VAGINAL DYSBIOSIS AND BIOFILMS

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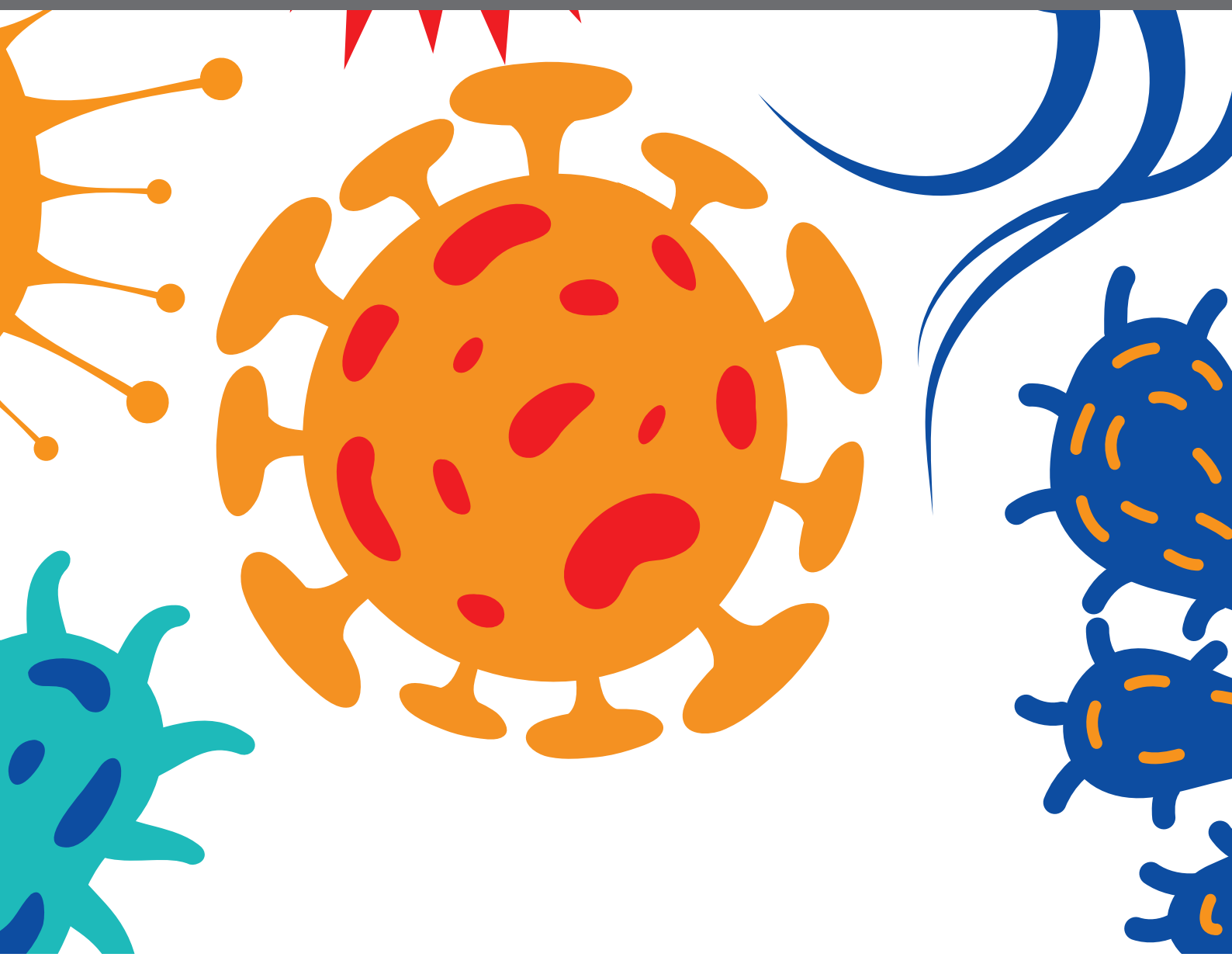
PUBLISHED IN: Frontiers in Cellular and Infection Microbiology



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ISSN 1664-8714
ISBN 978-2-83250-004-0
DOI 10.3389/978-2-83250-004-0

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VAGINAL DYSBIOSIS AND BIOFILMS

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Citation: Machado, A., Marangoni, A., Foschi, C., eds. (2022). Vaginal Dysbiosis and Biofilms. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-004-0

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Editorial
PUBLISHED 09 August 2022
DOI 10.3389/fcimb.2022.976057

OPEN ACCESS

EDITED AND REVIEWED BY
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SPECIALTY SECTION
This article was submitted to Biofilms, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 23 June 2022
ACCEPTED 18 July 2022
PUBLISHED 09 August 2022

CITATION
Machado A, Foschi C and Marangoni A (2022) Editorial: Vaginal dysbiosis and biofilms.
Front. Cell. Infect. Microbiol. 12:976057.
doi: 10.3389/fcimb.2022.976057

Editorial: Vaginal dysbiosis and biofilms

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KEYWORDS

biofilms, vaginal dysbiosis, reproductive health, vaginal microbiota, accurate diagnostics, novel treatments

Editorial on the Research Topic
Vaginal dysbiosis and biofilms

The vaginal microbiota is made up of a diversity of microorganisms (Pacha-Herrera et al.; Salinas et al., 2020), where commensal *Lactobacillus* species act as the first defense mechanism against the establishment of vaginal dysbiosis and vaginitis (Petrova et al., 2017) . When this balanced microbiota gets disrupted, the vaginal epithelium is less protected, and vaginal dysbiosis can succeed (Figure 1). It is characterized by a shift in microbial communities that include a progressive replacement of certain *Lactobacillus* species by pathogenic/opportunistic microorganisms (Machado and Cerca, 2015). This microbial shift can lead to bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), aerobic vaginitis (AV), among others. These vaginal dysbioses are characterized by an overgrowth of multiple pathogens and promoting mixed infections. Another fact is the ability of certain pathogens to develop biofilms (Machado and Cerca, 2015; Castro et al.). Biofilms represent the predominant mode of microbial growth in nature, leading to important public health problems, as infections and negative interactions affecting the host immune system and the reproductive health outcomes in women (Machado et al., 2017). Vaginal dysbiosis is also associated with an increased risk of acquiring human immunodeficiency virus (HIV), Herpes simplex type 2, and other sexually transmitted infections, as Chlamydia (Parolin et al., 2018; Ceccarani et al., 2019). Understanding these vaginal microbiota dynamics is the key to developing accurate diagnostics and novel treatments. Due to the heterogeneity of species within biofilms, it has been difficult to assess the relevance of individual species to the pathogenesis of vaginal dysbiosis.

This Research Topic focuses on several factors associated with vaginal dysbiosis and biofilm development. The first article reported the development of a novel peptide nucleic acid (PNA) probe targeting *Fannyhessea (Atopobium) vaginae* and validated with another *Gardnerella*-specific PNA probe. The authors showed a possible method for BV diagnosis evidencing excellent sensitivity and specificity for *F. vaginae-Gardnerella* biofilms (Sousa et al.). The second article by Zheng et al. reviewed the role

10.3389/fcimb.2022.976057

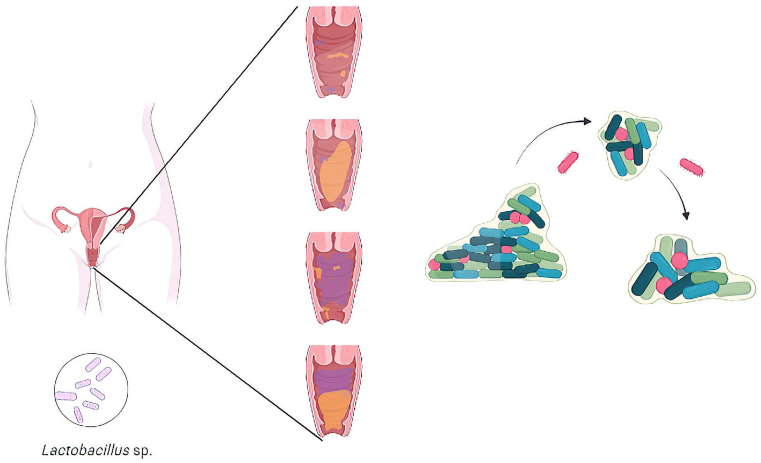

FIGURE 1
Representative illustration of the transition from a healthy vaginal microbiota with a high concentration of *Lactobacillus* species to the establishment of a vaginal dysbiosis in women.

of *Lactobacillus iners*, postulating its role as transitional species that colonizes after the vaginal microbiota is disturbed. *L. iners* offers an overall less protection against vaginal dysbiosis and leads to BV. Under certain conditions, *L. iners* is a genuine vaginal symbiont, but it can also act as an opportunistic pathogen.

Castro et al. evidenced that the crystal violet (CV) staining method, despite its widespread utilization, fails to properly quantify multiple species in BV-related biofilms, more exactly *Gardnerella vaginalis*, *F. vaginae*, and *Prevotella bivia*. Meanwhile, Ferreira et al. discussed sialidase activity in the cervicovaginal fluid (CVF) and its association with microscopic findings of BV. Through sequencing bacterial 16S rRNA gene in 140 vaginal samples, the authors demonstrated that 44 participants (31.4%) had molecular-BV, of which 30 (68.2%) had sialidase activity, suggesting that sialidase activity in molecular-BV is associated with changes in bacterial components of the microbiome. In the fifth article, Costantini et al. showed that vaginal microbiota dominated by lactobacilli protects women from sexually transmitted infections, in particular HIV type 1 (HIV-1), and partially this protection is mediated by *Lactobacillus*-released extracellular vesicles (EVs). These authors found that EVs released by lactobacilli protect human cervico-vaginal tissues *ex vivo* and isolated cells from HIV-1 infection by inhibiting HIV-1-cell receptor interactions. Also, they identified numerous EV-associated proteins involved in this protection.

Qin and Xiao reviewed the new genotyping of *Gardnerella* species (*G. leopoldii*, *G. piotii*, and *G. swidsinskii*) describing the genetic diversity when compared with *G. vaginalis* and reporting new findings on the correlation with BV. Furthermore, Xiao et al. reported 48 symptomatic patients with clinical diagnoses of VVC complicated with BV and the results obtained on their treatments with oral metronidazole combined with local clotrimazole, assessing the drug efficacy and vaginal microbiome alterations. Their results evidenced significant alterations on vaginal microbiome in BV+VVC mixed vaginitis patients and an enhanced treatment for BV. It is worth to be underlined that this is the first study to investigate multiple characteristics of the vaginal microbiome in patients with BV+VVC before and after drug treatment.

On the other hand, Pacha-Herrera et al. evaluated the probiotic activity promoted by individual and multi-microbial consortia of five vaginal lactobacilli (*Lactobacillus iners*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, and *Lactobacillus acidophilus*) among healthy women and women with BV or AV. The qualitative analysis through PCR assays was realized on 436 vaginal samples from a previous study (Salinas et al., 2020) and statistical analysis evaluated associations between lactobacilli and vaginal microbiota. Multi-microbial clustering model was also realized to determine the probiotic relationship between lactobacilli and vaginal dysbiosis. Concerning the individual effect, *L. acidophilus*, *L. jensenii*, and *L. crispatus* showed the highest normalized importance values against vaginal dysbiosis. *L. acidophilus* showed a significant prevalence on healthy microbiota against both dysbioses (BV, $p = 0.041$; and AV, $p = 0.045$). *L. jensenii* only demonstrated significant protection against AV ($p = 0.012$). Finally, the study evidenced a strong

multi-microbial consortium by *L. iners*, *L. jensenii*, *L. gasseri*, and *L. acidophilus* against AV ($p = 0.020$) and BV ($p = 0.009$), lacking protection in the absence of *L. gasseri* and *L. acidophilus* (Pacha-Herrera et al.).

Last, but not least, in the ninth article, Swidsinski et al. analyzed different types of clue cells (epithelial cells heavily covered with adherent bacteria), which are accepted as a key clue to BV diagnosis. The authors investigated adhesive and cohesive patterns of main microbiota groups in vaginal discharge using fluorescence *in situ* hybridization (FISH) on BV samples of 500 women. FISH analysis evaluated the spatial distribution of BV-related bacterial groups. The authors evidenced four patterns, such as dispersed (non-adherent bacteria), dispersed adherent bacteria, cohesive (non-adherent) bacteria, and cohesive adherent bacteria. Direct cohesive adherence to the epithelial cells representing true clue cells was unique for *Gardnerella* species. The study illustrated that taxon indifferent imaging is inadequate for the correct BV diagnosis, being BV constituted by a mix of at least two different conditions, more exactly biofilm vaginosis and bacterial excess vaginosis.

Author contributions

AMac wrote the first draft. CF, and AMar provided critical comments and editorial suggestions for revisions. All the authors agreed on the submitted version.

Acknowledgments

AMac would like to thank all the staff of the Microbiology Institute of USFQ and COCIBA, as well as the Research Office of Universidad San Francisco de Quito for their continuous research support. Likewise, AMac wishes to thank CF and AMar for accepting to collaborate on this Research Topic in Frontiers and for their expertise acknowledge during the editorial process. Finally, AMac also thanks Robert Josue Rodríguez-Arias for creating the figure for this editorial article.

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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frontiers in Cellular and Infection Microbiology

BRIEF RESEARCH REPORT
published: 18 November 2021
doi: 10.3389/fcimb.2021.779376

A New PNA-FISH Probe Targeting *Fannyhessea vaginae*

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Bacterial vaginosis (BV) is the most common vaginal infection in women of reproductive age and has been associated with serious health complications, mainly in pregnant women. It is characterized by a decrease in the number of *Lactobacillus* species in the healthy vaginal microbiota and an overgrowth of strict and facultative anaerobic bacteria that develop a polymicrobial biofilm. Despite over 60 years of research investigating BV, its etiology is not fully understood. *Gardnerella* spp. is a crucial microorganism that contributes to the formation of the biofilm and the development of BV, but the role of other BV-associated bacteria is not clear. Nevertheless, *Fannyhessea vaginae* (previously known as *Atopobium vaginae*) is a highly specific species for BV, and co-colonization with *Gardnerella* is thought to be a very specific diagnostic marker. The diagnosis of BV still presents some limitations, since currently used methods often fail to accurately detect BV. This work aims to develop a novel peptide nucleic acid (PNA) probe targeting *F. vaginae*. This probe was further validated in a multiplex assay, which included a *Gardnerella*-specific PNA probe, as a possible method for diagnosis of BV, and was compared with quantification by qPCR. The new PNA probe showed excellent sensitivity and specificity and could discriminate *F. vaginae*-*Gardnerella* biofilms, confirming the potential to be used for the detection of BV-associated pathogens.

Keywords: *Fannyhessea vaginae*, *Gardnerella vaginalis*, bacterial vaginosis, fluorescence *in situ* hybridization (FISH), peptide nucleic acid (PNA)

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Biofilms, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *18 September 2021*
Accepted: *01 November 2021*
Published: *18 November 2021*

Citation:
Sousa LGV, Castro J, França A, Almeida C, Muzny CA and Cerca N (2021) A New PNA-FISH Probe Targeting Fannyhessea vaginae. Front. Cell. Infect. Microbiol. 11:779376. doi: 10.3389/fcimb.2021.779376

INTRODUCTION

Bacterial vaginosis (BV) is the most common vaginal infection in women of reproductive age (Jung et al., 2017) affecting around 23% to 29% of women worldwide and it is associated with high healthcare costs (Peebles et al., 2019). BV is characterized by vaginal discharge and odor, an increase in the vaginal pH, as well as the presence of clue cells (Sobel, 2000; Livengood, 2009; Hay, 2014). BV has been associated with multiple health complications, including adverse birth outcomes (Svare et al., 2006; Isik et al., 2016). Microbiologically, BV is characterized by a decrease in commensal, protective lactobacilli and a dramatic increase in strict and facultative anaerobic bacteria which form a polymicrobial biofilm on the surface of the vaginal epithelial cells (Turovskiy et al., 2011; Rosca et al., 2020b).

The diagnosis of BV is usually performed using clinical criteria or microbiologically by interpretation of vaginal Gram-stains (Gutman et al., 2005). The most common method for BV diagnosis is Amsel's criteria, which is based on criteria related to the clinical signs of BV. These criteria include (i) homogeneous vaginal discharge, (ii) vaginal pH greater than 4.5, (iii) the release of a fishy smell on the addition of 10% potassium hydroxide to a drop of vaginal discharge, and (iv) the presence of clue cells (Amsel et al., 1983). BV is considered present when at least three of the four Amsel's criteria are detected (Turovskiy et al., 2011; Hay, 2014). An alternative method for the diagnosis of BV is the analysis of Gram-stains of vaginal fluid, proposed by Nugent *et al.*, (Nugent et al., 1991). According to the Nugent method, Gram-stain smears are classified by the presence of different bacterial morphotypes and scored on a 0 to 10 scale by the sum of the quantification (0 to 4+) of each morphotype (Nugent et al., 1991). A smear with a Nugent score of 0 to 3 is considered normal, a score of 4 to 6 is considered intermediate, and a score equal to or greater than 7 is considered positive for BV (Nugent et al., 1991). Due to the limitations of conventional methods of diagnosis, when comparing these two methods to one another, the Amsel criteria shows values of sensitivity between 37% and 70% and specificity between 94% and 99% (Schwebke et al., 1996; Sha et al., 2005; Modak et al., 2011). When evaluating the Nugent method using the Amsel criteria as reference, the values of sensitivity and specificity range from 78% to 94% and 67% to 94%, respectively, (Schwebke et al., 1996) and therefore, more reliable and accurate alternatives for the detection of bacteria associated with BV are needed to improve diagnosis (Africa, 2013; Redelinghuys et al., 2020).

Gardnerella spp. have been recognized as the most common bacteria present in BV and play an important role in the pathogenesis of BV (Muzny et al., 2019; Rosca et al., 2020b). Despite its high prevalence in cases of BV, *Gardnerella* is also found in women who do not have BV (Hickey and Forney, 2014). Importantly, *Gardnerella* spp. may exhibit several virulence factors that explain its pathogenic potential in women with BV (Janulaitiene et al., 2018), such as the ability to displace *Lactobacillus* adhered to vaginal epithelial cells (Castro et al., 2015), the high capacity to form biofilm (Harwich et al., 2010), greater cytotoxic activity by the production of vaginolysin (Patterson et al., 2010), which lyses vaginal epithelial cells (Gelber et al., 2008), and the presence of sialidase that leads to exfoliation of vaginal epithelial cells (Santiago et al., 2011; Lewis et al., 2013). These characteristics suggest that *Gardnerella* spp. is a crucial microorganism for the development of BV, which seems to adhere to the vaginal epithelial cells and initiate the formation of a biofilm to which other BVAB consequently attach and interact with each other, inducing the infection (Muzny et al., 2019). *Fannyhessea vaginae*, previously known as *Atopobium vaginae* (Nouioui et al., 2018), is highly specific for BV, as it is also found in most BV cases but is rarely present in the vaginal microbiota of healthy women (Bradshaw et al., 2006b). For this reason, *F. vaginae* is considered an indicator for abnormal vaginal microbiota and it is more specific to BV than *Gardnerella* spp. (Sehgal et al., 2021). The combination of *F. vaginae* and some *Gardnerella* spp. could be the best diagnostic method of BV (Menard et al., 2008). However, as a fastidious microorganism, *F. vaginae* is difficult to detect by culture-dependent methods (Trama et al., 2008). As such, new molecular approaches to identify *F. vaginae* in polymicrobial BV biofilms are necessary.

Molecular methods targeting nucleic acids are important approaches for BV diagnosis since they can identify multiple microorganisms associated with the infection (Coleman and Gaydos, 2018; Redelinghuys et al., 2020). Fluorescence *in situ* hybridization (FISH) is a molecular technique that uses probes specifically designed to target a microorganism of interest (Prudent and Raoult, 2019). Some advances in the development of FISH lead to an increase in the use of peptide nucleic acid (PNA) probes, which are polymeric neutral charged probes that bind to DNA or RNA without repulsion (Cerqueira et al., 2008). Peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH), which includes a PNA probe specifically designed to target a microorganism of interest, has been used as an alternative for the diagnosis of infections and detection of particular bacterial species (Almeida et al., 2011; Prudent and Raoult, 2019). The application of PNA-FISH methodology for the detection of bacteria related to BV has been proposed, however still with a limited number of targeted species. Previously, only one PNA probe targeting *Gardnerella vaginalis* (Machado et al., 2013) and three PNA probes targeting *F. vaginae* (Hardy et al., 2015) were designed and developed specifically for the study of BV. While the *Gardnerella* probe has been shown to have high sensitivity and specificity (Machado et al., 2013), the currently available *F. vaginae* probes have lower efficiency (Hardy et al., 2015). In this work, we aimed to develop a novel PNA probe targeting *F. vaginae*, in an attempt to improve BV diagnostic accuracy and further BV pathogenesis research. We also assessed whether the probe could be used in a multiplex assay, to discriminate species within a biofilm.

MATERIALS AND METHODS

In Silico Design of *F. vaginae* PNA Probe

To identify potential oligonucleotides for the *F. vaginae* probe, we selected a set of sequences from 16S and 23S collections, with lengths >1200 bp or >1600 bp, respectively, available at Arb-Silva database (https://www.arb-silva.de/search/). Only sequences with a quality score >90 were considered. Each set contained sequences from *F. vaginae* strains, as well as species from the same genus and other genera closely related to the bacterium of interest. The sequences were then aligned using the Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). Regions for potential probes were searched, showing the same sequence in the species of interest and one or more mismatches in the sequences belonging to other species. Theoretical sensitivity and specificity of the PNA probes were determined, as previously described (Almeida et al., 2013). The probes were evaluated using the TestProbe tool (https://www.arb-silva.de/search/testprobe/) with no mismatches allowed. Sequences with the highest theoretical

sensitivity and specificity, complementarity with a low number of non-interest sequences, GC content between 40% and 60%, high melting temperature (>50°C) (Almeida et al., 2011), and Gibbs free energy ranging from -13 kcal/mol to -20 kcal/mol (Yilmaz and Noguera, 2004) were selected as the best probes. The selected probe was then synthesized (Eurogentec, Seraing, Belgium) and the oligonucleotide N-terminus was linked to an Alexa Fluor molecule *via* a double 8-amino-3,6-dioxaoctanoic acid linker (*F. vaginae* probe: Alexa Fluor 488-OO-CGATGTGCGACTAAA).

Bacterial Growth Conditions

F. vaginae ATCC BAA-55, *F. vaginae* CCUG 42099, *F. vaginae* CCUG 44116, and 21 other *F. vaginae* strains, previously isolated from cases of BV (De Backer et al., 2006; De Backer et al., 2010; Santiago et al., 2012), were used to determine *F. vaginae* probe analytical sensitivity. Forty different bacterial species associated with BV or with the vaginal microbiota, were used to determine *F. vaginae* probe analytical specificity. The strains were grown in Columbia Blood Agar Base (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) of defibrinated horse blood (Oxoid) for 24 or 48 h, except for *Sneathia sanguinegens* which was maintained in chocolate agar supplemented with 10% (v/v) inactivated horse serum (Biowest, Nuaillé, France) for 48 h. *Actinomyces urogenitalis, Aerococcus christensenii, Bifidobacterium bifidum, Campylobacter ureolyticus, F. vaginae* strains, *Lactobacillus iners*, *Megasphaera micronuciformis*, *Mobiluncus curtisii*, *M. mulieris*, *Mycoplasma hominis*, *Peptostreptococcus anaerobius, Porphyromonas asaccharolytica, Prevotella bivia, Propionibacterium acnes, S. sanguinegens* and *Veillonella parvula* were kept at 37°C under anaerobic conditions (AnaeroGen Atmosphere Generation system, Oxoid). *Acinetobacter baumannii* was grown at 30°C and the remaining species were maintained at 37°C and 10% CO_2.

FISH Hybridization Procedure

For PNA-FISH experiments, a bacterial suspension was prepared in phosphate-buffered saline (PBS), in which we first adjusted it to an optical density (OD) at 620 nm ~ 0.1 and then we performed a 2-fold dilution. Afterward, 30 µL of the suspension was spread on epoxy coated microscope glass slides (Thermo Fisher Scientific, Lenexa, KS) and air-dried. Optimization experiments were performed to identify the optimal hybridization conditions which resulted in the best fluorescence signal. Variations in the hybridization temperature, ranging from 50°C-63°C, and time of 60 min and 90 min were tested using the strain *F. vaginae* ATCC BAA-55. At the optimized conditions, the cells were fixed with 100% (v/v) methanol (Thermo Fisher Scientific) for 15 min, 4% (w/v) paraformaldehyde (Thermo Fisher Scientific) for 10 min, followed by 50% (v/v) ethanol (Thermo Fisher Scientific) for 15 min, and allowed to dry. After, 20 µL of hybridization solution containing 10% (w/v) dextran sulfate (Sigma, Germany), 10 mM NaCl (Sigma), 30% (v/v) formamide (Thermo Fisher Scientific), 0.1% (w/v) sodium pyrophosphate (Thermo Fisher Scientific), 0.2% (w/v) polyvinylpyrrolidone (Sigma), 0.2% (w/v) Ficoll (Sigma), 5 mM disodium EDTA (Panreac, Spain), 0.1% (v/v) Triton X-100 (Thermo Fisher Scientific), 50 mM Tris–HCl (pH 7.5; Thermo Fisher Scientific), and 200 nM of PNA probe were applied to the slides and covered with a coverslip. The slides were placed in a moist and opaque container and incubated at the selected testing time/temperature. After that, the coverslips were removed, and the slides were immersed in the pre-warmed washing solution containing 5 mM Tris-base (Thermo Fisher Scientific), 15 mM NaCl (Sigma), and 1% (v/v) Triton-X (pH 10; Thermo Fisher Scientific) and incubated for 30 min at the same temperature as hybridization. Hybridization was performed at 56°C for 60 min. For the multiplex experiments in biofilms, a PNA probe specific for *Gardnerella* (Machado et al., 2013) was added to the hybridization solution at the same concentration of 200 nM.

Microscopic Analysis

Microscopic visualization was performed using an Olympus BX51 epifluorescence microscope (Olympus, Lisbon, Portugal) equipped with a FITC filter (BP 470-490, FT500, LP 516 sensitive to the Alexa Fluor 488 molecule). Filters that do not detect the probe fluorescence were used as controls to confirm if the cells did not have auto-fluorescence. For every experiment, a negative control was performed with hybridization solution without a probe. The experiments were performed with at least two independent assays.

Biofilm Formation and Confocal Laser Scanning Microscopy (CLSM)

Inoculums of *G. vaginalis* ATCC 14018 and *F. vaginae* ATCC BAA-55 were prepared in New York City III (NYCIII) broth (Rosca et al., 2020a) supplemented with 10% (v/v) inactivated horse serum and incubated for 24 h at 37°C under anaerobic conditions. After 24 h, the bacterial concentration was adjusted to 1×10^7 CFU/mL in NYCIII broth and biofilms were formed on eight-well chamber slides (Thermo Fisher Scientific™ Nunc™ Lab-Tek™, Rochester, NY, USA) by inoculating each of the respective species for single biofilms and both species for dual-species biofilms, for a final volume of 400 µL. Biofilms were incubated at 37°C in anaerobic conditions. After 24 h, the medium was removed, and the biofilm was washed once with NaCl and air-dried. Subsequently, fixation was performed (as described above) and PNA-FISH was conducted at 60°C for 90 min using the *F. vaginae* and *G. vaginalis* (Machado et al., 2013) probes. CLSM images were acquired using an Olympus™ Fluo-View FV1000 (Olympus) confocal laser scanning microscope. The experiments were performed in duplicate.

Bacterial Species Discrimination in Dual-Species Biofilms by PNA-FISH

The bacterial population within dual-species biofilms of *G. vaginalis* and *F. vaginae* was differentiated by PNA-FISH, as described previously (Castro et al., 2020). Briefly, non-adherent cells were removed by one gentle wash with PBS and, afterward, biofilms were scraped vigorously from the well. Then, 30 µL of each resuspended biofilm was spread on epoxy-coated microscope glass slides (Thermo Fisher Scientific) and the FISH procedure was performed as described above. Microscopic

visualization was performed using filters capable of detecting the PNA Gard162 probe (BP 530-550, FT 570, LP 591 sensitive to the Alexa Fluor 594 molecule) and the PNA FvagPNA651probe (BP 470-490, FT500, LP 516 sensitive to the Alexa Fluor 488 molecule). Twenty fields were randomly acquired in each sample. The number of bacteria was counted using *ImageJ* software (Rasband, 1997), applying automated counts and specific thresholds as indicated in a previous protocol (Labno). Biofilm assays were repeated three times on separate days.

Bacterial Species Discrimination in Dual-Species Biofilms by qPCR

Genomic DNA (gDNA) was extracted from the dual-species biofilms, as well as from pure cultures of the 2 species under study (for the qPCR calibration curves), using the DNeasy Ultraclean microbial kit (Qiagen), following the manufacturer instructions, with minor adaptations. In brief, bacterial samples were centrifuged at 14000 rpm for 5 min, the supernatant carefully removed, and the pellet frozen at -20°C, overnight. This step increased the DNA yield up to 2-fold. The cells were lysed in a BeadBug 6 Microtube Homogenizer (Benchmark Scientific, NJ, USA) using 2×3 cycles of 30 s at 4350 rpm, and samples were kept on ice between the 2 cycles. To assess the efficiency and variability of gDNA extraction between samples, 10 μL of luciferase cDNA, obtained as described before (Magalhães et al., 2019), were added to each sample, before transferring the lysate to the spin column. gDNA was eluted in 50 μL of DNase-free water. To determine the bacterial load, a calibration curve was generated with gDNA isolated from pure bacterial cultures with concentrations ranging from 1×10^9 CFU/mL to 5×10^6 CFU/mL. The primers used to quantify *G. vaginalis* (Fw CCTCATGCAAAATGTGATGC; Rv CCAAAACAGAAGCACGGAAT; amplifying the locus GAVG_1017, obtained from GenBank: AP012332.1) or *F. vaginae* (Fw CCTCATGCAAAATGTGATGC; Rv CCAAAACAGAAGCACGGAAT; amplifying the locus I6G91_00565, obtained from GenBank: CP065631.1) were designed with CLC genomics workbench version 21 (QIAGEN). Primer specificity was specifically designed to differentiate these two species in this controlled *in vitro* study and was first confirmed using Primer-BLAST and then experimentally determined by qPCR. All samples, including the standard curves, were diluted 10× in DNase-free water and then 2 μL of these solutions were mixed with 8 μL of reaction buffer containing 5 μL of Xpert Fast SYBR (Grisp, Porto, Portugal), 1 μL of primers mixture (at 10 μM) and 2 μL of water. All samples were analyzed in triplicates. Non-template controls were performed to evaluate reagent contamination. To assess the efficiency of gDNA extraction, and to calibrate data between qPCR runs, a control was used by adding 2 μL of cDNA luciferase to each qPCR plate. qPCR runs were performed in a CFX96™ (Bio-Rad, CA, USA) with the following cycle parameters: 95°C for 3 min, and 40 cycles of 95°C for 5 s and 60°C for 20 s. Melt analysis was performed to ensure the absence of unspecific products and primer-dimers. PCR amplification efficiency was determined from the slope of a standard curve and efficiencies of 82% for *G. vaginalis* primers and 79% for *F. vaginae* were obtained. Bacterial load in each sample was interpolated from the averaged standard curves. This experiment was repeated three times on separate days.

RESULTS

Design and *In Silico* Analysis of the *F. vaginae* PNA Probe

The alignment of the two sets of rRNA sequences gathered for the 16S and 23S rRNA evaluation revealed that the large subunit sequences presented conserved regions of potential interest (i.e. consistent among the *F. vaginae* sequences and with mismatches in the non-*F. vaginae* sequences) (**Supplementary Figure 1**). The candidate target region for probe design was then selected based on the number of target strains, the position of the mismatches in the close related strains used for the alignment, % of GC, melting temperature, and free energy. The probe was named FvagPNA651 considering the target starting position in the 23S rRNA (*E. coli* numbering).

In silico evaluation of *F. vaginae* probe performance was done using the TestProbe tool that searches for targets of the probe in an online database of rRNA sequences. A total of 157859 sequences, from the REF sequence collection, large subunit, 23S database (Arb-Silva) were analyzed. From those sequences, only six sequences corresponded to *F. vaginae* strains. The determination of sensitivity and specificity was done using the equations previously described by Almeida et al. (Almeida et al., 2013). The analysis of the probe resulted in a theoretical value of sensitivity of 100% and specificity of 99.9%; but it should be considered that the sensitivity value was estimated based on the very low available number of *F. vaginae* target sequences. The values of melting temperature and Gibbs free energy were also calculated *in silico*, resulting in 61.16°C and -17.71 kcal/mol, respectively.

Optimization of Experimental Conditions of FISH Procedure

PNA-FISH procedure can be affected by several factors that will influence the fluorescence signal of the probe. Factors such as pH, dextran sulfate, probe concentration (Rocha et al., 2016), fixation and permeabilization steps (Rocha et al., 2018), as well as the time and temperature of hybridization, are important in the outcome of the FISH method. As such, we first performed pilot experiments testing different times (60 and 90 min) and temperatures (50 to 63°C) of hybridization to obtain the best signal of the probe. The optimization assays (**Supplementary Table 1**) resulted in an optimal signal-to-noise ratio at a temperature of 56°C and 60 min, which was the selected temperature for the determination of the probe analytical sensitivity and specificity.

Determination of *F. vaginae* Probe Analytical Sensitivity and Specificity

F. vaginae probe analytical sensitivity was determined using 24 different isolates of *F. vaginae*. As described in **Table 1**, the

results of hybridization were qualitatively classified into four levels: absence (-), poor (+), moderate (++), and good (+++) hybridization. All tested *F. vaginae* isolates showed hybridization with the probe, although with different efficiencies, resulting in a value of analytical sensitivity of 100%. For the determination of analytical specificity, 40 different bacterial species associated with BV or with vaginal microbiota were used. Under the tested conditions, no hybridization was detected, which result in an analytical specificity of 100% (**Table 2**). **Figure 1** presents examples of the hybridization results for *F. vaginae* strains with either good or poor hybridization, as well as for some of the most common BV-associated bacteria, as well as *L. crispatus* which is typically associated with optimal vaginal microbiota. Results from the other tested species are available in **Supplementary Figure 2**.

Detection of *G. vaginalis* and *F. vaginae* in Dual-Species Biofilms

Since BV development has been strongly associated with *Gardnerella* and *F. vaginae* biofilms (Swidsinski et al., 2005; Hardy et al., 2016), we also tested if the probe could be used in a multiplex assay, to discriminate species within a biofilm. A mixed biofilm of *G. vaginalis* and *F. vaginae* was grown and the presence of both species was assessed using our novel probe and a previously developed *Gardnerella* probe (Machado et al., 2013). As shown in **Figure 2**, both probes were able to detect the respective species in mono- and dual-species biofilms. We further assessed if this method could be used to estimate the abundance of *F. vaginae* in this complex biofilm structure. PNA-FISH image quantification resulted in *F. vaginae* abundance of 50.0 ± 7.8% but when the same biofilms were quantified by qPCR, *F. vaginae* abundance was slightly lower, around 39.1 ± 3.8%.

TABLE 1 | Hybridization of *F. vaginae* probe with different strains of *F. vaginae* for determination of analytical sensitivity.

Strain	Reference	Hybridization result
Fannyhessea vaginae	ACS-043-V-Col2	++
Fannyhessea vaginae	ATCC BAA-55	+++
Fannyhessea vaginae	BVS064	++
Fannyhessea vaginae	BVS065	++
Fannyhessea vaginae	BVS067	++
Fannyhessea vaginae	BVS069	++
Fannyhessea vaginae	CCUG 42099	+++
Fannyhessea vaginae	CCUG 44116	++
Fannyhessea vaginae	FB010-06	+++
Fannyhessea vaginae	FB101-3C	++
Fannyhessea vaginae	FB106b	++
Fannyhessea vaginae	FB106B	++
Fannyhessea vaginae	FB106C	++
Fannyhessea vaginae	FB130-CNAB-2aD	++
Fannyhessea vaginae	FB145-BA-14A	+++
Fannyhessea vaginae	FB158-CNA-2C	+++
Fannyhessea vaginae	FB160-CNAB-7	++
Fannyhessea vaginae	FB160-CNAB-7A	++
Fannyhessea vaginae	PB2003/009-T1-4	++
Fannyhessea vaginae	PB2003/017-T1-2	++
Fannyhessea vaginae	PB2003/189-T1-4	+
Fannyhessea vaginae	VMF0907COL23	+++
Fannyhessea vaginae	VMF0914COL13	++
Fannyhessea vaginae	VMF0914COL43	++

Hybridization results were evaluated qualitatively according to the classification: (-) Absence of hybridization; (+) Poor hybridization; (++) Moderate hybridization; (+++) Good hybridization.

DISCUSSION

In clinical settings, BV is most commonly diagnosed using the highly subjective Amsel's criteria (Forsum et al., 2005). Conversely, laboratory diagnosis is often based on microscopic observation of vaginal fluid specimens which are Gram-stained, to determine the Nugent Score (Nugent et al., 1991). Both methods are neither highly specific nor sensitive (Forsum et al., 2005) and the concordance between the two methods varies between 80% to 90% (Livengood, 2009). Due to antibiotic resistance in BV and its impact on BV recurrence (Bradshaw et al., 2006a; Sobel et al., 2019), the necessity of developing more reliable diagnostic methods have emerged (Cartwright et al., 2012; Hilbert et al., 2016; Gaydos et al., 2017; Schwebke et al., 2020). Herein, we designed a new PNA probe specific for *F. vaginae* that can be used in combination with a *Gardnerella* probe for a highly accurate BV diagnosis in laboratory settings. The theoretical evaluation of the new probe showed a sensitivity and specificity of 100% and 99.9%, respectively. This excellent performance was confirmed experimentally using 24 strains of *F. vaginae* and 40 other culturable species associated with the vaginal microbiota. Furthermore, our probe was also efficient in discriminating species in a multispecies biofilm.

The study of BV biofilms using FISH methodology has been widely developed since the first study conducted by Swidsinskii and colleagues that showed the presence of a biofilm in the vaginal epithelium, composed of *Gardnerella* spp. and *F. vaginae*, using DNA-probes specific for these species (Swidsinski et al., 2005). Despite being more affordable, DNA-FISH probes often present some low permeability efficiency and affinity, resulting in weaker fluorescence signals. PNA probes overcome some of the disadvantages of DNA probes (Singh et al., 2020). PNA probes are synthetic nucleic acids analogs, where the negatively charged backbone characteristic of DNA structure is replaced by an uncharged polyamide backbone, formed by repetitive units of N-(2-aminoethyl) glycine (Stender et al., 2002; Singh et al., 2020). The synthetic backbone and consequently the lack of electrostatic repulsion, provides PNA probes unique hybridization characteristics such as improved thermal stability, allowing a stronger binding, higher specificity to complementary sequences and more rapid hybridization kinetics comparing to the traditional DNA probes (Perry-O'Keefe et al., 2001; Stender et al., 2002; Shakeel et al., 2006). PNA probes also hybridize under low salt concentrations which is ideal for targeting nucleic acids

TABLE 2 | Hybridization of *F. vaginae* probe with different species for determination of analytical specificity.

Species	Reference	Hybridization result
Acinetobacter baumanii	CCUG 59798	–
Actinomyces neuii	UM067	-*
Actinomyces urogenitalis	CCUG 44038	–
Aerococcus christensenii	CCUG 28826	-*
Bacillus firmus	UM034	–
Bifidobacterium bifidum	CCUG 59492	-*
Brevibacterium ravenspurgense	CCUG 42923	-*
Campylobacter ureolyticus	CCUG 44295	-*
Corynebacterium tuscaniense	UM137	–
Enterococcus faecalis	UM035	-*
Escherichia coli	UM056	–
Gardnerella leopoldii	UM034	-*
Gardnerella piotii	UM035	-*
Gardnerella swidsinskii	UM094	-*
Gardnerella vaginalis	ATCC 14018	-*
Gemella haemolysans	UM034	-*
Lactobacillus crispatus	EX533959VCO6	–
Lactobacillus gasseri	ATCC 9857	-*
Lactobacillus iners	ATCC 55195	–
Lactobacillus rhamnosus	CECT 288	-*
Lactobacillus vaginalis	UM062	–
Megasphaera micronuciformis	CCUG 45952T	-*
Mobiluncus curtisii	ATCC 35241	–
Mobiluncus mulieris	ATCC 35239	-*
Mycoplasma hominis	UM054	-*
Neisseria gonorrhoeae	CCUG 13281	-*
Nosocomiicoccus ampullae	UM121	-*
Peptostreptococcus anaerobius	ATCC 27337	-*
Porphyromonas asaccharolytica	CCUG 7834T	–
Prevotella bivia	ATCC 29303	–
Propionibacterium acnes	UM034	-*
Shigella spp.	UM137	–
Sneathia sanguinegens	CCUG 66076	-*
Staphylococcus epidermidis	UM066	-*
Staphylococcus haemolyticus	UM066	–
Staphylococcus hominis	UM224	-*
Staphylococcus saprophyticus	UM121	–
Staphylococcus simulans	UM059	–
Streptococcus agalactiae	UM035	–
Veillonella parvula	CCUG 59474	-*

*Hybridization results were evaluated qualitatively according to the classification: (-) Absence of hybridization; (+) Poor hybridization; (++) Moderate hybridization; (+++) Good hybridization. *These species showed some autofluorescence signal detected in the FITC filter.*

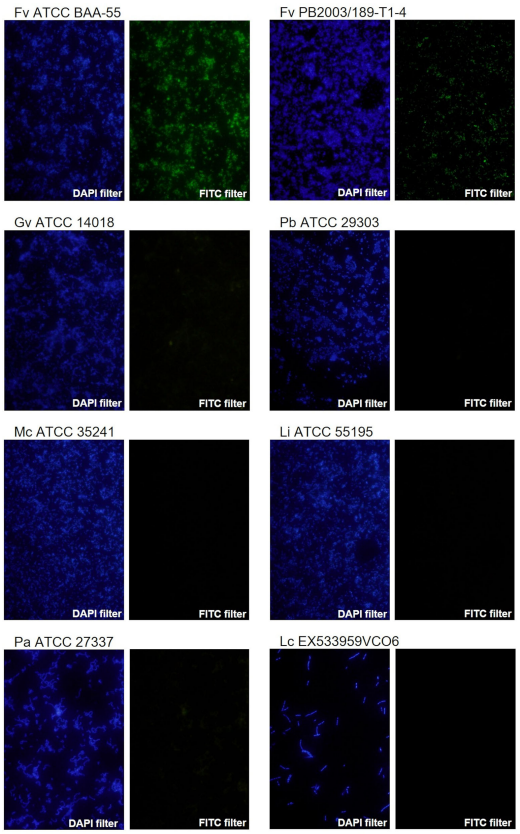

FIGURE 1 | Examples of fluorescence microscopy results using the novel PNA probe with *F. vaginae* strains and other BV-related species. *F. vaginae* ATCC BAA-55 (good hybridization), *F. vaginae* PB2003/189-T1-4 (poor hybridization), *G. vaginalis* ATCC 14018, *P. bivia* ATCC 29303, *M. curtisii* ATCC 35241, *L. iners* ATCC 55195, *P. anaerobius* ATCC 27337 and *L. crispatus* EX533959VCO6 (absence of hybridization). For each strain/species an image of DAPI staining (DAPI filter) and the correspondent signal of the probe (FITC filter) is shown. The images were acquired with a magnification of 400×.

with a high degree of secondary structures, as the absence of salts destabilizes the secondary structures (Perry-O'Keefe et al., 2001; Stender et al., 2002; Singh et al., 2020). The relative hydrophobic character of PNA probes allows the easy diffusion of the probe through the hydrophobic cell wall of fixed bacteria and yeasts (Stender et al., 2014). Furthermore, the unnatural backbone provides PNA probes resistance to the degradation by enzymes, such as nucleases and proteases (Stender et al., 2002; Singh et al., 2020). All these characteristics have given PNA a remarkable advantage over the use of DNA probes (Singh et al., 2020), and nowadays they are widely used in FISH methodology as means to improve its efficiency. The *F. vaginae* PNA-probe (AtoITM1) previously developed by Hardy and colleagues showed a sensitivity of 66.7% and specificity of 89.4%. However, their probe efficiency determination was not assessed against a large panel of pure cultures clinical isolates, as in this study, but instead by comparing PNA-FISH data with PCR data obtained by analyzing vaginal samples (Hardy et al., 2015), and as such, a direct comparison of probes efficacy is not possible.

One disadvantage of PNA-FISH detection, as compared with qPCR, is related to the sampling process and the heterogeneity of biofilms (Lopes et al., 2018). Furthermore, PNA-FISH does not allow high-throughput analysis, becoming more time consuming. Herein, we analyzed 20 images per biofilm. Although this is a significant number of images, it only represents a fraction of the biofilm. Conversely, qPCR data is obtained by homogenizing the whole biofilm and is more likely to be quantitatively accurate.

Overall, this work demonstrates an improved alternative for the detection of *F. vaginae* in BV biofilms, with very high specificity and sensitivity. Taking into consideration that *F.*

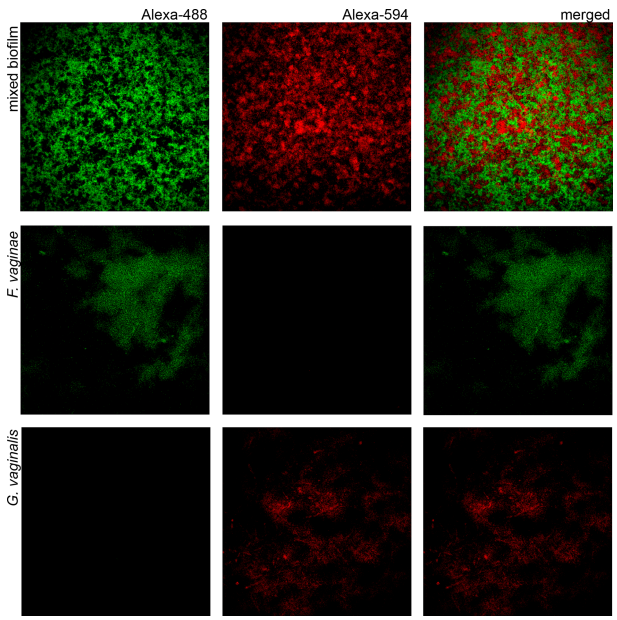

FIGURE 2 | Confocal Laser Scanning Microscopy images of single- and dual-species biofilms hybridization with Gard162 and FvagPNA651 probes. Gard162 probe detected *G. vaginalis* 24 h single-species biofilm and *F. vaginae* probe detected *F. vaginae* 24 h single-species biofilm. The probes were able to detect *G. vaginalis* (red) and *F. vaginae* (green) on 24 h dual-species biofilm. The images were acquired using 40× objective.

vaginae and *Gardnerella* co-culture has been considered the most specific marker for BV diagnosis (Menard et al., 2008), our multiplex approach might be a robust alternative for an accurate BV diagnosis, however, this needs to be determined in the future, by using clinical samples of women with BV. Furthermore, since this method is based on PNA-FISH methodology, it will also significantly contribute to other research studies that aim to study *in situ* BV biofilm structure, a unique advantage that non-FISH molecular methods lack.

