using probiotic species *L. rhamnosus* GR-1 and *L. reuteri* RC-14 was also developed as a positive control, as probiotics are more robust and more likely to survive in non-optimal environments (Corcoran et al., 2005). For a general term usage, we refer to these bacterial species as exogenous bacteria, whereas the normal vaginal bacteria of the mice are referred to as endogenous bacteria. Female mice were inoculated intravaginally once with a total of 10^7 CFU of RC-14 and GR-1 in equal concentrations, *L. crispatus* or *G. vaginalis*. PBS was administered as a negative control involving no exogenous bacteria. These groups of mice were then tracked for colonization using quantitative plating assays (Figure 1A). As expected, no exogenous bacteria were detected in the PBS inoculated mice (Figure 1B). 6/6 (100%) mice in the probiotics group (Figure 1C), 5/6 (83%) mice in the *L. crispatus* group (Figure 1D), and 4/9 (44%) mice in the *G. vaginalis* group (Figure 1E), were successfully colonized for at least 24 h. Mice inoculated with probiotic species were colonized for a minimum of 2 days and a maximum of 8 days, with an average duration of 4.2 days. Mice given *L. crispatus* were colonized for two days minimum and three days maximum, with an average duration of colonization of 2.6 days. *G. vaginalis* treated mice were colonized for one day minimum and two days maximum, with an average duration of colonization of 1.75 days.

To validate that the bacterial species being counted on the plates were indeed *L. reuteri* or *L. rhamnosus*, *L. crispatus*, or *G. vaginalis* and not endogenous mouse vaginal species, qPCR was used for validation. DNA was isolated from colonies of endogenous species, as well as probiotics, *L. crispatus* or *G. vaginalis* colonies plated from vaginal washes and distinguished by colony morphology (Figure 1A). Colonies identified as endogenous bacteria by plating did not show significant bacterial count in qPCR using probiotic specific primers (Figures 1F, G), *L. crispatus* specific primers (Figure 1H), or *G. vaginalis* specific primers (Figure 1I), indicating that the primers were specific, and colonies identified and counted as endogenous did not contain exogenous bacteria. For the probiotics inoculated mice, qPCR using RC-14 and GR-1 specific qPCR primers was performed. There was a significant difference between the Ct values for *Lactobacillus* colonies and RC-14 stock DNA when using RC-14 specific qPCR primers (Figure 1F), but no significant difference between the Ct values for *Lactobacillus* colonies and GR-1 stock DNA when using GR-1 specific primers (Figure 1G), indicating that the colonies being counted were predominantly *Lactobacillus rhamnosus* GR-1. Thus, among the probiotic mixture, GR-1 was the predominant bacteria colonizing the probiotic inoculated mice (Figure 1G). Likewise, there was no significant difference between Ct values for *L. crispatus* or *G. vaginalis* colonies and *L. crispatus* (Figure 1H) or *G. vaginalis* (Figure 1I) stock DNA, indicating the colonies counted as *L. crispatus* or *G. vaginalis* in the corresponding bacteria inoculated mice were indeed those bacteria.

The total bacterial load including endogenous and exogenous colonies in all groups was compared using quantitative plating assays by counting colonies of both endogenous and exogenous species. No significant increase in total bacteria was observed post inoculation, indicating that the exogenous bacteria were displacing the endogenous bacteria (Figure 1J). Total bacterial load remained consistently ~ 10^7-10^8 CFU/mL in all groups, suggesting that there is a finite niche for bacteria in the vaginal tract of mice.

Endogenous VMB did not dictate the ability for exogenous eubiotic or dysbiotic bacteria to colonize

Since we found differences in colonization success of the eubiotic species *L. crispatus* and the dysbiotic species *G. vaginalis*, we considered if the endogenous mouse VMB species played a role in the ability for eubiotic or dysbiotic species to colonize. Furthermore, we wanted to elucidate if specific endogenous species facilitate colonization by eubiotic or dysbiotic bacteria. To do this, we

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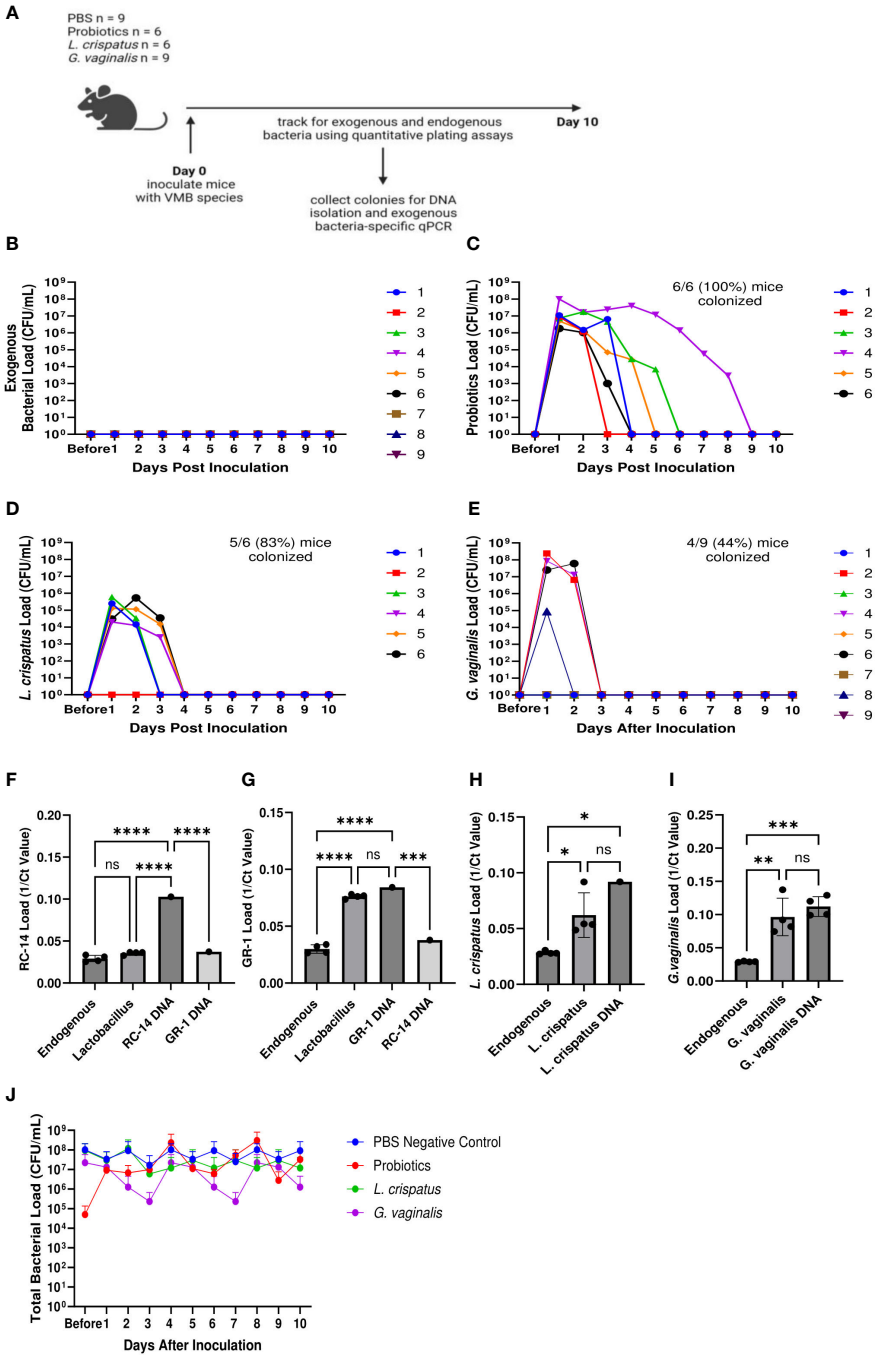

FIGURE 1
Normal mice were colonized with *Lactobacillus* probiotic species, *L. crispatus*, and *G. vaginalis*. Female mice were inoculated once with a total of 10^7 CFU *L. reuteri* RC-14 and *L. rhamnosus* GR-1 in equal concentrations, *L. crispatus*, *G. vaginalis*, or PBS as a negative control. Data are from n = 6-9 per group from one experiment representative of 3 independent experiments with similar results (schematic shown in **A**). Vaginal washes were collected up to 10 days post-inoculation, and bacterial colonies from the inoculated species types were counted for samples from PBS inoculated **(B)**, probiotics inoculated **(C)**, *L. crispatus* inoculated **(D)**, and *G. vaginalis* inoculated **(E)** groups on agar plates. Different mice are denoted by different colored points. The data was analyzed using a two-way ANOVA with Tukey's multiple comparisons, but no significance was found. From these plating assays, probiotic-looking colonies (n = 4 colonies), *L. crispatus*-looking colonies (n = 4 colonies), *G. vaginalis*-looking colonies (n = 4 colonies), and endogenous colonies (n = 4 colonies) were picked from the culture plates and DNA was isolated. Using this DNA, qPCR was performed with RC-14 specific qPCR primers **(F)**, GR-1 specific qPCR primers **(G)**, *L. crispatus* specific qPCR primers **(H)**, and *G. vaginalis* specific qPCR primers **(I)**, to determine what type of bacterial DNA was present. RC-14, GR-1, *L. crispatus*, and *G. vaginalis* stock DNA were used as positive controls. Data was analyzed using a one-way ANOVA (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05). The total vaginal bacterial load was plotted from all groups in panel **(J)**. The data was analyzed using a two-way ANOVA with Tukey's multiple comparisons, but no significant difference was found.

collected vaginal washes from mice one day before and after colonization with 10^7 CFU *L. crispatus*, *G. vaginalis*, or PBS as a negative control. DNA was isolated from the washes and qPCR was used to elucidate changes in endogenous and exogenous bacteria before and after colonization (Figure 2A). It has previously been found that the VMB of C57BL/6 mice is dominated by either *Staphylococcus* or *Enterococcus* (Vrbanac et al., 2018), with lower proportions of *Actinomyces* and *Corynebacterium*. Therefore, we

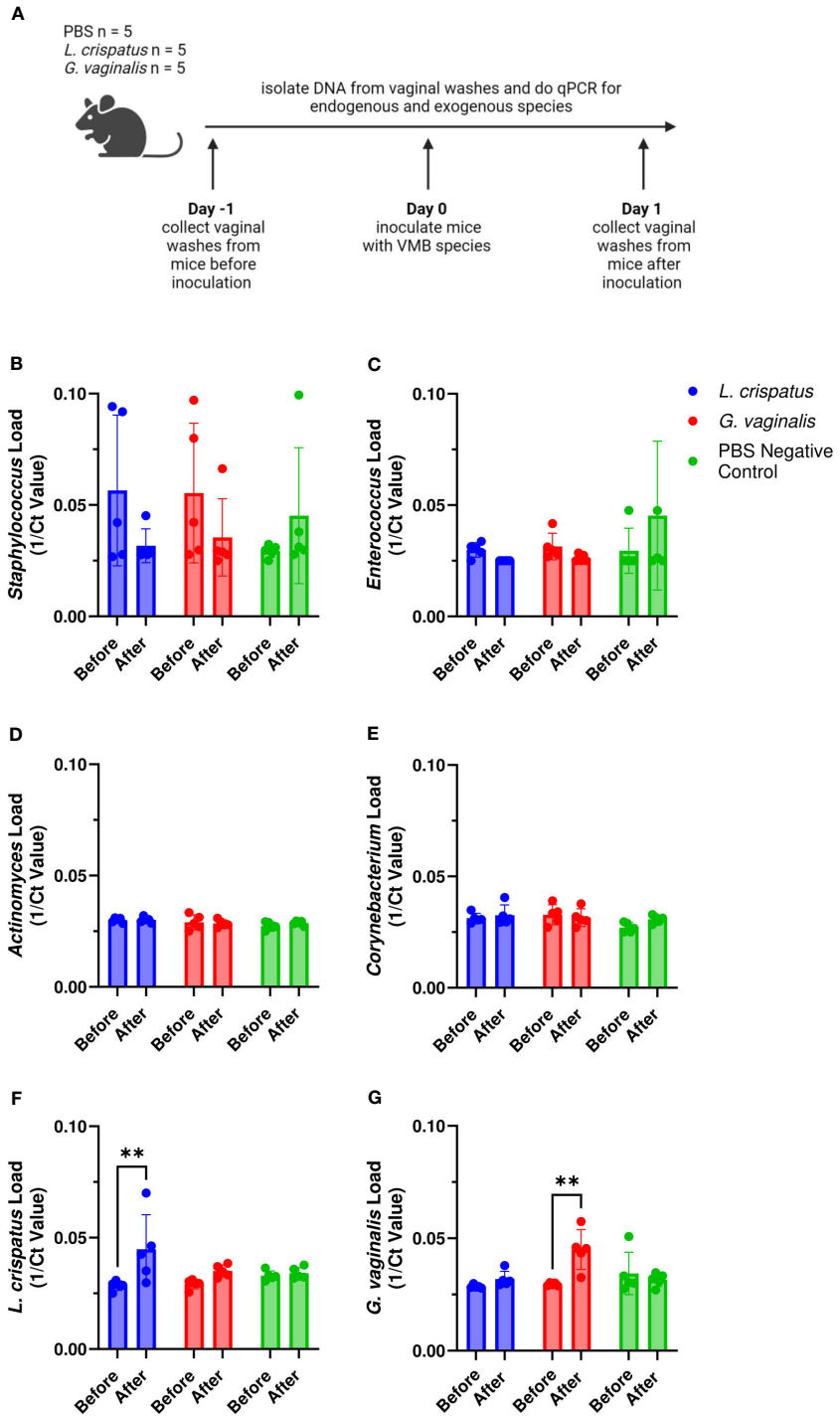

FIGURE 2
Endogenous VMB did not dictate the ability for *L. crispatus* or *G. vaginalis* to colonize. Vaginal washes were collected from mice one day before and after one intravaginal inoculation of 10^7 CFU *L. crispatus*, *G. vaginalis*, or PBS as a negative control. Data are from n = 5 per group representative of 1 independent experiment. DNA was isolated from vaginal washes and qPCR was performed. A schematic of the experiment is shown **(A)**. Endogenous genera were characterized using *Staphylococcus* specific qPCR primers **(B)**, *Enterococcus* specific qPCR primers **(C)**, *Actinomyces* specific qPCR primers **(D)**, and *Corynebacterium* specific qPCR primers **(E)**. Exogenous bacterial species were characterized using *L. crispatus* specific qPCR primers **(F)**, and *G. vaginalis* specific qPCR primers **(G)**. The data was analyzed using a two-way ANOVA with Tukey's multiple comparisons (**p<0.01).

screened for these genera in our study. Most of the mice had *Staphylococcus* (Figure 2B) or *Enterococcus* (Figure 2C) present in their VMB in significant amounts before inoculation with exogenous bacteria. After inoculation, there were trends towards decreasing *Staphylococcus* (Figure 2B) and *Enterococcus* (Figure 2C) levels in *L. crispatus* and *G. vaginalis* inoculated mice, but not PBS inoculated mice, indicating the exogenous bacteria were displacing the endogenous ones. *Actinomyces* (Figure 2D) and *Corynebacterium* (Figure 2E) were not present in any significant amount in these mice. When looking at the exogenous species, there was a significant increase in *L. crispatus* levels in *L. crispatus* inoculated mice (Figure 2F) and *G. vaginalis* levels in *G. vaginalis* inoculated mice (Figure 2G) post- inoculation. No increase was seen in *L. crispatus* load in *G. vaginalis* or PBS inoculated mice or *G. vaginalis* levels in *L. crispatus* or PBS groups. This increase occurred in all mice in their respective groups regardless of the type or levels of endogenous bacteria present, indicating the endogenous VMB did not dictate whether eubiotic or dysbiotic bacteria could colonize.

Exogenous mucin facilitated initial *G. vaginalis* colonization

Since not all mice were colonized equally with different bacteria, we wanted to test methods to improve colonization. Nutrient availability is an important determinant of what species colonize the VMB (Hood-Pishchany and Rakoff-Nahoum, 2021). Glycogen is a common nutrient source used by *Lactobacillus* species (Mirmonsef et al., 2014) and mucin is a common nutrient source used by *G.* vaginalis (Dupont, 2020; Vagios and Mitchell, 2021). Therefore, we tested if addition of mucin or glycogen at the time of inoculation could enhance colonization (Figure 3A). Supplementation with mucin or glycogen in probiotic (*L. reuteri* and *L. rhamnosus*) treated or *L. crispatus* treated mice did not significantly facilitate or hinder colonization (Figures 3B–E). 6/6 (100%) mice in the probiotics plus glycogen group and 5/6 (83%) mice in the probiotics plus mucin group were successfully colonized (Figures 3B, C), which is a similar colonization rate as no nutrient conditions (Figure 1C). The average duration of colonization in successfully colonized mice in the probiotics plus glycogen and mucin groups was 4 days and 2.2 days respectfully, indicating mucin might be decreasing the duration of colonization in mice. 5/6 (83%) mice in the *L. crispatus* plus glycogen group and 8/9 (89%) of the mice in the *L. crispatus* plus mucin group were colonized, with an average duration of colonization of 2.2 days and 2.3 days respectively (Figures 3D, E). These values are similar to no nutrient supplementation (Figure 1D), indicating nutrient supplementation did not help or hinder *L. crispatus* colonization. Glycogen supplementation did not enhance *G. vaginalis* colonization, with 2/9 (22%) of mice being initially colonized (Figure 3F). However, supplementation with mucin aided in initial *G. vaginalis* colonization, with 8/9 (89%) of mice being colonized for at least 24 hours (Figure 3G). This is double the number of mice compared to no mucin supplementation (Figure 1E), suggesting exogenous mucin administration could assist in *G. vaginalis* colonization in mice.

Mice were more likely to be colonized with exogenous bacteria in the estrus stage

Because significant differences were seen in successful initial colonization and duration of colonization between mice within the same treatment group, we considered other factors that could affect the colonization. The transition of women through menopause is marked by a gradual depletion of *Lactobacillus* species and an increase in anaerobic bacteria (Oliveira et al., 2022). Others have reported that mice given 17β-estradiol are more likely to be colonized by BV associated bacteria (Gilbert et al., 2019). Because of the identified relationships between hormones and the VMB, we first looked at the effect of the mouse estrus cycle on human VMB species colonization (Figure 4A). During the estrus stage (the estrogen high phase of the reproductive cycle), all mice in probiotics treated, *L. crispatus* treated, and *G. vaginalis* treated groups were colonized (Figures 4B, E, H). In the progesterone high diestrus phase, 4/6 (67%) of probiotics treated mice (Figure 4C), 3/6 (50%) of *L. crispatus* treated mice (Figure 4F), and 1/6 (17%) of *G. vaginalis* treated mice (Figure 4I) were colonized, indicating that overall, estrogen may be facilitating colonization. When looking at the average of all mice in estrus or diestrus, there were no significant differences in probiotics (Figure 4D) and *L. crispatus* (Figure 4G) inoculated mice, however there was a significant difference one day post-inoculation in the *G. vaginalis* group (Figure 4J). This is most probably due to the stark difference in initial colonization success between estrus and diestrus mice in the *G. vaginalis* group.

Hormone-depleted mice had decreased total bacterial load and were not colonized by exogenous human VMB species

We next examined the effect of eliminating endogenous sex hormones on the VMB of mice. Ovariectomies (OVX) were performed on normal mice to eliminate the effect of endogenous sex hormones. Vaginal washes were collected from mice before and 1, 2 and 3 weeks after ovariectomies and quantitative plating assays were performed (Figure 5A). There was a dramatic and significant decrease in the endogenous bacterial load of the mice after ovariectomy (Figure 5B). DNA was also isolated from vaginal washes collected from the same OVX mice before and 3 weeks post-OVX. qPCR using 16S rRNA gene specific primers to target total bacterial DNA was performed as a more quantitative measure of the amount of bacteria present before and after OVX. Vaginal washes showed a significantly decreased amount of bacterial DNA after ovariectomy compared to before (Figure 5C), validating the results from the plating assays.

We also wanted to determine the ability for human VMB species to colonize and persist in hormone-depleted mice. A single inoculation, as well as 3 consecutive daily doses of probiotic *Lactobacillus* species RC-14 and GR-1, *L. crispatus*, or *G. vaginalis* was delivered to OVX mice at 10^7 CFU. Probiotics, *L. crispatus*, and *G. vaginalis* colonies were counted for two days after the final inoculation (Figure 6A). In the probiotics inoculated mice, there was brief colonization one day post inoculation, and then a

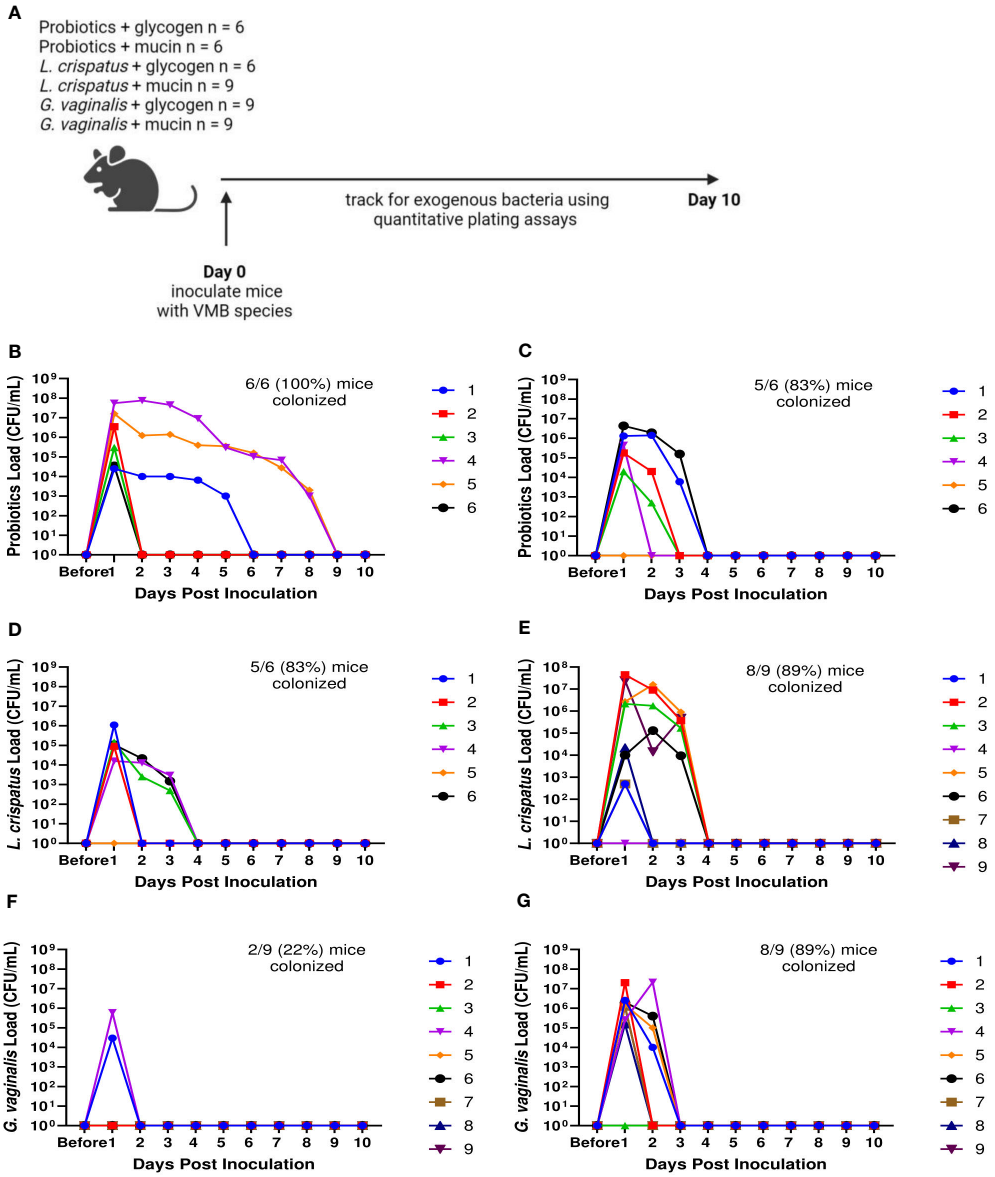

FIGURE 3
Mucin enhanced *G. vaginalis* colonization. Female mice were inoculated once with 10^7 CFU of equal parts *Lactobacillus* probiotic species *L. reuteri* RC-14 and *L. rhamnosus* GR-1 plus glycogen **(B)**, probiotic species plus mucin **(C)**, *L. crispatus* plus glycogen **(D)**, *L. crispatus* plus mucin **(E)**, *G. vaginalis* plus glycogen **(F)**, or *G. vaginalis* plus mucin **(G)**. Vaginal washes were collected up to 10 days post-inoculation and assessed by quantitative plating assays (schematic shown in **A**). Specific bacterial colonies derived from mice inoculated with different bacterial species with either glycogen or mucin were counted on agar plates. Data indicate n = 6-9 per group, representative of 3 independent experiments. Different mice are denoted by different colored points. The data was analyzed using a two-way ANOVA with Tukey's multiple comparisons, but no significance was found.

rapid decrease by day two post inoculation in both single and triple inoculated mice, indicating that probiotic bacteria were not able to colonize OVX mice for any significant length of time (Figures 6B, C). On the individual mouse level, 5/6 (83%) of the mice in the single inoculated group (Figure 6B) and 8/8 (100%) of mice in the triple inoculated group (Figure 6C) were colonized. The total bacteria count only reached a maximum concentration of ~10^3 CFU/mL, which is 100,000 times lower than the ~10^8 CFU/mL reached in normal mice (Figure 1J). Similar to probiotics inoculated mice, the *L. crispatus* group had transient colonization one day post-inoculation at ~10^3 CFU/mL, and no bacteria was detected on day two post inoculation in single and triple inoculated mice (Figures 6D, E). On the individual mouse level, 4/6 (83%) of the mice in the single (Figure 6D) and triple inoculated (Figure 6E) groups were colonized. In the *G. vaginalis* inoculated mice, no mice were colonized in the single inoculated group (Figure 6F) and bacteria was detected in 2 mice in the triple inoculated group, but once again, only for one day (Figure 6G). Overall, these results suggest the absence of hormones makes the vaginal environment inhospitable to both endogenous and exogenous bacteria, which could explain why the human VMB species we administered were not able to colonize, even temporarily.

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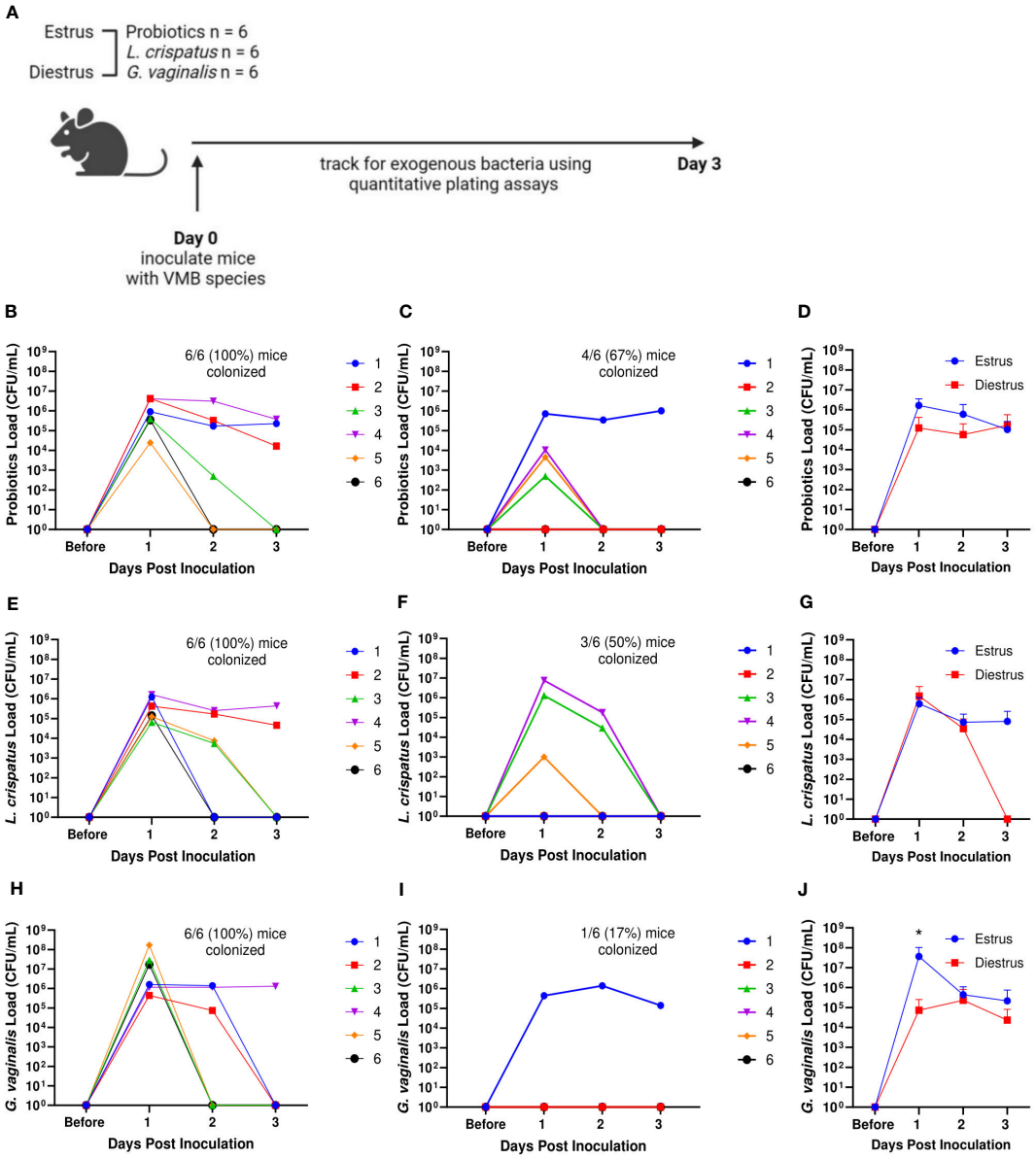

FIGURE 4
Mice were more likely to be successfully colonized by probiotic bacteria, *L. crispatus*, or *G. vaginalis* when they were in the estrus stage of the reproductive cycle. Probiotics load, *L. crispatus* load, or *G. vaginalis* load were assessed by quantitative plating assays before and after one vaginal administration of 10^7 CFU of *Lactobacillus* probiotics species RC-14 and GR-1 in equal proportions, *L. crispatus*, or *G. vaginalis* in mice in estrus or diestrus on the day of inoculation (n=6 per group, representative of 2 independent experiments) (schematic shown in **A**). Mice were monitored up to 3 days after inoculation and probiotics colonies in estrus **(B)**, diestrus **(C)**, and combined **(D)**, *L. crispatus* colonies in estrus **(E)**, diestrus **(F)**, and combined **(G)**, and *G. vaginalis* colonies in estrus **(H)**, diestrus **(I)**, and combined **(J)** groups were plotted. In panels **(B, C, E, F, H, I)** different mice are denoted by different colored points. The data in panels **(D, G, J)** were analyzed using a two-way ANOVA with Tukey's multiple comparisons (*p< 0.05).

Hormone-depleted mice treated with estrogen had significantly increased bacterial load and were colonized by human VMB species

We next examined the effects of 17β-estradiol or progesterone given to OVX mice to assess the effect of these hormones on the VMB in mice. Ten days after mice recovered from ovariectomies, either a 10 mg progesterone 21-day release pellet or a 0.01 mg 17β-estradiol 21-day release pellet was inserted subcutaneously into the mice to recapitulate hormone levels during the estrus cycle (Bhavanam et al., 2008; Bagri et al., 2020). Ten days after the hormone pellet insertion, mice were inoculated once with a total of 10^7 CFU probiotics RC-14 and GR-1, *L. crispatus*, *G. vaginalis*, or PBS as a no exogenous bacteria negative control (Figure 7A). The bacterial load of the mice before hormone treatments (OVX mice) was very low (Figures 7B, C), similar to what was seen in previous experiments (Figures 5, 6). After treatment with progesterone, there was a slight increase in bacterial load compared to OVX mice, but the difference was not statistically significant (Figure 7B). After

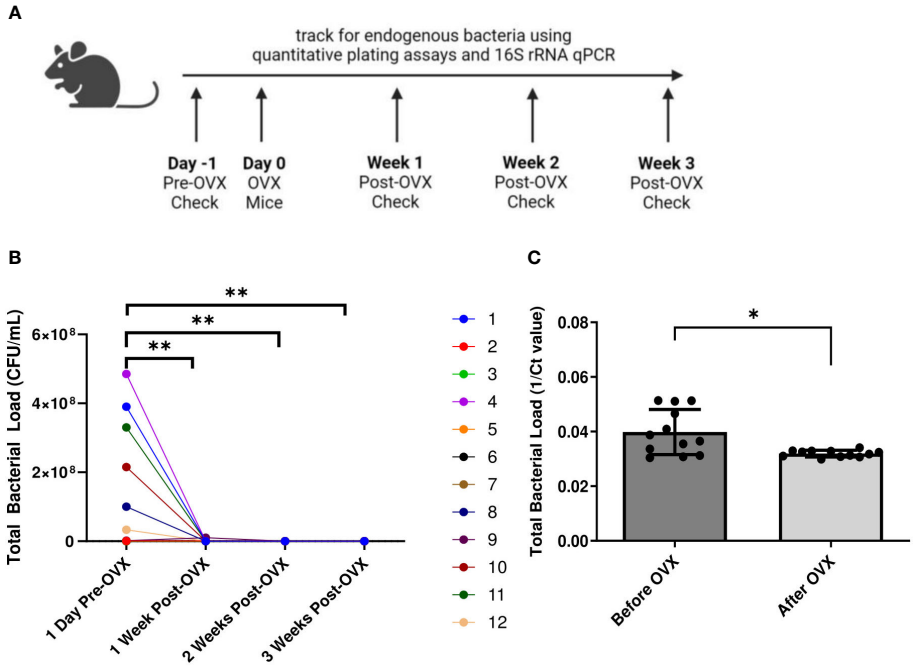

FIGURE 5
Mice showed decreased bacterial load post ovariectomies, as calculated by quantitative plating assays and qPCR. Vaginal washes were collected from individual mice 1 day before they were ovariectomized, as well as 1 week, 2 weeks, and 3 weeks post-OVX (n=12, representative of 2 independent experiments). A schematic of the experiment is shown in panel **(A)**. Total bacterial load was assessed via quantitative plating assays **(B)** or 16S rRNA-specific qPCR **(C)**. In panel **(B)**, different mice are denoted by different colored points. Data was analyzed using a two-way ANOVA with Tukey's multiple comparisons (**p<0.01) or t-test (*p<0.05).

treatment with 17β-estradiol, there was a statistically significant increase in bacterial load compared to OVX mice, indicating that 17β-estradiol may play a role in human VMB establishing in mice (Figure 7C). Of note, 17β-estradiol treated mice had a bacterial load in the ~10^7-10^8 CFU/mL range (Figure 7C), which is similar to normal mice (Figure 1).

As expected, no mice in the PBS treated group had any exogenous bacteria present (Figures 7D, E). Very few OVX mice treated with progesterone were colonized with any of the exogenous bacterial species administered for more than 24 h (Figures 7F, H, J). Specifically, 5/9 (56%) of probiotics inoculated mice (Figure 7F), 2/9 (22%) of *L crispatus* inoculated mice (Figure 7H), and 0/9 (0%) of the *G. vaginalis* inoculated mice (Figure 7J) were colonized in progesterone-treated mice. However, 17β-estradiol-treated OVX mice were colonized with all the exogenous bacterial species (Figures 7G, I, K) for similar durations of time as normal (untreated) mice (Figure 1). Specifically, 9/9 (100%) of probiotics inoculated mice (Figure 7G), 9/9 (100%) of *L crispatus* inoculated mice (Figure 7I), and 8/9 (89%) of the *G. vaginalis* inoculated mice (Figure 7K) treated with 17β-estradiol were successfully colonized, similar to rates seen in normal mice (Figure 1). This indicates that the presence of 17β-estradiol is sufficient for colonization of human VMB in mice. As with normal mice (Figure 1), the total bacterial load did not exceed ~10^7-10^8 CFU/mL after exogenous bacteria inoculation in the estradiol treated mice (Figure 7C), indicating the exogenous bacteria were displacing the endogenous bacteria and that there is a finite niche available in the vaginal environment. In progesterone treated mice, the available niche was much lower at ~10^4-10^5 CFU/mL (Figure 7B).

Glycogen was upregulated in 17β-estradiol treated mice and MUC-1 was upregulated in progesterone treated mice

Next, we examined a possible mechanism as to why 17β-estradiol promoted colonization with bacteria. We investigated at two nutrients that are known to assist in colonization of vaginal bacteria. As previously mentioned, glycogen is a common nutrient source used by *Lactobacillus* species (Mirmonsef et al., 2014) and mucin is a common nutrient source used by *G.* vaginalis (Dupont, 2020; Vagios and Mitchell, 2021). Mice were ovariectomized, treated with 17β-estradiol or progesterone, and 10 days after hormone treatment, vaginal washes were collected, and glycogen and Mucin-1 levels were measured (Figure 8A). 17β-estradiol-treated mice had significantly increased levels of glycogen compared to progesterone-treated, OVX, and normal mice (Figure 8B) and progesterone-treated mice had significantly increased Mucin-1 levels compared to 17β-estradiol-treated mice (Figure 8C). Tissue samples were also collected ten days after hormone treatment and Mucin-1 staining was performed. Progesterone-treated mice showed significantly more mucin staining on the surface of the vaginal epithelium compared to other groups (Figure 8D), validating the results from the ELISA.

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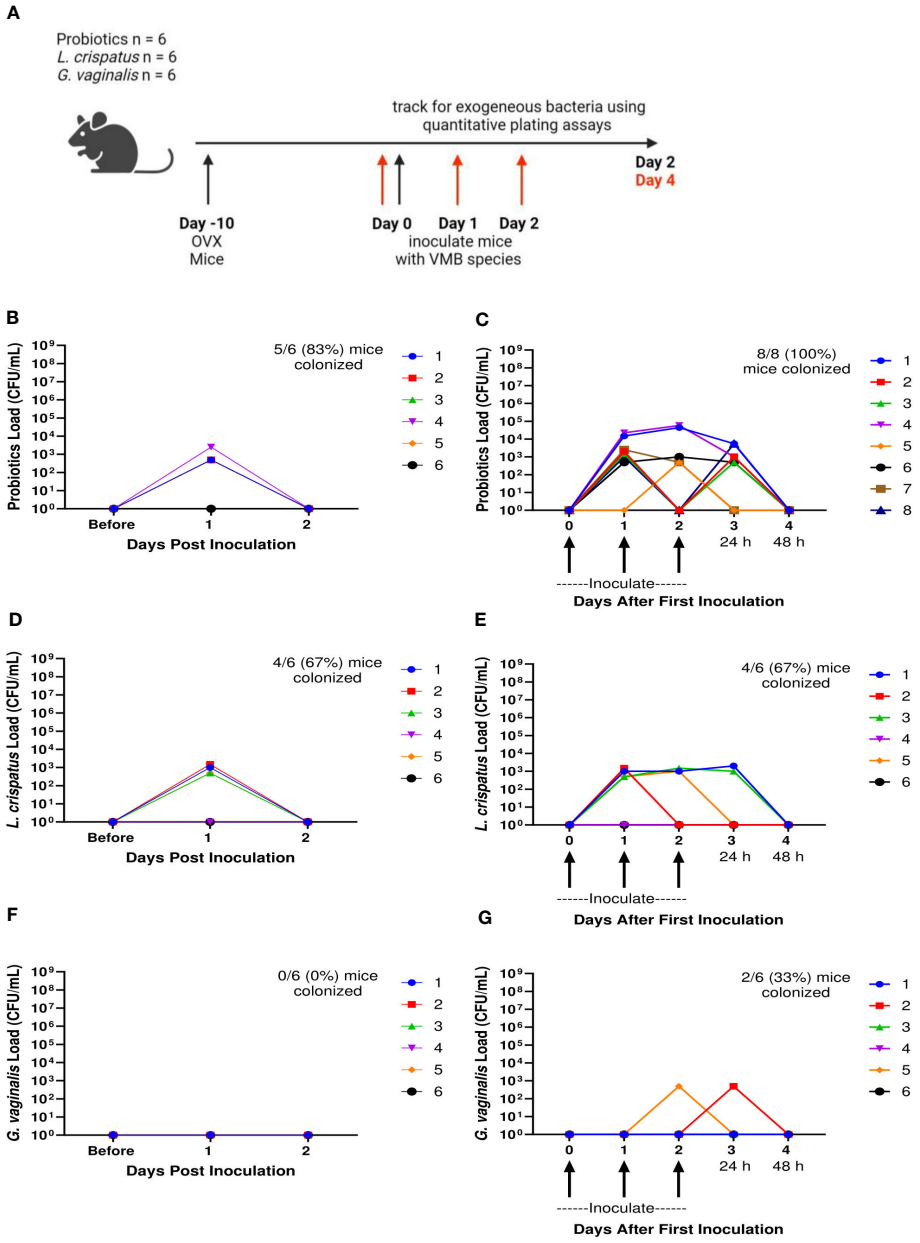

FIGURE 6
Ovariectomized mice did not get colonized with probiotic *Lactobacillus* species, *L. crispatus*, or *G. vaginalis* for more than 24 h after one inoculation and multiple inoculations. Female mice were ovariectomized and allowed to recover for 10 days. Mice were then inoculated once or three times consecutively every 24 h with a total of 10^7 CFU *L. reuteri* RC-14 and *L. rhamnosus* GR-1 in equal concentrations, *L. crispatus*, or *G. vaginalis* (n=6 per group, representative of 2 independent experiments). Vaginal washes were collected up to 2 days after the most recent inoculation, and probiotics load, *L. crispatus* load, or *G. vaginalis* load were assessed by quantitative plating assays. A schematic of the experiment is shown in panel **(A)**. Probiotics colonies in mice inoculated once **(B)** or three times **(C)**, *L. crispatus* colonies in mice inoculated once **(D)** or three times **(E)**, or *G. vaginalis* colonies in mice inoculated once **(F)** or three times **(G)** were counted and plotted. Different mice are denoted by different colored points. In panels **(C, E, G)**, 24 h or 48 h denote the time passed after the most recent inoculation. The data was analyzed using a two-way ANOVA with Tukey's multiple comparisons, but no significance was found.

Discussion

In the past decade, there has been a great deal of interest in developing human microbiota associated mouse models that are colonized with bacteria present in the human microbiota under optimal and dysbiotic conditions. These models can be used to study diseases and infections in the context of the microbiota composition and to understand the role microbiota health plays in disease outcomes (Arrieta et al., 2016). The majority of the studies conducted have been in the context of the gut microbiota. There are currently well-established animal models that mimic the human gut microbiota in mice (Hugenholtz and de Vos, 2018; Wrzosek et al., 2018). The establishment of these mouse models has been instrumental in advancing our understanding of the gut

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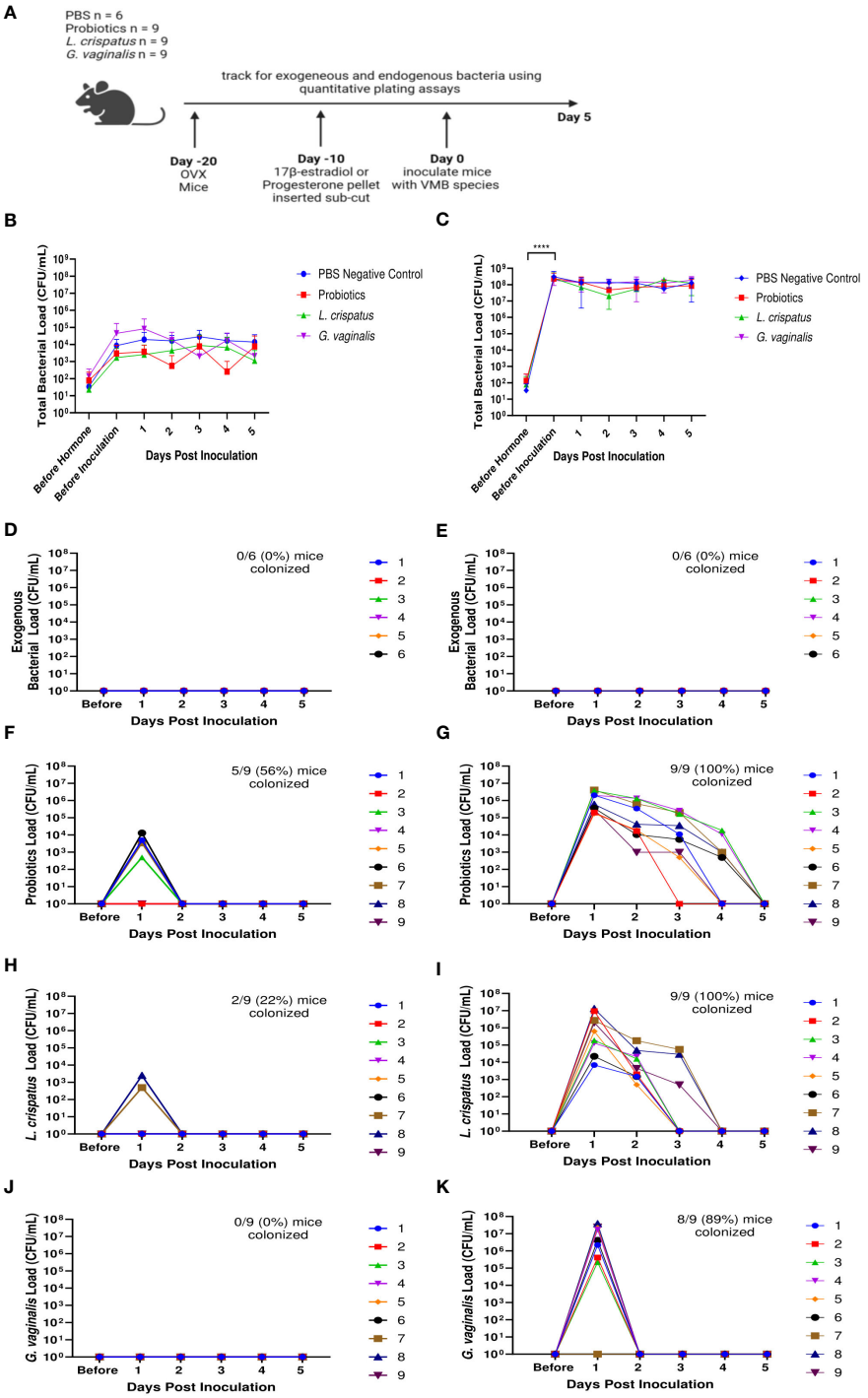

FIGURE 7
17β-estradiol treated OVX mice were fully colonized with human VMB species and had significantly higher bacterial loads compared to progesterone-treated OVX mice. Progesterone or 17β-estradiol 21-day release pellets were inserted subcutaneously into OVX mice 10 days after ovariectomies. Exogenous bacterial load and bacterial load were assessed by quantitative plating assays in mice before hormone treatment, 10 days after hormone treatment (before inoculation), and up to 5 days after one vaginal administration of a total of 10^7 CFU *L. reuteri* RC-14 and *L. rhamnosus* GR-1, *L. crispatus*, *G. vaginalis*, or PBS as a no exogenous bacteria negative control. Data are n=9 per group, except PBS n=6, and are representative of two independent experiments. A schematic of the experiment is shown in panel **(A)**. In the progesterone-treated mice, total colonies **(B)**, exogenous bacteria colonies in PBS inoculated **(D)**, probiotics colonies in probiotics inoculated **(F)**, *L. crispatus* colonies in *L. crispatus* inoculated **(H)**, and *G. vaginalis* colonies in *G. vaginalis* inoculated **(J)** groups were counted on agar plates. In the 17β-estradiol treated mice, total colonies **(C)**, exogenous bacteria colonies in PBS inoculated **(E)**, probiotics colonies in probiotics inoculated **(G)**, *L. crispatus* colonies in *L. crispatus* inoculated **(I)**, and *G. vaginalis* colonies in *G. vaginalis* inoculated **(K)** groups were counted on agar plates. In panels **(D–K)**, different mice are denoted by different colored points. The data was analyzed using a two-way ANOVA with Tukey's multiple comparisons (****p<0.0001).

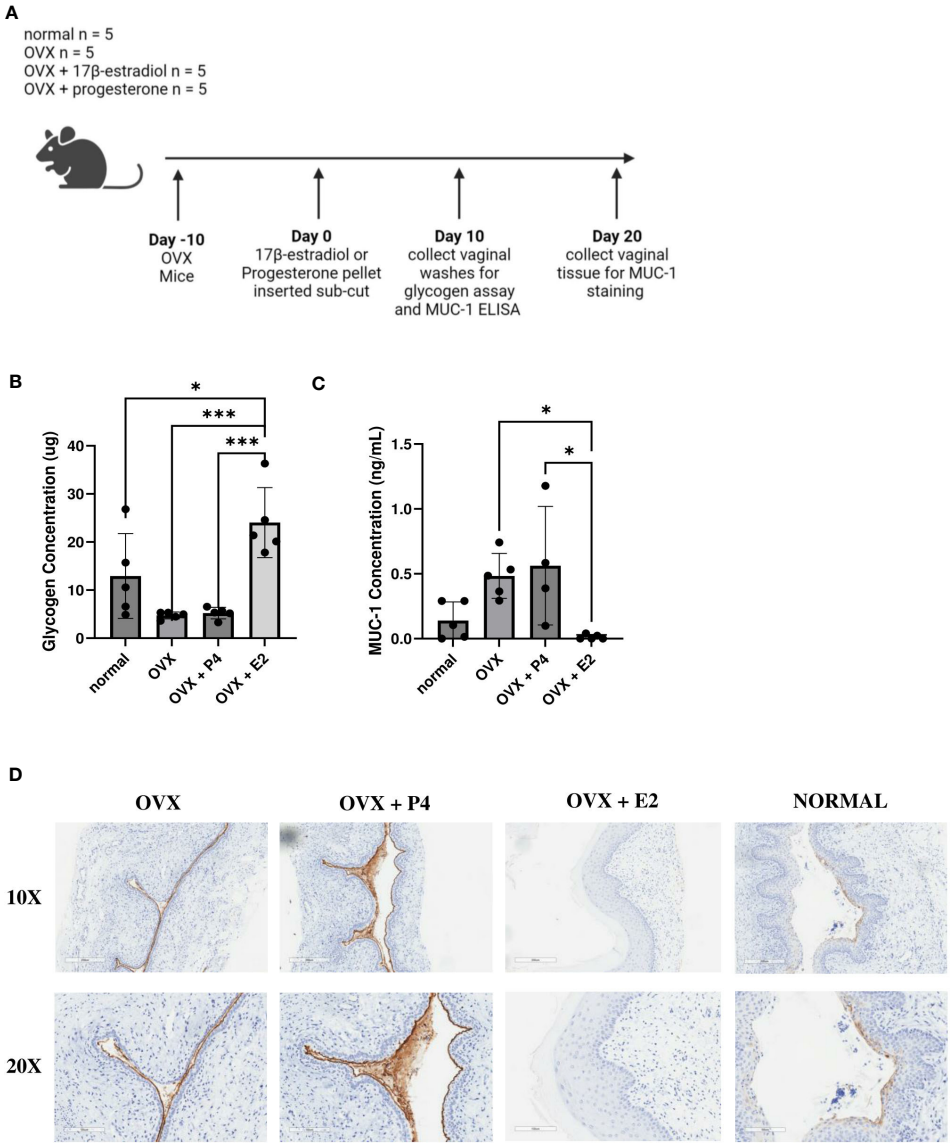

FIGURE 8
17β-estradiol-treated mice had increased glycogen levels and progesterone-treated mice had increased MUC-1 levels. 0.01 mg 17β-estradiol or 10 mg progesterone 21-day release pellets were inserted subcutaneously into female mice 10 days after ovariectomies. 10 days after hormone treatment, vaginal washes were collected from hormone-treated as well as OVX and normal mouse controls (n=5, representative of two independent experiments). A schematic of the experiment is shown in panel **(A)**. Glycogen **(B)** and MUC-1 **(C)** levels were measured using a glycogen assay or a MUC-1 ELISA. The data was analyzed using a one-way ANOVA with Tukey's multiple comparisons (***p<0.001 and *p<0.05). 20 days after hormone treatment, mice were sacrificed, and vaginal tracts were collected from hormone-treated as well as OVX and normal mouse controls (n = 6, representative of two independent experiments). Vaginal tracts were fixed in methacarn for 72 hours and MUC-1 staining was performed. Positive staining for MUC-1 is brown **(D)**.

microbiota and the role it plays in various diseases such as inflammatory bowel disease (Gkouskou et al., 2014) and Alzheimer's disease (Zhang et al., 2017). These models have also helped progress our understanding of the complex relationship between the gut microbiota and the immune system (Zheng et al., 2020). However, similar animal models of other human organ systems are greatly lacking. To study the female reproductive tract in the context of STIs, a similar mouse model that mimics the human VMB will be invaluable to study the mechanisms underlying VMB effect on STI susceptibility. Although a handful of studies have started to examine the establishment of such models (De Gregorio et al., 2015; Gilbert et al., 2019), this study is among the first to conduct an in-depth study in this area.

In this study, we successfully developed mouse models that mimic human eubiotic and dysbiotic VMB conditions. We first examined colonization in normal mice in their natural estrus cycle, to serve as a baseline. Other groups have also attempted to colonize hormone-unaltered mice with vaginal bacteria. One group attempted to colonize the mouse vaginal tract with human bacteria by inoculating vaginal swabs taken from women with BV into the vaginal canal of germ-free mice, however, the mice were not colonized with the bacterial species present in the swab from BV

patients (Wolfarth et al., 2020). Another group developed a BV model in mice by colonizing mice with *Gardnerella vaginalis* and *Prevotella bivia*, two species commonly seen in BV patients. They were able to demonstrate that this coinfection model recapitulates several aspects of human BV, including vaginal sialidase activity, which is a diagnostic BV feature, epithelial exfoliation, and *P. bivia* ascending infection (Gilbert et al., 2013; Gilbert et al., 2019). Another study inoculated the mouse vaginal canal of BALB/c mice with different *Lactobacillus* species, including probiotic species *L. rhamnosus* and *L. reuteri*, and was able to successfully detect viable bacterial species up to four days post inoculation (De Gregorio et al., 2012). The limited success of these few studies demonstrates that while it is feasible to establish these models, many more studies need to be conducted to understand the conditions and factors for successful colonization before clinically relevant research questions can be addressed. Here, we described the conditions for successful, albeit temporary, colonization of normal mice. Furthermore, we identified the conditions for a dysbiotic model colonized with *G. vaginalis*, as well as a eubiotic model colonized with *L. crispatus* and a probiotic model colonized with *Lactobacillus* probiotic species *L. rhamnosus* GR-1 and *L. reuteri* RC-14. To our knowledge, this is the first study where the eubiotic CST I human VMB species *L. crispatus* was successfully detected in the mouse vaginal tract after inoculation.

Because we saw variations in the extent and duration of colonization in our model, we further examined known modifiable factors that could improve colonization. A number of studies have looked at the changes in the VMB of women throughout various stages of their life, including during puberty, menstruation, pregnancy, and menopause (Kaur et al., 2019). The shifts between these gynecological stages are largely regulated by fluctuations in sex hormones, indicating an underlying relationship between hormones and the changes in the VMB throughout a woman's life. Therefore, we looked at the effect of hormones on bacteria colonization in our study and found many parallels with clinical data. We showed that removing endogenous hormones through OVX dramatically decreased VMB bacterial load compared to hormone-unaltered mice and neither eubiotic nor dysbiotic bacteria could colonize OVX mice for more than 24 h. Removing the effect of sex hormones through OVX may be considered congruent to menopause in women (Brzozowska and Lewiński, 2020). The transition of women through menopause has been shown to be marked by a gradual depletion of *Lactobacillus* species and an increase in anaerobic bacteria (Oliveira et al., 2022). Menopause is associated with a decrease in circulating hormones such as estrogen, which reduces glycogen deposition in the vaginal walls (Farage et al., 2010). Thus, it is likely that these changes lead to lower abundances of glycogen utilizing *Lactobacillus* in the vagina of postmenopausal women. Interestingly, estrogen hormone replacement therapy has been reported to increase *Lactobacillus* colonization in the VMB in post-menopausal women (Vitali et al., 2017; Geng et al., 2020), which is consistent with our findings. Treatment with 17β-estradiol restored the endogenous microbiota and colonization with eubiotic and dysbiotic bacteria in our mice. The results mentioned suggest the findings in our study recapitulate many aspects of the relationship between sex hormones and the VMB clinically, making it a reliable model to use in future studies.

The majority of the described effect of hormones on the VMB are from clinical studies. There are a few studies where sex hormones have been altered exogenously *in vivo* to assess the effect this has on the VMB. We have previously published showing humanized mice treated with medroxyprogesterone acetate (MPA), a synthetic progestin, have increased VMB diversity (Wessels et al., 2019). As increased VMB diversity is associated with dysbiosis (Ravel et al., 2011; Juliana et al., 2020), this study indicates a link between progesterone and decreased VMB health. A few other groups have also published in this area. In one study, young female mice were administered 17β-estradiol or progesterone exogenously and vaginal bacterial loads were examined. They found that 17β-estradiol-treated mice had an increase in vaginal bacterial load, and progesterone treated mice had a near disappearance of all vaginal bacteria (Taylor-Robinson, 1991). As mentioned previously, in our study, we found similar results; mice in estrogen high states such as during estrus and 17β-estradiol-treated mice had increased colonization with exogenous bacteria compared to mice in progesterone high states. Another study administered 17β-estradiol and MPA, to mice and determined VMB contents (De Gregorio et al., 2018). They found the predominant taxa to be *Enterobacteria* in all experimental groups. They also found that lactic acid producing bacteria and *Enterobacteria* were found in greater concentration than *Staphylococci* and *Enterococci* in the 17β-estradiol treated groups. Finally, they found that higher numbers of cultivatable bacteria were present in 17β-estradiol treated mice than MPA treated mice, which is a similar finding to the previous study and our study as well. Another study administered probiotic *Lactobacillus* species to mice and found a greater number of viable bacteria during the proestrus–estrous stage of the estrus cycle compared to the metestrus–diestrus phase (De Gregorio et al., 2012), which is similar to our study as well. In the BV model Gilbert et al. developed with *G. vaginalis* and *P. bivia*, they treated mice with 17β-estradiol prior to colonization with BV-associated bacteria (Gilbert et al., 2019), however we were able to show colonization without this step, although estradiol completely restored colonization of all bacteria after OVX. Overall, these studies suggest that sex hormones may alter the composition of the VMB, and that estrogen promotes overall bacterial load more than progestins *in vivo*. The results in our study align with previously published research in the area. To our knowledge, there are no comprehensive studies done where exogenous human eubiotic and dysbiotic bacterial species were administered to hormone-treated mice to show successful temporary colonization, which can be extended over time by repeated administration. To the best of our knowledge, this is the first study to optimize these conditions for prolonged colonization.

An additional optimization of our model was done by examining the effect of nutrient availability in the mouse vaginal canal, as this is an important determinant of what species colonize the VMB (Hood-Pishchany and Rakoff-Nahoum, 2021). As

mentioned previously, glycogen is a common nutrient source used by *Lactobacillus* species (Mirmonsef et al., 2014) and mucin is a common nutrient source used by *G. vaginalis* (Dupont, 2020; Vagios and Mitchell, 2021). We found mucin to facilitate initial colonization by *G. vaginalis*. Studies have shown that women with BV have higher concentrations of mucin-degrading enzymes, which in turn decreases the vaginal fluid viscosity (Olmsted et al., 2003). Sialidase positive bacteria, such as *Gardnerella vaginalis*, are able to catabolize sialic acid in the cervicovaginal mucins and use it as a nutrient source, which could explain why the addition of mucin aided in *G. vaginalis* colonization in our model. In our study, mice treated with progesterone had increased Mucin-1 levels, which is a common nutrient source used by BV-associated bacteria, which aligns with published literature indicating a link between progesterone and increased diversity in the VMB (Wessels et al., 2019). Previous studies have also indicated progestins are associated with decreased glycogen production (Wessels et al., 2019), which is also congruent to our study. Whilst glycogen did not improve or hinder *Lactobacillus* colonization in our model, other studies have proposed that increased glycogen levels can decrease bacterial diversity and promote *Lactobacillus* colonization (Wessels et al., 2019). 17β-estradiol-treated mice did however have significantly increased levels of glycogen, which aligns with previously published studies (Gregoire and Parakkal, 1972; Mirmonsef et al., 2016). The results of our study suggest nutrient availability is largely dictated by sex hormones, which suggests a potential mechanism of how sex hormones affect the VMB.

In conclusion, this study provides new and important insights into the conditions that can facilitate colonization of the mouse vaginal tract with bacteria found in the human VMB. While clinical studies have provided significant insights regarding the correlation between colonization by different vaginal bacteria and reproductive health, mechanistic studies examining cause-effect relationships will require animal models, which are currently under-developed. Based on our results, estrogen appeared to play a critical role in creating a hospitable environment for colonization by all human vaginal bacteria, while substrates like mucin selectively enhanced colonization by specific anerobic species. The current model can enable further studies that directly examine the effect of the VMB on STI susceptibility such as HSV-2 and HIV-1, as well as examine the effect on other reproductive outcomes such as inflammation and epithelial barrier integrity. In the future, human microbiota associated mouse models will be invaluable tools to help further elucidate the complex role the VMB plays in vaginal health.

Data availability statement

The original dataset is available from corresponding author upon reasonable request.

Ethics statement

The animal study was approved by the Animal Research Ethics Board at McMaster. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

NR: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review and editing. MFM: Investigation, Methodology, Supervision, Writing – review and editing. CK: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review and editing, Formal analysis. AN: Investigation, Methodology.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Human papillomavirus and cervical cancer in the microbial world: exploring the vaginal microecology

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The vaginal microbiota plays a crucial role in female reproductive health and is considered a biomarker for predicting disease outcomes and personalized testing. However, its relationship with human papillomavirus (HPV) infection and cervical cancer is not yet clear. Therefore, this article provides a review of the association between the vaginal microbiota, HPV infection, and cervical cancer. We discuss the composition of the vaginal microbiota, its dysbiosis, and its relationship with HPV infection, as well as potential mechanisms in the development of cervical cancer. In addition, we assess the feasibility of treatment strategies such as probiotics and vaginal microbiota transplantation to modulate the vaginal microbiota for the prevention and treatment of diseases related to HPV infection and cervical cancer. In the future, extensive replication studies are still needed to gain a deeper understanding of the complex relationship between the vaginal microbiota, HPV infection, and cervical cancer, and to clarify the role of the vaginal microbiota as a potential biomarker for predicting disease outcomes, thus providing a theoretical basis for personalized testing.

KEYWORDS

dysbiosis, probiotics, microbiota transplantation, biomarkers, personalized testing

1 Introduction

Cervical cancer (CC) is the most common malignant tumor in the female reproductive system and a significant global public health concern that seriously threatens women's health. Despite the implementation of human papillomavirus (HPV) vaccination and early screening for CC in reproductive-age women in most countries to prevent its occurrence, CC remains the fourth leading cause of cancer-related deaths in women. The GLOBOCAN 2020 database reported 604,000 new cases and 342,000 deaths worldwide (Sung et al., 2021), showing an

increasing trend in both incidence and mortality compared to the 2018 global CC statistics (Bray et al., 2018).

It is well known that the development of CC is closely associated with HPV infection. HPV is primarily transmitted through direct contact with lesions of infected individuals or indirectly through contact with virus-contaminated objects. Newborns can also acquire HPV through birth canal infections. HPV mainly affects the skin and mucous membranes, causing varying degrees of proliferative lesions, and plays a significant role in the development of genital warts, cervical precancerous lesions, and CC (Burchell et al., 2006; Koshiol et al., 2008). Most women are infected with one or more types of HPV at least once in their lifetime, but the majority of these infections are transient and can self-clear within 1-2 years. Only a small fraction of women may sustain infections and have an increased risk of developing cervical precancerous lesions or invasive CC under certain influencing factors (Sudenga and Shrestha, 2013; Brusselaers et al., 2019). However, the persistence and clearance of HPV may be related to the vaginal microbiota (Zeng et al., 2022).

The female reproductive system has its specific microbial communities that play essential roles in women's life processes and menstrual cycles (Chen et al., 2017), primarily maintaining vaginal health and protecting the vaginal environment from various genitourinary infections. The vaginal microbiota (VMB) interacts dynamically with the host and the environment, forming a dynamic ecosystem known as the vaginal microbiome, mainly maintained through interactions with the local microenvironment (Sharifian et al., 2023).

Research indicates that HPV is a necessary factor for CC and its precancerous lesions but not the sole determinant of CC development (Walboomers et al., 1999), suggesting that HPV alone is insufficient to induce cervical malignancy. Furthermore, the driving factors behind the transition states between HPV acquisition, clearance, persistence, and progression to cervical precancerous lesions remain unclear. However, some scholars have proposed that the VMB plays a significant role in the progression of cervical lesions induced by HPV infection (Gillet et al., 2011; Gillet et al., 2012; Audirac-Chalifour et al., 2016; Mitra et al., 2016; Brusselaers et al., 2019; Santella et al., 2022). Therefore, this paper reviews the impact of VMB on CC resulting from HPV infection, elucidating the role of VMB as a potential biomarker for predicting disease outcomes and providing a basis for personalized testing.

2 Healthy vaginal microbiome

The VMB plays a crucial role in protecting the host from infectious diseases and is considered a useful biomarker for predicting disease outcomes and personalized testing (Kyrgiou and Moscicki, 2022). A significant characteristic of a healthy vaginal microenvironment is the abundant colonization and dominance of *Lactobacillu*, primarily consisting of *Lactobacillus gasseri* (*L. gasseri*), *Lactobacillus crispatus* (*L. crispatus*), *Lactobacillus iners* (*L. iners*), *Lactobacillus jensenii* (*L. jensenii*), or *Lactobacillus vaginalis* (Younes et al., 2018; Chee et al., 2020; Fan et al., 2021). The composition of VMB varies during different life stages, including infancy, puberty, pregnancy, and menopause, due to hormonal changes, metabolic deposits, and antibiotic usage, with host estrogen levels having a significant impact on the vaginal environment (Das et al., 2023). Additionally, *Lactobacillus* spp. has a relatively low correlation with high risk-HPV (HR-HPV), cervical intraepithelial neoplasia (CIN), and CC (Wang et al., 2019).

2.1 Community state types typing

To describe the presence of different VMB profiles that may affect the development of cervical diseases, researchers have categorized them into Community State Types (CSTs). Using 16S rRNA gene amplicon sequencing, the VMB has been divided into five different CSTs: *L. crispatus* (CST I), *L. gasseri* (CST II), *L. iners* (CST III), low-*Lactobacillus* and bacterial vaginosis-associated bacteria (CST IV), and *L. jensenii* (CST V) (Ravel et al., 2011; Gajer et al., 2012; Mancabelli et al., 2021). CST IV is characterized by the augment in VMB diversity, mainly marked by a decrease in *Lactobacillus* abundance and an increase in anaerobic bacterial species. The most common bacteria in CST IV include *Gardnerella vaginalis*, *Megasphaera*, *Sneathia*, and *Prevotella* species (Castanheira et al., 2021).

The composition of VMB is dynamic and continuously changes in reproductive-age women. In healthy women, CST I and CST V are predominant. However, when infected with HPV, CST II significantly increases and promotes HPV clearance (Brotman et al., 2014b; Xu et al., 2020). A meta-analysis that examined the relationship between different CSTs and HPV infection showed that compared to *L. crispatus*, CSTs predominantly composed of 'low *Lactobacilli*' or *L. iners* had a 3-5 times higher risk of HPV infection and a 2-3 times higher risk of HR-HPV, cervical dysplasia, and CC occurrence (Norenhag et al., 2020). This suggests that VMB composition may potentially serve as a biomarker for HPV-related diseases, guiding clinical treatment.

2.2 Lactobacillus

Lactobacillus play a dominant role in the vaginal microbiota and contribute to maintaining vaginal microbiota balance, inhibiting the growth of pathogens, enhancing local vaginal immunity, and resisting tumors (Cheng et al., 2020).

The mechanisms through which *Lactobacillus* maintains the health of the female reproductive system include: (1) Lactic acid production and maintenance of vaginal acidity. *Lactobacillus:* primarily utilize carbohydrates on the vaginal mucosal epithelial cells as an energy source to produce lactic acid, maintaining the vaginal environment in a relatively acidic state. This inhibits the adhesion, colonization, and growth of pathogenic bacteria (Anahtar et al., 2018; Kroon et al., 2018). Additionally, L-lactic acid under acidic conditions can induce anti-inflammatory responses in cervical and vaginal epithelial cells while inhibiting the production of pro-inflammatory cytokines and chemokines induced by toll-like receptors (TLRs) (Delgado-Diaz et al., 2019).

(2) Destruction of essential epithelial proteins for vaginal barrier integrity by bacteriocins and H_2O_2. Myeloperoxidase (MPO), an enzyme expressed in neutrophils, catalyzes the production of hypochlorous acid in the presence of H_2O_2 produced by *Lactobacillus*, preventing the invasion of pathogens and HPV into cervical epithelial cells (Castelão et al., 2015). Furthermore, *Lactobacillus* produce protective proteins such as biosurfactants and bacteriocins, disrupting epithelial cells and forming the first line of defense against pathogen adherence in the vaginal environment (Zevin et al., 2016; Borgogna et al., 2020; Nieves-Ramírez et al., 2021). (3) Competition with pathogens for vaginal epithelial adhesion due to steric hindrance or specific obstruction at the receptor site. *Lactobacillus* compete for space and resources with other bacteria, either promoting or preventing the colonization of other bacteria. Vaginal mucus and epithelial cell receptors may play important roles in the colonization of certain bacteria (Hickey et al., 2012). (4) Regulation of local defense. Regulating VMB balance and enhancing local cervical immune function may reduce the occurrence of cervical lesions (Zheng et al., 2020). (5) Regulation of core fucosylation of vaginal mucosal epithelial cells. Fucosylation of mucosal epithelial cells is closely related to microbial colonization (Goto et al., 2016). Knocking out the core fucosyltransferase gene (Fut8) can promote CC proliferation and migration, while the lactic acid produced by *L. iners* activates the Wnt pathway through the lactic acid-gp81 complex, increasing epithelial cell fucosylation levels, and inhibiting CC proliferation and migration (Fan et al., 2021). (6) Autophagy in infected cells and promotion of clearance (Figure 1).

2.3 Factors influencing vaginal microbiota

The normal function of VMB is influenced by various factors, including ethnicity, genetic background, epigenetic changes, multiple pregnancies, lifestyle, hygiene habits, infections, antibiotic use, age at first sexual intercourse, number of sexual partners, smoking, and long-term use of contraceptives and hormonal medications (Busnelli et al., 2019; Kaur et al., 2020). Ravel et al. found that in Asian and white women, *Lactobacillus*-dominated VMB occurred in 80.2% and 89.7% of cases, respectively, while in black and Hispanic women, it occurred in 59.6% and 61.9% of cases, respectively (Ravel et al., 2011). This is consistent with the results of Xia Zhou et al., indicating potential differences in the quantity and types of VMB among women of different ethnicities (Zhou et al., 2007). Furthermore, a meta-analysis suggests that vaginal douching may increase vaginal bacterial diversity, potentially increasing the risk of CIN and CC (Zhang et al., 1997). Additionally, research has found that smoking (Brotman et al., 2014a) and sexual intercourse (Mändar et al., 2015) can decrease the abundance of *L. crispatus* and increase microbiota diversity. However, oral hormonal contraceptives may disrupt host hormone levels, leading to changes in the vaginal environment (Das et al., 2023). Dysbiosis in the VMB can lead to the overgrowth of opportunistic pathogens, ultimately leading to disease development.

3 HPV and cervical cancer

HPV is a class of small, non-enveloped, double-stranded circular DNA viruses. The viral particles have an icosahedral shape with a diameter of 50-55 nm and a molecular weight of 5×10^6 Da (Araldi et al., 2018). The HPV genome comprises 8 protein-encoding genes organized into three regions: (1) Non-coding region (NCR), containing the long control region (LCR) with promoters, enhancers, and silencers. This region is the least conserved segment of the HPV genome and is often used to describe relationships between variants. (2) Early region, including genes E1, E2, E4, E5, E6, and E7, which are involved in virus replication and transformation. (3) Late region, encoding the major (L1) and minor (L2) capsid proteins, participating in virus particle assembly (Tommasino, 2014; Sharifian et al., 2023). E1 is the initiation recognition protein for viral DNA replication. E2 is involved in viral DNA replication and transcription and can suppress the expression of E6 and E7. E4 interacts with the cellular cytoskeleton in the later stages of the virus lifecycle, and E5 may have a role in genome amplification. E6 and E7 mainly interfere with critical cell cycle checkpoints, such as inhibiting tumor protein p53 and retinoblastoma protein pRb

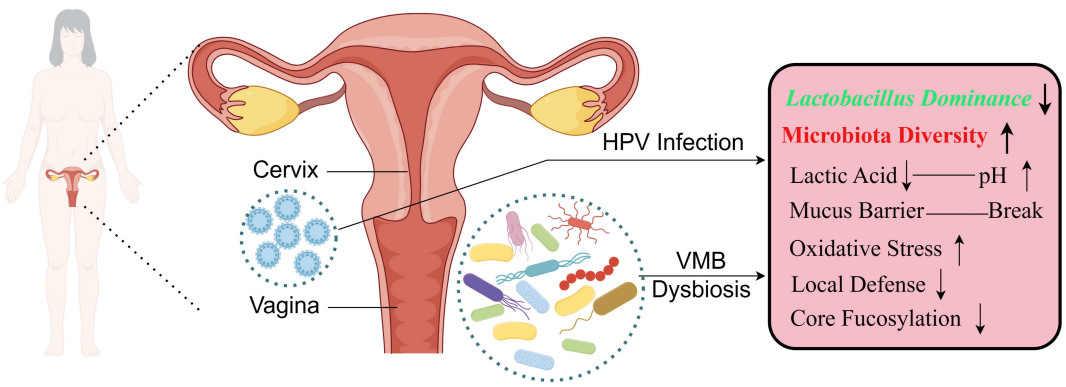

FIGURE 1
The alterations of the vaginal microenvironment when the dysbiosis of the VMB and HPV infection occur with cervical lesions.

(Haręża et al., 2022; Sharifian et al., 2023), forcing different epithelial cells to re-enter the cell cycle to increase virus production, thereby mediating HPV carcinogenesis (Pyeon et al., 2009; Lin et al., 2020). Recent literature reports that HPV E7 significantly inhibits the expression of host defense peptides, ultimately leading to VMB dysbiosis (Lebeau et al., 2022). Therefore, the expression of HPV E6 or E7 may be regulated by certain pathogens at different stages of cervical lesions (Liu et al., 2022).

Currently, more than 200 different HPV subtypes have been identified based on at least 10% sequence differences in the L1 gene sequence. Additionally, HPV is classified into low-risk types (LR-HPV) and HR-HPV based on the host diseases or cancers they cause. LR-HPV is responsible for mild benign proliferative lesions, while HR-HPV is more likely to lead to malignant changes. Among HR-HPV genotypes, HPV 16 and 18 are the most common and closely associated with the development of CC (Muñoz et al., 2006). S. Andersson et al. conducted a genetic typing of 131 cervical adenocarcinoma patients, and the results showed that HPV 18 and 16 had the highest infection rates, at 52% and 33%, respectively (Andersson et al., 2001). Furthermore, data indicates that out of 690,000 cancer cases caused by HPV infection, 570,000 were CC, with HPV 16 and 18 accounting for 72%, and HPV 31, 33, 45, 52, and 58 accounting for 17% (De Martel et al., 2020). This suggests that HPV 18 and 16 infections are major risk factors for cervical lesions.

Cervical lesions can be categorized into CIN I, II, and III based on the severity of cervical dysplastic cells (Brianti et al., 2017). Additionally, according to the Bethesda system, cervical precancerous lesions can be classified as low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL) (Ostör, 1993). While HPV infection is a necessary condition for CC, it is not the sole factor. Other contributing factors may include the number of sexual partners, pregnancy history, genetic background, epigenetic changes, immune system status, ethnicity, and VMB imbalance (Lin et al., 2015; Boda et al., 2018).

Recently, a cross-sectional study found differences in vaginal metabolites such as biogenic amines, glutathione, and lipids between women with cervical HPV positivity and those with HPV negativity. If a correlation between the incidence/persistence of HPV and reduced glutathione or oxidative glutathione can be demonstrated, antioxidant therapy may become a non-surgical intervention for HPV (Borgogna et al., 2020).

4 Vaginal microbiota, HPV, and cervical cancer

In recent years, research has shown that VMB can modulate HPV infection and play a role in the development of cervical lesions (Figure 1). The composition of VMB may act as a regulator of HR-HPV (Chao et al., 2019). Increased diversity in VMB and reduced abundance of *Lactobacillus*, leading to vaginal dysbiosis, may be associated with HPV infection and cervical lesions (Wu et al., 2022). The decrease in *Lactobacillus* may lead to a pro-inflammatory environment, increasing the expression of HPV E6 and E7 genes and malignant cell proliferation (Kyrgiou and Moscicki, 2022). In Mexican women, HPV infection leading to cervical squamous intraepithelial lesions (SIL) is mainly associated with changes in VMB composition (Nieves-Ramírez et al., 2021). SIL patients showed increased quantities of *L. jensenii* and *L. iners* (Gomez Cherey et al., 2023). Additionally, *L. crispatus* (CST I) and *L. gasseri* (CST II) were most common in HPV-negative women, while CST III and IV were associated with HPV infection and the development of CC (Audirac-Chalifour et al., 2016), closely related to VMB dysbiosis.

Cheng Weiye et al. used Illumina high-throughput sequencing to analyze the relationship between VMB composition and HPV infection. The results showed that *Lactobacillus* accounted for over 80% in healthy women, while HR-HPV-infected individuals exhibited increased VMB diversity, mainly characterized by an increase in *Gardnerella* and a decrease in *Lactobacillus*. Furthermore, *Chlamydia trachomatis* and *Ureaplasma urealyticum* may act synergistically with HPV in the development of CC (Cheng et al., 2020). Additionally, Zhai Qingzhi et al. investigated the VMB characteristics of 168 Chinese reproductive-age women with varying degrees of cervical lesions and HR-HPV positivity. They found that the healthy group (HR-HPV negative) was dominated by *Lactobacillus* and *Ignatzschineria*, while the disease group (HR-HPV positive) was mainly composed of *Gardnerella* and *Prevotella*. In the HR-HPV group, with the progression of cervical lesions, the content of *Lactobacillus* and *Ignatzschineria* continued to decrease (Zhai et al., 2021). These results suggest that the depletion of *Lactobacillus* and the overgrowth of anaerobic bacteria may be related to VMB dysbiosis. Additionally, vaginal dysbiosis may increase the risk of infections with pathogens such as *Chlamydia trachomatis* and *Gardnerella* (Balle et al., 2018; Di Pietro et al., 2019; Pekmezovic et al., 2019).

4.1 Chlamydia trachomatis

Chlamydia trachomatis, similar to HPV, is an obligate intracellular pathogen and a major cause of bacterial sexually transmitted diseases. *Chlamydia trachomatis* has unique biphasic developmental characteristics that aid in HPV stable invasion of the host, and it causes an estimated 128 million new infections annually (Rowley et al., 2019). *Chlamydia trachomatis* is a well-known common cause of cervicitis and urethritis, often presenting as asymptomatic infections. If left untreated, it can lead to severe reproductive sequelae such as pelvic inflammatory disease, ectopic pregnancy, tubal factor infertility, miscarriage, and preterm birth (Shaw et al., 2011). A case-control study found that women infected with *Chlamydia trachomatis* tend to have *L. iners* or multiple anaerobic bacteria dominating their cervical-vaginal microenvironment (Van Der Veer et al., 2017). Additionally, a meta-analysis reviewing the impact of *Chlamydia trachomatis* infection on VMB composition showed that individuals infected with *Chlamydia trachomatis* tend to have cervical-VMB dominated by *L. iners* or a mixture of facultative or strict anaerobes

(Di Pietro et al., 2022). Furthermore, a meta-analysis that firstly evaluated the relationship between VMB and infections such as HPV and *Chlamydia trachomatis* found a positive trend between a low *Lactobacillus*-dominated VMB and infections with HPV and *Chlamydia trachomatis* (Tamarelle et al., 2019). These studies suggest that genital infections caused by *Chlamydia trachomatis* have a significant impact on the composition of the cervical-VMB.