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

CA, CM, and NC designed the experiments. LS and CA performed the *in silico* design of the PNA probe. LS performed the PNA-FISH hybridization experiments. LS and NC performed the biofilm/CLSM experiments. JC and AF performed the PNA-FISH/qPCR comparison experiments. LS and NC drafted the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the National Institute of Allergy and Infectious Diseases (R01AI146065-01A1). It was also partially funded by the Portuguese Foundation for Science and Technology (FCT), under the scope of the strategic funding of unit (UIDB/04469/2020).

ACKNOWLEDGMENTS

LS acknowledges Fundação para a Ciência e Tecnologia the financial support of individual Grant 2020.04912.BD.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.779376/full#supplementary-material

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Conflict of Interest: CM has received research grant support from Lupin Pharmaceuticals, is a consultant for Lupin Pharmaceuticals and BioFire Diagnostics, and has received honoraria from Elsevier, Abbott Molecular, Cepheid, Becton Dickinson, Roche Diagnostics, and Lupin. She is currently on the scientific advisory board for Roche and PhagoMed.

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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frontiers in Cellular and Infection Microbiology

REVIEW
published: 22 November 2021
doi: 10.3389/fcimb.2021.792787

Contribution of *Lactobacillus iners* to Vaginal Health and Diseases: A Systematic Review

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Lactobacillus iners, first described in 1999, is a prevalent bacterial species of the vaginal microbiome. As *L. iners* does not easily grow on de Man-Rogosa-Sharpe agar, but can grow anaerobically on blood agar, it has been initially overlooked by traditional culture methods. It was not until the wide application of molecular biology techniques that the function of *L. iners* in the vaginal microbiome was carefully explored. *L. iners* has the smallest genome among known *Lactobacilli* and it has many probiotic characteristics, but is partly different from other major vaginal *Lactobacillus* species, such as *L. crispatus*, in contributing to the maintenance of a healthy vaginal microbiome. It is not only commonly present in the healthy vagina but quite often recovered in high numbers in bacterial vaginosis (BV). Increasing evidence suggests that *L. iners* is a transitional species that colonizes after the vaginal environment is disturbed and offers overall less protection against vaginal dysbiosis and, subsequently, leads to BV, sexually transmitted infections, and adverse pregnancy outcomes. Accordingly, under certain conditions, *L. iners* is a genuine vaginal symbiont, but it also seems to be an opportunistic pathogen. Further studies are necessary to identify the exact role of this intriguing species in vaginal health and diseases.

Keywords: bacterial vaginosis, dysbiosis, *Lactobacillus iners*, sexually transmitted infections, vaginal microbiota

OPEN ACCESS

Edited by:
Antonella Marangoni, University of Bologna, Italy

Reviewed by:
Barbara Giordani, University of Bologna, Italy
Olga Budilovskaya, D. O. Ott Research Institute of Obstetrics, Gynecology and Reproductology, Russia

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Specialty section:
This article was submitted to Biofilms, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *11 October 2021*
Accepted: *04 November 2021*
Published: *22 November 2021*

Citation:
Zheng N, Guo R, Wang J, Zhou W and Ling Z (2021) Contribution of Lactobacillus iners to Vaginal Health and Diseases: A Systematic Review. Front. Cell. Infect. Microbiol. 11:792787. doi: 10.3389/fcimb.2021.792787

INTRODUCTION

The vaginal microbiome plays an important role in determining human vaginal health. Using high-throughput metagenomic and 16S rRNA sequencing, over 250 bacterial species have been identified in the human vagina (Fredricks et al., 2005; Chen et al., 2020). Among them, *Lactobacillus* is the most frequently detected microorganism in the healthy vagina, and this includes *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus jensenii*, and *Lactobacillus gasseri* (Alonzo Martinez et al., 2021). For decades, *Lactobacillus* species have been regarded as beneficial to the vaginal econiche by preventing the invasion of pathogens through the production of organic acids, hydrogen peroxide (H_2O_2), bacteriocin, and other antimicrobial compounds (Petrova et al.,

2015). Predisposing factors, such as menstruation, pregnancy, sexual practices, vaginal douching, and uncontrolled use of antibiotics, can rapidly alter the microbial community (Chee et al., 2020). A disruption of the vaginal ecosystem is characterized by the depletion of *Lactobacillus* species and the overgrowth of non-*Lactobacillus* microbes. Typically, the overgrowth of anaerobic bacteria can result in aberrant conditions, such as bacterial vaginosis (BV) and sexually transmitted infections (STIs), as well as pregnancy-related complications (Fredricks et al., 2005; Bautista et al., 2016; Chang et al., 2020).

Since the advent of metagenome sequencing techniques, *L. iners* has been recognized as the most prevalent *Lactobacillus* species in the vaginal econiche (Spear et al., 2011; Campisciano et al., 2020). This species has been initially overlooked in past bacteriologically-based studies because of its fastidious requirements and inability to grow on de Man-Rogosa-Sharpe agar (MRS), a selective culture medium that isolates vaginal *Lactobacilli* (Falsen et al., 1999). Furthermore, *L. iners* has very unique characteristics compared with other symbiotic *Lactobacillus* species in the vaginal econiche (Vaneechoutte, 2017). Most vaginal *Lactobacillus* species exert a protective effect and play a role in the resistance of the vaginal tract to colonization by pathogens. However, the relationship between *L. iners* and vaginal health is somewhat complicated and ambiguous (Petrova et al., 2017). This review aims to present the overall characteristics, an overview of different arguments, and the dual roles of *L. iners* in the vaginal econiche.

CHARACTERISTICS OF *L. iners*

Culture Characteristics and Gram-Staining Properties

L. iners was first described by Falsen et al. in 1999 in vaginal and urinary tract specimens (Falsen et al., 1999). This species had escaped our attention for a long time due to its inability to grow on MRS agar under the same culture conditions as other *Lactobacillus* species (De Backer et al., 2007). Nevertheless, *L. iners* is characterized by small, smooth, circular, translucent, and non-pigmented colonies after 24 h of anaerobic incubation on blood agar (Falsen et al., 1999). Indeed, most *L. iners* isolates can grow on MRS agar upon the addition of 1–5% sheep and human blood (Vaneechoutte, 2017). In addition, Yoshimura et al. demonstrated that *L. iners* can grow on MRS agar under anaerobic conditions for a period of at least 7 days, which is evidently longer than that of other *Lactobacillus* species. In MRS broth with 0.5% cysteine as the reducing agent, which created the anaerobic conditions, *L. iners* grows slowly to its highest concentration of only 10^7 CFU/ml and then growth decreases after 12 h (Yoshimura et al., 2020).

L. iners was initially believed to be a Gram-positive, rod-shaped, non-spore-forming, and facultative anaerobic bacterium (Falsen et al., 1999). However, several studies have reported that, unlike other *Lactobacillus* species, *L. iners* does not always clearly stain as Gram-positive, and it seems to have a coccobacillary rather than a bacillary morphology (De Backer et al., 2007; Lebeer et al., 2008). Yoshimura et al. reported that *L. iners* was mostly Gram-negative with a very short rod shape and weak acid resistance, as it was non-viable in pH 3 medium (Yoshimura et al., 2020). This may be the reason why *L. iners* was initially overlooked by culture and microscopy methods. By transmission electron microscopy, Kim et al. revealed that the peptidoglycan (PG) layer in the cell wall of *L. iners* was thin enough to give an apparent Gram-negative morphology (Kim et al., 2020). This morphological characteristic and Gram-staining property of *L. iners* are clinically very important to consider, as Nugent scoring, which is based on the Gram-staining of vaginal smears, remains a common diagnostic tool in the assessment of vaginal health (Wang et al., 2021). The Gram-negative property of *L. iners* masks the fact that it is a *Lactobacillus* species and this may lead to the misdiagnosis of BV, which is a condition characterized by the depletion of *Lactobacillus* species in Gram-stained vaginal smears under microscopy (Vaneechoutte, 2017). This may help explain why as many as 50% of women diagnosed with BV by the Nugent score are asymptomatic (Klebanoff et al., 2004).

Genome Size and Function

L. iners has the smallest genome of ∼1.3 Mbp on a single chromosome among the *Lactobacillus* species identified so far, with its pangenome count of 2300 genes and average GC content of ∼33.3% (France et al., 2016). This low genome size is comparable to those of human symbionts and parasites, and is strongly indicative of a more parasitic, host-dependent lifestyle (Petrova et al., 2017). The genome of *L. iners* seems to have undergone rapid evolution events that resulted in large-scale gene loss and genome reduction, as well as the acquisition of genes, such as iron-sulfur genes, for specific adaption to the vaginal econiche (Macklaim et al., 2011).

Comparative genome analysis revealed that *L. iners* had a severely reduced number of genes related to carbohydrate and amino acid metabolism, whereas it maintained conserved genes for largely core metabolic proteins and membrane transport genes for essential compounds from the host or the community (Macklaim et al., 2011; Kim et al., 2020). Three potential core genes (inerolysin, ZnuA, and hsdR) were identified to be closely related to the specific adaption of *L. iners* to the vaginal environment (Kwak et al., 2020). Among them, inerolysin is an unusual pore-forming cholesterol-dependent cytolysin that is active in the acidic vaginal environment and creates aqueous pores within the cell membrane. It may be one of the essential *L. iners* genes required to stably obtain nutrients from the host (Rampersaud et al., 2011; France et al., 2016). High-affinity zinc uptake requires the binding protein ZnuA type I (ZnuA), which is essential for metal ion homeostasis in *L. iners*. ZnuA may be a key mediator of strong adhesion of *L. iners* to vaginal epithelial cells (Gabbianelli et al., 2011; McMillan et al., 2013). Type I restriction enzyme R protein (hsdR) was suggested to be involved in the defense mechanism against bacteriophage infection during BV (Miller-Ensminger et al., 2018). In addition, *L. iners* contains genes that encode all enzymes directly involved in PG synthesis and hydrolysis (Kim et al., 2020). The unique and thin PG layer of *L. iners* cell membranes may absorb nutrients or secrete proteins more easily than other

Lactobacillus species, which can provide essential nutrients or respond to rapid changes in the vaginal environment (Kim et al., 2020).

Ability to Produce Lactic Acid and H_2O_2

Lactobacillus species are the main lactic acid-producing bacteria in the vagina, and they reduce the vaginal pH and restrict the growth of potentially harmful bacteria (Jang et al., 2019). Nevertheless, this lactic acid-producing ability is different from the main *Lactobacillus* species found in the vaginal microbiome (Godovalov et al., 2019). *L. crispatus*, *L. gasseri*, and *L. jensenii* can produce D- and L-lactic acid by fermenting glycogen, whereas *L. iners* can produce only L-lactic acid because it lacks the gene that codes for D-lactate dehydrogenase in its genome (France et al., 2016). Because of the almost complete absence of D-lactic acid, the L/D lactic acid ratio is highest in *L. iners*. Witkin et al. reported that the isomers of lactic acid have different effects on the host immune system (Witkin et al., 2013). The L/D lactic acid ratio in the vagina may elevate extracellular matrix metalloproteinase inducer (EMMPRIN) and subsequently activate matrix metalloproteinase-8 (MMP-8), which facilitates the breakdown of the extracellular matrix, helps bacteria transverse the cervix, and initiates upper genital tract infections (Beghini et al., 2015). Additionally, D-lactic acid has been reported to have a greater inhibitory effect on exogenous bacteria than L-lactic acid (Tachedjian et al., 2017). Therefore, it seems that L-lactic acid renders *L. iners* less effective in preventing the invasion of pathogens (Basavaprabhu et al., 2020).

Srinivasan et al. reported that microbiomes abundant in *L. crispatus* were consistently strongly associated with low vaginal pH, but this was not the case for women with *L. iners* overgrowth (Srinivasan et al., 2012). In fact, *L. iners* was very weak in a vaginal environment with low pH, failing to maintain intravaginal acidity. A high vaginal pH is a characteristic of BV, a condition in which *Gardnerella vaginalis* and *L. iners* are generally found in the vaginal econiche but other *Lactobacillus* species are rarely found (Muzny et al., 2018; Pleckaityte, 2019). In addition, *L. iners* does not have the molecular and cellular machinery to produce H_2O_2 through pyruvate oxidation. The production of H_2O_2 is considered to be one of the mechanisms by which *Lactobacillus* species can prevent anaerobic bacteria from colonizing the vagina (Felten et al., 1999; Ojala et al., 2014). As such, when pathogenic bacteria challenge the vaginal environment, *L. iners* cannot resist the overgrowth of pathogenic bacteria and the increase of pH, whereas it may persist in dysbiosis (Chee et al., 2020).

Adhesive Capability of *L. iners*

The adherence of vaginal *Lactobacillus* species to host cells is believed to play a role in the exclusion of pathogenic microorganisms through a mechanism that involves the blocking of their binding sites on vaginal epithelial cells (Pino et al., 2019; Mane et al., 2020). Although *L. iners* lacks most of the main adhesion molecules of *Lactobacillus* species (Morris et al., 2012), it still shows a strong adhesive ability to vaginal epithelial cells (McMillan et al., 2013). Fibronectin is an insoluble glycoprotein in the extracellular matrix of the vaginal epithelium (Park et al., 2012). The *L. iners* genome encodes a fibronectin-binding protein that contains a motif (fibronectin-binding protein A) common to pathogenic strains of *Staphylococcus aureus*, thereby mediating the adhesion and the invasion of *S. aureus* to host cells (Macklaim et al., 2011; Macklaim et al., 2013). McMillan et al. demonstrated that *L. iners* bound significantly stronger to human fibronectin than other *Lactobacillus* species at a more neutral pH, which may contribute to the persistence of *L. iners* in the vagina despite the presence of pathogens or treatment with antibiotics (McMillan et al., 2013). An *in vitro* study reported that *L. iners* may increase the adhesion of BV-causing *G. vaginalis* (Castro et al., 2013). It was also demonstrated that *L. iners* produces inerolysin, a pore-forming protein typically found in pathogenic bacteria, which can enhance the adhesive ability (Rampersaud et al., 2011; Ragaliauskas et al., 2019). These findings indicate that the unique adhesive function of *L. iners* reduces the protection of the healthy vaginal microbiome from pathogenic bacteria.

Requirement of Nutrients From Exogenous Sources

L. iners has an unusually small genome with reduced metabolic capabilities, but it contains a broader array of genes that was probably acquired from foreign sources. The nutrient requirements of this species are more complex than those of other vaginal *Lactobacillus* species, thereby allowing *L. iners* to adapt to the diverse niche in the vagina (Macklaim et al., 2011). The fluctuation of hormones and other factors may affect the vaginal environment, resulting in changes in mucus and glycogen production, pH, and microbial species, which may provide essential nutrients for *L. iners* (Kwak et al., 2020). Genome analysis has indicated a higher dependence of *L. iners* on exogenous sources of amino acids (France et al., 2016). Furthermore, *L. iners* has the molecular and cellular machinery to ferment glucose, maltose, trehalose, and mannose, among which glucose and maltose are common glycogenolysis products (France et al., 2016). Macklaim et al. reported that *L. iners* genes for the uptake of mannose and maltose, genes for glycogen decomposition, as well as genes for mucin and glycerol transport, were strongly up-regulated in BV (Macklaim et al., 2013). Although no iron uptake system has been identified in *L. iners*, ferrochelatase, which is capable of catalyzing ferrous ion and binding protoporphyrin IX to form heme, was detected in *L. iners* (Macklaim et al., 2011).

The ability of *L. iners* to produce inerolysin may be one of the most important factors influencing its ability to acquire nutrients from the vaginal environment. *L. iners* is the only *Lactobacillus* species known to code for inerolysin, which is related to intermedilysin (69.2% similarity) and vaginolysin (68.4% similarity) produced by *Streptococcus intermedius* and *G. vaginalis*, respectively (Rampersaud et al., 2011). Over 10% of genes coding for inerolysin in *L. iners* are more highly expressed in dysbiosis than in balanced microbial environments (Macklaim et al., 2013). This cytolysin can liberate resources directly from host tissues or cells, which necessitates that *L. iners* acquire its nutrients from the host in a symbiotic way (Macklaim et al.,

2011). In other words, this characteristic may give *L. iners* a competitive advantage in the vaginal environment when nutrients are scarce, especially under potentially adverse conditions, such as BV, when other *Lactobacillus* species cannot colonize the vagina (Zozaya-Hinchliffe et al., 2010; Li and Ma, 2020).

L. iners AND WOMEN DISEASES

L. iners and Vaginal Dysbiosis

Compared with intestinal microflora, a typical feature of the vaginal microbial environment in healthy individuals is its extremely low bacterial diversity (Ravel et al., 2011; Collins et al., 2018). There are five major community-state types (CSTs) in healthy premenopausal women, namely, *L. crispatus*-dominated CST I, *L. gasseri*-dominated CST II, *L. iners*-dominated CST III, and *L. jensenii*-dominated CST V, whereas CST IV is characterized by the absence of *Lactobacillus* species (Wells et al., 2020). Vaginal dysbiosis, which is defined by a high bacterial diversity and a mixture of anaerobic bacteria, is frequently associated with a variety of gynaecological diseases (Eastment and McClelland, 2018; Chee et al., 2020; Chen et al., 2021).

L. iners can be predominant in the vagina of healthy women, or in those with vaginal dysbiosis, such as BV, or even in those receiving antimicrobial therapy (Ferris et al., 2007; Goodfellow et al., 2021). Many studies have reported that the presence of *L. crispatus* in the vagina is associated with good health, whereas communities dominated by *L. iners* fail to provide sufficient protection against vaginal dysbiosis (Petricevic et al., 2014; France et al., 2016; Tortelli et al., 2020). The existence of *L. iners* is related to higher levels of proinflammatory factors, such as interleukin-1α, interleukin-18, macrophage migration inhibitory factor, and tumor necrosis factor-α, which are responsible for the activation of an inflammatory response in the vagina (De Seta et al., 2019). *L. iners* is even believed to play a role in the onset of vaginal dysbiosis (Petrova et al., 2017), although the precise role of *L. iners* remains debated. However, it seems that the abundance of *L. iners* remains relatively constant, and *L. iners* is not easily displaced by pathogens or infectious conditions. In cases of BV, *L. iners*, rather than *L. crispatus*, usually coexists with other potentially harmful bacteria that colonize the vagina (Ferris et al., 2007; Zozaya-Hinchliffe et al., 2010). The ability of *L. iners* to adapt to dysbiosis, despite its small genome, may be related to its changes in genes involved in metabolism and cytolysis, as well as antibacteriophage defense genes, to changing conditions in the vagina (Borgdorff et al., 2016; Leizer et al., 2018). The remarkable ability of *L. iners* to survive under various conditions indicates that this species may be an important member of the host's defense and may be a persistent symbiotic *Lactobacillus* species that can maintain and restore the vaginal microbiome (Macklaim et al., 2011).

L. iners and BV

BV is the most common type of vaginitis in women of childbearing age. It is characterized by a significant reduction or disappearance of *Lactobacillus* species, accompanied by the emergence of more diverse microbiota dominated by anaerobic and facultative bacteria such as *Gardnerella* species, *Prevotella* species, and *A. vaginae* (Fredricks et al., 2005; Lee et al., 2020; Witkin et al., 2021). However, *L. iners* is usually the only vaginal *Lactobacillus* species coexisting with BV-associated bacteria that can be detected during BV (Macklaim et al., 2011; Santiago et al., 2012). It can persist under the drastically changing vaginal environment of BV due to its ability to respond and regulate its genomic functions (Macklaim et al., 2013). The increased gene expression of *L. iners* may lead to the production of succinate and other short-chain fatty acids and the increase in the pH value in the BV environment (Macklaim et al., 2013). To adapt to the BV environment, *L. iners* can increase the expression of inerolysin and mucin, and promote the production of glycerol and the expression of related metabolic enzymes, which ensures its acquirement of nutrients from foreign sources (Macklaim et al., 2013). In addition, bacteriophages were one of the reasons for the sudden decrease of *Lactobacillus* species during BV, whereas *L. iners* can upregulate defense systems such as the type I RM system and CRISPR, as well as its specific hsdR gene, to resist bacteriophage invasion during BV (Kwak et al., 2020). Nevertheless, a recent study discovered three active peptides of bacteriocin produced by a human intestinal strain named *Lactobacillus paragasseri*. These bacteriocins have strong selective inhibitory activity against *L. iners*, whereas *L. crispatus*, *L. jensenii*, and *L. gasseri* were only slightly inhibited, indicating that these *Lactobacillus*-derived effective inhibitors of *L. iners* can be combined with metronidazole to improve the current BV treatments (Nilsen et al., 2020).

As the coexistence of *L. iners* in BV is different from that of other *Lactobacillus* species, the prevalence of *L. iners* can be used as a microbial indicator to predict the onset of BV or the intermediate BV status (Basavaprabhu et al., 2020). Furthermore, *L. iners* is metronidazole-resistant, and it was found to be the predominant *Lactobacillus* species, even after treatment of BV with metronidazole (Ferris et al., 2007; Srinivasan et al., 2010; Mayer et al., 2015; Lehtoranta et al., 2020). Compared with other more protective *Lactobacillus* species, which hardly exist during BV, *L. iners* showed a stronger competitive advantage and coexisted in the disrupted microbiome (Nilsen et al., 2020). It has been proposed that *L. iners* facilitates the transition between BV and non-BV states (Shipitsyna et al., 2013; Petrova et al., 2015). Interestingly, it has been reported that even after BV treatment, the vaginal microbiome does not change from the *L. iners*-dominant state to the *L. crispatus*-dominated state (Lambert et al., 2013). Therefore, the persistence of *L. iners* may lead to long-term vaginal dysbiosis, especially after repeated treatment cycles of BV (Nilsen et al., 2020). Further studies are needed to clarify whether this species is only a biomarker of the vaginal microbiota transition or a contributing factor of BV.

L. iners and Biofilm Formation

Biofilms are bacterial structures tightly attached to a surface, and they are known to be more resistant to the host immune response and antibiotic therapy than planktonic cells (Hall-Stoodley et al.,

2012). It has been shown that biofilm formation on vaginal epithelial cells is strongly associated with vaginal infections (Costerton et al., 1999; Srinivasan and Fredricks, 2008). There is sufficient evidence that BV associates with the presence of a dense polymicrobial biofilm, in which *G. vaginalis* is the dominant bacterial strain on the vaginal epithelium (Machado et al., 2015; Rosca et al., 2020). It has been hypothesized that *Gardnerella* spp. initiate biofilm formation, which supports the attachment of other BV-associated bacteria (BVAB) to the vaginal epithelium, further enhancing the biofilm thickness (Muzny et al., 2019). Moreover, *Gardnerella* biofilms serve as barriers to antibiotics and function to protect other BVAB by preventing the penetration of antibiotics (Gustin et al., 2021). It is generally believed that the high rate of BV recurrence is due to the formation of biofilms that protect the bacteria from antibiotic treatment, and even serve as a reservoir for pathogen regrowth (Bradshaw et al., 2006; Gottschick et al., 2017).

Vaginal indigenous *Lactobacilli* are believed to prevent the colonization of pathogenic bacteria through steric hindrance or receptor masking in the mucosa (Zarate and Nader-Macias, 2006). Previous studies have used a *Lactobacillus* probiotic approach in an attempt to clear the polymicrobial biofilms, essentially impeding bacterial virulence and suppressing infection in the human vagina (Saunders et al., 2007; Hardy et al., 2017; Chee et al., 2020). *Lactobacillus plantarum* was reported to significantly reduce the adhesion of *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* in the HT-29 cell line, which made it a potential anti-biofilm agent for BV treatment (Liu et al., 2017). Saunders et al. reported that *G. vaginalis* biofilms grown *in vitro* were displaced with *Lactobacillus reuteri* RC-14, and to a limited extent with *L. iners* (Saunders et al., 2007). Castro et al. demonstrated that *L. crispatus* drastically reduced the adhesion of *G. vaginalis* strains, both from a healthy woman and a woman with BV, to cervical epithelial cells. Interestingly, *L. iners* significantly reduced the adhesion of *G. vaginalis* strains from a healthy woman, but markedly enhanced pathogenic *G. vaginalis* adhesion (Castro et al., 2013), suggesting that *L. iners* can cohabitate with BV-associated *G. vaginalis* and may contribute to *G. vaginalis*-dominated biofilm formation (Gottschick et al., 2017). In addition, it is well known that *Candida* species, mainly *C. albicans*, can form thick and tough biofilms, which greatly increases the tolerance to antifungal drugs during the treatment of recurrent vulvovaginal candidiasis (Taff et al., 2013). Mckloud et al. reported the ability of various *Lactobacillus* species to inhibit *C. albicans* biofilm formation and biofilm-related gene expression when cocultured (McKloud et al., 2021). *Lactobacillus rhamnosus* could down-regulate *C. albicans* biofilm-related gene expression. Conversely, coculture with *L. iners* resulted in an up-regulation of biofilm-related gene expression (ALS3 and ECE1), suggesting that the presence of *L. iners* may be indicative of a shift to vaginal dysbiosis; therefore, it should not be used as a probiotic intervention for *C. albicans* infection (Ponomarova et al., 2017). A further understanding of the interactions between vaginal commensal *Lactobacilli* and the structure and function of biofilms is of extreme importance to identify novel treatment approaches for biofilm-associated infections (Falconi-McCahill, 2019).

L. iners and STIs

Previous studies have reported that *L. crispatus*-dominated vaginal microbiomes associate with a lower prevalence of STIs, whereas BV associates with an elevated risk of STIs such as infection with *Chlamydia trachomatis*, human immunodeficiency virus (HIV), *Neisseria gonorrhoeae*, cytomegalovirus, and herpes simplex virus-2 (Bayigga et al., 2019; Gondwe et al., 2020; Redelinghuys et al., 2020). Van Houdt et al. reported that the vaginal microbiome dominated by *L. iners* at baseline significantly increased the risk of acquiring *C. trachomatis* infection one year later (van Houdt et al., 2018). A lack of D-lactic acid in the *L. iners*-dominated vaginal microbiome may increase the ability of HIV to transverse the cervicovaginal mucus by modulating cervical integrity (Witkin, 2015; Reimers et al., 2016; Hoang et al., 2020). Interestingly, Mehta et al. and Reimers et al. demonstrated that the vaginal microbiome did not differ between HIV-positive and HIV-negative black women in the United States (Mehta et al., 2015; Reimers et al., 2016). However, Spear et al. conversely observed that the percentage of *L. iners* was significantly higher in HIV-negative African Americans than in HIV-positive African Americans (Spear et al., 2011). The reason for these inconsistent results may be differences in the genetic background or complicated social and behavioral factors in black women, as black women without BV were more likely to have vaginal microbiomes dominated by *L. iners* (Fettweis et al., 2014; Wells et al., 2020). The precise role of *L. iners* in HIV infection should be further examined. Many studies reported a higher diversity of vaginal microbes and a lower abundance of *Lactobacillus* species among HPV-positive women (Lee et al., 2013; Oh et al., 2015; Reimers et al., 2016). Norenhag et al. showed that the vaginal microbiome dominated by *L. iners* was associated with high-risk HPV infection compared with *L. crispatus* (Norenhag et al., 2020). It can be speculated that vaginal dysbiosis may affect the host's innate immunity against HPV infection, resulting in dysplasia/cervical cancer (Kyrgiou et al., 2017). These findings indicate that *L. iners* may exhibit rapid changes in the composition of the vaginal microbiome similar to BV and could be a valuable biomarker of the dynamic vaginal environment under STIs (Ravel et al., 2013; van Houdt et al., 2018).

L. iners and Preterm Birth (PTB)

It is especially important to maintain the natural and healthy balance of *Lactobacillus* species in the vaginal microbiome during pregnancy (Zheng et al., 2019; Juliana et al., 2021). Earlier studies confirmed that high estradiol levels and the consequent high glycogen levels in the vagina during pregnancy result in stronger vaginal acidification, thereby promoting the prevalence of *Lactobacillus* species as gestation progresses (Aagaard et al., 2012; Basavaprabhu et al., 2020). However, many studies have indicated that the *L. iners*-

dominated vaginal microbiome was more likely to shift towards dysbiosis during pregnancy (Mls et al., 2019; Kumar et al., 2021; Sarmento et al., 2021). In our previous study, we found that the abundance of *L. iners* decreased significantly in the second and third trimester, whereas that of *L. crispatus* increased in the second trimester compared with the first trimester in healthy pregnant women (Zheng et al., 2019). In addition, we observed that the increase in the abundance of *L. iners*, but not that of *L. crispatus*, was related to the increase in vaginal cleanliness and positive leukocyte esterase activity, which is consistent with the results of a previous study (Vaneechoutte, 2017).

Increasing evidence indicates that BV is one of the major etiological causes for adverse pregnancy outcomes, especially PTB (Guerra et al., 2006; Basavaprabhu et al., 2020; Redelinghuys et al., 2020). The *L. iners*-dominated vaginal microbiome, a so-called 'intermediate microflora' and a typical feature of BV, is speculated to be a risk factor for PTB (Petricevic et al., 2014; Kindinger et al., 2017). Petricevic et al. suggested that the prevalence of *L. iners* detected in vaginal smears of healthy women in early pregnancy can associate with PTB (Petricevic et al., 2014). This was also demonstrated by Kindinger et al., who reported that the vaginal microbiome dominated by *L. iners* at 16 weeks of gestation is a risk factor for both a short cervix and early PTB (<34 weeks), whereas *L. crispatus* dominance is protective against PTB in a more ethnically diverse cohort (Kindinger et al., 2017). Recent studies from different countries also showed a significant association between *L. iners* and an increased prevalence of PTB (Elovitz et al., 2019; Aslam et al., 2020; Goodfellow et al., 2021; Kumar et al., 2021; Payne et al., 2021; Sarmento et al., 2021). However, several studies reported no significant association between *L. iners* and PTB (Callahan et al., 2017; Blostein et al., 2020; Witkin et al., 2021). *L. iners* was also demonstrated to be the most abundant *Lactobacillus* species among pregnant black women (Wells et al., 2020). However, most studies did not identify a significant relationship between the *Lactobacillus*-dominant vaginal microbiome and PTB in pregnant black women (Hyman et al., 2014; Nelson et al., 2016; Subramaniam et al., 2016; Stout et al., 2017). Conversely, three studies reported that *L. iners* was associated with a decreased risk of PTB (Fettweis et al., 2019; Tabatabaei et al., 2019; Park et al., 2021). Therefore, the association between *L. iners* and PTB risk is controversial (**Table 1**). Presently, it is believed that the vaginal microbiome in black women does not play an important role in the pathogenesis of PTB, as it does in Caucasians and Asians (Kindinger et al., 2017). Furthermore, the limited sample size, the time of sample collection, differences in the definition of PTB, ethnical and geographical variations, and differences in the methodology of strain identification, as well as complicated clinical conditions such as genetic abnormalities or a history of PTB, were confounding factors that impacted the results (Ravel et al., 2011; Jespers et al., 2012; Mehta et al., 2020; Wells et al., 2020).

According to the features of *L. iners* and its lack of protection against pathogens when it is the only *Lactobacillus* species in the vagina, some researchers have suggested that *L. iners* cannot be responsible for infections during pregnancy (Petricevic et al., 2014; Peelen et al., 2019). Indeed, because the vaginal microbiome dominated by *L. iners* is relatively unstable, there is a tendency for transition to BV-associated CST-IV during pregnancy (Verstraelen et al., 2009). In addition, the *L. iners*-dominated vaginal microbiome may increase the risk of PTB by modulating local tissue inflammation and cervical integrity, thereby disrupting chemical and mechanical mucosal protective barriers against ascending infections (Kindinger et al., 2017). Further studies are needed to clarify the potential mechanisms between the *L. iners*-dominated vaginal microbiome and PTB.

L. iners and Infertility

Previous studies have reported that up to 40% of patients who failed assisted reproduction by *in vitro* fertilization (IVF) had an abnormal reproductive tract microbiome (Fanchin et al., 1998; Moore et al., 2000; Koedooder et al., 2019). Vaginal dysbiosis, including an elevated pH value, increased flora diversity, BV, vulvovaginal candidiasis, and trichomonal vaginitis, are recognized as risk factors for infertility (Campisciano et al., 2017; Moumne et al., 2021). Campisciano et al. reported that the abundance of *L. iners* was associated with an increased infertility rate (Campisciano et al., 2020). Chen et al. also recently reported that the *L. iners*-dominated vaginal microbiome was associated with tubal infertility and *C. trachomatis* infection (Chen et al., 2021). As a transitional species, *L. iners* may facilitate the transition between an abnormal and a normal vaginal microbiome under treatment or artificially high estrogen levels that are needed for IVF (Kindinger et al., 2017; Kosti et al., 2020). It is believed that the *L. iners*-dominated vaginal microbiome is an unfavorable factor for pregnancy.mk

L. iners AND THE MENSTRUATION CYCLE

The Human Microbiome Project shows that in the microbial community for all body parts, including the vagina, within-subject variations over time are consistently lower than between-subject variations (Human Microbiome Project, 2012). The menstrual cycle is one of the most important factors disturbing the diversity of the vaginal microbiome (Chaban et al., 2014; Chen et al., 2021). *L. crispatus* usually dominates the vagina of reproductive-age women, whereas *L. iners* overgrows and replaces *L. crispatus* during the menstruation cycle (Gajer et al., 2012; Santiago et al., 2012). A recent study reported that *L. iners* was the most recurrent microbe in the follicular phase; *L. iners* and CST IV (microbial diversity) were predominant in the periovulatory phase; and in the luteal phase, the most frequent type was CST IV (Alonzo Martinez et al., 2021). Indeed, the abundance of *L. iners* remarkably increases during menses, frequently in conjunction with an increase of *G. vaginalis* and/or *Atopobium vaginae*; however, they subsequently decrease after menses without intervention (Jespers et al., 2012; Petrova et al., 2015). As dynamic changes in the vaginal econiche were characterized at different time points in the menstruation cycle within the same individual, the moment of sampling relative to the menstrual cycle is very important for vaginal community analysis.

TABLE 1 | Main results per study on *L. iners* and PTB.

Author (year)	Country	Time of sample collection	Sample size	Tools implied for identification	Main findings	References
Petricevic et al.	Austria	At 11-14 weeks of gestation	111 women (white European, Middle Eastern, Asian)	PCR-DGGE and sequencing	*L. iners* was the predominant vaginal *Lactobacillus* spp. in women who delivered preterm newborns. *L. iners* was predominantly present in 11/13 (85%) of women who delivered preterm newborns and in only 16/98 (16%) of women who delivered at term ($p < 0.001$).	(Petricevic et al., 2014)
Kindinger et al.	United Kingdom	At 16 weeks of gestation	161 women (30 Black, 104 Caucasian, 27 Asian)	16S rRNA gene sequencing at V1-V3 region	*L. iners* dominance of the vaginal microbial community at 16 weeks of gestation was significantly associated with both a short cervix <25 mm and early PTB (<34 weeks). By contrast, *L. crispatus* dominance was highly predictive of TB.	(Kindinger et al., 2017)
Callahan et al.	United States	Weekly	Low-risk cohort: 39 women; High-risk cohort: 96 women	16S rRNA gene sequencing at V4 region	*L. crispatus* was associated with the low risk of PTB in low- and high-risk cohorts, whereas no significant association was detected for *L. iners*. A subspecies clade of *Gardnerella vaginalis* explained the genus association with PTB.	(Callahan et al., 2017)
Tabatabaei et al.	Canada	At 8-13 weeks of gestation	94 spontaneous PTB cases, 356 term controls	16S rRNA gene sequencing at V4 region	*Lactobacillus gasseri*/*L. johnsonii*, *L. crispatus* (99%)/*L. acidophilus* (99%), *L. iners* (99%)/*Ralstonia solanacearum* (99%) and *Bifidobacterium longum*/*Bifidobacterium breve* were associated with a decreased risk of early but not late spontaneous PTB.	(Tabatabaei et al., 2019)
Elovitz et al.	United States	At 16–20 weeks, 20–24 weeks and 24–28 weeks of gestation	539 women (402 African American, 115 white, 22 other)	16S rRNA gene sequencing at V3-V4 region	In non-African American women, *L. iners* and *A. vaginae* were significantly associated with increased rates of spontaneous PTB.	(Elovitz et al., 2019)
Fettweis et al.	United States	At prenatal visit, at triage	45 spontaneous PTB cases and 90 term controls (African American predominantly)	16S rRNA gene sequencing at V1-V3 region	*L. crispatus* increased in abundance during pregnancy in women who delivered preterm newborns. Women who delivered at term exhibited significant decreases in the abundance of *A. vaginae* and *G. vaginalis*, and an increase in the abundance of *L. iners*.	(Fettweis et al., 2019)
Aslam et al.	Pakistan	Not available	8 term vaginal swabs, 8 preterm vaginal swabs, and 8 preterm placenta tissues	16S rRNA gene sequencing at V1-V2 region	Metagenomics data of vaginal swabs and placental tissues from severe PTB indicated that *L. iners* was the main difference between term and preterm deliveries. Overall, the lack of *Lactobacillus* species or the presence of rogue *Lactobacillus* species, such as *L. iners* and *L. vaginilis*, was associated with PTB.	(Aslam et al., 2020)
Blostein et al.	Peru	Before 16 weeks of gestation (9 weeks on average)	25 PTB cases and 100 term controls	16S rRNA gene sequencing at V4 region	Overall, no CST (diverse, *Lactobacillus*-dominated, or *L. iners*-dominated) was associated with PTB in crude or adjusted logistic models, whereas women with *Lactobacillus*-dominated CSTs were less likely to have PTB than those with diverse CST among women sampled before 12 weeks of gestation.	(Blostein et al., 2020)
Sarmento et al.	Brazil	In the second trimester	146 women	16S rRNA gene sequencing at V1-V3 region	*L. iners* was the dominant vaginal bacterium in 61.5% of women with spontaneous PTB but only in 31.2% of those who delivered at term ($p = 0.035$).	(Sarmento et al., 2021)
Kumar et al.	India	In each trimester of pregnancy	18 PTB cases and 20 term controls	16S rRNA gene sequencing at V3-V4 region	A significantly higher abundance of *L. iners* (all trimesters), *Megasphaera* sp (first trimester), *Gardnerella vaginalis* (second trimester), and *Sneathia sanguinegens* (second trimester) was identified in preterm samples, whereas a higher abundance of *L. gasseri* (third trimester) was observed in term samples.	(Kumar et al., 2021)
Witkin et al.	Brazil	In the second trimester	613 women	16S rRNA gene sequencing at V1-V3 region	Spontaneous PTB occurred in 9.6%, 9.3%, and 6.9% of women when *G. vaginalis*, *L. iners*, or *L. crispatus* was the dominant species, respectively, but the differences were not statistically significant.	(Witkin et al., 2021)
Goodfellow et al.	United Kingdom	At 15-22 weeks of gestation	109 high-risk women and 145 low-risk women	16S rRNA gene sequencing at V3-V4 region	*L. iners* achieved higher bacterial loads compared to the other *Lactobacillus* species and associated with early spontaneous PTB/PPROM recurrence.	(Goodfellow et al., 2021)
Park et al.	Korea	At 15-34 weeks of gestation	38 PTB cases and 56 term controls	Multiplex quantitative real-time PCR	Although most values for single bacteria were not statistically significant, the mean value of the total Bacillus class showed a significant difference between PTB and TB groups, in which the mean value of *L. iners* showed a significant increase in the TB group.	(Park et al., 2021)
Payne et al.	Australia	At 12-23 weeks of gestation	936 women (white race predominantly)	Quantitative PCR	Women who delivered at term had a higher level of *L. crispatus*, *L. gasseri*, or *L. jensenii* DNA in their vaginal swabs. In the remaining women, a specific microbial DNA signature was identified, which was strongly predictive of spontaneous PTB risk, consisting of *G. vaginalis*, *L. iners*, and *Ureaplasma parvum* DNA.	(Payne et al., 2021)

PTB, preterm birth; TB, term birth; PPROM, preterm prelabor rupture of membranes; DGGE, denaturing gradient gel electrophoresis.

ANTI-MICROBIAL AND IMMUNE-INDUCING ACTIVITY OF *L. iners*

L. iners is the most common and persistent vaginal symbiotic *Lactobacillus* species with good adaptability to the complex and dynamically changing vaginal environment (Borgdorff et al., 2016; Kwak et al., 2020). Under fluctuating environmental conditions, other *Lactobacillus* species may not survive, while *L. iners* persists with relatively constant abundance due to its ability to respond and regulate its genomic functions, such as specific carbohydrate uptake, fibronectin-binding protein activity, bacteriophage defense, and inerolysin synthesis (Rampersaud et al., 2011; Macklaim et al., 2013; McMillan et al., 2013; Kwak et al., 2020). This remarkable ability to survive under a range of conditions contributes to *L. iners*' being a dominant species when the microbiome is in a transitional stage (Jakobsson and Forsum, 2007), suggesting that *L. iners* may be an important member of the host defense mechanism as a persistent mutualistic lactobacilli, and even promote the restoration of a healthy vaginal microbiome (Ravel et al., 2011; France et al., 2016).

In fact, several studies have confirmed many probiotic characteristics of *L. iners*. It has many ecological functions, such as lactic acid production, that are similar to those of other *Lactobacillus* species (Linhares et al., 2011; O'Hanlon et al., 2011). Although the genome of *L. iners* lacks most of the adhesion factors of other *Lactobacillus* species, it can still adhere strongly to vaginal epithelial cells (Morris et al., 2012; McMillan et al., 2013).Thus, *L. iners* shows the similar phenotypic traits of colonization and host interaction, as well as excluding pathogens, as other vaginal *Lactobacillus* species (Osset et al., 2001). Shipitsyna et al. reported the loss of *L. iners* during BV and suggested that it was not the key pathogen causing the disease (Shipitsyna et al., 2013). *L. iners* can destroy or replace *G. vaginalis* to form biofilms *in vitro* (Hummelen et al., 2010; Zhou et al., 2010). Macklaim et al. demonstrated that some specific functions of *L. iners*, such as the expression of cytokines, absorption of exogenous sources, and bacteriophage defense, facilitated the harsh conditions in the vagina, including BV (Macklaim et al., 2013). *L. iners* prevents harmful bacteria from obtaining important nutrients, such as iron, and inhibits their sustained growth by triggering the innate immune system in vaginal epithelial cells (Vaneechoutte, 2017). In addition, the anti-inflammatory effects of *L. iners* were observed through specific molecular interactions between vaginal epithelial cells (Rose et al., 2012).

The *L. iners*-dominated vaginal microbiome was associated with the induction of a stress response in the vaginal epithelium (Vaneechoutte, 2017; Linhares et al., 2019). Doerflinger et al. discovered that *L. iners*, but not *L. crispatus*, significantly upregulated the pattern-recognition receptor signaling pathway in human primary vaginal epithelial cells and increased the mRNA expression of tumor necrosis factor, indicating that the vaginal microbiome regulates the host immune response species-specifically (Doerflinger et al., 2014). It has been suggested that, in response to stress, *L. iners* can activate the toll-like receptor signaling pathway in vaginal epithelial cells, increase heat shock protein 70 expression, and inhibit autophagy, which would destroy the homeostasis between vaginal epithelial cells and reduce the ability of these cells to recognize and respond to potential pathogens (Doerflinger et al., 2014; Feng et al., 2015). Conversely, many compounds involved in the antimicrobial defense of vaginal epithelial cells, such as neutrophil gelatinase-associated lipocalin, calprotectin, and hyaluronan, were also preferentially induced by *L. iners* (Leizer et al., 2018). These findings support the fact that *L. iners* can fight non-physiological threats, and maintain and promote the recovery to a healthier state, as well as exhibit proinflammatory qualities and act less like a commensal microbe under different conditions (Levine et al., 2011).

CONCLUSIONS

In conclusion, *L. iners* is a unique and intriguing *Lactobacillus* species with extraordinary characteristics. Its small genome and concurrent nutrient dependency are conducive to its high adaptation to both the low and the high pH vaginal environment, as well as both BV-positive and BV-negative conditions. Therefore, *L. iners* is often classified as a transitional species that colonizes the vagina after an ecological disturbance. However, whether *L. iners* is beneficial or pathogenic for the host's microbiome remains controversial. Most researchers are inclined to believe that *L. iners* offers limited protection against vaginal colonization by pathogens and may contribute to the onset and maintenance of vaginal dysbiosis. *L. iners* may also be a risk factor for sexually transmitted infections and adverse pregnancy outcomes. A greater understanding of the roles of *L. iners* in health and diseases in individuals of different races and ethnicities is warranted. In addition, further studies are required to clarify the role of *L. iners* in vaginal mucosal immune regulation, and to further clarify whether it can be used as a novel biomarker to detect the existence or prognosis of vaginal inflammation and guide subsequent clinical treatment.

AUTHOR CONTRIBUTIONS

NZ, RG, JW, WZ, and ZL discussed the contents, wrote, reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This present work was funded by the grants of the National Natural Science Foundation of China (81771724, 31700800, 81790631), the Taishan Scholar Foundation of Shandong Province (tsqn202103119), the Nutrition and Care of Maternal & Child Research Fund Project of Guangzhou Biostime Institute of Nutrition & Care (2019BINCMCF045), the National S&T Major Project of China (2018YFC2000500), and the Foundation of China's State Key Laboratory for Diagnosis and Treatment of Infectious Diseases.

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frontiers in Cellular and Infection Microbiology

PERSPECTIVE
published: 05 January 2022
doi: 10.3389/fcimb.2021.795797

Crystal Violet Staining Alone Is Not Adequate to Assess Synergism or Antagonism in Multi-Species Biofilms of Bacteria Associated With Bacterial Vaginosis

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OPEN ACCESS

Edited by:
António Machado, Universidad San Francisco de Quito, Ecuador

Reviewed by:
Camila Marconi, Federal University of Paraná, Brazil
David Esteban Pacha-Herrera, University of Debrecen, Hungary

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Specialty section:
This article was submitted to Biofilms, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *15 October 2021*
Accepted: *15 December 2021*
Published: *05 January 2022*

Citation:
Castro J, Lima Â, Sousa LGV, Rosca AS, Muzny CA and Cerca N (2022) Crystal Violet Staining Alone Is Not Adequate to Assess Synergism or Antagonism in Multi-Species Biofilms of Bacteria Associated With Bacterial Vaginosis. Front. Cell. Infect. Microbiol. 11:795797. doi: 10.3389/fcimb.2021.795797

Bacterial Vaginosis (BV) involves the presence of a multi-species biofilm adhered to vaginal epithelial cells, but its in-depth study has been limited due to the complexity of the bacterial community, which makes the design of *in vitro* models challenging. Perhaps the most common experimental technique to quantify biofilms is the crystal violet (CV) staining method. Despite its widespread utilization, the CV method is not without flaws. While biofilm CV quantification within the same strain in different conditions is normally accepted, assessing multi-species biofilms formation by CV staining might provide significant bias. For BV research, determining possible synergism or antagonism between species is a fundamental step for assessing the roles of individual species in BV development. Herein, we provide our perspective on how CV fails to properly quantify an *in vitro* triple-species biofilm composed of *Gardnerella vaginalis*, *Fannyhessea (Atopobium) vaginae*, and *Prevotella bivia*, three common BV-associated bacteria thought to play key roles in incident BV pathogenesis. We compared the CV method with total colony forming units (CFU) and fluorescence microscopy cell count methods. Not surprisingly, when comparing single-species biofilms, the relationship between biofilm biomass, total number of cells, and total cultivable cells was very different between each tested method, and also varied with the time of incubation. Thus, despite its wide utilization for single-species biofilm quantification, the CV method should not be considered for accurate quantification of multi-species biofilms in BV pathogenesis research.