In young, unmarried, sexually active women with multiple sexual partners who use oral contraceptives, co-infection with *Chlamydia trachomatis* and HPV may be a crucial risk factor for CC (Suehiro et al., 2021; Mangieri et al., 2023). Approximately 50%-80% of sexually active individuals can be co-infected with both *Chlamydia trachomatis* and HPV, with about half of them harboring oncogenic HPV types. This co-infection may persist silently as long as the local equilibrium of VMB, immune responses, and hormones is maintained (Gargiulo Isacco et al., 2023). Italian researchers found that women co-infected with *Chlamydia trachomatis* and HPV had higher microbiota diversity in their cervical microbiota compared to the healthy group (Di Pietro et al., 2018). Furthermore, studies have shown that women who are HPV-positive and infected with *Chlamydia trachomatis* have a higher prevalence of cervical lesions compared to HPV-negative women, which may lead to the development of CC (Mosmann et al., 2021). While a relationship between *Chlamydia trachomatis*, HPV, and CC has been discovered, further research and validation experiments are still needed to clearly define the relationship between these factors and provide a scientific basis and theoretical foundation for the prevention and treatment of CC.

4.2 Gardnerella

Gardnerella, an anaerobic pathogen, is a predominant group within CST IV and a major pathogenic bacterium in bacterial vaginosis. It is often detected at higher rates in women who are HR-HPV positive (Gao et al., 2013). A cross-sectional analysis found that HR-HPV infection rates were significantly higher in women with *Gardnerella* vaginalis-associated VMB (72.73%) compared to those dominated by *Lactobacillus* (44.72%) (P=0.04). *Gardnerella*, along with the *Prevotella* genus, was identified as the highest-risk combination for HPV-positive women (Lin et al., 2022). Meanwhile, a prospective longitudinal cohort study suggested a positive correlation between *Gardnerella* and CIN2-CIN3, possibly due to increased VMB diversity (Usyk et al., 2020).

Gardnerella produces sialidase (SNA), an enzyme that releases sialic acid from the terminal polysaccharides on mucous secretions and mucosal cell surfaces (Severi et al., 2007). Pathogens can adhere to cells using sialic acid and alter the normal mucous barrier and immune responses (Vick et al., 2014). The genes encoding SNA include nanH1, nanH2, and nanH3, with nanH3 being associated with SNA activity (Robinson et al., 2019). Researchers have found that nanH3 levels are higher in women with persistent cervical HPV16 infection for 12 months compared to those who cleared HPV16 (P=0.007) (Novak et al., 2023). Elevated SNA levels are associated with susceptibility to CC (Govinden et al., 2018). In addition, studies have shown that the prevalence of SNA-producing *Gardnerella* and *Prevotella* is higher in HR-HPV infection and CIN groups, suggesting that SNA may play an important part in the development of HPV infection to cervical lesions (Lin et al., 2022). Therefore, further research is needed to understand the mechanisms by which *Gardnerella* influences HPV infection and cervical lesions, potentially identifying biomarkers that could guide the treatment of CC.

5 Prevention and treatment of HPV and microbiota dysbiosis

HPV infection cannot be detected within 1-2 years after infection (Markowitz and Unger, 2023), and there are currently no effective drugs or supplements to clear HR-HPV infection, so HPV infection and related lesions are mainly effectively prevented by vaccines. Based on the viruslike particles of the major capsid protein L1 pentamers, HPV vaccines are divided into bivalent, quadrivalent and nine-valent vaccines (Markowitz and Unger, 2023). Among them, bivalent vaccine mainly protects against HPV16 and 18 (Bonanni et al., 2009). Quadrivalent vaccine protects against two HR-HPV (HPV16 and 18) and two LR-HPV (HPV6 and 11) (Bonanni et al., 2009). The nine-valent vaccine, which protects against HPV6, 11, 16, 18, 31, 33, 45, 52 and 58, prevents 97% of high-grade precancerous lesions, more effective than the quadrivalent vaccine (Joura et al., 2015; Markowitz and Unger, 2023). It is important to note that the HPV vaccine does not protect against all HPV types that can progress to CC (Lei et al., 2020). Therefore, regular cervical screening is also mandatory for those vaccinated with HPV.

In addition, studies have shown that the use of α-glucans, short chain and low molecular weight polysaccharides may eradicate HPV (Cazzaniga et al., 2022). Active Xerose Correlated Compound (AHCC, a particular mixture of α-glucans) has been reported to be effective in supporting the host immune system to clear persistent HR-HPV infection (Smith et al., 2019) and is well tolerated. Results showed that IFN-β suppression to less than 20 pg/ml was associated with an increase in T lymphocytes and IFN-γ and durable clearance of HPV infection in women receiving AHCC supplementation (Smith et al., 2022).

Currently, there are two main treatment strategies for regulating the VMB, which are probiotics and vaginal microbiota transplantation (VMT). These two methods primarily aim to promote the healthy VMB to prevent and treat diseases related to HPV infection and CC.

Probiotics, referred to by the World Health Organization as "live microorganisms" (Ebner et al., 2014), include *Bifidobacteria*, *Lactobacillus*, and *Streptococci*. They help balance VMB, enhance host immune responses, and complement standard antibiotics, thereby strengthening treatment and preventing recurrence (Sharifian et al., 2023). Verhoeven et al. found that HPV-infected patients in the probiotics group had a higher HPV clearance rate compared to the control group, with the clearance rate of HPV-related cellular abnormalities being twice that of the control group (Verhoeven et al., 2013). While probiotics have a certain positive role in treating microbiota dysbiosis, their efficacy has not met the

expected results. At present, there are still many questions that need to be studied, such as whether probiotics should be used at the same time of antibiotics? What is the dose of probiotics to maintain VMB balance? Does high frequency of probiotic use increase the colonization rate of VMB?

VMT is another new method for treating microbiota dysbiosis, primarily involving the transplantation of healthy microbiota isolated from donors into the patient's vaginal environment (Ma et al., 2019). However, VMT not only requires specific conditions in the patient's vaginal environment but also places high demands on the health of the donor's microbiota. If the donor's microbiota contains drug-resistant microorganisms, hidden pathogens, or sperm, it may introduce unnecessary complications to the patient (Gargiulo Isacco et al., 2023). Therefore, the VMT method is not yet mature, and further research is needed to determine its efficacy and adverse reactions to address its shortcomings (Lev-Sagie et al., 2019).

6 Prospects and conclusions

VMB and host maintain their health and homeostasis through co-innate. Some studies have shown that VMB components are considered as potential biomarkers for predicting disease outcomes and individualized monitoring (Kyrgiou and Moscicki, 2022). At present, most studies are focused on the relationship between VMB, HPV infection and CC, but the underlying molecular mechanism of VMB in HPV infection and cervical disease is still challenging. Therefore, large sample longitudinal cohort studies are needed to explore the effect of VMB composition changes on HPV infection and cervical disease outcomes, and analyze its role as a microbial marker of disease, which is expected to provide reasonable targets for the development of new prevention and treatment drugs.

VMB plays a significant role in HPV infection, persistence, and clearance. The occurrence and progression of HPV infection or cervical lesions are closely related to the imbalance of the VMB, primarily characterized by a decrease in the abundance of *Lactobacillus* and an increase in the diversity of the microbiota. Understanding the composition and changes in the VMB and evaluating the potential of the VMB as a novel adjunctive biomarker for predicting HPV infection and persistence will aid in the prevention and reduction of CC incidence and enhance CC prognosis. In the future, it is imperative to explore the exact molecular mechanism of VMB in the process of HR-HPV infection and cervical lesions, and to clarify the role of probiotics, prebiotics, VMT, new antibacterial agents and biofilm disrupting agents in clinical practice, so as to restore healthy VMB.

Author contributions

ZZ: Writing – original draft, Writing – review & editing. QM: Writing – original draft, Writing – review & editing. LZ: Writing – original draft, Writing – review & editing. LM: Writing – original draft. DW: Writing – original draft. YY: Writing – review & editing. PJ: Writing – review & editing. YW: Writing – review & editing. FW: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The role of sialidases in the pathogenesis of bacterial vaginosis and their use as a promising pharmacological target in bacterial vaginosis

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Bacterial vaginosis (BV) is an infection of the genital tract characterized by disturbance of the normally *Lactobacilli*-dominated vaginal flora due to the overgrowth of *Gardnerella* and other anaerobic bacteria. *Gardnerella vaginalis*, an anaerobic pathogen and the major pathogen of BV, produces sialidases that cleave terminal sialic acid residues off of human glycans. By desialylation, sialidases not only alter the function of sialic acid-containing glycoconjugates but also play a vital role in the attachment, colonization and spread of many other vaginal pathogens. With known pathogenic effects, excellent performance of sialidase-based diagnostic tests, and promising therapeutic potentials of sialidase inhibitors, sialidases could be used as a biomarker of BV. This review explores the sources of sialidases and their role in vaginal dysbiosis, in aims to better understand their participation in the pathogenesis of BV and their value in the diagnosis and treatment of BV.

KEYWORDS

bacterial vaginosis, *Gardnerella vaginalis*, sialidase, vaginal dysbiosis, pathogenesis

1 Introduction

Bacterial vaginosis (BV) is caused by a disturbance to the vaginal flora in which *Gardnerella* and other anaerobic bacteria replace the normal vaginal microbiota dominated by *lactobacilli* (Ravel et al., 2011). Lactic acid, H2O2, bacteriocins, and biosurfactants, which are antimicrobial and anti-inflammatory products produced by *lactobacilli*, decreases along with the health-promoting *lactobacilli*. The increased pH of the vagina creates advantages for the proliferation of facultative and obligate anaerobes, including *Gardnerella*, *Atopobium*, *Mobiluncus*, *Prevotella*, *Streptococcus*, *Ureaplasma*, *Megasphaera* etc (Amabebe and Anumba, 2018). Meanwhile, the concentrations of short chain fatty acids (SCFAs, such as acetate, malonate and succinate) and amines (such as putrescine, cadaverine, and tyramine) produced by the overgrown anaerobes increase in parallel with bacterial abundance and species

biodiversity (Srinivasan et al., 2015; Vitali et al., 2015). A recent study found that a combination of vaginal microbiota metabolites representing BV increased basal and toll-like receptor (TLR) -induced production of TNF-α, demonstrating their immune regulatory effects (Delgado-Diaz et al., 2020).

As a major obstetrical and gynecological concern, BV is associated with many negative health outcomes, such as infertility (Ravel et al., 2021), preterm delivery (Honest et al., 2004; Cauci and Culhane, 2011; Manns-James, 2011), pelvic inflammatory disease (Taylor et al., 2013; Ravel et al., 2021), and sexually transmitted infections (Bautista et al., 2016; Armstrong and Kaul, 2021). Pathogenesis of BV involves degradation of the mucus layer on the surface of vaginal epithelium, exfoliation and detachment of the epithelial cells (Cauci et al., 2003), which in turn facilitates bacterial adhesion and biofilm formation (Swidsinski et al., 2005; Varki, 2009; Varki and Gagneux, 2012). Sialidases play a key role in the processes mentioned above, making sialidase activity measurement useful in the diagnosis and management of BV (Javed et al., 2019; Mabugana et al., 2023). Of course, mucus degradation is such a complex process that there are other glycosidases, proteases, and sulphatases involved in (Wiggins, 2001). For example, prolidase is a kind of proteolytic enzymes associated with BV, which shows a negative association with interleukin (IL)–8 levels in female CVF (Cauci et al., 2002) and can predict low birth weight and preterm birth with combination of vaginal pH and vaginal sialidase (Cauci et al., 2005).

As a major virulence factor of *Gardnerella* spp (Schellenberg et al., 2016; Kurukulasuriya et al., 2021), sialidases are important glycoside hydrolases that cleave sialic acid residues off of terminal glycans (Lewis et al., 2013; Robinson et al., 2019). Sialic acids are 9-carbon monosaccharides found in glycoconjugates such as glycoproteins and glycolipids, as well as at the distal end of *N*- and *O*-linked carbohydrate chains, also named glycans (Ghosh, 2020). As a part of glycoconjugates and substrates of sialidases, glycans have been found in human cervicovaginal fluid (CVF) (Moncla et al., 2015; Moncla et al., 2016; Wang et al., 2015) and surface of vaginal epithelial cells (Agarwal et al., 2023). Glycans heavily coat the surface of mammalian epithelial cells (Ochs et al., 2020; Argüeso et al., 2021), making them the frequent primary point of interaction between microorganisms and mucosal barriers (Poole et al., 2018). Through hydrolysis of sialic acids, which are highly electronegative carbohydrates, sialidases participate in many physiological and pathological pathways by lowering the surface charge of the whole cell, exposing glycoconjugates' binding sites, changing the conformation of the glycoproteins, and eventually altering the functions of sialic acid-containing glycoconjugates (Pshezhetsky and Ashmarina, 2013).

Sialic acids support the defense barriers through a delicate balance between sialylation and desialylation (Cohen and Varki, 2010; Cao and Chen, 2012). Sialylation, mediated by sialyltransferases, is the addition of sialic acids to the end of oligosaccharides and glycoproteins, while desialylation, mediated by sialidases, is the removal of sialic acids. Sialoglycoproteins, composed of glycoproteins and sialic acids, are important defense components of the mucosal surface that create a physical barrier against pathogens (Lewis and Lewis, 2012). With a weight percentage of almost 16% sialic acids, mucins provide a dense physical barrier that disrupt the interactions between pathogens and epithelial cells (Slomiany et al., 1996; Moran et al., 2011). Moreover, sialylation also plays a role in immune response by altering the functions of immunoglobulins and regulating inflammation (Yoo and Morrison, 2005; Anthony and Ravetch, 2010).

Sialidases, also known as neuraminidases, have been detected in CVF and elevated level of sialidase activity is associated with BV (Briselden et al., 1992; Myziuk et al., 2003). In a 1992 study, women with BV had higher levels of sialidase activity in their vaginal secretions than those without (Briselden et al., 1992). Over the next three decades, many more studies produced similar results (Howe et al., 1999; Smayevsky et al., 2001; Cauci et al., 2003; Lewis et al., 2012). A recent study suggests that women with BV have higher sialic acid depletion and lower levels of sialylation (Agarwal et al., 2023), which could be explained by elevated sialidase activity as sialylation breaks down and depletes sialoglycans. Another study also detected roughly 3-fold lower amounts of total sialic acids and 3.5-fold greater amounts of free sialic acids in BV samples compared with normal samples using high-performance liquid chromatography (HPLC) (Lewis et al., 2012). However, the exact mechanism of sialidases causing BV is not fully understood, as the current understanding of the roles of vaginal epithelial glycans is still limited.

Besides BV, sialidases are involved in a broad spectrum of diseases within the human body as they can be produced by not only bacteria but also viruses, mammals, and protozoa. Bacterial sialidases also participate in host-bacteria interactions, coinfections, and dysbiosis in oral cavity, gastrointestinal tract and respiratory system (Siegel et al., 2014; Huang et al., 2015; Wong et al., 2018). Influenza A and B viruses can also produce sialidases, which in turn facilitates the development of influenza (Zambon, 2001). In mammals, sialidases are involved in a wide range of health issues, including cancers (SoÈnmez et al., 1999; Zhou et al., 2020), diabetes (Natori et al., 2013), neurodegenerative disorders (Liao et al., 2020; Khan et al., 2021), fibrosing diseases (Karhadkar et al., 2022) and heart diseases (Zhang et al., 2018; Chen et al., 2021).

As the catalytic activity of sialidases is essential to the colonization and dissemination of several pathogenic microorganisms, sialidases could be used as a promising diagnostic marker for BV (Briselden et al., 1992; Smayevsky et al., 2001). This article aims to review relevant literature to explore the characteristics of sialidases in CVF, their contributions to vaginal dysbiosis, and their clinical use in BV diagnosis and treatment.

2 Sources of sialidase activity

So far research has reported *in vitro* sialidase activity in some BV-associated bacteria (BVAB), such as isolates of *Prevotella*, *Bacteroides*, and *Gardnerella* (Briselden et al., 1992). Studies have illustrated the ability to produce sialidases by every strain of *Prevotella bivia*, while only some *G.vaginalis* isolates produce sialidases (Briselden et al., 1992; Lopes Dos Santos Santiago et al., 2011). However, *G.vaginalis* is able to produce higher levels of sialidases, demonstrated in a study of C57BL/6 mouse models where *Prevotella* models showed similar levels of sialidase activities with *G.vaginalis* in a 100 times infection titer compared to *Gardnerella*-colonized models (Gilbert et al., 2019).

Apart from the abundance of bacteria themselves, other factors, such as sialidase expression levels, individual heterogeneity, and interactions between bacteria, might also affect sialidase activity in the CVF. Furthermore, sialidase produced by possible viruses and the host should be taken into consideration though there are few studies about this.

Among genotypes of *G.vaginalis*, the expression levels of sialidases are highly heterogeneous (Schellenberg et al., 2016). Based on quantitative polymerase chain reaction (qPCR) targeting clade-specific genes, *Gardnerella* is divided into four clades, clade 1 (encoding putative a-L-fucosidase), clade 2 (encoding a hypothetical protein), clade 3 (encoding thioredoxin) and clade 4 (encoding CIC family chloride transporter) (Balashov et al., 2014). They are different in sialidase activity: clade 2 have the highest activity followed by clade 1, clade3, and clade 4 (Qin and Xiao, 2022). In clade 4, the proposed sialidase encoding gene *sialidase A* gene is not detected (Shipitsyna et al., 2019).

Three sialidase homologs, NanH1 (also known as sialidase A), NanH2, and NanH3, have been identified in *G.vaginalis* (Janulaitiene et al., 2018; Robinson et al., 2019). Sialidase activity in *G.vaginalis* was initially thought to derive from sialidase encoding gene *nanH1* (Lopes Dos Santos Santiago et al., 2011), while a more recent study concludes that *nanH2* and *nanH3* are the primary sources of sialidase activity in *G.vaginalis* (Robinson et al., 2019). Schellenberg et al (Schellenberg et al., 2016). found that using a filter spot assay, the presence of *nanH1* was not indicator of sialidase activity: only 36 of the 77 *G.vaginalis* isolates that tested positive for *nanH1* actually produced sialidases. Meanwhile in another test done by polymerase chain reaction (PCR), sialidase activity in a collection of 34 isolated *G.vaginalis* strains was consistent with the detection of *nanH2* or *nanH3* (Robinson et al., 2019). The main functional distinction between NanH2 and NanH3 is that, NanH2 cleaves 9-O-acetylated sialic acid substrates far more efficiently than NanH3, either *in vitro* or *in vivo* (Robinson et al., 2019). In addition, *nanH3* is more commonly present than *nanH2* (Cauci et al., 2003). These results suggest that NanH2 and NanH3 are more likely to be the primary sources of sialidase activity in *G.vaginalis* in human CVF, whereas NanH1 contributes little.

Studies propose that the absence of sialidase activity by *nanH1* could be due to transcriptional regulation (Janulaitiene et al., 2018) and a lack of signal sequence, suggesting an intracellular localization of *nanH1* (Kurukulasuriya et al., 2021). However, limited evidence supports these hypotheses. Additionally, elevated *nanH1* gene levels have been found to be associated with both high-risk human papillomavirus (HPV) (Di Paola et al., 2017) and BV (Hardy et al., 2017). Thus, more research is needed to better understand the roles of the sialidase encoding genes besides sialidases expression.

3 Pathogenicity of sialidases

The host mucosal defense barrier, which is important in the identification, integration, and elimination of pathogens, can be destroyed by desialylation of glycoconjugates such as mucins, cellular receptors, and immunoglobulins, which in turn facilitates bacterial adherence, colonization, invasion, and tissue breakdown (Briselden et al., 1992; Cauci et al., 2003, 1998; Cauci and Culhane, 2011). Sialidases' participation in the pathogenesis of *G.vaginalis* and BV is discussed below (Figure 1).

3.1 Source of nutrition in bacteria

Bacteria can use free sialic acids, a hydrolysate of glycoconjugates catalyzed by sialidases, as a source of carbon for their nutrition and colonization (Figure 1A) (Lewis et al., 2013; Agarwal et al., 2020; Agarwal and Lewis, 2021). Evidence from mouse models shows that free sialic acids released by sialidases promote the growth of group B *Streptococcus* and the spread of ascending vaginal tract infections (Pezzicoli et al., 2012; Gilbert et al., 2013). Bacteria lacking sialidase encoding genes can also benefit from sialoglycan in the vagina via sialidase producers such as *G.vaginalis* (Agarwal et al., 2020). Some bacteria, such as *Fusobacterium nucleatum* (Haines-Menges et al., 2015; Agarwal et al., 2020) and group B *Streptococcus* (Pezzicoli et al., 2012), have sialic acid transport or catabolic pathways despite being sialidase-negative themselves. Moreover, *F.nucleatum* can reinforce sialidase activity produced by *G.vaginalis* in both *ex vivo* and *in vitro* coculture studies. *G.vaginalis* titers exhibit a dose-dependent increase with higher inocula of *F.nucleatum* or increasing proportions of its cell-free supernatant in an *in vitro* coculture system of *F.nucleatum* and *G.vaginalis*, in which *G.vaginalis* could not survive itself. This suggests that *F.nucleatum* may secrete factors to facilitate *G.vaginalis* growth. Additionally, in comparison to cocultures with *F.nucleatum*, monocultures of *G.vaginalis* needed at least a 20,000-fold greater inoculum to be viable after an overnight incubation (Agarwal et al., 2020). Therefore, *F.nucleatum* and *G.vaginalis* form a mutually beneficial relationship based on their glycan cross-feeding mode, which promotes their colonization and contributes to vaginal dysbiosis.

There have also been reports of the cross-feeding between commensal bacteria in the gut. For example, *Bifidobacterium breve* UCC2003, which contains a functional Nan cluster for sialic consumption, can use the sialic acid produced by *Bifidobacterium bifidum* PRL201048 (Egan et al., 2014). Similarly, in the oral cavity, *Streptococcus gordonii* employs sialic acids as their only carbon source (Byers et al., 1996). During the coinfection of influenza and *Streptococcus pneumoniae* in the respiratory tract, sialic acids produced by influenza accelerate bacterial replication *in vivo* and stimulate pneumococcal proliferation (Siegel et al., 2014).

3.2 Exposure of receptor binding sites

Sialidases can also promote infections by damaging the protective physical and biochemical barriers against pathogens through exposure of receptor binding sites for adhesins and toxins. In the oral cavity, adhesion of *S.gordonii* to oral epithelial cells is greatly increased by the presence of *Streptococcus oralis* in a sialidase-dependent manner through exposure of cryptic receptors binding sites (Beighton and Whiley, 1990; Wong et al., 2018).

Sialic acids are typically found at the terminal position of glycans. They can shield the underlying sugars (mostly galactose

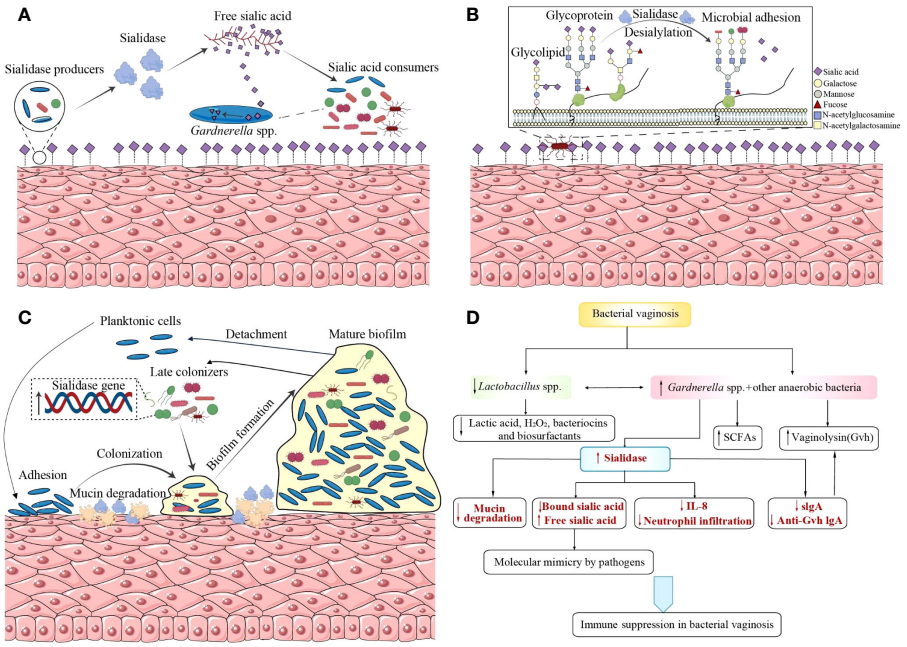

FIGURE 1
Sialidases' participation in the pathogenesis of *Gardnerella vaginalis* and bacterial vaginosis. **(A)** Sialidase producers catalyze sialic acids from glycoconjugates as nutrition source for sialic acid consumers. **(B)** Desialylation of glycoconjugates by sialidases exposes new glycan epitopes for bacterial recognition and adhesion. **(C)** *G.vaginalis* and BVAB bacteria establish synergistic interactions based on sialidases during the formation of a polymicrobial biofilm. **(D)** Sialidases participate in the immune regulation of BV, supported by other hydrolytic enzymes, virulence, and immunomodulatory metabolites. BVAB, bacterial vaginosis-associated bacteria; SCFAs, short chain fatty acids; Gvh, *Gardnerella vaginalis* hemolysin; IL, interleukin; Anti-Gvh IgA, immunoglobulin A against *Gardnerella vaginalis* hemolysin.

residues) from recognition, and then breakdown or adherence. Sialidases in the vagina may reveal glycan epitopes by the depletion of sialic acids and the exposure of underlying sugars to the surface (Figure 1B). In both *N*- and *O*-linked glycans, sialic acids cap Gal residues bound to N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc), which is not accessible on the epithelial surface unless treated with exogenous sialidases or using cells from BV-positive specimens (Agarwal et al., 2023). Desialylation of glycoconjugates by sialidases causes loss of or reveal of new glycan epitopes, affecting microbe binding and host immunological recognition (Varki and Gagneux, 2012). Bacterial adhesion occurs when terminal sugars are exposed with the degradation of glycans, in which process carbohydrate-binding proteins like lectins, previously predicted in *Gardnerella*, serve as mediums (Bonnardel et al., 2021). According to genome screening, a greater repertory of carbohydrate-binding proteins is produced by vaginal bacterial species that are linked to infection and inflammation, which may allow them to bind a greater variety of glycans in the vagina. Compared with commensals like *Lactobacillus crispatus*, the mean number of lectins per strain is approximately 2-fold higher among those regarded as potential and confirmed pathogens (including *Lactobacillus iners*, *G.vaginalis*, *Prevotella*, group B *Streptococcus*, and *Escherichia coli*) (Bonnardel et al., 2021). With the deepening of research on the surface polysaccharide structure of the vagina and the bacterial carbohydrate-binding proteins, comprehensive insights into host–microbe interactions will be reached.

3.3 Biofilms formation

A biofilm is an organized community of microorganisms encased in a extracellular matrix made of proteins, polysaccharides, and nucleic acids, that attaches to a biological surface (Flemming et al., 2016; Jung et al., 2017) and contributes to the survival of bacterial infections (Del Pozo, 2018). Vaginal biofilms contribute to the persistence and recurrence of BV, as well as antibiotic resistance (Swidsinski et al., 2008; He et al., 2021). According to a recent research, 11 of the 24 *G.vaginalis* strains were able to form biofilms, providing themselves with advantages to evade host defense mechanisms and survive against antibiotics (Ma et al., 2022). An *in vitro* study suggests that most of the BVAB have a tendency to grow biofilms, and *G.vaginalis* has greater propensity to form a biofilm, enhancing its virulence potential through increased adhesion and cytotoxicity of epithelial cells compared to other anaerobes (Alves et al., 2014).

The lifecycle of biofilm formation is considered to include several stages: (i) adhesion to a surface, (ii) production of extracellular matrix, bacterial aggregation and biofilm accumulation until the development of a mature biofilm structure, and finally (iii) detachment (Joo and Otto, 2012). The initial adherence to vaginal epithelial cells has been acknowledged to be a necessary process to elicit BV (Swidsinski et al., 2005). As a dominant component of BV biofilm, *Gardnerella* spp. replaces pre-dominant *L.crispatus*, initiate bacterial colonization on vaginal epithelium and then serve as a scaffold for the attachment of other BVAB, including *Atopobium*

vaginae (found in 80% of the samples and compromises 40% of the biofilm mass) and other heterogeneously mixed bacteria belonging to the *Bacteroides*, *Corynebacterium*, *Lactobacillus*, *Staphylococcus*, Streptococcus genera and so on (Castro et al., 2015; Castro et al., 2019; Swidsinski et al., 2005; Verstraelen and Swidsinski, 2013; Schwebke et al., 2014). The process is known as coaggregation (Figure 1C). Sialidases serve as a trigger at this initial stage of colonization. By means of its mucinase activity, the enzymes alter the characteristics of mucus discharges, catalyze them as a meal for bacteria and expose adhesion receptors on polysaccharides to promote bacterial colonization, increasing the potential for *G.vaginalis* to contact closely with the epithelium. Then early biofilm forms with the aggregation of other BVAB and the accumulation of extracellular matrix (Verstraelen and Swidsinski, 2013). Though *sialidase A* gene is not found to be associated with sialidase activity, it has been found to be associated with the presence of *G.vaginalis* biofilms, suggesting its possible contribution to biofilm formation (Hardy et al., 2017). There is still a lack of research comparing the expression levels of sialidase in biofilms and planktonic cells, which can provide us with deeper insights into the role of sialidase in biofilm formation. What's more, interactions between the microorganisms within vaginal biofilms are worth investigating as sialidase activity may be affected by those sialidase-negative bacteria. Besides the finding that *F.nucleatum* and *G.vaginalis* benefit from each other, an *in vitro* dual-species biofilm model demonstrates that other BVAB, such as *Actinomyces neuii* and *Enterococcus faecalis*, can upregulate sialidase and vaginolysin expression in *G.vaginalis* to reinforce its virulence (Castro et al., 2019).

Similar findings of the involvements of sialidases in biofilm formation also presents in infections of other systems. In the early phases of pulmonary infection, *Pseudomonas aeruginosa* and its sialidases, existing on the highly sialylated surfaces of the upper respiratory tract, can target bacterial glycoconjugates, promote cell-cell interactions, and initiate biofilm formation (Soong, 2006). Viral sialidase inhibitors have demonstrated the ability to block the process of biofilm formation in clinical *in vitro*, suggesting a potential novel pharmacological target in bacterial pneumonia prevention (Soong, 2006). In *Porphyromonas gingivalis*, the main pathogenic bacterium in chronic periodontitis, the sialidase encoding gene shows a higher expression level than that in planktonic cells (Lo et al., 2009). Sialidase-deficient strains also demonstrates less and discontinuous biofilm formation compared with wild-type *P.gingivalis* strains (Xu et al., 2017).

3.4 Immune regulation

The host-mucosa-sialidase can be regarded as a whole because sialidase functions on the mucosa. Sialidase is central to the suppression and overwhelm of host immune response. Meanwhile, it is also supported by other hydrolytic enzymes, vaginolysin, and immunomodulatory metabolites (Figure 1D) (Amabebe and Anumba, 2022). Sialylation of glycoconjugates, such as mucins, immunoglobulins (especially secretory immunoglobulin A, sIgA), and cytokines, cleave the molecules' terminal sialic acids and uncover their carbohydrate residues to all kinds of glycosidases, thus making them more susceptible to proteolytic degradation and hampering immune response against bacteria (Cauci et al., 2003; Cauci, 2004). For example, during the incubation of sIgA and BV vaginal specimens, the release of products with lower molecular weight into the extracellular environment are observed and the phenomenon can be reproduced by adding three exogenous enzymes: sialidase, β-galactosidase and hexosaminidase, which suggests the deglycosylation and proteolysis of sIgA in BV (Lewis et al., 2012).

In BV-positive women with a specific IgA immune response against *G.vaginalis* hemolysin (Gvh, vaginolysin), increased cleavage of IgA and a 5-fold higher sialidase activity is observed compared to those with a weaker IgA response (Cauci et al., 1998). Later, another study reconfirmed that elevated sialidase and prolidase levels reduce this mucosal adaptive immune response. Vaginolysin, another virulence factor of *G.vaginalis*, is a cholesterol-dependent cytolysin (CDC) which forms pores on cell membranes, free host intracellular contents and disrupts genital epithelial cells (Morrill et al., 2023). The immunosuppression allows vaginolysin to fully carry out its cytolytic action, which results in the detachment and destruction of the vaginal epithelial cells that eventually produce clue cells (Castro et al., 2019).

High sialidases and prolidases levels are also associated with elevated vaginal IL-1β, leading to tissue damage and increased susceptibility to sexually transmitted infections (STIs) (Cauci et al., 2008). Despite that IL-1β stimulates IL-8 secretion, sialidase level is also inversely correlated to vaginal IL-8 and neutrophils, which inhibits neutrophil infiltration and the proinflammatory cascade (Cauci et al., 2008). According to *in vivo* research, BVAB can evade the immune response by either secreting molecules that aid in the breakdown of IL-8 or by suppressing the generation and stability of IL-8 (Santos et al., 2018). These findings suggest that in BV-positive women, sialidases contribute to the suppression of innate mucosal immunity.

However, BVAB induced the secretion of IL-6, IL-8, G-CSF, IP-10, MIP-1β, RANTES, and Gro-α, while *lactobacilli* did not in another study that used a coculture model to characterize the response of vaginal epithelial cells to a series of vaginal bacteria, including commensal *lactobacilli* and BVAB such as *G.vaginalis*, *A.vaginae*, *Mobiluncis curtisii*, and *P.bivia* (Eade et al., 2012). The results is consistent with that *A.vaginae* induces a robust proinflammatory response by elevating transcript levels of IL-6, IL-8, and antimicrobial peptide β-defensin 4 (Libby et al., 2008). It seems that BVAB trigger mucosal innate immune response, increasing production of cytokines and defensins to eliminate pathogens. But excessive inflammatory response might lead to a disturbance of the vaginal immunological barrier and increasing susceptibility to STIs (Doerflinger et al., 2014).

Furthermore, bacterial surface sialylation may serve as an immunological mask (Ram et al., 2017; Vimr and Lichtensteiger, 2002). It has been proposed that bacteria might be passed for host cells and evade the host's immune system by incorporating the cleaved sialic acids into their cell surface structures (Varki and Gagneux, 2012). Differentiation between self-sialic-acids and close mimics is achieved through intrinsic lectins such as sialic acid-

binding immunoglobulin-like lectins (Siglecs) anchored on most immune cells (Duan and Paulson, 2020). By engaging inhibitory Siglec-5 and Siglec-9, group B *Streptococcus* can escape from host immune responses (Carlin et al., 2007). *Neisseria gonorrhoeae* transfers sialic acid residues to its surface lipooligosaccharide (LOS) to achieve molecular simulation, which contributes to its serum resistance and complement resistance in all three pathways (classical, lectin, and alternative) (Ram et al., 2017). A study reports that vaginolysin is able to release the contents of cervical epithelial cells, promote gonococcal LOS acquisition of sialic acids, and evade complement attack through increased binding of the regulatory protein factor H (Morrill et al., 2023), suggesting that sialidases and vaginolysin are both crucial in the regulation of the LOS sialylation level and its pathogenic ability. Meanwhile, another study reports that desialylation of gonococcal LOS by sialidases in women promotes increased transmission of infection to men (Ketterer et al., 2016). These findings suggest that sialylation and desialylation may to have unique functions during the invasion of pathogens.

4 Sialidase and bacterial vaginosis

4.1 Sialidase and characteristics of BV

Elevated sialidase activity has been observed in BV CVF, suggesting that sialidases could be used as a promising biomarker for BV (Briselden et al., 1992). The presence of *sialidase A* gene was detected in all 24 *G.vaginalis* samples in a recent study (Ma et al., 2022), while another study reports an association between sialidase activity in molecular-BV (community state type IV, CST IV) and changes in the bacterial components of the local microbiome, assessed by using V3–V4 16S rRNA sequencing (Ferreira et al., 2022). *Gardnerella*, *Atopobium*, and *Prevotella* were among BV-associated the genera that were more prevalent in women with high sialidase activity (Ng et al., 2021). Increased sialidase may be attributed to the higher abundance of some BVAB that can produce sialidases by themselves, such as *Prevotella* (Briselden et al., 1992). At the same time, sialidases can impair the vaginal mucosal immune system, which creates a beneficial environment for the overgrowth of BVAB over the *Lactobacillus* spp. and increases bacterial diversity (Lewis et al., 2013).

4.2 Sialidase and diagnosis of BV

As a biomarker for BV, sialidases could be used to develop new diagnostic tests as cheaper and quicker alternatives to the current standard clinical diagnostic tools. Current clinical diagnosis of BV is often based on the Nugent scoring system (Nugent et al., 1991) or the Amsel criteria (Amsel et al., 1983), both of which require microscopy and trained professionals. On the contrary, enzyme-based simple assays may be cheaper and quicker (Robinson et al., 2019; Wu et al., 2019; Cortés-Sarabia et al., 2020; Rodríguez-Nava et al., 2021; Avila-Huerta et al., 2023; Liu et al., 2023). Several new tests have been developed to detect sialidases. A comparison of their clinical diagnostic performance is shown in Table 1. The most widely used is BVBlue test, a microscopy-independent bedside test that detects sialidase activity using ≥7.8 U as the cut-off value for diagnosis of BV (Myziuk et al., 2003; Bradshaw et al., 2005; Permsak et al., 2005; Madhivanan et al., 2014; Foessleitner et al., 2021). *OSOM® BVBLUE® Test* is a commercial chromogenic test that can rapidly detect elevated vaginal fluid sialidase activity, with excellent sensitivity and specificity compared to Gram Stain, and it is widely used in many parts of the world. Similarly, a sensitive colorimetric bioactive paper that changes its color from white to dark purple in the presence of sialidases demonstrates a quick reaction time and strong storage stability (Zhang and Rochefort, 2013), though its clinical performance in BV diagnosis was not evaluated. Although sialidase activity tests are performed clinically, the results are currently only used as references and not as a diagnostic criterion.

4.2.1 PCR

The *nanH3* gene expression could be used for PCR detection of BV as its level differs in normal microbiota and BV cervicovaginal fluid samples (Novak et al., 2023). PCR detection of *nanH2* or *nanH3* has a sensitivity of 80.95% and a specificity of 78.26% in differentiating between *Lactobacillus*-dominance and BV, as determined by Nugent scoring (Robinson et al., 2019). However, the test only detects sialidase produced by *G.vaginalis*, limiting its applicability to other BV pathogens.

4.2.2 Fluorescence

Fluorescence could also be used to visualize sialic acids on cell membranes. The first test developed and adopted for BV diagnosis was turn-on tetravalent sialic acid-coated tetraphenylethene luminogen (Liu et al., 2018). Later on, a biochemiluminescent sialidase assay using a firefly luciferin derived substrate was developed, in which luciferins released by cleavage of the substrate subsequently oxidize and generate a light signal indicating relative sialidase concentration (Wu et al., 2019). More recently, a novel boron and nitrogen codoped fluorescent carbon dots (BN-CDs) was developed based on fluorescence spectrometry, in which sialidases can restore the fluorescence by interfering with the selective recognition interaction between the sialic acid and phenylboronic acid groups on the surface of BN-CDs, limiting fluorescence emission (Liu et al., 2023). The probe is comparable to Amsel criteria in its diagnosis of BV, indicating promising use for clinical diagnosis and therapy (Liu et al., 2023).