Keywords: bacterial vaginosis, anaerobic bacteria, biofilm quantification, microtiter plates, crystal violet staining

INTRODUCTION

Biofilms are widely present in the environment (Hall-Stoodley et al., 2004), industry settings (Galié et al., 2018), and are causative agents of human infections (Vestby et al., 2020). A biofilm can be defined as a three-dimensional microbial community that grows on an abiotic or biotic surface, and is surrounded by an exopolymer matrix composed of bacterial- and environmental-derived

molecules (Flemming et al., 2016). This matrix composition can vary with time and is dependent on the bacterial species present in the biofilm, as well as environmental conditions (Flemming and Wingender, 2010). The biofilm matrix is an important protective barrier against external stimuli, including antimicrobial agents (Sharma et al., 2019). However, the matrix is not solely responsible for antimicrobial tolerance, with biofilm heterogeneity (Hall and Mah, 2017) and reduced metabolism (Crabbé et al., 2019) other key factors.

It is widely acknowledged that a polymicrobial biofilm is the hallmark of bacterial vaginosis (BV) (Verstraelen and Swidsinski, 2019), the leading vaginal infection in women of childbearing age (Redelinghuys et al., 2020). BV can lead to serious obstetric and gynecological complications. Furthermore, Women with BV are at increased risk for acquisition of HIV (Atashili et al., 2008) and other STIs, including *Chlamydia trachomatis* and *Trichomonas vaginalis* (Abbai et al., 2016), *Neisseria gonorrhoeae* (Brotman et al., 2010), *Mycoplasma genitalium* (Lokken et al., 2017), human papilloma virus (HPV) (Brusselaers et al., 2019), and herpes simplex virus type-2 (HSV-2) (Abbai et al., 2018). Despite its importance, BV etiology remains undetermined and a matter of controversy (Chen et al., 2021) and the study of polymicrobial biofilms associated with BV is still in its infancy. It has been hypothesized that virulent strains of *Gardnerella* spp. initiate the formation of the biofilm on vaginal epithelial cells and become a scaffolding to which other BV-associated bacteria (BVAB) can attach thereafter (Machado and Cerca, 2015). In fact, an earlier study showed that *Gardnerella* spp. produce amino acids through their metabolism, which can be used by *Prevotella bivia* as its nutrient source which results in the production of ammonia, which in turn is used by *Gardnerella* spp. (Pybus and Onderdonk, 1997). It has also been recently hypothesized that, as a result of these initial bacterial interactions, the vaginal epithelium might be damaged by losing the protective mucous layer, being more favorable for the adherence of other BV-associated bacteria (Muzny et al., 2019). To validate this hypothesis, the experimental determination of synergistic or antagonistic interactions within multi-species BV biofilms is fundamental.

Due to the pivotal role of *Gardnerella* spp. in BV biofilms (Swidsinski et al., 2005), we have sought to quantify *in vitro* BV-associated biofilms, by using a model that first allows *Gardnerella* spp. to establish a biofilm, followed by the addition of other BVAB to the pre-formed *Gardnerella vaginalis* biofilm. Until recently (Rosca et al., 2020) we have not assessed single-species biofilm formation by other BVAB beyond *G. vaginalis*, as this is not a naturally occurring phenomenon. In an early dual-species study using this model, we have identified possible synergism and antagonism between several BVAB (Castro and Cerca, 2015). However, this assessment was only performed using the crystal violet (CV) staining method that, despite being the most widely used technique to quantify biofilms, is not without its flaws (Azeredo et al., 2017). Moving forward to studying triple-species biofilms, we observed that CV staining failed to predict important interactions occurring within these consortia (Castro et al., 2021). Since there is a lack of critical studies comparing the different methodological approaches to quantifying multi-species biofilms (Magana et al., 2018), we aimed to provide a perspective on the lack of feasibility of the CV method to properly assess possible synergism or antagonism between individual BV-associated bacteria growing as triple-species biofilms. For this purpose, we quantified single-species biofilms formed by three BVAB thought to play significant roles in the pathogenesis of incident BV (Muzny et al., 2019), namely *G. vaginalis*, *Fannyhessea vaginae* (previously known as *Atopobium vaginae*) (Nouioui et al., 2018), and *P. bivia.*

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

G. vaginalis strain ATCC 14018^T, *F. vaginae* strain ATCC BAA-55^T, and *P. bivia* strain ATCC 29303^T were used in this study. Each inoculum was grown in New York City III broth (NYC III) [1.5% (wt/vol) Bacto proteose peptone no. 3 (BD, NJ, USA); 0.5% (wt/vol) glucose (Thermo Fisher Scientific, KS, USA); 0.24% (wt/vol) HEPES (VWR, NV, USA); 0.5% (wt/vol) NaCl (VWR); 0.38% (wt/vol) Yeast extract (Liofilchem, Roseto degli Abruzz, Italy)], supplemented with 10% (vol/vol) inactivated horse serum (Biowest, Nuaillé, France) for 24 h at 37°C under anaerobic conditions (AnaeroGen Atmosphere Generation system, Oxoid, United Kingdom), as optimized before (Rosca et al., 2020).

Single- and Multi-Species BV Biofilm Formation Model

Single-species biofilms were initiated by inoculating a 10^7 CFU.mL^{-1} bacterial suspension of each tested bacterial species into 24-well tissue culture plates (Orange Scientific, Braine L'Alleud, Belgium) and incubating the plates for 24 or 48 h at 37°C under anaerobic conditions. Of note, we first adjusted the bacterial concentration of the bacterial suspension to 9×10^7 CFU.mL^{-1} due to the limit of detection of the microplate reader, and then diluted it to 1×10^7 CFU.mL^{-1}. At 620 nm, 9×10^7 CFU.mL^{-1} of *G. vaginalis* corresponds to an optical density (OD) of 0.15; *F. vaginae* an OD of 0.11, and *P. bivia* an OD of 0.16 (Castro et al., 2021). Multi-species biofilms were also initiated by inoculating a 10^7 CFU.mL^{-1} bacterial suspension of *G. vaginalis* into 24-well tissue culture plates and incubating the plates for 24 h at 37°C under anaerobic conditions. After 24 h, planktonic cells were removed, and 10^7 CFU.mL^{-1} of *F. vaginae* and *P. bivia* were inoculated in the pre-formed *G. vaginalis* biofilms, followed by another 24 h of incubation (**Supplementary Figure 1**). As a control, single-species biofilms of *G. vaginalis* were grown for 24 and 48 h, in which fresh medium was added to the respective wells after the first 24 h of biofilm formation (for the 48-h control). These assays were repeated at least three times on separate days.

Biofilm Biomass Quantification by the Crystal Violet (CV) Method

To quantify the biomass of single- and multi-species biofilms, we used the CV method (Peeters et al., 2008). In brief, after the fixation step with 100% (vol/vol) methanol (Thermo Fisher Scientific) for 20 min, biofilms were stained with CV solution at 1% (vol/vol) (Merck, Darmstadt, Germany) for 20 min. Each

well was washed twice with PBS, and bound CV was released with 33% (vol/vol) acetic acid (Thermo Fisher Scientific). To estimate total biofilm biomass, the OD of the resulting solution was measured at 595 nm. Biofilm assays were repeated at least three times on separate days, with four technical replicates assessed each time.

Quantification of Total Number of Cells in the Biofilm Using Acridine Orange Through Epifluorescence Microscopy

Prior to the quantification of total biofilm cells, several optimizations were performed. First, we prepared fresh suspensions of each bacterial species from Columbia Blood Agar (CBA) plates and then adjusted the bacterial concentration to 10^8 CFU.mL^{-1}. We subsequently performed several dilutions in PBS 1×, aiming to determine the number of fields needed to obtain linearity among the different dilutions (**Supplementary Figure 2**). A minimum of 13 images per sample resulted in a very high correlation between bacterial counts and bacterial concentration. After this first optimization, we quantified the total number of cells from the single- and multi-species biofilms. In brief, the biofilms were carefully washed with 0.9% (wt/vol) NaCl, and 1 mL of PBS 1× was added to each well. The biofilms were then scrapped, and a pool of the different wells was obtained. Afterward, 30 µL of each bacterial suspension dilution was spread on epoxy-coated microscope glass slides (Thermo Fisher Scientific), and the slides dried at 60°C. Next, cells were fixed at room temperature with 100% (vol/vol) methanol for 20 min, followed by 4% (wt/vol) paraformaldehyde (Thermo Fisher Scientific) for 10 min, and 50% (vol/vol) ethanol (Thermo Fisher Scientific) for 15 min. After the fixation step, the samples were covered with 20 µL of acridine orange (0.01 mg.mL^{-1}) for 5 min. The excess of acridine orange was removed and the slides were air-dried in the dark at room temperature. Microscope visualization was performed using filters capable of detecting acridine orange (BP 470-490, FT500, LP 516). The number of bacterial cells was manually counted, at the appropriate dilution (<100 bacteria per field). These assays were repeated three times on separate days.

Enumeration of Total Culturable Bacteria in the Biofilm Using the CFU Counting Method

Regarding the culture plate counting method, serial dilutions ranging from 10^{-1} to 10^{-6} were performed on the resuspended biofilm in 0.9% (wt/vol) NaCl. After homogenization, 10 µL of each dilution was spread onto CBA plates. The plates were incubated at 37°C under anaerobic conditions for 72 h. This process was carried out with two replicates in at least three independent assays. More details are explained in the Supplementary Materials and Methods.

Discrimination of Bacterial Populations in Multi-Species Biofilms by PNA-FISH

The bacterial population within the 48 h multi-species biofilms was discriminated using the peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) method, as previously described (Castro et al., 2021). Briefly, after fixing the biofilm suspension, a PNA probe specific for *G. vaginalis* (Gard162) and for *F. vaginae* (AtoITM1) were added to each well of epoxy-coated microscope glass slides (Thermo Fisher Scientific). An additional staining step was done at the end of the hybridization procedure, covering each glass slide with DAPI (2.5 µg.mL^{-1}). Microscopic visualization was performed using filters capable of detecting the PNA Gard162 probe (BP 530-550, FT 570, LP 591 sensitive to the Alexa Fluor 594 molecule attached to the Gard162 probe), the PNA AtoITM1 probe (BP 470-490, FT500, LP 516 sensitive to the Alexa Fluor 488 molecule attached to the AtoITM1 probe), and DAPI (BP 365–370, FT 400, LP 42). The number of bacteria was counted using *ImageJ Software* (Rasband, 1997). These assays were repeated three times on separate days.

Statistic Analysis

The data were analyzed using GraphPad Prism version 7 (La Jolla,CA, USA) by unpaired t-test, or non-parametric Wilcoxon matched-pairs signed-rank test. A $P < 0.05$ were considered statistically significant. Data are presented as mean (of all independent assays) ± standard deviation (s.d.).

RESULTS AND DISCUSSION

To better understand how different BVAB are affected by standard biofilm quantification, we first characterized 24 and 48 h single-species biofilms to assess how each technique reflects biofilm growth. The total biofilm biomass was determined by the CV method, while cell culturability was detected anaerobically in the appropriate medium and total cells were quantified by epifluorescence microscopy. For all three tested species, we observed that the total biofilm biomass and bacterial concentration obtained by epifluorescence microscopy significantly increased after 48 h of biofilm formation in batch conditions, compared to a 24 h-biofilm ($p<0.05$) (**Figure 1A**). However, the same was not true for bacterial culturability, wherein only *P. bivia* was able to increase its bacterial concentration from 24 to 48 h biofilms. In contrast, *F. vaginae* significantly decreased its bacterial culturability after 48 h of biofilm formation, while no CFU was able to grow from 48 h-*G. vaginalis* biofilms in the tested conditions.

Since it was previously shown that *G. vaginalis* lost 1 log cell culturability when manipulated (after anaerobic growth) in a regular biosafety cabinet (Turovskiy et al., 2012), we verified if this significant loss of culturability could be a result of bacterial manipulation in the presence of atmospheric oxygen. Two approaches were tested (controlled delay after biofilm scrapping and after CFU plating – see Supplementary Methods and **Supplementary Figure 3**). As shown in **Supplementary Figure 4**, the reduction of bacterial culturability was more affected by the delay after plating the suspension onto CBA plates. Still, by performing all manipulations under 30 min, we were able to reduce the loss of viability under 20%, which does not explain the observed significantly higher loss of culturability.

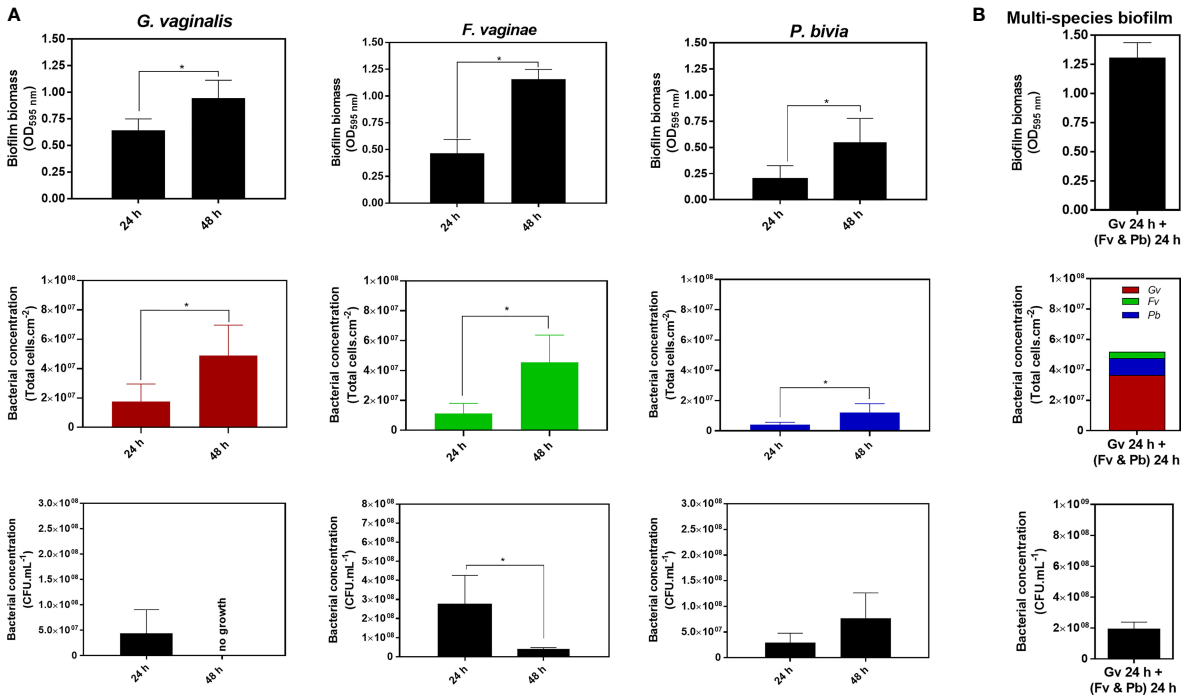

FIGURE 1 | Quantification of 24 h and 48 h single-species biofilms of *G. vaginalis*, *F. vaginae* and *P. bivia* **(A)** or a multi-species biofilm composed of all three species **(B)** using the crystal violet method, total cell counts by epifluorescence microscopy and the colony-forming units (CFU) method. The colors selected for the epifluorescence microscopy data reflect the fluorophore detection spectra. Each data point represents the average ± s.d. of three experiments. *Values are significantly different between 24 h and 48 h of biofilm formation without changing the growth medium (batch system) (unpaired t-test, $P < 0.05$).

As recently pointed out for bacterial species in the gut microbiota, a possible reason that a greater proportion of the bacterial community was not cultured when the fresh sample was exposed to O_2 might be the fact that oxygen-sensitive cells were in the viable but not culturable (VBNC) state, or either injured or dead (Bellali et al., 2019). While similar observations have been reported elsewhere (Li et al., 2014; Lv et al., 2020), to our knowledge, this has not been determined in *G. vaginalis* biofilms. As such, we performed another experiment, wherein we used the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit. It is important to highlight that this staining system has some limitations, as pointed by Netuschil and colleagues, mainly when used in multi-species biofilms (Netuschil et al., 2014). However, in this study, we only used the LIVE/DEAD kit for the examination of single-species biofilms, in which carrying out adequate controls allows obtaining reliable interpretations (Robertson et al., 2019). By using this kit, we were able to observe viable cells within the 48 h *G. vaginalis* biofilms, although the majority of cells had damaged cell walls (**Supplementary Figure 5**). The presence of this state has been associated with longer periods of biofilm formation, nutritional resource limits, and deposits of metabolic waste (Ayrapetyan et al., 2018; Carvalhais et al., 2018). Interestingly, we could prevent VBNC in 48 h *G. vaginalis* biofilms by replacing the growth media after 24 h (**Supplementary Figure 5**).

Interestingly, after comparing the quantification of 24 or 48 h biofilms by the three different techniques, we observed that each species had significantly different quantification yields, depending on the technique used. Clearly, the total biomass (cells plus matrix) produced by the different species varied among species and with the time of incubation (**Table 1A**). Such a fact is not surprising, given that these techniques measure different features of the biofilm (Stiefel et al., 2016). To better highlight the differences found in the quantification of each single-species biofilm, we calculated the ratio of biofilm total biomass formation by $1{\times}10^7$ total cell.cm^{-2} or by $1{\times}10^7$ CFU.mL^{-1}. Curiously, our findings indicated that under our tested conditions, *G. vaginalis* produced the lowest biofilm biomass by each $1{\times}10^7$ total cell.cm^{-2}, which became more pronounced at 48 h. In contrast, *P. bivia* produced more biofilm biomass per bacteria (**Table 1B**). While the CV method for the quantification of the biofilm biomass is widespread (Azeredo et al., 2017), it has been suggested that direct comparison of total biofilm biomass between species might not be feasible, as different species may have distinct biofilm matrices (Haney et al., 2018). This is supported by the results of this study. To make comparisons even more challenging, the ratio of biofilm biomass produced at different incubations times might not be constant, at least for *G. vaginalis* and *F. vaginae* in our tested conditions.

The results of this study raise the question of how the CV staining method of a multi-species biofilm could in fact reflect its bacterial composition. As shown in **Table 1C**, multi-species biofilms had very distinct CV/total cells, or CV/CFU ratios, further suggesting that simply quantifying a multi-species

TABLE 1 | Different ratios resulted from quantification using the three different methodologies.

PANEL A | Ratios obtained from data of the quantification of 48 h and 24 h single-species biofilms by three different methodologies

	G. vaginalis	*F. vaginae*	*P. bivia*
Biofilm biomass (48 h/24 h)	1.47	2.48	2.81
Total cell counts by epifluorescence microscopy (48 h/24 h)	2.77	4.03	2.95
CFU counting (48 h/24 h)	0.0	0.15	2.59

PANEL B | Ratios obtained from data of the quantification of total cells or CFUs (both expressed in 1×10^7) in relation to biofilm biomass for each time point assessed for single-species biofilms

	G. vaginalis	*F. vaginae*	*P. bivia*
Biofilm biomass/Total cell counts by epifluorescence microscopy (24 h)	0.36	0.41	0.47
Biofilm biomass/Total cell counts by epifluorescence microscopy (48 h)	0.19	0.25	0.45
Biofilm biomass/CFU counting (24 h)	0.15	0.02	0.07
Biofilm biomass/CFU counting (48 h)	n.d.*	0.28	0.07

PANEL C | Ratios obtained from data of the quantification of total cells or CFUs (both expressed in 1×10^7) in relation to biofilm biomass for multi-species biofilms

	Multi-species biofilm
Biofilm biomass/Total cell counts by epifluorescence microscopy	0.25
Biofilm biomass/CFU counting	0.07

**n.d., not determined – Since the value of cfu.mL^{-1} for the 48 h G. vaginalis biofilm was zero, it was not possible to determine the ratio.*

biofilm by the CV method will not provide a reliable quantification of the biofilm. While both the absolute CV (**Figure 1B**) staining and the CV/total cells ratio were similar to the 48 h *F. vaginae* biofilm, it is very unlikely that this multi-species biofilm would be solely composed by *F. vaginae*. Furthermore, the CV/total cells ratio did not match the *F. vaginae* profile. With this in mind, we analyzed the bacterial composition in the multi-species by PNA-FISH differentiation, using specific probes for *G. vaginalis* and *F. vaginae* and DAPI, counterstaining to quantify total cells (Castro et al., 2021). Under our tested conditions, *G. vaginalis* represented 70.3 ± 1.2% of the multi-species biofilm, followed by *P. bivia* (21.4 ± 1.0%) and *F. vaginae* (8.3 ± 0.9%).

CONCLUSIONS AND PERSPECTIVES

CV staining quantification has proven extremely useful as a cellular estimate for biofilm formation, mainly because both Gram-positive and Gram-negative bacterial cells are able to take up the CV. The dye will freely pass from the cell during the decolorization process, allowing for the quantification of CV *via* spectroscopy (Peeters et al., 2008; Magana et al., 2018). However, it has been noted that in polymicrobial consortia, accurate biofilm quantification becomes more complex (Røder et al., 2016).

As shown here, our three key BVAB produced different biofilms with different profiles (i.e. cells and matrix), which varied with time (with the exception of *P. bivia*). The relationship between total biofilm biomass/total cells is unique to each tested species, in specific environmental conditions, and as such, a direct comparison between single- and multi-species biofilms using the CV method alone is unlikely to be without bias. If we could assume that, for a specific period of incubation, each individual species could maintain the same biofilm production profile, when growing alone or in consortia, it might be possible to interpolate the measured data to be adjusted by the relative contribution of the species in the multi-species biofilm. However, when growing in consortia, the biofilm matrix components produced by each species might be affected, since the matrix composition is highly dependent on environmental conditions (Karygianni et al., 2020). Thus, we proposed that when comparing single to multiple-species biofilms, an increased or decreased CV staining should not be taken as an accurate measure of bacterial synergism or antagonism, as we have mistakenly done before (Castro and Cerca, 2015). A lower total biomass might in fact reflect an increase in cell concentration. On the other hand, an increase in total biomass might provide an advantage to the cells within the biofilm, by providing better protection against antibiotics (Sharma et al., 2019), even if the total bacterial load is reduced. Due to this complexity, we argue that to properly analyze a BV-associated, or in that matter any other multi-species biofilms, a multiple-technical approach should be used when quantifying these consortia, in order to circumvent the caveats of individual techniques alone. This multiple technical approach will provide a more compressive picture of the biofilm consortia associated with BV, and will contribute in furthering BV pathogenesis research.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

NC and CM designed the experiments. JC, ÂL, LS, and AR performed the experiments. JC and NC drafted the manuscript. All authors critically reviewed and approved the final manuscript.

FUNDING

This research was partially funded by the Portuguese Foundation for Science and Technology (FCT) by the research project (PTDC/BIA-MIC/28271/2017), under the scope of COMPETE 2020 (POCI-01-0145-FEDER-028271), by the strategic funding of unit (UIDB/04469/2020). It was also partially funded by the National Institute of Allergy and Infectious Diseases (R01AI146065-01A1). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.795797/full#supplementary-material

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Conflict of Interest: CM has received research grant support from Lupin Pharmaceuticals, Gilead, and Abbott Molecular, is a consultant for Lupin Pharmaceuticals, PhagoMed, and BioFire Diagnostics, and has received honoraria from Elsevier, Abbott Molecular, Cepheid, Becton Dickinson, Roche Diagnostics, and Lupin.

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frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 13 January 2022
doi: 10.3389/fcimb.2021.813520

Sialidase Activity in the Cervicovaginal Fluid Is Associated With Changes in Bacterial Components of *Lactobacillus*-Deprived Microbiota

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Edited by:
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University of Bologna, Italy

Reviewed by:
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University of Bologna, Italy
Nneka Agbakoba,
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Specialty section:
This article was submitted to Biofilms, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 11 November 2021
Accepted: 06 December 2021
Published: 13 January 2022

Citation:
Ferreira CST, Marconi C, Parada CMGL, Ravel J and Silva MGd (2022) Sialidase Activity in the Cervicovaginal Fluid Is Associated With Changes in Bacterial Components of Lactobacillus-Deprived Microbiota. Front. Cell. Infect. Microbiol. 11:813520. doi: 10.3389/fcimb.2021.813520

Introduction: Sialidase activity in the cervicovaginal fluid (CVF) is associated with microscopic findings of bacterial vaginosis (BV). Sequencing of bacterial 16S rRNA gene in vaginal samples has revealed that the majority of microscopic BV cases fit into vaginal community-state type IV (CST IV), which was recently named "molecular-BV." Bacterial vaginosis-associated bacterial species, such as *Gardnerella* spp., may act as sources of CVF sialidases. These hydrolases lead to impairment of local immunity and enable bacterial adhesion to epithelial and biofilm formation. However, the impact of CVL sialidase on microbiota components and diversity remains unknown.

Objective: To assess if CVF sialidase activity is associated with changes in bacterial components of CST IV.

Methods: One hundred forty women were cross-sectionally enrolled. The presence of molecular-BV (CST IV) was assessed by V3–V4 16S rRNA sequencing (Illumina). Fluorometric assays were performed using 2-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUAN) for measuring sialidase activity in CVF samples. Linear discriminant analysis effect size (LEfSe) was performed to identify the differently enriched bacterial taxa in molecular-BV according to the status of CVF sialidase activity.

Results: Forty-four participants (31.4%) had molecular-BV, of which 30 (68.2%) had sialidase activity at detectable levels. A total of 24 bacterial taxa were enriched in the presence of sialidase activity, while just two taxa were enriched in sialidase-negative samples.

Conclusion: Sialidase activity in molecular-BV is associated with changes in bacterial components of the local microbiome. This association should be further investigated, since it may result in diminished local defenses against pathogens.

Keywords: vaginal microbiota, sialidases, bacterial vaginosis, Gardnerella, 16S rRNA

INTRODUCTION

Bacterial vaginosis (BV) has been acknowledged as the most prevalent dysbiotic condition of the vaginal microbiota, affecting nearly one-third of women of reproductive age (Spiegel, 1991; Marconi et al., 2015). Microscopic BV is often diagnosed using Nugent criteria and is characterized by replacement of *Lactobacillus* spp. dominance by a wide array of other bacterial types (Nugent et al., 1991; Spiegel, 1991). The presence of BV was already associated with poor pregnancy outcomes and with increased risk for several sexually transmitted infections (STI), including the human immunodeficiency virus (HIV) (Leitich and Kiss, 2007; Gallo et al., 2012; Mitchell et al., 2013). The exact components of the vaginal microbiota were described utilizing sequencing hypervariable regions of the bacterial 16s rRNA gene (Ravel et al., 2011). Studies based on this molecular approach have shown that the vaginal microbiota of virtually all reproductive-aged women fits into five bacterial community state types (CSTs), of which four (CST I, CST II, CST III, and CST V) present dominance of certain *Lactobacillus* spp. CST IV is *Lactobacillus* spp.-deprived and comprises most of the cases of BV detected by microscopy; thus, it has been named as "molecular-BV" (Ravel et al., 2011; McKinnon et al., 2019; Marconi et al., 2020).

Sialidase activity is often detected in the cervicovaginal fluid (CVF) of women with microscopic BV (Marconi et al., 2013; Santos-Greatti MM de et al., 2016). These hydrolases have a negative effect on cervicovaginal immunity since they degrade local immunoglobulin A (IgA) and vaginal mucins, likely contributing to the diminished viscosity of local secretion which results in increased vulnerability to pathogens (Lewis et al., 2012; Schwebke et al., 2014; Muzny and Schwebke, 2016). The sialidase CVF concentration is correlated with local levels of IL-1beta which may lead to tissue damage increasing the vulnerability to STIs (Marconi et al., 2013; Mitchell et al., 2013). Additionally, sialidases cleave sialic acid from the terminal glycans of glycoproteins present in the vaginal mucosa, allowing bacterial adhesion to the epithelial cells (Briselden et al., 1992). Several BV-associated species may produce sialidases. However, *Gardnerella* spp. are considered the main source of this enzyme in the cervicovaginal environment (Hardy et al., 2017; Kurukulasuriya et al., 2020). In fact, *Gardnerella* spp. have been proposed as the scaffold on vaginal mucosa for the attachment of other bacterial species, such as *Prevotella* and *Atopobium*, leading to biofilm formation (Hardy et al., 2015; Muzny and Schwebke, 2016).. Thus, by cleaving the sialic acid of epithelial cells, sialidases may facilitate the adhesion of *Gardnerella* spp. to the underlying glycan-binding sites enabling biofilm formation (Varki and Gagneux, 2012; Castro et al., 2019). Vaginal biofilms have been considered the hallmark of BV and are particularly troublesome as they hinder antibiotic action leading to persistence of BV-associated bacteria after treatment (Swidsinski et al., 2008).

Thus, considering the deleterious effects of sialidases for cervicovaginal immune defense, particularly related to local biofilm formation, as well as the intricate relation between hosts' defenses and components of their microbiota, the aim of this study was to investigate if presence of sialidase activity in CVF is associated with differences in the bacterial composition of molecular-BV.

MATERIALS AND METHODS

Ethics Statement and Study Population

The Ethics Review Board of the Botucatu Medical School (São Paulo State University) approved this study and consent procedures (approval number 3.095.119) and by the National Commission of Ethics in Research (Comissão Nacional de Ética em Pesquisa, approval number 294.202). The aims and procedures of the study were explained to all participants, after which each of them signed a consent term for the participation in the study. This cross-sectional study included 140 non-pregnant reproductive-aged women attending a primary healthcare clinic in Botucatu, São Paulo, Brazil, for a comprehensive study on the composition of the vaginal microbiome of Brazilian women (Marconi et al., 2020).

Women were only considered for enrollment if they were 18 years old or older, were menstruating monthly, and had their last menstrual period of at least for 5 days. Approached women were asked if they had HIV infection, intrauterine device (IUD), urinary loss, therapy with antibiotics in the prior 30 days, and sexual intercourse/vaginal douching in the prior 48 h. In case of any positive response, they were not considered for study enrollment. Women who fulfilled the inclusion criteria were interviewed face to face by a member of the research team that used a structured form that included questions for assessment of sociodemographic, behavioral, and clinical characteristics.

Sampling Procedures

During the physical exam, nurses previously trained for this study procedures assessed vaginal pH by the direct contact of commercial pH strips (range 4.0–7.0, Merck, Darmstadt, Germany) with the vaginal wall. The mid-third portion of the vaginal wall was sampled using two swabs. The first vaginal swab was kept into Amies liquid medium (Copan, Brescia, Italy) at -80°C for molecular analysis. Another vaginal swab was smeared onto glass slides for microscopic interpretation of the vaginal microbiota using the Nugent scoring system (Nugent et al., 1991). Samples of CVF were obtained by washing the vaginal wall and posterior fornix with 3 ml of sterile NaCl 9.5% [w/v] solution, as previously standardized for measurement of sialidase activity (Marconi et al., 2013).

Molecular Analysis of Vaginal Microbiota

Frozen vaginal samples inoculated in transport medium were thawed on ice and shaken vigorously, and the swabs were then discarded. DNA extraction was performed using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA), according to the manufacturer's protocol. Molecular analysis was performed at the Institute for Genomic Sciences of University of Maryland (Baltimore, MD), according to Fadrosh et al. (2014) and a previous study (Marconi et al., 2020). All samples were submitted to amplification of the V3–V4

hypervariable region of the 16S rRNA gene using dual-indexed 319F and 806R sets of primers. Amplicon libraries were sequenced using the 300 PE protocol on a MiSeq equipment (Illumina, San Diego, CA). Sequences were demultiplexed and quality trimmed in QIIME (version 1.8.0), as previously described (Ramírez-Guzmán et al., 2010; Marconi et al., 2020). Taxonomic assignments were performed using an in-house fifth-order Markov chain model and a pre-compiled database containing all bacterial species previously observed in vaginal microbiota (Ravel et al., 2011; Gajer et al., 2012). Samples were clustered into CSTs I to V using taxonomic information, taxa abundance, and the Jensen–Shannon divergence metrics (Marconi et al., 2020).

Measurement of Sialidase Activity

Measurement of CVF sialidase levels was performed by the conversion of the fluorogenic substrate 2-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUAN; Sigma-Aldrich, St. Louis, MO), according to methods previously optimized for CVF samples (Marconi et al., 2013). Aliquots of 50 µl of CVF supernatants were transferred to a 96-well plate (OptiPlate-96F, PerkinElmer, Waltham, MA). A volume of 50 µl of 0.35% MUAN (wt/vol) in 3 mM sodium acetate (pH 4.5) was added to the samples and kept at 37°C for 30 min. A standard curve was constructed with 10 dilution points ranging from 1000.0 to 0.1 ng/ml of purified *Clostridium perfringens* neuraminidase (Sigma-Aldrich, St. Louis, MO). Fluorescence signals were detected at 450 nm emission and 365 nm excitation and filtered at the 420-nm cutoff (Epoch instrument, Biotek, Winooski, VT). Samples were considered as positive for sialidase activity when the fluorescence was above the detection limit of the assay (set at 0.1 ng/ml corresponding to the lowest point of the standard curve).

Data Analyses

Sialidase levels in CVF were compared across the CSTs using the non-parametric Kruskal–Wallis test in Stata software (StataCorp, College Station, TX) with p-value <0.05 considered as statistically significant. The Shannon–Weiner index was calculated for assessing alpha-diversity based on median rarefied taxa reads using the *vegan* package in R (Oksanen et al., 2012) and compared between sialidase-positive and -negative samples using the Mann–Whitney test ($p < 0.05$ considered as significant). Relative abundances of bacterial taxa identified in molecular-BV (i.e., CST IV) were compared using linear discriminant analysis effect size (LEfSe) according to the status of CVF sialidase activity (Segata et al., 2011). Only relative abundances of microbial taxa with >0.05% population-wide representativeness were included at LEfSe.

RESULTS

The characteristics of the 140 participants of the study are detailed in **Supplementary Table 1**. The median age of participants was 33 years (range: 18–51). Most participants were living in a steady relationship upon study enrollment (63.6%, n = 89). A small fraction of participants reported having more than one sex partner in the prior 12 months (10%, n = 14) or having a new sex partner within the prior 4 months (16.4%, n = 23). Consistent use of condoms during the intercourse was reported by only 15.7% (n = 22) of the participants. Current use of hormonal contraceptives was reported by 42.9% (n = 60) participants. Cervical infections by *C. trachomatis* and *N. gonorrhoeae* were detected in 8 (5.7%) and 2 (7.1%) participants. As also detailed in **Supplementary Table 1**, a microscopic assessment of vaginal microbiota using the Nugent scoring criteria showed that 48 (34.3%) participants had microscopic BV.

As displayed in **Table 1**, sialidase activity was detected in 34 (24.3%) out of 140 CVF samples. The measured values of vaginal pH of sialidase-positive participants were statistically superior (median: 4.9; range: 4.7–5.8) to those with no sialidase activity (median: 4.7; range: 4.0–5.5), ($p < 0.001$). When observing the categories of vaginal microbiota retrieved from microscopic Nugent analysis, sialidase activity was almost exclusive to BV (91.2%, n = 31) (**Table 1**).

A molecular analysis of 140 vaginal samples resulted in 1,938,832 reads with a total 116 bacterial taxa identified. A total of 96 participants were clustered into *Lactobacillus*-dominant CSTs: *L. crispatus*-dominated CST I (n = 40), *L. gasseri*-dominated CST II (n = 4), *L. iners*-dominated CST III (n = 51), or *L. jensenii*-dominated CST V (n = 1). A total of 44 (31.4%) participants had molecular-BV (*Lactobacillus*-depleted CST IV). Molecular-BV was diagnosed in 41 (85.4%) out of the 48 microscopic-BV cases detected in the study (**Supplementary Figure 1**). As displayed in **Table 2**, nearly all participants with detectable levels of CVF sialidases had molecular-BV (n = 30 out of 34) ($p < 0.0001$). Also, significantly increased sialidase levels (expressed in ng/mL of CVF sample) were observed in molecular-BV (median 10.3, range: 0.0–818.9) when compared to *L. iners*-CST III (median: 0.0; range 0.0–84.4) ($p = 0.0001$).

In order to identify the bacterial taxa differently abundant in the presence of sialidase activity, the 44 samples with molecular-BV (CST IV) were tested using LEfSe. The relative abundances of 80 bacterial taxa that were the most representative population-wide (>0.05%) were used at this analysis. The list of all bacterial taxa and their respective overall abundances is provided in **Supplementary Table 2**. **Figure 1** depicts the result of LEfSe

TABLE 1 | Covariates of vaginal microbiota by the status of sialidase activity in cervicovaginal fluid samples.

Characteristics	Sialidase activity		
	Negative (n = 106)	Positive (n = 34)	p-value
Vaginal pH; median (range)	4.7 (4.0–5.5)	4.9 (4.7–5.8)	<0.0001[a]
Nugent score categories; n (%)			<0.0001[b]
0–3 (normal)	75 (70.8)	0 (0.0)	
4–6 (intermediate)	14 (13.2)	3 (8.8)	
7–10 (bacterial vaginosis)	17 (16.0)	31 (91.2)	

[a]*Mann–Whitney non-parametric test.*
[b]*Chi-squared test.*
p-values <0.05 considered as statistically different.

TABLE 2 | Presence of sialidase activity in the cervicovaginal fluid, as well as measured levels of sialidases, by molecularly defined community state types (CSTs).

	CST I (n = 40)	CST III (n = 51)	Molecular-BV (CST IV) (n = 44)	CSTs II and V[a] (n = 5)	p-value
Sialidase activity					<0.0001[b]
Negative	40 (100.0)	48 (94.1)	14 (31.8)	4 (80.0)	
Positive	0 (0.0)	3 (5.9)	30 (68.2)	1 (20.0)	
Sialidases (ng/mL), median (range)	nd	0.0 (0.0–84.9)	10.5 (0.0–818.9)	0.0 (0.0–1.8)	0.0001[c]

[a]*CST II (n = 4) and CST V (n = 1) were merged into one category and excluded from analysis, as they were underrepresented in the study population.*
[b]*Chi-squared test for comparison within CSTs I, III, and IV.*
[c]*Mann–Whitney non-parametric test for comparison between CSTs III and IV.*
nd, not detected.

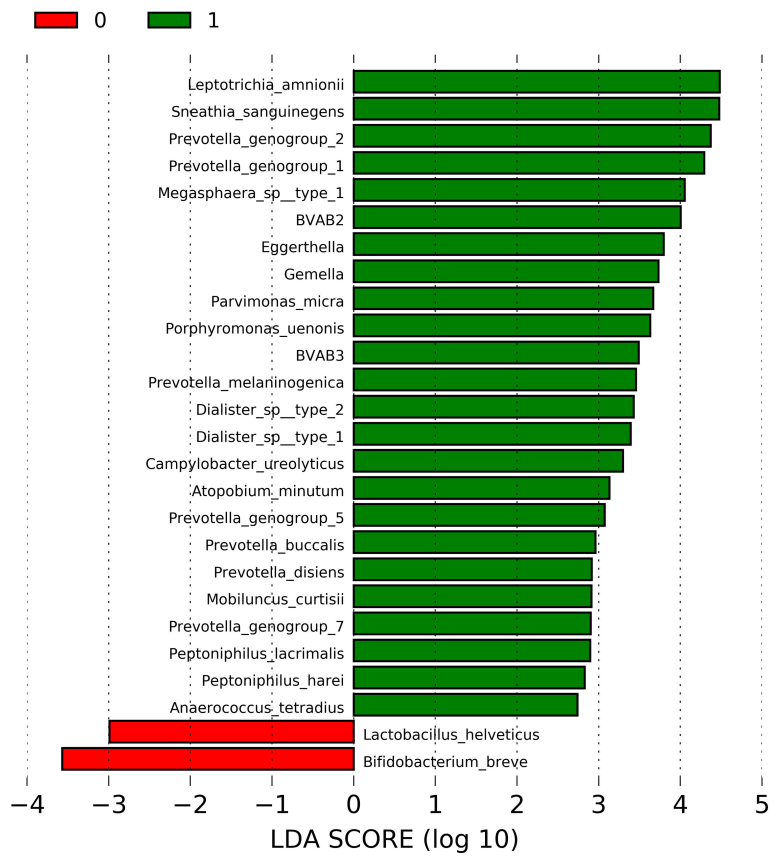

FIGURE 1 | Linear discriminant analysis (LDA) effect size (LEfSe) for comparison of taxa abundance among CST IV-positive samples, according to the presence (n = 30) or absence (n = 14) of detectable levels of sialidase activity in cervicovaginal fluid. LDA scores of significantly enriched taxa in the presence of sialidase activity are represented by green bars, while red bars are referent to LDA scores of enriched taxa in sialidase-negative samples. p-values <0.05 were considered as significant.

showing a total of 24 enriched taxa in the presence of sialidase activity, of which 7 (29%) belong to *Prevotella* genera. Other well-acknowledged BV-associated bacteria taxa were also enriched in sialidase-positive samples, such as *Leptotrichia amnionii*, *Megasphaera* sp., *Mobiluncus curtisii*, and BVAB3 (the novel *Mageeibacillus indolicus*). Only two taxa were enriched in sialidase-negative samples: *L. helveticus* and *Bifidobacterium breve*. *Gardnerella* spp. was not figured out among the differently enriched taxa. A comparison of the Shannon–Weiner index showed that bacterial diversity was significantly increased in the presence of sialidase activity (median: 1.90; range: 1.27–2.77) in relation to sialidase-negative samples (median: 1.16; range: 0.51–2.21) (p = 0.0001).

DISCUSSION

The impairment of cervicovaginal immunity due to the presence of bacterial sialidases has been investigated by several studies, while the impact of these hydrolases on local microbiota remains poorly known (Lewis et al., 2012; Muzny and Schwebke, 2016; Hardy et al., 2017; Castro et al., 2019). Thus, the current study

adds to the literature novel information about the changes in the microbial components of molecular-BV when in the presence of sialidase activity. The population enrolled in this study consisted of women of reproductive age in order to minimize the well-known impact of low estrogen levels on the vaginal microbiota (Hillier and Lau, 1997).

The current results showing the association between microscopic BV and presence of sialidase activity reinforce similar reports in the literature (Briselden et al., 1992; Smayevsky et al., 2001). In fact, several BV-associated bacteria, such as *Gardnerella* spp., *Prevotella* spp., and *Bacteroides* spp., were already shown as capable of sialidase production, corroborating with this hypothesis (Briselden et al., 1992; Smayevsky et al., 2001). Despite the relevance of previous and current findings on the relation between bacterial sialidases and microscopic-BV, microscopy provides very limited information regarding the actual components of the microbiota (Briselden et al., 1992; Marconi et al., 2013). Thus, this study was based in 16S rRNA sequencing and CST assignments to classify the vaginal microbiota. These strategies provide more precise information on the bacterial taxa present and their association change with sialidase activity. Particularly, these results focused on the *Lactobacillus*-deprived CST IV, which has been acknowledged as molecular-BV (McKinnon et al., 2019). The bacterial composition of CSTs observed in this study population was described in detail in our prior study and is consistent with the recent nearest centroid classification named VALENCIA (France et al., 2020; Marconi et al., 2020).

Sialidase activity was almost exclusively observed in molecular-BV, as only 4 out of 34 sialidase-positive samples were molecularly classified as non-CST IV. These findings were not unexpected as CST IV "molecular-BV" comprises most of the cases of microscopic-BV detected by Nugent criteria (Ravel et al., 2011; Marconi et al., 2020). Although this study design does not allow to establish a causal and effect link between CVF sialidases and microbiota components, it does show that several (n = 24) bacterial taxa of molecular-BV are enriched in the presence of sialidase activity, thus contributing to a significantly increased alpha-diversity. Interestingly, although *Gardnerella* spp. are recognized as the main sialidase producers in the cervicovaginal environment (Hardy et al., 2017; Kurukulasuriya et al., 2020), they were not figured out among the differently enriched taxa. Recently, it has been demonstrated that *Gardnerella* sialidase-encoding genes nanH2 and nanH3 are more restricted to some strains than previously thought (Kurukulasuriya et al., 2020). Thus, despite the fact that sialidases contribute for *Gardnerella* adherence and biofilm formation, these species are also highly abundant in sialidase-negative CFV samples (Hardy et al., 2017).

There are several plausible hypotheses for the increased relative abundance of certain taxa in the presence of CVF sialidase activity. Firstly, non-*Gardnerella* species might also be acting as local sources of sialidases, such as *Prevotella*. In fact, the current results show that *Prevotella* genogroups were the most frequent taxa among those enriched in the presence of sialidases (7 out of 24). Thus, increased sialidases may also be due to the higher abundance of *Prevotella* sialidase-producing strains. In fact, an early study by Briselden et al. (1992) showed that the majority of *Prevotella* sp. isolates from vaginal cultures are sialidase producers (Briselden et al., 1992). Interestingly, the latter study also showed that few *G. vaginalis* isolates are sialidase producers, which also corroborates with recent findings showing that few *Gardnerella* strains present the sialidase-encoding genes.

Increased bacterial diversity in the presence of sialidases may also be due to their impairment of local immunity, allowing the overgrowth of several bacterial species that overcome the host's defenses (Lewis et al., 2012; Lewis et al., 2013; Schwebke et al., 2014). Also, the increased proinflammatory cytokine levels observed in the presence of CVF sialidase activity may lead to increased risk for HIV acquisition (Mitchell et al., 2013). Moreover, sialidases are associated with the early stages of vaginal biofilm formation, as they enable the adherence of *Gardnerella* sp. to glycan-binding sites of epithelial cells uncovered by the removal of sialic acids (Varki and Gagneux, 2012; Muzny and Schwebke, 2016; Hardy et al., 2017). Biofilm-forming *Gardnerella* sp. would then act as scaffold for attachment of other BV-associated species, such as *Atopobium vaginae* and *Prevotella* sp., among others. Current knowledge on microbial composition of vaginal biofilms points out to a predominance of *Gardnerella* sp. and *A. vaginae*. However, other BV-associated bacteria such as those enriched in sialidase activity may also be figured out as secondary components of vaginal biofilms, warranting future investigations (Hardy et al., 2015; Castro et al., 2019). A recent study by Castro et al. (2019) showed that several BV-associated bacteria increase the expression of the *Gardnerella* sp. sialidase-encoding gene in an *in vitro* dual-species biofilm model (Castro et al., 2019). However, the latter study did not test the bacterial taxa that were enriched in the presence of sialidase in the current study, the exception being for *P. bivia*. Therefore, further studies based on polymicrobial biofilm models should target other bacterial types associated with dysbiotic vaginal microbiota, especially those enriched in the presence of sialidase activity.

Regarding the only two taxa that were enriched in sialidase-negative samples, one belongs to *Lactobacillus* genera, *L. helveticus*, which is widely accepted as beneficial to vaginal microbiota. Despite the characteristic paucity of *Lactobacillus* spp. in CST IV, these organisms are frequently detected in low abundances in this community (Ravel et al., 2011; Marconi et al., 2020). Additionally, *Bifidobacterium breve* was enriched in samples with no sialidase activity. Despite being typically associated with gut microbiota, some *Bifidobacterium* spp., including *B. breve*, were already shown as frequent and abundant colonizers of the vaginal microbiota (Freitas and Hill, 2017). Also, vaginal isolates of *B. breve* produce L-lactic acid *in vitro* (Freitas and Hill, 2017), which may contribute to a low pH inhibiting the growth of sialidase-producing BV-associated bacteria.

In conclusion, this study shows that the presence of CVF sialidase activity is associated with changes in bacterial composition of molecular-BV characterized by increased bacterial diversity and abundance of several BV-associated bacteria, but not *Gardnerella* spp. Thus, these results may serve as basis for the better understanding on how virulence factors produced by major constituents of vaginal biofilms (i.e., *Gardnerella* spp.) may affect the local microbiota.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found as follows: http://hdl.handle.net/11449/215315.

ETHICS STATEMENT

This study involving human participants was reviewed and approved by the Ethics Review Board of the Botucatu Medical School (São Paulo State University) (approval number 3.095.119) and by the National Commission of Ethics in Research (Comissão Nacional de Ética em Pesquisa, approval number 294.202). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CSTF: enrolled study participants, performed laboratory analysis, wrote the first version of the manuscript; CM: designed the study, performed statistical analysis, reviewed the first version of the manuscript, CMLP: enrolled study participants, critically reviewed the data analysis and display; JR: performed the molecular analysis, critically reviewed the manuscript; MGS: coordinated the study, critically reviewed the data analysis and display.

FUNDING

Financial support was granted by São Paulo Research Foundation (FAPESP) (grants #2012/16800-3 and #2012/10403-2) to MS and CM and by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil – Finance code 001 by providing a doctorate scholarship to CF.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.813520/full#supplementary-material

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frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 25 January 2022
doi: 10.3389/fcimb.2021.822882

Extracellular Vesicles Generated by Gram-Positive Bacteria Protect Human Tissues *Ex Vivo* From HIV-1 Infection

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Biofilms, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *26 November 2021*
Accepted: *30 December 2021*
Published: *25 January 2022*

Citation:
Costantini PE, Vanpouille C, Firrincieli A, Cappelletti M, Margolis L and Ñahui Palomino RA (2022) Extracellular Vesicles Generated by Gram-Positive Bacteria Protect Human Tissues Ex Vivo From HIV-1 Infection. Front. Cell. Infect. Microbiol. 11:822882. doi: 10.3389/fcimb.2021.822882

Vaginal microbiota dominated by lactobacilli protects women from sexually transmitted infection, in particular HIV-1. This protection is, in part, mediated by *Lactobacillus*-released extracellular vesicles (EVs). Here, we investigated whether EVs derived from other Gram-positive bacteria also present in healthy vaginas, in particular *Staphylococcus aureus*, *Gardnerella vaginalis*, *Enterococcus faecium*, and *Enterococcus faecalis*, can affect vaginal HIV-1 infection. We found that EVs released by these bacteria protect human cervico-vaginal tissues *ex vivo* and isolated cells from HIV-1 infection by inhibiting HIV-1-cell receptor interactions. This inhibition was associated with a diminished exposure of viral Env by steric hindrance of gp120 or gp120 modification evidenced by the failure of EV-treated virions to bind to nanoparticle-coupled anti-Env antibodies. Furthermore, we found that protein components associated with EV's outer surface are critical for EV-mediated protection from HIV-1 infection since treatment of bacteria-released EVs with proteinase K abolished their anti-HIV-1 effect. We identified numerous EV-associated proteins that may be involved in this protection. The identification of EVs with specific proteins that suppress HIV-1 may lead to the development of novel strategies for the prevention of HIV-1 transmission.

Keywords: vaginal microbiota, gram positive bacteria, HIV-1, extracellular vesicles (EVs), *Gardnerella vaginalis*, *Staphylocccus aureus*, *Enteroccoccus faecalis*, *Enteroccoccus faecium*

INTRODUCTION

Lactobacillus spp. in the vaginal niche of premenopausal women, when dominant, protect from several gynaecological infections (O'Hanlon et al., 2013; Parolin et al., 2015; Nardini et al., 2016; Gosmann et al., 2017; Siroli et al., 2017; Parolin et al., 2018). Indeed, a shift from *Lactobacillus* dominance to non-*Lactobacillus* dominance increases the colonization and/or overgrowth of pathogenic or opportunistic bacteria such as *Gardnerella* spp., *Prevotella*, and *Clostridium*, which

are often found during bacterial vaginosis (Fredricks and Marrazzo, 2005; Fredricks et al., 2005; Oakley et al., 2008; Srinivasan and Fredricks, 2008; Ravel et al., 2013). Like bacterial vaginosis, aerobic vaginitis is featured by a marked rearrangement in the microbial community. In this case, the decrease in abundance of lactobacilli is associated with the overgrowth of aerobic bacteria mainly belonging to the genera *Staphylococcus*, *Streptococcus*, *Escherichia*, and *Enterococcus* (Donders, 2007). Nevertheless, in small amounts, these opportunistic/pathogenic bacteria can be present in healthy vaginal microbiota (Ravel et al., 2011).

Numerous studies have reported that vaginal lactobacilli protect from HIV-1 infection through acidification of the vaginal niche, stimulation of an anti-HIV-1 immune response, and capture of HIV-1 virions by membrane lectins (Atashili et al., 2008; Petrova et al., 2013; Gosmann et al., 2017; Ñahui Palomino et al., 2017). Recently, another mode of lactobacilli-mediated protection against HIV-1 infection was discovered, that is the release of extracellular vesicles (EVs) (Ñahui Palomino et al., 2019).

EVs are considered to be important mediators for cell–cell communications and constitute nanosized proteolipid particles carrying numerous bioactive molecules and covered by a lipid bilayer membrane (Nagakubo et al., 2019; Ñahui Palomino et al., 2021). The complexity of EVs reflects their multifunctionality, since EVs can shuttle numerous bioactive molecules that are involved during bacterial–bacterial interactions such as antibiotic resistance, stress resistance, horizontal gene transfer, and competition with other microorganisms; bacterial EVs are also involved during bacterial–host interactions, such as immune modulation and delivery of virulence factor, among others (Mashburn and Whiteley, 2005; Oakley et al., 2008; Manning and Kuehn, 2011; Kim et al., 2015; Stubbendieck and Straight, 2016; Domingues et al., 2017; Ñahui Palomino et al., 2021). Despite several functions described for bacterial EVs in the last decade, little is known about their possible interactions with viruses. In our previous work, we reported on the HIV-1 inhibition by *Lactobacillus*-derived EVs (*L. crispatus* BC3 and *L. gasseri* BC12) in both human cell lines and *ex-vivo* tissues (Ñahui Palomino et al., 2019). This inhibition was associated with the binding of *Lactobacillus*-derived EVs to HIV-1 Env protein, resulting in a reduction of HIV-1 entry and binding to target cells (Ñahui Palomino et al., 2019).

Here, we investigated whether EVs derived from four opportunistic human pathogenic bacteria (*S. aureus*, *E. faecium*, *E. faecalis* and *G. vaginalis*) affect HIV-1 infection of human cell cultures *in vitro* as well of human cervico-vaginal tissues *ex-vivo*. *Ex vivo* tissue cultures allow to maintain the complex cytoarchitecture of the tissue and thus constitute an adequate experimental model to study HIV-1 pathogenesis (Grivel and Margolis, 2009).

Our results demonstrate that bacterial EVs derived from Gram-positive (*S. aureus*, *E. faecium*, *E. faecalis*) and Gram-variable (*G. vaginalis*) bacteria inhibit HIV-1 infection of human lymphoid T-cell line and human cervico-vaginal tissues *ex vivo*. This protection is exerted by the protein components exposed on the EV surface and is associated, at least in part, with obstruction of the gp120 protein of the viral envelope.

MATERIALS AND METHODS

Bacterial Strains and Extracellular Vesicle Isolation and Characterization

In this study, we used the Gram-variable bacteria *Gardnerella vaginalis* ATCC14018 and the following Gram-positive bacteria: *Staphylococcus aureus* ATCC12600, *Enterococcus faecium* ATCC19434, and *Enterococcus faecalis* ATCC19433. All bacterial strains were cultured at 37°C in de Man, Rogosa, and Sharpe (MRS) broth (Difco, Detroit, MI) pre-filtered with 0.1-µm filters to decrease the number of particles that are present in the medium. *G. vaginalis* was cultured in anaerobic conditions in jars containing Gaspak EZ (BD, Franklin Lakes, NJ), while the remaining strains (*S. aureus, E. faecium*, and *E. faecalis*) were cultured in aerobic conditions. The optical density (OD) of overnight cultures were measured using the spectrophotometer (Biophotometer, Eppendorf, Germany) and the bacterial concentration was calculated using an OD_{600} nm conversion factor of 0.4, corresponding to a concentration of 10^8 colony-forming units (CFU)/mL. Bacterial EVs were isolated from the supernatants of bacterial cultures by ultracentrifugation (Théry et al., 2006; Ñahui Palomino et al., 2019). Briefly, tubes containing 50 mL of bacterial cultures were centrifuged at $2{,}800 \times g$ for 15 min at 4°C. The supernatants obtained were filtered with 0.22-µm filters to remove any remaining bacteria. Then, bacterial EVs were isolated by ultracentrifugation at $100{,}000 \times g$ for 70 min at 4°C (Ultracentrifuge WX ultra 80, Thermo Fisher Scientific). EV-free supernatant was removed and collected for further experiments, while the pellet, containing bacterial EVs, was resuspended in 150 µL of PBS and stored at 4° C. A similar approach was used to isolate EVs from MRS medium.

Bacterial EVs were characterized in terms of size and concentration through nanoparticle tracking analysis (NTA) using the NanoSight NS300 (Malvern instruments Ltd, Malvern, UK) equipped with a 405-nm laser. The isolated bacterial EVs were diluted 1:100 in PBS and loaded in 1-mL syringes placed in a syringe pump controller. The samples were analyzed for 60 seconds three times with camera level = 13, detect threshold = 6, and syringe pump speed = 15. NTA results were analyzed using NTA software, version 3.1.54 (Malvern instruments Ltd, Malvern, UK).

Cell and Human Cervico-Vaginal Tissue Cultures

Human T-lymphocytes MT-4 (NIH AIDS Reagent Program, Germatown, MD, catalog number 120) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco BRL, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS).

Human cervico-vaginal tissues were received as anonymized samples, obtained from routine hysterectomies through the National Disease Research Interchange (NDRI, Philadelphia, PA). The protocol was approved by the NDRI IRB#5 of the University of Pennsylvania. NDRI maintains a Federal Wide Assurance (FWA00006180) agreement with the DHHS, Office

for Human Research Protections to comply with federal regulations concerning research involving human subjects. Tissues were dissected as described previously (Grivel and Margolis, 2009) with slight modifications. Briefly, the mucosa layers from ecto- and endo-cervix tissues were cut in blocks of 2 mm^3, and blocks were placed onto collagen sponge gel (Gelfoam, Pfizer, New York, NY) at the air–liquid interface and cultured in RPMI 1640 medium supplemented with FBS at 15%, 1 mM non-essential amino acids, 1 mM sodium pyruvate, amphotericin B at 2.5-µg/mL, and gentamycin sulfate at 50-µg/mL.

Anti-HIV-1 Assays

Anti-HIV-1 properties of bacterial supernatants and bacterial-derived EVs were evaluated in human T-lymphocyte MT-4 cell line.

Anti-HIV-1 effect was tested in MT-4 cells infected with a prototypic X4 HIV-1 isolate, LAI.04 (HIV-$1_{LAI.04}$; Rush University Virology Quality Assurance Laboratory, Chicago, IL) as described earlier (Ñahui Palomino et al., 2019). Briefly, 30 µL of HIV-$1_{LAI.04}$ viral stock (350 ng $p24_{gag}$/mL) was treated with 1×10^{10} bacterial EVs (derived from *S. aureus*, *E. faecium*, *E. faecalis*, or *G. vaginalis*), or with bacterial supernatants (*S. aureus*, *E. faecium*, *E. faecalis*, *G. vaginalis*) at 0.5%, or with bacterial EV-free supernatants at 0.5% (after ultracentrifugation, *S. aureus*, *E. faecium*, *E. faecalis*, *G. vaginalis*), or with 1×10^{10} particles isolated from MRS medium, or controls (PBS, MRS medium), for 1 h, at 37°C. EV/HIV-$1_{LAI.04}$ or EV-free/HIV-$1_{LAI.04}$ mixtures were then used to infect 3×10^5 MT-4 cells for 1 h at 37°C under constant agitation at 400 rpm. Following infection, cells were washed three times with 1 mL of PBS and centrifuged for 5 min at 400 g to eliminate free virions. Infected cells were resuspended in 3 mL of RPMI medium containing the corresponding 1×10^{10} EVs/mL, or PBS, or MRS medium. Cells were transferred to 24-well plates (1 mL/well, 1×10^5 cells/well; Sigma-Aldrich, St. Louis, MO) and incubated for 3 days at 37°C. Experiments of the same type were conducted to address the concentration-dependent antiviral activity of bacterial EVs derived from *S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis*, using 1×10^7, 1×10^8, 1×10^9, or 1×10^{10} bacterial EVs/mL.

To digest the EV protein surface, 150 µL of bacterial EVs (stock 1×10^{10} EVs/mL) were treated with proteinase K (PK) at a final concentration of 0.2 mg/mL (ThermoFisher Scientific, Waltham, MA) for 1 h. PK-treated EVs were resuspended with PBS to a final volume of 30 mL. Then, bacterial EVs were isolated by ultracentrifugation at $100{,}000\times g$ for 70 min at 4°C (Ultracentrifuge WX ultra 80, Thermo Fisher Scientific). The pellet, containing bacterial EVs, was resuspended in 150 µL of PBS. In control experiments, PK alone was used. The anti-HIV-1 effect of EVs previously treated with PK (1×10^{10} EVs/mL derived from *S. aureus*, *E. faecium*, *E. faecalis*, or *G. vaginalis*) was evaluated in MT-4 cells as described above.

To study whether bacterial EVs induce anti-HIV-1 responses in host cells, 3×10^5 MT-4 cells were cultured in cell media containing or not containing 1×10^{10} bacterial EVs derived from *S. aureus*, *E. faecium*, *E. faecalis*, or *G. vaginalis*, for 24 h at 37°C. Then, cells were washed three times with 1 mL of PBS and centrifuged for 5 min at 400 g to eliminate bacterial EVs. Thirty µL of HIV-$1_{LAI.04}$ (stock 350 ng $p24_{gag}$/mL) was then used to infect MT-4 cells for 1 h at 37°C under constant agitation at 400 rpm. After infection, cells were washed three times with 1 mL of PBS and centrifuged for 5 min at 400 g to eliminate free virions. Infected cells were resuspended in 3 mL of RPMI medium, transferred at 1 mL/well to a 24-well plate, and incubated for 3 days at 37°C.

Also, the anti-HIV-1 effect of bacterial EVs was evaluated in human cervico-vaginal tissues *ex vivo*. To infect human cervico-vaginal tissues *ex vivo*, tissue blocks were infected with the prototypic R5 HIV-1 isolate BaL (HIV-1_{BaL}; Rush University Virology Quality Assurance Laboratory). HIV-1_{BaL} viral stock (400 µL, with 120 ng $p24_{gag}$/mL) was preincubated with 1×10^{10} bacterial EVs (derived from *S. aureus*, *E. faecium*, *E. faecalis*, or *G. vaginalis*), or with 1×10^{10} particles isolated from MRS medium, or with PBS only (control), for 1 h at 37°C. Then, cervico-vaginal tissues were infected with an EV/HIV-1_{BaL} or EV-free/HIV-1_{BaL} mixture for 2 h at 37°C in agitation. Afterward, the infected cervico-vaginal tissue blocks were washed three times with PBS and transferred at the liquid–air interface onto Gelfoam (nine blocks/well) containing 1 mL of RPMI medium supplemented or not with 1×10^{10} bacterial EVs/mL. The tissue blocks were kept for 12 days at 37°C, replacing and collecting the medium containing or not containing bacterial EVs (1×10^{10} EVs/mL) every 3 days.

Measurement of HIV-1 Replication

In all the experiments, we evaluated HIV-1 replication by measuring the HIV-1 capsid protein, p24gag antigen, released in cell or tissue culture medium, using an immunofluorescent cytometric bead-based assay by Luminex (Biancotto et al., 2009). Briefly, 15 µL/well of each sample were initially transferred to a 96-well plate and lysed with 135 µL/well of Luminex lysis buffer (PBS containing 1% Triton X-100, 0.02% Tween20, 0.02% BSA, and 20 mM Tris-HCl pH 6) for 30 min at 37°C. The lysed samples were mixed 1:1 (50 µL:50 µL) with a solution containing p24 antibodies coupled to magnetic beads at a concentration of 1×10^3 beads/mL and incubated for 1 h at room temperature, in agitation at 400 rpm. Magnetic beads were previously coupled with anti-p24 antibody. Then, each plate was washed twice with 200 µL of Luminex wash buffer (PBS containing 0.02% Tween20, 20 mM Tris-HCl pH 6) using an ELx405 magnetic microplate washer (BioTek, Winooski, VT); then 100 µL of the detection antibody RD1-anti-p24 (Beckman Coulter, Indianapolis, IN) was added to each well (final concentration 0.5 µg/mL), followed by 1 h of incubation at room temperature, in agitation at 400 rpm. The plates were washed twice, as described above. To measure the p24 concentration, 100 µL/well of Luminex buffer was added (PBS containing 0.02% Tween20, 0.02% BSA, 20 mM Tris-HCl pH 6). The latter was performed on a Luminex 200 (BioRad, Hercules, CA) using its Bioplex manager software version 6.0.

Cell Viability

We performed cell viability assays in MT-4 cells using the automated cell counting system Cellometer Auto 2000 (Nexcelom bioscience, San Diego, CA). We determined the numbers of total and dead cells in control cultures, in bacterial EV-treated cultures, and in bacterial supernatant cultures, staining the cells with a dual-fluorescence acridine orange/

propidium iodide (AOPI, Nexcelom bioscience, San Diego, CA). Acridine Orange stains all nucleated cells to generate green fluorescence. Propidium iodide stains all dead nucleated cells to generate red fluorescence. Briefly, cells were seeded to 24-well plates at a final concentration of 1×10^5 cells per well and treated with 1×10^{10} EVs/mL, or bacterial supernatants at 0.5%, or PBS. After day 3 of cell culture, 20 µL of cell suspensions was mixed with 20 µL of AOPI staining solution. Cell viability was expressed as percentage of viable cells in EV-free or EV-treated cells or in bacterial supernatants treated cells.

HIV-1 Capture Assays

HIV-1 capture experiments were performed to study the interactions of bacterial-derived EVs (*S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis*) with the HIV-1 envelope proteins gp120 and gp41.

Human monoclonal PG9 antibody (stock 1 mg/mL; Polymun Scientific, Austria) was used to capture HIV-1 by binding the trimeric form of viral gp120. Human monoclonal VRC01 antibody (stock 1 mg/mL; NIH AIDS Reagent Program, Germatown, MD) was used to capture HIV-1 through its CD4 binding site on gp120. Human monoclonal 4B3 antibody (stock 1 mg/mL; Polymun Scientific, Austria) was used to capture HIV-1 virions with gp41. The antibodies were previously coupled to 15-nm carboxyl-terminated magnetic iron oxide nanoparticles (MNPs) according to manufacturer's protocol (Ocean NanoTech, San diego, CA). Towards this goal, 50 µL of HIV-$1_{LAI.04}$ was incubated with 50 µL of bacterial EVs at a concentration of 1×10^{10} particles/mL or with PBS (control) for 1 h at 37°C under constant agitation at 400 rpm. Thereafter, viral particles were captured by adding 50 µL of MNPs coupled to monoclonal antibody PG9, VRC01, or 4B3 and incubated for 1 h at 37°C, under constant agitation at 400 rpm. To evaluate the number of viruses captured, the virions captured with PG9, VRC01, or 4B3 MNPs were separated from free virions using magnetic columns inserted in a high field MACS magnet (Miltenyi Biotech, Auburn, CA). Columns were then washed 3 times with 600 µL of washing buffer (0.5% BSA, 1 mM EDTA), demagnetized for 5 min, and eluted with 600 µL of Luminex lysis buffer (PBS containing 1% Triton X-100). The p24gag antigen concentration in the eluate was measured as described above. Experiments of a similar type were performed to capture HIV-1 virions with PG9-MNPs or VRC01-MNPs after exposure of HIV-1 to PK-treated bacterial EVs (1×10^{10} EVs).

Proteomic Analysis

Three independent replicates from EVs derived from *S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis* were analyzed for proteomics with mass spectroscopy (BGI Americas, San Jose, CA) as follows:

Sample lysis: About 100 µL of bacterial EVs were mixed with 700 µL of lysis buffer (9M urea, 0.5% Waters Rapigest, pH 8.5). Samples were waterbath-sonicated for 30 min followed by centrifugation for 10 min at 14,000 *g*. Protein concentration of lysates were measured by BCA assay (Cat No: A53225, Thermo Fisher Scientific).

Proteomics sample preparation: About 20 µg of each sample was taken from the lysate and normalized to the same volume with lysis buffer. Samples were reduced in 10 mM dichlorodiphenyltrichloroethane (DTT) for 25 min at 60°C, and then reduced samples were alkylated in 20 mM iodoacetamide (IAM) in a dark environment for 20 min at room temperature. Excess IAM in the samples were quenched by adding 100 mM DTT solutions. Deionized water and HEPES pH 8.5 were added to each sample, so that final urea concentration was diluted to 1.6 M, and the final pH was about 8 for enzymatic digestion. Try/LysC (1 µg) (Cat No: A41007, Thermo Fisher Scientific) was added to each sample. Samples were incubated overnight at 37°C for 12 h. An additional 1 µg of Tryp/LysC was added to each sample the next day, and samples were incubated for 4 h to complete the enzymatic digestion; 10% trifluoroacetic acid (TFA) was added into each digested peptide sample to a final concentration of 1% TFA. Then, the acidified samples were desalted using C18 stage tips (Cat # PTR-92-05-18, Biotage), and the samples were ready for LC/MS analysis.

LC/MS analysis: All samples were analyzed by using nano flow HPLC (Ultimate 3000, Thermo Fisher Scientific) followed by Thermo Orbitrap Mass Spectrometer (Q-Exactive HF-X). Nanospray Flex™ Ion Source (Thermo Fisher Scientific) was equipped with Column Oven (PRSO-V2, Sonation) to heat up the nano column (PicoFrit, 100 µm x 250 mm x 15 µm tip, New Objective) for peptide separation. The nano LC method is water acetonitrile-based, lasts 150 minutes with a 0.25-µL/min flowrate. For each LC/MS run, all peptides were first engaged on a trap column (Cat. No: 160454, Thermo Fisher) and then were delivered to the separation nano column at the mobile phase. A protein profiling specific data dependent acquisition– (DDA) based mass spectrometry method on QE HF-X was used to sequence digested peptides that were eluted from the nano column. For the full MS, a 60,000 resolution was used with 3E6 AGC target or 50ms IT, and the scan range was 400 m/z–1,600 m/z. For the dd-MS2(MS/MS), a 15,000 resolution was used with 1E5 AGC target or 40ms IT. The isolation window was 1.4 Da. Normalized Collision Energy (NCE) was set to 27 with a 10-cycle loop.

Bioinformatic analysis pipeline overview: Collected LC-MS data were analyzed by Proteome Discoverer 2.5 (Thermo Fisher Scientific). Area under the curve (AUC) quantitative proteomics searches were performed. All searches were performed in Sequest HT node with mass tolerance of 20 ppm MS1 and 0.05 Da for MS2. Multiple databases (UP000310049, UP000005269, UP000001415, UP000033074, UP000002371, UP000030761) from Swiss-Prot were used to search the corresponding species of exosomes. Perculator node was used for peptide FDR filtering (Strict: 001, relaxed: 0.05). Peptide abundances were normalized by total peptide abundance and quantified by MS1 level AUC.

Filtering and identification of orthologue proteins in *S. aureus*, *G. vaginalis*, *E. faecium*, and *E. faecalis* EV proteomes: For each strain, the EV-derived proteomic data were initially filtered out to remove proteins that i) were identified as contaminants, i.e. of human origin, ii) were not detected in all the replicates. Redundant proteins were also excluded by keeping only the BLAST Reciprocal Best Hits (RBH) (https://github.com/

peterjc/galaxy_blast/tree/master/tools/blast_rbh) that were identified from comparisons of each EV-derived proteome (predicted using the Proteome Discover UniProt database) with the corresponding NCBI RefSeq strain-specific proteome (*S. aureus*: GCF_001879295.1, *G. vaginalis*: GCF_004336685.1, *E. faecium*: GCF_900447735.1, *E. faecalis*: GCF_000392875.1). These resulting curated EV-derived proteomes were further analyzed using OrthoFinder (Emms and Kelly, 2019) to identify orthologue proteins shared among the four strains.

Gene Ontology annotation functional enrichment: Gene ontology (GO) annotation was performed using eggNOG-mapper v2 against the database eggNOG v5.0 (Cantalapiedra et al., 2021). The R package topGO was used to identify the molecular function GO terms that were enriched in the shared EVs orthologue dataset (test set) as compared with each strain EV-derived proteome (universe set). Significantly enriched GO terms were detected by application of the 'weight01' algorithm and the Fisher test implemented in topGO (Alexa and Rahnenfuhrer, 2019). TopGO results were finally manually curated to remove redundant GO terms including the same set of proteins.

Identification of cytoplasmic, membrane, and extracytoplasmic proteins: TMHMM v. 2.0 was used in combination with SignalP v5.0 to distinguish cytoplasmic, membrane and extracytoplasmic proteins. Proteins without trans-membrane (TM) domains but with a signal peptide were identified as putatively extracytoplasmic, which could correspond to either periplasmic or extracellular proteins. Conversely, proteins with TM motifs and a signal peptide were marked as membrane.

RESULTS

Gram-Positive Bacteria Secrete EVs

EVs were isolated from the following four opportunistic human pathogens: *Staphylococcus aureus* ATCC12600, *Enterococcus faecium* ATCC19434, *Enterococcus faecalis* ATCC19433, and *Gardnerella vaginalis* ATCC14018. EVs were isolated from bacterial cultures by ultracentrifugation followed by its characterization in terms of size and concentration using nanoparticle tracking analysis (NTA). Our results demonstrated that all bacterial strains secrete EVs of similar size ranging from 179.07 ± 17.22 nm (*G. vaginalis*) to 231.97 ± 11.24 nm (*S. aureus*) (**Figure 1A**). *E. faecalis* released EVs of around one order of magnitude higher than the other strains, $2.27 \pm 0.88\times10^{12}$ particles/mL (**Figure 1B**). *S. aureus* released $3.52 \pm 1.79\times10^{11}$, *E. faecium* released $3.78 \pm 1.61\times10^{11}$, and *G. vaginalis* released $5.42 \pm 1.65\times10^{11}$ particles/mL (**Figure 1B**).

Gram-Positive–Derived EVs Suppress HIV-1 Infection in Human MT-4 Cells

To investigate whether Gram-positive bacteria secrete anti-HIV-1 compounds, we tested the anti-HIV-1 activity of the supernatants derived from overnight cultures of the four pathogenic bacteria on HIV-1 replication in human lymphoid MT-4 cells. MT-4 cells infected with HIV-$1_{LAI.04}$ were cultured in cell culture medium supplemented with bacterial supernatants at 0.5% (diluted 1:200 in cell culture medium). We found that HIV-$1_{LAI.04}$ replication was reduced by 81.26 ± 18.52% (*S. aureus*, $p < 0.0001$, $n = 4$), 63.93 ± 33.40% (*E. faecium*, $p = 0.0004$, $n = 4$), 71.14 ± 17.40% (*E. faecalis*, $p = 0.0001$, $n = 4$), and 91.77 ± 2.85% (*G. vaginalis*, $p < 0.0001$, $n = 3$) (**Figure 2A**) compared with controls. Also, there was no inhibition of HIV-1 in MT-4 cells treated with MRS, the medium used to grow bacteria (8.91 ± 4.43%, $p = 0.9272$, $n = 4$).

These data show that *S. aureus, E. faecium, E. faecalis*, and *G. vaginalis* secrete antiviral factors extracellularly. On the basis of previous findings (Ñahui Palomino et al., 2019), we then isolated EVs from bacterial supernatants and tested their effects on HIV-1 infection in MT-4 cells. Our results showed that in the presence of any of the pathogen-derived EVs, HIV-$1_{LAI.04}$ replication was significantly reduced compared with the control (**Figure 2B**). In particular, HIV-$1_{LAI.04}$ replication was reduced when MT-4 cells were treated with 1 x 10^{10} bacterial EVs by 93.28 ± 5.38% (*S. aureus*, $p <$

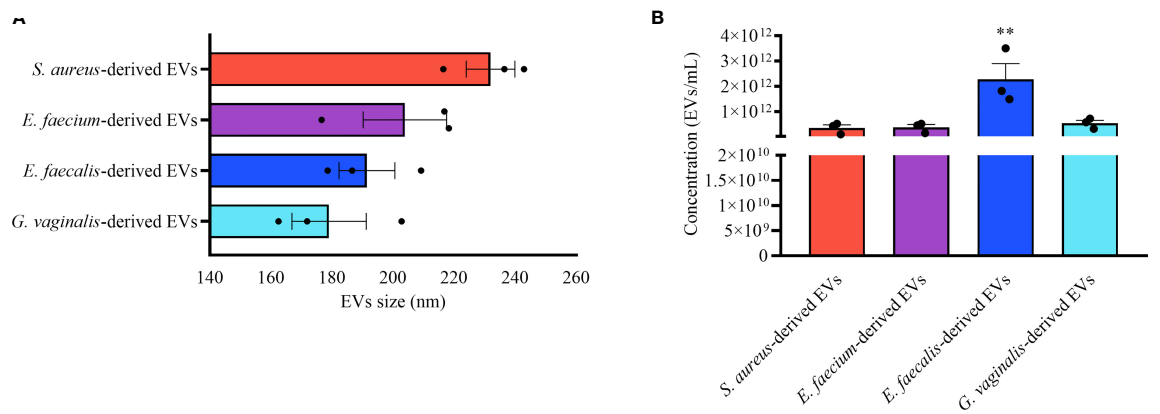

FIGURE 1 | Bacterial EV-size and concentration. Nanoparticle tracking analysis of EVs derived from *S. aureus, E. faecium, E. faecalis*, and *G. vaginalis*. **(A)** EV-sizes expressed as mean ± SEM of particle diameter (nm). **(B)** Mean ± SEM of the EV concentration (particles/mL). Presented are the results of three independent measurements. **p < 0.01.

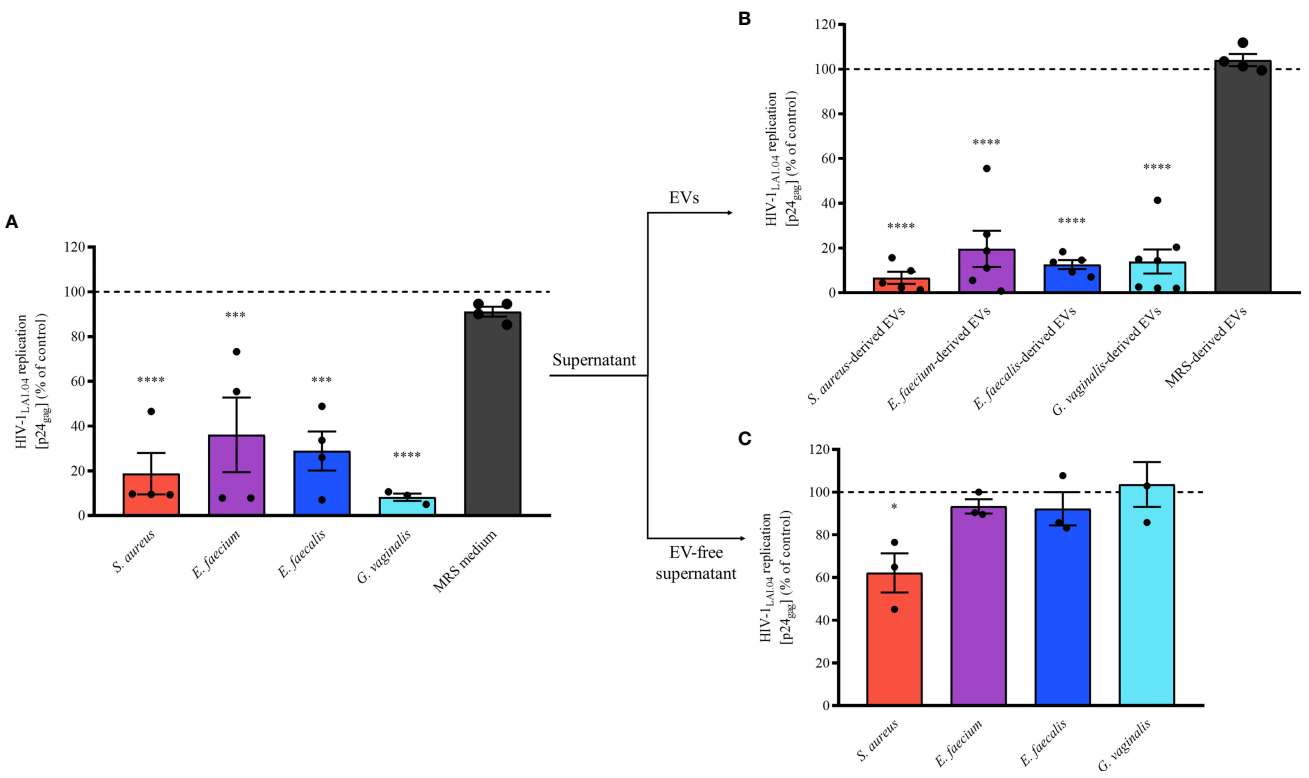

FIGURE 2 | Antiviral effect of bacterial-derived supernatants, EVs, and EV-free supernatants. A mixture of HIV-$1_{LAI.04}$ and bacterial-derived supernatants (0.5%), EVs (1×10^{10} EVs/mL), or EV-free supernatants (0.5%) incubated for 1 h was added to MT-4 cell cultures. Cells were washed and cultured for 3 days in the presence of bacterial supernatants, EVs, or EV-free supernatants. In control experiments, EV-free, particles isolated from fresh MRS medium, and MRS medium were tested as well. Replication of HIV-1 was evaluated from measurements of the capsid protein $p24_{gag}$, in the cell culture medium; data are presented as percentages of HIV-1 replication compared with untreated controls. **(A)** The effects of bacterial supernatants (0.5%) derived from *S. aureus*, *E. faecium*, *E. faecalis*, *G. vaginalis*, and MRS medium (0.5%) on HIV-1 replication in MT-4 cells. **(B)** The effects of 1×10^{10} EVs/mL bacterial EVs derived from *S. aureus*, *E. faecium*, *E. faecalis*, *G. vaginalis*, and MRS medium-derived particles on HIV-1 replication in MT-4 cells. **(C)** The effects of bacterial EV-free supernatants (0.5%, after pulling down EVs by ultracentrifugation) derived from *S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis* on HIV-1 replication in MT-4 cells. Presented are means ± SEM from at least three independent measurements. Asterisks indicate statistical significance by one-way ANOVA multiple comparison with Dunnett's correction (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$).

0.0001, $n = 5$), 80.33 ± 18.09% (*E. faecium*, $p < 0.0001$, $n = 6$), 87.37 ± 3.97% (*E. faecalis*, $p < 0.0001$, $n = 5$), and 86.02 ± 13.13% (*G. vaginalis*, $p < 0.0001$, $n = 7$). On the contrary, as expected, particles isolated from MRS medium did not reduce HIV-1 replication ($p = 0.9723$, $n = 4$).

To determine whether antiviral factors other than bacterial EVs were present in the bacterial supernatants, we tested the effects of bacterial EV-depleted supernatants (0.5% of EV-free supernatants) on HIV-1 replication in MT-4 cells. As shown in **Figure 2C**, the depletion of EVs from bacterial supernatants caused a significant loss of their anti-HIV activities. Indeed, HIV-1 replication in MT-4 cells cultured in EV-free supernatant were similar to control experiments (100%), at 93.32 ± 5.80% (*E. faecium*, $p = 0.9115$, $n = 3$), 92.24 ± 13.52% (*E. faecalis*, $p = 0.8628$, $n = 3$), and 92.24 ± 18.14% (*G. vaginalis*, $p = 0.9893$, $n = 3$). Nevertheless, EV-free supernatant derived from *S. aureus* still significantly decreased HIV-1 replication by 37.82 ± 15.86% ($p = 0.0141$, $n = 3$), suggesting the presence of other antiviral factors in the supernatant of *S. aureus*.

Next, we assessed the concentration-dependent anti-HIV-1 effect of bacterial EVs by testing different EV concentrations, 1×10^7, 1×10^8, 1×10^9, and 1×10^{10} EVs/mL. As shown in **Figure 3**, the anti-HIV-1 effect exerted by bacterial EVs from each bacterial species was in a concentration dependent manner. HIV-1 replication in MT-4 cells was reduced by 47.45 ± 45.89% when incubated with *S. aureus*-derived EVs starting at 1×10^9 EVs/mL (**Figure 3A**, $p = 0.0094$, $n = 6$), or by 44.69 ± 50.45% by *E. faecium*-derived EVs starting at 1×10^8 EVs/mL (**Figure 3B**, $p = 0.0378$, $n = 6$), or by 28.51 ± 20.92% by *E. faecalis*-derived EVs starting at 1×10^8 EVs/mL (**Figure 3C**, $p = 0.0199$, $n = 6$), or by 45.24 ± 34.35% by *G. vaginalis*-derived EVs starting at 1×10^8 EVs/mL (**Figure 3D**, $p = 0.0005$, $n = 6$). On the other hand, decreasing the amount of bacterial EVs below 1×10^7 EVs/mL led to a loss of anti-HIV-1 activity, demonstrating that the antiviral effect of pathogen-derived EVs is dose-dependent (**Figure 3**).

EV Suppression of HIV-1 Replication Is Not Due to Cell Cytotoxicity

To exclude the possibility that the bacterial EV- or bacterial supernatant- anti-HIV-1 effects were due to cell cytotoxicity, we evaluated the possible cytotoxic effect of bacterial supernatants (diluted 1:200 in cell culture medium) and EVs in MT-4 cells. As

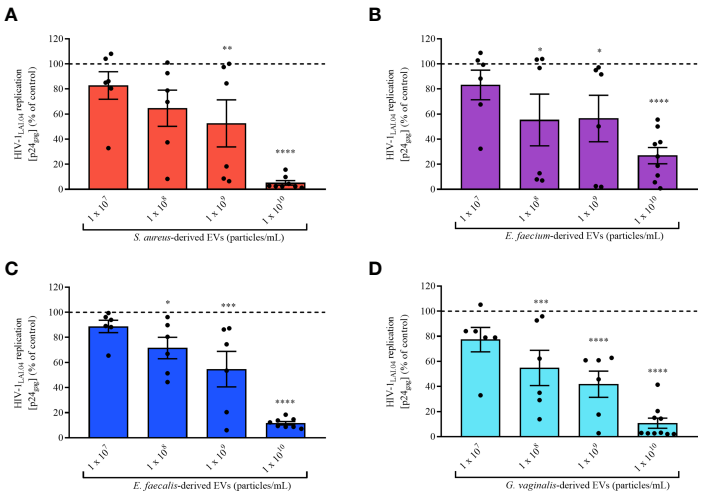

FIGURE 3 | Concentration effect of bacterial EVs on HIV-1 replication. MT-4 cells infected with HIV-$1_{Lai.04}$ were cultured in presence of bacterial EVs [*S. aureus* **(A)**, *E. faecium* **(B)**, *E. faecalis* **(C)**, and *G. vaginalis* **(D)**] at different EV concentrations, $1{\times}10^7$, $1{\times}10^8$, $1{\times}10^9$, and $1{\times}10^{10}$ EVs/mL. After 3 days of culture, HIV-1 replication was evaluated from measurements of the concentration of $p24_{gag}$ in the cell culture medium. The data are presented as percentages of HIV-1 replication compared with untreated controls. Presented are means ± SEM from six independent measurements. Asterisks indicate statistical significance by one-way ANOVA multiple comparison with Dunnett's correction (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

shown in **Figure 4**, there was no decrease in cell viability after treatment with bacterial supernatants (**Figure 4A**) or bacterial EVs (**Figure 4B**) in comparison with untreated control. All the bacterial supernatants tested, as well as MRS medium, were not cytotoxic for MT-4 cells. Indeed, cell viability treated with bacterial supernatants was statistically not different from that in control experiments (100%), ranging from 92.93 ± 2.21% (*E. faecium*, $p = 0.8841$, $n = 3$) to 98.87 ± 0.38% (*E. faecalis*, $p = 0.0107$, $n = 3$) (**Figure 4A**). Similarly, bacterial EV-treatment of MT-4 cells did not alter cell viability compared with controls, ranging from 88.93 ± 6.85% (*E. faecalis*-derived EVs, $p = 0.8603$, $n = 6$) to 92.13 ± 6.01% (*G. vaginalis*-derived EVs, $p = 0.9999$, $n = 6$) (**Figure 4B**).

Gram-Positive-Derived EVs Suppress HIV-1 Infection in Human Cervico-Vaginal Tissues *Ex Vivo*

On the basis of the EVs'anti-HIV-1 effect observed in MT-4 cells, we tested the effect of bacterial EVs against HIV-1 replication in an *in vivo*–like system of human cervico-vaginal tissues *ex vivo*. Toward this goal, human cervico-vaginal tissues infected with HIV-1_{BaL} were cultured in medium containing or not containing

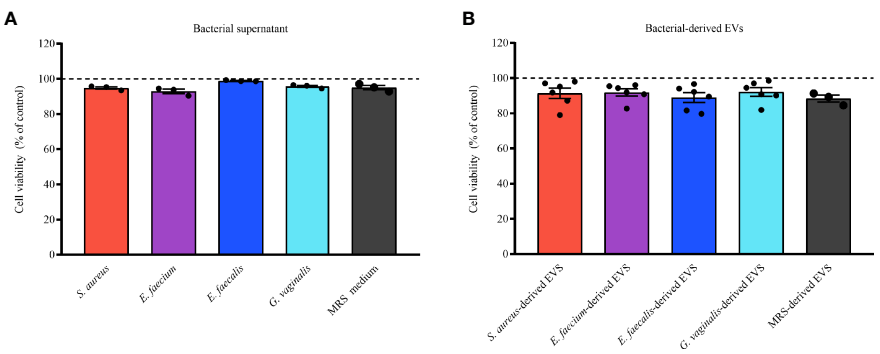

FIGURE 4 | Cell viability of cells treated with bacterial supernatants or EVs. MT-4 cells were treated or not treated for 3 days with bacterial supernatants (0.5%) or EVs ($1{\times}10^{10}$ EVs/mL) derived from *S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis*. MRS medium or particles derived from MRS were used as controls. The numbers of viable and dead cells were counted according to orange acridine/propidium-iodide-based assay. Results are expressed as percentages of cell viability in EV-free or EV-treated cells. Presented are means ± SEM from five independent measurements. Cell viability in presence of bacterial supernatants **(A)** or EVs **(B)** derived from *S. aureus*, *E. aecium*, *E. faecalis*, and *G. vaginalis* is shown. Results are expressed as percentages of viable cells treated or not treated with bacterial supernatant or EVs. Presented are means ± SEM from at least three independent measurements.

bacterial EVs (1x10^{10} EVs/mL). We found that EVs derived from all bacteria significantly reduced HIV-1 replication (**Figure 5**). In particular, *S. aureus*-derived EVs decreased HIV-1 replication by 17.17 ± 6.52% ($p = 0.009$, $n = 5$), *E. faecium*-derived EVs did so by 30.04 ± 7.47% ($p < 0.0001$, $n = 4$), *E. faecalis*-derived EVs did so by 34.20 ± 11.65% ($p < 0.0001$, $n = 6$), and *G. vaginalis*-derived EVs did so by 37.01 ± 10.26% ($p < 0.0001$, $n = 5$).

Cell Pre-Exposure With Bacterial EVs Does Not Prevent HIV-1 Infection

To understand whether bacterial EVs inhibit HIV replication directly or indirectly by inducing host cell responses, we pre-incubated MT-4 cells with bacterial EVs for 24 h, washed off the EVs, and infected MT-4 cells with HIV-1$_{LAI.04}$. As shown in **Figure 6**, there was no statistically significant decreasing effect on HIV-1$_{LAI.04}$ infection when cells were pre-treated with 1x10^{10} bacterial EVs derived from *S. aureus* ($p = 0.2516$, $n = 3$), *E. faecium* ($p = 0.1968$, $n = 3$), *E. faecalis* ($p = 0.6834$, $n = 4$), or *G. vaginalis* ($p = 0.2468$, $n = 4$), or with particles derived from MRS medium ($p = 0.9970$, $n = 3$).

Bacterial EVs Prevent HIV-1 Infection Affecting Viral Env, gp120

To investigate whether bacterial EVs derived from *S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis* directly affect the HIV-1 viral envelope, in particular gp120 or gp41, we incubated 1×10^{10} bacterial EVs for 1 h with HIV-1$_{LAI.04}$. Then, virions were captured using antibodies conjugated to magnetic nanoparticles (MNPs). We used PG9-MNPs, which recognize preferentially the HIV-1 trimeric envelope proteins gp120. VRC01-MNPs recognize the CD4 binding site (CD4bs) on the gp120 subunit. 4B3-MNPs recognize the viral gp41 subunit. Our results show that the incubation of HIV-1$_{LAI.04}$ with bacterial EVs led to a significant reduction in the number of HIV-1 virions captured with PG9-MNP antibodies in comparison with the control (free of bacterial EVs) (**Figure 7A**). The numbers of virions captured decreased by 52.56 ± 9.23% ($p < 0.0001$, $n = 5$) when incubated with *S. aureus*-derived EVs, by 54.83 ± 14.78% ($p < 0.0001$, $n = 5$) when incubated with *E. faecium*-derived EVs, by 54.01 ± 15.33% ($p < 0.0001$, $n = 5$) when incubated with *E. faecalis*-derived EVs, and by 42.64 ± 9.32% ($p < 0.0001$, $n = 6$) when incubated with *G. vaginalis*-derived EVs (**Figure 7A**).