4.2.3 Immunosensing

A new microfluidic paper-based analytical tool based on a monoclonal antibody that has a high specificity for sialidase recognition for BV diagnosis was described (Avila-Huerta et al., 2023). Taking advantage of a surface coated with graphene oxide as a fluorescence quencher, they developed a Y-shaped strip, consisting of an entrance, a control, and a test zone (Avila-Huerta et al., 2023). The apparatus can achieve a prompt and sensitive response within 20 minutes for the identification of BV, making it economically accessible and convenient for large scale use (Avila-Huerta et al., 2023).

TABLE 1 Sialidase-based tests for BV and their clinical diagnostic performance.

Methods	Technique	Diagnostic Criteria	References	Sample Size	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
BVBlue test (Myziuk et al., 2003)	Chromogenic test	Sialidase activity≥7.8 U	the Nugent scoring	57	91.7%	97.8%	91.7%	97.8%
			the Amsel criteria		50.0%	100%	100%	88.2%
BVBlue test (Bradshaw et al., 2005)			the Nugent scoring	288	88%	95%	/	/
			the Amsel criteria		88%	91%	/	/
BVBlue test (Permsak et al., 2005)			the Nugent scoring	173	94%	96%	86%	98%
BVBlue test (Madhivanan et al., 2014)			the Nugent scoring	266	38%	95%	90%	54%
			the Amsel criteria	323	51%	94%	82%	78%
BVBlue test (Foessleitner et al., 2021)			the Nugent scoring	200	81%	100%	100%	98.1%
A sensitive colorimetric bioactive paper (Zhang and Rochefort, 2013)	Colorimetric biosensor	The changes of paper color from white to dark purple	/	/	/	/	/	/
PCRs of nanH2 or nanH3 (Robinson et al., 2019)	PCR	The dictation of nanH2 or nanH3 gene	the Nugent scoring	67	80.95%	78.26%	/	/
A turn-on tetravalent sialic acid-coated tetraphenylethene luminogen (TPE4S) (Liu et al., 2018)	Fluorescence response	Based on the relative fluorescence intensities (I/I_0) monitored at 510 nm of experimental groups (I) and control group (I_0) added 20 μM TPE4S, the samples are graded as normal (grade 1, $0< I/I_0 \leq 5$), sialidase weak positive (grade 2, $5< I/I_0 \leq 10$), and sialidase strong positive (grade 3, $I/I_0 > 10$).	BVBlue test	150	92.5%	91.8%	/	/
A biochemiluminescent sialidase assay (Agarwal et al., 2020)	Biochemiluminescence	A cutoff value of 400,000 relative light units when a Helios 2000 luminometer is used.	the Amsel criteria	423	95.40%	94.94%	83%	98.76%
Boron and nitrogen codoped fluorescent carbon dots (BN-CDs) (Liu et al., 2023)	Fluorescence spectrometry	Sialidase concentration>1.25 U/mL	the Amsel criteria	6	/	/	/	/
Nanophotonic sialidase immunoassay (Rodríguez-Nava et al., 2021)	Immunosensing	Sialidase concentration>25.194 ng/mL	the Amsel criteria	162	96.29%	96.29%	92.86%	98.11%

(Continued)

TABLE 1 Continued

Methods	Technique	Diagnostic Criteria	References	Sample Size	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
A novel microfluidic paper-based analytical device (Avila-Huerta et al., 2023)	Immunosensing	Sialidase concentration>25.1ng/mL	the Nugent scoring&the Amsel criteria	14	/	/	/	/

/: the data was not provided.

Another research team recently designed and manufactured a monoclonal antibody (mAb) targeted against *G.vaginalis* sialidases (Cortés-Sarabia et al., 2020). They further developed a single-step quantitative biosensing system for BV diagnosis, using graphene oxide-coated microwells and mAb-decorated quantum dots (Rodríguez-Nava et al., 2021). Sialidase activity in vaginal swab samples detected by this method has a 96.29% specificity and 96.29% sensitivity, using Amsel criteria for the identification of BV (Rodríguez-Nava et al., 2021).

4.3 Sialidase and treatment of BV

With the understanding of the molecular mechanism of sialidases and its association with the pathogenesis of BV, sialidases can be used as not only a promising diagnostic marker but also a pharmaceutical target through activity blockage using inhibitors (Keil et al., 2022). Sialidase inhibitors include transition-state analogue inhibitors, mechanism-based inhibitors, suicide substrate inhibitors, product analogue inhibitors, and natural product inhibitors (Keil et al., 2022), which can act on virus, bacteria, human and protozoa sialidases. Numerous natural compounds have been identified and examined for their ability to inhibit sialidases from human, bacteria and influenza viruses. As for bacteria sialidases, three novel compounds as potent inhibitors are isolated from *Lespedeza bicolor* and effect in a dose-dependent manner, among them the best inhibitor has an IC_{50} (represents the compound concentration that causes 50% enzyme activity loss) of 0.09 μM (Woo et al., 2011). A recent discovery is a curcumin analogue against *S.pneumoniae* Nan A, whose IC_{50} value is 0.2 ± 0.1 μM, exhibiting a 3-fold increase in inhibitory efficacy compared to curcumin (Kim et al., 2018). Natural products provide us with an alternate source for creating novel bacterial sialidases inhibitors and treating sepsis caused by bacteria infections, which are worth exploring for BV treatment. In our discussion of potential treatment options for BV, with *G.vaginalis* being the major pathogen, we will be focusing on bacterial sialidase inhibitors (Keil et al., 2022) and salic acid analogs (Agarwal and Lewis, 2021).

4.3.1 Sialidase inhibitors

Among them the most studied is the influenza virus sialidase inhibitors. Influenza sialidase (usually called neuraminidase) is required for the infection cycle to continue because it releases the freshly generated virus from the host cell, contributing to its spreading and preventing self-aggregation of the viral particles (Glanz et al., 2018). Currently, there are three antiviral drugs that target the glycoprotein neuraminidase on the surface of the influenza virus, including oseltamivir, zanamivir, and peramivir. They are essentially transition-state analogue inhibitors and work by inhibiting the neuraminidase enzyme's activity and preventing the virus from exiting the infected cells (Javanian et al., 2021).

As bacterial and viral sialidases share the same sialic acid interaction sites, the ASP boxes (Roggentin et al., 1989), influenza virus sialidase inhibitors can be used to block the bacterial sialidase active site and prevent the formation of biofilms (Hayden et al., 1999). Evidence shows that influenza virus sialidase inhibitors oseltamivir and peramivir can block *P.aeruginosa* biofilm formation in a dose-dependent manner (Soong, 2006). Similarly, the desialylation of sIgA during incubations with BV samples and can be inhibited by deoxy-dehydro-sialic acid (DDSia), a synthetic sialidase inhibitor (Lewis et al., 2012). Zanamivir impairs the virulence of the BV-associated pathogen *G.vaginalis* through a reduction of 30% in *G.vaginalis* sialidase activity and 50% in its ability to invade host cells (Govinden et al., 2018). It's interesting that the medicine for influenza treatment associates with BV. Anyway, they provide us with a new prospective to treat BV, despite neuraminidase inhibitor sensitivity varies throughout mammalian, microbial, and viral neuraminidases.

4.3.2 Sialic acid analogs

The two major forms of sialic acid in mammals, N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc), differ by a single oxygen atom, with Neu5Ac being the most prevalent form of sialic acid in mammalian cells (Schauer and Kamerling, 2018). The enzyme needed to synthesize Neu5Gc from Neu5Ac is called CMP-N-acetylneuraminic acid (CMP-NeuAc) hydroxylase, which is inactive in human, so Neu5Gc is a non-human derived sialic acid (Lewis et al., 2013). Once Neu5Ac is released by sialidases in the vagina, transport, uptaking and catabolism of them are proceeded within cells. The intracellular process is mediated by Neu5Ac lyase/aldolase and the substrates are catalyzed into N-acetylmannosamine (ManNAc) and pyruvate without accumulation (Lewis et al., 2013). An inherent biological mechanism for regulating enzyme processes is feedback inhibition through end-product inhibition of upstream enzymes. Through feedback inhibition, free Neu5Ac is a weak inhibitor of sialidases (Schauer and Kamerling, 1997). While a high-affinity transport mechanism in *G.vaginalis* has a preference for Neu5Ac, *G.vaginalis* sialidase does not appear to have strong preferences between Neu5Ac and Neu5Gc as substrates (Byers et al., 1999). This means that the uptake and breakdown of sialic acids are

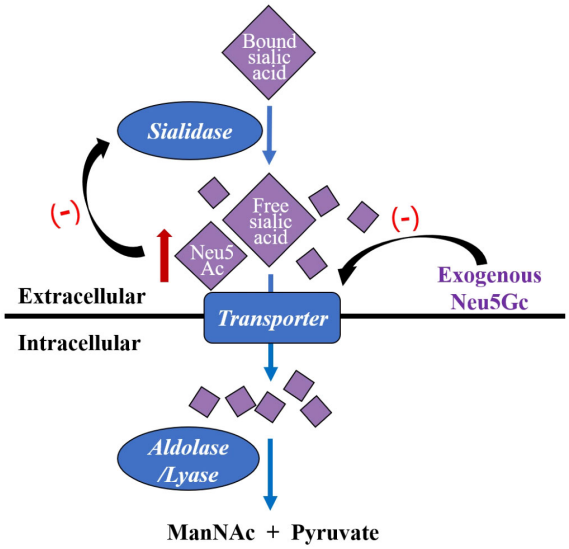

FIGURE 2
Gardnerella vaginalis captures free Neu5Ac hydrolyzed by sialidases, pumps them into the cell by a transporter, and then catalyze them into ManNac and pyruvate by intracellular aldolase/lyase. Exogenous Neu5Gc is a kind of sialic acid analogues, which inhibits *G.vaginalis* transporter and results in extracellular Neu5Ac accumulation. Neu5Ac is a weaker inhibitor of sialidases base on feedback mechanism. Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; ManNac, *N*-acetylmannosamine.

substrate-dependent and occur far more slowly and incompletely, when there is a substantial concentration of Neu5Gc (Figure 2). Later a study confirmed that *G.vaginalis* could liberate free Neu5Ac from IgA but fails to consume them with the presence of Neu5Gc, which further indicates Neu5Gc's potential as an inhibitor to reduce Neu5Ac transport into *G.vaginalis* (Lewis et al., 2013). These findings are consistent with a prior discovery that in the bacterium *S.oralis*, Neu5Gc inhibits the uptake of Neu5Ac (Byers et al., 1999). Despite that Neu5Gc shows sialidase inhibitory activity, its effects for anti-BV are not verified and its effectiveness and safety still need experimental verification.

5 Conclusion

This study explores the role of sialidases in vaginal dysbiosis, pathogenesis of BV, and promising diagnostic and treatment options for BV. Although the composition and dynamics of the human vaginal microbiome are being studied more and more, we still know little about the mechanisms underlying the development of vaginal dysbiosis and the critical factors that influence it. As a main virulence factor of *Gardnerella* spp. and an important glycoside hydrolase enzyme, sialidases cleave sialic acid from terminal glycans, also known as desialylation. The process facilitates the destruction of mucosal defense barrier, as well as bacterial adhesion, colonization, and invasion into the vaginal epithelia through provision of nutrient sources, exposure of receptor binding sites, biofilms formation, and immunity regulation. However, not all *G.vaginalis* strains can produce sialidases and the contribution of sialidases to BV is just part of the pathogenesis of *G.vaginalis*. There are still other BVAB, virus, and even the human body itself can produce sialidases. Moreover, the use of sialidases as a biomarker for predicting treatment outcomes and the prognosis of BV still needs to be tested in clinical studies. Future research should focus on understanding the pathogenesis of sialidases produced by different strains of *G.vaginalis* and other sources, as well as the association between sialidases and the persistence and recurrence of BV, to provide new insights to improve diagnosis and treatment of BV.

Author contributions

LC: Writing – original draft, Writing – review & editing. JL: Writing – review & editing. BX: Conceptualization, Writing – review & editing, Funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Synergistic *in vitro* activity and mechanism of KBN lotion and miconazole nitrate against drug-resistant *Candida albicans* biofilms

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Background: In the face of increasing antifungal resistance among *Candida albicans* biofilms, this study explores the efficacy of a combined treatment using Kangbainian lotion (KBN) and miconazole nitrate (MN) to address this challenge.

Methods: Using UPLC-Q-TOF/MS Analysis for Identification of Active Compounds in KBN Lotion; FICI for synergy evaluation, XTT and ROS assays for biofilm viability and oxidative stress, fluorescence and confocal laser scanning microscopy (CLSM) for structural and viability analysis, and real-time fluorescence for gene expression.

Conclusion: Our study indicates that the combined application of KBN and MN somewhat impacts the structural integrity of *Candida albicans* biofilms and affects the expression of several key genes involved in biofilm formation, including *ALS1*, *ALS3*, *HWP1*, *HSP90*, and *CSH1*. These preliminary findings suggest that there may be a synergistic effect between KBN and MN, potentially influencing not only the structural aspects of fungal biofilms but also involving the modulation of genetic pathways during their formation.

KEYWORDS

Candida albicans, biofilms, KBN, miconazole nitrate, synergistic

1 Introduction

Vaginal vulvovaginal candidiasis (VVC) is a common and complex vaginal infection caused by fungi of the *Candida* genus, with symptoms influenced by host physiology, fungal biology, and immune responses, affecting the health and quality of life of millions of women worldwide. Among these, *C. albicans* is the primary pathogen of VVC.

It invades the host and contributes to disease progression through various virulence factors (Tamura et al., 2007), such as adherence ability, biofilm formation, production of extracellular hydrolytic enzymes, hyphal morphogenesis, and phenotypic switching (Pereira et al., 2021).

The formation of biofilms is one of the major virulence factors of *C. albicans*. Unlike planktonic cells, *C. albicans* biofilms exhibit unique phenotypic characteristics, including significantly increased resistance to antifungal drugs, host defense mechanisms, and physical and chemical stress (Donlan and Costerton, 2002). The colonization and biofilm formation of *C. albicans* on vaginal mucosa is a key reason why conventional antifungal treatments are becoming increasingly difficult. These biofilms show growing resistance to widely used antifungal drugs, such as amphotericin B (Fernandes et al., 2015) and fluconazole (Taff et al., 2013), which may prevent the complete eradication of the pathogen from the vagina. This can lead to frequent recurrences of VVC and further progression of the disease.

In recent years, studies have shown that natural plants and their compounds, such as phenols, essential oils, terpenes, lectins, and alkaloids (Bersan et al., 2014; Fabri et al., 2021), may inhibit biofilm formation and disrupt mature biofilm structures of *C. albicans* by downregulating the expression of biofilm-related genes (Rodrigues de Araújo et al., 2019). Various natural compounds have also been found to enhance the antifungal effects of azole drugs. When fluconazole is combined with eugenol or cinnamaldehyde, its activity against *C. albicans* biofilms is enhanced, demonstrating synergistic effects (Khan and Ahmad, 2012; Pemmaraju et al., 2013). Further research has shown that thymol and menthol, when used in combination with fluconazole, exhibit synergistic inhibitory effects. These lipophilic compounds can penetrate cell membranes and disrupt the ergosterol biosynthesis pathway (Ahmad et al., 2011). Berberine has been found to exhibit a synergistic effect with miconazole against *C. albicans* biofilms (Wei et al., 2011). *Park* et al. further demonstrated that berberine enhances cell membrane permeability by inhibiting sterol 24-methyltransferase, a key enzyme in ergosterol biosynthesis (Park et al., 1999). Additionally, berberine can activate the Krebs cycle and inhibit ATP synthase activity, ultimately leading to oxidative cell damage through increased production of reactive oxygen species (ROS) (Ghannoum and Rice, 1999; Xu et al., 2009; Shi et al., 2010). *De Cremer* et al. screened a library of repurposed compounds to identify molecules that could enhance the effects of miconazole nitrate (MN), discovering three compounds—hexachlorophene, pyrvinium pamoate, and artesunate—that show synergistic effects with MN in treating mature *C. albicans* biofilms (De Cremer et al., 2015).

The plant-based preparation Kang Bai Nian (KBN) lotion has been shown to effectively inhibit the growth of susceptible *C. albicans in vitro*. Electron microscopy examinations have revealed that KBN can disrupt the organelles, cell membrane, and cell wall of susceptible *C. albicans* cells (Huang et al., 2020). In efficacy tests using a mouse model of VVC, KBN lotion was found to reduce the vaginal *C. albicans* load and inhibit hyphal growth (Chen et al., 2021). MN is a widely used clinical treatment for VVC, but the issue of increasing resistance during treatment is becoming more serious. Whether the combined use of MN and KBN lotion can combat resistant *C. albicans* by inhibiting the biofilm virulence factor warrants further in-depth research.

In this study, we focus on exploring the efficacy of KBN in combination with MN in inhibiting the biofilms of *C. albicans*. Through *in vitro* experiments and RT-PCR tests, we aim to preliminarily validate the potential mechanisms targeting biofilm-inhibiting genes, providing a foundational experimental basis for the future clinical use of KBN combined with MN in treating fungal infections.

2 Materials and methods

2.1 Preparation of KBN lotion

Take 75 g of Coptidis Rhizoma (Huanglian), 37.5 g of Herba Taraxaci (Sanbaicao), 20 g of Folium Isatidis (Daqingye), 20 g of Flos Lonicerae (Jiguanhua), 12.5 g of Herba Moslae (Xiangru), 12.5 g of Sophorae Flavescentis Radix (Kushen), 12.5 g of Radix Scutellariae (Baibu), 10 g of Radix Gentianae (Longdan), 5 g of Caryophylli Flos (Dingxiang), and 1 g of Borneolum Syntheticum (Bingpian), totaling 206 g. Except for Borneol, each herb was refluxed with 6 to 4 times the amount of 80% ethanol for 1 hour each time. The extract was filtered through a 200-mesh sieve and the filtrate was combined. Concentrate under reduced pressure to obtain a concentration of 1.0 g crude drug/mL. Dissolve 1 g of Borneo in a small amount of ethanol, then add it to the mixture and mix thoroughly to obtain KBN lotion. Borneol was purchased from Yunnan Linyuan Spice Co., Ltd. (Yunnan, China), the rest of the botanical drugs were purchased from Guangzhou Zhixin Chinese Medicine Pieces Co., Ltd. (Guangzhou, China) (Figure 1).

2.2 UPLC-Q-TOF/MS

Weigh 0.50 g of KBN lotion and ultrasonically extract it in 10 ml of 50% methanol-water for 1 hour. Subsequently, filter out the residue. Centrifuge the extract at 4°C and 12,000 rpm for 15 minutes, then filter the supernatant through a 0.22 μm membrane. Dilute the filtrate 5 times before performing UPLC-Q-TOF/MS (SCIEX TripleTOF® 6600+ LC-MS/MS, USA) analysis. The composition of the two mobile phases was 0.1% (v/v) formic acid in water (A) and acetonitrile (B): 0–15 min, 5%–35% B; 15–20 min, 35%–90% B; 20–28 min, 90%–5% B; 28–34 min, 5% B. The separations were performed with a constant flow rate of 0.3 ml/min. Scanning Mode: +ESI and -ESI; Acquisition Range: Mass 50-1500 M/Z. Capillary Voltage ISVF: 5500V; Cone Voltage CE: ± 35V; Ion Source Temperature: 550°C. Cone Gas Flow: 50 L/H. Injection Volume: 1 μL.

2.3 Preparation of activated strains and fungal suspensions of *C. albicans*

Inoculate white *C. albicans* on Sabouraud Dextrose Agar medium (SDA) and incubate in a constant temperature incubator

FIGURE 1
The voucher specimens of the plants used in the KBN lotion.

at 35°C for 24 hours. Pick a single colony (with a diameter greater than 1mm) and inoculate it onto Yeast Extract Peptone Dextrose medium (YEPD). Adjust the yeast concentration using a McFarland turbidity tube (Huankai, China), then incubate on a constant temperature shaker at 30°C with shaking at 200 rpm for 16 hours to reach the logarithmic growth phase. Use a hemocytometer for counting and adjust the concentration of the bacterial suspension to $1*10^3$CFU/ml with RPMI1640 liquid medium(Gibco, USA). In this study, the *C. albicans* quality control strain CMSS(F)98001 was obtained from the National Institutes for Food and Drug Control (NIFDC) of China. Additionally, the clinically isolated drug-resistant strains *C. albicans 901*, *904*, *953*, and *311* were generously provided by Professor Yuanying Jiang from the Second Military Medical University.

2.4 Minimum inhibitory concentration test

The antifungal susceptibility test for KBN lotion against *C. albicans* was conducted using the microbroth dilution method according to the M27-A3 standard protocol recommended by the Clinical and Laboratory Standards Institute (CLSI) of the United States ((CLSI), C. a. L. S. I, 2008). A sterile 96-well cell culture plate was used for the assay (Figure 2). In each row, 100 μL of blank RPMI 1640 liquid medium was added to the first well to serve as a blank control. Wells 3 to 12 in each row were inoculated with 100 μL of a fungal suspension. The second well in each row received a mixture of x μL of the detergent stock solution and (200-x) μL of the *C. albicans* suspension. A two-fold serial dilution was performed from the second to the eleventh well in each row using a multichannel pipette, and then 100 μL of liquid was removed from the eleventh well. This procedure was repeated three times. The 96-well plate was incubated at 37°C for 48 hours in a constant temperature incubator. Optical density (OD) values at 630 nm were measured using a microplate reader (BioTek, Winooski, Vermont, USA).

2.5 Fractional inhibitory concentration index

To prepare the experimental setup, a sterile 96-well cell culture plate was used (Figure 2). The first well of each row was filled with 100 μL of RPMI 1640 liquid medium to serve as a blank control. In well A2, 2x μL of KBN lotion was combined with (200-2x) μL of the fungal suspension. For wells A3 to A11, (200-x) μL of the fungal suspension was mixed with x μL of KBN lotion. The remaining wells were inoculated with 100 μL of the fungal suspension. A two-fold serial dilution was performed from wells A2 to A11 down to wells F2 to F11 using a pipette, and 100 μL of liquid was removed from wells F2 to F11. In wells A2 to G2, y μL of miconazole nitrate solution was added along with (100-y) μL of the fungal suspension. In well H2, z μL of KBN lotion was added along with (100-z) μL of the fungal suspension. Another two-fold serial dilution was performed from well 2 to well 11 in each row, with 100 μL of liquid removed from well 11 in each row. The susceptibility plate was incubated at 37°C for 48 hours, and the results were interpreted as previously described. The experiment was repeated three times. The evaluation of drug combination effects was conducted using the Fractional Inhibitory Concentration Index (FICI) model.

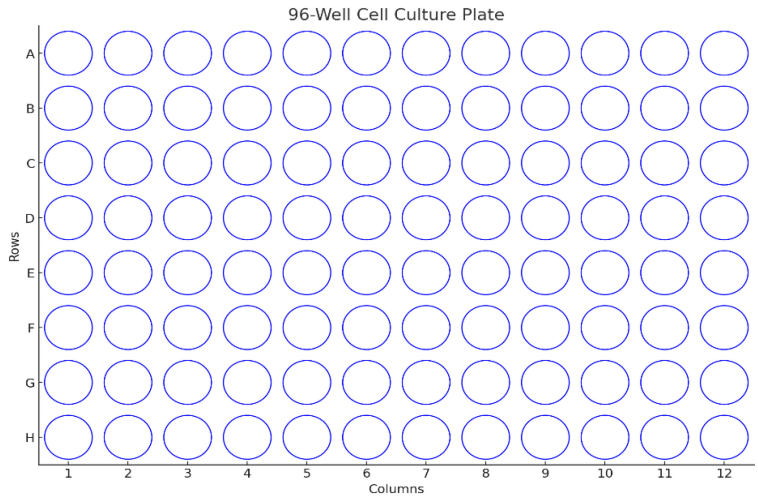

FIGURE 2
The schematic diagram of a 96-well cell culture plate.

2.6 XTT assay

Add 100 μL of fungal suspension to a sterile 96-well cell culture plate and incubate at 37°C for 1.5 hours (for early biofilm formation) or 24 hours (for mature biofilm) to allow *C. albicans* to adhere. After adhesion, aspirate the medium and rinse twice with PBS buffer. Then, add 100 μL of the following treatments: KBN 6.25 mg/mL, KBN 0.78 mg/mL + MN 0.25 μg/mL, KBN 0.39 mg/mL + MN 0.25 μg/mL, KBN 0.19 mg/mL + MN 0.25 μg/mL, MN 4 μg/mL, and a blank control of RPMI 1640 liquid medium. Incubate statically in a constant temperature incubator at 37°C for 24 hours. Afterwards, aspirate the supernatant and rinse twice with PBS buffer. In the dark, add 200 μL of XTT/menadione solution (sigma, USA) to each well, cover with aluminum foil to protect from light, and incubate statically in a constant temperature incubator at 37°C for 1 hour. Then, transfer 100 μL of the supernatant to a new sterile 96-well cell culture plate and measure the OD value at 492 nm to calculate the biofilm formation inhibition rate (Koban et al., 2012).

2.7 ROS assay

Quantitative determination of intracellular reactive oxygen species levels using the oxidation-sensitive DCFH-DA (sigma, USA) dye. Add 100 μL of fungal suspension to a sterile black 96-well cell culture plate and incubate at 37°C for 1.5 hours (for early biofilm formation), 24 hours (for mature biofilm formation), and 48 hours (for late-stage mature biofilm formation) to allow *C. albicans* to adhere. After adhesion, aspirate the culture medium and rinse twice with sterile PBS buffer. Then, add 100 μL of the following treatments: KBN at 6.25 mg/mL, KBN at 0.78 mg/mL + MN at 0.25 μg/mL, KBN at 0.39 mg/mL + MN at 0.25 μg/mL, KBN at 0.19 mg/mL + MN at 0.25 μg/mL, MN at 4 μg/mL, and a blank control of RPMI 1640 medium. Incubate statically at 37°C for 24 hours, then add DCFH-DA dye (10 μM, 30 μL) and incubate in the dark at 37°C for 40 minutes. Remove the fungal suspension and wash twice with sterile PBS buffer to remove any DCFH-DA dye not internalized by the cells, then re-add 200 μL of sterile RPMI 1640 liquid medium. Finally, measure fluorescence intensity under ex-citation at 488 nm and emission at 522 nm.

2.8 Fluorescence microscopy

Sterile 1 cm × 1 cm cover slips are soaked overnight in fetal bovine serum and prepared for use. Place the cover slips in a sterile 24-well cell culture plate, adding 1 mL of fungal suspension to each well, and incubate at 37°C for 1.5 hours, 24 hours, and 48 hours to form early, mature, and late-stage biofilms of *C. albicans*, respectively. Wash three times with sterile PBS to remove planktonic cells. For the experimental groups, add 0.5 mL of the following treatments to each well: KBN 6.25 mg/mL, KBN 0.78 mg/mL + MN 0.25 μg/mL, KBN 0.39 mg/mL + MN 0.25 μg/mL, KBN 0.19 mg/mL + MN 0.25 μg/mL, and MN 4 μg/mL. The blank control group receives 0.5 mL of blank RPMI 1640 liquid medium. Continue to incubate at 37°C for 24 hours, then wash three times with sterile PBS to remove planktonic cells. Add 20 μL of FITC-conA (sigma, USA) to each well and stain in the dark at room temperature for 1 hour, followed by three washes with cold PBS. Observe under a fluorescence microscope(Leica DM2500 LED Optical microscope, Germany).

2.9 CLSM

Use sterile cell culture dishes, adding 2 mL of fungal suspension to each dish, and incubate at 37°C for 1.5 hours, 24 hours, and 48 hours to form early-stage and mature biofilms of *C. albicans*, respectively. Wash three times with cold sterile PBS to remove planktonic cells. To each dish, add 2 mL of the following treatments: KBN 6.25 mg/mL, KBN 0.78 mg/mL + MN 0.25 μg/mL, KBN 0.39 mg/mL + MN 0.25 μg/mL, KBN 0.19 mg/mL + MN 0.25 μg/mL,

and MN 4 μg/mL. The blank control group receives 2 mL of blank RPMI 1640 liquid medium. Continue incubating at 37°C for 24 hours, then wash three times with sterile PBS to remove planktonic cells. Following the instructions of the SYTO 9/PI Live/Dead Bacterial Double Stain Kit(MK, shanghai, China), add 1.5 μL of SYTO-9 and 1.5 μL of PI to each culture dish (Pakkulnan et al., 2019; Yu et al., 2022). Incubate in the dark at room temperature for 30 minutes and observe by CLSM (TCS SP8, Leica, Germany).

2.10 RT-PCR

Use a sterile, enzyme-free 6-well cell culture plate, adding 2 mL of fungal suspension to each well, and incubate at 37°C for 1.5 hours, 24 hours, and 48 hours to form early-stage and mature biofilms of *C. albicans*, respectively. Wash three times with cold sterile PBS to remove planktonic cells. To each well, add 2 mL of the following treatments: KBN 6.25 mg/mL, KBN 0.78 mg/mL + MN 0.25 μg/mL, KBN 0.39 mg/mL + MN 0.25 μg/mL, KBN 0.19 mg/mL + MN 0.25 μg/mL, and MN 4 μg/mL. The blank control group receives 2 mL of blank RPMI 1640 liquid medium. After continuing the incubation for 24 hours, use a sterile, enzyme-free pipette tip to aspirate the culture medium, then scrape off the biofilms adhered to the wells with a biofilm scraper, collect the scraped cells, centrifuge to remove the supernatant, wash once with DEPC water, centrifuge again and discard the supernatant for later use. Total RNA of each group was extracted by Trizol method. The RNA sample was reverse transcribed into cDNA according to the instructions of PrimeScript™ One Step RT-PCR Kit Ver.2 (Takara Bio Inc.). For the design of primers, refer to Table 1.

2.11 Statistical analysis

RT-PCR data were expressed by ¯x± S, and statistical analysis was performed using SPSS 21.0. The data were first tested for normal distribution. If they conformed to a normal distribution, a homogeneity of variance test was performed. When variances were homogeneous and sample sizes were equal across groups, pairwise comparisons were conducted using Tukey's test in one-way ANOVA. When variances were homogeneous but sample sizes were unequal, Scheffé's test was used for pairwise comparisons. If variances were not homogeneous, Dunnett's T3 test in one-way ANOVA was employed for pairwise group comparisons. A significance level of $P<0.05$ was considered indicative of statistical differences. Statistical analyses were conducted using GraphPad Prism 8.0. All experiments were triplicated and repeated three times on different days.

TABLE 1 Specific primer sequence and PCR product length.

Target gene	Target gene sequence(5′-3′)	Product
ALS1 F	GTGGATCTGTTACTGGTGGAGC	154bp
ALS1 R	ATGAATGTGTTGGTTGAAGGTGA	
ALS3 F	GAGTGGAAGCAGCTGTGGAAG	140bp
ALS3 R	TGTTCCAACAACTGAAAGTGAGG	
HWP1 F	TTTCTACTGCTCCAGCCACTG	118bp
HWP1 R	ACTTCAGATTCGGTACAAGAGCT	
HSP90 F	GACCGTTAAGGACTTGACCACT	116bp
HSP90 R	ATCCCAAGGCAATCAATCTGT	
CSH1 F	GGTTCCGTACTTTCGATACTGCT	154bp
CSH1 R	GAACTGTCTTCTGCGTCGTCT	
18S RNA-F	ATTGCGATAACGAACGAGACC	110bp
18S RNA-R	TGCCTCAAACTTCCATCGACT	

3 Results

2.1 UPLC-Q-TOF/MS

The active compounds in KBN lotion were identified using UPLC-Q-TOF/MS analysis, which also provided the total ion chromatograms (Figures 3A, B). Information on the chemical constituents of KBN lotion was collected and organized by searching databases such as CNKI, Medline, PubMed, and NIST. A total of 44 compounds were identified, including 16 alkaloids, 13 flavonoids, 4 phenols, 2 essential oils, 2 caffeoylquinic acids, 1 organic acid, 1 lignan, 1 tannin, 1 nucleosides, and 1 sugar (Table 2). According to the results of UPLC/Q-TOF MS, the main components of KBN lotion are alkaloids and flavonoids, many of which exhibit antibacterial activity.

2.2 MICs of KBN and MN against *C. albicans*

As shown in Table 3, the results of the *in vitro* antifungal drug susceptibility test show that the MICs of Fluconazole (FLC), Ketoconazole (KET), and MN against the quality control sensitive *C. albicans* 98001 are 1 μg/mL, <0.015 μg/mL, and <0.12 μg/mL, respectively. Against resistant *C. albicans* strains (901, 904, 311), the MICs are >64 μg/mL, >8 μg/mL, and 2 μg/mL or 1 μg/mL, respectively (Table 3). The results suggest that the resistant *C. albicans* strains (901, 904, 311) are resistant to Fluconazole and Ketoconazole, consistent with the standards recommended by the Clinical and Laboratory Standards Institute (CLSI) in the M27-A3 protocol.

The *in vitro* antifungal drug susceptibility test results indicate that, for both sensitive and resistant *C. albicans*, the MICs of the KBN are consistently 6.25 mg/mL. This indicates that KBN has the same antifungal activity against both sensitive and resistant *C. albicans*.

2.3 Inhibitory effect of KBN combined with MN on *C. albicans*

The MIC values of KBN for *C. albicans* strains *901*, *904*, *953*, and *311* are 6.25 mg/mL, and the MIC values for MN against these

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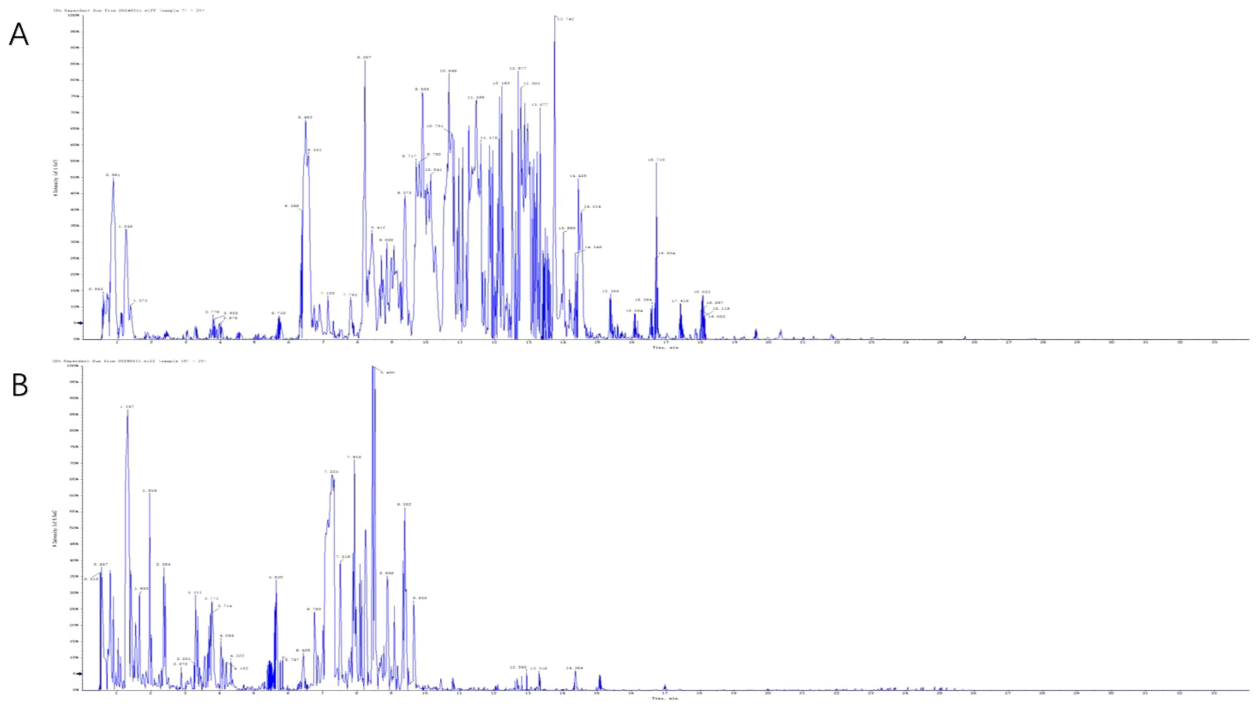

FIGURE 3
The total ion chromatogram obtained in the positive **(A)** and negative **(B)** modes.

TABLE 2 Components of KBN lotion.

No	RT (min)	Extracting ions	Measured mass (m/z)	Formula	Identity	Error (ppm)	Main secondary fragment ions (MS/MS)	Category	Cas no
1	0.6241333	[M-H]-	341.108	C12H22O11	Sucrose	-2.8	179,89,119	Sugar	57-50-1
2	0.7882167	[M+H]+	265.1921	C15H24N2O2	13a-Hydroxylupanin	3.8	148,150,247	Alkaloids	15358-48-2
3	0.9240333	[M+H]+	247.1801	C15H22N2O	Sophocarpine	-1.6	245,179,136	Alkaloids	6483-15-4
4	1.018617	[M-H]-	169.0095	C7H6O5	Gallic acid	-26.6	125	Tannins	149-91-7
5	1.132767	[M-H]-	133.011	C4H6O5	Malic acid	-22.6	115,71	Organic acids	6915-15-7
6	1.17335	[M+H]+	249.1974	C15H24N2O	Matrine	5.2	148,150	Alkaloids	519-02-8
7	1.264683	[M+H]+	265.1938	C15H24N2O2	Oxymatrine	14.3	247,205,148,136	Alkaloids	16837-52-8
8	1.4005	[M+H]+	268.1068	C10H13N5O4	Adenosine	10.4	136,119	Nucleosides	58-61-7
9	1.430583	[M-H]-	151.0361	C8H8O3	4-hydroxyphenylacetic acid	-20.5	123,107	Phenols	156-38-7
10	2.37285	[M-H]-	153.0151	C7H6O4	Protocatechuic acid	-27.6	109,108	Phenols	99-50-3
11c	3.809183	[M+H]+	377.1431	C16H24O10	loganic acid	8.2	213,169	Terpenes	22255-40-9
12a	4.73045/ 14.24/ 17.023	[M+H]+	165.0929	C10H12O2	Isoeugenol	11.5	91,77	Essential oils	97-54-1
13	5.308917	[M+H]+	338.1981	C18H27NO5	platyphylline	-5.6	236,264	Alkaloids	480-78-4

(Continued)

TABLE 2 Continued

No	RT (min)	Extracting ions	Measured mass (m/z)	Formula	Identity	Error (ppm)	Main secondary fragment ions (MS/MS)	Category	Cas no
14a	5.707/ 6.88765	[M+H]+	355.1013	C16H18O9	Chlorogenic Acid	-3.0	235,205,319	Caffeoylquinic Acids	327-97-9
15ac	5.716383/ 6.89	[M+H]+	355.1042	C16H18O9	chlorogenic acid	3.2	235,205,259,319	Caffeoylquinic Acids	327-97-9
16	6.530334	[M+H]+	342.1727	C20H23NO4	Isocorydine	7.9	279,265,237	Alkaloids	475-67-2
17	6.858217	[M-H]-	137.0204	C7H6O3	Salicylic acid	-29.3	93	Phenols	69-72-7
18	7.454433	[M+H]+	195.0656	C10H10O4	Vanillin acetate	2.1	77,91,103	Essential oils	881-68-5
19	7.800583	[M+Na]+	379.1007	C16H20O9	Gentiopicrin	1.8	158,200,217	Terpenes	20831-76-9
20	7.91575	[M+Na]+	349.1379	C20H22O4	Licarin A	-6.0	137,272,314	Lignans	51020-86-1
21	8.696016	[M+H]+	625.1718	C28H32O16	Isorhamnetin 3-glucoside-7-rhamnoside	2.9	343,445,463,313	Flavonoids	17331-71-4
22	8.90575	[M-H]-	359.0692	C18H16O8	Rosmarinic acid	-22.4	161,197,179	Phenols	20283-92-5
23	9.063	[M+H]+	340.1571	C20H21NO4	Papaverine	8.1	324,309	Alkaloids	58-74-2
24	9.154333	[M+H]+	187.0883	C11H10N2O	Deoxyvasicinone	9.1	187,118,120	Alkaloids	530-53-0
25	9.40765	[M+NH4]+	372.1823	C21H22O5	Xanthohumol	4.7	222,189,161	Flavonoids	6754-58-1
26	9.545466	[M+H]+	287.056	C15H10O6	Kaempferol	3.5	153,165,213	Flavonoids	520-18-3
27	9.6158	[M+H]+	285.0789	C16H12O5	Calycosin	-3.8	270,168,140	Flavonoids	20575-57-9
28	9.639133	[M]+	337.1278	C20H18NO4	Berberine	-9.1	321,292,278	Alkaloids	2086-83-1
29	10.0696	[M+H]+	324.1256	C19H17NO4	Stylopine	7.9	309,294,266	Alkaloids	84-39-9
30a	10.086/ 16.7185	[M+H]+	352.1183	C20H17NO5	Oxoglaucine	0.8	322,337,294,308	Alkaloids	5574-24-3
31a	10.0921/ 16.707	[M+H]+	352.1199	C20H17NO5	Oxyberberine	5.5	336,308,322,294	Alkaloids	19716-60-0
32	10.15977	[M+H]+	338.1374	C20H19NO4	Dihydroberberine	-3.8	322,307,306	Alkaloids	483-15-8
33	10.15977	[M+Na]+	397.1128	C16H22O10	Swertiamarin	7.1	301,235,205	Alkaloids	17388-39-5
34	10.18243	[M+H]+	354.1724	C21H23NO4	Dihydropalmatine	6.7	338,323	Alkaloids	26067-60-7
35	10.48223	[M+H]+	303.0499	C15H10O7	Quercetin	-0.1	229,153	Flavonoids	117-39-5
36	10.90972	[M+H]+	317.0668	C16H12O7	Isorhamnetin	3.8	302,153,170	Flavonoids	480-19-3
37	14.0955	[M+H]+	285.0705	C16H12O5	Maackiain	1.7	270,168	Flavonoids	2035-15-6
38	14.68963	[M+H]+	269.0817	C16H12O4	Formononetine	-1.4	197,253,213,237	Flavonoids	485-72-3
39	15.38308	[M+H]+	285.0786	C16H12O5	Wogonin	10.0	168,140	Flavonoids	632-85-9
40	16.5545	[M+H]+	263.0821	C16H10N2O2	Indirubin	0.4	235,219,206,132	Alkaloids	479-41-4
41	16.7185	[M+H]+	455.2128	C26H30O7	Kushenol I	6.2	179,303,153, 285,313	Flavonoids	99119-69-4
42	17.023333	[M+H]+	165.0913	C10H12O2	Eugenol	1.8	109,124,137,81	Flavonoids	97-53-0
43	18.98185	[M+H]+	453.225	C27H32O6	2'-O-methyl-Kurarinone	11.0	179,329,303	Flavonoids	270249-38-2
44	21.89415	[M+H]+	439.209	C26H30O6	Kurarinone	-2.3	179,303	Flavonoids	34981-26-5

TABLE 3 The MIC of KBN lotion and MN against *C. albicans*.

C. albicans Strains	KBN(mg/mL)	MN(μg/ml)	KET(μg/ml)	FLC(μg/ml)
98001	6.25	<0.12	<0.015	1
901	6.25	2	>8	>64
904	6.25	2	>8	>64
953	6.25	2	>8	>64
311	6.25	2	>8	>64

strains are 2 μg/mL. When KBN concentrate and MN are used in combination against the drug-resistant strains *901*, *904*, *953*, and *311*, the FICI values are all 0.2498, indicating a synergistic interaction (Table 4). This suggests that the combination of KBN and MN is effective in restoring the sensitivity of drug-resistant strains. The experiment found that a low dose of KBN could restore the sensitivity of drug-resistant strains to MN, with significant and stable synergistic effects observed even at concentrations below 0.39 mg/mL (Table 4).

The evaluation method for drug combination effects employs the Fractional Inhibitory Concentration Index (FICI) model. This model is based on the Loewe Additivity (LA) theory. The LA theory is predicated on the assumption that a drug does not interact with itself and requires that equivalent therapeutic effects are achieved for comparison purposes. Specifically, the FICI model compares the concentration of drugs used in combination to those used individually, as follows:

$$FICI = FICI_A + FICI_B$$

$$= (MIC\ A_{comb})/(MIC\ A_{alone}) + (MIC\ B_{comb})/(MIC\ B_{alone})$$

When FICI ≤ 0.5, it indicates a synergistic effect; when 0.5 < FICI ≤ 1, it indicates an additive effect; when 1 < FICI ≤ 4, it indicates no interaction; and when FICI > 4, it indicates an antagonistic effect.

2.4 The XTT assay evaluates the inhibitory effect on biofilms of the combination of KBN and MN

The XTT reduction assay is used to quantitatively determine the effect of the combination of KBN and MN on the biofilms and planktonic cells of *C. albicans*. The inhibitory effect on biofilms is calculated as a percentage of the metabolic activity compared to the blank control group. Three combined concentrations, as well as the MIC concentrations of KBN and MN, significantly reduce the metabolic activity of *C. albicans*. The concentration of KBN at 0.78 mg/mL combined with MN at 0.25 μg/mL demonstrates a significant and consistent inhibitory effect on both early and mature biofilms (Figure 4, Tables 5, 6).

2.5 Evaluation of the impact of combined use of KBN lotion and MN on *C. albicans* biofilms at early and mature stages by ROS assay

Utilizing DCFH-DA as a probe, the study assessed the accumulation of reactive oxygen species (ROS) in *C. albicans* biofilms at both early and mature stages across varying treatment concentrations. Observations revealed that for both initial and mature biofilm stages, fluorescence intensity significantly increased across all five treatment concentrations compared to the control group, indicating marked intracellular ROS production and accumulation. The combination therapy of KBN and MN, across high, medium, and low dosage tiers, matched the fluorescence intensities observed at the MIC levels of KBN and MN when used independently. Notably, the combined dosage of KBN at 0.78 mg/mL and MN at 0.25 μg/mL elicited the most pronounced increase in fluorescence intensity for both biofilm stages. This indicates that this specific concentration of combined therapy significantly enhanced ROS accumulation, inducing oxidative stress and effectively promoting fungal cell death (Figure 5, Tables 7–9)

TABLE 4 The MIC values and FICI values of KBN and MN against *C. albicans*.

C. albicans Strains	KBN(mg/mL)	MN (μg/ml)	combined use		FICI
			KBN	MN	
901	6.25	2	0.78	0.25	0.2498
904	6.25	2	0.78	0.25	0.2498
953	6.25	2	0.78	0.25	0.2498
311	6.25	2	0.78	0.25	0.2498

*When FICI ≤ 0.5, it indicates a synergistic effect; when 0.5 < FICI ≤ 1, it indicates an additive effect; when 1 < FICI ≤ 4, it indicates no interaction; and when FICI > 4, it indicates an antagonistic effect.

TABLE 5 The impact of KBN lotion in combination with MN on early biofilms (1.5 hours).

Groups / Biofilm Inhibition %	901	904	953	311
KBN 12.5mg/mL	58.88 5.14	67.42 3.87	65.25 4.51	81.97 1.70
KBN 6.25mg/mL	50.70 8.93	57.30 3.73	65.25 2.52	84.86 1.92
KBN 0.78mg/mL+ MN 0.25μg/mL	56.78 7.39	68.66 3.09	64.77 4.32	83.10 3.50
KBN 0.39mg/mL+ MN 0.25μg/mL	43.69 5.50	70.16 3.26	66.58 4.53	82.25 1.34
KBN 0.19mg/mL+ MN 0.25μg/mL	35.28 6.11	72.16 3.40	68.87 3.08	84.30 1.93
MN 2μg/mL	69.63 4.98	83.52 3.85	60.17 0.72	78.66 2.18

(¯x ± s,n=6).

2.6 Detecting the effect of combined use of KBN and MN on early and mature *C. albicans* biofilms by fluorescence microscopy

In this study, we used FITC-conA fluorescent dye to label the extracellular polysaccharides in *Candida albicans* biofilms, allowing us to observe the effects of drug treatment on their biofilm structure. FITC-conA binds to the extracellular polysaccharides in the *C. albicans* biofilm and exhibits a bright green fluorescence under a fluorescence microscope, enabling a clear visual assessment of the biofilm's thickness and structural integrity. As the culture time increases, the biofilm in the control group progressively thickens, forming clumps characterized by buds encased in secretions and a network of intertwined fungal hyphae, resulting in dense, flaky biofilms and hyphal structures visible under the microscope. However, the combined application of KBN and MN significantly disrupts the biofilm structure of *C. albicans*, primarily revealing scattered spores of the fungus. These spores exhibit irregular morphology and size variation, with only a few inhibited hyphae visible and no dense biofilm formations observed.

Across all stages of biofilm development—early, mature, and late mature—the combined use of KBN and MN at varying doses achieves the effectiveness of the MIC levels seen with individual applications of KBN and MN. Notably, the combination of KBN at 0.78 mg/mL and MN at 0.25 μg/mL demonstrates superior efficacy in disrupting the biofilm structure of *C. albicans* compared to the individual MIC levels of either agent. This tailored approach not only showcases the enhanced disruption of fungal biofilms but also highlights the potential for more effective antifungal strategies through combined therapeutic dosing (Figure 6).

2.7 Detecting the effect of combined use of KBN and MN on the viability of *C. albicans* within early and mature biofilms by CLSM

Confocal laser scanning microscopy (CLSM) has further validated the antimicrobial efficacy of the KBN and MN combination. In this study, we employed the SYTO 9/PI Live/Dead Bacterial Double Staining Kit for cell viability assessment. Propidium iodide (PI), which can only infiltrate cells with compromised membranes, marks dead cells with a red fluorescence. In contrast, live cells, characterized by intact membranes, emit a bright green fluorescence. A greater proportion of damaged fungi results in a more pronounced orange hue due to the overlay of these fluorescent signals.

In the untreated control group, early and mature stages of *C. albicans* biofilms were observed under the microscope as dense clusters emitting bright green fluorescence, with negligible red fluorescence. For the samples treated solely with KBN detergent, solely with MN, and with a combination of KBN and MN at three different dosages, the *C. albicans* biofilms predominantly consisted

TABLE 6 The impact of KBN lotion in combination with MN on mature biofilms (24 hours).

Groups / Biofilm Inhibition %	901	904	953	311
KBN 12.5mg/mL	58.88 5.14	67.42 3.87	65.25 4.51	81.97 1.70
KBN 6.25mg/mL	50.70 8.93	57.30 3.73	65.25 2.52	84.86 1.92

(¯x ± s,n=6).

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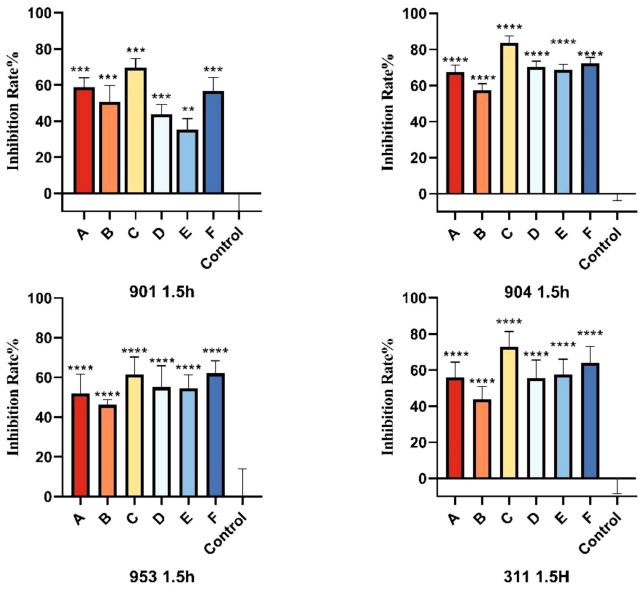
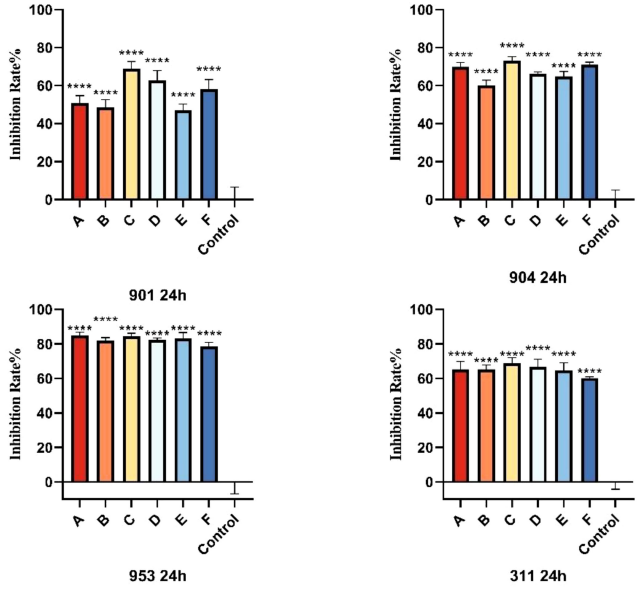
of dispersed yeast cells and pseudohyphae, with an almost complete absence of true hyphae and no dense structural formations. There was a noticeable increase in red fluorescence, indicating cell damage, alongside a significant decrease in green fluorescence, indicating fewer live cells. The combination treatment of KBN at 0.78 mg/mL and MN at 0.25 μg/mL showed the highest proportion of red fluorescence, suggesting the most effective biofilm disruption (Figure 7).



(a)



(b)

FIGURE 4
(A) The inhibition rates of early biofilms (1.5h) of *C. albicans 901*, *904*, *953*, and *311*; **(B)** The inhibition rates of mature biofilms (24h) of *C. albicans 901*, *904*, *953*, and *311*. A: KBN at 6.25 mg/mL, B: KBN at 0.78 mg/mL + MN at 0.25 μg/mL, C: KBN at 0.39 mg/mL + MN at 0.25 μg/mL, D: KBN at 0.19 mg/mL + MN at 0.25 μg/mL, and E: MN at 2 μg/mL. ** P ≤0.01; *** P ≤0.001; **** P ≤0.0001.

TABLE 7 Fluorescence values of KBN in combination with MN on early biofilms (1.5 hours).

Groups / Fluorescence value	901	904	953	311
Control	11233.67± 46.36	11141.00 117	10939.00 67.45	10084.67 409.06
KBN 6.25mg/mL	13457.00 325.12	12709.00 392.53	13378.33 500.31	12975.67 727.93
KBN 0.78mg/mL+ MN 0.25μg/mL	15101.67 1251.76	15384.67 150.28	15841.33 380.42	13713.00 1256.81
KBN 0.39mg/mL+ MN 0.25μg/mL	14631.33 505.38	14513.33 831.44	14421.67 233.29	13374.67 895.04
KBN 0.19mg/mL+ MN 0.25μg/mL	13946.67 672.07	14523.33 247.25	14316.33 216.77	12193.33 45.06
MN 2μg/mL	14036.33 333.98	14272.33 629.21	13561.00 303.79	13063.00 342.52

(¯x ± s,n=3).

TABLE 8 Fluorescence values of KBN in combination with MN on mature biofilms (24 hours).

Groups / Fluorescence value	901	904	953	311
Control	8479.67± 177.86	7922.33± 231.53	8113.33± 156.59	7616.00± 275.80
KBN 6.25mg/mL	10478.00± 505.37	13070.67± 244.11	9545.67± 287.03	12191.33± 938.05
KBN 0.78mg/mL+ MN 0.25μg/mL	10991.00± 73.30	13157.000± 413.49	10916.67± 235.09	12621.00± 185.43
KBN 0.39mg/mL+ MN 0.25μg/mL	10884.33± 165.19	12551.33± 495.70	10824.33± 353.74	12491.33± 254.69
KBN 0.19mg/mL+ MN 0.25μg/mL	10475.33± 75.57	11283.33± 369.27	10345.00± 173.25	11413.00± 76.30
MN 2μg/mL	10756.67± 145.50	11965.67± 94.32	10572.67± 398.01	12476.33± 280.39

(¯x ± s,n=3).

Utilizing Image J software for fluorescence intensity quantification from confocal laser scanning microscope images, the ratio of green to red fluorescence provided an accurate measure of biofilm alterations post-drug treatment. In the control group, the SYTO 9/PI fluorescence ratio demonstrated high fungal vitality within resistant *C. albicans* biofilms across early and mature stages, underlining minimal cell mortality protected by biofilm. In contrast, drug-treated groups exhibited a notable decline in green fluorescence alongside an uptick in red fluorescence, indicating a substantial increase in cell mortality and a decrease in viable cell

TABLE 9 Fluorescence values of KBN in combination with MN on mature biofilms (48 hours).

Groups / Fluorescence value	901	904	953	311
Control	11399.33±398.31	11220.33±299.37	11287.67±141.08	10767.00±83.72
KBN 6.25mg/mL	16418.00±473.34	18428.00±300.65	16788.67±673.47	17758.67±528.51
KBN 0.78mg/mL+ MN 0.25μg/mL	21015.33±218.22	21399.33±1162.9	20919.00±1126.09	20015.33±304.67
KBN 0.39mg/mL+ MN 0.25μg/mL	16246.33±90.94	19570.67±553.02	17756.00±180.03	19014.67±66.29
KBN 0.19mg/mL+ MN 0.25μg/mL	15615.67±275.95	19731.33±224.26	16616.33±421.92	16408.67±363.15
MN 2μg/mL	16823.33±362.15	17947.00±646.31	16540.33±392.27	15225.33±157.98

(¯x ± s,n=3).

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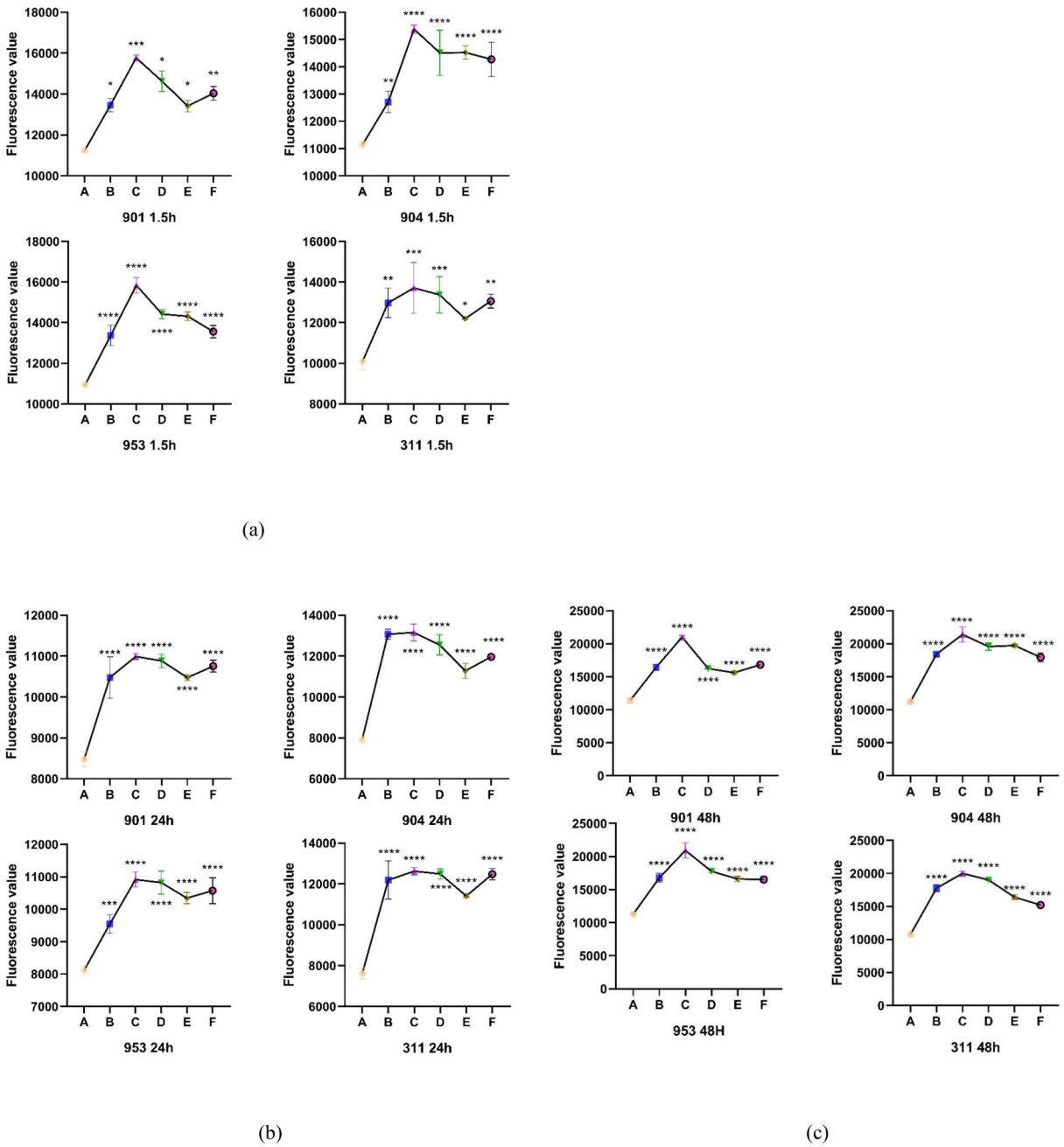


FIGURE 5
(A) The Fluorescence value of early biofilms (1.5h) of *C. albicans 901, 904, 953,* and *311*; **(B)** The Fluorescence value of mature biofilms (24h) of *C. albicans 901, 904, 953,* and *311*. **(C)** The Fluorescence value of mature biofilms(48h) of *C. albicans 901, 904, 953,* and *311*. A: KBN at 6.25 mg/mL, B: KBN at 0.78 mg/mL + MN at 0.25 μg/mL, C: KBN at 0.39 mg/mL + MN at 0.25 μg/mL, D: KBN at 0.19 mg/mL + MN at 0.25 μg/mL, and E MN at 2 μg/mL. * P ≤0.05; ** P ≤0.01; *** P ≤0.001; **** P ≤0.0001.

count. Particularly, the drug combination of KBN 0.78mg/mL and MN 0.25μg/mL showcased the highest red fluorescence ratio, implying superior efficacy in biofilm disruption and fungal cell death. This effect was consistent across all biofilm stages, with early biofilm red fluorescence ratios for strains 901, 904, 953, and 311 at 51.75%, 55.06%, 51.90%, and 49.29% respectively; at the mature stage (24h), ratios were 53.53%, 57.13%, 50.56%, and 56.66% respectively; and at the mature stage (48h), they reached 57.85%, 58.68%, 53.24%, and 61.92% respectively. These findings underscore the combination therapy's potent impact on biofilm structural integrity and fungal mortality, highlighting its synergistic effect against biofilms (Figure 8).

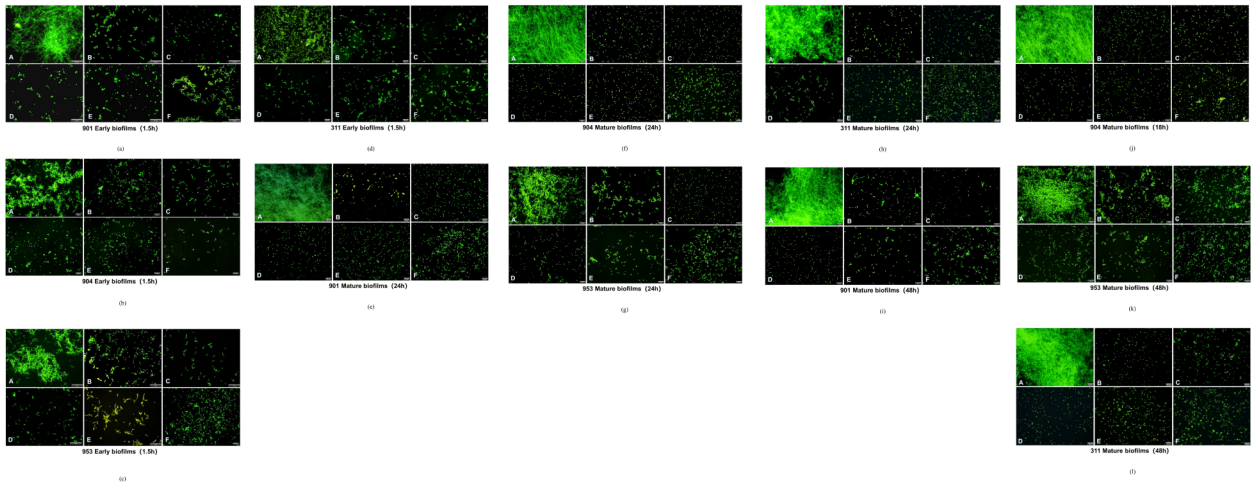


FIGURE 6
Fluorescence microscopy images of *C. albicans* after early stage (1.5h) **(A–D)** and mature stages [24h **(E–H)**, 48h **(I–L)**] treatment with A: blank RMPI 1640 liquid medium, B: KBN at 6.25 mg/mL, C: KBN at 0.78 mg/mL + MN at 0.25 μg/mL, D: KBN at 0.39 mg/mL + MN at 0.25 μg/mL, E: KBN at 0.19 mg/mL + MN at 0.25 μg/mL, and F: MN at 2 μg/mL. The scale bar represents 50 micrometers.

2.8 Expression analysis of KBN and MN on *ALS1*, *ALS3*, *HWP1*, *HSP90* and *CSH1* of *C.albicans* biofilm formation by RT-PCR

Given the observed inhibitory impact of KBN and MN on *C. albicans* biofilms, we delved into the expression patterns of several key genes known to play roles in hyphal development and adherence. The findings from RT-PCR revealed that post-treatment, there was a notable decrease in the expression levels of *ALS1*, *ALS3*, *HWP1*, *HSP90*, and *CSH1*. The efficacy observed at the three combined concentrations matched that of the MIC values when KBN and MN were used individually. Notably, the combined dose of KBN at 0.78 mg/mL and MN at 0.25 μg/mL exhibited the most pronounced effect in terms of gene suppression, surpassing the downregulation observed with the individual MIC doses of either KBN or MN (Figure 9).

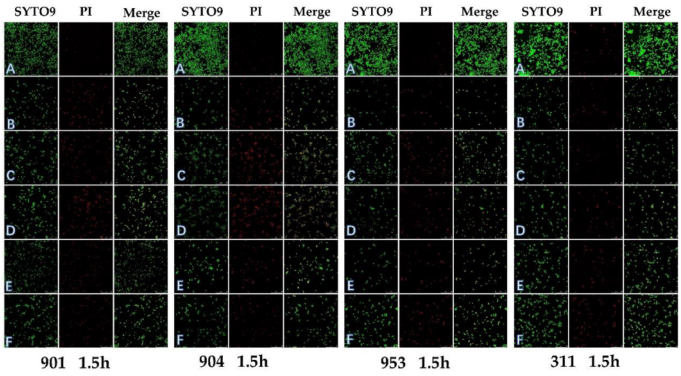
4 Discussion

Candida albicans, typically commensal microorganisms colonizing the skin, vaginal, gastrointestinal, and pharyngeal cavities, can transition to opportunistic pathogens under certain conditions, leading to infections ranging from superficial to life-threatening systemic candidiasis. The virulence of these infections is significantly enhanced by the formation of biofilms, which protect the fungal cells from antifungal agents and immune responses, contributing to high mortality rates, particularly in immunocompromised patients (Atiencia-Carrera et al., 2022b; Yu et al., 2022). Biofilms increase the resistance of *Candida albicans* to first-line antifungals, complicating treatment efforts (Zhu et al., 2022).

In tackling antifungal resistance, especially in the context of *C. albicans* biofilms, our study adds to the essential discussion on the effectiveness of combination therapies. The synergistic interaction between KBN and MN observed in our research is consistent with recent findings advocating for combination therapies to improve antifungal efficacy (Atiencia-Carrera et al., 2022a; Atiencia-Carrera et al., 2022b). This strategy is not only a current focal point but also represents a significant trend in future therapeutic developments (Liu et al., 2014; Czechowicz et al., 2021).

Alternative treatments targeting biofilm formation and maintenance are crucial. These approaches, including new antifungal agents and combination therapies (Wang et al., 2021), are essential for reducing morbidity and mortality associated with biofilm-related infections (Guo et al., 2008). The demonstrated effectiveness of KBN and MN combination therapy in our study underscores the potential of these approaches to surpass the limitations of existing treatments and offer more robust solutions for combating *Candida* infections.

The KBN lotion is composed of Huanglian, Sanbaicao, Daqingye, Jiguanghua, Xiangru, Kushen, Baibu, Longdan, Dingxiang, and Bingpian. It possesses the efficacy of clearing heat, drying dampness, and killing insects to alleviate itching. Huanglian in the prescription contains berberine, palmatine, berberine alkaloid, and epiberberine. Its bacteriostatic main components are berberine and berberine alkaloid. Among them, berberine can inhibit the formation of *C. albicans* biofilms by downregulating the expression levels of *EFG1*, *HWP1*, *ALS1*, and *ECE1* genes. *EFG1* regulates the transition to hyphal growth, essential for biofilm development. *HWP1* and *ALS1* are involved in adhesion, crucial for biofilm stability, while *ECE1* contributes to biofilm maturation and immune evasion. By suppressing these genes, berberine disrupts biofilm formation, reducing the pathogen's virulence and resistance to antifungal treatments. This inhibitory effect of berberine hydrochloride on *C. albicans* biofilm formation was demonstrated in the study by Huang et al. (Huang et al., 2020). The main active ingredient in Sanbaicao is Sauchinone, which exhibits anti-inflammatory effects by inhibiting the activity of NF-κB and reducing TNF-α expression in macrophages (Hwang et al., 2003). Daqingye's



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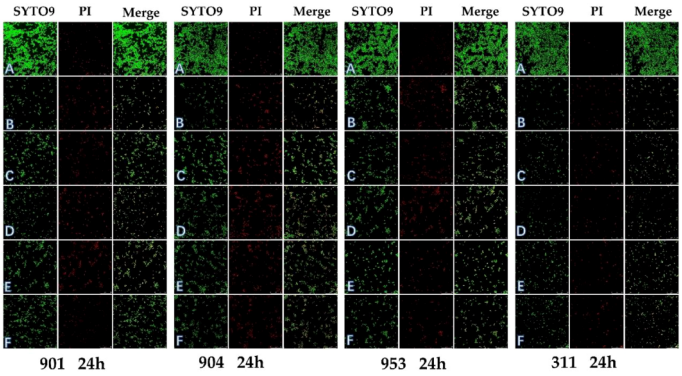

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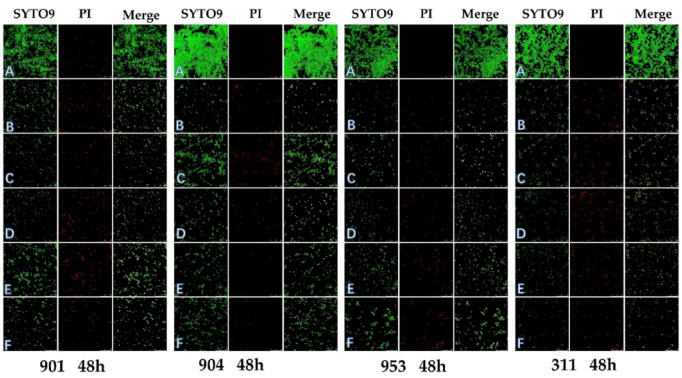

(c)

FIGURE 7
CLSM images of (*C. albicans* after early (1.5h) **(A)** and mature stages [24h **(B)**, 48h **(C)**] treatment with A: blank RMPI 1640 liquid medium, B: KBN at 6.25 mg/mL, C: KBN at 0.78 mg/mL + MN at 0.25 μg/mL, D: KBN at 0.39 mg/mL + MN at 0.25 μg/mL, E: KBN at 0.19 mg/mL + MN at 0.25 μg/mL, and F: MN at 2 μg/mL. SYTO 9 emits green fluorescence indicating live cells within intact biofilms; PI emits red fluorescence indicating dead cells within damaged biofilms; the combined orange fluorescence intensity represents the proportion of live to dead cells. The scale bar represents 50 micrometers.

effective antibacterial component is indirubin, which can effectively inhibit the formation of Candida albicans mixed biofilms and significantly suppress the activity of *C.albicans* mixed biofilm formation, demonstrating good efficacy in the prevention and treatment of VVC (Ahmad et al., 2010; Ponnusamy et al., 2010). The main component of Jiguanghua, kaempferol, has strong anti-inflammatory effects, regulating the activity of pro-inflammatory enzymes and the expression of inflammation-related genes (Devi et al., 2015). The primary antibacterial active ingredient in Dingxiang is eugenol, which can disrupt the fungal cell membrane (He et al. 2007; Zanul Adibin et al. 2023).

The noteworthy reduction in MIC values achieved through the concurrent application of KBN and MN underscores a potent synergistic effect, resonating with the work of Czechowicz, P et al.

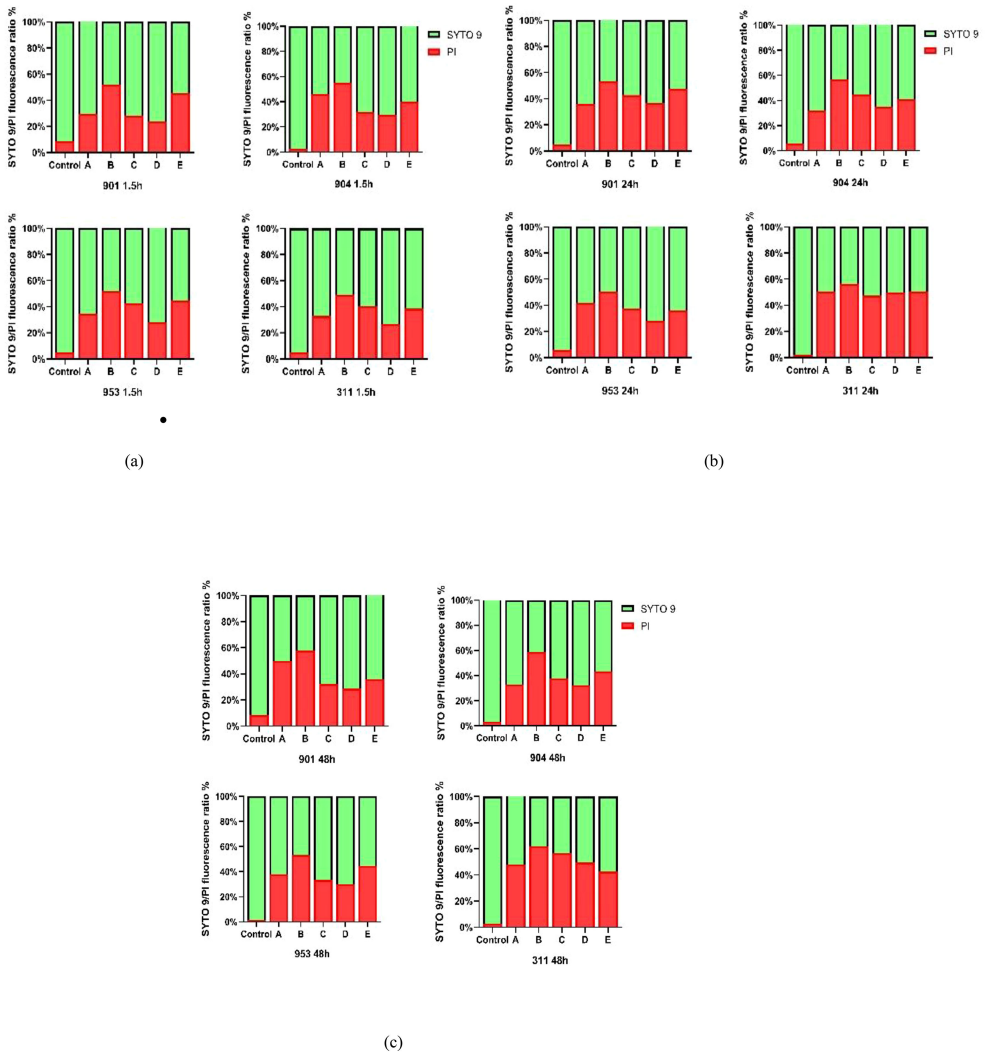

FIGURE 8
(A) The SYTO 9/PI fluorescence ratio of early biofilms (1.5h) of (*C. albicans 901, 904, 953*, and *311*; **(B)** The SYTO 9/PI fluorescence ratio of mature biofilms (24h) of *C. albicans 901, 904, 953*, and *311*.**(C)** The SYTO 9/PI fluorescence ratio of mature biofilms (48h) of (*C. albicans 901, 904, 953*, and *311*. A: KBN at 6.25 mg/mL, B: KBN at 0.78 mg/mL + MN at 0.25 μg/mL, C: KBN at 0.39 mg/mL + MN at 0.25 μg/mL, D: KBN at 0.19 mg/mL + MN at 0.25 μg/mL, and E: MN at 2 μg/mL.

(Kim and Lee, 2021), who documented enhanced biofilm disruption with low-dose combination treatments. This approach highlights the potential of sub-inhibitory concentrations to target and dismantle biofilm-associated resistance mechanisms, thereby restoring fungal susceptibility to antifungal agents.

Furthermore, several studies have highlighted the significant role of oxidative stress in the antifungal action against Candida albicans, particularly within biofilms. Yanjiao Ding et al. (Hwang et al., 2012) demonstrated that D319 induces ROS-mediated apoptosis by inhibiting isocitrate lyase, leading to mitochondrial dysfunction and cell death. Ji Hong Hwang et al. (Tian et al., 2019) showed that (+)-Medioresinol promotes ROS accumulation, triggering mitochondria-mediated apoptosis. Similarly, Heesu K et al. (Ding et al., 2022) emphasized the role of nanoparticles in inducing oxidative stress, disrupting fungal cell wall integrity, and promoting cell death through ROS production. These findings support our observations of the KBN and MN combination's efficacy, reinforcing oxidative stress as a critical mechanism for reducing fungal viability and enhancing treatment outcomes.