Similar results were obtained using VRC01 as capture antibody. The numbers of virions captured upon treatment with bacterial EVs were reduced by 35.67 ± 10.95% (*S. aureus*-derived EVs, $p < 0.0001$, $n = 3$), 30.52 ± 4.03% (*S. faecium*-derived EVs, $p < 0.0001$, $n = 3$), 30.52 ± 4.03% (*S. faecalis*-derived EVs, $p = 0.0262$, $n = 3$), and by 24.46 ± 9.40% (*G. vaginalis*-derived EVs, $p = 0.0002$, $n = 4$), compared with the EV-free control (**Figure 7B**).

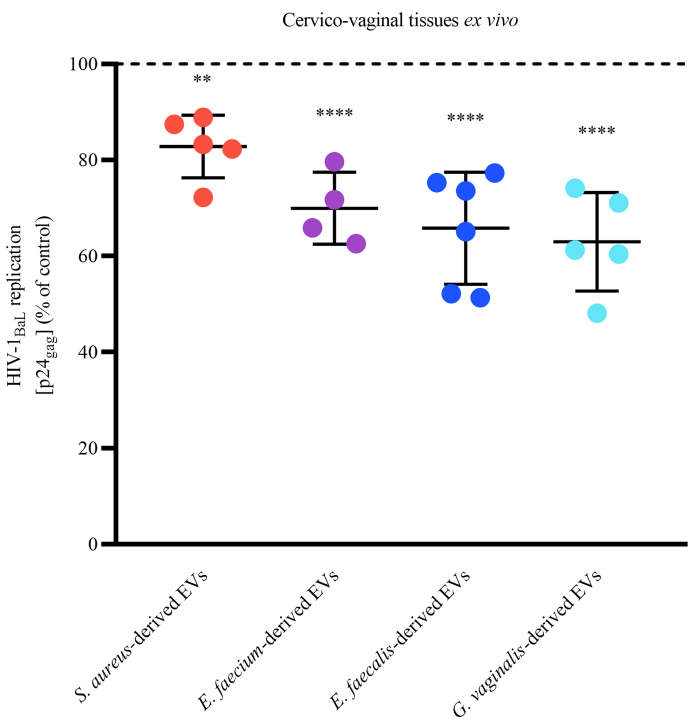

FIGURE 5 | Anti-HIV-1 effect of bacterial EVs in human cervico-vaginal tissues *ex vivo*. Cervico-vaginal tissue blocks were infected with EV-pretreated HIV-1$_{BaL}$ and cultured for 12 days, with replacement every 3 days of tissue culture medium containing or not containing 1×10^{10} EVs/mL derived from *S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis*. Replication of HIV-1 was evaluated from measurements of the capsid protein p24$_{gag}$ in tissue culture medium and is represented as a percentage of HIV-1 replication in untreated control. Presented are means ± SEM from tissues of at least four donors. Asterisks indicate statistical significance by one-way ANOVA multiple comparison with Dunnett's correction (**p < 0.01, ****p < 0.0001).

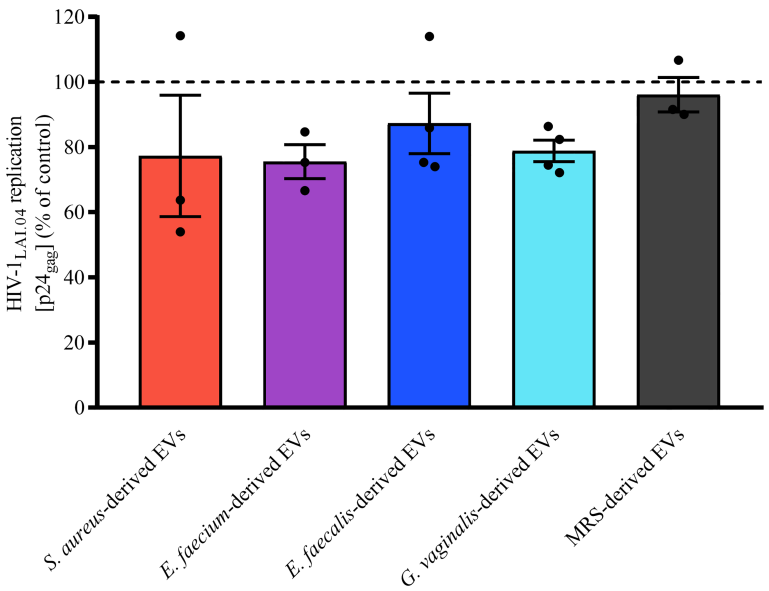

FIGURE 6 | Effect of cell pre-exposure with bacterial EVs on HIV-1 infection. MT-4 cells were preincubated with bacterial EVs for 24 h, washed off, infected with HIV-$1_{LAI.04}$, and incubated for 3 days. HIV-1 replication was evaluated from measurements of the capsid protein $p24_{gag}$ in cell culture medium and is represented as a percentage of HIV-1 replication in untreated control. Presented are means ± SEM from at least three independent measurements.

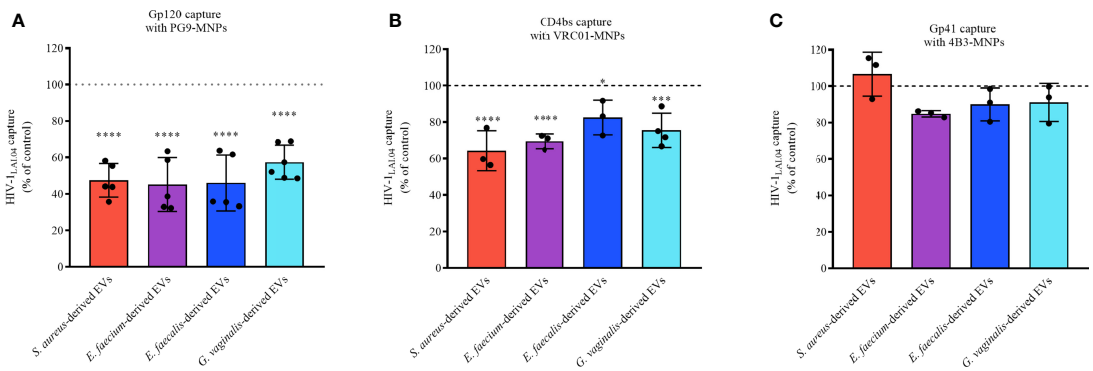

FIGURE 7 | HIV-1 capture. HIV-$1_{LAI.04}$ was pre-treated with 1×10^{10} EVs derived from *S. aureus*, *E. faecium*, *E. faecalis*, or *G. vaginalis*, or with particles derived from MRS medium, or with PBS (control) for 1 h. Next, HIV-$1_{LAI.04}$ virions were captured with PG9 **(A)**, VRC01 **(B)**, or 4B3 **(C)** antibodies coupled to magnetic nanoparticles (MNPs). PG9 antibody recognizes HIV-1 trimeric gp120 proteins, VRC01 recognizes the CD4 binding site on the viral gp120, and 4B3 antibodies recognize the viral gp41. Data are presented as percentage of $p24_{gag}$ concentration compared with the control. Presented are means ± SEM from three independent measurements. Asterisks indicate statistical significance by one-way ANOVA multiple comparison with Dunnett's correction (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$).

No statistically significant reduction in the number of captured viruses was observed with 4B3-MNPs when treated with EVs derived from *S. aureus* ($p = 0.9082$, $n = 3$), *E. faecium*-derived EVs ($p = 0.2405$, $n = 3$), *E. faecalis*-derived EVs ($p = 0.6281$, $n = 3$), or *G. vaginalis*-derived EVs ($p = 0.7270$, $n = 3$) (**Figure 7C**).

The Protein Component of Pathogen EV Is Essential for HIV-1 Inhibition

Since our results suggest that bacterial-derived EVs inhibit HIV-1 infection by interacting with viral gp120, we wondered whether proteins exposed on the EV surface are responsible for this interaction. Toward this goal, EVs derived from *S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis* were treated with PK to digest any proteins on the EV surface.

As shown in **Figures 8A, B**, upon treatment with PK the capacities of bacterial-derived EVs to interact directly with viral gp120 were lost, as the number of virions captured with PG9-MNPs (**Figure 8A**) and VRC01-MNPs (**Figure 8B**) were similar to that in the EV-free control (100%). Indeed, capturing virus with PG9-MNPs (**Figure 8B**), we found no statistically significant effect

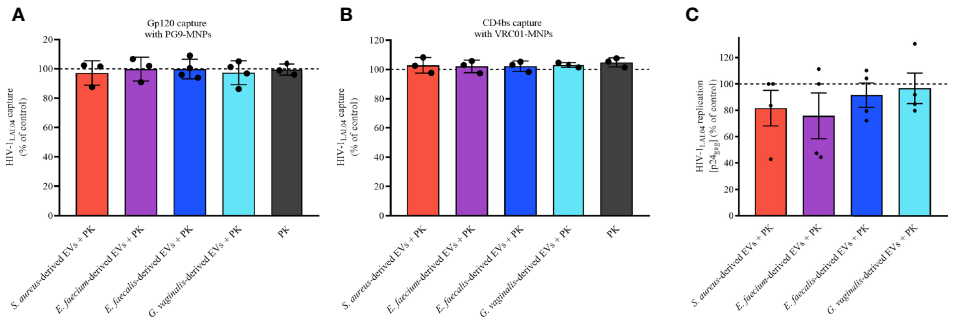

FIGURE 8 | Bacterial EV-associated protein role during HIV-1 infection. The proteins associated to bacterial EVs were digested with PK followed by an ulterior ultracentrifugation to obtain bacterial EVs free of surface proteins. HIV-$1_{LAI.04}$ was pre-treated with 1×10^{10} PK-treated EVs derived from *S. aureus*, *E. faecium*, *E. faecalis*, or *G. vaginalis*, or with PK (PBS treated with PK and purified by ultracentrifugation), or with PBS (control) for 1 h. Next, HIV-$1_{LAI.04}$ virions were captured with PG9 **(A)** or VRC01 **(B)** antibodies. Also, the antiviral effect of PK-treated bacterial EVs in MT-4 cells infected with $HIV_{LAI.04}$ was evaluated **(C)**. The amount of virus captured and the amount of virus present on cell culture medium were determined from measurement of the levels of viral $p24_{gag}$. Data are presented as percentages of $p24_{gag}$ concentration compared with the control (PBS). Presented are means ± SEM from three independent measurements. Statistical analysis was performed with one-way ANOVA multiple comparison.

on HIV-$1_{LAI.04}$ envelope when virions were treated with PK-treated EVs derived from *S. aureus* ($p = 0.9994$, $n = 3$), *E. faecium* ($p > 0.9999$, $n = 3$), *E. faecalis* ($p = 0.9994$, $n = 4$), or *G. vaginalis* ($p > 0.9999$, $n = 4$), or with PK ($p > 0.9999$, $n = 3$). Similarly, capturing virus with VRC01-MNPs (**Figure 8B**), no statistically significant effect on HIV-$1_{LAI.04}$ envelope was observed when virions were treated with PK-pretreated EVs derived from *S. aureus* ($p = 0.9967$, $n = 3$), *E. faecium* ($p > 0.9994$, $n = 3$), *E. faecalis* ($p = 0.9999$, $n = 2$), or *G. vaginalis* ($p = 0.9937$, $n = 3$), or with PK ($p > 0.9967$, $n = 3$).

We also tested the anti-HIV-1 activities of bacterial EVs treated with PK in MT-4 cells. As shown in **Figure 8C**, the anti-HIV-1 activities of all bacterial EVs tested were completely lost after proteinase K treatment. Indeed, our results demonstrated that HIV-$1_{LAI.04}$ replication was not reduced significantly in the presence of PK-treated bacterial EVs derived from *S. aureus* ($p = 0.9654$, $n = 4$), *E. faecium* ($p = 0.8498$, $n = 4$), *E. faecalis* ($p = 0.9994$, $n = 4$), or *G. vaginalis* ($p = 0.9998$, $n = 4$), compared with the control experiments (**Figure 8C**).

These results suggest that proteins exposed on bacterial EV surface are involved in the antiviral effect by obstructing the trimeric region and the CD4 binding site of viral gp120 protein.

Proteins Associated With Bacterial EVs

Using liquid chromatography–mass spectroscopy, we investigated the proteins associated to bacterial EVs. We identified 390, 960, 1,158, and 118 bacterial proteins associated to EVs derived from *S. aureus, E. faecium, E. faecalis*, and *G. vaginalis*, respectively (**Table S1**). In terms of cellular localization by gene ontology (GO) terms, more than 90% of the EV-associated proteins were predicted to have cytoplasmatic and trans-membrane localizations, with less than 10% of them being extracytoplasmic (**Figure 9A**). According to the molecular function by the GO terms, EVs derived from *S. aureus, E. faecium*, and *E. faecalis* shared most of their molecular functions. However, the EV-derived proteome of *G. vaginalis* showed some differences from the other strains. In this regard, EV-derived proteins with binding activity, transferase activity, transporter activity, hydrolase activity, and nucleotide/nucleic acid binding activity were shared among all the strains under analysis (**Figure 9B**), while an additional five molecular functions (protein and DNA binding, kinase and peptidase activity, and transporter activity) were shared only among *S. aureus*, *E. faecium* and *E. faecalis*. (**Figure 9B**). On the other hand, proteins with structural molecule activity and translation factor activity were detected only in the *G. vaginalis* EV-related proteome (**Figure 9B**).

Comparative analysis of EV-derived proteomes identified a total of 30 clusters of orthologue (OG) proteins shared among *S. aureus*, *E. faecium*, *E. faecalis* and *G. vaginalis* strains (**Table 1**). These clusters included proteins mostly involved in basal cellular functions such as translation (OG25, OG50, OG49, OG55, OG15 and OG30), transcription (OG51, OG56, OG69, OG68), chaperones and chaperonins (OG66, OG60 OG54, OG52), components of the ABC transporters (OG00 and OG28), and enzymes of the glycolytic pathways (OG53, OG48, OG11 and OG09). Significantly enriched GOs were identified from comparisons of the molecular functions of the EV-derived proteins included in the shared OG clusters with the whole EV-derived proteome from each strain. As a result, significantly enriched GO terms within the shared proteins corresponded to molecular functions involved in the binding of unfolded proteins (GO:0051082), nucleoside (GO:0003938) and nucleotide (GO:0000774) catalytic activity, interaction with the host cells (GO:2001065; GO:0035375), and catalytic activities of the glycolytic pathway (GO:0004743; GO:0004340; GO:0004365; GO:0004634) (**Figure 10**).

DISCUSSION

Based on the establishment of complex interactions, the microbial communities that populate the human body are well-balanced and perfectly distributed with the host, other

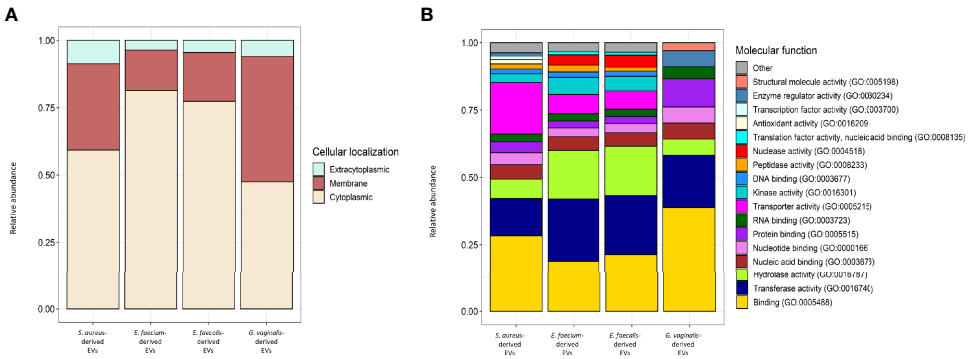

FIGURE 9 | Cellular localization and molecular function GOs of *S. aureus*, *E. faecium*, *E. faecalis* and *G.vaginalis*-derived EV proteome. **(A)** Relative abundance of the proteins predicted to have a cytoplasmic, extracytoplasmic, or intracellular localization within each strain-derived EV proteome. Detailed results of TMHMM and SignalP analysis used to predict TM and signal peptide motifs in the EV-related proteins are reported in **Tables S1–S4**. **(B)** Relative abundance of the proteins belonging to each molecular function's GO within each strain-derived EV proteome; 'other' includes GO terms with a relative abundance < 1%. Molecular functions were extracted from strain-derived EV proteomes, analyzed in topGO, and categorized in CateGOrizer (Hu et al., 2008) against the GOSlim2 database. Raw data are reported in **Tables S1–S11**; sa and gv indicate molecular functions that were identified only in the *S. aureus* and *G. vaginalis*-derived EVs proteome, respectively.

microorganisms, and the environment. The variations of a single bacterial component may perturb the entire ecosystem and lead to a deep rearrangement in the community composition, with possible onset of different diseases.

TABLE 1 | Description of EV-derived proteins included in orthogroups (OG) shared between *S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis*.

Shared OG[a]	Description
OG69	DNA-directed RNA polymerase subunit beta'
OG68	DNA-directed RNA polymerase subunit beta
OG67	Acetate kinase
OG66	Protein GrpE
OG64	ATP synthase gamma chain
OG63	Ferritin-like protein
OG62	ATP synthase subunit beta
OG60	Chaperone protein DnaK
OG58	60 kDa chaperonin
OG56	DNA-directed RNA polymerase subunit alpha
OG55	50S ribosomal protein L4
OG54	10 kDa chaperonin
OG53	Pyruvate kinase
OG52	Trigger factor
OG51	Elongation factor G
OG50	50S ribosomal protein L10
OG49	50S ribosomal protein L21
OG48	Enolase
OG47	Glucokinase
OG46	30S ribosomal protein S10
OG30	Serine-tRNA ligase
OG28	ABC transporter, substrate-binding protein[b]
OG25	Elongation factor Tu
OG24	Glutamine synthetase
OG15	Isoleucine-tRNA ligase
OG11	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase
OG10	Lipoprotein[b]
OG09	L-lactate dehydrogenase
OG08	Inosine-5'-monophosphate
OG00	ABC transporter ATP-binding protein[b]

[a]*The full list of proteins from each strain included in OG is reported in* ***Table S5****.*
[b]*For OGs including heterogeneous names, the protein family name was used.*

Lactobacillus species in vaginas of healthy pre-menopausal women represent the first barrier against numerous urogenital pathogens. Indeed, it has been shown that several *Lactobacillus* strains, such as *L. crispatus*, *L. gasseri*, and *L. jensenii*, reduce the possibility of acquisition of sexually transmitted infections, such as HIV-1 (Petrova et al., 2013). We reported that vaginal lactobacilli exert anti-HIV-1 activity by multiple mechanisms, including lactic acid production, acidification of the niche, and more recently by the release of nano-sized EVs (Ñahui Palomino et al., 2017; Ñahui Palomino et al., 2019).

Although *Lactobacillus* dominates in healthy vaginas, other bacteria that are considered pathogenic, such as *Gardnerella* spp., *Enterococcus*, *Staphylococcus*, etc., are present as well but are kept under control (Petrova et al., 2013; Gosmann et al., 2017). Here, we found that some of these bacteria secrete vesicles that confer protection against HIV-1 transmission.

In the last decade, the number of studies on bacterial EVs has been rapidly evolving, as demonstrated by their implication in bacteria–bacteria and bacteria–host interactions, either promoting health or causing various pathologies (Ñahui Palomino et al., 2021).

Specifically, we investigated whether three strains of Gram-positive bacteria (*S. aureus*, *E. faecium*, and *E. faecalis*) and the Gram-variable bacteria *G. vaginalis*, which can be present in the vaginas of healthy women (Ravel et al., 2011), release EVs capable of counteracting HIV-1 infection. We found that *S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis* release EVs similar in size (~ 200 nm) and comparable to those previously reported for other bacteria (Askarian et al., 2018; Wagner et al., 2018a; Kim et al., 2019; Shishpal et al., 2020).

We investigated whether, as with several *Lactobacillus* strains (Ñahui Palomino et al., 2019), EVs released by *S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis* may contribute to a protective effect against HIV-1 infection. Towards this goal, we tested the anti-HIV-1 effect of bacterial EVs in immortalized human lymphoid cells and in human cervico-vaginal tissues *ex vivo*. *Ex vivo* human tissues faithfully reflect many aspects of the

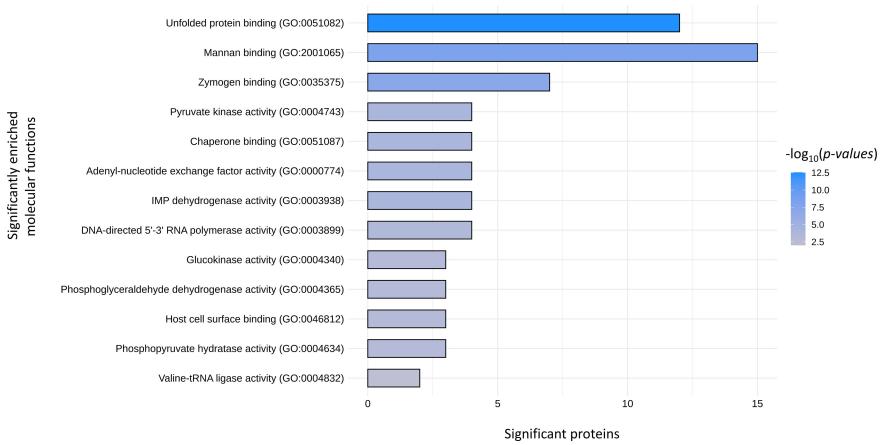

FIGURE 10 | Molecular function GOs significantly enriched in the EV derived proteins that are shared among *S. aureus*, *E. faecium*, *E. faecalis* and *G.vaginalis*. Raw data used for the plot are reported in **Tables S1–S11**.

tissues *in vivo*, where critical events of HIV-1 pathogenesis and transmission occur. In particular, they retain the key cell-surface molecules to support HIV-1 infection. Tissues *ex vivo* have been used widely to study host–pathogen interaction during HIV-1 infection (Grivel and Margolis, 2009; Ñahui Palomino et al., 2017; Ñahui Palomino et al., 2019).

Our results demonstrate that all tested bacteria release antiviral factors, as their cell supernatants inhibited HIV-1 replication in human T cells. The strength of this inhibition was comparable with that reported earlier for the supernatant of vaginal lactobacilli (*L. crispatus* BC3, *L. crispatus* BC5, *L. gasseri* BC12, and *L. gasseri* BC13) (Ñahui Palomino et al., 2017).

Next, we checked whether EVs that are present in the supernatants from the bacterial cultures can protect human cervico-vaginal tissues *ex vivo* as well as T-cell line from HIV-1 infection. Indeed, we found that EVs isolated from *S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis* largely protected human cervico-vaginal tissues *ex vivo* as well as a T-cell line from HIV-1 infection. The protection was observed using two HIV-1 strains which recognise different co-receptors for cell entry (CXCR4 for HIV-$1_{LAI.04}$ and CCR5 for HIV-1_{BaL}), suggesting a common and co-receptor–independent antiviral effect exerted by bacterial EVs. The anti-HIV-1 effect of EVs was concentration-dependent, and the inhibition of HIV-1 replication was not due to EV-induced cell death. Moreover, these anti-HIV-1 activities were comparable to the anti-HIV-1 activity exerted by other Gram-positive bacteria–derived EVs (*L. crispatus* BC3-derived EVs, *L. gasseri* BC12-derived EVs) in human T-cell lines and human tissues *ex vivo* (tonsillar and cervico-vaginal tissues) (Ñahui Palomino et al., 2019).

In principle, EVs may directly affect the infectivity of HIV-1 virions or may indirectly inhibit HIV-1 infection by interacting with viral target cells, or both. Pre-incubation of bacterial EVs with cells, at least for 24 h, did not result in HIV-1 inhibition. Therefore, we did not investigate further the bacterial EV-host cell interactions. In contrast, we found that the reduction of HIV-1 replication can be related to the alteration of HIV-1 virions by all bacterial-derived EVs tested.

One meaningful strategy to prevent HIV-1 infection is to target the viral gp120, which facilitates viral entry into the target cell. Steric hindrance or blocking the gp120 trimeric region and CD4 binding site on HIV-1 envelope is a key aspect of such strategy. Accordingly, numerous broadly neutralizing antibodies, nanobodies, or small molecules targeting viral gp120 have been developed. A small molecule that directly interacts with gp120 protein, Fostemsavir, was recently approved for medical treatment in the United States (2020) and in Europe (2021).

It seems that *S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis* use a similar anti-HIV-1 strategy as virions exposed to EVs released by these bacteria were no longer being recognized by MNP-coupled PG9 antibodies, which bind to the functional trimeric form of viral gp120. Similar results were observed when VRC01 antibodies, which recognize the CD4bs on the gp120, were used. These observations indicate that bacterial EVs interact specifically with the gp120 subunit of the HIV-1 envelope, as the virus treated with bacterial EVs and its subsequently capture with 4B3 antibodies, which recognize gp41, did not alter the amount of virus captured. Altogether, our results obtained in the present study are comparable with those previously reported for *Lactobacillus*-derived EVs (Ñahui Palomino et al., 2019), suggesting that HIV-1 inhibition by steric hindrance of gp120 or gp120 modification is a common mechanism of Gram-positive bacteria.

Here, we showed that proteins associated to bacterial EVs are important for this anti-HIV-1 effect. Toward this goal, we treated bacterial EVs with proteinase K (PK). PK-treated EVs lost their ability to inhibit HIV-1 replication in human T cells, suggesting that proteins exposed on the external surface of EVs are involved in the EV-mediated anti-HIV-1 mechanism. In agreement with these results, PK-treated EVs did not alter viral capture by PG9 or VRC01 antibodies, compared with controls.

We identified proteins associated to bacterial EVs that may be responsible for their anti-HIV-1 effect. Among the proteins detected in all the bacterial EVs, 30 orthogroup proteins (clusters of orthologue proteins) were common to the EV-proteome of all four strains. Similar EV-associated proteins were found in EVs released by *S. aureus*, *E. faecalis*, and *G. vaginalis*, as reported by others (Wagner et al., 2018b; Shishpal et al., 2020; Tartaglia et al., 2020). None of these bacterial 30 orthogroup proteins were previously reported to interact with HIV-1 or other viruses. However, human homologues of these proteins, such as chaperone protein DnaK (Hsp70), enolase (ENO), elongation factor TU (EFTU), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are involved during HIV-1 infection (Cimarelli and Luban, 1999; Kumar et al., 2011; Kishimoto et al., 2012; Li et al., 2015; Kishimoto et al., 2020). In particular, Hsp70 decreases HIV-1 replication by inhibiting viral gene expression (Kumar et al., 2011). Likewise, *Mycobacterium* Hsp70 has been shown to interact with CCR5, abrogating HIV-1 infection of human $CD4^+$ T cells (Babaahmady et al., 2007). Overexpression of ENO in HIV-1 target cells decreases HIV-1 infection by inhibiting viral integration and replication (Kishimoto et al., 2020). EF1α (eukaryotic homologue of EFTU) and GAPDH oppositely impact the reverse transcription of viral RNA: EF1α positively influences the reverse transcription by interacting with 5'UTR of HIV-1, while GAPDH negatively regulates the reverse transcription of viral RNA (Kishimoto et al., 2012; Li et al., 2015). Also, EF1α interacts with HIV-1 gag proteins and can be incorporated in the HIV-1 virion (Cimarelli and Luban, 1999). Furthermore, in EVs with anti-HIV-1 activity, such as EVs derived from *L. gasseri*, ENO-2, EFTU, ATP synthase gamma chain, and 60 kDa chaperonin (Ñahui Palomino et al., 2019; Costantini et al., 2021) have also been found. Whether HIV-1 inhibition by bacterial EVs is directly or indirectly exerted by one or more of these proteins remain to be elucidated.

It has been reported that changes in microbial composition in the vaginal environment from *Lactobacillus* dominance to non-*Lactobacillus* species such as *G. vaginalis* increase the susceptibility to HIV-1 infection (Sha et al., 2005; Ravel et al., 2011; Klatt et al., 2017). However, the exact mechanisms of this phenomenon still need to be fully elucidated. The ability of each bacterium to increase or decrease the susceptibility to HIV-1 infection will depend on the bacteria itself, release of virulence factors, capabilities to disrupt the host epithelial barrier, their capacities to induce pro-inflammatory environment that attract HIV-1 target cells, etc (Petrova et al., 2013). The final effect of vaginal microbiota on HIV-1 infection may be a balance of all the effects. Here, we focused on the study of EVs produced by *S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis*. If our results are extrapolated to *in vivo* HIV-1 transmission, it would be important to determine the amount of EVs produced by bacteria *in vivo*. A limiting factor for the comprehension of the real impact exerted by EVs is a lack of specific tools for the isolation of bacterial EVs released *in vivo*. Identification of antiviral molecules present in EVs, as well as their mechanism of action, will be fundamental for understanding HIV-1 protection mediated by bacterial EVs *in vivo*.

Although antiviral properties have never been reported before for *S. aureus*, *E. faecalis*, and *G. vaginalis*, *E. faecium* has been shown to exert antiviral activity against transmissible gastroenteritis coronavirus (TGEV), swine influenza A, astrovirus, and rotavirus A in *in vivo* and in *in vitro* studies (Kreuzer et al., 2012; Chai et al., 2013; Wang et al., 2013). Also, *E. faecium*, induced the upregulation of proinflammatory cytokines (IL-6, IL8) and nitric oxide on epithelial swine testicle cells, with a consequent reduction of viral infectivity. Moreover, *E. faecium* was able to trap TGEV virions, as observed with electron microscopy, decreasing viral infectivity (Kreuzer et al., 2012; Chai et al., 2013; Wang et al., 2013). Now, it should be investigated whether EVs generated by these bacteria mediate these activities.

In conclusion, EVs released by Gram-positive bacteria (*S. aureus*, *E. faecium*, *E. faecalis*) and *G. vaginalis*, protect human cervicovaginal tissues *ex vivo* as well as isolated human cells from HIV-1 infection. This protection is not due to a cytotoxic effect of bacterial EVs but rather to steric hindrance of gp120 or gp120 modifications. EVs carry numerous bacterial proteins which may be associated with the anti-HIV-1 effect. Identification of the bacterial EV molecules responsible for this anti-HIV-1 effect may help to the development of new therapeutic agents that prevent HIV-1 vaginal transmission.

DATA AVAILABILITY STATEMENT

The datasets regarding relative abundance of proteins identified in bacterial EVs and presented in the study are reported in the **Supplementary Material** and are also available upon a request to the authors.

AUTHOR CONTRIBUTIONS

PC and RÑ designed and performed the experiments, analyzed the data, and wrote the manuscript. LM and CV designed experiments, analyzed the data, and wrote the manuscript. AF and MC analyzed the proteomic data. All the authors contributed to data interpretation. All the authors read, reviewed, and approved the final manuscript.

FUNDING

This work was supported by the NICHD Intramural Program.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.822882/full#supplementary-material

Supplementary Table 1-11 | Functional annotation and cellular organization of EVs-related proteins quantified through proteomic analysis in *S. aureus*, *E. faecium*, *E. faecalis* and *G. vaginalis*.

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ORIGINAL RESEARCH
published: 08 March 2022
doi: 10.3389/fcimb.2022.860589

Correlation Analysis of Vaginal Microbiome Changes and Bacterial Vaginosis Plus Vulvovaginal Candidiasis Mixed Vaginitis Prognosis

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Mixed vaginitis is the result of the simultaneous presence of different pathogenic processes mediated by at least two types of vaginal pathogens. Among the various types of mixed vaginitis presentations, bacterial vaginosis (BV) plus vulvovaginal candidiasis (VVC) presents to be the most prevalent form. Mixed vaginitis affects the health of women of all ages worldwide. However, few studies have focused on clinical manifestations, pathogenesis, diagnostic criteria, or therapy of mixed vaginitis. We recruited 48 symptomatic patients with clinical diagnoses of VVC complicated with BV, they were treated with oral metronidazole combined with local clotrimazole and followed to assess the drug efficacy and vaginal microbiome alterations before and after treatment. The vaginal microbiome in BV+VVC mixed vaginitis patients was altered significantly after the combined drug treatment within a unique form different from a simple overlay mode of BV and VVC, the key bacteria including *Gardnerella* and *Atopobium*, *Lactobacillus*. The combined drug therapy for the mixed vaginitis in this study was effective and enhanced treatment for BV may be more favorable because of more difficulty in dealing with BV according to the treatment outcome. The abundance of *Lactobacillus* in patients with mixed vaginitis affects the recovery of the vaginal microbiome as well as the prognosis, and the abundance should be actively restored. This is the first study to investigate the composition, diversity, and other characteristics of the vaginal microbiome in patients with BV+VVC mixed vaginitis before and after drug treatment, our results provide clues to improving the cure rate and reducing recurrences.

Keywords: mixed vaginitis, vulvovaginal candidiasis (VVC), vaginal microbiome, *Gardnerella*, *Atopobium*, *Lactobacillus*, bacterial vaginosis (BV)

OPEN ACCESS

Edited by:
Claudio Foschi,
University of Bologna, Italy

Reviewed by:
Manpreet Kaur,
Guru Nanak Dev University, India
Gregory A. Buck,
Virginia Commonwealth University, United States

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Specialty section:
This article was submitted to Biofilms, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *23 January 2022*
Accepted: *15 February 2022*
Published: *08 March 2022*

Citation:
Xiao B, A D, Qin H, Mi L and Zhang D (2022) Correlation Analysis of Vaginal Microbiome Changes and Bacterial Vaginosis Plus Vulvovaginal Candidiasis Mixed Vaginitis Prognosis. Front. Cell. Infect. Microbiol. 12:860589. doi: 10.3389/fcimb.2022.860589

INTRODUCTION

Mixed vaginitis, a syndrome combining symptoms of different pathogenic processes is mediated by at least two types of vaginal pathogens that cause vaginal inflammation. Common vaginitis includes bacterial vaginosis (BV), vaginal trichomoniasis (TV), vulvovaginal candidiasis (VVC), and aerobic vaginitis or desquamative inflammatory vaginitis (AV/DIV) (Paavonen and Brunham, 2018). Although some microorganisms always exhibit asymptomatic colonization, such as *Candida*,

they are actually opportunistic pathogens and able to cause clinical symptoms when the immune system of the host is weakened. Mixed vaginitis presentations may be divided into various types such as BV+VVC, BV+TV, and AV+VVC according to their causative pathogenic processes, they require multiple therapies targeting all pathogens causing symptoms. Few studies on mixed vaginitis have focused on clinical manifestations, pathogenesis, diagnostic criteria and therapy. Mixed vaginitis needs to be distinguished from vaginal coinfections, which present clinical manifestations and signs caused by a single pathogen with asymptomatic concomitant colonization by another pathogen and, therefore, require treatments targeting only the pathogen causing symptoms (Sobel et al., 2013; Benyas and Sobel, 2022).

Attention has been drawn to the increasing prevalence of mixed vaginitis in recent years. According to a literature review summarizing mixed vaginitis advances, the proportion of symptomatic women with mixed vaginitis, most frequently presenting as genital itching, burning pain, and changes in the characteristics of discharge (odor, color, consistency), ranges from 4.44% to 35.06%; moreover, BV+VVC is the most prevalent form (Qi et al., 2021). During the past decade, the overall prevalence of mixed vaginitis in different regions of China varied greatly, fluctuating within a range from 7.33% to 41.87%, with a prevalence for the most common BV+VVC reaching 20.95% to 74.89% (Zhang and Liu, 2020). Regardless of the regional differences in feminine hygiene and the lack of global epidemiological studies, mixed vaginitis seems to have become much more common.

Even after considering mixed vaginitis as a separate vaginal infectious disease, many relevant facts remain unknown, especially the corresponding vaginal microbiome. The vaginal microbiome is characteristic of specific vaginitis types and determines both pathogenic and therapeutic outcomes; for instance, BV is a polymicrobial disorder of the vaginal microbiome that is characterized by the absence of vaginal lactobacilli (Paavonen and Brunham, 2018) and VVC by a *Candida* dominant vaginal microbiome (Kalia et al., 2020). Whether the vaginal microbiome of BV+VVC mixed vaginitis consists of a simple overlay microbiome or presents unique characteristics is unclear. For this study, we enrolled patients with BV+VVC mixed vaginitis and described their vaginal microbiome before and after treatment with oral metronidazole and local vaginal clotrimazole to provide evidence for improving the cure rate and reducing recurrences (van Schalkwyk and Yudin, 2015; Cooperative Group of Infectious Disease, 2021b; Workowski et al., 2021).

MATERIALS AND METHODS

Study Design and Study Population

We selected record data from 48 18-to-50-year-old patients clinically diagnosed as having VVC complicated with BV, who were admitted to The First Hospital of Peking University from January 2017 to December 2020. BV was diagnosed using Amsel's diagnostic criteria and identification of a Nugent score ≥7 from a vaginal Gram stain. Referring to the modified Amsel's diagnostic criteria, BV was diagnosed when three of the following were present: a thin homogeneous discharge, elevated vaginal pH above 4.5, release of amines on the addition of 10% potassium hydroxide to vaginal fluid, and the presence of "clue" cells. VVC was diagnosed by identification of budding yeasts, hyphae, or pseudo-hyphae in a wet preparation (saline, 10% KOH) of vaginal discharge, or by identification of a culture or Gram staining yielding a positive result for a yeast species (Kalia et al., 2015; Xiao et al., 2016; Workowski et al., 2021). Two experienced microscopists blinded to the patients' clinical information scored all the vaginal smears independently. The exclusion criteria included patients younger than 18 years or older than 50, *Trichomonas vaginalis* or AV/DIV, cervicitis or pelvic inflammation caused by *Neisseria gonorrhoeae* or *Chlamydia trachomatis*, pregnancy or lactation, menopause, innate or acquired immunodeficiency, and use of corticosteroid or antibiotics during the prior month.

No international medical standards for mixed vaginitis have been developed yet due to the lack of adequate studies. Referring to the latest version of *Sexually Transmitted Infections Treatment Guidelines, 2021* and the *Expert Consensus on Diagnosis and Treatment of Mixed vaginitis*, we adopted oral metronidazole combined with local clotrimazole to treat the BV+VVC mixed vaginitis (Cooperative Group of Infectious Disease, 2021a; Workowski et al., 2021). All patients were treated with metronidazole (400 mg orally twice per day for 7 days) combined with clotrimazole (500 mg intravaginally once per day on days 1, 4, and 7). We obtained informed consents from patients to perform follow-ups 7 ± 3 days after treatment termination; in addition, we also performed secondary follow-ups of patients with incomplete remission of symptoms after their second treatment. The follow-up observation indicators included a general examination and detailed anamnesis, a routine gynecological examination, a routine examination of discharge, vaginal discharge pH determination, Nugent scoring, and microecological detection of vaginal discharge.

Vaginal samples were collected before and after the drug treatment as well as at the time of follow-up. Two vaginal swabs were placed into the vagina at a standard anatomical site (one third of the lateral vaginal wall) and rubbed against the vaginal wall at each visit, one for microecological detection of vaginal secretions and the other for subsequent vaginal microbiota analysis. At every visit, a specimen was taken from each patient for a vaginal fungal culture and drug sensitivity test. To evaluate drug efficacy, we used fungal microscopic examinations for VVC (cured patients had negative results while the uncured ones had positive results) and Amsel's diagnostic criteria combining Nugent scores for BV (scores between 0 and 3 identified cured patients, scores between 4 and 6 identified those with intermediate BV, scores between 7 and 10 identified uncured patients).

16S rRNA Gene Sequencing

Total genomic DNA was extracted from samples using the SDS method (Lim et al., 2016). 16S rRNA genes (V3-V4) were

amplified using specific primers with a barcode. PCR products were mixed at equal ratios. Next, the PCR products mix was purified using a Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using a TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations, and index codes were added. The library was sequenced on an Illumina NovaSeq using 2 x 250 base paired-end technology on an SP flow cell. This process generated an average of 93,000 reads per sample. Paired-end reads were merged using FLASH (VI.2.7, http://ccb.jhu.edu/software/FLASH/) (Magoč and Salzberg, 2011), and the splicing sequences were called raw tags. High quality tags were obtained (Bokulich et al., 2013) in a quality controlled process of QIIME (V1.9.1, http://qiime.org/scripts/splitlibrariesfastq.html) (Caporaso et al., 2010). The tags were compared with the reference database(Silva database, https://www.arb-silva.de/) using UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html) (Edgar et al., 2011) to detect chimera sequences, and then the chimera sequences were removed (Haas et al., 2011). Then the Effective Tags finally obtained.

UHPLC-MS/MS Analysis

UHPLC-MS/MS analyses were performed using a Vanquish UHPLC system (ThermoFisher, Germany) coupled with an Orbitrap Q ExactiveTM HF mass spectrometer (Thermo Fisher, Germany). Samples were injected onto a Hypesil Goldcolumn (100×2.1 mm, 1.9 μm) using a 17-min linear gradient at a flow rate of 0.2 mL/min. The Orbitrap Q ExactiveTM HF mass spectrometer was operated in positive/negative polarity modes. The raw data files generated by the UHPLC-MS/MS were processed using Compound Discoverer 3.1 (CD3.1, Thermo Fisher) to perform peak alignment, peak picking, and quantitation for each metabolite. Statistical analyses were performed using the statistical software R (R version R-3.4.3), Python (Python 2.7.6 version) and CentOS (CentOS release 6.6). We attempted normal transformations using an area normalization method for non-normally distributed data.

Metabolite Analysis

The samples were placed into EP tubes and resuspended with pre-chilled 80% methanol using a vortex. Next, the samples were incubated on ice for 5 min and centrifuged at 15,000 *g* (4°C for 20 min). The supernatants were diluted to a final concentration containing 53% methanol by LC-MS grade water. The samples were subsequently transferred to fresh Eppendorf tubes and were then centrifuged at 15,000 *g*, 4°C for 20 min. Finally, the supernatants were injected into the LC-MS/MS system analysis (Want et al., 2006; Barri and Dragsted, 2013).

Metabolites were annotated using the KEGG (https://www.genome.jp/kegg/pathway.html), HMDB (https://hmdb.ca/metabolites) and LIPIDMaps (http://www.lipidmaps.org/) databases. Principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed with metaX (Wen et al., 2017) (a flexible and comprehensive software for processing metabolomics data). Volcano plots were used to filter metabolites of interest based on $\log_2$ (FoldChange), a $\log_{10}$ (*P*-value) of metabolites by ggplot2 in R language. For clustering heat maps, the data were normalized using z-scores of the intensity areas of differential metabolites and were plotted using a Pheatmap package in R language.

Statistical Analysis

SPSS19.0 software was used for statistical analyses. We analyzed changes in vaginal pH and Nugent scores using *t* tests, the improvement of main symptoms and efficacy were analyzed by χ^2 and *U* tests (all conducted using bilateral tests). In addition to the main efficacy indicators, pairwise comparisons were not carried out between groups for other indicators, and $P<0.05$ indicated significant differences between groups. The description of quantitative indicators is a calculation of the mean, standard deviation, median, minimum, and maximum. Classification indicators and grade indicators are described in terms of the number and percentage of each category.

For community clustering analyses, the clustering of communities was based on community composition and abundance using complete linkage hierarchical clustering with four clusters using R (version 4.0.0) (Zhou et al., 2007). The PCoA and heatmap of main genus abundance figures of vaginal microbial communities were generated using the ade4 and ComplexHeatmap package in R (version 4.0.0). For alpha diversity analyses, the Shannon index at the operational taxonomic units (OTU) level was calculated with QIIME (Version 1.9.1). We used a Wilcoxon rank sum test for comparing Shannon index differences between groups. Marker vaginal bacteria analyses were performed using a differential abundance of bacteria by LDA Effect Size (Segata et al., 2011), and we used a *t*-test to compare bacteria abundances between groups. Only genera with LDA scores >3 and $p<0.05$ were regarded as significant. Statistical analyses were performed using R (version 4.0.0).

For marker metabolite analyses, we applied univariate analyses (*t*-tests) to calculate the statistical significance (*P*-value) between groups. The metabolites with VIP > 1 and *P*-values < 0.01 and $\log_2$ fold changes ≥1 or ≤ -1 were considered to be differential metabolites. Volcano plots were used to filter metabolites of interest based on $\log_2$ (FoldChange) and $\log_{10}$ (*P*-value) of metabolites by ggplot2 in R (version 4.0.0). Genera–metabolome correlations were obtained by computing pairwise correlation coefficients using Spearman's correlation coefficients between marker genera and marker metabolites for each group. We focused on genera–metabolite pairs for which the correlation coefficients were greater than or equal to 0.4 (Benjamin–Hochberg adjusted p <0.05).

RESULTS

Grouping Information and Clinical Characteristics of the Study Population

We enrolled 48 patients in this study. We obtained data from all patients before and after treatment and at follow-ups, 39 patients did not undergo recurrences (groups M1–M4 in **Table 1**) but 9 patients experienced recurrences (group MR in **Table 2**).

TABLE 1 | Grouping information of the study population without recurrences.

Group	VVC	BV	Total population (*n*)
M1	Cured	Cured	11
M2	Cured	Not cured	16
M3	Not cured	Not cured	9
M4	Not cured	Cured	3

Recurrence in our study was defined as that either of BV or VVC relapsed after drug treatment, while merely the recurrence of VVC was observed in our study.

The mean age of the 48 patients was 32.92 ± 6.17 years; 38 patients had had previous VVC episodes (24 with a history of RVVC) and 13 patients had had previous BV episodes, while 9 patients had both. We observed that the symptoms and signs of patients with BV+VVC decreased significantly after treatment, indicating that this treatment plan can effectively relieve symptoms. The abundance of *Lactobacillus* and dominant bacteria in vaginal samples changed with the remission of symptoms. The vaginal pHs were lower than 4.5 after treatment (and significantly lower than the pHs before treatment).

Analysis and Comparison of Microecology Between Groups Before and After Treatment

We analyzed the scores of clinical symptoms and signs before treatment, during follow-up and recurrence, as well as the results of microscopic microecological evaluations, and we found similar indicators before treatment and Nugent score decreases after treatment. Overall, the BV cure rate was only 47.9% (23/48) while that of VVC reached 56.3% (27/48), this significant difference ($p<0.05$) indicates that this treatment may not be sufficient against bacterial vaginosis. Further analysis of the BV treatment outcomes showed that Nugent scores increased in 4 individuals, remained unchanged in 9, and decreased in 35 individuals after treatment, suggesting that 72.9% of patients with BV presented improvement after treatment (**Supplementary Tables 1–3**).