In this study, the use of FITC-conA (Galdiero et al., 2021) for the visualization of *C. albicans* biofilm disruption through the combined application of KBN and MN dovetails with the growing body of research highlighting the susceptibility of biofilms to antifungal agents. Our findings underscore the effectiveness of synergistic antifungal combinations in compromising biofilm integrity, particularly evident in the significant disruption caused by the KBN 0.78 mg/mL and MN 0.25 μg/mL dosage. This synergy is further validated by our confocal CLSM analysis, which, akin to the approach by Lee and Park (Li et al., 2019; Ma et al., 2020), utilizes SYTO 9/PI staining to differentiate between viable and compromised fungal cells within biofilms. The notable increase in red fluorescence in treated samples

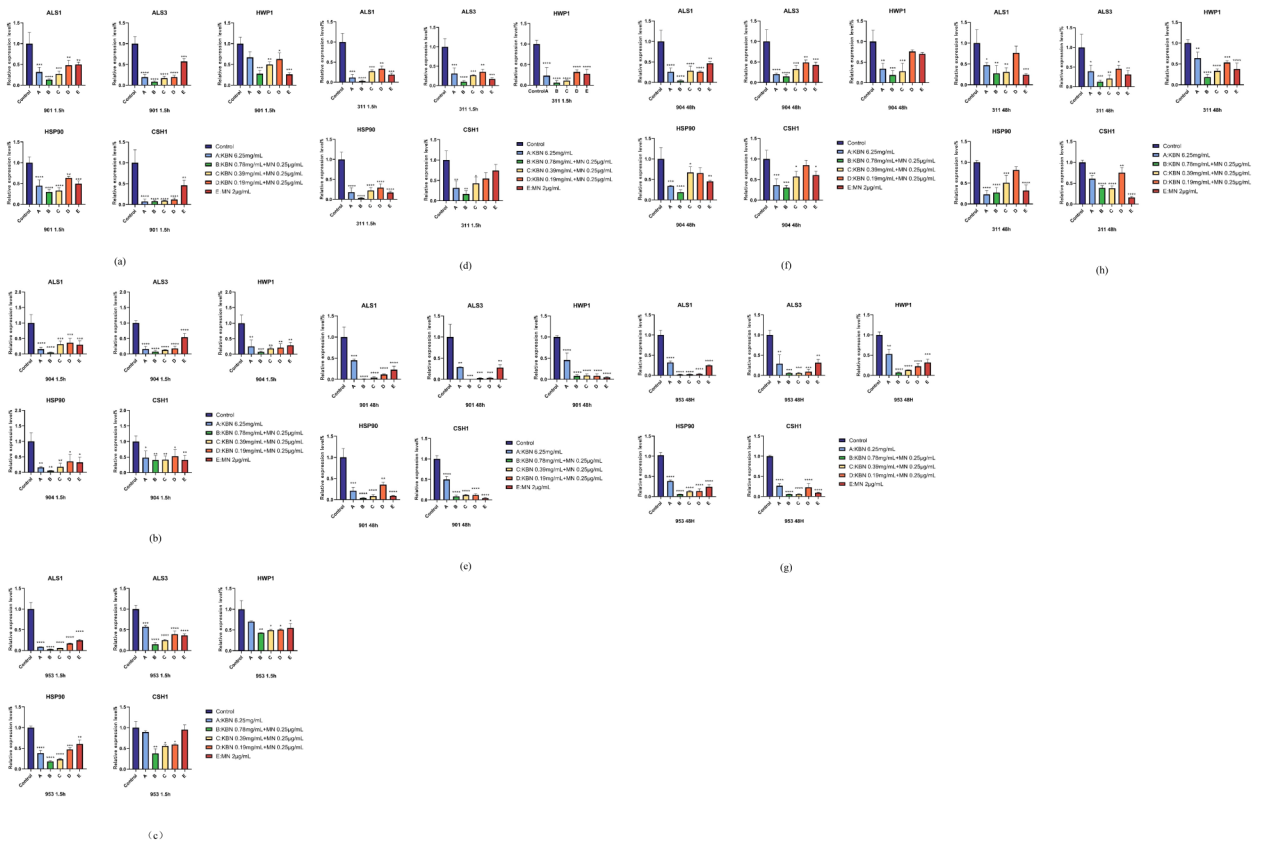

FIGURE 9
Comparison of the relative expression levels of the *ALS1*, *ALS3*, *HWP1*, *Hsp90*, *CSH1*gene, associated with the formation of *C. albicans* biofilms, after early (1.5 hours) **(A–D)** and mature (48 hours) **(E–H)** stages treatment with KBN at 6.25 mg/mL, KBN 0.78 mg/mL + MN 0.25 μg/mL, KBN 0.39 mg/mL + MN 0.25 μg/mL, KBN 0.19 mg/mL + MN 0.25 μg/mL, MN at 2 μg/mL, and a control of blank 1640 liquid medium. Expression of the individual genes was normalized to the expression of *18s*, a reference gene, and is shown relative to untreated control cells. * P ≤0.05; ** P ≤0.01; *** P ≤0.001; **** P < 0.0001.

—indicative of enhanced cellular damage—positions this combination therapy as a viable antifungal strategy, supporting the conclusions drawn by Patel and Wright (Gonçalves et al., 2015; Qian et al., 2020; Bonvicini et al., 2021) in their review of fungal biofilm resistance mechanisms. This alignment with contemporary research (Hoyer et al., 1998) underscores the potential of our findings to contribute to the development of more effective treatments against fungal biofilms, with the caveat that further studies are needed to explore the broader implications of these synergistic treatments across various fungal pathogens.

In the context of our findings on the inhibitory effects of KBN combined with MN on *C. albicans* biofilms, we further explored the expression of genes implicated in hyphal formation and adhesion. The results from RT-PCR revealed a significant downregulation of *ALS1*, *ALS3*, *HWP1*, *HSP90*, and *CSH1* following treatment. The efficacy observed at the three combined concentrations matched that of the MIC values when KBN and MN were used individually, with the combination of KBN at 0.78 mg/mL and MN at 0.25 μg/mL showing the most pronounced effect on gene suppression. This downregulation surpasses the effects seen with individual applications of KBN and MN at their respective MIC concentrations.

The significant downregulation of *ALS1*, *ALS3*, *HWP1*, *HSP90*, and *CSH1* post-treatment aligns with the hypothesis that targeting specific pathways related to biofilm integrity and fungal virulence can enhance the therapeutic efficacy against *C. albicans*. The *ALS* gene family, particularly *ALS1* and *ALS3* (Liu and Filler, 2011; Roudbarmohammadi et al., 2016), plays a crucial role in the adhesion process, essential for biofilm formation and maintenance (Murad et al., 2001). *HWP1* is integral to hyphal development, another critical component of biofilm structure (Murzyn et al., 2010a; Oh et al., 2010b; Salehipour et al., 2021). *Hsp90*, a heat shock protein, is known to stabilize several host proteins and has been implicated in stress response pathways that contribute to anti-fungal resistance (Yan et al., 2019; Huang et al., 2020). Murzyn, A, Yan, Y, et al. have also demonstrated a positive correlation between *CSH1* expression and biofilm formation (Wall et al., 2019; Lu et al., 2021). The observed downregulation of these genes suggests that the combination therapy not only disrupts biofilm formation but also impacts the fungal cell's ability to adhere and form hyphae, key factors in its pathogenicity and resistance.

Our study provides insights into the potential molecular mechanisms behind *C. albicans* ' resistance, suggesting that combination therapy, particularly through gene expression modulation, could offer a promising approach to address fungal infections. The observed synergistic effect in downregulating genes essential for biofilm formation and virility, achieved with KBN and

MN, points towards a strategic avenue that might help in navigating the complexities of antifungal resistance. This prompts a call for further explorations into the specific molecular interactions spurred by this therapy, with the goal of uncovering new therapeutic targets (Romo et al., 2017; Wall et al., 2019) that could enhance antifungal strategies. Aligning with the evolving landscape of current re-search (Pohl, 2022), there appears to be a consensus on the potential benefits of pursuing combination therapies against fungal biofilms. Recent discussions in the field (Chen et al., 2020), as outlined by reviews, emphasize a nuanced understanding of biofilm dynamics and the identification of molecular targets as essential steps towards crafting more nuanced antifungal treatments (Xu et al., 2014). Our research adds to the dialogue on addressing the challenge of biofilm-mediated antifungal resistance, highlighting the prospective value of innovative therapeutic approaches in the broader context of combating fungal infections.

5 Conclusions

To address the challenge of antifungal resistance, especially against *C. albicans* biofilms, our study conducted *in vitro* experiments on the combined use of KBN and MN against *C. albicans* biofilms, demonstrating a synergistic effect in combating these biofilms. Through detailed analysis using FITC-conA for biofilm visualization and real-time RT-PCR for gene expression, our findings indicate that this combination therapy not only disrupts the integrity of the biofilms but also significantly downregulates key genes related to fungal adhesion and hyphal formation. These observations suggest that targeting specific fungal pathways and mechanisms through combined therapeutic strategies can significantly improve the management of fungal infections, offering a promising route to overcome the complexities of antifungal resistance. This also provides experimental support for future clinical applications of KBN in combination with antibiotics to treat infections caused by drug-resistant *C. albicans*. Despite the promising *in vitro* results of our study on the synergistic effect of KBN and MN against *C. albicans* biofilms, our experiment did not validate these effects in drug-resistant *C. albicans* VVC cell and animal models. Furthermore, the study did not investigate the specific mechanisms behind the synergy between KBN and MN or the interactions that might impact efficacy and safety. Future research should address these limitations to confirm the findings and understand the therapeutic potential of this combination.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

XC: Conceptualization, Methodology, Writing – original draft. NX: Data curation, Formal analysis, Resources, Writing – original draft. JH: Investigation, Software, Visualization, Writing – original draft. LL: Investigation, Writing – original draft. LZ: Software, Writing – original draft. JZ: Funding acquisition, Project administration, Supervision, Writing – review & editing. FW: Funding acquisition, Project administration, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2024.1426791/full#supplementary-material

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Prevalence and clinical correlates of *Gardnerella* spp., *Fannyhessea vaginae*, *Lactobacillus crispatus* and *L. iners* in pregnant women in Bukavu, Democratic Republic of the Congo

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Background: *Gardnerella* is a key pathogen in bacterial vaginosis (BV), but the role of the different *Gardnerella* species remains unclear. We investigated the role of four *Gardnerella* species, as well as *Fannyhessea vaginae*, *Lactobacillus crispatus* and *L. iners* in BV.

Methods: From 331 pregnant women from the Democratic Republic of the Congo, BV was diagnosed using Nugent scoring and a cervicovaginal lavage was used to quantify *G. leopoldii*, *G. piotii*, *G. swidsinskii*, *G. vaginalis*, *F. vaginae*, *L. crispatus and L. iners* by qPCR. Univariate associations between these species and clinical outcomes were assessed. A logistic regression model and ROC curves were calculated to determine the best diagnostic marker for BV.

Results: Here, *L. iners* (75.8%) was the most prevalent species and *G. vaginalis* (36.0%) the most common *Gardnerella* species. All investigated *Gardnerella* spp. were prevalent (50.9-57.9%) in women with (asymptomatic) BV. Univariate analysis revealed no significant associations with clinical symptoms of BV, while *F. vaginae* (positive Whiff test, high pH), *G. vaginalis* (high pH) and *L. crispatus* (low pH) were associated with signs of BV. *G. piotii* was associated with markers of urinary tract infection. Women with *L. iners* had higher odds of delivering preterm. ROC analyses showed that *F. vaginae* was the best marker for BV (AUC 0.81), and the combined model further increased the diagnostic performance (AUC 0.90).

Conclusion: All *Gardnerella* species were involved in BV, although none were associated with the most important clinical symptoms of BV and none emerged as a superior molecular marker for BV.

KEYWORDS

bacterial vaginosis, *Gardnerella*, molecular diagnosis, preterm birth, low birthweight, Democratic Republic of the Congo

Introduction

Bacterial vaginosis (BV) is the most common gynecological condition among women of reproductive age worldwide (Allsworth and Peipert, 2007), with a prevalence among the general population of approximately 25% both globally (Peebles et al., 2019) and in sub-Saharan Africa (Park et al., 2024). Besides being a discomforting and often recurrent/chronic condition, BV is also considered a significant risk factor for adverse pregnancy outcomes such as preterm birth (PTB) and low birthweight (LBW) (Hillier et al., 1995). Furthermore, BV has been associated with an increased risk for the acquisition of sexually transmitted infections, such as human immunodeficiency virus (HIV) (Atashili et al., 2008), herpes simplex virus-2 (HSV-2) (Cherpes et al., 2005), *Trichomonas vaginalis*, *Neisseria gonorrhoeae* and *Chlamydia trachomatis* (Brotman et al., 2010).

Microbiologically, BV is characterized by a dysbiosis of the healthy vaginal microbiome (VMB) (Muzny et al., 2019), normally characterized by the predominance of only a single *Lactobacillus* species, mostly *L. crispatus* or *L. iners* (Vaneechoutte, 2017a). The vaginal microbiome can be categorized into community-state types (CSTs), with CST-I representing a vaginal microbiome dominated by *L. crispatus*, CSTI-II dominated by *L. gasseri*, CST-III dominated by *L. iners*, CST-IV representing a diverse microbiome and finally CST-V dominated by *L. jensenii* (Ravel et al., 2011). The lactobacilli-dominated environment has a low pH due to production of lactic acid by lactobacilli, making the vagina a hostile environment for many pathogens, although it is generally known that *L. iners* is less protective than *L. crispatus* (Vaneechoutte, 2017a). In BV, the VMB is replaced by a polymicrobial VMB with *Gardnerella* as the key marker (Muzny et al., 2018). However, also *L. iners* has been found among women with BV (Vaneechoutte, 2017b). Additionally, *Gardnerella* has also been isolated from healthy women in several studies (Hickey and Forney, 2014). *G. vaginalis* was long considered the single species within the *Gardnerella* genus. This is because the 16S rRNA gene, which forms the basis for bacterial taxonomy and microbiome studies (Clarridge, 2004), lacks resolution needed to distinguish the different *Gardnerella* species (Vaneechoutte et al., 2019). In 2019, Vaneechoutte and coworkers showed the existence of minimum thirteen genomospecies in the genus (Vaneechoutte et al., 2019), of which six have been named validly: *Gardnerella greenwoodii*, *G. leopoldii*, *G. pickettii*, *G. piotii*, *G. swidsinskii* and *G. vaginalis* (Vaneechoutte et al., 2019; Sousa et al., 2023).

Gardnerella species mostly cooccur in the VMB rather than being present as a single species (Hill and Albert, 2019; Munch et al., 2024; Schuster et al., 2024) and their clinical relevance has been under investigation for several years. In non-pregnant reproductive-aged Canadian women, Hill and coworkers (2019) already demonstrated, using a cpn60 sequencing approach, that *G. vaginalis* and *G. swidsinskii*, but not *G. leopoldii* and *G. piotii*, were associated with vaginal discharge and malodor, typical symptoms of BV (Hill and Albert, 2019). Turner and coworkers showed that, in American women with recurrent BV, persistently high concentrations of genomospecies 12 (as defined by Vaneechoutte and colleagues (Vaneechoutte et al., 2019)) were associated with refractory responses after metronidazole treatment of BV, and persistently low concentrations of genomospecies 12 and *G. swidsinskii*/*G. leopoldii* with remission (Turner et al., 2021). Munch and coworkers recently documented that American non-pregnant BV-negative women colonized with three or more *Gardnerella* species had higher chance for incident BV within 100 days compared to women colonized with fewer *Gardnerella* species (Munch et al., 2024). In a cohort of pregnant women at high risk of recurrent preterm birth, *G. leopoldii*, but not *G. piotii*, *G. swidsinskii* or *G. vaginalis*, was associated with spontaneous preterm birth (Schuster et al., 2024).

However, the different *Gardnerella* spp. have not yet been studied in African women, known to harbor a VMB that can differ significantly compared to Caucasian women (Fettweis et al., 2014). Therefore, we aimed to investigate the distribution of *G. leopoldii*, *G. piotii*, *G. swidsinskii* and *G. vaginalis* and the correlates with clinical signs and symptoms and adverse pregnancy outcomes using species-specific quantitative PCR (qPCR) assays in a population of pregnant women from the Democratic Republic of the Congo.

Abbreviations: BV, bacterial vaginosis; BVAB-2, bacterial vaginosis-associated bacterium 2; CI, confidence interval; CVL, cervicovaginal lavage; DRC, Democratic Republic of the Congo; GE/mL, genome equivalents per milliliter; LBW, low birthweight; OR, odds ratio; PRHB, Provincial Referral Hospital of Bukavu; PTB, preterm birth; qPCR, quantitative polymerase chain reaction; UTI, urinary tract infection; VMB, vaginal microbiome; WGA, weeks of gestational age.

Materials and methods

Ethical approval

Ethical approval was obtained by the Internal Review Board of the Catholic University of Bukavu (reference number UCB/CIE/NC/016/2016), the Ministry of Public Health (reference number 062/CD/DPS/SK/2017) and the Ethical Committee of Ghent University Hospital (reference number PA2014/003). All pregnant women that participated in this study signed an informed consent form.

Study design and population

This research was part of the AVEONS (acronym for Angamiza Vizuri (Swahili for 'stop') Early Onset Neonatal Sepsis) study. The AVEONS project had the overall aim to study the prevalence and clinical correlates of vaginal infections in a population of pregnant women from Bukavu, Democratic Republic of the Congo (DRC). The prevalence (26.3%), risk factors and adverse pregnancy outcomes of second trimester BV [assessed by microscopy (Nugent score)], as well as the study design and population have been described elsewhere (Mulinganya et al., 2021). Briefly, the AVEONS study was a prospective observational study where pregnant women were seen between 16 and 20 weeks of gestation [visit 1 (V1)], between 36 and 38 weeks (V2) and at delivery. Participants were recruited from January to October 2017 at the Provincial Referral Hospital of Bukavu (PRHB). Pregnant women visiting the PRHB for antenatal care were asked whether they were interested in participation, whereafter eligible women were individually informed about the study details. Women were considered eligible when they (i) were between 16 and 20 weeks pregnant, (ii) accepted to be followed by a referral hospital team, (iii) were willing to deliver at PRHB, and (iv) agreed to be contacted by phone or other means. Women could not be included in the study (i) if they planned to move out of the study area during their pregnancy, (ii) in case of genital bleeding, (iii) in case of twin pregnancies or a visible malformation of the fetus at ultrasound examination, or (iv) in case they used antibiotics during the two weeks before recruitment. The research described here only reports data on the VMB and clinical correlates at V1, and pregnancy outcomes (such as PTB and LBW).

Routine antenatal care and delivery procedures

At V1, a questionnaire on sociodemographics, reproductive health history, sexual activity, vaginal practices and vaginal complaints was completed by each participant. A general physical examination, including anthropometric measurements, and a gynecological examination, including a speculum examination with a sterile non-moistened speculum, were performed. The vaginal mucosa and cervix were inspected for the presence of sores and tumors, and a diagnosis of vaginal infection was made according to the syndromic-based protocol for the management of pregnancy of the Ministry of Public Health of DRC (Ministère de la Santé RDdC, 2006), which is based on previous WHO recommendations (World Health, 2007). When a vaginal infection was diagnosed, women were treated empirically with a combination (in one vaginal ovule) of clotrimazole (200 mg) (against candidiasis) and clindamycin (100 mg) (against BV) once a day for six days, in accordance with the local protocol. In case a woman was allergic to clindamycin, this was replaced by metronidazole (in a vaginal ovule). During the gynecological examination, the vaginal pH was also determined by means of indicator pH papers (Hilo Indicator® pH paper, Sigma Aldrich). In addition, an ultrasound examination was performed to determine the viability of the fetus and to measure cervical length. Furthermore, five mL of total blood was collected in a VacuTube® (Becton Dickinson) red tube without anticoagulant for HIV, malaria and hemoglobin testing. Next, midstream urine was collected in a sterile container to investigate the presence of nitrite and white blood cells (indicative for urinary tract infections or bacteriuria) using dipsticks (Multistix dipsticks®, Siemens). Lastly, three vaginal swabs were taken from the midportion of the lateral vaginal wall, and a cervicovaginal lavage (CVL) sample was collected by rinsing the cervical mucosa with 5 mL of sterile physiological water and collecting as much lavage as possible into a VacuTube®. These CVL samples were stored at -20°C for shipment on dry ice to the Laboratory Bacteriology Research (LBR) (Ghent University, Ghent, Belgium) respecting the cold chain. All participants followed routine antenatal care and they received a single dose of mebendazole (500 mg) against intestinal worm infections and a single dose of sulfadiazine-pyrimethamine (500 mg) against malaria. At delivery, the labor was monitored, and delivery features and pregnancy outcomes were collected by nurses and the senior assistant.

Study specific laboratory procedures

Microscopic examinations

At the PRHB, a wet mount slide was prepared within 20 minutes after collection of a vaginal swab. Saline (0.5 mL) was added to the swab and one droplet of this was put on a glass slide and covered with a cover slip. The presence of *Trichomonas vaginalis*, *Candida*, white blood cells and clue cells was determined with microscopy.

A second vaginal swab was rolled on a glass slide and fixated by briefly passing the back of this slide through a flame. Subsequently, these smears were stored and shipped to the LBR, where they were Gram-stained at the Department of Laboratory Medicine (Ghent University Hospital, Ghent, Belgium) using an automated PolyStainer (IUL). These Gram-stained slides were used to diagnose BV according to Nugent as described previously (Mulinganya et al., 2021).

DNA extraction

A random selection was made of 331 CVLs to be analyzed by qPCR. DNA was extracted from CVLs using the RNeasy PowerMicrobiome Kit (Qiagen) according to manufacturer's instructions, with minor modifications (i.e. DNase treatment was omitted). The DNA extracts were then stored at -20°C until use.

qPCR assays

Species-specific qPCR assays were used to quantify *Fannyhessea* (*Atopobium*) *vaginae*, *G. leopoldii*, *G. piotii*, *G. swidsinskii*, *G. vaginalis*, *L. crispatus* and *L. iners*. The species-specific qPCR assays for *G. leopoldii*, *G. piotii*, *G. swidsinskii* and *G. vaginalis* were designed and validated in-house (Latka et al., 2022). All reactions were carried out in a total volume of 10 µL, containing 1X LightCycler 480 SYBR Green I master mix (Roche), forward and reverse primer (listed in Table 1) and 2 µL of DNA extract, DNA standard (positive control and calibration) or molecular water (negative control). DNA was extracted from cultured *F. vaginae* (CCUG 38953^T), *G. leopoldii* (UGent 09.48), *G. piotii* (UGent 18.01^T), *G. swidsinskii* (GS10234), *G. vaginalis* (GvB LMG7832^T), *L. crispatus* (LMG 9479^T) and *L. iners* (FB123-CNA-4) using the High Pure PCR Template Preparation Kit (Roche) as previously described (De Keyser, 2020) and used to make a tenfold standard dilution series to generate qPCR standard curves.

Reactions were carried out on a LightCycler 480 (Roche). qPCRs were performed for *F. vaginae* by pre-incubation for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 20 s at 62°C and 40 s at 72°C, for *Gardnerella* species by pre-incubation 5 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 56°C and 30 s at 72°C, for *L. crispatus* by pre-incubation for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C, and for *L. iners* by pre-incubation for 10 min at 95°C, followed by 40 cycles of 10s at 95°C, 20 s at 50°C and 4 s at 72°C.

High resolution melting curves were generated by first melting all amplified dsDNA at 95°C for 5 s, followed by renaturating the DNA for 30 s at 50°C (*F. vaginae*), 1 min at 55°C (*Gardnerella* species), or 1 min at 60°C (*L. crispatus* and *L. iners*), whereafter the temperature was increased to 97°C at a ramp rate of 0.02°C/s. Specific target amplification was defined based on a melting temperature within a range of 1°C of the mean of the standard dilution series of the respective assays. All raw data was analyzed with the standard LightCycler 480 Software Version 1.5 (Roche). All bacterial concentrations were expressed in genome equivalents/mL (GE/mL).

TABLE 1 Primers used in this study.

Species	Primer	Sequence (5′-3′)	Final concentration (µM)	Target gene	Reference
Fannyhessea vaginae	AV_F	CCCTATCCGCTCCTGATACC	0.7	16S rRNA	(Menard et al., 2008)
	AV_R	CCAAATATCTGCGCATTTCA	0.7		(Menard et al., 2008)
Gardnerella leopoldii	GldnaG_F	GATACTGCACTGTATCGA	0.5	dnaG	(Latka et al., 2022)
	GldnaG_R	CAGTATCAATACCAGCC	0.5		(Latka et al., 2022)
Gardnerella piotii	GpdnaG_F	AGCTGCTTACGATTATAGT	0.5	dnaG	(Latka et al., 2022)
	GpdnaG_R	TTACTCATTCTAAGCTTAATAG	0.5		(Latka et al., 2022)
Gardnerella swidsinskii	GsdnaG_F	ATTTAGTTAGATATTTGGCAA	0.5	dnaG	(Latka et al., 2022)
	GsdnaG_R	ATAGTCATATATTCCGCGC	0.5		(Latka et al., 2022)
Gardnerella vaginalis	GvdnaG_F	TATTATAACTAAAGCTGCTG	0.5	dnaG	(Latka et al., 2022)
	GvdnaG_R	TCGCCACTATAGTCG	0.5		(Latka et al., 2022)
Lactobacillus crispatus	Lcris_F	AGCGAGCGGAACTAACAGATTTAC	0.1	16S rRNA	(Byun et al., 2004)
	Lcris_R	AGCTGATCATGCGATCTGCTT	0.1		(Byun et al., 2004)
Lactobacillus iners	Liners_F	GTCTGCCTTGAAGATCGG	0.2	16S rRNA	(De Backer et al., 2007)
	Liners_R	ACAGTTGATAGGCATCATC	0.2		(De Backer et al., 2007)

Data analysis

First, the presence of each bacterial species as assessed by qPCR was defined as a binary variable and was used to calculate prevalences and univariate associations. The prevalence of each bacterial species was documented together with the corresponding 95% confidence interval (CI), which was calculated using the Wilson method. The median and range of (log-transformed) concentrations of the different species among positive women were documented in boxplots. Next, the Pearson correlation coefficients between the different bacterial concentrations were calculated with the R programming language to document the correlations between the species and were illustrated in a correlation matrix created with heatmapper.ca (http://www.heatmapper.ca/pairwise/). The Pearson correlation coefficients were defined as negligible (0.00-0.10), weak (0.10-0.39), moderate (0.40-0.69), strong (0.70-0.89), or very strong (0.90-1.00) (Schober et al., 2018). To identify the best molecular markers for BV, receiver-operator characteristic (ROC) curves were created based on the log-transformed bacterial concentrations and the categorization of BV based on Nugent (healthy and intermediate scores seen as negative for BV) using the R package pROC (Robin et al., 2011). A logistic regression model was made to investigate the best combination of markers.

The log-transformed bacterial concentrations were used as continuous variables to create VMB profiles and were visualized in a heatmap using the R function pheatmap() (Kolde, 2019). On this heatmap, clusters of VMB profiles were visually defined after hierarchical clustering. Species were also hierarchically clustered on the heatmap. We defined clusters by cutting the tree at a height (indicating dissimilarity) of 110 using the function cutree(). This height was chosen because it yielded two expected clusters of healthy VMB (one dominated by *L. crispatus* and one dominated by *L. iners*).

Subsequently, univariate associations between the bacterial species and clusters on the one hand, and clinical signs and symptoms and pregnancy outcomes on the other hand were determined. For this, the presence of each bacterial species/cluster was defined as the independent variable in each univariate analysis, and clinical signs and symptoms of mother and neonate, as well as pregnancy outcomes, were defined as dependent variables. PTB was defined as birth before 37 completed weeks of gestation and low birthweight as a birthweight below 2500 g. Fisher exact tests were performed to determine which of these associations were significant (p-value <0.05) and odds ratios (OR) were reported for each binary variable. For the parameters that showed a significant p-value when comparing clusters, specific T-tests were performed to compare all clusters against a reference cluster. Here, cluster 6, dominated by *L. crispatus* and mainly containing women with a healthy VMB according to Nugent, was selected as reference cluster for these one-on-one comparative analyses. All statistical analyses described above were performed with R Studio (version 2024.09.1 + 394) or GraphPad Prism [version 10.0.3 (217)].

Results

Participant flow and sociodemographics

The flowchart depicted in Figure 1 describes the number of pregnant women and neonates withheld at each visit. A total of 750 women were screened, of which 533 were found eligible and included in the study (at V1). Roughly one fifth of these women withdrew from the study before V2, mostly due to rejection of the study by some opinion leaders (who wrongly believed participants were given a substantial imbursement) and the instable socio-political situation in Bukavu during the study period. Of the 533 women included, a second trimester pregnancy loss happened in 26 women (4.9%), and 49 neonates (9.2%) were born preterm. Of the 354 women who completed V2, 66 (18.6%) withdrew from the study because they decided to not deliver at PRHB. The 288 other neonates (81.4%) were all born at term at PRHB.

The complete AVEONS study population has previously been described in detail (Mulinganya et al., 2024). The demographics are summarized in Table 2. There were no statistically significant differences in terms of demographics and Nugent score at V1 in the 331 participants whose CVLs were selected for qPCR analysis compared to the overall study population (data not shown). Among these 331 women, 172 gave birth at term and 30 PTBs were observed.

Distribution and concentration of the bacterial species

The prevalence, overall and across VMB categories, and the median and range of the (log_{10}-transformed) concentrations of each species among positive women are presented in Figure 2. Overall, *L. iners* was the most prevalent species (75.8%) and had the highest median concentration (8.31 log_{10} GE/ml). We found that 36.0% of women were positive for *G. vaginalis*, making it the most common *Gardnerella* species in this population. *G. leopoldii* was the least prevalent *Gardnerella* species, with a prevalence of 14.5%. *G. swidsinskii* had the highest median concentration among *Gardnerella* species (8.04 log_{10} GE/ml) while *G. piotii* had the lowest median concentration (6.48 log_{10} GE/ml).

In total, 157 women (47.4%) were not colonized with *Gardnerella* species, 82 women (24.8%) were colonized with only a single *Gardnerella* species, 62 women (18.7%) were colonized with two *Gardnerella* species, 29 women (8.8%) were colonized with 3 *Gardnerella* species and one woman (0.3%) was colonized with all four *Gardnerella* species. Overall, *Gardnerella* and *F. vaginae* occurred mostly among women with BV, more specifically 54.2% of all *G. leopoldii* was found among women with BV, and this was 57.9% for *G. piotii*, 55.7% for *G. swidsinskii*, 50.9% for *G. vaginalis* and 55.0% for *F. vaginae*. In contrast, *L. crispatus* and *L. iners* were most abundant among women with a healthy VMB (66.2% and 56.2% of *L. crispatus* and *L. iners*, respectively).

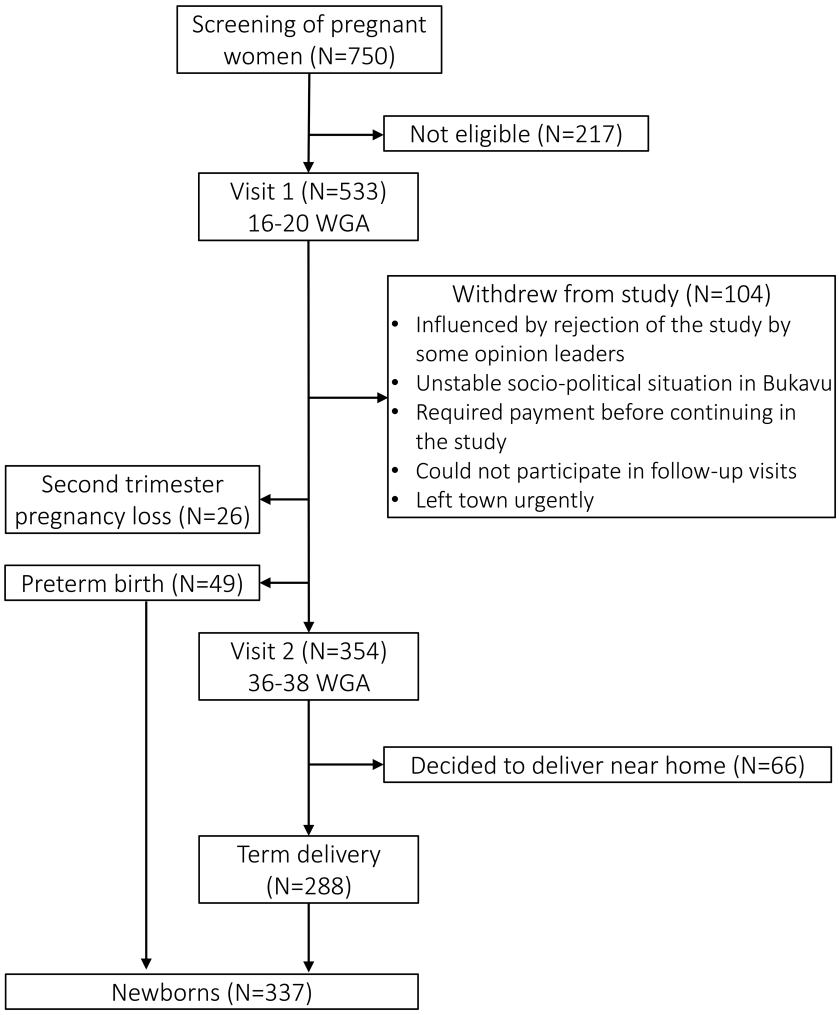

FIGURE 1
Flowchart of the study. CVL, cervicovaginal lavage; WGA, weeks of gestational age.

Correlations between the different species

Figure 3 presents the Pearson correlation coefficients between the concentrations of the different species. The strongest correlation was seen between *G. swidsinskii* and *F. vaginae* (r = 0.403, moderate correlation). There was a significantly weak negative correlation between *L. iners* and *L. crispatus* (r = -0.172), and a significantly weak positive correlation between *L. iners* and *G. piotii* (r = 0.143). Furthermore, a significantly weak positive correlation was seen between *G. swidsinskii* and *G. vaginalis* (r = 0.155), *G. swidsinskii* and *G. piotii* (r = 0.234), *F. vaginae* and *G. leopoldii* (r = 0.139), and *F. vaginae* and *G. piotii* (r = 0.126).

ROC analysis and logistic regression model

In Figure 4 the ROC curves and in Table 3 the area under the curve (AUC), the Youden's index, sensitivity and specificity are shown for each species and the multiple logistic regression model. Off all investigated species, *F. vaginae* had the largest area under the curve (0.81), followed by *G. vaginalis* (0.72), *G. piotii* (0.68), *G. swidsinskii* (0.64) and *G. leopoldii* (0.60) The logistic regression model gave the following combined result as optimal to diagnose BV: -1.46 + (0.14 × *G. swidsinskii*) + (0.10 × *G. vaginalis*) + (0.15 × *G. leopoldii*) + (0.15 × *G. piotii*) + (0.29 × *F. vaginae*) - (0.26 × *L. crispatus*) - (0.13 × *L. iners*).

Characterization of clusters based on vaginal microbiome profiles

In Figure 5 a heatmap with hierarchical clustering of VMB profiles and bacterial species, annotated by Nugent score categorization, is shown. Six different clusters (1 to 6) were defined after cutting the dendrogram of these profiles at a height of 110. Four of these clusters mainly contained women with Nugent BV (cluster 1-4), while the other clusters contained mainly women with a healthy VMB according to Nugent (cluster 5-6). Each cluster can be characterized by a distinct distribution pattern of species. The prevalence of the species among the different clusters is

TABLE 2 Sociodemographic characteristics and pregnancy outcomes of the study population stratified by vaginal microbiota categorization.

Characteristics of pregnant women	Number 525 (%)	Vaginal microbiota		
		Healthy microbiome 285 (54.3)	Intermediate microbiome 102 (19.4)	Bacterial vaginosis 138 (26.3)
Age at recruitment				
<20 years	26 (5.0)	13 (4.6)	5 (4.9)	8 (5.8)
20-24 years	113 (21.5)	65 (22.8)	17 (16.7)	31 (22.5)
25-29 years	172 (33)	94 (33.0)	36 (35.3)	43 (31.2)
30-34 years	135 (25.7)	72 (25.3)	30 (29.4)	33 (23.9)
≥35 years	78 (14.9)	41 (14.4)	14 (13.7)	23 (16.7)
Tribe				
Shi	374 (71.2)	203 (71.2)	83 (81.4)	88 (63.8)
Rega	57 (10.9)	32 (11.2)	8 (7.8)	17 (12.3)
Other tribes[1]	94 (17.9)	50 (17.5)	11 (10.8)	33 (23.9)
Religion[2]				
Christians	492 (93.7)	266 (93.3)	94 (92.2)	132 (95.7)
Not Christian	33 (6.3)	19 (6.7)	8 (7.8)	6 (4.4)
Educational level[3]				
Primary	62 (11.8)	35 (12.3)	11 (10.8)	16 (11.6)
Secondary	273 (52.0)	151 (53.0)	54 (52.9)	68 (49.3)
Higher	190 (36.2)	99 (34.7)	37 (36.3)	54 (39.1)
Quality of life[4]				
Poor	381 (72.6)	209 (73.3)	74 (72.6)	98 (71.0)
Not poor	144 (27.4)	76 (26.7)	28 (27.5)	40 (29.0)
Employment status				
Employed or self-employed	89 (17.0)	57 (20.0)	17 (16.7)	15 (10.9)
Unemployed	436 (83.1)	228 (80.0)	85 (83.3)	122 (89.1)
Marital status				
Married	501 (95.4)	273 (95.8)	100 (98.0)	128 (92.8)
Unmarried	24 (4.6)	12 (4.2)	2 (2.0)	10 (7.3)
Clinical status[5] at first visit				
Symptomatic	254 (48.4)	126 (44.2)	55 (53.9)	73 (52.9)
Asymptomatic	271 (51.6)	159 (55.8)	47 (46.1)	65 (47.1)

[1]Tembo, Fuliru, Hunde, Nyanga, Hutu, Nande, Vira, Bembe, each with a proportion < 2.5%; [2]Christian represents Catholics, Protestants, Anglicans, Kimbanguistes and Christian Revival Church; not Christian represents Muslims, Animists, and atheists; [3]Participants who not yet ended their level were included in that level; [4]Taking into account local parameters, poverty was calculated considering the type of the floor, water source, electricity and commodities in the house. The total score ranged from 4 to 17, a score < 10 was considered under the threshold of poverty, a score ≥10 was considered above the threshold of poverty. We did not include income in the score calculation because it is very unstable and depends mainly on the informal sector, [5]Participants were symptomatic if they presented with abnormal vaginal discharge, vaginal itching, burning vaginal sensation after sexual intercourse and/or foul smell from vagina.

listed in Table 4. Cluster 1 was the only cluster not containing *L. iners*. Also in cluster 1, almost no *G. leopoldii* was present. In cluster 2, also almost no *G. leopoldii* was present, while *G. swidsinskii* and *L. iners* were found in all women of this cluster. Cluster 3 was characterized by 100% abundance of *G. vaginalis*, and an absence of *G. swidsinskii*, while *G. leopoldii* was found among approximately one fifth of the women in this cluster. In cluster 4, *G. leopoldii* was 100% abundant, while *G. swidsinskii* was hardly present, and *G. piotii* and *L. crispatus* were found in one quarter of the women in this cluster. In cluster 5,

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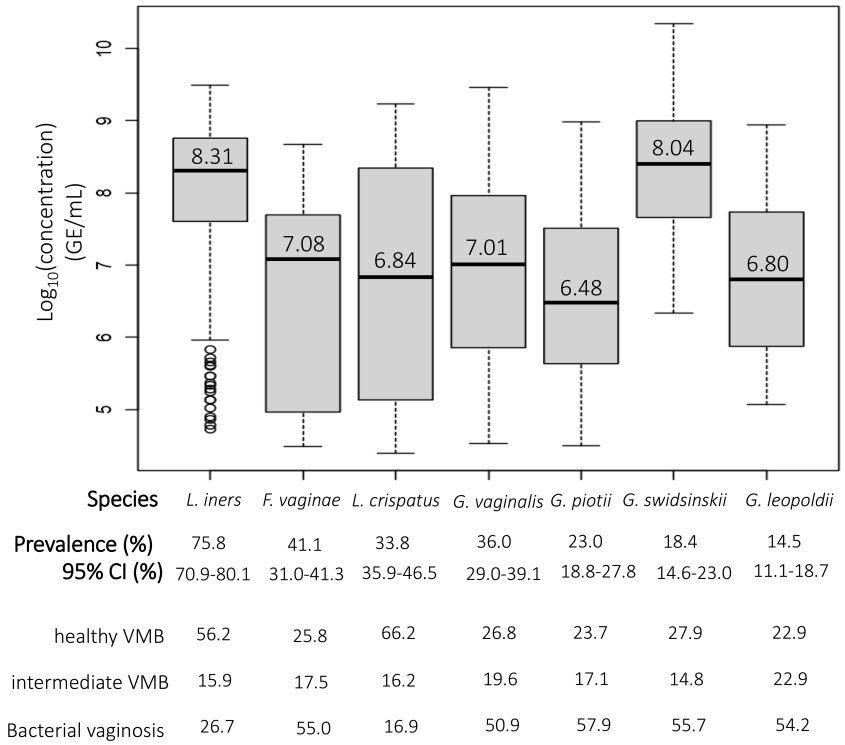

FIGURE 2
The prevalence and median concentrations of the different species. The black horizontal line in each boxplot represents the median concentration of each species among women positive for that species, which is also shown above this line; the bottom and top edge of the grey boxes represent the first (25% of the data is below this value) and third quartile (75% of the data is below this value), respectively; bottom and top end of the dotted line represent minimum and maximum, respectively; open circles represent outliers.