All vaginal fungal cultures were *Candida albicans* sensitive to clotrimazole. The microecological indicators of the patients in the MR group after relapse did not change significantly compared to those after treatment, therefore, we could not identify the microecological changes that promoted fungal relapses at a microscopic level.

Drug Treatment Affected the Composition of the Vaginal Microbiome

We classified the vaginal microbiome of all patients into clusters based on correlations between the members of their microbiome at the species level. As shown in **Figure 1A**, we divided each individual vaginal microbiome into four clusters with significant differences in their composition based on the species abundances at the species level. The heat map in **Figure 1B** shows the distribution of the horizontal abundance of each species in the four main clusters converted to $\log_{10}$. **Figure 1C** shows the bacterial Shannon diversity index distribution in different clusters. Among the four clusters, the highest diversity index was found in cluster 3 (**Figure 1C**, Cluster3), which was mainly enriched in *Prevotella* (**Figure 1B**, Cluster3). The Shannon diversity index of Cluster 1 was the lowest (**Figure 1C**, Cluster1), resulting in no enrichment of any single dominant genus (**Figure 1B**, Cluster1). By contrast, the Shannon diversity index of clusters 2 and 4 were higher than that of cluster 1 (**Figure 1C**, Cluster2, Cluster4), and both clusters were dominated by *Lactobacillus* microorganisms. Cluster 2 was mainly enriched in *Lactobacillus iners* (**Figure 1B**, Cluster2), while Cluster 4 was enriched in *Lactobacillus crispatus* (**Figure 1B**, Cluster4).

Figure 2A shows the vaginal microbiota distribution changes in different groups of patients after drug treatment. M1, M2 and M3 groups at different status were respectively represented as M1.1, M2.1, M3.1 (before treatment) and M1.2, M2.2, M3.2 (after treatment). MR group at different status were represented as MR.1 (before treatment), MR.2 (after treatment) and MR.3 (after recurrence). The microbiomes in patients of the M1 group were dominated by the genus Cluster2 and Cluster3 before treatment, while Cluster3 predominated in other groups of patients before the drug treatment (M2.1, M3.1, and MR.1). However, the vaginal microbiota changed significantly after the drug treatment and showed significant decreases in the proportions of Cluster3 in patient groups M1.2, M2.2, M3.2, and MR.2. In addition, the proportion of Cluster2 increased in patients of the M3 group after treatment (M3.2). By contrast, in the case of the patients of the relapse group (MR.1, MR.2, and MR.3), Cluster3 was predominant before treatment (MR. 1) and its proportion decreased or disappeared after treatment, while the proportions of Cluster2 and Cluster4 (dominated by *Lactobacillus*) increased significantly. However, after the relapse (MR.3), the proportion of Cluster 3 increased and the proportion of Cluster 2 decreased significantly.

Figure 2B shows the variation in vaginal microbial abundance distributions at the genus level among all groups of patients. We found significant vaginal microbial composition imbalances before drug treatment in all subgroups (M1.1, M2.1, M3.1, and MR.1) with low levels of *Lactobacillus* and high levels of BV-associated microorganisms, such as *Atopobium*, *Prevotella*, and *Gardnerella*. The vaginal microbiome genus distributions changed significantly after drug treatment. The abundance of *Lactobacillus* increased significantly while that of BV-related *Atopobium* and *Prevotella* decreased in all subjects after drug treatment. However, the abundance of *Gardnerella* decreased only in the M1 group (M1.2) and did not improve significantly in the other groups; in fact the abundance of *Gardnerella* increased in the M2 group (M2.1, M2.2).

TABLE 2 | Specific information of the study population with recurrences.

Group	VVC	BV	Population (*n*)	Total population (*n*)
MR	Recurrent	Cured	6	9
		Not cured	3	

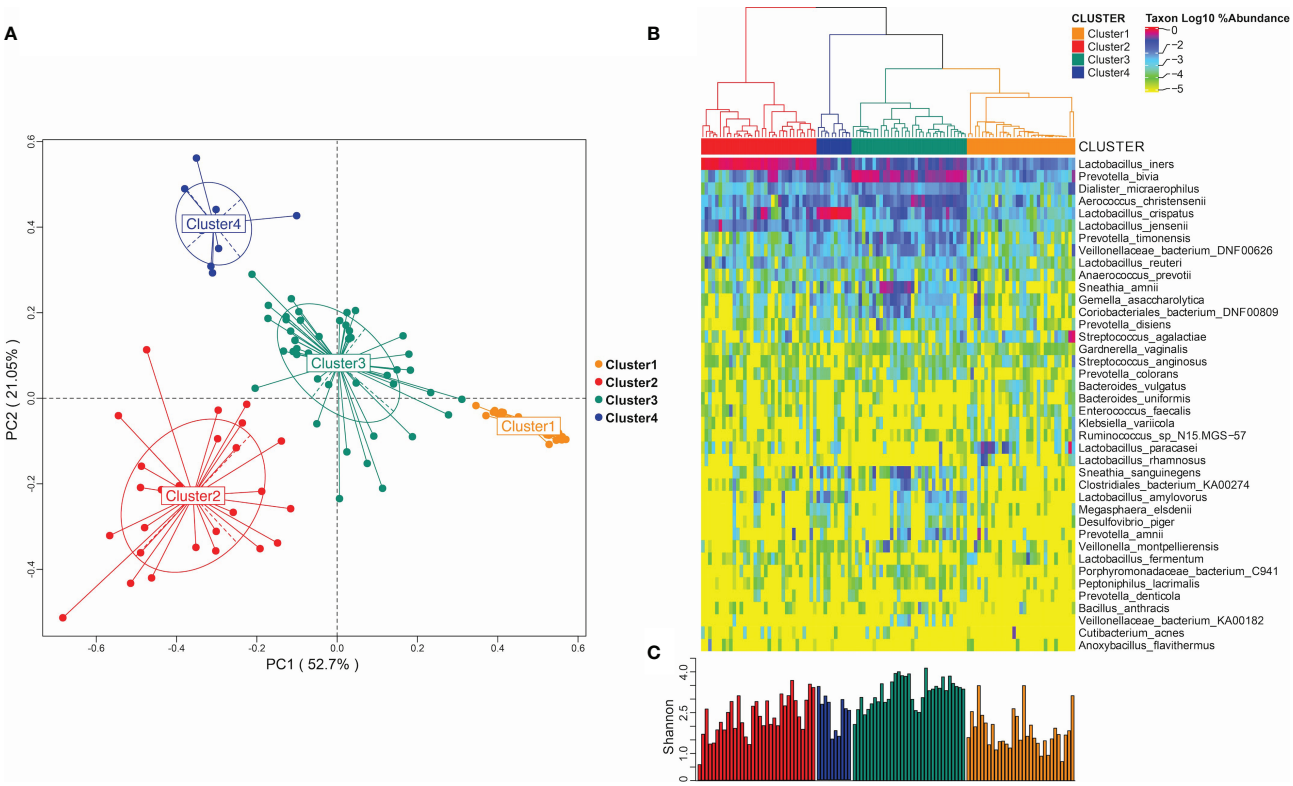

FIGURE 1 | Microbiome of vaginal bacterial community. **(A)** Microbiome distribution obtained by PCoA clustering of the vaginal microbiota of all subjects; **(B)** heat maps of vaginal microbiome abundance distribution and the distribution of microbial community obtained by hierarchical clustering. **(C)** Shannon diversity index distribution in different microbial groups.

Drug Therapy Changes the Vaginal Microbiome Diversity

We conducted Shannon diversity analyses based on the distribution of at the vaginal species OTU for all patients, **Figure 2** shows the Shannon diversity index changes for different patient groups before and after drug treatment. The Shannon diversity results of all patients differed before and after drug treatment, and were mainly manifested by a decreased diversity of vaginal bacteria after drug treatment; in addition, we found a statistically significant difference between the results of the M2 group (**Figure 2C**, M2.1 *vs.* M2.2, p=0.0148) and those of the MR group (**Figure 2C**; MR.1 *vs.* MR.2; p=0.0116). By contrast, we found differences in the vaginal microbiota diversity in different groups of patients on the basis of their treatment outcomes. As shown in **Figure 2C**, the diversities of M1 group patients (M1.1 and M1.2) were higher than that of the M2 group patients (M2.1 and M2.2) before and after treatment. **Figure 2C** shows that the diversity of the M1 group patients (M1.1 and M1.2) was lower than that of the M3 group patients (M3.1 and M3.2) before and after treatment, but the difference was not statistically significant; in addition, the diversities of M3 group patients before and after treatment were similar (M3.1 *vs.* M3.2, p=0.3045). As shown in **Figure 2C**, the bacterial diversities of the MR group patients decreased significantly after treatment (MR.1 *vs.* MR.2, p=0.0116), but increased after relapse, and we found no significant differences when compared to those before treatment (MR.1 *vs.* MR.3, p=0.0803).

Key Vaginal Microorganism Changes Associated With the Effect of Drug Therapy

We used linear discriminant analysis (LDA) and *t*-tests to analyze the changes in key vaginal microorganisms on the basis of different treatment outcomes and on the microbial abundance distributions at the genus and species levels for the patients' groups (**Figure 3**).

As shown in **Figure 3A**, *Prevotella* and *Gardnerella* were predominant in M1.1 group patients before treatment, while *Lactobacillus* was predominant after treatment in M1.2 group patients. *Prevotella* and *Atopobium* were predominant in M2.1 group patients before treatment, while the *Prevotella* abundance decreased significantly after treatment in M2.2 group patients, but that of *Gardnerella* increased. Compared with the abundance in M2.1 group patients, the abundance of *Lactobacillus* was higher in M1.1 patients, suggesting that the failure to cure the BV may have been associated with the low baseline abundance of *Lactobacillus*. In addition, the abundance of *Lactobacillus*,

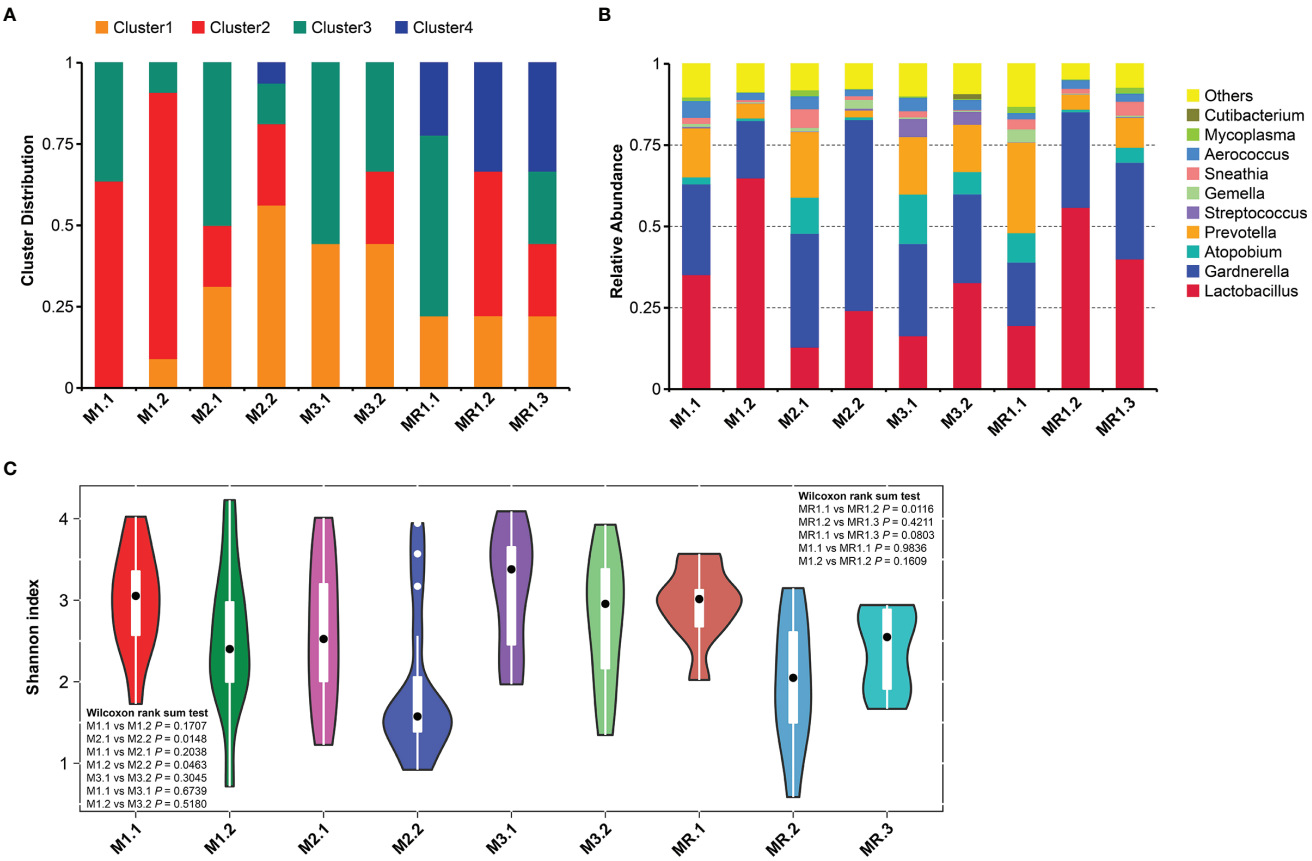

FIGURE 2 | Changes in bacterial vaginal compositions in different patient groups. **(A)** Microbiota distributions before and after drug treatment. **(B)** Genus abundances before and after drug treatment. **(C)** Vaginal microbiome Shannon diversity indexes before and after drug treatment.

especially those of *Lactobacillus iners*, *Lactobacillus jensenii* and *Lactobacillus crispatus*, was higher in M1.2 group patients than in M2.2 group patients with predominant *Gardnerella*. Moreover, the high abundance of *Gardnerella* probably correlates with the failure to cure BV.

As shown in **Figure 3B**, we found similar microbiome compositions in M3 group patients before and after treatment (M3.2 *vs*. M3.1), suggesting that the drug treatment failed to significantly change the vaginal microbiome of the patients in this group. The abundance of *Lactobacillus*, especially that of *Lactobacillus iners*, was lower in M3 group patients (M3.1 and M3.2) than in M1 group patients (M1.1 and M1.2) before and after treatment, suggesting a possible association between the treatment failure and a low *Lactobacillus* abundance. In addition, the abundance of *Atopobium* was high in M3 group patients before treatment (M3.1).

As shown in **Figure 3C**, in the recurrence group, the abundances of *Prevotella* and *Atopobium* were significantly decreased after treatment (MR.2) compared with those before treatment (MR.1); however, after the recurrence (MR.3), the abundance of *Atopobium* increased to a level similar to that before treatment (MR.1). By contrast, patients in the MR group (MR.1, MR.2 and MR.3) had lower *Lactobacillus* abundances (especially lower *Lactobacillus iners* and *Lactobacillus jensenii* abundances) and a higher *Atopobium* abundance than patients in the M1 group (M1.1 and M1.2) before and after treatment.

Correlation Between Metabolites and Key Vaginal Microbiota Associated With the Therapy Outcomes

The key bacteria found to be associated with the treatment outcomes included *Lactobacillus*, *Atopobium*, *Prevotella* and *Gardnerella*. In addition, we identified metabolites that were differentially expressed in vaginal discharge of different patients, including 2-hydroxyvaleric acid, tyramine, acetophenone, styrene, LysoPE, oleoyl ethanolamide, 16–hydroxyhexadecanoic acid, N–acetylmethionine, propionyl–L –carnitine, glycerol 1–hexadecanoate, α -linolenoyl ethanolamide, 4 –hydroxybutyric acid (GHB), sedanolide, and thymidine (**Supplementary Figures 1–5**). We conducted correlation analyses to assess the potential association between key vaginal bacteria (*Lactobacillus*, *Atopobium*, *Prevotella* and *Gardnerella*) associated with the treatment outcomes and the differential metabolites in vaginal secretions (**Figure 4**).

As shown in **Figure 4A**, in the patients completely cured (M1.1 and M1.2) and in those with cured VVC and untreated BV (M2.1 and M2.2), the association of differential metabolites with

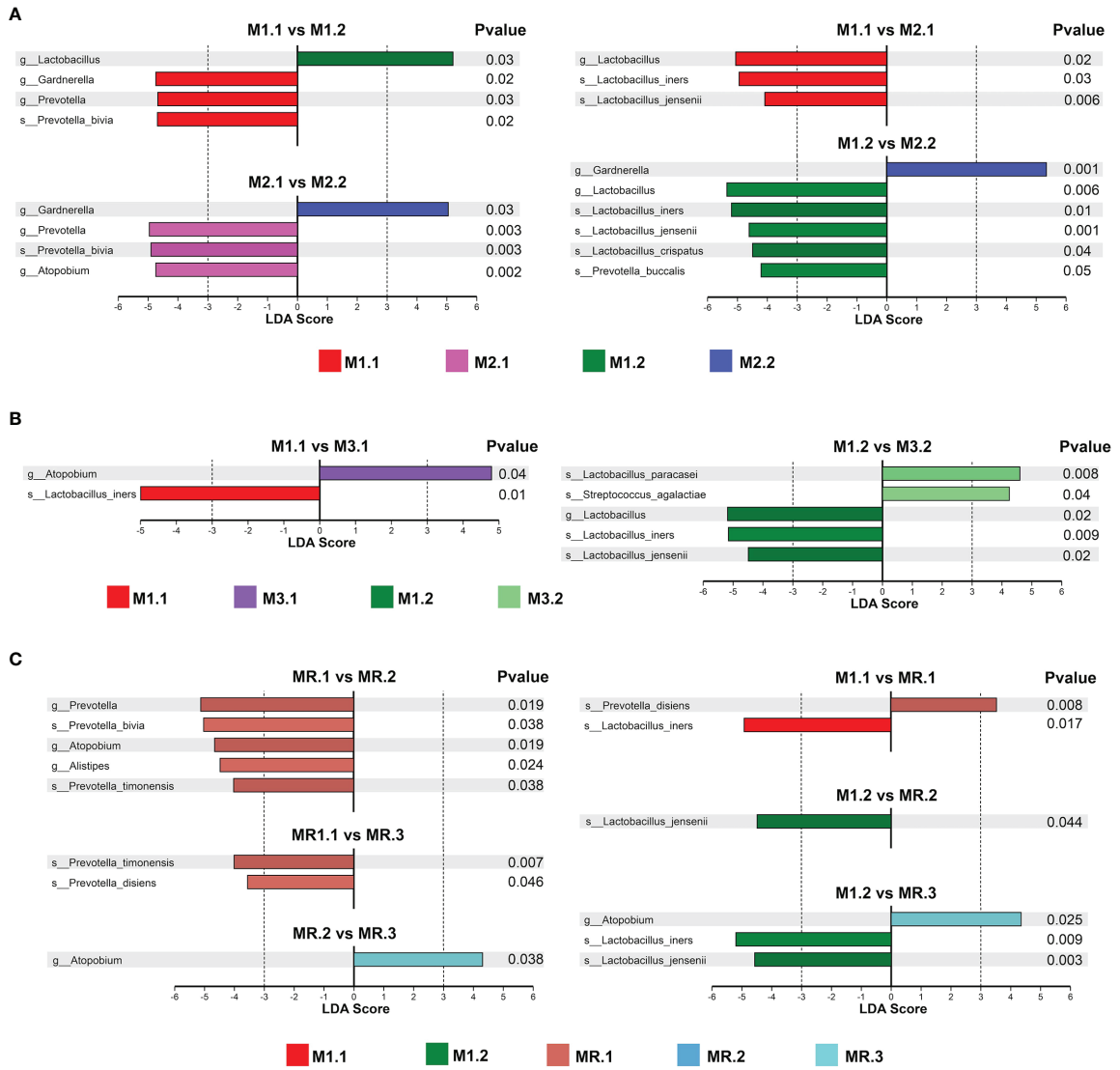

FIGURE 3 | Key vaginal microorganism changes before and after treatment. LDA and T-test identified the most differentially abundant taxons between **(A)** patients completely cured (M1.1, M1.2) and patients with cured VVC plus uncured BV (M2.1, M2.2), **(B)** patients completely cured(M1.1, M1.2) and patients with cured BV plus uncured VVC (M3.1, M3.2), **(C)** patients completely cured (M1.1, M1.2) and patients with recurrence (MR.1, MR.2, MR.3).

the presence of key vaginal microorganisms showed that metabolites including tyramine, acetophenone, styrene, LysoPE, oleoyl ethanolamide, 16– hydroxyhexanoic acid, 2 –hydroxyvaleric acid, thymidine, glycerol 1–hexadecanoate, N–acetylaspartic acid, and N–acetylmethionine were positively correlated with the presence of BV-related microorganisms including *Atopobium*, *Prevotella* and *Gardnerella*. In addition, 2–hydroxyvaleric acid, α-linolenoyl ethanolamide, 4–hydroxybutyric acid (GHB), sedanolide, thymidine, and N–acetylmethionine were negatively correlated with the presence of *Lactobacillus*. Moreover, 2-hydroxyvaleric acid was not only positively correlated with the presence of *Prevotella* but also negatively correlated with *Lactobacillus* and *Lactobacillus iners*. By contrast, we found amine metabolites, benzoones and acids such as tyramine, acetophenone, 16–hydroxyhexadecanoic acid, and 2–hydroxyvaleric acid to be positively correlated with the presence of *Prevotella*. Thymidine and N–acetylmethionine were also positively correlated with the presence of *Gardnerella* and negatively correlated with the presence of *Lactobacillus*, especially of *Lactobacillus crispatus*. *Gardnerella* was the predominant microorganism after treatment in the patients with cured VVC and untreated BV.

In completely cured patients (M1.1 and M1.2) and in uncured patients (M3.1, M3.2), the association analysis of differential metabolites with key vaginal microorganisms (**Figure 4B**) showed that 2–hydroxyvaleric acid was positively correlated with the presence of *Prevotella* and negatively correlated with the presence of *Lactobacillus* and *Lactobacillus iners*, further

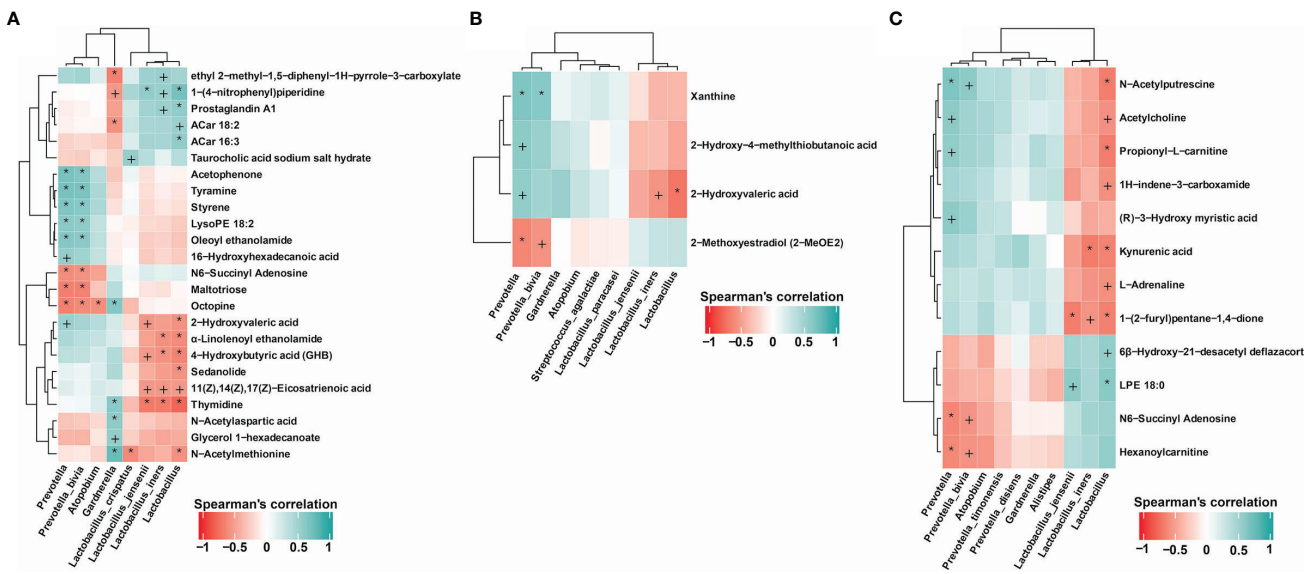

FIGURE 4 | Correlation between metabolites and key vaginal microbiota associated with the drug therapy outcomes. **(A)** Association of key vaginal microorganisms and metabolites in patients completely cured (M1.1, M1.2) and in patients with cured VVC and untreated BV (M2.1, M2.2). **(B)** Associations between key vaginal microorganisms and metabolites in completely cured patients (M1.1, M1.2) and in uncured patients (M3.1, M3.2). **(C)** Association of key vaginal microorganisms and metabolites in completely cured patients (M1.1, M1.2) and in patients with recurrence (MR.1, MR.2, MR.3). P value of Spearman's correlation: $^{+}$<0.05, *<0.01.

illustrating the important role of 2−hydroxyvaleric acid in the treatment of BV and VVC. By contrast, in completely cured patients (M1.1 and M1.2) and in patients with recurrences (MR.1, MR.2, and MR.3), our association analysis of differential metabolites and differential species revealed positive correlations between N−acetylputrescine, acetylcholine, and propionyl−L−carnitine and the presence of *Prevotella* and negative correlations with the presence of *Lactobacillus* (**Figure 4C**).

DISCUSSION

For this study, we investigated the composition, diversity and other characteristics of the vaginal microbiome in patients with BV+VVC mixed vaginitis before and after drug treatment. As mentioned, mixed vaginitis and vaginal co-infections are different entities. Based on the literature and our own results, patients presenting BV and VVC simultaneously have their own characteristic symptoms and signs of vaginitis rather than a simple overlay microbiome, *Lactobacillus iners* and *Prevotella* predominate in their vaginal microbiome and the abundances of *Gardnerella* and *Atopobium* are also higher than those in healthy women. Previous studies have focused on single types of vaginitis or in asymptomatic co-infections with multiple pathogenic micro-organisms. For example, in 2005, Fredricks et al. (Fredricks et al., 2005) found that compared with the vaginal microbiome of healthy women, that of women with BV had a significant increase in its diversity, with low abundance of *Lactobacillus crispatus* (significantly decreased to almost inexistent) but detection of *Lactobacillus iners* in most patients. In addition, the abundances of *Gardnerella*, *Atopobium*, *Megasphaera*, *Leptotrichia*, and BVAB 1-3 were significantly increased and correlated with the presence of BV (p <0.001). McKloud et al. (McKloud et al., 2021) found that the vaginal microbiome of women with recurrent VVC was dominated by bacteria of the genus *Lactobacillus* with a significant increase in the *Candida* abundance compared with the abundance in healthy women. The levels of bacterial diversity and the abundances of vaginal anaerobic bacteria such as *Gardnerella* and *Prevotella* were similar, but slightly higher in healthy women. The abundances of *Lactobacillus crispatus* and *Lactobacillus jensenii* dropped from 44% in healthy women to 30% in women with RVVC, while the abundance of *Lactobacillus iners* increased from 19% in healthy women to 40% in women with RVVC. The abundances of *Lactobacillus iners* in the two groups were significantly different.

On the basis of the high abundances of BV-related pathogens in the treatment failure and recurrence groups in this study, we believe that BV-related pathogens play a major pathogenic role in patients with BV+VVC mixed vaginitis and determine their treatment outcome and the occurrence of relapses. We found that *Gardnerella* abundance increased in the VVC cured but BV uncured group after treatment (M2.2); patients in the MR recurrence group had lower *Lactobacillus* abundances (especially *Lactobacillus iners* and *Lactobacillus jensenii*) and a higher *Prevotella and Atopobium* abundance than patients in the M1 group before and after treatment. *Gardnerella*, *Prevotella*, and *Atopobium* were the main pathogenic bacteria of BV in our study, and other studies have identified synergistic effects between them, allowing them to form multi-strain biofilms and to further lead to refractory infections and recurrences. Moreover, each bacterium plays a different role during

the infections. *Prevotella* can secrete proteases to degrade host antibodies and transfer ammonia to *Gardnerella*, leading to excessive secretion of ammonia in the host vagina, reducing mucosal immunity (Xie et al., 2020), and promoting the growth of other anaerobic bacteria. However, in the course of our limited follow-up, we found that the abundance of *Prevotella* decreased significantly after metronidazole treatment and did not increase again, a finding consistent with the reported high drug sensitivity of *Prevotella* to metronidazole (Ulger Toprak et al., 2018). Thus, we believe that *Prevotella* probably did not play a significant role in recurrences. *Gardnerella*, the main bacteria in the formation of BV biofilms, can adhere to vaginal epithelial cells and acts as a scaffold for the formation of biofilms. Dense biofilms can tolerate high concentrations of H_2O_2 and lactic acid, and have enhanced resistance to antibiotics and host mucosal immune defenses (Hardy et al., 2017). The presence of this specific type of biofilm can lead to resistance of *Gardnerella* to metronidazole, causing BV treatment difficulties and recurrences (He et al., 2021). In the BV pathogenesis model (Muzny et al., 2020), *Gardnerella* and *Prevotella*, as initially colonizing bacteria, are highly abundant in the vagina of patients with BV, but the formation of a biofilm does not induce a strong inflammatory response in vaginal epithelial cells and instead promotes immune escape. The secondary colonizer *Atopobium* is more likely to effectively stimulate the host to produce a strong immune response against the BV, leading to the corresponding signs and symptoms. A synergistic effect may exist between the two types of colonizers (Castro et al., 2019; Vestby et al., 2020). In addition, previous studies have found that the sensitivity of different clinical isolates of *Atopobium* to metronidazole varies greatly and more than half of the isolates display drug resistance to metronidazole (Schuyler et al., 2015; Shaskolskiy et al., 2016; Mendling et al., 2019), which suggests that targeted drugs with high sensitivity to *Atopobium* are needed to reduce recurrences during the treatment of patients with BV+VVC mixed vaginitis.

Although multi-species biofilms play a role in recurrences, the type and abundance of *Lactobacillus* also had a considerable influence on infection and recurrences (Ceccarani et al., 2019; Redelinghuys et al., 2020). The results of this study showed that before treatment the completely cured patients (M1.1) had a higher abundance of *Lactobacillus iners* than the patients with recurrences (MR.1), whereas the significantly higher abundance of *Lactobacillus jensenii* was established earlier in the completely cured patients after treatment (M1.2) than in the patients with recurrences (MR.2), suggesting that the abundance of *Lactobacillus iners* and *Lactobacillus jensenii* correlate with the recurrence of BV+VVC mixed vaginitis after a cure. On the basis of existing findings and our results, we can speculate that the absence of *Lactobacillus crispatus* and other protective *Lactobacillus* from the vaginal microenvironment of patients with mixed vaginitis, *Lactobacillus iners* may tend to act as the protector of the vaginal epithelium. Verwijs et al. (Verwijs et al., 2020) assessed the vaginal microbiota of 68 women with BV who received metronidazole treatment and found results similar to ours: *Lactobacillus iners* was the most common *Lactobacillus* species before and after treatment, the abundance of *L. jensenii* was also significantly increased after treatment, and that of *Lactobacillus crispatus* was significantly decreased before treatment and only slightly increased after treatment. After analyzing the vaginal microbiota of patients treated with metronidazole gel, *Lactobacillus iners* was the only dominant strain 30 days after treatment, indicating that *Lactobacillus iners* was resistant to metronidazole, while *Lactobacillus crispatus* was probably more susceptible to metronidazole in the BV environment (Petrova et al., 2017). Moreover, due to the ineffectiveness of metronidazole against *Lactobacillus iners*, the removal of BV-related pathogens with therapy results in a vaginal microbiome with predominant *Lactobacillus iners*. Therefore, combining with our results, assessing the level of *Lactobacillus iners* in the vagina of patients with mixed vaginitis before treatment may have a predictive value for the treatment outcome and, promoting the recovery of the *Lactobacillus* abundance in the vagina may help to enhance the treatment effects beyond antibiotic treatment.

This study was limited mainly by the size of the patient cohort, and larger prospective studies are needed to confirm our results. This is our first study to explore the vaginal microbiome of the patients with BV+VVC mixed vaginitis, so we merely measured the relative abundances. Apart from this, all our fungal culture results showed *Candida albicans*, preventing us from studying more on various vaginal fungus. In addition, the final cure rates of BV and VVC in this study were 47.92% (23/48) and 56.25% (27/48), respectively, revealing a better therapeutic effect for VVC than for BV. The cure rates in our study were low probably owing to our therapy targeting VVC with more potency than BV, future studies should apply longer periods of BV-targeted treatments.

In conclusion, we observed significant vaginal microbiome alterations in patients with BV+VVC mixed vaginitis before and after drug treatment, with the key causative bacteria including *Gardnerella*, *Atopobium*, and *Lactobacillus*. We have found some special bacterial in this study and we will consider proceeding with further quantitative research in the subsequent studies. The therapy of oral metronidazole with local clotrimazole to treat BV+VVC mixed vaginitis was effective in our study. And to enhance treatment for BV may be more favorable for prognosis of patients with BV+VVC mixed vaginitis. The abundance of *Lactobacillus* in women with mixed vaginitis has a great influence on the recovery of a normal vaginal microbiome and on the prognosis, and it should be actively restored.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI SRA database, accession PRJNA801439 and EMBL-EBI MetaboLights database, accession MTBLS4222.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of Peking University First Hospital (V2.0/201504.20). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

BX and DZ conceived the study design. BX, LM, and DZ recruited volunteers and collected samples. LM and DZ were responsible for performing the laboratory assays. BX, DA, and HQ performed the data analysis. DA wrote the initial manuscript. BX and DZ revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the grants of the National Natural Science Foundation of China (No. 81971342 and No. 81200411).

ACKNOWLEDGMENTS

We would like to thank all the participants for their support and cooperation. We would also like to thank all the researchers, clinicians, and technicians involved in our study for their participation and technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.860589/full#supplementary-material

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REVIEW
published: 25 March 2022
doi: 10.3389/fcimb.2022.858155

Research Progress on the Correlation Between *Gardnerella* Typing and Bacterial Vaginosis

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Bacterial vaginosis (BV) is the most common infectious disease of the reproductive tract in women of childbearing age. It often manifests as an imbalance in the vaginal microbiome, including a decrease in *Lactobacillus* and an increase in anaerobic bacteria. While *Gardnerella* spp. are considered a major cause of BV, they are also detected in the vaginal microbiome of healthy women. *G. vaginalis* was the only recognized species of *Gardnerella* until a recent study characterized three new species, *G. leopoldii*, *G. piotii*, and *G. swidsinskii*. This review describes the different types and genetic diversity of *Gardnerella*, as well as new findings on the correlation between different *Gardnerella* spp. and BV.

Keywords: *Gardnerella*, bacterial vaginosis, clinical outcomes, subtypes, qPCR, cpn-60, whole genome sequencing

OPEN ACCESS

Edited by:
António Machado, Universidad San Francisco de Quito, Ecuador

Reviewed by:
Alexander Swidsinski, Charité Universitätsmedizin Berlin, Germany
Daniela Marlene Da Silva Machado, Universidade Católica Portuguesa, Portugal

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Specialty section:
This article was submitted to Biofilms, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *19 January 2022*
Accepted: *01 March 2022*
Published: *25 March 2022*

Citation:
Qin H and Xiao B (2022) Research Progress on the Correlation Between Gardnerella Typing and Bacterial Vaginosis. Front. Cell. Infect. Microbiol. 12:858155. doi: 10.3389/fcimb.2022.858155

INTRODUCTION

Bacterial vaginosis (BV) is the most common lower genital tract infection affecting approximately 30% of women in the general population and 50% of African American women (Ravel et al., 2011; Kenyon et al., 2013; Morrill et al., 2020). Variations in prevalence exist among different races and ethnicities (Allsworth and Peipert, 2007). BV is associated with a disruption of the optimal vaginal microbiota characterized by a decreased proportion of lactic acid-producing bacteria and an increased proportion of a wide array of strict and facultative anaerobes (Ravel et al., 2021). Bacteria commonly associated with BV include *Gardnerella vaginalis* (*G. vaginalis*), *Megasphaera* spp., *Fannyhessea vaginae* [previously known as *Atopobium vaginae*, (Rodriguez Jovita et al., 1999; Nouioui et al., 2018)], *Dialister* spp., *Mobiluncus* spp., *Sneathia amnii*, *Sneathia sanguinegens*, *Porphyromonas* spp., and Prevotella spp (Muzny et al., 2018; Rosca et al., 2020). Although BV is frequently asymptomatic, women with BV are more likely to report vaginal odor, itching, and discharge than those without (Klebanoff et al., 2004). In addition, most women do not report BV symptoms to their providers, even when there are clinical signs (Masson et al., 2019). Serious adverse health outcomes have been associated with BV, including increased risk of infertility (Ravel et al., 2021), adverse pregnancy outcomes (Giakoumelou et al., 2016; Tabatabaei et al., 2019), pelvic inflammatory disease (PID) (Wiesenfeld et al., 2002; Ravel et al., 2021) and sexually transmitted

infections (STIs), including chlamydia (Shipitsyna et al., 2020), gonorrhea (Bautista et al., 2017), human papilloma virus (HPV) (Usyk et al., 2022) and human immunodeficiency virus (HIV) (Atashili et al., 2008).

BV etiology is controversial and not yet fully understood. According to Catlin'research, the G. vaginalis-associated vaginal syndrome was earlier called nonspecific vaginitis (NSV) in recognition of the absence of recognized agents of vaginitis (Catlin, 1992). In 1955, Gardner and Dukes showed that 90% of NSV cases were caused by a single microbe, *Haemophilus vaginalis* (*H. vaginalis*), and the name was changed to *H. vaginalis* vaginitis (Gardner and Dukes, 1955). However, subsequent studies found that because *H. vaginalis* does not require heme or nicotinamide adenine dinucleotide for growth, it may not be a member of the *Haemophilus* genus. *H. vaginalis* shows uncertainty in gram staining, also a characteristic of the *Corynebacteria*. As a result, *H. vaginalis* was reassigned to the *Corynebacterium* genus and renamed *Corynebacterium vaginale* (Zinnemann and Turner, 1963). This name also proved to be inaccurate because these bacteria are catalase-negative and do not have arabinose in their cell wall (Catlin, 1992). In 1980, two large taxonomic studies using DNA hybridization, electron microscopy, and various biochemical methods showed that the bacterium lacked close similarity to any previously established genus (Greenwood and Pickett, 1980; Piot et al., 1980). This resulted in the development of a new genus, *Gardnerella*, and *Corynebacterium* vaginitis was renamed *Gardnerella* vaginitis. Gardner believed that *Gardnerella* vaginitis was a specific vaginal infection with a clear cause and that most vaginitis previously classified as "nonspecific" was likely caused by *Gardnerella* spp. In 1984, the name was officially changed to "BV" because vaginitis is suggestive of an inflammatory response in the vaginal epithelium, which is usually absent in women with BV (Catlin, 1992). It wasn't until 1984 that BV was officially named (Workshop on Anaerobic Curved Rods and Bacterial Vaginosis, Stockholm, January, 1984) (Bump et al., 1984).

Since *Gardnerella* spp. is highly detected in BV, it appears to have a special role in vaginal microbiota dysbiosis (Reid, 2018). While *Gardnerella* spp. is found in 95% to 100% of BV cases (Muzny et al., 2019), colonization does not always lead to BV (Hickey and Forney, 2014; Machado et al., 2015). Indeed, the role of *Gardnerella* spp. in BV has remained controversial because it is present in both healthy vaginal microbiota and in BV (Zozaya-Hinchliffe et al., 2010). As a result, researchers have speculated about whether there are different *Gardnerella* spp. (Cornejo et al., 2018) with distinct pathogenicities that can lead to different clinical outcomes (Janulaitiene et al., 2018). It is possible that while healthy women are colonized by a less virulent strain of *Gardnerella* spp., other more virulent strains promote the development of BV. Studies have used several *Gardnerella* spp. typing tests to explore the clinical characteristics of different species. Indeed, the taxonomy of *Gardnerella* spp. will need to be more completely defined in order to fully understand the mechanism of *Gardnerella* spp. in BV pathogenesis (Castro et al., 2019). This study reviews recent literature to explore the characteristics of *Gardnerella* spp., the typing methods used, and their clinical significance.

CHARACTERISTICS OF *GARDNERELLA*

G. vaginalis was the first recognized *Gardnerella* species (Harwich et al., 2010), with its closest relatives in the Bifidobacterium genus (Harwich et al., 2010; Castro et al., 2019). The cells are small, nonmotile, nonencapsulated, non-spore-forming, pleomorphic rods with average dimensions of 0.4×1.0~1.5μm (Onderdonk et al., 2016). The cells are small, nonmotile, nonencapsulated, non-spore-forming, pleomorphic rods with average dimensions of 0.4×1.0~1.5μm (Sadhu et al., 1989). Known as a facultative anaerobic microorganism, *G. vaginalis* was also described as fastidious, as it grew better at 37°C in complex media in an atmosphere with 5–10% of carbon dioxide (CO2) or in a candleflame extinction jar (Catlin, 1992; Cereija et al., 2013). Nevertheless, it was demonstrated that certain *G. vaginalis* strains are strict anaerobes (Malone et al., 1975). The cellular surface of *G. vaginalis* is covered with fimbriae, which are responsible for the attachment of *G. vaginalis* to vaginal epithelial cells (Scott et al., 1989; Onderdonk et al., 2016). More recent biochemical testing has shown that *G. vaginalis* is catalase-, oxidase-, and b-glucosidase-negative (Catlin, 1992; Turovskiy et al., 2011). It can ferment starch, dextrin, sucrose, glucose, fructose, ribose, maltose and raffinose. Some strains can also ferment xylose and trehalose. Conversely, *G. vaginalis* is unable to ferment rhamnose, melibiose, mannitol and sorbitol (Harwich et al., 2010).

The important pathogenic characteristics of *Gardnerella* spp. include the production of sialidase, an enzyme that degrades cervical and vaginal mucus, and vaginolysin, a lysoid that induces the lysis of vaginal epithelial cells (Castro et al., 2019). Sialidase is associated with the degradation of key mucosal protective factors, such as mucin, that lead to the shedding of vaginal epithelial cells (Lewis et al., 2013; Hardy et al., 2017). *Gardnerella* spp. has three genes that encode sialidase, NanH1 (sialidase A gene), NanH2, and NanH3. NanH2 and NanH3 appear to be the main sources of sialidase activity in *Gardnerella* spp (Robinson et al., 2019). Vaginal hemolysin is a cholesterol-dependent cell hemolysin that was first discovered in the culture medium of *Gardnerella* spp. It has cytotoxic activity against human erythrocytes and can induce the dissolution of human erythrocytes, epithelial cells, and polymorphonuclear leukocytes (Zilnyte et al., 2015). Vaginal hemolysin and sialidase are the most widely studied virulence factors of *Gardnerella* spp. (Santiago et al., 2011; Janulaitiene et al., 2018), and are assumed to play a substantial role in the pathogenesis of BV (Pleckaityte et al., 2012). Swidsinski et al. used fluorescence *in situ* hybridization (FISH) specific for *Gardnerella* spp. and were the first to show that these species form biofilms on vaginal epithelium in women with BV. This finding revealed the nature of clue cells, epithelial cells covered by a *Gardnerella* spp. biofilm, and their etiological role in *Gardnerella* spp. infection (Swidsinski et al., 2005).

GARDNERELLA SPP. TYPING

Biotyping

Gardnerella spp. was first divided into eight biotypes based on its metabolic characteristics (Piot et al., 1984). In 1894, Piot et al. established a simple and repeatable method for *Gardnerella* spp. biotyping, known as Pilot typing, that is based on the reaction of lipase, hippurate hydrolysis, and β-galactosidase. Benito et al. identified 17 *Gardnerella* spp. biotypes based on these characteristics in addition to fermentation of xylose, arabinose, and galactose (Benito et al., 1986). This method is based on enzyme reactions, however, so there is a risk that the wrong biotypes will be produced when conditions change. Thus, biotyping is not recommended to understand the epidemic etiology of *Gardnerella* spp. (Ingianni et al., 1997).

Genotyping

Amplified Ribosomal DNA Restriction Analysis

Four genotypes of *Gardnerella* spp. were identified by amplified ribosomal DNA restriction analysis (ARDRA) using different restriction enzymes (Ingianni et al., 1997). In 1997, Ingianni et al. first used ARDRA to identify 34 *Gardnerella* spp. strains. This method is more accurate than biotyping but still relies on the isolation and purification of *Gardnerella* spp. Each subtype has a distinct growth and metabolic pattern, however, and preferentially isolated strains may misrepresent the clinical distribution of *Gardnerella* subtypes.

qPCR

Quantitative polymerase chain reaction (qPCR) typing can be used on uncultured clinical specimens, allowing for quantitative assessment of bacterial load and qualitative identification of *Gardnerella* subtypes (Balashov et al., 2014). Since the isolation of living bacterial cells is not involved, this approach can be performed on different types of samples, including frozen DNA or vaginal swab samples collected and stored under sub-optimal conditions. In 2012, Ahmed et al. conducted a comparative genomic analysis of 17 *Gardnerella* spp. clinical isolates and suggested that it would be more accurate to define *Gardnerella* spp. as four separate species (Ahmed et al., 2012). Balashov and his colleagues developed multiplex single-tube qPCR targeting genes encoding putative a-L-fucosidase (clade 1), a hypothetical protein (clade 2), thioredoxin (clade 3) and CIC family chloride transporter (clade 4), which was proven to be clade-specific and capable of strain typing and identification of the four *G. vaginalis* clades in noncultured clinical vaginal specimens (Balashov et al., 2014).

Cpn-60 Sequencing

Chaperonin-60 is a molecular chaperone required for the folding and assembly of proteins and protein complexes in all eubacteria and in the plastids and mitochondria of eukaryotes (Hill et al., 2005). A 549-567 bp fragment of the cpn-60 gene was amplified by universal PCR primers, and the sequencing results were relatively stable (Jayaprakash et al., 2012). *Gardnerella* spp. comprises four subgroups A, B, C, and D, based on cpn60 barcode sequences and whole-genome sequences (Jayaprakash et al., 2012; Schellenberg et al., 2016). Cpn60 sequencing was also applied to vaginal microbes in a larger sample of African commercial sex workers (Schellenberg et al., 2011). Profiling of vaginal microbiomes using cpn60 barcode sequencing, and application of clade-specific PCR showed that the vagina is often colonized by multiple *Gardnerella* spp. subgroups simultaneously (Khan et al., 2019).

Whole Genome Sequencing

Whole genome sequencing refers to the analysis of the whole genome sequence of *Gardnerella* using digital DNA-DNA hybridization and average nucleotide identity. In 2019, Vaneechoutte et al. analyzed the whole genome sequence of 81 *Gardnerella* spp. and confirmed the existence of 13 subgroups, GSP01-GSP13, all of which are sufficiently different to be classified as independent species. Two subgroups were found in subtypes clade 1, clade 2, and clade 4, and three undefined subgroups were found in subtype clade 3 (Vaneechoutte et al., 2019). This was an important breakthrough in this field that resulted in an amended description of *G. vaginalis* and the characterization of three additional *Gardnerella* species, *G. leopoldii, G. piotii*, and *G. swidsinskii* (Potter et al., 2019).