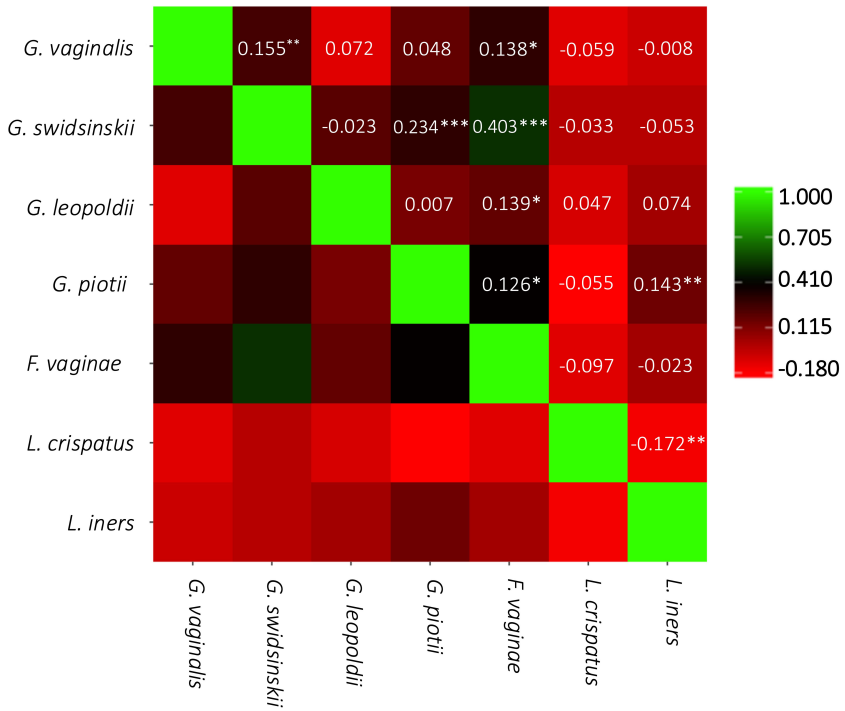

FIGURE 3
Pairwise correlation matrix. Pearson correlation coefficients are represented by the color scale and are shown in the squares. ***p<0.001; **p<0.01; *p<0.05.

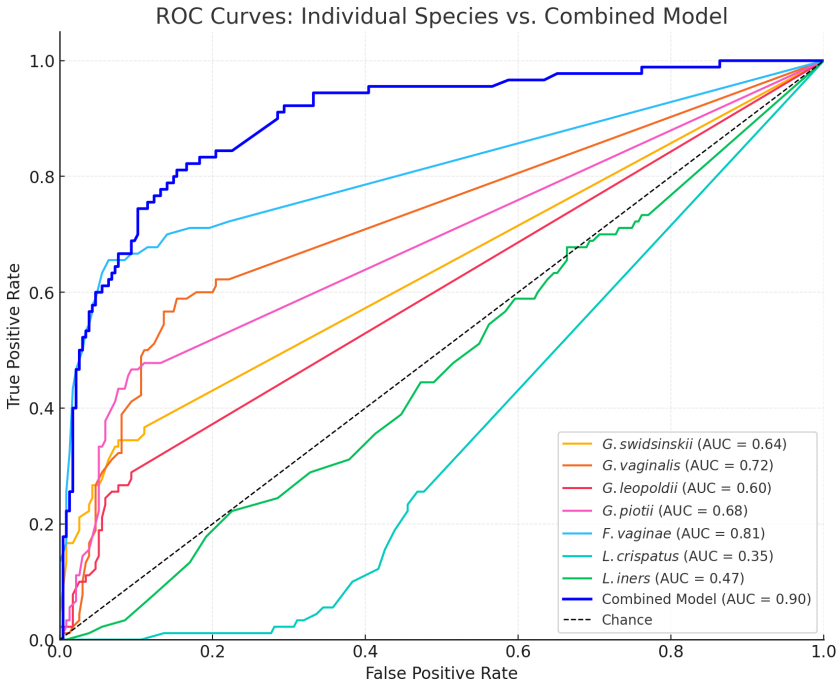

FIGURE 4
Receiver operator characteristics (ROC) curves for all investigated species and the multiple logistic regression model. AUC, area under the curve. The dashed diagonal line represents random performance (AUC = 0.50).

L. iners was 100% abundant, with hardly any *L. crispatus*, and *G. piotii* as the only *Gardnerella* species. In cluster 6, both *L. iners* and *L. crispatus* were highly abundant, while (almost) no *G. swidsinskii*, *G. leopoldii* and *G. piotii* were found among this cluster.

Univariate associations of the different species and clusters with laboratory findings and clinical signs and symptoms

The statistically significant univariate associations of the presence of the different species with laboratory findings and clinical signs and symptoms are summarized in Table 5. Further results of these univariate analyses (which were not statistically significant) for the different species are shown in Supplementary Information 1 (SI1) to SI7. The results of the univariate analysis across all clusters are shown in SI8.

All species except for *L. iners* were significantly associated with the Nugent score and the corresponding categorization of the VMB. Furthermore, *G. piotii* was positively associated with clinical symptoms and markers of urinary tract infections, i.e. dysuria (OR: 2.18; 95% CI: 1.20-3.92), nitrite levels measured with urine dipstick (OR: 7.43; 95% CI: 1.92-34.77) and white blood cell levels measured with urine dipstick. Also, women colonized with *G. piotii* had higher odds for anemia compared to women not colonized with *G. piotii* (OR: 2.68; 95% CI: 1.01-6.48). *G. vaginalis* showed a positive association with the Whiff test (OR: 2.98; 95% CI: 1.32-6.91). Women colonized with *F. vaginae* also showed increased odds for a positive Whiff test

TABLE 3 Diagnostic performance of each investigated species and the multiple logistic regression model for bacterial vaginosis.

Species/Model	AUC	Youden Index	Threshold	Sensitivity (%)	Specificity (%)
F. vaginae	0.81	0.59	6.6	65.6	93.6
G. leopoldii	0.60	0.20	5.1	28.9	90.6
G. piotii	0.68	0.37	5.0	46.7	90.6
G. swidsinskii	0.64	0.27	7.3	34.4	92.3
G. vaginalis	0.72	0.44	5.5	58.9	84.7
L. crispatus	0.35	0.00	10.2	0.0	1.0
L. iners	0.47	0.01	6.7	67.8	33.6
Combined model	0.90	0.66	0.51	81.1	84.7

AUC, area under the curve. Threshold values are in logarithmic scale.

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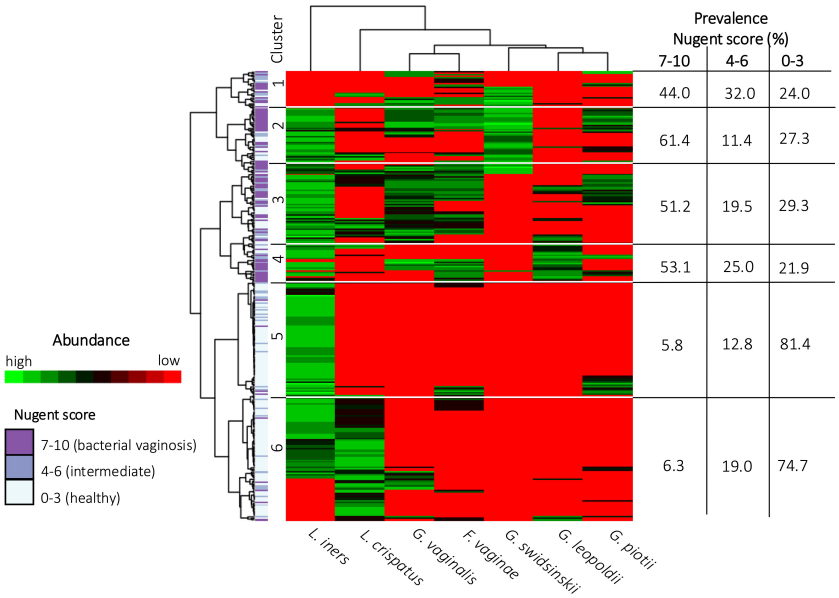

FIGURE 5
Heatmap of the vaginal microbiome. Each row depicts a woman's vaginal microbiome profile and each column represents a bacterial species. The hierarchical clustering of vaginal microbiome profiles and bacterial species is based on log-transformed concentrations of bacterial species. The color scale ranges from red, indicating the absence of bacterial species, to green, indicating a high concentration of bacterial species. The categorization of the vaginal microbiome in healthy, intermediate or BV is shown on the left.

compared to women not colonized with *F. vaginae* (OR: 3.68; 95% CI: 1.61-8.85). Furthermore, *F. vaginae* was significantly associated with an increase in vaginal pH and white bloods cell levels determined on wet mount as well as with urine dipstick. *L. crispatus*, on the other hand, showed a significant association with a decrease in vaginal pH. For *L. iners* a significant positive association was found with maternal fever at V1 (OR: 3.71; 95% CI: 1.11-19.47) and *Candida* on wet mount (OR: 1.94; 95% CI: 1.00-3.98).

After one-on-one comparison with reference cluster 6, significant differences were found for Whiff test with cluster 2, with cluster 3 and with cluster 4. Cluster 6 and cluster 2 differed significantly with regard to *Candida* on wet mount. For Nugent score and VMB-categorization according to Nugent, cluster 1 to cluster 4 differed significantly from cluster 6. For fever the significant difference was between clusters other than reference cluster 6.

Univariate associations of the different species and clusters with pregnancy outcomes

The associations between the different species and pregnancy outcomes are shown in SI1-7. A significant positive association was found between LBW and two species, i.e. *G. vaginalis* (OR: inf; 95% CI: 3.03-inf, based on all seven women with a LBW baby being colonized with *G. vaginalis*) and *F. vaginae* (OR: 9.50; 95% CI: 1.12-444.16, based on six of the seven women with a LBW baby being positive for *F. vaginae*) (SI4 and SI5). Furthermore, women colonized with *L. iners* showed an almost four times higher odds for delivering a baby preterm compared to women not colonized with *L. iners* (OR: 3.73; 95% CI: 1.07-20.08) (SI7). However, also 70.6% of women with term birth were colonized by *L. iners*, although the mean *L. iners*

TABLE 4 Prevalence of species in the clusters. N, number of participants.

Cluster (N)	*Lactobacillus iners* (%)	*Lactobacillus crispatus* (%)	*Gardnerella vaginalis* (%)	*Fannyhessea vaginae* (%)	*Gardnerella swidsinskii* (%)	*Gardnerella leopoldii* (%)	*Gardnerella piotii* (%)
1 (26)	0.0	26.9	46.2	65.4	57.7	3.8	23.1
2 (45)	100.0	28.9	60.0	60.0	100.0	4.4	48.9
3 (42)	100.0	47.6	100.0	83.3	0.0	21.4	52.4
4 (32)	87.5	25.0	46.9	46.9	3.1	100.0	25.0
5 (87)	100.0	1.3	0.0	13.9	0.0	0.0	16.5
6 (79)	66.7	100.0	18.4	17.2	0.0	4.6	5.7

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TABLE 5 Summary of univariate associations between bacterial species and clinical signs and symptoms.

	Bacterial species present	Bacterial species absent	p-value	Odds ratio (95% CI)
Gardnerella leopoldii (48 positive, 283 negative)				
Vaginal microbiome characterization				
Healthy VMB, n (%) (N=176)	11 (22.92)	165 (59.35)	<0.001	REF
Intermediate VMB, n (%) (N=59)	11 (22.92)	48 (17.27)		5.95 (2.66-14.17)
Bacterial vaginosis, n (%) (N=91)	26 (54.17)	65 (23.38)		3.42 (1.26-9.29)
Mean Nugent score	5.69	2.98	0.001	NA
Gardnerella piotii (76 positive, 255 negative)				
Dysuria, n (%) (N=86)	29 (39.19)	57 (22.80)	0.007	2.18 (1.20-3.92)
Anemia, n (%) (N=24)	10 (13.51)	14 (5.49)	0.038	2.68 (1.01-6.84)
Nitrite urine dipstick, n (%) (N=12)	8 (10.67)	4 (1.57)	0.001	7.43 (1.92-34.77)
Vaginal microbiome characterization				
Healthy VMB, n (%) (N=176)	18 (24.00)	158 (62.95)	<0.001	REF
Intermediate VMB, n (%) (N=59)	13 (17.33)	46 (18.33)		8.14 (4.16-16.49)
Bacterial vaginosis, n (%) (N=91)	44 (58.67)	47 (18.73)		2.47 (1.03-5.80)
White blood cells urine dipstick				
≥ 25, n (%) (N=19)	3 (4.00)	16 (6.27)	0.019	REF
≥ 50, n (%) (N=45)	12 (16.00)	33 (12.94)		0.52 (0.08-2.32)
≥ 75, n (%) (N=70)	25 (33.33)	45 (17.65)		0.34 (0.06-1.36)
Negative, n (%) (N=196)	35 (46.67)	161 (63.14)		0.86 (0.15-3.26)
Mean Nugent score	5.92	2.62	<0.001	NA
Gardnerella swidsinskii (61 positive, 270 negative)				
Vaginal microbiome characterization				
Healthy VMB, n (%) (N=176)	17 (28.33)	159 (59.77)	<0.001	REF
Intermediate VMB, n (%) (N=59)	9 (15.00)	50 (18.80)		5.54 (2.77-11.45)
Bacterial vaginosis, n (%) (N=91)	34 (56.67)	57 (21.43)		1.68 (0.62-4.28)
Mean Nugent score	5.57	2.89	<0.001	NA
Gardnerella vaginalis (112 positive, 219 negative)				
Positive whiff test, n (%) (N=31)	18 (16.07)	13 (6.02)	0.005	2.98 (1.32-6.91)
Vaginal microbiome characterization				
Healthy VMB, n (%) (N=176)	30 (27.52)	146 (67.28)	<0.001	REF
Intermediate VMB, n (%) (N=59)	22 (20.18)	37 (17.05)		8.08 (4.40-15.17)
Bacterial vaginosis, n (%) (N=91)	57 (52.29)	34 (15.67)		2.88 (1.41-5.85)
Mean Nugent score	5.47	2.33	<0.001	NA
Fannyhessea vaginae (120 positive, 211 negative)				
Positive whiff test, n (%) (N=31)	20 (16.95)	11 (5.24)	0.007	3.68 (1.61-8.85)
Vaginal microbiome characterization				
Healthy VMB, n (%) (N=176)	31 (26.27)	145 (69.71)	<0.001	REF
Intermediate VMB, n (%) (N=59)	21 (17.80)	38 (18.27)		12.19 (6.50-23.61)
Bacterial vaginosis, n (%) (N=91)	66 (55.93)	25 (12.02)		2.57 (1.26-5.23)

(Continued)

TABLE 5 Continued

	Bacterial species present	Bacterial species absent	p-value	Odds ratio (95% CI)
Fannyhessea vaginae (120 positive, 211 negative)				
White blood cells urine dipstick				
≥ 25, n (%) (N=19)	7 (5.88)	12 (5.69)	0.017	REF
≥ 50, n (%) (N=45)	19 (15.97)	26 (12.32)		0.80 (0.22-2.72)
≥ 75, n (%) (N=70)	35 (29.41)	35 (16.59)		0.59 (0.17-1.84)
Negative, n (%) (N=196)	58 (48.74)	138 (65.40)		1.39 (0.44-4.05)
Mean number of white blood cells on wet mount per field	9.82	8.35	0.045	NA
Mean Nugent score	5.56	2.14	<0.001	NA
Mean vaginal pH	6.07	5.87	0.001	NA
Lactobacillus crispatus (136 positive, 195 negative)				
Vaginal microbiome characterization				
Healthy VMB, n (%) (N=176)	90 (66.67)	86 (45.03)	<0.001	REF
Intermediate VMB, n (%) (N=59)	22 (16.30)	37 (19.37)		0.32 (0.18-0.58)
Bacterial vaginosis, n (%) (N=91)	23 (17.04)	68 (35.60)		0.57 (0.29-1.08)
Mean Nugent score	2.43	4.05	<0.001	NA
Mean vaginal pH	5.88	5.98	0.043	NA
Lactobacillus iners (251 positive, 76 negative)				
Maternal fever, n (%) (N=37)	34 (13.77)	3 (4.11)	0.022	3.71 (1.11-19.47)
Candida on wet mount, n (%) (N=91)	77 (30.80)	14 (18.67)	0.041	1.94 (1.00-3.98)

CI, confidence interval.
NA, not applicable.

concentration was twice as high in women with PTB compared to women who delivered at term (p<0.05) (data not shown). For the different clusters, no significant associations were found with any of the pregnancy outcomes.

Discussion

Prevalence and cooccurrence

In this study we investigated the distribution and clinical correlates of four *Gardnerella* species, *L. crispatus*, *L. iners* and *F. vaginae* related to women's reproductive health. To our best knowledge, the different *Gardnerella* species have not yet been studied in African women. In our study population of Congolese pregnant women, *G. vaginalis* was the most prevalent *Gardnerella* species (33.8%), followed by *G. piotii* (23.0%), *G. swidsinskii* (18.4%), and *G. leopoldii* (14.5%). This is in line with a study among Dutch women where *G. leopoldii* was also the least prevalent species (22.2%) (Schuster et al., 2024). Also, if colonized with *Gardnerella*, the proportion of women harboring one *Gardnerella* species was approximately equal to the proportion of women carrying more than one *Gardnerella* species. This is in line with a study by Berman et al., who, based on sequences derived from vaginal swab samples from three demographically distinct cohorts of pregnant women, showed that metagenomes in which *Gardnerella* was present often contained more than one species (mean 3.92) (Berman et al., 2024), and with other previous work that has shown the majority of women are being colonized by more than one *Gardnerella* species (Munch et al., 2024; Schuster et al., 2024). Also noteworthy here is that *G. swidsinskii* had a median concentration around ten times higher compared to the other *Gardnerella* species.

In 2019, Hill and coworkers investigated the distribution of *Gardnerella* species among *Gardnerella*-positive non-pregnant Canadian women based on cpn60 deep sequencing (Hill and Albert, 2019). They reported *G. vaginalis* to be the most prevalent *Gardnerella* species (68.4%), followed by *G. swidsinskii* (49.2%), *G. leopoldii* (26.2%) and *G. piotii* (25.2%). These prevalences are considerably higher compared to ours, likely because only *Gardnerella*-positive women (72.9% of their total study population) were analyzed. In their study, they also reported a statistically significant co-occurrence between *G. swidsinskii* and *G. vaginalis*, and *G. piotii* and genomospecies 3, while *G. swidsinskii* and *G. leopoldii* significantly did not occur together. This is partly in line with findings reported here, since we also observed a positive correlation between *G. swidsinskii* and *G. vaginalis*. We did, however, also find a statistically significant correlation between *G. swidsinskii* and *G. piotii*.

In our study population, *L. iners* was the most prevalent species (75.8%), which is in line with results from numerous previous studies in African populations (Vaneechoutte, 2017a) (Vaneechoutte,

2017b). We found a negative correlation between *L. iners* and *L. crispatus* (r = -0.172), which is also in line with most literature, documenting single species dominance of lactobacilli in the healthy VMB (Vaneechoutte, 2017a). Only very weak correlations were seen between *L. crispatus* and *Gardnerella* species, while a positive correlation did exist between *L. iners* and *G. piotii* (r = 0.143). For *F. vaginae* a co-occurrence was reported with *G. swidsinskii* (r = 0.403), with *G. leopoldii* (r = 0.139) and with *G. piotii* (r = 0.126), which is in line with the general knowledge that *F. vaginae* often occurs together with *Gardnerella* (Bradshaw et al., 2006).

Diagnostic markers

BV-associated species such as *G. vaginalis*, *F. vaginae*, *Megasphaera* type 1, bacterial vaginosis-associated bacterium-2 (BVAB-2) and/or species associated with health, such as *Lactobacillus* spp., are commonly utilized as qPCR targets in both commercial [e.g. BD MAX™ Vaginal Panel (BD), Xpert Xpress MVP (Cepheid)] and in-house assays for BV detection. However, some of these markers, for example BVAB-2, have been shown to differ in prevalence in women found positive for BV depending on race (Srinivasan et al., 2012).

In our study population, we showed that *F. vaginae* was the best species marker for BV according to ROC analysis, with a sensitivity of 66% and specificity of 94%, using Nugent-BV as reference. This is in line with several previous studies that have shown that *F. vaginae* is a more specific marker for BV compared to the different *Gardnerella* species investigated (De Backer et al., 2007; Marconi et al., 2012; Vaneechoutte, 2017b). When considering combinations of species, our logistic regression model increased the AUC to 0.90, with a sensitivity and specificity of 81% and 85%, respectively.

Other studies using similar approaches of combining species markers to diagnose BV reported higher sensitivities and specificities (Fredricks et al., 2007; Hilbert et al., 2016; Munch et al., 2024). This might partly be due to the fact that we did not consider markers such as *Megasphaera* and BVAB-2, which have been found to have sensitivities and specificities of around 96% and 94%, respectively, compared to Nugent-BV (Fredricks et al., 2007). However, the same study also documented that *Fannyhessea* alone had a sensitivity and specificity of 96% and 85%, respectively, outperforming our combined model (Fredricks et al., 2007). Also in the study of Hilbert and coworkers, *Fannyhessea* alone had a sensitivity and specificity of 87% and 91%, respectively, and the overall model had a sensitivity and specificity of 92% and 95%, respectively (Hilbert et al., 2016). Taken together, above findings might suggest that optimal (combined) molecular BV-markers are population dependent.

The AUCs for the investigated *Gardnerella* species were similar (ranging from 0.60 for *G. leopoldii* to 0.72 for *G. vaginalis*), with *G. vaginalis* having the highest sensitivity but the lowest specificity. *G. vaginalis* was also suggested as the best marker among the different *Gardnerella* species by Munch and colleagues (Munch et al., 2024). Furthermore, all *Gardnerella* species and *F. vaginae* were positively, and *L. crispatus* negatively associated with the Nugent score and Nugent categorization, which confirms their role as key markers of (Nugent) BV also in our study population of women from Bukavu (DRC).

Univariate species associations

None of the seven investigated species showed a significant association with the symptoms typically associated with BV (i.e. discharge and malodor), or less typically associated with BV (itching and burning). Not stratifying our data for *Candida* carriage here might explain this observation, since this is a known confounder of BV and we previously showed that in *Candida*-negative women BV was significantly associated with malodor, while in *Candida*-positive women BV was not significantly associated to any typical symptom (De Keyser, 2020).

We did find a statistically significant association between *G. piotii* and both symptoms (dysuria) and laboratory markers (nitrite and white blood cells on dipstick) of urinary tract infections (UTIs). *Gardnerella* has previously been identified as a pathogen causing UTIs (Woolfrey et al., 1986) and, in a mouse model, *G. piotii* has been shown to facilitate *Escherichia coli* UTIs (O'Brien et al., 2020). *G. piotii*, in contrast to other *Gardnerella* spp., possesses the gene for extracellular sialidase activity (Kurukulasuriya et al., 2021), which in *Bifidobacterium bifidum* has been shown to enhance mucosal adhesion in *in vitro* experiments (Nishiyama et al., 2017). Hence, this trait could enhance bladder mucosal adhesion of *G. piotii* or other pathogens. Facilitation of adherence by other pathogens might be the more likely explanation, given that *Gardnerella* is thought to be negative for nitrate reduction (Taylor-Robinson, 1984), although this has not been investigated for the different species.

A significant positive association was also seen for *G. piotii* with anemia (hemoglobin levels <12.0 g/dL). Verstraelen and coworkers documented that subclinical iron-deficiency in Belgian pregnant women was an independent predictor for BV (Verstraelen et al., 2005) and it has also been demonstrated that *Gardnerella* can use human hemoglobin as a source of iron (Jarosik et al., 1998). Brabin and coworkers, in contrast, showed that iron-deficient women from Burkina Faso were more likely to have a normal VMB compared to iron-replete women, but also that the prevalence of vaginal discharge was significantly higher among iron-deficient women (Brabin et al., 2017).

Both *G. vaginalis* and *F. vaginae* were found to show a positive association with a positive Whiff test, one of the four Amsel criteria used for the clinical diagnosis of BV (Amsel et al., 1983). In a Whiff test, 10% potassium hydroxide (KOH) is added to vaginal discharge, and a fishy smell (positive Whiff test) is caused by aromatization of aromatic amines by anaerobes associated with BV. *In vitro* and bio-informatic analyses suggest that *Prevotella* but not *Gardnerella* is involved in the production of these amines (Nelson et al., 2015), but this has not yet been studied using various *Gardnerella* species.

Univariate cluster associations

All *Gardnerella* species were found among the clusters containing mainly Nugent-BV-positive women (i.e. cluster 1 to cluster 4), suggesting that not a single *Gardnerella* species but rather an interplay between different species plays a role in (asymptomatic) BV. This is in line with previous studies showing

the majority of women are colonized by more than one *Gardnerella* species (Berman et al., 2024; Munch et al., 2024; Schuster et al., 2024). *G. swidsinskii* and *G. leopoldii* were not and nearly not (4.6%), respectively, observed among clusters with mainly women without Nugent-BV (i.e. cluster 5 and cluster 6), confirming the above described finding that these species are more specific markers for BV than *G. vaginalis*.

We hypothesized that distinct *Gardnerella* species specific clusters within women with Nugent BV existed and were associated to different degrees with clinical signs and symptoms and/or pregnancy outcomes. However, we showed that, although clusters could be defined based on the *Gardnerella* species composition, no signs and symptoms, with the exception of Whiff test, nor pregnancy outcomes were associated with these clusters. This is in line with the fact that we also did not find single *Gardnerella* species to be associated with clinical signs and symptoms and/or pregnancy outcomes (with the exception of *G. vaginalis* being associated with pH, and with LBW, albeit with a very broad 95% CI for the OR).

In cluster 5, which was dominated by *L. iners* and mostly contained women with a healthy Nugent score (74.7%), we saw no statistically significantly lower pH compared to the clusters 1-4, which is somewhat unexpected since this cluster represents mostly healthy women, but is in line with a report stating that the pH is not always lowered in case of *L. iners* predominance (Vaneechoutte, 2017b). Likewise, for cluster 6, which was dominated by *L. crispatus* and also mainly represented healthy women according to Nugent (81.4%), no statistically significant difference in pH was found compared to clusters 1-4.

A previous meta-analysis showed a twofold higher odd for PTB in women with BV (Vandenwyngaerden et al., 2020). In our study population, however, we previously showed that Nugent-BV was not significantly associated with PTB, but with LBW (Mulinganya et al., 2021). Here, using *Gardnerella*-species specific qPCR, offering absolute quantification and a higher taxonomic resolution compared to the Nugent scoring system, we showed that there was no association between different key markers of BV (*Gardnerella*, *F. vaginae*) and a healthy VMB (*L. crispatus*), and PTB. This is in contrast to a study in a cohort of pregnant Dutch women at high risk of recurrent preterm birth, where *G. leopoldii* was in fact associated with spontaneous preterm birth (Schuster et al., 2024).

In contrast, here, we did find that women with PTB had a significantly nearly four times higher odds of *L. iners* compared to women giving birth at term, and we see a higher concentration of *L. iners* among women with preterm birth than among the *L. iners*-carrying women with term birth. These findings are in line with results presented by Petricevic and colleagues, who studied pregnant Austrian women and reported a significantly higher prevalence of *L. iners* among women with PTB (85%) compared to women with term birth (16%) (Petricevic et al., 2014). In another study (among English women with an increased risk of PTB), *L. iners* dominance at 16 weeks of gestation, but not vaginal dysbiosis, was found to be significantly associated with PTB (<34 + 0 weeks) (Kindinger et al., 2017). Fettweis and colleagues, on the other hand, demonstrated that women who delivered at term showed a significant increase in the prevalence of *L. iners* (Fettweis et al., 2019).

Study limitations

Our study was subject to several limitations. First, approximately one third of study participants dropped out between the first visit and follow up, causing the number of women who delivered at the PRHB hospital to be less than foreseen. Second, women with complaints were treated after the first visit, which could have masked certain correlations. Lastly, we only studied a limited scope of seven key species, excluding other potential marker species such as *Prevotella* spp., *L. gasseri*, *L. jensenii* and *L. vaginalis*.

Conclusion

G. vaginalis was the most prevalent *Gardnerella* species in pregnant women from Bukavu (DRC), and *G. leopoldii* was the least prevalent species. Our results suggest that all *Gardnerella* species are involved in BV, although none were associated with the most clinically important BV symptoms. However, *G. piotii* was associated with markers of urinary tract infection. *F. vaginae* was the best single species diagnostic marker for BV.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Internal Review Board of the Catholic University of Bukavu (reference number UCB/CIE/NC/016/2016), Ministry of Public Health (reference number 062/CD/DPS/SK/2017) and Ethical Committee of Ghent University Hospital (reference number PA2014/003). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

LH: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. GM: Conceptualization, Data curation, Funding acquisition, Investigation, Writing – review & editing. TR: Investigation, Methodology, Validation, Writing – review & editing. GB: Investigation, Writing – review & editing. FK: Investigation, Writing – review & editing. YK: Investigation, Writing – review & editing. JM: Investigation, Writing – review & editing. IM: Investigation, Writing – review & editing. SC: Conceptualization,

Funding acquisition, Writing – review & editing. MV: Conceptualization, Resources, Validation, Writing – review & editing. PC: Conceptualization, Data curation, Methodology, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2024.1514884/full#supplementary-material

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Reproductive tract microbiome dysbiosis associated with gynecological diseases

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Female health and the microbiota of the reproductive tract are closely associated. The research scope on reproductive tract microbiota extends from the vaginal to the upper reproductive tract and from infectious diseases to various benign and malignant gynecological and obstetrical diseases. The primary focus of this paper was to evaluate the most recent findings about the role of reproductive tract microbiota in gynecological diseases, including endometrial polyps, uterine fibroids, endometriosis, adenomyosis, endometrial hyperplasia, and endometrial carcinoma. Different stages of gynecological diseases have diverse microbiota in the female reproductive tract, and some specific bacteria may help the disease progress. For example, *Fusobacterium* may exacerbate endometriosis, while treatments that target microbiota, such as antibiotics, probiotics, and flora transplantation, showed some efficacy in the experiment. These findings indicate the wonderful prospect of this field. Additionally, we have discussed how microbiome research can improve our understanding of the interactions between reproductive tract microorganisms and hosts, aid in the screening and diagnosis of gynecological diseases, and direct the development of preventive and therapeutic strategies aimed at maintaining and restoring a healthy reproductive tract microbiota when combined with other technologies like transcriptome and proteome, *in vitro* cultured cells, and animal models.

KEYWORDS

reproductive tract, microbiome, gynecological diseases, vaginal microbiome, endometrial microbiome, endometrial cancer, endometriosis (EM)

1 Introduction

Research on the microbiota of the female reproductive system was mainly focused on the lower reproductive tract in the era when technology based on culture was used to detect the composition of microorganisms (Medina-Bastidas et al., 2022). *Lactobacillus* makes up most of the vaginal microbiota in healthy women of childbearing age, which prevents the

growth of pathogenic microorganisms by maintaining a low vaginal pH, competing for nutrients, preventing adhesion, producing antimicrobial metabolites, and regulating the local immune response (Verstraelen et al., 2022). During this time, the research on the connection between vaginal microbiota and health was mainly focused on a few prevalent infectious diseases, such as vulvovaginal candidiasis and bacterial vaginosis (Pereira et al., 2021).

Two innovation points in the research of reproductive tract microbiota emerged with the advancement of genomics, particularly meta-genomics and next-generation sequencing (NGS) technologies (Li et al., 2018). First, researchers became aware of the microbiota in the upper reproductive tract, which was previously believed to be sterile. According to studies, the uterus of healthy people is microbially colonized, and the health of the female reproductive system is directly correlated with the microbiota of the upper reproductive tract (Rampersaud et al., 2012). Due to ethical considerations and limitations in sampling methods (Yuan et al., 2024), studies on the endometrial microbiome of healthy individuals without any gynecological or obstetric disorders are indeed scarce. Although there is still debate on the makeup of a healthy endometrial microbiota, mounting research suggests that *Lactobacillus* is the most prevalent genus in a relatively healthy uterus (Chen et al., 2017; de Medeiros Garcia Torres and Lanza, 2024; Peric et al., 2019). In addition, the focus of study on reproductive tract microbiota has shifted from infectious diseases to a range of obstetrical and gynecological diseases, including endometrial cancer, endometriosis, and infertility. These discoveries might help with the pathogenesis of the disease, diagnosis, and treatment. This paper primarily evaluated recent developments in the research of reproductive tract microbiota in gynecological diseases and examined the relationship and potential interactions between microbiota and common gynecological diseases. It also discussed the possibility of using the microbiome as an adjunct to current treatments and a potential biomarker for identifying gynecological diseases.

2 Reproductive tract microbiota in gynecological diseases

2.1 Endometrial polyps

Endometrial polyps (EP), a prevalent benign gynecological disease, with an incidence of 7.8%-34.9% (Anonymous, 2012; Salim et al., 2011), is characterized by excessive growth of glands, stromal cells and blood vessels in the endometrium, which is usually associated with abnormal uterine bleeding (AUB). According to some studies, EP is associated with chronic endometritis (CE) (El-Hamarneh et al., 2013), whereas CE may result from an overgrowth of *Ureaplasma urealyticum* or common bacteria (Cicinelli et al., 2009). Thus, the EP may be related to the prolonged existence of certain microorganisms. The vaginal and uterine microbiomes of patients with EP, patients with EP and CE, and healthy women were compared in a 2016 study with 10 subjects in each group. It was discovered that while *Proteobacteria*, *Firmicutes*, and *Actinobacteria* dominated the intrauterine microbiota of all samples, the proportion of *Proteobacteria* was much lower in patients with EP than that of healthy women, while the proportion of *Firmicutes* was significantly higher than that of healthy women. In addition, the proportion of *Pseudomonas* was significantly lower in patients with EP, while the proportion of *Lactobacillus*, *Gardnerella*, *Bifidobacterium*, *Streptococcus*, and *Alteromonas* was significantly higher than the control group. Patients with EP generally had a more diverse intrauterine microbiome than the control group. Whether EP patients were with CE or not had no significant effect on their intrauterine microbiome (Fang et al., 2016). It is worth noting that this study included healthy women as the control group, which few other studies did. These women underwent hysteroscopy and laparoscopy because of infertility of their male partners, while they had regular menstrual cycles and no structural abnormalities of the uterus. This eliminates the interference, making it possible to figure out the characteristic bacteria of the disease.

Liang et al. prospectively collected vaginal, cervical and uterine microbiological samples from 134 infertile patients. They found that the microbiota in the reproductive tracts of patients with CE and EP differed from that of the healthy control group. For example, compared with the control group, the distribution of *Firmicutes* throughout the reproductive tract of EP patients was significantly increased. In contrast, the distribution of *Proteobacteria* was significantly lower, which is consistent with the findings of previous studies. In contrast to previous studies, the abundance of *Lactobacillus* in the EP group was lower than that in the control group (Liang et al., 2023). Their study was an essential reference for subsequent research because it included a sufficient sample size and adequate sampling locations. Future research on the microbiome of endometrial polyps may focus on specific clinical problems, such as recurrence and response to treatment, to identify suitable biomarkers or therapeutic targets.

2.2 Leiomyoma

Leiomyomas, or uterine fibroids, are the most prevalent benign gynecological neoplasms. They are characterized by muscle and fibrous tissue growths that can cause infertility, heavy or prolonged menstrual bleeding, anemia (Donnez and Dolmans, 2016; Zepiridis et al., 2016), and significant personal and social problems (Millien et al., 2021). There is an increasing understanding of hormones, genetics, epigenetics, and growth factors in the development and progression of uterine fibroids (Torres-de-la-Roche et al., 2017); however, the exact pathogenesis is still unclear. According to some research, bacteria may cause inflammation that contributes to the pathogenesis of uterine fibroids. A study reported that the TLR4/MyD88/NFKB signaling pathway in primary cultured human fibroblasts from leiomyomas was activated under *E. coli* LPS treatment, which suggested that bacteria may be involved in the pathogenesis of leiomyomas by inducing cell proliferation through inflammation (Guo et al., 2015).

According to a study comparing the microbiota of the vagina, cervix, endometrium, and pouch of Douglas in 20 patients with leiomyoma and controls, *Lactobacillus* sp. was found to be less abundant in the vaginal and cervical samples from leiomyoma patients, but *L. iners* was more abundant in the cervix (Chen et al., 2017). There was no significant difference in microbial diversity between patients with leiomyoma and controls in another study that examined the vaginal and cervical microbiomes of 29 patients with leiomyoma and 38 healthy women. Interestingly, alpha diversity decreased in patients with leiomyoma as the number of fibroids increased. At the phylum level, an increase in *Firmicutes* in the vagina and cervix was observed in patients with leiomyoma. *Erysipelotrichaceae* UCG-003 and *Sporolactobacillus* were significantly less common in patients with leiomyoma, whereas *Erysipelatoclostridium*, *Mucispirillum*, and *Finegoldia* were significantly enriched in the differential analysis of relative abundance. Moreover, the microbial co-occurrence networks exhibiting lower connectivity and complexity in patients with leiomyoma suggested decreased interactions and stability of the microbiota in these patients compared to healthy individuals (Mao et al., 2023). The research mentioned above revealed the characteristic microbiome of the reproductive tract associated with leiomyoma, provided clear evidence of microbial dysbiosis in patients with leiomyoma, and restricted the scope of screening for associated microorganisms potentially implicated in the pathogenesis of leiomyoma. More research is required to fill the knowledge gap of microbial dysbiosis and leiomyoma interaction.

2.3 Endometriosis

Endometriosis, a gynecological disease that affects 6%-10% of women of childbearing age worldwide, is characterized by the growth of endometrial tissue outside the uterus, including glands and stroma. This disease results in progressive secondary dysmenorrhea and difficulty in having sexual intercourse as well as infertility (Dai et al., 2018). The pathogenesis of endometriosis has not been clarified yet, and scientists are primarily interested in the retrograde menstruation hypothesis. However, 90% of the women have retrograde menstruation, while only 10% have the disease (Akiyama et al., 2019; Bellelis et al., 2019; Gueuvoghlanian-Silva et al., 2018; Khan et al., 2018; Zhang et al., 2018), indicating that other factors may play a role in the pathogenesis of endometriosis. Research has demonstrated that the abundance of bacterial colonization in menstrual blood and endometrial tissue of patients with endometriosis is higher than that of average female (Khan et al., 2018, 2016). Therefore, it is essential to investigate if the microbiome of the reproductive tract is involved in the pathogenesis of endometriosis.

There is more clinical research on endometriosis and microbiome than on uterine fibroids and endometrial polyps (Akiyama et al., 2019; Ata et al., 2019; Chang et al., 2022; Chao et al., 2021; Chen et al., 2020; Hernandes et al., 2020; Huang et al., 2021; Le et al., 2022; Lu et al., 2022; Oishi et al., 2022; Perrotta et al., 2020; Wei et al., 2020; Wessels et al., 2021). In terms of vaginal microbiome, it was discovered that *Lactobacillus* was less common in the vaginal microbiome of patients with endometriosis than in the control group (Chao et al., 2021; Findeklee et al., 2023; Hernandes et al., 2020; Lu et al., 2022). The vaginal microbiome of patients with stages III or IV endometriosis and healthy women were compared by Ata et al (Ata et al., 2019). Patients with endometriosis did not have any detections of *Gemella* and *Atopobium* spp. Another research studied community state type (CST) in greater detail (Perrotta et al., 2020). They used machine learning methods to construct a classification model based on random forest to predict the Revised American Society for Reproductive Medicine (r-ASRM) stage of endometriosis. The study discovered that the *Anaerococcus* from the *Firmicutes* phylum could predict whether patients had stage I-II or III-IV endometriosis after accounting for the menstrual cycle. As the vaginal sample is convenient to obtain, the above research findings have great clinical application potential.