The Relationship Between Different *Gardnerella* Subtypes

The commonly used *Gardnerella* typing methods include ARDRA, cpn-60, qPCR, and whole genome sequencing (Castro et al., 2019). Since ARDRA typing of *Gardnerella* results does not specifically correspond to clinical status (Piot et al., 1984; Benito et al., 1986), it is not detailed here. Cpn-60 gene sequencing was used to divide *Gardnerella* into four subtypes A, B, C and D, based on the phylogenetic relationship between each cpn60 gene subsequence (Jayaprakash et al., 2012). Similarly, qPCR divided *Gardnerella* isolates into four clades 1, 2, 3 and 4 (Balashov et al., 2014). Schellenberg et al. compared cpn60 UT sequences from 112 *G. vaginalis* isolates from three continents with cpn60 UT sequences extracted from previously published whole genome sequences. All new isolates and previously published genomes fall into four cpn60 subgroups (Jayaprakash et al., 2012), including 17 isolates belonging to clades 1- 4 (Ahmed et al., 2012). These results indicate that similar phylogenetic resolution can be achieved using a partial single gene sequence (552 bp) as 473 full-length gene sequences common to all 17 genomes. Cpn60 subgroups A, B, C, and D correspond to clade 4, 2, 1, and 3, respectively (Schellenberg et al., 2016). Using a retrospective comparative analysis of 103 publicly available genomes and meta-transcriptomic bacterial vaginosis datasets, Potter et al. identified 9 genome species of *Gardnerella*, GS01-09 (Potter et al., 2019). *Gardnerella* species, labeled with names or numbers, were defined by analysis of 81 genome sequences by digital DNA-DNA hybridization, average nucleotide identity (ANI), and MALDI-MS protein signatures (Vaneechoutte et al., 2019). The specific corresponding relationship is shown in **Table 1**.

TABLE 1 | The relationship between different *Gardnerella* species.

Cpn-60	qPCR	*Gardnerella* genomospecies	*Gardnerella* species
Subgroup A	Clade 4	GS03	*G. swidsinskii*, *G. leopoldii*
Subgroup B	Clade 2	GS02 & GS06	*G. piotii*, Gsp03
Subgroup C	Clade 1	GS01	*G. vaginalis* (Gsp01), Gsp02
Subgroup D	Clade 3	GS05	Gsp08, Gsp09, & Gsp10
		GS04	Gsp07
		GS06	Gsp11
		GS07	Gsp12
		GS08	Gsp13
		GS09	

Four subgroups of Gardnerella have been defined by sequencing polymorphisms within the cpn60 gene and by qPCR detection of clade-specific genes (clades 1, 2, 3 and 4). These subtypes were designated as genovars by genome sequencing of 17 isolates. Genomospecies (GS) were defined by integrating four sequence comparison methods of 103 genomic sequences. Gardnerella species, labelled with names or numbers, were defined by analysis of 81 genome sequences by digital DNA-DNA hybridization, average nucleotide identity (ANI) and MALDI-MS protein signatures.

CORRELATION BETWEEN BACTERIAL VAGINOSIS AND *GARDNERELLA* SUBTYPES

Gardnerella and Bacterial Vaginosis

Gardnerella is detected in both the vaginal secretions of women with BV and healthy women. Using two qPCR assays, Balashov et al. analyzed *G. vaginalis* bacterial loads and clade distribution in 100 clinical vaginal-swab samples and showed that the prevalence of *G. vaginalis* was 100% of BV patients and 97% in healthy women; however, the *G. vaginalis* concentration was significantly lower in non-BV samples. The detection frequency of clades 1, 2, 3 and 4 was 53%, 25%, 32% and 83%, respectively. In addition, 70% of BV vaginal swab samples had multiple subtypes of *Gardnerella* (Balashov et al., 2014). An assessment of the vaginal microbiota of 413 non-pregnant, reproductive-age Canadian women showed that the number of *Gardnerella* spp. detected per *Gardnerella*-positive sample ranged from 1 to 10. Multiple *Gardnerella* spp. were detected in 63.8% of samples, consistent with a previous report of multiple *Gardnerella* clades in 70% of samples from women (Balashov et al., 2014), and the majority contained one or two species (Hill et al., 2019). Multiple subtypes of *Gardnerella* had a significant positive correlation with BV, suggesting that the co-occurrence of multiple subtypes may be one of the risk factors for BV.

Clinical Symptoms Associated With *Gardnerella* Subtypes

There was no correlation between *Gardnerella* spp. biotyping and clinical symptoms (Pleckaityte et al., 2012). According to Hill and Albert's research, the relative abundances of the more frequently occurring species (*G. vaginalis*, *G. swidsinskii*, *G. leopoldii*, *G. piotii*, and genome species) among groups based on clinical Nugent scores (negative, intermediate, and BV) and self-reported symptoms in the 2 weeks prior to the swab collection (odor, irritation, and discharge) were compared (Hill and Albert, 2019). Whole genome sequence analysis revealed a strong relationship between abnormal odor and discharge with higher relative abundance of *G. vaginalis* and *G. swidsinskii*. The cooccurrence of these *Gardnerella* spp. also showed proportionality, suggesting that their abundance is correlated. Thus, it remains unclear whether one of these species or both are associated with vaginal symptoms (Hill and Albert, 2019).

Gardnerella Subtypes Distribution

Early studies on *Gardnerella* typing indicated that biotypes 1, 2, and 5 were more common in females with BV (Piot et al., 1984), however subsequent studies could not confirm these findings. Balashov et al. found a positive correlation between BV and clade 1 and clade 3 in vaginal samples from 60 American women. Meanwhile, clade 2 was positively correlated with vaginal microbiota in an intermediate state of BV and C4 had no correlation with infection (Balashov et al., 2014). Janulaitiene et al. performed qPCR on vaginal swab samples from 109 Lithuanian women and confirmed that the microbial status of the vaginal microbiota was associated with the clade 1 and clade 2 subgroups (Nugent score 7-10). The clade 3 and clade 4 showed no association with high Nugent Scores (Janulaitiene et al., 2017). However, the results from a study on the vaginal microbiota of 299 Russian women of reproductive age were different from those of previous studies. Quantifying the four *Gardnerella* subtypes could more accurately distinguish BV from healthy microbiota than detecting the sialidase A gene and clade 4 was closely related to the status of the BV microbiome (Shipitsyna et al., 2019).

Plummer et al. studied the relationship between infection with the clade 1, clade 2, and clade 3 subtypes and Nugent scores in 101 Australian women of reproductive age. Multiple *Gardnerella* subtypes and the clade 1 subtype alone were associated with the absence of *Lactobacillus* in the vaginal microbiome. Clade 4 was not associated with BV or the absence of *Lactobacillus*, supporting the existence of symbiotic and pathogenic subtypes of *Gardnerella* spp (Plummer et al., 2020). Hill et al. used whole genome sequencing to compare the species abundance of *Gardnerella* subgroups between healthy women and those with BV. The relative abundance of *G. vaginalis, G. swidsinskii*, and *G. piotii* correlated significantly with Nugent scores. In addition, the association between *G. piotii* (B subgroup/clade 2) and the "intermediate state" microbiome was observed by cpn60 sequencing and qPCR (Hill and Albert, 2019). *Gardnerella* typing results from different studies are shown in **Table 2**.

TABLE 2 | Different *Gardnerella* subtypes predict distinct BV clinical outcomes.

	BV	BV Intermediate	Healthy	Methods
Jayaprakash et al., 2012	Subgroup B	–	–	Cpn-60
Balashov et al., 2014	Clades 1 and 3	Clade 2	Clade 4	qPCR
Janulaitiene et al., 2017	Clades 1 and 2	–	–	clade-specific PCR
Shipitsyna et al., 2019	Clade 4	–	–	clade-specific PCR
Plummer et al., 2020	Clades1, 2, 3 and multiple subtypes	Clade 3	Clade 4	qPCR
Hill and Albert, 2019	*G. vaginalis, G. swidsinskii*, and *G. piotii*	–	–	Whole Genome Sequencing

In summary, current studies remain unable to determine the relationship between different *Gardnerella* genotypes and clinically relevant BV status.

Gardnerella Subtypes Drug Resistance

Metronidazole resistance by *Gardnerella* isolates is likely responsible for refractory or recurrent BV. Women with BV are typically infected with multiple *Gardnerella* spp. subtypes, so metronidazole may eliminate sensitive *Gardnerella* subtypes but allow drug-resistant subtypes to survive. This could explain the presence of *Gardnerella* in the vaginal microbiota even after metronidazole treatment. BV is characterized by a thick vaginal multi-species biofilm, in which *Gardnerella* spp. is the predominant species. Since standard antibiotics, like metronidazole, are unable to fully eradicate the vaginal biofilm, this may explain the high recurrence rates of BV (Machado et al., 2015; Verstraelen and Swidsinski, 2019). Early identification of metronidazole-resistant *Gardnerella* subtypes helps to predict the prognosis of BV and inform an appropriate treatment plan. To explore the sensitivity of different *Gardnerella* subtypes to metronidazole, Schuyler et al. collected 88 *Gardnerella* strains and divided them into four groups, clades 1, 2, 3 and 4, by qPCR sequencing. Metronidazole resistance was defined as a minimum inhibitory concentration ≥32 μg/mL. A high correlation was found between metronidazole resistance and *Gardnerella* typing. While clade 3 and clade 4 strains showed 100% resistance, while clade 1 and clade 2 showed 35% and 7.1% resistance, respectively (Schuyler et al., 2016).

Clinical Outcomes of *Gardnerella* Subtypes

In 2017, Hilbert et al. conducted a short-term longitudinal study of 149 non-pregnant Canadian women of reproductive age. Vaginal swab samples were collected at first diagnosis, 7 days after treatment, and 40-45 days after follow-up to detect the abundance of different *Gardnerella* subtypes by qPCR. Higher prevalence of clade 1 and clade 4 were found in vaginal samples than clade 2 and clade 3. The abundance of each subtype increased as the Nugent score, or the severity of BV, worsened (Hilbert et al., 2017). The abundance of clade 1 and clade 4 decreased after clinical treatment regardless of the clinical and microbiological outcome, and clade 2 decreased in women who continued treatment for 40-45 days. Recurrent BV is characterized by increased clade 1 and clade 2 levels after treatment. The clade 1 and clade 4 subgroups were the dominant strains in vaginal specimens. While the clade abundance of *Gardnerella* was generally higher in vaginal samples that met the four Amsel criteria than those that did not, clade 1 was an exception. Thus, differences in qPCR subtype abundance were associated with Nugent score, Amsel criteria, sensitivity to treatment, and BV recurrence.

A recent study by Turner et al. associated sustained high abundance of the *Gardnerella* Gsp07 subtype with a refractory BV response and sustained low abundance of the *Gardnerella* Gsp07 subtype and *G. Swidsinskii/G. Leopoldii* with BV remission. In most patients with BV relapse or remission, the abundance of *Lactobacillus* species increased 4–14 days after initiation of treatment, and the increase was more obvious and sustained in patients with BV remission. These findings confirmed that Gardner's Gsp07 subtypes and *G. swidsinskii/G. leopoldii* coinfection correlate with poor clinical outcomes. Alternatively, direct or indirect inhibition of lactic acid bacteria strains may interfere with clinical recovery. Treatment by clinicians targeting these marker subtypes of adverse outcomes may improve clinical outcomes in patients with BV (Turner et al., 2021).

Virulence Factors of *Gardnerella* Subtypes

Gardnerella pathogenicity is primarily mediated through vaginal hemolysin (VLY), sialidase and biofilm formation (Pleckaityte et al., 2012). Previous studies have shown that pathogenicity differs by *Gardnerella* subspecies (Janulaitiene et al., 2018). Zilnyte et al. found that VLY activity is dependent on the complement regulatory molecule, CD59, and showed that higher CD59 expression in hamsters correlated with increased vaginal hemolysin-soluble cell sensitivity (Zilnyte et al., 2015). In the cell culture model, the expression level of vaginal hemolysin was correlated with the level of cytotoxicity, but there was no any correlation between VLY production level and *G. vagina*lis genotype/biotype (Pleckaityte et al., 2012).

Sialidase lyses the terminal sialic acid residues of sialoglycan in the vaginal environment and plays a key role in providing nutrition for *Gardnerella* spp. through sialic acid catabolism, providing a site for bacteria adhesion to the epithelium, facilitating biofilm formation, and modulating immune responses (Lewis et al., 2013; Schellenberg et al., 2017). Harwich et al. (2010) and Janulaitiene et al. (2018) found significant differences in the sialidase activity of *Gardnerella* clades, however, with clade 2 having the highest levels followed by clade 1, and clade 4 having the lowest. In addition, the gene coding for sialidase was detected in all isolates of clade 1 and clade 2, but not in clade 4 isolates (Schellenberg et al., 2016; Janulaitiene et al., 2017). Shipitsyna also holds that clade 4 strains mostly lack the sialidase A gene (Shipitsyna et al., 2019). Sialidase activity is considered a marker of BV. Indeed, more than 50% of

BV is asymptomatic, which may be caused by *Gardnerella* subspecies that lack sialidase (Janulaitiene et al., 2017). Sialidase acts on sugar chains with sialic acid residues, which are abundant on the mucosal surface of the reproductive tract. Sialidase activity can be used as a diagnostic marker of BV (Janulaitiene et al., 2018) and rapid clinical detection using products like BVBlue® (Sekisui Diagnostics, L.L.C., Birmingham, AL, USA) (Hill and Albert, 2019).

The exfoliated vaginal epithelial cells in BV patients are covered with multi-bacterial biofilms dominated by *Gardnerella* (Vestby et al., 2020). Biofilm formation is not only associated with increased antimicrobial resistance and disease recurrence but also increased risk of sexual transmission. However, there is no significant difference in biofilm quantity between *Gardnerella* subtypes (Janulaitiene et al., 2018).

CONCLUSIONS

In summary the relationship between the different subtypes of *Gardnerella* and bacterial vaginosis is unclear. A large number of studies show that different *Gardnerella* subtypes are possibly represent different drug resistance, virulence, bacterial load and indicate the clinical outcomes of BV. And the clinical significance of asymptomatic BV remains unclear, one possible explanation for its occurrence is the presence of high numbers of nonpathogenic *Gardnerella* or other morphologically similar species. This is especially likely given that *Gardnerella* is one of the key predictors of the Nugent score. Furthermore, metronidazole treatment for BV cure rate is not ideal, and the proportion of refractory and recurrent BV continues to rise. This study reviewed the relationship between *Gardnerella* subtypes species and BV clinical outcomes and evaluated patient prognosis according to *Gardnerella* typing. This is particularly important so that appropriate treatment can be given to improve the BV clinical cure rate and reduce adverse obstetric and gynecological complications as well as disease recurrence. Given the current diversity of *Gardnerella* phenotypes, especially virulence factors, genotypic diversity, and *Gardnerella* prevalence in women, understanding the clinical significance of these different strains is critical.

AUTHOR CONTRIBUTIONS

HQ and BX discussed the contents, wrote, reviewed, and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This present work was funded by the grants of the National Key Research and Development Program of China (2021YFC2301000) and the National Natural Science Foundation of China (81971342).

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frontiers | Frontiers in Cellular and Infection Microbiology

BRIEF RESEARCH REPORT
published: 11 May 2022
doi: 10.3389/fcimb.2022.863208

Clustering Analysis of the Multi-Microbial Consortium by *Lactobacillus* Species Against Vaginal Dysbiosis Among Ecuadorian Women

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OPEN ACCESS

Edited by:
Nuno F. Azevedo, University of Porto, Portugal

Reviewed by:
Daniela Marlene Da Silva Machado, Universidade Católica Portuguesa, Portugal
Joana Castro, University of Minho, Portugal

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Specialty section:
This article was submitted to Biofilms, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *26 January 2022*
Accepted: *18 March 2022*
Published: *11 May 2022*

Citation:
Pacha-Herrera D, Erazo-Garcia MP, Cueva DF, Orellana M, Borja-Serrano P, Arboleda C, Tejera E and Machado A (2022) Clustering Analysis of the Multi-Microbial Consortium by Lactobacillus Species Against Vaginal Dysbiosis Among Ecuadorian Women. Front. Cell. Infect. Microbiol. 12:863208. doi: 10.3389/fcimb.2022.863208

The vaginal microbiota plays vital protection in women. This probiotic activity is caused not only by individual *Lactobacillus* species but also by its multi-microbial interaction. However, the probiotic activity promoted by multi-microbial consortia is still unknown. The aim of this study was the individual and collective analysis on the prevalence of five vaginal lactobacilli (*Lactobacillus iners*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, and *Lactobacillus acidophilus*) among healthy women and women with bacterial vaginosis (BV) or aerobic vaginitis (AV). PCR assays were realized on 436 vaginal samples from a previous study. Chi-square, univariable, and multivariable logistic regression analyses with the Benjamini–Hochberg adjustment evaluated associations between these lactobacilli and vaginal microbiota. Multi-microbial clustering model was also realized through Ward's Minimum Variance Clustering Method with Euclidean squared distance for hierarchical clustering to determine the probiotic relationship between lactobacilli and vaginal dysbiosis. Concerning the individual effect, *L. acidophilus*, *L. jensenii*, and *L. crispatus* showed the highest normalized importance values against vaginal dysbiosis (100%, 79.3%, and 74.8%, respectively). However, only *L. acidophilus* and *L. jensenii* exhibited statistical values (p = 0.035 and p = 0.050, respectively). *L. acidophilus* showed a significant prevalence on healthy microbiota against both dysbioses (BV, p = 0.041; and AV, p = 0.045). *L. jensenii* only demonstrated significant protection against AV (p = 0.012). Finally, our results evidenced a strong multi-microbial consortium by *L. iners*, *L. jensenii*, *L. gasseri*, and *L. acidophilus* against AV (p = 0.020) and BV (p = 0.009), lacking protection in the absence of *L. gasseri* and *L. acidophilus*.

Keywords: hierarchical clustering analysis, *Lactobacillus* species, vaginal microbiota, bacterial vaginosis, aerobic vaginitis

INTRODUCTION

Vaginal microbiota balances the health state of women through its ability to prevent potential dysbiosis or infections (Pacha-Herrera et al., 2020; Joseph et al., 2021). Healthy women usually show a diversity of anaerobic and aerobic microorganisms in the vaginal epithelium (Borges et al., 2013), in which lactobacilli are the dominant species and act as a protective barrier to prevent pathogenic colonization (Di Cerbo et al., 2016; Scillato et al., 2021). However, the vaginal colonization by different lactobacilli species depends also on their ability to produce antimicrobial compounds, such as hydrogen peroxide, lactic acid, and bacteriocin-like substances (Borges et al., 2013; Castillo-Juárez et al., 2022). These antimicrobial compounds are extremely important in the impairment of colonization by pathogens associated with different types of vaginitis or dysbiosis, such as bacterial vaginosis (BV), vulvovaginal candidiasis (VC), and aerobic vaginitis (AV) (Vaneechoutte, 2017b; Vaneechoutte, 2017a). Vaginal dysbiosis increases public health costs and affects women of reproductive age who will develop chronic infections and more serious outcomes (Van De Wijgert et al., 2014; Walker, 2016), such as infertility, miscarriage, chronic pelvic inflammation, and an augmented HIV transmission (Onderdonk et al., 2016; Oostrum et al., 2018).

Different *Lactobacillus* species are usually found in the vaginal microbiota of healthy women, such as *Lactobacillus iners*, *Lactobacillus crispatus*, and *Lactobacillus gasseri* (Cribby et al., 2008; Vaneechoutte, 2017b). Despite that *L. iners* is found in the vaginal microbiota of healthy women, this bacterial species is also associated with transient or BV-associated microbiota, as previously discussed (Petrova et al., 2017). It is also well-known that significant differences in lactobacilli composition on the vaginal tract among women of different countries, races, and ethnicities are commonly found (Zhou et al., 2007; Madhivanan et al., 2014; Van De Wijgert et al., 2014; Borgdorff et al., 2017). Likewise, variations on microbial consortia among women with different vaginitis or dysbiosis are frequently reported (Demba et al., 2005; Borgdorff et al., 2017). However, most studies on Latin American mainly focus on determining BV prevalence (Kenyon et al., 2013; Krauss-Silva et al., 2014), and little is still known about the lactobacilli composition and their prevalence in Latin American women (Salinas et al., 2018; Peebles et al., 2019). Therefore, our main goal is to characterize the prevalence of five well-known lactobacilli species (*L. iners*, *L. crispatus*, *L. gasseri*, *Lactobacillus jensenii*, and *Lactobacillus acidophilus*) in the vaginal microbiota of native Ecuadorian women from our previous epidemiological study (Salinas et al., 2020). The present study assessed the presence of these lactobacilli using PCR amplification of *16S* and *23S* rRNA genes, and further multiple comparisons evaluated potential correlations between *Lactobacillus* species and sociodemographic factors and different types of vaginal microbiota (healthy microbiota, intermediate microbiota, and vaginal dysbioses, such as BV and AV) through chi-square, univariable, and multivariable logistic regression analyses with the Benjamini–Hochberg (BH) adjustment. Finally, a multi-microbial clustering model was also realized through Ward's Minimum Variance Clustering Method with Euclidean squared distance for hierarchical clustering fed to determine any potential symbiotic or antagonistic relationship between these *Lactobacillus* species against both cases of vaginal dysbiosis.

MATERIALS AND METHODS

Study Design

This study was conducted in the Microbiology Institute at the Universidad San Francisco de Quito (USFQ) from June 2017 to November 2018. As previously reported (Salinas et al., 2020), 436 Ecuadorian women of Hispanic ethnicity between 18 and 56 years old volunteered to be part of the epidemiological study. Briefly, all women received a kit containing an informed consent approved by the Bioethics Committee of the USFQ, a standardized medical survey, and a vaginal transport swab system (Stuart's transport media swabs; Copan Diagnostics Inc., Brescia, Italy). Volunteers were excluded if they reported having had sexual intercourse within the last 48 h, antimicrobial treatment in the last 3 months, or any evidence of bleeding. The study was supervised by a physician, a psychologist, and a full-time researcher from the USFQ. This investigation adopted a cross-sectional study design to determine the association between the presence of five well-known lactobacilli species and vaginal microbiota or opportunistic pathogens (such as *Gardnerella* spp., *Fannyhessea vaginae* previously known as *Atopobium vaginae*, *Mobiluncus* spp., *Escherichia coli*, and *Candida albicans*) previously diagnosed/detected in our last publication (Salinas et al., 2020), more exactly, healthy microbiota, intermediate microbiota, and vaginal dysbioses (AV and BV, and VC).

Ethics Statement

The present study was approved by the Ethics Committee of the USFQ (Protocol code: 2016-023IN by MSP-VGVS-2016-0244-O review board).

DNA Extraction

DNA extraction was realized through standard procedure following Peng and colleagues' direct boiling point method (Peng et al., 2013). Briefly, the stored aliquots (0.9% NaCl) of 1 ml were incubated at 100°C in a water bath for 15 min and then immediately frozen at −20°C for 15 min. Next, the samples were centrifuged at 13,000 rpm for 15 min, and supernatants were aliquoted into 500-µl volumes. DNA quantification was performed with a NanoVue spectrophotometer (GE Healthcare Life Science, Marlborough, MA, USA), samples were eluted at 20 ng/µl with molecular grade water and stored at −20°C until the PCR analysis was performed. The quality of DNA was evaluated by measuring the concentration of phenolic compounds or the presence of salts (260/230) and protein contaminants (260/280). This procedure was adapted from Money (Money, 2005).

Identification of *Lactobacillus* Species by PCR

From our previous study (Salinas et al., 2020), 436 vaginal samples were selected for molecular characterization by PCR in a Bio-Rad Thermocycler (Bio-Rad, Hercules, CA, USA). DNA quantification was performed with a NanoVue spectrophotometer (GE Healthcare Life Science) to ensure the presence of amplifiable DNA. Concentrations of DNA in ng/µl were measured, as well as the phenolic contaminants (260/230) and the protein contaminants (260/280). Aliquots of DNA between 10 and 20 ng/µl were used for PCR analysis. Before lactobacilli detection was realized, all samples were analyzed for *16S* conserved rRNA genes (fDD2-CCGGATCCGT CGACAGAGTTTGATCITGGCTCAG; rPP2-CCAAGC TTCTAGACGGITACCTTGTTACGACTT) by PCR, ensuring the absence of PCR inhibitors on samples, as previously described (Borja-Serrano et al., 2020). All samples were analyzed with a total of five primer pairs, targeting five *Lactobacillus* species (*L. acidophilus*, *L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. iners*). Single-template PCR assays were performed for each primer set. The sequence, amplicon size, target species, and temperature of annealing for each primer pair are described in **Supplementary Table 1**. A final volume of 20 µl was used according to the reference protocols (Galán et al., 2006; Fredricks et al., 2007; Sepehri et al., 2009; Henriques et al., 2012; DTU- National Food Institute, 2014), which included 0.5 U of Go Taq® DNA Polymerase (Promega, Madison, WI, USA), 1× of Green GoTaq® Flexi Buffer (Promega), 0.25 mM of $MgCl_2$ (Promega), 200 µM of dNTP mix (Promega), 0.5 µM of each primer and target template DNA concentration of approximately 4 ng/µl, and the remaining volume with molecular grade H_2O. The PCR thermal cycling consisted of initial denaturation at 94°C for 2 min, followed by 29 cycles of denaturation at 94°C for 30 s, annealing at each primer pair temperature for 30 s and extension at 72°C for 1 min, and final extension of 5 min at 72°C. The respective use of negative (without DNA sample and samples with other related bacteria) and positive (collection of identified strains of each species through DNA sequencing) controls were used in each PCR assay. These positive controls were provided by the Microbiology Institute at USFQ. All samples were randomly performed in triplicate with different negative and positive controls. After PCR amplification, a volume of 4 µl from each PCR product was visualized in 1.5% (w/w) agarose (Promega) gel electrophoresis using 0.1% ethidium bromide staining. The DNA analysis was performed under permit No. MAE-DNB-CM-2016-0046 (De Backer et al., 2007; Garg et al., 2009; Tsai et al., 2010; Zhang et al., 2012).

Statistical Analysis

A multivariate logistic regression model was used to calculate the odds ratios (OR) of the clinical outcomes that included demographic variables (age, sex, city, and marital status), socioeconomic variables (occupation and level of education), personal habits (sex relationships, hygiene, and other habits), and the type or number of vaginal *Lactobacillus* species associated with the presence or absence of vaginal infection using logistic regression. These data were also considered categorical variables. Firstly, the variable of vaginal infection in the samples was categorized as the presence and absence, so a comparison of the different risk factors of both groups can be performed. After further statistical analysis, the study was defined by the type of vaginal dysbiosis (BV and AV) for testing differences in the previously analyzed factors and vaginal microbiota. The chi-square test was used to evaluate associations between the prevalence of vaginitis with the other risk factors. A value of $p < 0.05$ and 95% CIs were considered significant for the test. Logistic regression was also performed to calculate crude ORs for each variable mentioned; adjusted ORs were produced for variables with statistical significance in both tests applied for association (Ozaydin et al., 2013; Porras et al., 2014; Syam et al., 2015). Therefore, the chi-square test was used as a test of association, while the OR was then used as a measure of association (Kim, 2017). The statistical analysis of association with risk factors was performed for each type of vaginal infection but negative for the remaining types of vaginal infection to observe a significant difference between those populations. Each type of vaginal infection, normal or healthy microbiota, and intermediate microbiota were classified as dependent variables against sociodemographic and behavioral variables or the presence of *Lactobacillus* species as independent variables. All initial values of $p < 0.05$ obtained by univariable logistic regression, chi-square, and multivariable logistic regression analyses were then evaluated through the BH adjustment to detect false discovery rate (FDR) for conducting multiple comparisons. All statistical analyses were performed using SPSS version 22.0 (SPSS Statistics for Windows Version 22.0, IBM Corp, Armonk, NY, USA), except for the BH adjustment. The BH adjustment was realized using Seed-based d Mapping software (SDM, version 6.21, https://www.sdmproject.com, formerly "Signed Differential Mapping") (Radua and Mataix-Cols, 2009; Radua et al., 2012). A clustering model was realized through Ward's Minimum Variance Clustering Method with Euclidean squared distance to perform hierarchical clustering fed by a dimensionality reduction algorithm Principal Component Analysis (PCA) implemented in RStudio software (version 1.3.1073; https://rstudio.com/), using the option method = "ward" of the hclust function from the stats base R package (Package stats version 4.1.0) (Murtagh and Legendre, 2014).

RESULTS

Description of Study Population

A total of 436 women volunteered in our last study (Salinas et al., 2020), with their vaginal samples and epidemiologic data selected for lactobacilli characterization in the present study. The stored samples were chosen for the molecular analysis by PCR. As shown in **Table 1**, our population set was constituted by Ecuadorian women between 18 and 56 years old, with 76.3% between 18 and 28 years old. Briefly, 66.1% of the women have healthy vaginal microbiota, 10.8% have an intermediate microbiota, and finally, 23.1% showed vaginal dysbiosis or

TABLE 1 | Identification of the main vaginal *Lactobacillus* species among the population set of the study realized by Salinas and colleagues (2020).

	***Lactobacillus iners* N (%)**		***Lactobacillus jensenii* N (%)**		***Lactobacillus acidophilus* N (%)**		***Lactobacillus crispatus* N (%)**		***Lactobacillus gasseri* N (%)**		**Total N (%)**
	Absence	**Presence**	**Absence**	**Presence**	**Absence**	**Presence**	**Absence**	**Presence**	**Absence**	**Presence**	
Total incidence	162 (37.2)	274 (62.8)	310 (71.1)	126 (28.9)	314 (72.0)	122 (28.0)	391 (89.7)	45 (10.3)	316 (72.5)	120 (27.5)	436 (100.0)
Vaginal microbiota†											
Healthy microbiota	101 (23.2)	187 (42.9)	195 (44.7)	93 (21.3)	194 (44.5)	94 (21.6) *	258 (59.2)	30 (6.9)	199 (45.6)	89 (20.4) *	288 (66.1)
Intermediate microbiota	23 (5.3)	24 (5.5)	34 (7.8)	13 (3.0)	39 (8.9)	8 (1.8)	42 (9.6)	5 (1.1)	40 (9.2)	7 (1.6)	47 (10.8)
Bacterial vaginosis	10 (2.3)	14 (3.2)	19 (4.4)	5 (1.1)	21 (4.8)	3 (0.7)	23 (5.3)	1 (0.2)	20 (4.6)	4 (0.9)	24 (5.5)
Aerobic vaginitis	22 (5.0)	31 (7.1)	45 (10.3)	8 (1.8)	43 (9.9)	10 (2.3)	47 (10.8)	6 (1.4)	40 (9.2)	13 (3.0)	53 (12.2)
Candidiasis	0 (0.0)	7 (1.6)	5 (1.1)	2 (0.5)	4 (0.9)	3 (0.7)	6 (1.4)	1 (0.2)	3 (0.7)	4 (0.9)	7 (1.6)
Coinfections	6 (1.4)	11 (2.5)	12 (2.8)	5 (1.1)	13 (3.0)	4 (0.9)	15 (3.4)	2 (0.5)	14 (3.2)	3 (0.7)	17 (3.9)
Age											
≤21	59 (13.5)	109 (25.0)	115 (26.4)	53 (12.2)	126 (28.9)	42 (9.6)	156 (35.8)	12 (2.8)	125 (28.7)	43 (9.9)	168 (38.5)
22–28	53 (12.2)	112 (25.7)	118 (27.1)	47 (10.8)	109 (25.0)	56 (12.8) *	137 (31.4)	28 (6.4) **	118 (27.1)	47 (10.8)	165 (37.8)
29–35	16 (3.7)	18 (4.1)	27 (6.2)	7 (1.6)	22 (5.0)	12 (2.8)	30 (6.9)	4 (0.9)	25 (5.7)	9 (2.1)	34 (7.8)
36–42	12 (2.8)	14 (3.2)	17 (3.9)	9 (2.1)	19 (4.4)	7 (1.6)	25 (5.7)	1 (0.2)	16 (3.7)	10 (2.3)	26 (6.0)
43–49	3 (0.7)	6 (1.4)	5 (1.1)	4 (0.9)	7 (1.6)	2 (0.5)	9 (2.1)	0 (0.0)	6 (1.4)	3 (0.7)	9 (2.1)
≥50	5 (1.1)	7 (1.6)	9 (2.1)	3 (0.7)	9 (2.1)	3 (0.7)	12 (2.8)	0 (0.0)	8 (1.8)	4 (0.9)	12 (2.8)
Did not answer	14 (3.2)	8 (1.8)	19 (4.4)	3 (0.7)	22 (5.0)	0 (0.0)	22 (5.0)	0 (0.0)	18 (4.1)	4 (0.9)	22 (5.0)
Ethnicity											
Hispanic	134 (30.7)	251 (57.6) **	270 (61.9)	115 (26.4)	271 (62.2)	114 (26.1)	345 (79.1)	40 (9.2)	272 (62.4)	113 (25.9)	385 (88.3)
Indigenous	2 (0.5)	4 (0.9)	5 (1.6)	1 (0.2)	6 (1.4)	0 (0.0)	6 (1.4)	0 (0.0)	6 (1.4)	0 (0.0)	6 (1.4)
Caucasian	2 (0.5)	4 (0.9)	4 (0.9)	2 (0.5)	4 (0.9)	2 (0.5)	5 (1.1)	1 (0.2)	5 (1.1)	1 (0.2)	6 (1.4)
Afro-Ecuadorian	1 (0.2)	0 (0.0)	1 (0.2)	0 (0.0)	1 (0.2)	0 (0.0)	1 (0.2)	0 (0.0)	1 (0.2)	0 (0.0)	1 (0.2)
Did not answer	23 (5.3)	15 (3.4)	30 (6.9)	8 (1.8)	32 (7.3)	6 (1.4)	34 (7.8)	4 (0.9)	32 (7.3)	6 (1.4)	38 (8.7)
Occupation											
Housewife	1 (0.2)	6 (1.4)	4 (0.9)	3 (0.7)	6 (1.4)	1 (0.2)	7 (1.6)	0 (0.0)	5 (1.1)	2 (0.5)	7 (1.6)
Student	98 (22.5)	208 (47.7) **	210 (48.2)	96 (22.0)	216 (49.5)	90 (20.6)	273 (62.8)	33 (7.6) *	226 (51.8)	80 (18.3)	306 (70.2)
Unprofessional	12 (2.8)	13 (3.0)	21 (4.8)	4 (0.9)	19 (4.4)	6 (1.4)	25 (5.7)	0 (0.0)	15 (3.4)	10 (2.3)	25 (5.7)
Professional	36 (8.3)	38 (8.7)	55 (12.6)	19 (4.4)	50 (11.5)	24 (5.5)	62 (14.2)	12 (2.8)	50 (11.5)	24 (5.5)	74 (17.0)
Did not answer	15 (3.4)	9 (2.1)	20 (4.6)	4 (0.9)	23 (5.3)	1 (0.2)	24 (5.5)	0 (0.0)	20 (4.6)	4 (0.9)	24 (5.5)
Civil status											
Married	21 (4.8)	31 (7.1)	37 (8.5)	15 (3.4)	38 (8.7)	14 (3.2)	48 (11.0)	4 (0.9)	34 (7.8)	18 (4.1)	52 (11.9)
Divorced	4 (0.9)	5 (1.1)	7 (1.6)	2 (0.5)	8 (1.8)	1 (0.2)	9 (2.1)	0 (0.0)	5 (1.1)	4 (0.9)	9 (2.1)
Single with partner	51 (11.7)	132 (30.3) **	120 (27.5)	63 (14.4)	119 (27.3)	64 (14.7) **	156 (35.8)	27 (6.2) *	124 (28.4)	59 (13.5) *	183 (42.0)
Single without partner	67 (15.4)	93 (21.3)	121 (27.8)	39 (8.9)	122 (28.0)	38 (8.7)	148 (33.9)	12 (2.8)	128 (29.4)	32 (7.3)	160 (36.7)
Free union	4 (0.9)	4 (0.9)	5 (1.1)	3 (0.7)	4 (0.9)	4 (0.9)	6 (1.4)	2 (0.5)	5 (1.1)	3 (0.7)	8 (1.8)
Did not answer	15 (3.4)	9 (2.1)	20 (4.6)	4 (0.9)	23 (5.3)	1 (0.2)	24 (5.5)	0 (0.0)	20 (4.6)	4 (0.9)	24 (5.5)
Sexual partner											
With partner	77 (17.7)	169 (38.8) **	164 (37.6)	82 (18.8) *	163 (37.4)	83 (19.0) ***	213 (48.9)	33 (7.6) *	165 (37.8)	81 (18.6) **	246 (56.4)
Without partner	71 (16.3)	97 (22.2)	127 (29.1)	41 (9.4)	129 (29.6)	39 (8.9)	156 (35.8)	12 (2.8)	133 (30.5)	35 (8.0)	168 (38.5)
Did not answer	14 (3.2)	8 (1.8)	19 (4.4)	3 (0.7)	22 (5.0)	0 (0.0)	22 (5.0)	0 (0.0)	18 (4.1)	4 (0.9)	22 (5.0)
Education level											
≤Basic	2 (0.5)	4 (0.9)	6 (1.4)	0 (0.0)	5 (1.1)	1 (0.2)	6 (1.4)	0 (0.0)	5 (1.1)	1 (0.2)	6 (1.4)
Secondary	109 (25.0)	221 (50.7) **	230 (52.8)	100 (22.9)	233 (53.4)	97 (22.2) **	298 (68.3)	32 (7.3)	240 (55.0)	90 (20.6)	330 (75.7)
≥University	37 (8.5)	40 (9.2)	54 (12.4)	23 (5.3)	53 (12.2)	24 (5.5)	64 (14.7)	13 (3.0)	52 (11.9)	25 (5.7)	77 (17.7)
Did not answer	14 (3.2)	9 (2.1)	20 (4.6)	3 (0.7)	23 (5.3)	0 (0.0)	23 (5.3)	0 (0.0)	19 (4.4)	4 (0.9)	23 (5.3)
Birth control methods											
Condom	24 (5.5)	50 (11.5)	53 (12.2)	21 (4.8)	45 (10.3)	29 (6.7)	62 (14.2)	12 (2.8)	52 (1.9)	22 (5.0)	74 (17.0)
Other than condom	57 (13.1)	121 (27.8) **	121 (27.8)	57 (13.1)	123 (28.2)	55 (12.6) **	157 (36.0)	21 (4.8) **	121 (27.8)	57 (13.1)	178 (40.8)
None	62 (14.2)	90 (20.6)	112 (25.7)	40 (9.2)	118 (27.1)	34 (7.8)	145 (33.3)	7 (1.6)	118 (27.1)	34 (7.8)	152 (34.9)
Did not answer	19 (4.4)	13 (3.0)	24 (5.5)	8 (1.8)	28 (6.4)	4 (0.9)	27 (6.2)	5 (1.1)	25 (5.7)	7 (1.6)	32 (7.3)

*N, number of women who responded in the survey within each category; %, assigned percentage for each classification within each category. The chi-square test was used to evaluate associations between the prevalence of each Lactobacillus sp. with the other risk factors. $p < 0.05$ and 95% CIs were considered significant for the test: *$p \leq 0.05$; **$p \leq 0.02$; ***$p \leq 0.001$.*
†Vaginal microbiota diagnoses, sociodemographic, and behavioral variables among the population set based on the previous study by Salinas et al. (2020).

infections. Among women with vaginal dysbiosis or infection, AV was the main vaginal dysbiosis being diagnosed in 52.5% (53/101), followed by BV (23.8%; 24/101) and VC (6.9%; 7/101). Eighty-four women were diagnosed with a single type of dysbiosis (83.2%), and the remaining 17 had vaginal coinfections (16.8%). The most common coinfections found in women were BV and AV (12/17), followed by BV and VC (3/17), AV and VC (1/17), and lastly, all studied vaginal infections (1/17).

Approximately 87.2% of the population set was constituted by undergraduate students or young professionals (380/436). The categories of professionals included the following: health professionals (23.0%), administrative clerks (20.3%), educators (14.9%), and general employees with college degrees (18.0%).

The remaining professions without specialization or need for college degrees were classified as unprofessional careers. Most of the volunteers were single women (78.7%) and of Hispanic ethnicity (88.3%). Among the participants, 56.4% had a steady sexual partner, and 38.5% reported not having any sexual partner. Concerning birth control methods, 17.0% of participants reported using a condom, 40.8% reported the use of other birth control methods, and the remaining women did not use any birth control method (34.9%) or merely did not answer (7.3%). Alternative birth control methods included hormone treatment or other forms of protection (e.g., spermicides, diaphragm, cervical cap, and sterilization), intrauterine device (IUD), and natural methods (abstinence, fertility awareness method (FAM), and withdrawal). In our study, the most used alternative contraceptive method was hormonal, through oral contraceptives (46.7%) and local implants (6.6%).

When the results of the chi-square test in the prevalence of each *Lactobacillus* sp. between healthy microbiota, intermediate microbiota, and vaginal dysbiosis were analyzed, each group showed statistically significant differences in the presence of *L. acidophilus*, *L. jensenii*, and *L. gasseri*, as shown in **Figure 1A**. However, only *L. acidophilus* showed simultaneous statistical differences between healthy and intermediate microbiota (p = 0.026) and between healthy microbiota and vaginal dysbiosis (p = 0.015). The prevalence of both *L. jensenii* and *L. gasseri* was statistically different between healthy microbiota and vaginal dysbiosis (p = 0.017 and p = 0.020, respectively). No statistically significant differences were observed in these lactobacilli presence between intermediate microbiota and vaginal dysbiosis (see **Figure 1A**). Finally, *L. iners* and *L. crispatus* did not demonstrate statistical differences among these groups of the vaginal microbiota.

Further statistical analysis was then realized between healthy microbiota and specific types of vaginal dysbiosis (more exactly AV and BV), as well as between vaginal dysbioses. As shown in **Figure 1B**, some statistically significant differences were found on certain *Lactobacillus* species when comparing healthy microbiota against BV and AV, but no statistical differences were found between BV and AV. *L. acidophilus* showed statistically significant differences in its prevalence on healthy microbiota against both dysbioses (BV, p = 0.041; and AV, p = 0.045), while *L. jensenii* only showed statistically significant differences between healthy microbiota and AV cases (p = 0.012).

To evaluate if the statistically significant differences found in the prevalence of lactobacilli could have a protective effect against the development of vaginal dysbiosis, univariable logistic regression analyses were then performed. As shown in **Figure 2A**, each *Lactobacillus* species was normalized according to the importance of their presence against the vaginal dysbiosis establishment, showing *L. acidophilus*, *L. jensenii*, and *L. crispatus* importance of 100%, 79.3%, and 74.8%, respectively. However, only *L. acidophilus* and *L. jensenii* exhibited statistically significant differences (p = 0.035 and 0.050, respectively), suggesting a potential protective effect against the development of vaginal dysbiosis.

In addition, little is still known about epidemiological factors and lactobacilli colonization among women (Vaneechoutte, 2017a; Auriemma et al., 2021). Therefore, multiple chi-square analysis was also performed to evaluate possible correlations with each individual *Lactobacillus* species. As shown in **Figure 2B**, *L. iners* (p = 0.017) and *L. gasseri* (p = 0.013) were more prevalent in women with previous antimicrobial treatment in their clinical background. *L. crispatus* (p = 0.048) was associated with the presence of vaginal secretion among women. However, *L. iners* was also related to other

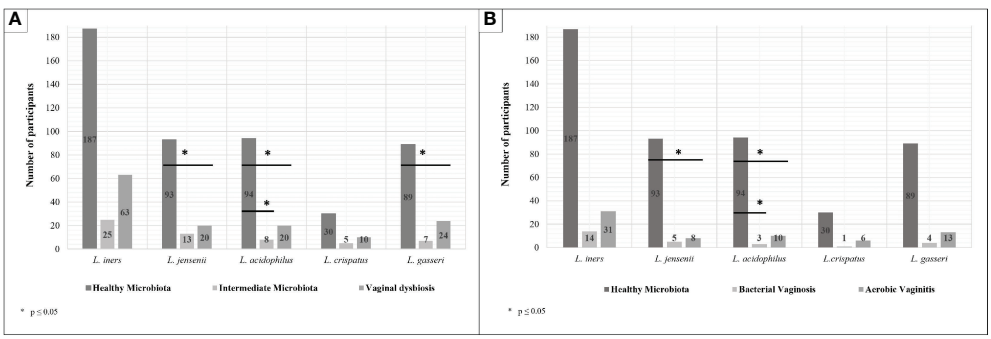
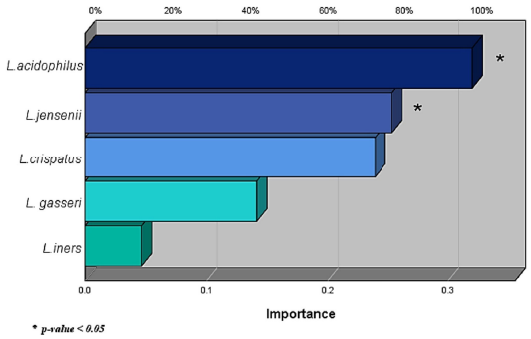


FIGURE 1 | Prevalence of each *Lactobacillus* species according to the type of vaginal microbiota. **(A)** Lactobacilli prevalence in healthy microbiota, intermediate microbiota, and vaginal dysbiosis. **(B)** Lactobacilli prevalence in healthy microbiota, bacterial vaginosis (BV), and aerobic vaginitis (AV). Chi-square tests were performed among the prevalence of each *Lactobacillus* species in the presence of healthy microbiota, intermediate microbiota, or vaginal dysbiosis **(A)** and then BV and aerobic vaginitis (AV) **(B)**. **(A)** The results show statistically significant differences between healthy and intermediate microbiota in *Lactobacillus acidophilus* (p = 0.026) and *Lactobacillus gasseri* (p = 0.020). Meanwhile, statistically significant differences between healthy microbiota and vaginal dysbiosis were shown in presence of *Lactobacillus jensenii* (p = 0.017) and *L. acidophilus* (p = 0.015). However, no statistically significant differences were established between intermediate microbiota and vaginal dysbiosis. **(B)** The results show statistically significant differences between healthy microbiota and AV in *L. jensenii* (p = 0.012) and *L. acidophilus* (p = 0.045). Meanwhile, only statistically significant differences between healthy microbiota and BV were shown in presence of *L. acidophilus* (p = 0.041); no statistically significant differences were established between AV and BV.