In terms of cervical microbiome, Akiyama et al. demonstrated that compared with the control group, patients with endometriosis had higher levels of *Corynebacterium*, *Enterobactericaea*, *Flavobacterium*, *Pseudomonas*, and *Streptococcus* in their cervical microbiota (Akiyama et al., 2019). NGS results were further confirmed using a real-time polymerase chain reaction to quantify bacteria. They found a statistically significant difference in the abundance of *Enterobacteriaceae* and *Streptococcus* between endometriosis and control. Furthermore, patients with stage I-II endometriosis and those with stage III-IV endometriosis exhibit differences in cervical microbiomes. For stages I and II, the researchers suggested *L. jensenii* or members of the *Corynbacteriales*, *Porphyromonadaceae*, and *Ruminococcaceae* as potential microbial biomarkers; for stages III and IV, they suggested *Bifidobacterium breve* and members of *Streptococcaceae* (Chang et al., 2022). Studies on vaginal and cervical microbiota of patients with endometriosis have shown an increase in the abundance of some low-abundance genera, including *Fannyhessea*, *Prevotella*, *Streptococcus*, *Bifidobacterium*, and *Veillonella*. The degree of endometriosis is correlated with the ratio of the cervical to vaginal abundance of these genera (Yang et al., 2023), indicating that specific genera may migrate between the vagina and cervix and contribute to the pathogenesis of endometriosis.

In the aspect of endometrial microbiome, a study included 12 patients with endometriosis and 9 subjects without endometriosis. It was found that among individuals with endometriosis, the abundance of the *Tepidimonas* genus and *Oxalobactaceae* and *Streptococcaceae* families increased, whereas the *Ralstonia* genus and *Burkholderiaceae* family decreased (Wessels et al., 2021). Another study included 36 patients with endometriosis and 14 control subjects and discovered a significant increase in *Pseudomonadaceae* in endometriosis. Notably, there was a significant increase in *Sphingobium* sp. and *Pseudomonas viridiflava* in the endometrium and peritoneal fluid in endometriosis (Wei et al., 2020), indicating that these could be potential biomarkers.

According to some researchers, an imbalance in microbiota may disrupt the immune system, which could increase pro-inflammatory cytokines, disrupt immune surveillance, and change

the profiles of immune cells. This immune disorder could become chronic inflammation, creating a vicious cycle leading to endometriosis (Jiang et al., 2021). Thus, research is being done on how to treat endometriosis by regulating the balance of microbiota. Patients with endometriosis had a significantly higher positive *Fusobacterium* rate in their endometrium than the control group. Following *Fusobacterium* infection of endometrial cells, the transforming growth factor-β signal pathway was activated, resulting in the transformation of quiescent fibroblasts into transgelin-positive myofibroblasts, which obtained the ability of adhesion, migration and proliferation *in vitro*. In the mouse model, the inoculation of *Fusobacterium* increased the number and weight of lesions of endometriosis, while therapy with metronidazole and chloramphenicol decreased the lesion. These results showed that endometriosis pathogenesis might involve *Fusobacterium* infection (Muraoka et al., 2023). Another study using animal models showed that the use of antibiotics or vaginal flora transplants in the vagina effectively treated endometriosis in mice (Lu et al., 2022). In this study, endometriosis mouse models were first established, then 10 mice received vaginal microbiota transplants from healthy mice, and 10 served as controls. The group receiving the vaginal microbiota transplant exhibited smaller endometriotic lesions compared to the control group ($p < 0.05$). The expression levels of cell proliferation marker Ki-67 and macrophage marker Iba-1 were both reduced in the transplant group. In terms of inflammatory cytokines, the levels of IL-1β, IL-6, and TNF-α in the peritoneal fluid of the microbiota-transplanted mice were lower than those in the control group. Additionally, the expression of TLR4, MyD88, and p65/p-p65 (NF-κB signaling pathway) in the lesions of the microbiota-transplanted mice was downregulated. Taken together, these results suggest that vaginal microbiota transplantation may modulate immune responses by regulating signaling pathways such as NF-κB and influencing the levels of inflammatory cytokines, thereby inhibiting the progression of endometriosis. Furthermore, this study implies that reproductive tract microbiota transplantation may have potential therapeutic effects not only for infections such as bacterial vaginosis but also for gynecological disorders like endometriosis. In patients with stage III and IV endometriosis, oral *Lactobacillus gasseri* OLL2809 relieved pain, according to a placebo-controlled randomized clinical trial (Khan et al., 2018). Another study discovered oral levofloxacin decreased cell proliferation, inflammation, and angiogenesis in endometrium and endometriosis lesions (Khan et al., 2021). The abovementioned studies demonstrated the promising future of the therapy targeted dysbacteriosis in treating endometriosis.

2.4 Adenomyosis

Adenomyosis is a benign disease characterized by the invasion of endometrial glands and stroma into the myometrium, resulting in uterine enlargement, increased menstrual bleeding, prolonged menstruation, and progressively deteriorating dysmenorrhea. It is common among women of childbearing age. The reproductive tract microbiome of adenomyosis is currently the topic of many studies. A study analyzed the vaginal, cervical and uterine microbiota of patients with adenomyosis, patients with endometriosis, patients with both adenomyosis and endometriosis, and healthy women. It revealed that *Atopobium* was more abundant in patients with both adenomyosis and endometriosis simultaneously than in the other three groups (Chen et al., 2020). A cross-validated random forest model based on operational taxonomic units (OTUS) from the vagina, cervix, or uterus was developed to differentiate patients with and without adenomyosis (Chen et al., 2017). Studies with larger sample sizes have revealed that patients with adenomyosis had a higher alpha diversity of vaginal microbiota than the control group. In adenomyosis, the abundance of *Alloscardovia*, *Oscillospirales*, *Ruminoccoccaceae*, etc. increased, while the abundance of *Megaspehera*, *Fastidiosipila*, *Hungateiclostridiaceae*, and *Clostridia* decreased. While CST-III and IV were dominant in adenomyosis, CST-IV dominated the control group (Kunaseth et al., 2022). Another study focused on the endometrial microbiome and found that *Citrobacter freundii*, *Prevotellacopri*, and *Burkholderiacepacia* are the most promising microbial biomarkers for adenomyosis (Lin et al., 2023). The clinical application of these findings and the clarification of the adenomyosis pathogenesis require further study.

2.5 Endometrial hyperplasia

Endometrial hyperplasia, characterized by an increase in the number of dilated glands and the ratio of glands to stroma, is commonly diagnosed worldwide, which can coexist or gradually progress into endometrial cancer (Sanderson et al., 2017). Some patients with endometrial hyperplasia have irregular uterine bleeding, while others may not exhibit any symptoms at all and are only diagnosed during routine physical examinations. In terms of pathogenesis, this disease occurs by a microenvironment high in estrogen and a heightened inflammatory response (Kubyshkin et al., 2016). Atypical hyperplasia, a subtype of endometrial hyperplasia, is a premalignant lesion of endometrial carcinoma, receiving clinical attention. Because of the heterogeneity of endometrial hyperplasia, it is necessary to make a sensitive and accurate diagnosis of the actual premalignant lesions and the appropriate course of treatment are required. The disease must primarily be distinguished from normal proliferative endometrium and endometrial carcinoma. The main non-invasive auxiliary examination is an ultrasound; invasive surgery is required to collect the histopathology results to confirm the diagnosis. Clinically, ultrasonic measurement is significantly influenced by the technology and the operator, and it is more challenging to monitor in situations like obesity. The extent of endometrial hyperplasia is frequently restricted; hence, the ultrasound readings can occasionally be inaccurate. In clinical practice, it is easy to underestimate and overestimate the lesions. This clinical situation needs to be fixed. On the one hand, invasive procedures such as endometrial sampling or uterine curettage for women with simple hyperplasia or even normal proliferative endometrium is a type of excessive medical treatment, which not only results in a waste of medical resources but also the potential complications of these operations, such as uterine adhesion and infection, may harm these women, especially since the majority of these women are of childbearing age. On the other hand,

misdiagnosing endometrial cancer as endometrial hyperplasia, or even misdiagnosing endometrial cancer and endometrial hyperplasia as normal endometrium, may result in errors in the selection of treatment and delay in the initiation of necessary treatment, which in the former case will prompt over-the-top medical care. Therefore, there is no doubt that the study of reproductive tract microbiota of endometrial hyperplasia is meaningful, which may help clarify the pathogenesis of endometrial hyperplasia and find suitable biomarkers for clinical diagnosis.

At present, most reproductive tract microbiome studies on endometrial hyperplasia studied endometrial carcinoma at the same time, and some studies even combined the two diseases into one group (Chao et al., 2022). A study published in 2016 included 4 patients with endometrial hyperplasia, 17 with endometrial carcinoma, and 10 with other benign gynecological diseases (Walther-António et al., 2016). The uterine α diversity in the hyperplasia group was similar to that in the carcinoma group, and was significantly higher than that in the benign group. Significant differences in the overall microbial flora structure among the three groups, mainly in the uterus rather than the vagina, were consistent with the α diversity analysis. The paired comparison of uterine samples showed no difference between endometrial hyperplasia and endometrial carcinoma. Simultaneously, there was a difference between the two groups and benign group respectively. According to the microbiome perspective, endometrial hyperplasia is the transitional stage from normal endometrium to endometrial cancer. The difference in the microbiota of endometrial hyperplasia from endometrial carcinoma and benign diseases makes exploring its characteristic microbial biomarkers possible. It is important to note that although correlation does not imply causation, the fact that two microorganisms found to be closely related to endometrial cancer, *A. vaginae* and *Porphyromonas* sp., did not exist in the vagina in endometrial hyperplasia, but exist in the uterine of half of the patients with endometrial hyperplasia, supported the role of these two microorganisms in early diseases, when taking into account the distance from the sample sites to the location of the disease. The primary shortcoming of this study is the insufficient number of patients with endometrial hyperplasia. Hence, the credibility of the relevant conclusions is low. Second, because the menopause and menstrual cycle were not considered in the design of the study, and because the factors mentioned above can affect the microbiome by hormone levels, it is crucial to evaluate the findings of this study carefully.

Another study included 26 patients with AUB and endometrial thickening identified by transvaginal ultrasound. Twelve of these patients had endometrial hyperplasia, and 14 had proliferative endometrium. In contrast to the previous study, it was discovered that the diversity of the vaginal microbial community in endometrial hyperplasia was much smaller than that in proliferative endometrium. This discrepancy may have resulted from differences in sample size, race, age, and other factors (Moosa et al., 2020). There were significant differences in the abundance of *Lactobacillus* in 15 genera, and if its abundance is used in the differential diagnosis of endometrial thickening, the sensitivity is 93%, and the specificity is 75% (Zhang et al., 2021). This study has a relatively sufficient sample size, and the choice of the control group is suitable for addressing clinical issues. Still, it ignores the impact of menstruation and menopause on the microbiome. According to the microbiome structure, endometrial hyperplasia can generally be distinguished from proliferative endometrium, suggesting that the microbiome may play a role in the development of the disease or that the difference may be a series of physiological changes in the local microenvironment brought on by the disease.

Even though the most recent study had a bigger sample size, it still mixed up pre-menopausal and post-menopausal patients, and its focus was only on the cervix and vagina (Barczyński et al., 2023). These research findings may aid in early detection and screening of endometrial hyperplasia using non-invasive techniques, given the accessibility of vaginal and cervical samples. However, the investigation of endometrial microbiota is more likely to further our understanding of pathogenesis. In general, we still know very little about the microbiome of endometrial hyperplasia and its subtypes, and the current research still has room to learn from the microbiome research of other diseases in terms of research design, as there are still a lot of open questions.

2.6 Endometrial cancer

Endometrial cancer is a common malignant tumor of the female reproductive system. Its pathogenesis has not been thoroughly elucidated, and genetics can only account for 20% of the incidence of endometrial cancer (Hampel et al., 2006). The remaining 80% of the causes could be related to hormones (Beral et al., 2005), diabetes (Friberg et al., 2007), and other potential pathogenic factors, and studies on these factors are intended to establish focused preventive interventions. It is proposed that dysbiosis may contribute to the activation of inflammatory pathway in endometrial cancer. Therefore, considerable research has investigated the interaction between the reproductive tract microbiota and endometrial cancer (Table 1).

A paper published in 2016 found that *Firmicutes*, *Actinobacteria* and *Bacteroidetes* were more common in endometrial cancer than in benign uterine diseases. The sensitivity of combination of *A. vaginae* and the *Porphyromonas* sp. to diagnose endometrial carcinoma was 73%-93%, and the specificity was 67%-90% (Walther-António et al., 2016). Although there are a few drawbacks, it pioneers the way for future research. In a study released in 2019 that further examined how host variables influence the microbiome characteristics in endometrial cancer, and the post-menopausal state was found to be the primary driver of the network connected to endometrial cancer. When *P. somerae* was considered a predictive biomarker of endometrial cancer in all patients, the study found that the sensitivity of vaginal *P. somerae* was 74% and the specificity was 63%. In post-menopausal and obese patients, who were generally thought to be at high risk for endometrial cancer, the positive predictive value increased to 0.86. The study also focused on type I and type II endometrial cancers, two pathophysiological subtypes.

TABLE 1 Studies in reproductive tract microbiota and endometrial cancer.

Reference	Year	Inclusion criteria	Exclusion criteria	Sample size for group A	Sample size for group B	Sample size for group C	Sample site	Methods	Findings
Walther-António et al. (2016)	2016	18 years or older; undergoing hysterectomy	pregnant or nursing; antibiotics within two weeks; using morcellation during the hysterectomy	10 (benign uterine conditions)	4 (endometrial hyperplasia)	17 (endometrial cancer)	Vaginal, cervical, uterine, fallopian, ovarian, peritoneal, and urine samples	16S rDNA V3-V5 region	*Firmicutes*, *Spirochaetes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* are significantly enriched in endometrial cancer; the simultaneous presence of *Atopobium vaginae* and an uncultured representative of the *Porphyromonas* sp. (99 % match to *P. somerae*) is associated with endometrial cancer, especially if combined with a high vaginal pH (>4.5).
Walsh et al. (2019)	2019	total/ subtotal hysterectomy	intravaginal infections within 3 months, allergy, autoimmune diseases, pregnancy, previous history of cancer	75 (benign uterine conditions)	7 (endometrial hyperplasia)	66 (endometrial cancer, 56 with type I, 10 with type II)	Vaginal, cervical, uterine, fallopian, ovarian, peritoneal, and urine samples	targeted qPCR	postmenopausal status is the main driver of a polymicrobial network associated with endometrial cancer; *Porphyromas somerae* is the most predictive microbial marker of endometrial cancer
Gonzalez-Bosquet et al. (2021)	2021	NA	NA	12 (normal)	112 (serous ovarian cancers)	62 (endometrioid endometrial cancers)	Tumor or normal fallopian tubes	Bacterial, archaea, and viral transcript (BAVT)	93 BAVTs differentially expressed between endometrioid endometrial cancer and serous ovarian cancer; endometrioid endometrial cancer BAVT expressions are between ovarian cancers and normal tubes
Lu et al. (2021)	2021	aged between 18 and 75 years, undergoing hysterectomy	pregnant or nursing, antibiotics or micro-ecologies within 3 months, infectious disease or genital tract medication within 3 months, preoperative chemotherapy or radiotherapy	25 (benign uterine conditions)	25 (endometrial cancer)	NA	Endometrial tissue	16S rRNA gene amplicon sequencing, real-time qPCR, Western blot.	*Micrococcus* was more abundant in the endometrial cancer, and *Pseudoramibacter_Eubacterium*, *Rhodobacter*, *Vogesella*, *Bilophila*, *Rheinheimera*, and *Megamonas* were less; rank correlation analysis showed a positive correlation between the relative abundance of *Micrococcus* and mRNA of IL-6 and IL-17
Li et al. (2021)	2021	aged between 40 and 69 years; undergoing hysterectomy	pregnant or nursing, antibiotics within three months, genital tract infection or medication within three months, preoperative chemotherapy or radiotherapy	10 (benign uterine conditions)	30 (stage I endometrial cancer)	NA	Endometrial tissue	16S rRNA sequencing	*Pelomonas* and *Prevotella* were more abundant in the endometrial cancer; the abundance of *Prevotella* and was positively corelated with serum D-dimer (DD) and fibrin degradation products (FDPs); transcriptome analysis identified 8 robust associations between *Prevotella* and fibrin degradation-related genes expressed within endometrial cancer; *Prevotella*, DD and FDPs showed a high potential to predict the onset of endometrial cancer (AUC = 0.86)

(Continued)

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TABLE 1 Continued

Reference	Year	Inclusion criteria	Exclusion criteria	Sample size for group A	Sample size for group B	Sample size for group C	Sample site	Methods	Findings
Hakimjavadi et al. (2022)	2022	18 years or older	neoadjuvant chemotherapy, douching within 14 days, vaginal cream or lubricant within 14 days, antibiotics within 14 days, or sexual intercourse within 5 days	11 (benign gynecologic disease)	30 (low-grade endometrial carcinoma)	20 (high-grade endometrial carcinoma)	vagina	shotgun metagenomic sequencing	*Fusobacterium nucleatum* was more abundant in group C than in group A; a significant increase in diversity from benign to HG disease; group A clustered in CST1, while group B clustered in CST2, and group C into both CST3 and CST4
Wang et al. (2022)	2022	Endometrioid adenocarcinoma; menopausal	current autoimmune diseases and gastrointestinal disorders, or a history of gastrointestinal surgery; genital tract infection and/or antimicrobial treatments to the genital area within 3 months; preoperative chemotherapy or radiotherapy; systemic antibiotics, corticosteroids, or any other immunosuppressive therapy within 6 months; smoking index >400; daily ethanol intake≥20 g in the past 5 years or ≥80 g in the past 2 weeks	28 (endometrial cancer)	NA	NA	Tumor tissue and adjacent non-endometrial cancer tissue	16S rRNA sequencing	The α diversity significantly increased in tumor tissues than those in adjacent non-endometrial cancer tissues. *Lactobacillus* and *Gardnerella* were the main bacterial genera in both tissues; *Prevotella*, *Atopobium*, *Anaerococcus*, *Dialister*, *Porphyromonas*, and *Peptoniphilus* were more abundant in the tumor tissue
Kaakoush et al. (2022)	2022	Postmenopausal; undergoing hysterectomy; body mass index (BMI) of either <27 kg/m^2 (lean) or >30 kg/m^2 (obese)	recipient of an investigational new drug within prior 6 days; antibiotics within 3 weeks	lean women with benign disease (n = 18), obese women with benign disease (n = 12)	lean women with endometrioid adenocarcinoma (n = 17), obese women with endometrioid adenocarcinoma (n = 23)	NA	endometrial tissues	16S rRNA transcript amplicon sequencing	obesity was not related to the type of microbial community in mouse endometrium in humans, the abundance of *Lactobacillus* in endometrial carcinoma was decreased, and obesity did not have association with it in mice, the abundance of *Lactobacillus* was positively correlated with normal uterine histology
Hawkins et al. (2022)	2022	Postmenopausal; Stage I endometrioid adenocarcinoma undergoing hysterectomy	preoperative chemotherapy or radiotherapy; antibiotics within 3 months	16 women in benign uterine conditions (2 Black, 13 White, 1 Other race).	95 women in the endometrioid adenocarcinoma group (23 Black, 72 White).	NA	Tumor tissues	16S rRNA sequencing	microbial diversity was decreased, and *Firmicutes*, *Cyanobacteria* and *OD1* were less in endometrial cancer from White versus Black women; *Dietzia* and *Geobacillus* was more abundant in tumors of obese White versus obese Black women

(Continued)

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TABLE 1 Continued

Reference	Year	Inclusion criteria	Exclusion criteria	Sample size for group A	Sample size for group B	Sample size for group C	Sample site	Methods	Findings
Barczyński et al. (2023)	2023	undergoing hysterectomy	intravaginal infection within 3 months, allergy, autoimmune diseases, pregnancy, previous history of cancer	27 (benign conditions)	21 (endometrial hyperplasia)	48 (endometrial cancer)	vaginal fornix and endocervical canal	Quantitative and qualitative real-time PCR analysis of DNA	*Lactobacillus iners* was significantly more frequent in group A, while *Dialister pneumosintes* and *Mobiluncus curtisii* were more frequent in cancer
Gonzalez-Bosquet et al. (2023)	2023	NA	NA	36 (normal)	112 (serous ovarian cancers)	62 (endometrioid endometrial cancers)	Tumor or normal endometrium	RNA sequencing and whole genome sequencing	*Desulfobacter*, *Desulfomicrobium*, *Parabacteroides*, and *Proteus* predicted endometrial cancer with AUC of 1.00; *Rhodopseudomonas* and *Proteus* had the most significant correlations with significant genes harboring SNVs
Byrd et al. (2023)	2023	NA	NA	528 (endometrial cancer)	NA	NA	NA	NA	The abundance of 11 common microbes changed with different MSI status in endometrial carcinoma, colorectal cancer and stomach adenocarcinoma

Type I endometrial carcinoma, the endometrioid carcinoma, accounts for 70% to 80% of endometrial carcinoma, which frequently develops due to endometrial hyperplasia (Bansal et al., 2009). Type II typically has a *p53* gene mutation, which is more aggressive and has a worse prognosis. Early detection is crucial because Type II endometrial carcinoma sometimes lacks early symptoms like post-menopausal bleeding. Type I endometrial carcinoma is estrogen-dependent, while type II is not sensitive to estrogen. The study found that *P. somerae* was present in the vagina of all patients with type II endometrial cancer and 57% of patients with endometrial hyperplasia (Walsh et al., 2019), indicating that it is a suitable microbial marker for endometrial cancer. However, the use of *P. somerae* in diagnosing pre-menopausal and peri-menopausal endometrial cancer should be emphasized as constrained.

In a study of bacterial, archaea, and viral transcript (BAVT) in the tumor tissues of serous ovarian cancer and endometrioid endometrial cancer, and healthy fallopian tubes, the authors found that 93 BAVTs were expressed differentially in these three groups. Furthermore, the expression of 12 independently expressed BAVT in endometrioid endometrial cancer was higher than that in ovarian cancers and lower than that in healthy fallopian tubes. Potential targets for cancer therapy in the future may include the pathways connected to these BAVT (Gonzalez-Bosquet et al., 2021). Further study found that microbial communities of the uterus correlated with genetic variation (Gonzalez-Bosquet et al., 2023). The study validated these results in separate datasets, the Cancer Genome Atlas (TCGA) and Gene Expression Omnibus databases, strengthening the reliability and universality of the research conclusions. However, it did not control confounding variables like age and menopause. Another study explored the relationship between endometrial microbiota, the expression of inflammatory cytokines, mRNA and proteins of interleukin (IL)-6, IL-8 and IL-17, and disease state. It was noted that there were notable differences in 12 genera between the endometrial cancer group and the benign uterine disease control group. *Micrococcus* was more abundant in the endometrial cancer group, while other genera were less in the endometrial cancer group. In terms of inflammatory factors, this study found that endometrial cancer had higher mRNA expression of IL-6, IL-8 and IL-17, and higher levels of IL-6 protein than control. Rank correlation analysis showed a positive correlation between the relative abundance of *Micrococcus* and mRNA of IL-6 and IL-17 (Lu et al., 2021). This study has a substantial number of samples, but unfortunately the menopausal status of patients was not adequately considered in the study design, and there was a statistical difference in age between the endometrial cancer group and the control group. The range of applications for the results must be determined by additional research. Another article published in 2021 examined for the first time the correlation between endometrial microbiota and tumor transcriptome in endometrial cancer and the relation between endometrial microbiota and blood coagulation indicators. *Pelomonas* and *Prevotella* were more abundant in the endometrial cancer than the benign uterine disease control. Further study showed that *Prevotella* abundance was positively correlated with serum D-dimer (DD) and fibrin degradation products (FDPs). The microorganism-related transcriptome of tumor tissue provided an explanation for the

correlation. *Prevotella*, combined with DD and FDPs, had excellent potential for predicting endometrial cancer existence (Li et al., 2021). It should be carefully considered whether this conclusion may be generalized to younger pre-menopausal and peri-menopausal patients with endometrial cancer because the patients included in this study were relatively old.

A study focused on the vaginal microbiota at various endometrial cancer malignancy levels. It reported that *Fusobacterium nucleatum* was more abundant in high-grade endometrial carcinoma than in benign gynecologic disease. The diversity increased greatly from benign to high-grade endometrial carcinoma and the benign condition, low-grade endometrial carcinoma and high-grade clustered into CST1, CST 2, and both CST3 and CST4, respectively (Hakimjavadi et al., 2022). The limitation of this study is that there were significant differences across groups in several clinical and demographic characteristics, including age and ethnicity. Another article in 2022 focused on postmenopausal endometrioid adenocarcinoma. It compared and analyzed the microbiota of tumor tissue and paracancerous tissue, and discovered that the α diversity and evenness of tumor tissue were significantly higher than those of paracancerous tissue, and *Prevotella*, *Atopobium*, and *Porphyromonas* were more abundant in the tumor tissue (Wang et al., 2022).

Lean and obese mice models were utilized to test the hypothesis that the endometrial microbiota is associated with obesity and endometrial cancer. It was found that obesity was unrelated to the type of microbial community in mouse endometrium. In humans, the abundance of *Lactobacillus* in the endometrium of postmenopausal patients with endometrial carcinoma was decreased compared with patients without endometrial carcinoma, but obesity did not have association with its abundance. In mice, the abundance of *Lactobacillus* was positively correlated with healthy uterine histology (Kaakoush et al., 2022). These findings suggested that *Lactobacillus* may preserve the endometrium, whereas endometrial carcinoma and obesity may impact the microorganisms living within the endometrium. Another study looked into how race as a demographic characteristic affected the microbiota. In contrast to black women, white women had lower *Firmicutes*, *Cyanobacteria*, and OD1 levels in their endometrial cancers, according to this study (Hawkins et al., 2022). TCGA database was used creatively in this study, which future researchers should note. A study based on TCGA database found that the abundance of 11 common microbes changed with different microsatellite instability (MSI) status in endometrial carcinoma, colorectal cancer and stomach adenocarcinoma (Byrd et al., 2023) which indicated that the intratumor microbiota may be different with various MSI status and affect the tumor microenvironment.

Despite the availability of numerous technological techniques, there is still a lack of research on premenopausal endometrial cancer. It is important to note that in clinical practice, the pathology of endometrial biopsy or curettage may be inconsistent with the pathology of hysterectomy, which makes it challenging for a portion of young patients to choose the appropriate therapy, as they may desire to preserve reproductive function. In the present, they can only rely on inaccurate biopsy pathological results when making treatment choices. Therefore, if we can figure out suitable microbial biomarkers to assist in determining determinthe pathological subtype of premenopausal endometrial carcinoma, whether to retain reproductive function or not will be decided with more certainty.

3 Discussion

We summarized findings of variations in the microbiota of the female reproductive tract between different disease states and healthy women (Figure 1). Comparing the microbiota of the same disease in several regions of the reproductive tract longitudinally, we found that when there was a change in one microorganism, its trend in the entire reproductive tract was frequently the same. For instance, in endometrial polyps, the level of *Proteobacteria* in the entire reproductive tract was lower than that in healthy women, and *Lactobacillus* showed a decreasing trend throughout the entire reproductive tract of endometrial carcinoma. Not surprisingly, there were some differences in the changes of microbiota in different parts of the reproductive tract of the same disease, just as there are varied flora composition patterns in diverse locations in healthy women (Chen et al., 2017). By comparing various diseases horizontally, we found that the increase or decrease of *Lactobacillus* was related to all the diseases we reviewed. Specifically, the decrease of vaginal *Lactobacillus* was associated with leiomyoma (Chen et al., 2017), endometriosis (Chao et al., 2021; Lu et al., 2022), and endometrial cancer (Kaakoush et al., 2022). The increase in the abundance of *Lactobacillus* in the reproductive tract was associated with endometrial polyps (Fang et al., 2016), adenomyosis (Lin et al., 2023), and endometrial hyperplasia (Zhang et al., 2021). This demonstrated that *Lactobacillus*, one of the dominating phyla in all regions of the female reproductive tract, was closely related to the health status of women. If its abundance changes noticeably, it is important to be vigilant about the gynecological diseases. Besides, *Firmicutes* (Fang et al., 2016; Hawkins et al., 2022; Hua et al., 2022; Kaakoush et al., 2022; Liang et al., 2023; Medina-Bastidas et al., 2022) and *Streptococcus* (Gonzalez-Bosquet et al., 2021; Kaakoush et al., 2022; Lee et al., 2021; Liang et al., 2023) always increased in abundance when women experienced various gynecological conditions, suggesting that these bacteria can be harmful to the health of women. If an increase in these bacteria is detected, it is important to be aware of the potential diseases.

There is some basic research concerning the mechanisms of genital microbiome in gynecological diseases. Under normal conditions, the vagina maintains an acidic pH (3.0–4.5), which is primarily due to the secretion of lactic acid and hydrogen peroxide by *Lactobacillus* species. *Lactobacillus* species not only produce bacteriocins, which, with the acidic pH, discourage the growth of pathogens, but they also adhere to epithelial surfaces, preventing the adhesion of pathogenic microorganisms (O'Hanlon et al., 2011; Ravel et al., 2011). They also promote autophagy of infected cells, eliminating viruses, bacteria, and protozoa (Ghadimi et al., 2010), and modulate inflammatory responses, particularly during pregnancy (Aldunate et al., 2015). Loss of *Lactobacillus* dominance, coupled with an increase in microbial diversity, is often associated with immune and epithelial homeostasis alterations. This may be induced by a series of mechanisms, such as: (a) the

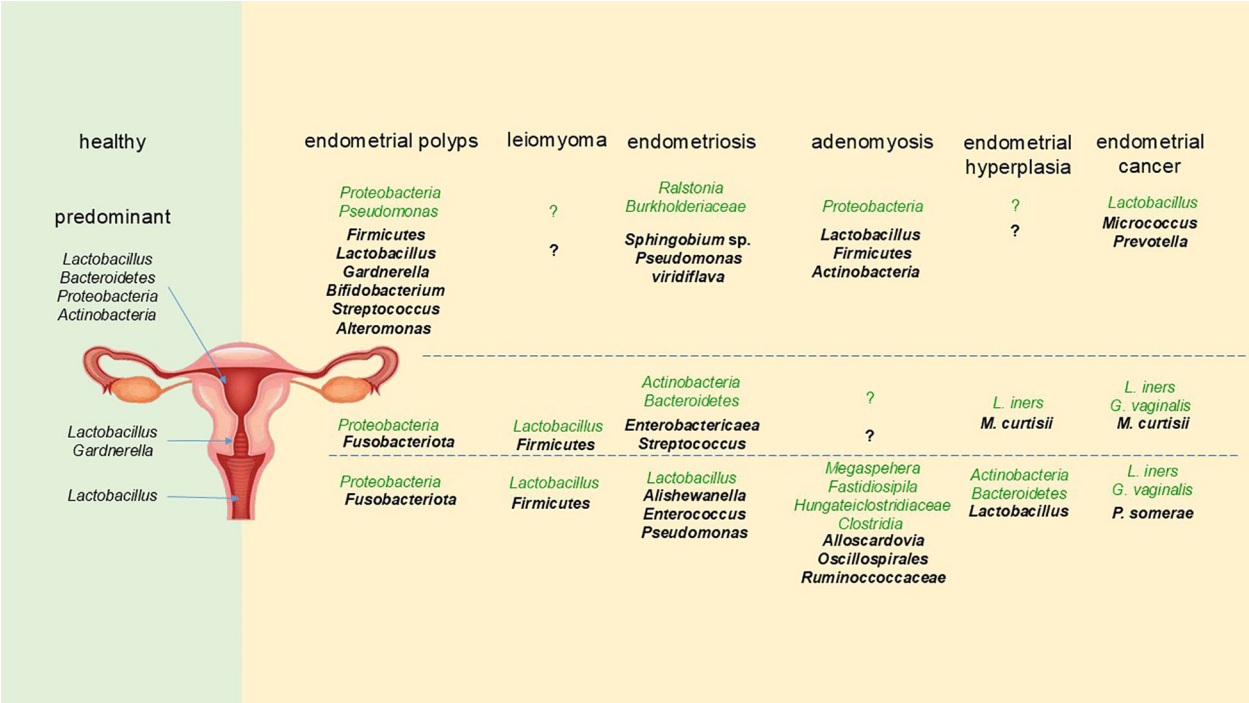

FIGURE 1
The changes of female reproductive tract microbiota in different gynecological diseases. The format of the font represents different trends compared with healthy women, with the bold and darker fonts representing an increase, and the normal and lighter fonts representing a decrease.

production of pro-inflammatory cytokines and chemokines, (b) the recruitment of immune cells, and (c) a reduction in the viscosity of cervicovaginal fluid due to the activity of mucin-degrading enzymes, including sialidase, fucosidase, galactosidase, N-acetyl-glucosaminidase, and various aminopeptidases (Moncla et al., 2015; Olmsted et al., 2003). Dysbiosis may also have far-reaching effects on immune and metabolic signaling, potentially influencing the development of gynecological diseases such as cervical, endometrial, and ovarian cancers. These pathophysiological changes can contribute to chronic inflammation, epithelial barrier dysfunction, alterations in cell growth and apoptosis, genomic instability, angiogenesis, and metabolic dysfunction (Wu et al., 2015). Emerging evidence suggests that reproductive organ dysfunction, in conjunction with specific bacteria, could play an active role in the development, progression, and metastasis of gynecologic malignancies, possibly through mechanisms such as the regulation of estrogen metabolism (Łaniewski et al., 2020). In benign gynecological diseases, studies in non-human primates (*Papio anubis*) have been conducted. One study found that induction of endometriosis in primates resulted in a systemic inflammatory response, characterized by a decrease in peripheral Tregs and an increase in Th17 cells, both of which are markers of systemic inflammation. Following the induction of endometriosis, the diversity and abundance of the microbiome in the vagina were altered, suggesting that shifts in the mucosal microbial community may contribute to ongoing inflammation by producing inflammatory mediators (Le et al., 2022).

Because of the tremendous diversity of the microbiota and the enormous variances across patients, an effective analytical method is required to describe the overall alterations in the microbiota more concisely. The application of CST filled in the gaps by summarizing the characteristics of the microbiota. This technique categorizes microorganisms, which is convenient for researchers to analyze the broad aspects of changes in microbiota (Brooks et al., 2017; Ravel et al., 2011).

There have generally been few statistically significant findings about endometrial microbiota in various gynecological diseases, which can be ascribed to several factors. First, the difficulties of sampling may have prevented adequate meaningful exploration. In addition, some studies conducted the research with only a few positive findings revealed, which was potentially caused by the low microbial biomass in the uterine cavity. As a consequence, the results were easily affected by sample contamination. Moreover, low abundance made sequencing more challenging (Molina et al., 2021). Therefore, future research should pay attention to standardizing sampling, preservation, extraction, and sequencing procedures, and strictly controlling contamination at every step of the operation process to acquire statistically significant results reflecting the changes in endometrial microbiota.

This paper reviewed the most recent research on the reproductive tract microbiota in common benign and malignant gynecological diseases, including endometrial polyps, uterine fibroids, endometriosis, adenomyosis, endometrial hyperplasia, and endometrial carcinoma. Numerous studies have demonstrated that the microbiota of the female reproductive tract is different in different health states. Additionally, specific bacteria may promote the development of the certain diseases, and the therapy targeting microbiota showed certain efficacy in the experiment. Simultaneously, it is acknowledged that we do not

thoroughly comprehend the mechanism of interaction between the human body and the microbiota of the reproductive tract.

An examination of the literature revealed that, despite the relatively advanced technology, the knowledge of microbiota in various diseases varied greatly in different diseases. For instance, there are more studies on endometriosis and endometrial carcinoma than adenomyosis and endometrial hyperplasia, some of which have even explored the subtypes of the diseases (Yang et al., 2023). The discrepancy in disease incidence and the level of clinical concern of diseases could cause the variance. Since endometriosis is more common than adenomyosis, finding patients who match the inclusion requirements is simpler. Additionally, other factors can make it more challenging to collect enough samples. For instance, women of childbearing age comprise most adenomyosis patients. Gathering enough uterine samples for research is difficult because only a small portion of them chooses hysterectomy. Some of the issues can be resolved by inter-institutional collaboration, and patients without surgical plans may be subjected to alternate sampling techniques, including endometrial sampling brushes for uterine microbiota.

Some studies describe and analyze the sampling results of reproductive tract microbiome in common gynecological diseases, but these results can only prove the correlation. There are few studies on the interaction between microbiota and disease state. Future research can make use of several established techniques, including simultaneously examining the transcriptome and proteome (Li et al., 2021), exploring the pathways and mechanisms underlying the interaction between the human body and the microbiota using primary cultured tissues *in vitro* (Guo et al., 2015), and utilizing animal models to investigate the causal relationship between alterations in microbiota and the onset of disease (Lu et al., 2022). Lastly, return to the clinic and conduct clinical trials of treatments targeting microbiota (Khan et al., 2018). Numerous studies and efforts are required to identify significance and clarify the possible mechanism.

The research of reproductive tract microbiome may contribute to resolving several clinical issues, including the causes of endometrial polyps recurrence, which patients with endometrial hyperplasia are more likely to develop endometrial cancer, and the considerations that should be made when young patients with endometrial cancer opt for fertility-preserving treatment. In large clinical studies, particularly in long-term follow-up cohort studies and randomized controlled trials, the inclusion of reproductive tract microbiome research will help to acquire more comprehensive and detailed results.

In the future, more high-quality microbiome studies will provide information for the prevention, screening, diagnosis and treatment of common gynecological diseases. In particular, because the endometrial microbiome is frequently closer to the site of the lesion and is more likely to directly interact with the disease, it is more likely to be significant for the study of the pathogenesis of the disease, which may help inform the development of prevention and treatment strategies. Because of the convenience of vaginal sampling, the results of vaginal microbiome studies are more suitable for disease screening and diagnosis. The cervix locates halfway between the two sites, combining both the advantages and disadvantages. We should base our choice of research location on clinical problems we hope to resolve.

After being properly validated, it is anticipated that treatments aimed at the microbiome, such as antibiotics, probiotics, and flora transplantation, will be exploited in clinical practice to alleviate the suffering of patients.

4 Conclusion

The study of human reproductive tract microbiota has been a rapidly developing and promising field recently. It has been demonstrated that in different gynecological disease states, the female reproductive tract microbiota is different. Additionally, some bacteria may induce the disease progress, and therapies targeted microbiota, such as antibiotics, probiotics, and flora transplantation, have been successful in the trial. These findings highlight the significance of symbiotic microbiota in the reproductive tract in preserving health. By merging other technologies, such as transcriptome and proteome, *in vitro* cultured cells, and animal models, we can comprehend how reproductive tract microbes interact with their hosts to protect health or cause disease. The information obtained from more high-quality microbiome studies can contribute to screening and diagnosis of gynecological diseases as well as the development of preventative and therapeutic strategies aimed at preserving or reestablishing healthy reproductive tract microbiota.

Author contributions

QZ: Conceptualization, Writing – original draft, Writing – review & editing. TS: Conceptualization, Investigation, Supervision, Writing – review & editing. XL: Investigation, Resources, Writing – review & editing. LZ: Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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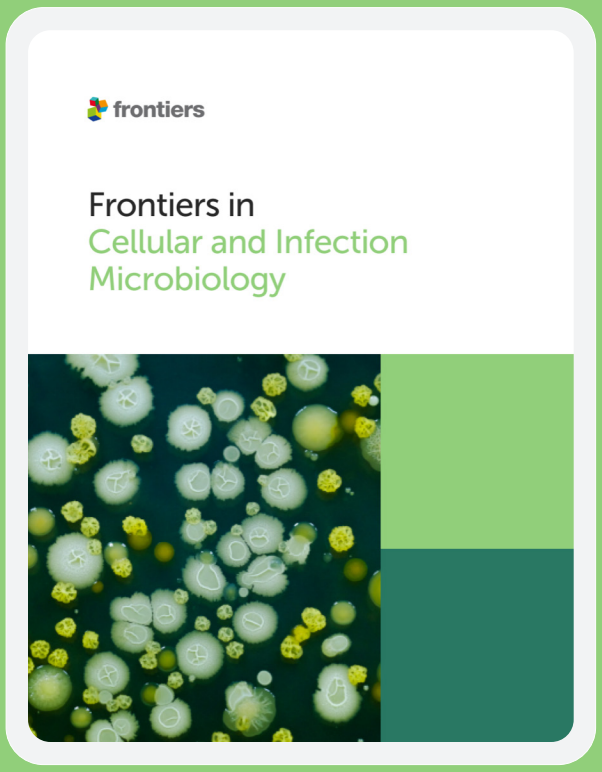

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