A



B

Epidemiological Factor	*Lactobacillus* species	*P*-value
Occupation	*L. iners*	0.009
Sexual Partner	*L. iners*	0.022
	L. acidophilus	0.021
	L. gasseri	0.007
	L. crispatus	0.044
Education level	*L. iners*	0.013
Contraceptive use	*L. acidophilus*	0.032
	L. gasseri	0.029
	L. crispatus	0.013
Vaginal secretion	*L. crispatus*	0.048
Previous medical treatment	*L. iners*	0.017
	L. gasseri	0.013

FIGURE 2 | Protective effect of the identified *Lactobacillus* sp. against the development of vaginal dysbiosis **(A)** and their statistical significance with epidemiological factors **(B)** evaluated in the study. The effect of each *Lactobacillus* sp. against the presence of a vaginal dysbiosis case was evaluated using logistic regression, where *Lactobacillus acidophilus*, *Lactobacillus jensenii*, and *Lactobacillus crispatus* show the greatest importance. However, only *L. acidophilus* and *L. jensenii* featured a significant *p*-value, more exactly, 0.035 and 0.050, respectively. Meanwhile, *Lactobacillus gasseri* and *Lactobacillus iners* show a null protective effect against the infection when they are evaluated independently.

epidemiological factors, such as occupation (p = 0.009) and education level (p = 0.013), showing statistical differences in its distribution among women in these categories. More exactly, a higher prevalence of *L. iners* was found in women with a secondary level of education (see **Table 1**). Finally, other factors, such as having a sexual partner and contraceptive use, demonstrated statistically significant values in relation to multiple *Lactobacillus* species (*L. acidophilus*, *L. gasseri*, *L. crispatus*, and *L. iners*) differing only in the absence of *L. iners* in contraceptive use. Although these results evaluated the species' individual probiotic role in the vaginal microbiota, it is well known that a probiotic microbiota is characterized by a multi-microbial effect character and is not caused merely by an individual effect (Vaneechoutte, 2017b; Wieërs et al., 2020). Therefore, a multi-microbial analysis was performed to evaluate a potential symbiotic or antagonistic relationship between these *Lactobacillus* species against both cases of vaginal dysbiosis.

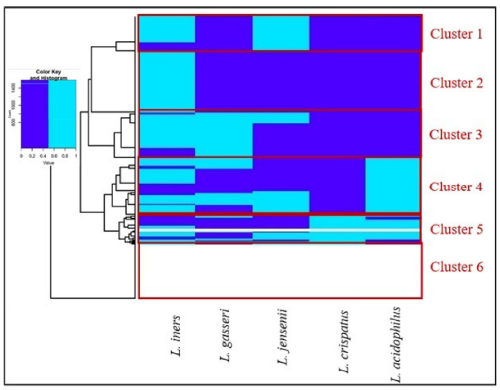
Analysis of *Lactobacillus* Species Association by Clustering Model

Nowadays, it is well known that the probiotic activity provided by a certain microbiota is caused not just by the effect of an individual *Lactobacillus* species but also by its multi-microbial interaction. So further analysis was also done through the clustering model of these *Lactobacillus* species against vaginal dysbiosis (by itself and then AV and BV) and epidemiological factors. A clustering model was realized through Ward's Minimum Variance Clustering Method evidencing multiple clusters. The clustering of samples was developed according to the presence of different *Lactobacillus* species in vaginal samples. As shown in **Figure 3A**, six clusters were selected for multiple chi-square analysis to evaluate statistically significant differences ($p \leq 0.05$). Cluster 1 was characterized by the presence of *L. iners* and *L. jensenii*, while Cluster 2 was only formed by *L. iners*. Cluster 3 was constituted by *L. iners*, *L. jensenii*, and *L. gasseri*. Cluster 4 assembled four *Lactobacillus* species, more exactly, *L. iners*, *L. jensenii*, *L. gasseri*, and *L. acidophilus*. Finally, Cluster 5 gathered all studied *Lactobacillus* species, and Cluster 6 evidenced no lactobacilli presence.

As shown in **Figure 3B**, only Clusters 3 and 4 demonstrated statistically significant differences against Clusters 1 and 6 in the establishment of vaginal dysbiosis. Clusters 3 and 4 shared *L. iners*, *L. jensenii*, and *L. gasseri*, but Cluster 4 also comprised *L acidophilus*. Both clusters evidenced a multi-species effect, being more notorious in Cluster 4 due to the obtained statistical values ($p < 0.030$). Interestingly, Cluster 1 also gathered *L. iners* and *L. jensenii* as Clusters 3 and 4; however, the absence of *L. gasseri* and *L. acidophilus* led to the lack of probiotic protection in vaginal dysbiosis establishment. The absence of *Lactobacillus* species in Cluster 6 was expected to relate to vaginal dysbiosis. It is also important to highlight that no statistical differences were found between Cluster 3 and 4 or even between Cluster 1 and 6, suggesting a potential probiotic connection among them. When individually evaluating each vaginal dysbiosis, greater statistically significant differences were found in the presence of AV (three p-values ≤0.05) than BV (one p-value ≤0.05). In AV cases, clustering analysis showed statistically significant differences in Clusters 3 and 4 when compared to Cluster 6 (p = 0.022 and p = 0.020, respectively), but only Cluster 3 showed statistical difference against Cluster 1 (p = 0.048). However, in BV cases, only Cluster 4 showed a statistically significant difference against Cluster 6 (p = 0.009), which is characterized by the absence of *Lactobacillus* species. In AV and BV cases, the combination of *L. iners*, *L. jensenii*, *L. gasseri*, and *L. acidophilus* from Cluster 4 reflected a multi-microbial consortium with statistical differences in the establishment of both dysbioses.

Finally, multiple chi-square analysis was also performed to evaluate the epidemiological factors related to the presence of each Cluster. As shown in **Figure 3C**, only Clusters 2 and 6 showed statistically significant differences among epidemiological factors. Both clusters shared statistically significant differences in women

A



B

Vaginal Dysbiosis					
***P*-value**	**Cluster 1**	**Cluster 2**	**Cluster 3**	**Cluster 4**	**Cluster 5**
Cluster 2	0.4636	1			
Cluster 3	**0.0299**	0.3222	1		
Cluster 4	**0.0224**	0.3889	0.9451	1	
Cluster 5	0.6902	0.8389	0.1149	0.1203	1
Cluster 6	1	0.4776	**0.0334**	**0.0262**	0.7061

Aerobic Vaginitis					
***P*-value**	**Cluster 1**	**Cluster 2**	**Cluster 3**	**Cluster 4**	**Cluster 5**
Cluster 2	0.8517	1			
Cluster 3	**0.0477**	0.1760	1		
Cluster 4	0.0548	0.2825	0.7904	1	
Cluster 5	0.9916	1	0.0798	0.1083	1
Cluster 6	0.7990	0.5757	**0.0217**	**0.0201**	0.6523

Bacterial Vaginosis					
***P*-value**	**Cluster 1**	**Cluster 2**	**Cluster 3**	**Cluster 4**	**Cluster 5**
Cluster 2	0.3601	1			
Cluster 3	0.4200	1	1		
Cluster 4	0.2084	1	1	1	
Cluster 5	0.2886	1	1	1	1
Cluster 6	0.5702	0.1332	0.1353	**0.0371**	0.0752

C

Epidemiological Factor	Clusters	*P*-value
Sexual Partner	Cluster 2	**0.044**
	Cluster 6	**0.001**
Contraceptive use	Cluster 2	**0.013**
	Cluster 6	**0.014**
Vaginal secretion	Cluster 2	**0.048**
Previous medical treatment	Cluster 6	**0.023**

FIGURE 3 | Clustering of the vaginal samples according to the prevalence of *Lactobacillus* sp. **(A)** Clusters obtained by Ward's Minimum Variance Clustering Method. **(B)** Chi-square analysis between clusters in presence of vaginal dysbiosis, aerobic vaginitis, and bacterial vaginosis. **(C)** Epidemiological factors related to each cluster. Six clusters were chosen according to the presence of different *Lactobacillus* species in vaginal samples using Ward's Minimum Variance Clustering Method. The following clusters are in panel **(A)**, *Cluster 1* was characterized by the presence of *Lactobacillus iners* and *Lactobacillus jensenii*; *Cluster 2* only showed *L. iners*; *Cluster 3* was constituted by *L. iners*, *L. jensenii*, and *Lactobacillus gasseri*; *Cluster 4* was formed by *L. iners*, *L. jensenii*, *L. gasseri*, and *Lactobacillus acidophilus*; *Cluster 5* is a mixture of all *Lactobacillus* species; and *Cluster 6* shows the absence of all of them. The dark blue color indicates the absence of a *Lactobacillus* species; meanwhile, the light blue color indicates the presence of the *Lactobacillus* species. In panel **(B)**, chi-square tests were performed to assess the statistical differences between clusters. The *p*-values where statistically significant differences were found are shown in bold. Finally, in panel **(C)**, multiple chi-square tests were performed to evaluate the epidemiological factors related to the presence of each cluster; the significant values are featured in the corresponding table in bold.

with a sexual partner and no contraceptive use (see **Figure 3C**), evidencing an association between these epidemiological behaviors and the lack of lactobacilli apart from *L. iners*. However, only Cluster 2 was associated with vaginal secretion in women ($p = 0.048$), more exactly, the presence of *L. iners* and the absence of the remaining analyzed lactobacilli, while Cluster 6 was correlated to women with a previous clinical history of antibiotic treatment for vaginal dysbiosis ($p = 0.023$), suggesting a potential correlation between treatments and eradication of vaginal lactobacilli.

Association Between the Presence of *Lactobacillus* sp. and Opportunistic Pathogens

To determine the relationship between the presence of any opportunistic pathogens and each *Lactobacillus* species analyzed in this study or the clusters formed by them, multiple chi-square tests were realized (see **Supplementary Tables 2**, **3**). The opportunistic pathogens previously identified in our last study were *Gardnerella* spp., *F. vaginae* (previously known as *A. vaginae*) and *Mobiluncus* spp. related with BV, *E. coli* related with AV, and *C. albicans* related with candidiasis (Salinas et al., 2020). As shown in **Supplementary Table 2**, every opportunistic pathogen showed a statistical significance for at least one *Lactobacillus* species, except for *Gardnerella* genus.

Interestingly, the presence of *L. iners* was statistically correlated with the absence of *Mobiluncus* species ($p = 0.033$). On the contrary, the absence of *L. jensenii* was statistically associated with the absence of *C. albicans* ($p = 0.034$), while the absence of *L. acidophilus* evidenced the same association with *F. vaginae* ($p < 0.001$) and *E. coli* ($p = 0.015$). Meanwhile, *L. crispatus* showed multiple statistical associations with *F. vaginae*, *C. albicans* (both $p < 0.001$), and *E. coli* ($p = 0.005$), where its absence was correlated with the absence of these opportunistic microorganisms. Finally, no statistical correlation was found in the presence or absence of *L. gasseri* in the vaginal epithelium among Ecuadorian women.

To better understand the multispecies probiotic activity of lactobacilli, multiple chi-square tests were further studied in the lactobacilli clusters. The results showed significant values among clusters 1, 2, 4, and 6, as shown in **Supplementary Table 3**. As previously stated, these clusters represent the presence of *L. iners* and *L. jensenii* (Cluster 1), only *L. iners* (Cluster 2), all lactobacilli except for *L. crispatus* (Cluster 4), and the absence of *Lactobacillus* sp. (Cluster 6). As shown in **Supplementary Table 3**, Cluster 1 evidenced an inhibition of the presence of *Gardnerella* genus ($p = 0.04$). Cluster 2 is particularly interesting showing multiple statistical associations, illustrating a proliferation of *F. vaginae* ($p = 0.001$) and an inhibition of *C. albicans* ($p = 0.001$) and *E. coli* ($p = 0.006$). *Mobiluncus* spp.

showed an opposite effect with the presence of Cluster 4, while the absence of *Lactobacillus* sp. in Cluster 6 could be inhibiting the proliferation of *F. vaginae* ($p = 0.05$).

DISCUSSION

The vaginal microbiota plays a vital role in modulating the risk of vaginal dysbiosis (Salinas et al., 2020). The protective role of *Lactobacillus* species in maintaining a healthy vaginal state in women is well known (Pacha-Herrera et al., 2020). However, this probiotic protection is caused not just by the individual effect of *Lactobacillus* species but also by its multi-microbial interaction (Graf et al., 2019). Little is still known about this multi-microbial dynamic among lactobacilli. Herein, we evaluated the individual and collective analyses of the prevalence of five lactobacilli (*L. iners*, *L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. acidophilus*) among healthy women and women with vaginal dysbiosis, more exactly, BV and AV.

The chi-square, univariable, and multivariable logistic regression analyses with the BH adjustment allowed us to evaluate the possible associations between each *Lactobacillus* species and vaginal microbiota. According to the univariable logistic regression analysis for determining the protective effect against vaginal dysbiosis, *L. acidophilus*, *L. jensenii*, and *L. crispatus* demonstrated excellent normalized importance values of 100%, 79.3%, and 74.8%, respectively. Moreover, *L. acidophilus* and *L. jensenii* exhibited statistically significant values, more exactly, $p = 0.035$ and $p = 0.050$, respectively. However, only *L. acidophilus* showed statistically significant differences in its prevalence on healthy microbiota against both dysbioses (BV, $p = 0.041$; and AV, $p = 0.045$), whereas *L. jensenii* only showed statistically significant differences between healthy microbiota and AV cases ($p = 0.012$). Although these findings are in agreement with previous studies (Hütt et al., 2016; Chee et al., 2020), *L. acidophilus* evidenced a higher probiotic effect than the vaginal consortia previously described by Chee and colleagues, and both *L. acidophilus* and *L. jensenii* showed a significant probiotic effect against AV development, which was not previously reported, to the best of our knowledge.

Furthermore, the multi-microbial clustering model done by Ward's Minimum Variance Clustering Method with Euclidean squared distance for hierarchical clustering allowed us to estimate the symbiotic relationship between these *Lactobacillus* species against both cases of vaginal dysbiosis. Our results evidenced a plausible strong probiotic multi-microbial consortium by *L. iners*, *L. jensenii*, *L. gasseri*, and *L. acidophilus* against AV ($p = 0.020$) and BV ($p = 0.009$). In addition, the absence of *L. gasseri* and *L. acidophilus* in other lactobacilli clusters leads to the lack of probiotic protection in vaginal dysbiosis establishment. These results are also in concordance with the predominance of bacterial consortia in our previous exploratory analysis among Ecuadorian teenagers against BV establishment (Salinas et al., 2018) and complementing information about lactobacilli combinations in probiotic formulas for the vaginal health against urogenital pathogens by Nader-Macías and colleagues (Nader-Macías et al., 2021).

Overall, this study shows the multi-microbial probiotic protection of these lactobacilli (*L. jensenii*, *L. gasseri*, and *L. acidophilus*) against both dysbioses. *L. jensenii* showed an individual probiotic effect against AV. Although the protective effect of *L. gasseri* against BV is well known (Scillato et al., 2021), its individual effect is overlapped when other *Lactobacillus* species are present in the same cluster, in particular *L. iners* and *L. crispatus*. However, the present study has several limitations, such as the absence of longitudinal analysis between vaginal infections and sociodemographic/behavioral variables or lactobacilli, only one vaginal sample was collected of each volunteer, and the lack of quantitative data. Therefore, the results of the present study could lead to an underestimation of the prevalence of opportunistic pathogens or even an overestimation of the probiotic activity of lactobacilli. Further studies should be conducted in Ecuador to quantify lactobacilli in different vaginal microbiota types verifying their probiotic activities among women.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Universidad San Francisco de Quito (USFQ) (Protocol code: 2016-023IN by MSP-VGVS-2016-0244-O review board). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Experimental research: DP-H, MPE-G, DC, MO, PB-S, and CA. Methodology: AM. Validation: DP-H, MPE-G, DC, MO, PB-S, and CA. Formal analysis: DP-H, ET, and AM. Resources: AM. Data curation: DP-H, ET, and AM. Writing—original draft preparation: DP-H and AM. Writing—review and editing: DP-H, ET, and AM. Supervision: AM. Project administration and funding: AM with Universidad San Francisco de Quito (USFQ) Chancellor Grants. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This work is supported by Chancellor Grants 2018 and Colegio de Ciencias Biológicas y Ambientales research budget from Universidad San Francisco de Quito, under the Project ID: 5456 entitled "Caracterización de la microbiota vaginal y sus factores de riesgos en mujeres ecuatorianas." The funders had no role in study design, data collection, analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We would like to thank all the staff of the Microbiology Institute of USFQ and COCIBA, as well as the Research Office of Universidad San Francisco de Quito.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.863208/full#supplementary-material

Supplementary Table 1 | PCR primers used in this study.

Supplementary Table 2 | Evaluation of potential associations between the presence of *Lactobacillus* sp. and opportunistic pathogens. Multiple chi-square tests were performed to evaluate the absence or presence of each pathogen during the presence of each *Lactobacillus* sp., showing the *P*-values with statistically significant differences as bold values.

Supplementary Table 3 | Evaluation of potential associations between opportunistic pathogens with clustering of *Lactobacillus* sp. Multiple chi-square tests were performed to evaluate the absence or presence of each pathogen during the presence of each cluster, showing the *P*-values with statistically significant differences as bold values.

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frontiers | Frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 27 May 2022
doi: 10.3389/fcimb.2022.905739

Clue Cells and Pseudo Clue Cells in Different Morphotypes of Bacterial Vaginosis

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Introduction: Clue cells (epithelial cells heavily covered with adherent bacteria) are an accepted clue to the diagnosis of bacterial vaginosis. However, the exact morphologic criteria of clue cells and bacterial adherence were never elaborated.

Materials and Methods: We investigated adhesive and cohesive patterns of main microbiota groups in vaginal discharge using fluorescence *in situ* hybridization (FISH). Samples from 500 women diagnosed with bacterial vaginosis and positive for clue cells with classic microscopy were collected from 42 gynecologic practices in Berlin and reexamined in our FISH laboratory for the spatial distribution of Bifidobacteriaceae, *Gardnerella*, *Fannyhessea vaginae* (*Atopobium*); low G+C (guanine+cytosine) bacteria, lactobacilli, *Lactobacillus iners*; *Lactobacillus crispatus*, Gamma-Proteobacteria; and Enterobacteriaceae, *Prevotella–Bacteroides*, *Veillonella*, and *Coriobacterium* groups.

Results: Bacterial taxa present in vaginal smears were not accidentally assembled according to their relative abundance but were built in group-specific distribution patterns, which can be well described by two features: cohesiveness to each other and adherence to epithelial cells. Accordingly, four patterns can be distinguished: dispersed (non-adherent bacteria), dispersed adherent bacteria, cohesive (non-adherent) bacteria, and cohesive adherent bacteria. Direct cohesive adherence to the epithelial cells representing true clue cells was unique for *Gardnerella* species and observed only in 56% of the investigated samples. In the remaining vaginal samples, the epithelial cells were mechanically entrapped in bacterial masses, and the composition was unrelated to the epithelial cell surface, building non-adherent pseudo clue cells. The proportion of women with true clue cells in their samples from different gynecologic practices varied from 19% to 80%.

Discussion: Taxon indifferent imaging is inadequate for the exact analysis of the microbial layer adjacent to the vaginal epithelial cells. Morphologically seen bacterial vaginosis is a mix of at least two different conditions: biofilm vaginosis and bacterial excess vaginosis.

Keywords: dysbiosis, FISH, clue cells, bacterial vaginosis, biofilm vaginosis, bacterial excess vaginosis, polymicrobials

OPEN ACCESS

Edited by:
António Machado, Universidad San Francisco de Quito, Ecuador

Reviewed by:
Bingbing Xiao, Peking University, China
Joana Castro, National Institute for Agricultural and Veterinary Research (INIAV), I.P., Portugal

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Specialty section:
This article was submitted to Biofilms, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *27 March 2022*
Accepted: *27 April 2022*
Published: *27 May 2022*

Citation:
Swidsinski A, Loening-Baucke V, Swidsinski S, Sobel JD, Dörffel Y and Guschin A (2022) Clue Cells and Pseudo Clue Cells in Different Morphotypes of Bacterial Vaginosis. Front. Cell. Infect. Microbiol. 12:905739. doi: 10.3389/fcimb.2022.905739

INTRODUCTION

Excessive vaginal discharge, which is troublesome, is the most frequent complaint in sexually active women visiting gynecologists (Redelinghuys et al., 2020). Only a small portion of such cases are attributed to sexually transmittable or other exactly defined mono-infections (Peebles et al., 2019). In most cases of bacterial vaginosis (BV), a marked increase in bacterial diversity and concentrations is observed without apparent cause. The composition of microbiota in such overgrowth dysbiosis is highly variable in patients, and even samples were taken from the same patient at different time points (Peebles et al., 2019; Muzny et al., 2019; Redelinghuys et al., 2020).

Gardner and Dukes in 1955 described epithelial cells covered with a bacterial layer in vaginal discharge of symptomatic women, which were absent in healthy women, and called them clue cells for their central role in recognizing the condition (Gardner and Dukes, 1955). Since then, many other researchers confirmed the vital role of clue cells in the diagnosis of the dysbiotic condition that was later named bacterial vaginosis. Despite broad acceptance and more than 60 years passing since the first description, no consensus exists about what should be regarded as a clue cell (Muzny et al., 2019; Redelinghuys et al., 2020). Exact criteria defining clue cells were never elaborated. Each investigator has his own highly subjective and even individually not reproducible feeling of what a clue cell already is and what it is still not. The main difficulty is the high heterogeneity of the microbial cover in wet smear or Gram/Pap stain microscopy. Transient findings between an obvious clue cell and a definitively normal epithelial cell are much more numerous than the both "unmistakable" extremes and still impossible to assign according to classic definition. As a solution, it was proposed to accept as clue cells only findings in which 20% of all visualized epithelial cells were covered with bacteria. But such a solution is not better and has the same deficiency as the detection of a "significant number" of clue cells.

The aim of the present study was to investigate how different bacterial groups contribute to the clue cell adhesion using fluorescence *in situ* hybridization (FISH).

PATIENTS, MATERIALS, AND METHODS

The study was based on vaginal samples from 500 (19- to 51-year-old women with a mean age of 28 years) symptomatic white women. Probes were randomly selected from 42 Berlin gynecologic practices with previous observation of positive clue cells with classic microscopy, Amsel criteria confirmed BV, and exclusion of sexually transmitted diseases or candidiasis. No other exclusion criteria including the previous therapy were applied since the most important fact for us was the presence of clue cells, diagnosed by other physicians. Therefore, usual exclusion criteria like previous therapy, and racial and social heterogeneities were not important or applied.

Nine gynecologic practices delivered more than 20 samples each, resulting in 271 samples. The number of samples from the remainder offices varied between 3 and 17 (mean 7).

ESwab™ 493C02 (COPAN Diagnostics, Murrieta, CA, USA) was used for the collection of vaginal smears and stored at 4°C between 12 and 48 h prior to FISH, whereby 43/500 samples were forwarded to FISH analysis within 12 h and 122/500 within 24 h after sampling. The samples were fixated with Carnoy's solution according to the protocol (Swidsinski and Loening-Baucke, 2017).

Amies smears were gently vortexed. The suspension measuring 100 µl was fixated by adding 1,000 µl of Carnoy's solution (alcohol/chloroform/acetic acid 6/3/1 by volume) (Meier et al., 1999) for 10 min. The samples were then centrifuged at 6,000 RPM for 8 min and stored in 50 µl of Carnoy's solution.

Fluorescence *In Situ* Hybridization

Fields of 10 mm × 10 mm were marked on SuperFrost slides (Langenbrinck, Emmendingen, Germany) with a PAP pen (Kisker-Biotech, Steinfurt, Germany). Aliquots of vortexed fixed vaginal swab suspension measuring 5 µl were dropped onto the marked field. The slides were dried for 60 min at 50°C before FISH analysis.

The exact protocols with single steps and solutions are also presented at http://www.swidsinski.de/zusatzdateien/fishmethode/fishmethode.htm.

The following probes were hybridized with all vaginal samples:

Bif 164 (Bifidobacteriaceae), Gard662 (*Gardnerella*), Ato291 (*Atopobium*) (Swidsinski et al., 2019),

LGC35 (low guanine+cytosine bacteria including Mycoplasmatales, Firmicutes, Bacillales, Lactobacillales) (Meier et al., 1999), Lab158 (lactobacilli) Liner23-2 (*Lactobacillus iners*), Lcrips16-1 (*Lactobacillus crispatus*) (Swidsinski et al., 2019), GAM42a (Gammaproteobacteria) (Amann et al., 1990), Ebac1790 (Enterobacteriaceae) (Bohnert et al., 2000), Bact1080 (*Prevotella–Bacteroides*) (Doré et al., 1998), Veil223 (*Veillonella*) (Harmsen et al., 2002), and Cor653 (Coriobacteriaceae) (Harmsen et al., 2000).

For multicolor analysis, each of the applied oligonucleotide probes was synthesized with a carbocyanine Cy3 (orange) and Cy5 (dark red) fluorescent dye. The hybridizations were performed at 50°C as previously described (Swidsinski and Loening-Baucke, 2017). DAPI stain was used to visualize the DNA-rich structures of bacteria and eukaryotic cells.

The Cy3-stained probe served as the evaluation of the targeted bacterial cluster, Cy5-stained probe served as a reference to the taxonomically broader microbiome group.

Despite previously tested and reported specificity of the FISH probes, we do know from our own experience that the reported specificity is never absolute since the specificity testing usually involves only isolated cultured strains. However, the real diversity of naturally occurring microorganisms is much higher than that of the cultured representatives. Therefore, it was important to support the hybridization results through a comparison of hybridization data of related and taxonomically unrelated FISH probes in multicolor FISH. Only hybridizations in which bacteria hybridized with Bif universal and *Gardnerella* probes, but did not hybridize with FISH probes representing other specificities, were regarded as genuine. Within the sets of

our experiment, we did not observe any cross hybridizations of bacteria with unrelated microbial probes.

A Nikon e600 fluorescence microscope was used. The images were photo-documented with a Nikon DXM 1200F color camera and software (Nikon, Tokyo, Japan).

RESULTS

Patterns of Bacterial Distribution in Vaginal Smears

In the majority of the samples, the microbial groups within the vaginal smear were not accidentally mixed and evenly distributed over glass slides; on the contrary, each taxon demonstrated its own difference from other spatial profiles and relation to the epithelial cells. According to these, bacteria could be divided into the following: diffusely distributed with clear distances between each other, only seldom building clumps of about 30 bacteria or less; bacteria cohesively attached to each other without spaces between them and preferentially organized in groups of 30 or more bacteria (up to 1,000–2,000) each. The transition from dispersed to cohesive condition was not abrupt. In each case, a considerable part of the cohesive bacteria was also distributed in the surroundings and vice versa. As cutoff, we regarded bacteria as cohesive only when at least 10 clumps with 30 bacteria each or one or more clumps together exceeded 300 coherent bacteria and were detected in the whole microscopic surface available for evaluation. Bacteria could be divided into the desquamated epithelial cells in non-adherent (having similar or higher concentrations in the distance than on the epithelial cell surface) and adherent bacteria (attached to epithelial cells in concentrations exceeding at least twice the concentrations of the same bacteria in the surroundings). In the case of very-high-density cohesive bacteria (with no spaces between microorganisms), exact concentrations could not be quantified. In such cases, a surface completely occupied by bacteria in the area of 10 μm adjacent to the epithelial surface was compared to regions at a distance of more than 20 μm from the epithelial surface. The total surface of the cohesive microbial layer attached to the epithelial cell surface and adjacent areas had to exceed the surface not connected to the epithelial cells clumps at least by a factor of two.

The adherence does not involve all epithelial cells. To make data numerically comparable, we arbitrarily defined bacteria as adherent when at least 10 epithelial cells within a smear demonstrated clear adherence as described above.

The investigations were performed at resolutions between ×100 and ×1,000. The combination of cohesiveness and adherence revealed four patterns of spatial distribution: dispersed (non-adherent), dispersed-adherent, cohesive (non-adherent) sludge-like, and cohesive-adherent or biofilm-like.

For convenience, we do not mention "non-adherent" in the following presentation, when not necessary for understanding.

Cohesive-adhesive growth was typical for biofilms attached to the epithelial cell surface and built true "clue cells" (**Figure 1**). The microbial cover of such cells was identically assembled all over the microscopy surface but varied in intensity from cell to cell, leaving some epithelial cells completely free. However, a finding of at least 10 epithelial cells with identical structured cohesive adherent cover was sufficient and unmistakable for the identification of a cohesive-adherent pattern, even in cases where the overall number of bacteria and epithelial cells on the glass slide was moderate (**Figure 1**, top-left image).

In contrast, dispersed and cohesive non-adherent growing microorganisms embedded epithelial cells only secondarily (**Figure 1**, bottom images). Even when highly concentrated and completely enclosing multiple or even most of the available epithelial cells through surrounding microbial growth, the resulting microbial cover of single epithelial cells remained unique for each epithelial cell and was not reproducible.

Graph 1 and **Table 1** demonstrate taxon-specific patterns of spatial microbial distribution.

Dispersed distribution was the most common and could be observed for all main representatives of the vaginal microbiota (**Table 2**). *Fannyhessea vaginae* (*Atopobium*), Enterobacteriaceae, *Prevotella*, *Veillonella*, and *Coriobacterium* groups were nearly exclusively dispersed. Cohesiveness was typical for *Gardnerella* and progressively declined from low GC bacteria to lactobacilli *L. iners* and *L. crispatus*, with Enterobacteriaceae *F. vaginae* and *Prevotella* being only exceptionally cohesive. Although low guanine+cytosine bacteria/lactobacilli and specifically *L. iners* could grow to extended cohesive fields covering large surfaces and include multiple epithelial cells in this growth, no cohesive adherence was observed in these groups. The highest concentrations of bacteria were always located outside of the epithelial cell or regions adjacent to them, building secondary pseudo clue cells; such a bacterial cover was definitively non-adherent.

A cohesive-adhesive pattern of distribution was unique for Bifidobacteriaceae (exclusively represented by *Gardnerella*) and comprised 56% of all investigated vaginal samples. The fluorescence of the Bif164 probe was however higher and visually more perceptible.

The difference between Bifidobacteriaceae and *Gardnerella* was altogether small and increased from coherent non-adherent to disperse-adherent and dispersed growing *Gardnerella*, indicating that in these cases other than *Gardnerella*, Bifidobacteriaceae species or other *Gardnerella* genotypes could be involved (**Table 2**).

The discrimination between cohesive adherent *Gardnerella* biofilms primarily enwrapping true clue cells and all other spatial patterns of microbial distribution was straightforward and unmistakable (**Figure 1**). These were especially obvious in cases with mainly dispersed growing bacteria such as *F. vaginae* (*Atopobium*). Although *F. vaginae* bacteria were often concomitant to cohesive *Gardnerella* and preferentially located within clue cell cover, different from *Gardnerella*, they always grew dispersedly or distributed in small islands within more abandoned *Gardnerella* conglomerates and never adhered directly to the epithelial cell surface (not shown).

On the other hand, the agreement between FISH data and the taxon unrelated methods was low, and we found true clue cells only in 56% of the vaginal samples, which were previously

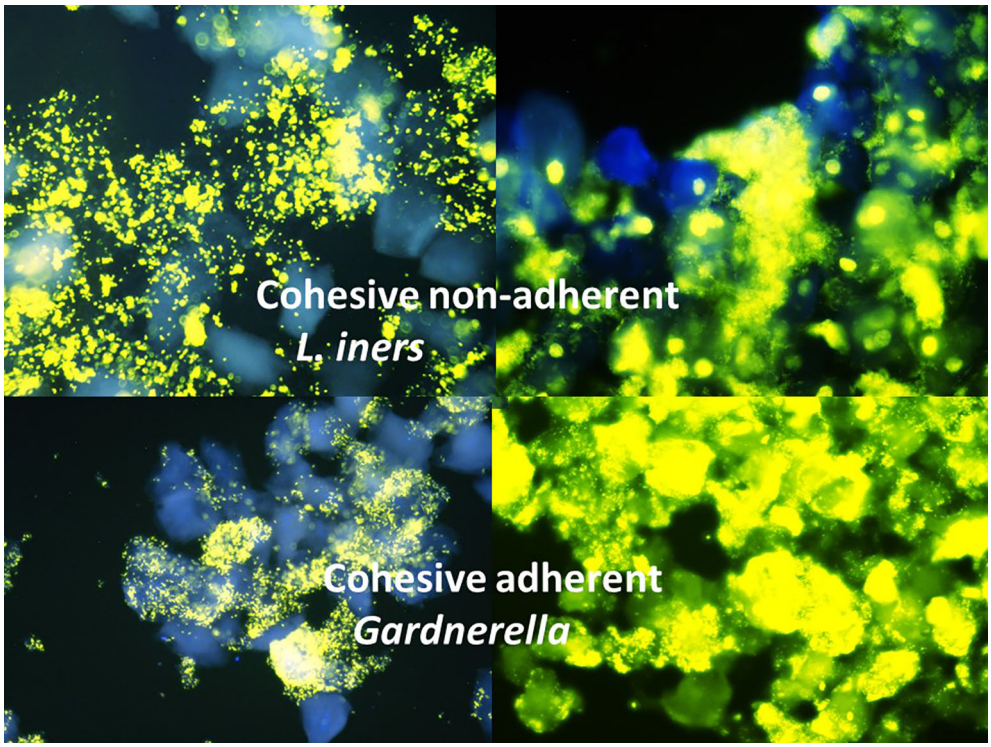
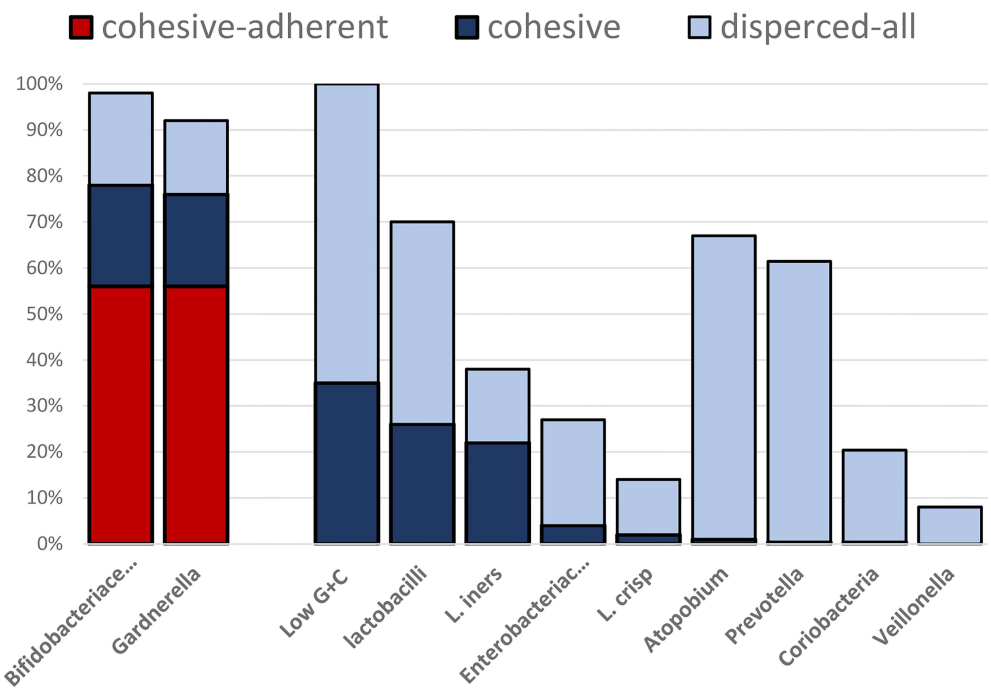


FIGURE 1 | Four examples of cohesive adherent (*Gardnerella with moderate and high density*) and cohesive non-adherent (*Lactobacillus iners* with moderate and high density) spatial distribution of bacteria; C3 yellow fluorescence against blue DAPI counterstain background, ×400.



GRAPH 1 | Percent of samples demonstrating cohesive adherent, cohesive non-adherent, and dispersed patterns of spatial distribution for each of the investigated microbial groups. The exact numbers are presented in **Table 1** (as additional material only).

TABLE 1 | Number of patients/samples demonstrating different patterns of spatial distribution for each of the investigated groups.

	Dispersed	Dispersed adherent	Cohesive	Cohesive adherent	All
					500
Bif164	47	52	109	281	489
Gard662	34	45	98	278	455
LGC35	223	101	176		500
Lab158	120	100	131		351
Liners23-2	41	39	108		188
Lcrisp16-1	34	25	12		71
*Gam42a	84	31	21		136
Ebac1790	84	31	21		136
Ato291	205	124	4		333
Bacto1080	269	37	2		308
Cor653	102	2	2		106
Veil223	42	2			44

**Gam42a and Ebac1790 probes hybridized with the same bacteria indicating that except for Enterobacteriaceae, no other Gammaproteobacteria are present in vaginal samples.*

identified as positive for clue cells with classic microscopy. Moreover, the proportion of cohesive adherent "clue cells" in samples sent from different gynecologic practices varied highly, ranging between 19% and 80% (**Graph 2**).

DISCUSSION

Our data demonstrate that unfortunately there is no "cheap reliable and convenient" way to recognize clue cells by classic microscopy. The definition of "stippled bacterial cover obscuring epithelial cell margins" is highly subjective. The uniform appearance of bacterial aggregates is erroneous. Taxonomic decoding of microorganisms by FISH unravels the highly differentiated structure of the bacterial cover and indicates the existence of at least two principally different modes of its formation: characteristic adherent growth of cohesive *Gardnerella* species on the surface of the epithelial cells leading to the development of true "clue cells" and sludge-like growth of individually arranged bacterial groups in the vaginal slime, which appeared to be "pseudo clue cells." They appeared to be secondarily entrapped (enveloped) and were in the way of epithelial cells in regions of microbial excess.

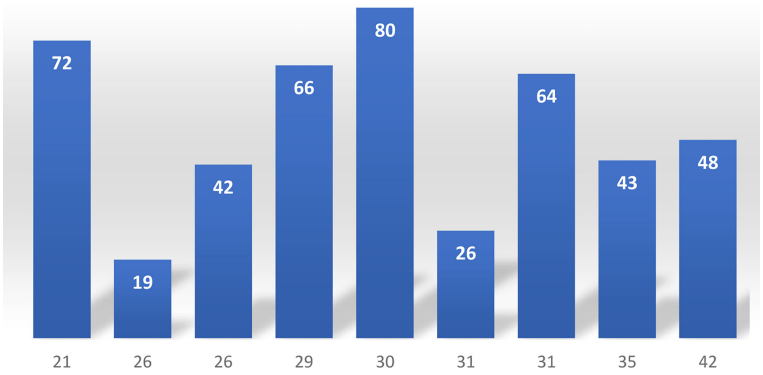
The characteristic adherent growth of cohesive *Gardnerella* species on the surface of the epithelial cells represented biofilm growth and should be probably more accurately called biofilm vaginosis (BiV). A proper name for the second condition could be a bacterial excess vaginosis (BeV). For a better overview, we present the proposed definitions in **Table 1** separately. The extension of the name by "excess" might be reasonable since it allows a separation of high microbial load conditions from aerobic and desquamative vaginal dysbiosis with overall low microbial counts independent of the presence or absence of pseudo clue cells.

The BiV was highly consistent in its morphologic appearance, while bacterial overgrowth vaginosis was mainly a mismatch of diversely distributed microbial groups. However, within the latter, some bacterial groups may dominate the picture with Enterobacteriaceae covering the microscopic surface in 4% of samples, building an eye-catching blanket or with *L. iners* arranged in 22% of samples in extensive cohesive conglomerates with a characteristic appearance. Obviously, these microorganisms did not need a firm surface for proliferation and preferred to grow freely suspended in slime. A situation could be basic and rooted in the natural history of polymicrobial communities of which some were specialized to grow attached to firm surfaces and form biofilms like stromatolites or vaginal biofilms (Swidsinski et al., 2014) and others were swimming free in fluids forming activated sludge (Mesquita et al., 2013). It is therefore and also imaginable that BeV could be further subdivided into etiologically relevant subgroups according to organisms leading the sludge-like microbial proliferation, of which cohesive *L. iners* could be of special interest not only for its high frequency in vaginal dysbiosis but also for the increasingly reported clinical significance (Zheng et al., 2021). A possible name for such could be *L. iners* excess vaginosis.

TABLE 2 | Morphological forms of bacterial vaginosis (BV) based on this study.

	Mode of growth	FISH appearance	Taxa
Biofilm vaginosis (BiV)	Epithelium attached biofilm growth	Cohesive biofilm adherent to and presumably growing on epithelial cells or true clue cells	*Gardnerella* spp.
Bacterial excess vaginosis (BeV)	Sludge like growth within vaginal slime	Cohesive and diffuse bacteria mainly growing on its own and only secondarily incorporating epithelial cells into the bacterial masses building pseudo clue cells	*Lactobacillus iners* and other lactobacilli Enterobacteriaceae
BV modifications	Diffuse or in isolated islands mixed with other bacteria	Non-coherent growing microorganism using overgrowth of other bacteria for own propagation	*Atopobium* (*Fannyhessea vaginae*), probably *Mobiluncus*, *Mycoplasma* spp., *Candida*, etc., not investigated in this study

FISH, fluorescence in situ hybridization.



GRAPH 2 | Percent of patients (vertical column) with cohesive-adhesive clue cells in samples from 9 gynecologic practices, delivering more than 20 samples each (N = horizontal line).

We found no other taxon-characteristic appearance features in our series, but the number of 500 samples may be too small to uncover a variety of dysbiotic morphology.

However, hybridization with the LGC35 probe demonstrated the highest occurrence of cohesive non-adherent bacteria that formed large sludge aggregates. These findings are less relevant than the hybridization with the *L. iners* probe. The low guanine +cytosine group is (similarly to Gram stain) very broad and includes Mycoplasmatales, Firmicutes, Bacillales, and Lactobacillales (Meier et al., 1999). The cohesive non-adherent bacteria detected by the LGC probe might be in reality a composite mishmash of non-prevalent bacterial groups being truly cohesive only in the case of *Lactobacillus* species. However, the absence of adhesive coherent growth in all vaginal samples when investigated with LGC35-FISH stresses the importance of *Gardnerella* species as the only microorganisms important for biofilm formation.

In contrast to the LGC35 probe, the use of the broad Gam42a probe that covered the phylum of Gammaproteobacteria added nothing to the hybridizations with the Enterobacteriaceae-specific probe, indicating that besides Enterobacteriaceae, no other Gammaproteobacteria were involved in shaping vaginal dysbiosis conglomerates.

Whichever terms and classifications ultimately prevail in the future, one thing is for certain: the syndrome currently diagnosed as BV is actually highly heterogeneous and made up of morphologically different conditions. All of the samples included in our study and obtained in different gynecologic practices contained clue cells routinely diagnosed with classic microscopy. Although we confirmed true clue cells in 56% of the samples, this value cannot be generalized for smaller groups and groups selected for specific criteria. Unfortunately, the comparison of nine practices that delivered more than 20 samples revealed an occurrence rate of true clue cells that ranged between 19% and 80%.

This heterogeneity could be an explanation for the previously observed inconsistencies in results of clinical studies regarding sexual transmissibility, severity, and incidence of associated complications, and also inconclusive therapeutic outcomes in women with BV (Muzny et al., 2019; Coudray and Madhivanan, 2020). The composition in contrasting vaginosis subtypes would predictably differ depending on the clinical focus, selection criteria, country, and even region of the performed study. Future clinical investigations should differentiate the cohesive-adhesive microbiome subtypes in their groups to ensure that they are not comparing apples and oranges.

Concluding Comments and Limitations

Unfortunately, FISH is not available in all laboratories, which limits the broad application of such a diagnostic method. FISH would probably remain indispensable for such differentiation, as long as the exact biomarkers responsible for cohesive adherent biofilm growth of *Gardnerella* are unknown.

However, for routine use, a one-step multicolor hybridization with Bif164-C3 probe together with LGC-C5 probe should be sufficient to distinguish the main morphotypes.

The use of the universal for Bifidobacteriaceae Bif164 probe for such diagnostic is preferable for the following reasons: Bifidobacteriaceae other than *Gardnerella* are numerically marginal in the vaginal microbiome. The accidental presence of *Bifidobacteria* other than *Gardnerella* in the vagina cannot therefore bias the final results. On the other hand, the genetic and taxonomic heterogeneities of *Gardnerella* species involved in BV are enormous. Each year, new yet unknown sequences of isolated species are reported. It would be important to include them all with routine testing. The Bif164 probe demonstrates a high luminescence and does not cross hybridize with other vaginal bacteria that positively hybridize with other probes used in the series including the ***F. vaginae*** (*Atopobium*) probe. Although the Bif164 probe often hybridizes with dispersed *Gardnerella*, the same is true for the *Gardnerella* probe. It demonstrates that genotypes and/or specific genes responsible for cohesive adherent growth are still to be found.

We also consider the dark red C5-stained LGC35 probe as a valuable addition for routine FISH diagnostics. The LGC35 probe covers all lactobacilli and also many other non-*Gardnerella* vaginal microbiota and is therefore preferable for the detection of cohesive non-adherent bacterial overgrowth. For specific

questions, the LGC probe can be substituted by Liners23-2 or by an extended set of other available FISH probes.

The costs of consumables for one-shot FISH analysis are the same as for classic Gram stain; however, for manual performance, 30 additional minutes must be taken into account. The one-time costs for procurement of microscope and camera of about 40,000 € are likewise necessary (present in each large laboratory). Investigators experienced in saline wet mount or Gram stain microscopy would have no difficulties in using FISH.

Some of the results of our study could be partially due to the long storage time of some samples, exceeding 24 h; the proportion of true clue cells in 102 samples delivered on the same day was 54% and was the same as in all other samples. However, this limitation had to be taken into account to access the cross section of all gynecologic practices in Berlin.

One important restraint of all FISH studies is the still limited number of available in open-access FISH probes. For some of the important species such as *Mycoplasma*, no satisfactory working FISH probes have been developed thus far. One can hope that such limitations are temporary and that the range offered for species-specific FISH probes will increase.

Finally, the clinical significance of our morphologic FISH microscopy-based subtypes of BV is unknown, but our findings emphasize the heterogeneity of this common syndrome.

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 human participants were reviewed and approved by the ethical committee of the Charité approval number EA1/088110. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Each author named in the byline participated actively and sufficiently in the study. AS, AG, and YD designed the study. AS and SS conducted the study. JS and VL-B critically revised the manuscript. AS and YD performed the FISH. AS and SS analyzed the data. All authors contributed to the conception of the work, revising the data, shaping the manuscript, and approving the final draft submitted.

FUNDING

The study was supported by the Charité University research promotion grant (2016) and the German Federation of Industrial Research Associations ZIM Project ZF4143701AJ5.

DISCLAIMER

Both funding sources were not involved in the study design, collection, analysis, and interpretation of data, in writing a report, or the decision to submit the article for publication.

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Conflict of Interest: Author SS was employed by MDI Limbach GmbH.

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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